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The interplay of short-term mesophyll and stomatal conductance responses under variable environmental conditions

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Abstract

Understanding the short-term responses of mesophyll conductance (g_m) and stomatal conductance (g_{sc}) to environmental changes remains a challenging yet central aspect of plant physiology. This review synthesises our current knowledge of these short-term responses, which underpin CO_2 diffusion within leaves. Recent methodological advances in measuring g_m using online isotopic discrimination and chlorophyll fluorescence have improved our confidence in detecting short-term g_m responses, but results need to be carefully evaluated. Environmental factors like vapour pressure deficit and CO_2 concentration indirectly impact g_m through g_{sc} changes, highlighting some of the complex interactions between the two parameters. Evidence suggests that short-term responses of g_m are not, or at least not fully, mechanistically linked to changes in g_{sc} , cautioning against using g_{sc} as a reliable proxy for g_m . The overarching challenge lies in unravelling the mechanistic basis of short-term g_m responses, which will contribute to the development of accurate models bridging laboratory insights with broader ecological implications. Addressing these gaps in understanding is crucial for refining predictions of g_m behaviour under changing environmental conditions.

KEYWORDS

chlorophyll fluorescence method, g_m , g_{sc} , isotope discrimination method, mesophyll conductance

1 | INTRODUCTION

Plants take CO_2 from the atmosphere to photosynthesise organic carbon in the chloroplast of the mesophyll cells, the location of the primary carboxylating enzyme Rubisco. CO_2 moves by passive diffusion along the concentration gradient between the outside air and the chloroplast, crossing the stomatal pore and mesophyll space until the Rubisco active site is reached. The ease with which CO_2

crosses the stomatal pore and the mesophyll space is referred to as stomatal conductance to CO_2 diffusion (g_{sc}) and mesophyll conductance (g_m), respectively. Both conductances adjust to environmental changes in short-term responses, usually in the order of minutes to hours for g_{sc} and seconds to minutes for g_m . These short-term responses modify the path that CO_2 and water vapour must cross in the exchange between the atmosphere and the leaf, thereby impacting photosynthesis rates.

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Efficiently easing CO₂ diffusion to the chloroplast is crucial for plants because, for a given amount of Rubisco active sites, Rubisco's activity and thus net CO₂ assimilation rate (*A*) is largely dependent on the CO₂ concentration inside the chloroplast (*c_c*) (Farquhar et al., 1980). Plants open stomatal pores to increase *g_{sc}*, allowing CO₂ to diffuse into the leaf and increasing the CO₂ concentration in the substomatal cavity (*c_i*), while Rubisco activity fixing CO₂ decreases *c_c*. Mesophyll conductance to CO₂ (*g_m*) bridges these two concentrations, facilitating the diffusion of CO₂ from the substomatal cavity to the chloroplast.

Consequently, environmental factors and plant properties that affect *c_c* and Rubisco activity, such as light intensity, CO₂ concentration, Rubisco content, *g_{sc}* and *g_m*, determine the value of *A*. For instance, stomatal closure in response to environmental stress can limit CO₂ availability in the mesophyll, decreasing the photosynthetic rate. Conversely, when stomata are open, mesophyll conductance must be sufficiently high to facilitate the movement of CO₂ to the chloroplasts for photosynthesis to occur at high rates.

To elucidate the importance of some interaction between *g_{sc}* and *g_m*, consider the common scenario taken for analysing the *A/c_c* relation, where *g_m* is assumed infinite (implying *c_c* = *c_i*). In this case, *g_{sc}* is the only limitation to CO₂ diffusion. However, under realistic conditions where *g_m* is finite, to obtain the same *A/c_c* ratio, a larger *g_{sc}* would be needed since *c_c* < *c_i*. In other words, *c_c* is overestimated if *g_m* is assumed to be infinite. Because CO₂ diffusion via open stomata incurs a water loss whereas diffusion through the mesophyll does not, for a given *A/c_c* ratio, the plant's water cost decreases when the *g_m/g_{sc}* ratio increases. This interplay between *g_{sc}* and *g_m* is a pivotal factor in plant carbon gain and water use efficiency (Buckley & Warren, 2014) and crucial for a plant's ability to adapt to variable atmospheric conditions, optimising the trade-off between carbon assimilation and water conservation (Evans et al., 2009; Flexas et al., 2008, 2012).

Due to their significant implications for understanding plant physiological processes, enhancing agricultural methods, and responding to climate change impacts, many studies have focused on exploring the physiology underlying *g_{sc}* and *g_m*. In this regard, even though predicting a value for stomatal conductance from atmospheric conditions is challenging (Buckley, 2005, 2023; Cowan, 1978), the general short-term stomatal responses to environmental conditions are well-known (Buckley & Mott, 2013; Farquhar & Sharkey, 1982; Lawson & Matthews, 2020). For example, stomata are expected to open when the light intensity increases, when the vapour pressure deficit (VPD) or CO₂ concentration decreases, and close under opposite conditions. The molecular signalling and mechanisms behind regulating stomatal conductance are complex (Murata et al., 2015), but short-term changes in stomatal conductance are essentially a consequence of varying the stomatal aperture. Thus, stomatal conductance to CO₂ refers to the physical implication of gases crossing through the stomatal pore.

On the other hand, assessing the causes of a variable *g_m* is a more complex task (Xiong, 2023). Mesophyll conductance to CO₂ is a composite of different biochemical and anatomical properties of the

leaf that, in combination, ultimately determine the diffusion of CO₂ inside the mesophyll (Evans & von Caemmerer, 1996). Given that *g_m* represents the ease of CO₂ diffusion through the mesophyll, it can be understood analogously as its inverse: mesophyll resistance to CO₂ diffusion (*r_m* = 1/*g_m*) is the sum of leaf properties that impede CO₂ diffusion. Briefly, *r_m* (and therefore *g_m*) is the composite of the leaf air space resistance, the liquid volume resistance, and the enzymatic reactions that influence CO₂ diffusion, such as carbonic anhydrase (CA) (Evans et al., 2009). The liquid path comprises the cell wall, cell membrane, cytosol, chloroplast envelope, and stroma (Evans, 2021). In practice, these mechanisms and structures respond independently to environmental changes and at different time scales, impacting the estimation of *g_m* in various ways (Busch, Holloway-Phillips, et al., 2020; Flexas et al., 2013). For instance, the physical resistance to diffusion given by the cell wall structure is not expected to be responsive to short-term variations of environmental conditions, while enzymatic reactions may be. Thus, short-term variations in *g_m* are triggered to different and variable degrees under different environmental conditions by enzymatic responses (Ogée et al., 2018), membrane permeability changes (Zhao et al., 2017), gas solubility and diffusivity variations in the liquid volume, and others. Ideally, one would like to evaluate each mechanism independently; however, experimentally assessing each component of *g_m* is challenging.

Even though state-of-the-art gas exchange instruments that allow for chlorophyll fluorescence measurements have simplified *g_m* estimation, quantifying *g_m* is still not a standard measurement. Thus, the data available for interpreting the mechanisms behind *g_m* is not as abundant as for its counterpart *g_{sc}*, and the complexity behind the physiology of *g_m* makes finding a clear trend to infer and model the short-term response of *g_m* to environmental conditions difficult. This is also partially driven by the complications of measuring *g_m* responses, its possible influence on other measurements and the cost associated with obtaining reliable measurements. For instance, the equipment required for more reliable methods, such as online isotope discrimination measurements (Busch, Holloway-Phillips, et al., 2020; Evans et al., 1986; Holloway-Phillips et al., 2019) are significantly more costly and complex than less reliable but simpler methods (Pons et al., 2009). The chlorophyll fluorescence method (Di Marco et al., 1990; Harley et al., 1992) is accepted as reliable at standard atmospheric conditions, but debate still exists on parameterising it under variable environmental conditions (Evans, 2021) or whether it is as reliable as the isotopic method (Gu & Sun, 2014; Yin & Struik, 2009).

These difficulties have led to practices that may become uncertain knowing *g_m* is variable, such as deriving a constant *g_m* from fitting CO₂ response curves (Sharkey et al., 2007), obtaining *g_m* values from the literature or using *g_{sc}* as a proxy for *g_m* behaviour (see, e.g., Gong et al., 2022; Ma et al., 2021; Sun et al., 2023). However, *g_m* is an essential parameter for predicting *A*, and there is a high risk of misinterpreting photosynthetic trends under variable environmental conditions if *g_m* is unknown or its environmental sensitivity is incorrectly attributed (Cano et al., 2013; Yin et al., 2020). Furthermore, a change in one environmental condition generates

changes in mechanisms other than g_m , and stomatal aperture is one of the most recognisable factors in such effects. These variations may indirectly affect g_m by modifying other internal leaf conditions without necessarily being linked as a coordinated response. Therefore, it is vital to study the mechanisms behind the short-term responses of g_m and the physiological processes directly and indirectly affecting g_m as a whole.

In this review, we aim to comprehensively analyse the short-term dynamics of g_m in response to environmental changes. We discuss current methods for estimating g_m and the limitations that arise when interpreting short-term responses. Further, we address the critical factors influencing c_i and their implications for g_m estimation. The possible mechanisms underlying the short-term variability of g_m , including enzymatic processes and anatomical adaptations, and the latest advancements in modelling these responses are explored. Additionally, as some studies have employed stomatal conductance as a proxy for g_m , we examine the evidence that suggests this does not accurately reflect mesophyll conductance, discussing both the potential misalignments and the limitations of such an approach. By examining these aspects, this paper seeks to summarise the current understanding of g_m dynamics and to highlight areas where further inquiry is necessary.

2 | CONSIDERATIONS ON THE ESTIMATION OF SHORT-TERM g_m RESPONSES

Aligned with Fick's First Law, g_m can be characterised by A along with the CO_2 concentration gradient between the intercellular air space (c_i) and the chloroplast (c_c) as

$$g_m = A/(c_i - c_c). \quad (1)$$

It is important to note that when Fick's First Law is used to estimate g_m , the embedded assumptions are that the medium between c_i and c_c is uniform, has a constant diffusion coefficient, and no chemical reactions involving CO_2 occur in the path (Fick, 1855, 1995). These assumptions are not met during changes in environmental conditions; thus, the estimation will result in an apparent g_m , and the variation on uniformity, diffusion coefficient, or chemical reactions in the path will have an impact in the form of an apparent variable conductance.

It is currently not possible to directly measure c_c for an outright calculation of g_m and, thus, for a direct determination of the variability of g_m with environmental conditions. Methods for estimating g_m all depend on models that relate measurable quantities to an 'apparent' g_m value and consequently require assumptions associated with some degree of uncertainty. For a detailed discussion of methods used to estimate g_m and the corresponding assumptions, refer to, e.g., Pons et al. (2009) and Holloway-Phillips et al. (2019). The two methods commonly used to estimate short-term responses of g_m in *planta* are the variable J method (Harley et al., 1992) and the

isotope discrimination method (Evans et al., 1986), which rely on gas exchange measurements coupled with chlorophyll fluorescence or carbon isotope discrimination measurements, respectively.

The first method is based on the estimation of the actual rate of photosynthetic electron transport (J) from chlorophyll fluorescence and gas exchange measurements (Harley et al., 1992) as

$$g_m = \frac{A}{c_i - \frac{\Gamma^*[J + 8(A + R_d)]}{J - 4(A + R_d)}}, \quad (2)$$

where R_d is the respiration rate in the light and Γ^* is the CO_2 compensation point in the absence of R_d . In Equation (2), J is an unknown that can be estimated from chlorophyll fluorescence using Equation (3),

$$J = \alpha\beta I_{inc} \Phi_{PSII}, \quad (3)$$

where Φ_{PSII} is the photochemical quantum yield of photosystem II (PSII) obtained from fluorescence, α is the leaf absorptance, β is the fraction of photons absorbed by PSII, and I_{inc} is the incident light intensity. Thus, the value of J depends on the assumptions for leaf absorptance, the fraction of photons absorbed by Photosystem II (Φ_{PSII}), fluorescence emissions from Photosystem I (PSI) and the strength of other electron sinks (see Pons et al., 2009).

While α can be measured or estimated reasonably accurately (Evans & Poorter, 2001), β is challenging to obtain and is usually assumed to be 0.5. However, this value can vary between species and environmental conditions. A significant issue is the unknown contribution of PSI to chlorophyll fluorescence (Franck et al., 2002), as its underestimation leads to an overestimation of g_m . This is especially important at variable ambient temperatures and high light intensities, where the signal-to-noise ratio is decreased in fluorescence measurements, intensifying PSI's role. Significant discrepancies can arise from alternative electron sinks, such as nitrate reduction (Laisk et al., 2002), which may be substantial (Bloom et al., 1989; Busch et al., 2018). To overcome some of these problems, measurements under low oxygen conditions to minimise photorespiration are used but introduce complexities, and even low rates of photorespiration can affect the g_m estimation. Concern also arises from the possibility of a non-representative sampling of chloroplasts by fluorimeters, capturing only part of the whole leaf profile. These elements collectively significantly impact the reliability of g_m estimations, where an error of just 5% in J estimation can lead to errors exceeding 30% in g_m values (Pons et al., 2009).

The other parameter not measured directly from gas exchange is Γ^* , which is usually assumed constant and taken from the literature. In C_3 plants, Γ^* has been reported to vary between species by only up to ~20% at 25°C (Hermida-Carrera et al., 2016); thus, minor errors in g_m estimations are expected from the approximation of Γ^* near that temperature. However, the temperature response of Γ^* varies significantly between species (Hermida-Carrera et al., 2016; Orr et al., 2016), and, potentially, growth environments. Thus, it can be risky to obtain Γ^* from literature and modelled temperature corrections (Crous et al., 2013), since when assigned incorrectly, it

can affect g_m estimates by up to 50% or even make g_m incomputable (van der Putten et al., 2018; Yin & Struik, 2009). In addition, the value of Γ^* may vary dynamically with the amount of carbon exported from the photorespiratory pathway (Busch et al., 2018; Busch, 2020). Accounting for all these considerations, if Γ^* is measured, albeit with small inaccuracies, minor errors are expected in g_m computation under normal conditions.

Advances in the fluorescence technique have been presented in the study by van der Putten et al. (2018), offering a significant contribution to the understanding of g_m estimation using the fluorescence method. Their research addresses various procedural challenges mentioned above. A key takeaway from their study is the critical role of calibration based on non-photorespiratory condition measurements in the accuracy of g_m estimates. van der Putten et al. (2018) demonstrate that without proper calibration, the multiphase flash method fails to produce realistic g_m estimates, thereby emphasising the importance of calibration for precision. Additionally, the study shows that the single saturation pulse method can yield reasonable g_m estimates, but this accuracy depends on the proper calibration implementation.

The second method (isotopic method) allows for estimating g_m under varying environments by measuring the distinctive isotopic signature imprinted during CO_2 assimilation, which arises from Rubisco's carbon isotope discrimination characteristic (Farquhar & O'Leary, & Berry, 1982). This effect can be employed to derive g_m values from gas exchange and carbon isotope measurements by comparing the modelled isotopic discrimination assuming infinite g_m (Δ_i) against the observed isotopic discrimination (Δ_{obs}) (Evans et al., 1986). The most current model to estimate g_m in this way was presented by Busch, Holloway-Phillips, et al. (2020), where R_d is isotopically disconnected from the Calvin-Benson-Bassham (CBB) cycle (see Equation (4)). The estimation of g_m from the isotope discrimination observed in plants is then

$$g_m = \frac{1+t}{1-t} \frac{A \left(b - a_m - \frac{R_d}{A} \frac{\alpha_b}{\alpha_{e'} + \frac{R_d}{A}} e' \right)}{c_a (\Delta_i - \Delta_{\text{obs}})} \quad (4)$$

where t is a ternary correction factor dependent on the rate of transpiration and the conductance to CO_2 diffusion in air, a_m , b and e' are the isotopic fractionations associated with diffusion through water, Rubisco carboxylation and respiration, respectively. α_b and $\alpha_{e'}$ are the isotope effects of Rubisco carboxylation and respiration, respectively (see Busch, Holloway-Phillips, et al., 2020 for a full description of each parameter).

Previous models assumed that R_d and the CBB cycle are isotopically connected (Evans et al., 1986; Farquhar & Cernusak, 2012), leading to erratic estimations of g_m near the light and CO_2 compensation points. While Equation (4) is still somewhat sensitive to values selected as fractionation factors, Equation (4) is much more robust to inaccuracies in their values than previous models (Busch, Holloway-Phillips, et al., 2020). In practice, Busch, Holloway-Phillips, et al. (2020) tackle a significant limitation in

previous carbon isotope discrimination models for estimating g_m , particularly under low photosynthesis rates, where such models often yield implausible g_m results. Their updated model, presented in Equation (4), solves this issue by revising assumptions related to the isotope effect of mitochondrial respiration. A critical aspect of their approach involves treating the carbon pool associated with respiration as distinct from the pool of primary assimilates. This distinction leads to a model that consistently returns more plausible and accurate g_m estimates. This representation significantly enhances the accuracy of g_m estimates in scenarios of low assimilation rates, which is crucial for understanding short-term g_m responses. It also implies that previously established models for g_m estimation are less reliable under these conditions.

There is still considerable debate on how closely the fluorescence and isotope methods deliver a 'true' value of g_m , with mixed results being reported in the literature (Evans, 2021; Gu & Sun, 2014; Th eroux-Rancourt & Gilbert, 2017); however, they are considered the most trustworthy methods for estimating g_m (Pons et al., 2009).

3 | SHORT-TERM RESPONSE OF g_m

Over the past two decades, many studies have contributed information about the short-term response of g_m to environmental changes (e.g., Gago et al., 2016; Knauer et al., 2022; Sugiura et al., 2020; Th eroux-Rancourt & Gilbert, 2017; Tosens et al., 2012; Veromann-J urgenson et al., 2020). In general, they have demonstrated consistent trends of g_m responses for CO_2 and light in many plant species, but trends in response to changes in temperature and drought have been less consistent.

Figures 1–3 show the general behaviour of short-term responses of g_m and g_{sc} obtained from experiments where both sets of data are available for paring g_m and g_{sc} responses to CO_2 concentrations, light intensity and temperature variations. Note that data that could not be recomputed with the most current model for isotopic discrimination was not included in the figures, although it is considered in the discussion. In general, the behaviour presented for g_m to each environmental variable is consistent with that of other studies where g_{sc} was not reported. We analyse each response in detail below.

3.1 | Response to CO_2 concentrations

Many studies have shown short-term g_m responses to changes in CO_2 concentration within the leaf presenting similar behaviours to those presented in Figure 1 (Busch, Holloway-Phillips, et al., 2020; Flexas et al., 2007; Hassiotou et al., 2009; M arquez et al., 2023; Tazoe et al., 2011; Vr abl et al., 2009; Xiong et al., 2015). To reiterate and caution, analyses made near the compensation point using the isotope technique performed with the old model by Farquhar et al. (1980) bring large uncertainties (Busch, Holloway-Phillips, et al., 2020; Pons et al., 2009). However, the general responses are similar and present equivalent behaviours when using the isotope discrimination

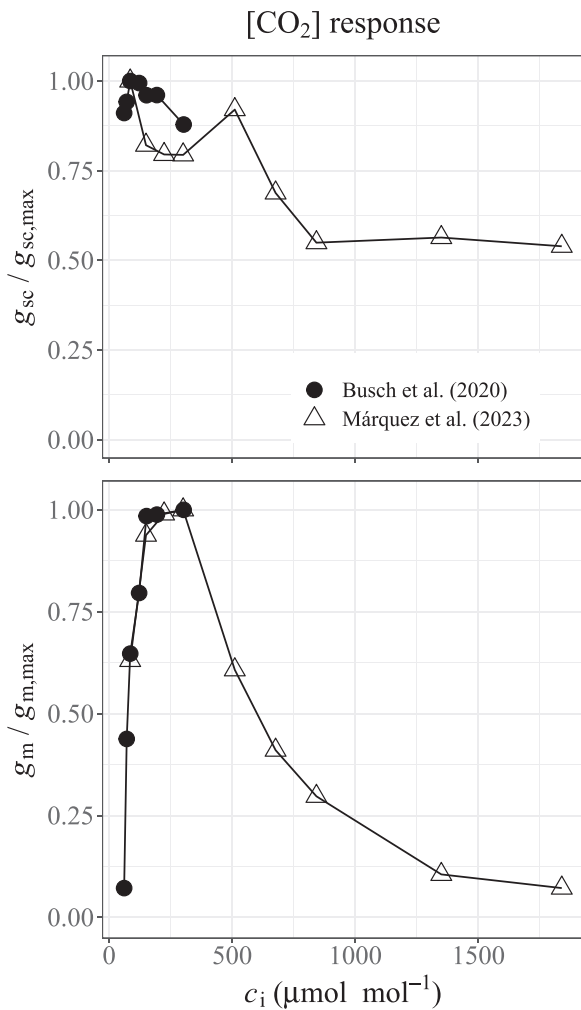


FIGURE 1 Proportional g_{sc} and g_m responses to changes in CO_2 concentration. Data from Busch, Holloway-Phillips, et al. (2020) used the isotope method in *Triticum aestivum* and Márquez et al. (2023) used the fluorescence method in *Gossypium hirsutum*.

model by Busch, Holloway-Phillips, et al. (2020) and the variable J method, as shown in Figure 1 (e.g., Flexas et al., 2007). Broadly, g_m tends to remain stable at CO_2 concentrations near ambient growth conditions, but g_m decreases as CO_2 concentration increases or decreases.

3.2 | Response to light intensity

The magnitude of g_m tends to increase with short-term increases in light intensity from low to high light (Figure 2), up to a maximum where g_m is not further responsive to increases in light intensity (Busch, Holloway-Phillips, et al., 2020; Carriquí et al., 2019; Ellsworth et al., 2018; Gauthier et al., 2018; Shrestha, Buckley, et al., 2019; Thérroux-Rancourt & Gilbert, 2017). That is, the increase of g_m during short-term responses to increased irradiation is discernible only until a threshold from which the g_m is insensitive to further light increase (Yamori et al., 2010). Other studies have reported g_m to be almost

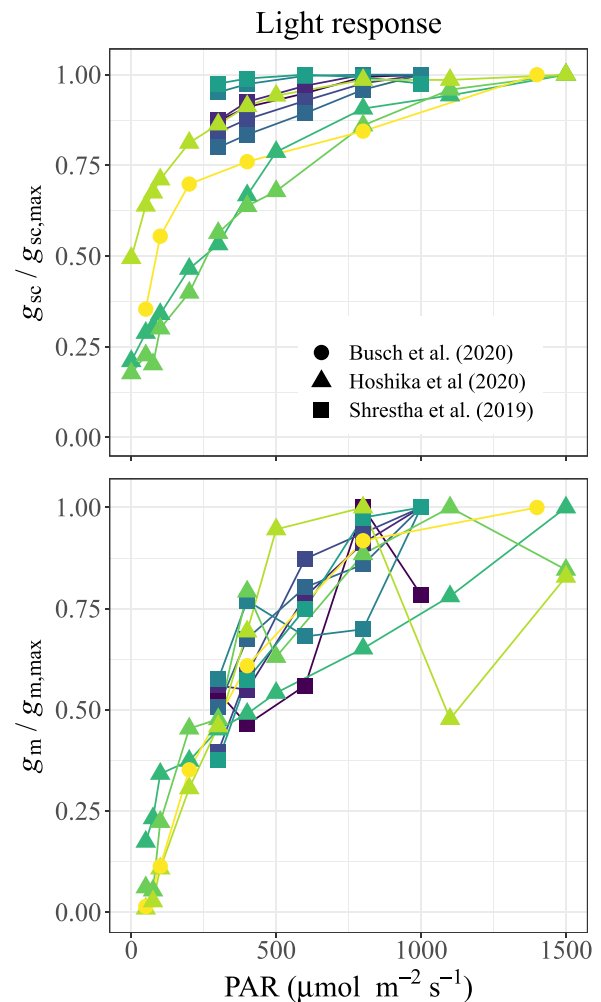


FIGURE 2 Proportional g_{sc} and g_m responses to changes in light intensity. Data from Busch, Holloway-Phillips, et al. (2020) used the isotope method in *Triticum aestivum*, Shrestha, Buckley, et al. (2019) used the isotope method in *Cicer arietinum* and Hoshika et al. (2020) used the fluorescence method in *Fagus crenata*. [Color figure can be viewed at wileyonlinelibrary.com]

insensitive to light increases, which Evans (2021) has referred to as a type B g_m response to light. This prompts whether g_m in certain plants is unaffected by light variations or if they reach an unresponsive threshold at lower light intensities than what was measured.

3.3 | Response to temperature

Several publications provide evidence that temperature affects short-term responses of g_m (von Caemmerer & Evans, 2015; Diao et al., 2024; Huang et al., 2022; Li et al., 2020; Qiu et al., 2017; Shrestha, Song, et al., 2019; Walker et al., 2013). Various trends in g_m response to temperature have been observed, also influenced by the assumptions made during the calculations (Evans & von Caemmerer, 2013). Generally, g_m tends to increase with temperature initially and sometimes collapses at very high temperatures, as shown

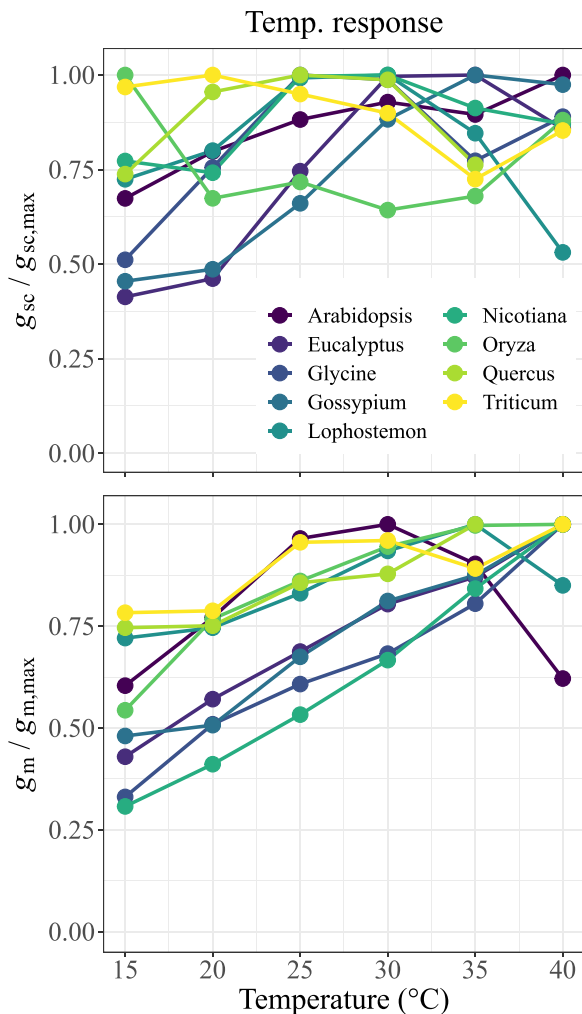


FIGURE 3 Proportional g_{sc} and g_m responses to changes in leaf temperature. Data from von Caemmerer and Evans (2015) used the isotope method in *Eucalyptus pauciflora*, *Quercus engelmannii*, *Lophostemon confertus*, *Nicotiana tabacum*, *Oryza sativa*, *Triticum aestivum*, *Gossypium hirsutum*, *Glycine max* and *Arabidopsis thaliana*. [Color figure can be viewed at wileyonlinelibrary.com]

in Figure 3. Still, the degree of increment seems species-dependent (Evans, 2021).

3.4 | Response to drought and air saturation deficit

Wong et al. (2022) showed that g_m remains unchanged during variations in air VPD, even when unsaturation is induced in the substomatal cavity. Along the same line, Warren (2008b) showed that g_m was not responsive to VPD changes if g_{sc} does not vary significantly. Further evidence, using ABA-deficient mutants, in which stomata remain open, showed that g_m does not exhibit a short-term response to drought and VPD (Mizokami et al., 2015). This is consistent with what is expected from measurements of water stress not affecting photosynthesis (Wong et al., 1985). Nevertheless, some studies have reported changes in g_m due to water stress (Cano

et al., 2013; Olsovska et al., 2016), but the cause of the variation seems to be the stomatal closure as the variation follows the c_i decrease, as other studies have noted (Loucos et al., 2017; Théroux-Rancourt et al., 2014).

In terms of water relations, there are reports of g_m sometimes correlating with leaf water potential (Loucos et al., 2017; Théroux-Rancourt et al., 2014). Leaf water potential has even been suggested as a possible proxy for g_m , but the mechanisms behind the correlation are still unknown (see Flexas et al. (2013) for a comprehensive analysis). Li et al. (2020) explored the temperature and leaf water potential responses, demonstrating that while maintaining a constant leaf water potential, g_m continues to respond to temperature fluctuations but not vice versa. Further, Warren (2008b) and Li et al. (2020) showed that changes in stomatal conductance led by leaf water potential fluctuations are responsible for the observed variations in g_m . This finding indicates that alterations in leaf water potential alone do not seem to be sufficient to initiate changes in g_m . Further research is needed on the effect of VPD and leaf water potential, which presents significant challenges to be evaluated *in planta* due to the difficulty of determining c_i under drought and VPD stress (for instance, see corrigendum for Roig-Oliver et al., 2023 and Flexas et al., 2002).

4 | CRITICAL CONSIDERATIONS ABOUT c_i

As g_m deals with the conductance to CO_2 diffusion between c_i and c_c , a correct estimation of c_i from gas exchange measurements is essential for calculating g_m . This is particularly critical when assessing g_m under water stress conditions and high VPD as stomata conductance tends to be low and accepted assumptions in gas exchange measurements start to weaken.

Estimations of c_i are normally performed assuming the leaf air space is saturated with water vapour (Gaastra, 1959), using leaf temperature as a proxy to estimate water content in the substomatal cavity (w_i). Recent studies have confirmed this assumption is incorrect at mild or high VPD, finding w_i equal to 80% and 90% of relative humidity (Cernusak et al., 2018; Wong et al., 2022). Failing to estimate w_i correctly will affect the estimation of g_{sc} and, consequently, c_i and g_m . Wong et al. (2022) showed how unsaturation within the leaf can affect our estimation of g_m if not accounted for in the calculations (Figure 4). If saturated conditions are assumed, stomatal conductance is underestimated as VPD increases and, as a consequence, c_i is underestimated and a larger g_m is computed. However, when w_i is assessed properly, g_m remains practically constant with changes in VPD.

Another concern at high VPD or under water stress is the occurrence of areas where the stomata remain closed, a phenomenon called patchiness. Patchiness effectively decreases the photosynthesising leaf area, making it different from the one used in the c_i calculations (Mott & Buckley, 2000; Terashima et al., 1988). Unfortunately, patchiness and unsaturation are difficult to identify and distinguish during measurements, but it is fundamental to

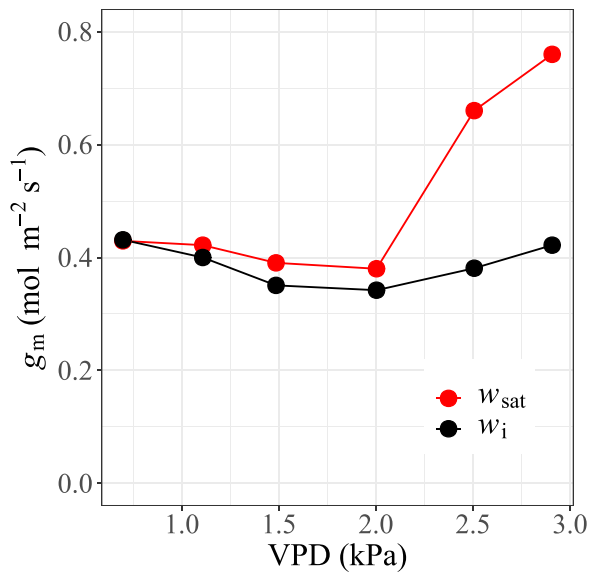


FIGURE 4 Steady-state mesophyll conductances to CO_2 (g_m) responses to changes in vapour pressure deficit (VPD), assuming saturation within the leaf air space (w_{sat} , red line and circles) and accounting for unsaturation in the intercellular air space (w_i , black line and circles). Data from Wong et al. (2022) used the isotope method in *Gossypium hirsutum*. [Color figure can be viewed at wileyonlinelibrary.com]

account for them for the accuracy of gas exchange parameter estimations (Rockwell et al., 2022). The analysis proposed by Laisk (1983) using commercial gas exchange systems can help identify patchiness but not unsaturation. The method proposed by Márquez et al. (2023) allows us to identify both patchiness and unsaturation but requires a gas exchange system capable of independently measuring adaxial and abaxial gas exchange. Developing mechanistic models that allow us to foretell unsaturation and patchiness in the leaf is still challenging and a gap in our current knowledge on gas exchange.

Another important consideration is the effect of small fluxes, cuticular conductance and ternary corrections on c_i estimates, especially when transpiration or stomatal conductance to water (g_{sw}) are low ($g_{\text{sw}} < 160 \text{ mmol m}^{-2} \text{ s}^{-1}$) (Márquez, Stuart-Williams, et al., 2021). Although small fluxes are commonly ignored, it has been shown that neglecting them can generate overestimations of c_i up to $100 \mu\text{mol mol}^{-1}$ (Boyer, 2015; Boyer et al., 1997; Márquez, Stuart-Williams, et al., 2021). In particular, using stable isotope techniques, it has been shown that incorporating a ternary correction in the calculations improves the reliability of g_m results (Farquhar & Cernusak, 2012). It is necessary to properly assess cuticular conductance to water (e.g., by applying the Red-light method proposed by Márquez, Stuart-Williams, et al., 2021) and use models of gas exchange that can account for it, especially when plants experience low stomatal conductance (Márquez, Stuart-Williams, et al., 2021).

Note that this analysis suggests that when accounting for the correct estimation of c_i , VPD does not directly affect short-term

responses of g_m (Figure 4). However, not many reports of g_m responding to VPD changes and drought have accounted for unsaturation, patchiness, small fluxes, cuticular conductance and ternary corrections as Wong et al. (2022) did. Still, more research is needed to confirm that the invariability of g_m to changes in VPD is a general behaviour.

5 | POSSIBLE MECHANISMS INVOLVED IN THE SHORT-TERM RESPONSE OF g_m

Several different mechanisms have been proposed to influence or correlate with g_m . However, it has been challenging to single out a mechanism that could conclusively describe the observed responses to all environmental factors or even one factor. Here, we discuss the mechanisms that have been suggested to be linked to short-term responses of g_m , summarised in Table 1.

5.1 | Changes in chloroplast cover

It is well-established that within a species and under steady-state conditions, the surface area of chloroplasts exposed to the intercellular air space per unit leaf area (S_c/S ; $\text{m}_{\text{chlor}}^2 \text{ m}^{-2}$) correlate reasonably well with g_m (Evans, 2021; Evans et al., 1994). This observation can be interpreted as a long-term adaptation of g_m . Put simply, a larger S_c/S increases the probability of a CO_2 molecule finding its way into a chloroplast, thereby increasing g_m . More recently, it was proposed that a change in chloroplast cover, for example, induced by chloroplast movement or shape, could alter the resistance experienced by CO_2 diffusing through the mesophyll in the short term (Flexas & Diaz-Espejo, 2015; Shrestha, Song, et al., 2019; Tholen et al., 2008). Variations of S_c/S in response to changes in light quality were shown to be in the order of 20% in *Arabidopsis*, corresponding to similar changes in g_m (Tholen et al., 2008). However, other studies have shown that chloroplast cover did not explain the short-term responses of g_m (Shrestha, Song, et al., 2019), and in general, there is still no direct evidence of chloroplast cover and arrangement causing a short-term g_m response.

5.2 | Aquaporins

Some classes of aquaporins, abundant proteins that transport water across plasma membranes, have also been shown to transport CO_2 (Uehlein et al., 2003). They have been shown to help maintain the permeability to CO_2 of membranes by creating paths in areas of the membrane typically hindered by structures that obstruct CO_2 movement (Kai & Kaldenhoff, 2014). Thus, some long-term effects in g_m may be expected by altering their natural abundance in the CO_2 path. Experimental evidence related to long-term observations of g_m regarding the

TABLE 1 Mechanisms suggested as possibly being involved in one or more short-term responses of g_m to environmental changes of CO₂ concentration, light intensity and leaf temperature.

Mechanism/response	[CO ₂]	Light	Temp.	References
Changes in chloroplast cover	-	/	-	Evans et al. (1994); Flexas and Diaz-Espejo (2015); Shrestha, Song, et al. (2019); Tholen et al. (2008)
Aquaporins	/	-	-	Clarke et al. (2022); Ermakova et al. (2021); Flexas et al. (2006); Heckwolf et al. (2011); Israel et al. (2021); Kai and Kaldenhoff (2014); Kromdijk et al. (2020); Terashima et al. (2006); Uehlein et al. (2003); Zhou et al. (2023)
Membrane and cell wall permeability	-	-	/	Evans and von Caemmerer (2013); Evans (2021); Shrestha, Song, et al. (2019)
Structures across leaf profiles	-	+	-	Evans et al. (2009); Th��roux-Rancourt and Gilbert (2017)
Variable fluxes along the CO ₂ diffusion pathway	+	+	-	Busch (2020); Busch, Holloway-Phillips, et al. (2020); Parkhurst (1994); Tholen et al. (2012); Yin and Struik (2017)
Enzymatic involvement	+	-	-	Cowan (1986); Enns (1967); Majeau and Coleman (1996); M��rquez et al. (2023); Momayyezi et al. (2020); Raven and Glidewell (1981)

Note: Symbols indicate that experimental evidence has been found for (+) or against (-) an existing link, or conflicting or no direct evidence exists (/).

physiological relevance of aquaporins in CO₂ transport has been mixed. Some long-term effects of aquaporins on g_m were observed in antisense and overexpression lines of the tobacco aquaporin NtAQP1 (Flexas et al., 2006). Arabidopsis plants with a mutated aquaporin suggest a functional role of aquaporins (Heckwolf et al., 2011) and Arabidopsis PIP2;5 was shown to be permeable to CO₂ when tested in yeast, which suggested it is aiding g_m *in planta* (Israel et al., 2021). Similarly, the expression of a *Setaria italica* plasma membrane intrinsic aquaporin was shown to increase g_m in the C₄ species *Setaria viridis* (Ermakova et al., 2021). In contrast, g_m was unaffected in Arabidopsis aquaporin knockout lines (Kromdijk, G  wacka, & Long, 2020) or transgenic tobacco lines expressing Arabidopsis aquaporins (Clarke et al., 2022).

One possible explanation for the observed inconsistencies in aquaporin involvement in facilitating g_m might be attributable to interactions between aquaporins and some carbonic anhydrases (CA), an enzyme whose role in g_m is elaborated upon below. Studies have shown that CA can bind to certain aquaporins within guard cells (Hu et al., 2015) and more recently, this interaction has also been identified in the chloroplast envelope and mesophyll cell membranes (Zhou et al., 2023). Alterations in the quantity of aquaporins could, therefore, influence the distribution and localisation of CA, subsequently impacting g_m . This interaction suggests that if aquaporins under investigation are functionally connected to CA, their genetic manipulation—either through knockout or overexpression—might affect g_m indirectly by paths other than the aquaporin's CO₂ permeability. This effect could be mediated by altering the proximity of CA to the membrane and potentially by influencing the amount of CA. However, it is important to note that this hypothesis, proposing a direct link between aquaporin modifications and changes in g_m through CA interactions, remains untested and speculative at this stage.

As aquaporins abundance or conductance to CO₂ could be rapidly altered, it was also suggested they could contribute to the short-term variability of g_m (Terashima et al., 2006). However, if aquaporins were directly contributing to the short-term variability of g_m , the molecular mechanism through which aquaporins could be regulated in response to CO₂ and O₂ concentration, light intensity or temperature would also have to be elucidated. One reason that argues against the involvement of aquaporins in g_m variability in the short-term response to temperature was pointed out by von Caemmerer and Evans (2015): If membrane permeability were high due to CO₂ permeable aquaporins, g_m would be expected to be relatively insensitive to temperature if the activation energy for CO₂ transport follows that of H₂O transport through aquaporins. It could thus not explain the observed temperature sensitivity of g_m . To date, we lack evidence of possible short-term regulation of the CO₂ conductance of aquaporins *in planta* that could account for the fast changes in g_m observed.

In this regard, it is important to be aware that aquaporins significantly influence plants' stomatal behaviour (Hu et al., 2015). Thus, varying aquaporin content tends to lead to changes in stomata conductance under various conditions, consequently varying c_i even if c_a is the same. The more common practice to perform as a comparison experiment is a common c_a rather than c_i , but, as shown in Figure 1, changes in c_i directly affect the short-term response of g_m . Therefore, to effectively evaluate the impact of aquaporins, or any other factor, on CO₂ transport within plants, it is crucial to focus on the variability of g_m at a constant c_i rather than at a constant c_a or analysing CO₂ response curves of g_m .

5.3 | Membrane and cell wall permeability

It has been suggested that the sensitivity of g_m to changes in temperature might be related to the physical properties of the

conductivity through the liquid volume and membranes, namely the plasma membrane and chloroplast envelopes (Evans & von Caemmerer, 2013). While conductivity via the liquid volume tends to decrease slightly with rising temperatures (attributable to alterations in CO_2 diffusivity and its solubility in water), the conductivity across membranes is anticipated to increase as a result of enhanced membrane permeability (Evans & von Caemmerer, 2013). This behaviour difference between liquid volume and membrane conductivity has been suggested to explain the observed variability in g_m 's temperature sensitivity across different species (von Caemmerer & Evans, 2015). Nevertheless, other factors known to change with temperature such as enzymatic activity, may affect g_m as well. Thus, the short-term responses to temperature are complex and also influenced by growth conditions, age, and species (von Caemmerer & Evans, 2015). Our current understanding of the mechanisms underlying g_m 's short-term response to temperature changes remains incomplete (Evans, 2021; Shrestha, Song, et al., 2019). Furthermore, this theoretical framework does not adequately address the sensitivities of g_m to variations in light intensity or CO_2 concentration.

5.4 | Structures across leaf profiles

The hypothesis that the observable fluctuations in g_m might not necessarily indicate variations in the leaf environment's diffusive resistances has been proposed. This hypothesis suggests that variations in light intensity in the leaf profile could lead to apparent changes in g_m , which has been subjected to a more in-depth theoretical analysis (Evans et al., 2009; Thérroux-Rancourt & Gilbert, 2017). Considering the diffusive resistances as constants, the perceived variability in g_m could result from the leaf's three-dimensional properties. The light absorption by chloroplasts, dependent on their position within the leaf's structure, can influence the CO_2 drawdown from the intercellular air space to the chloroplast stroma (c_i – c_c). This would cause different leaf layers to contribute different degrees to leaf-level photosynthesis, depending on the light intensity affecting the average g_m . However, this effect would be small if the profile of light absorption matches the profile of photosynthetic capacity (Evans et al., 2009), which has been shown to be the case (Evans & Vogelmann, 2003; Vogelmann & Evans, 2002). This hypothesis also does not provide a mechanistic explanation for the variability of g_m to CO_2 or temperature.

5.5 | Variable fluxes along the CO_2 diffusion pathway

Following Fick's First Law assumptions, Parkhurst (1994) pointed out some time ago that conductances become variable when there are CO_2 sources or sinks along the diffusion pathway, or in

other words when the CO_2 flux is not constant along the entire diffusion pathway from c_i to c_c . Tholen et al. (2012) later argued that the net flux of CO_2 along the part of the pathway across the cell wall and plasma membrane is equal to A , while it should be assumed equal to the Rubisco carboxylation rate (V_c) along the part crossing the chloroplast envelope and stroma. This difference in fluxes results from photorespiratory and respiratory CO_2 being released from the mitochondria and mixing with the incoming CO_2 somewhere inside the cytosol, affecting g_m by adding another source of CO_2 between c_i and c_c (Tholen et al., 2012). Yin and Struik (2017) later argued that some proportion of the (photo)respired CO_2 should enter the chloroplast directly due to the location of the mitochondria (usually adjacent to the chloroplast), so only part of this flux should mix with the incoming CO_2 in the cytosol.

Under both scenarios described above, g_m is expected to decrease to zero at the CO_2 compensation point (Busch, 2020; Tholen et al., 2012; Yin & Struik, 2017), making this a distinctive and testable feature. Recent work using a new isotope discrimination model has demonstrated this (Busch, Holloway-Phillips, et al., 2020), providing evidence that this mechanism plays some part in the short-term variability of g_m . However, it fails to accurately describe, e.g., the decrease of g_m towards high CO_2 concentrations, highlighting that our understanding of the environmental response of g_m is at the very least incomplete.

5.6 | Enzymatic involvement (CA)

Márquez et al. (2023) highlighted significant fluctuations in the CO_2 gradient from c_i to the area around the photosynthetically active mesophyll cells (c_w) in response to changes in atmospheric CO_2 levels. Moreover, the study observed that changes in c_a were accompanied by decreased apparent conductance to CO_2 in the mesophyll air space, as deduced from comparative analyses of g_m using c_i and c_w values. These phenomena challenge the explanation of simple diffusion from c_w to c_c , which theoretically should result in a negligible gradient between c_i and c_w due to the considerably lower diffusivity in liquid than in air—by three orders of magnitude—and it would be independent of any helper to diffusion through the membranes. This discrepancy suggests that the diffusion gradient is not merely a function of direct movement through the membranes or the impact of Rubisco activity, which, while decreasing c_c and raising the c_w – c_c gradient, would not alter the observed resistance between c_i and c_w .

Turning to enzymatic processes for an explanation, CA and the facilitated transfer process (Cowan, 1986; Enns, 1967) emerge as plausible candidates to explain these short-term responses of g_m (Momayyezi et al., 2020) and the apparent mesophyll air space conductance. CA interconverts dissolved CO_2 and bicarbonate, facilitating carbon transport, and bicarbonate is converted into CO_2 near Rubisco (Raven & Glidewell, 1981). It has been shown that plant growth at high

CO₂ concentrations presents lower CA activity and content than growth at ambient or low c_a , and that CA activity is decreased when CO₂ concentration is lower than the original growth conditions (Majeau & Coleman, 1996). On the other hand, when Rubisco activity is not high enough to decrease c_c significantly from c_w , the CA activity will not facilitate the movement of carbon, as the concentration of CO₂ in the chloroplast would already be high, as at high c_i .

Thus, the observed behaviour in the g_m short-term response to CO₂ is similar to that expected of the facilitated transfer process to changes in CO₂ concentration (see Enns, 1967). That is, the variation in the apparent air space conductance and the liquid volume conductance observed by Márquez et al. (2023) cannot be explained if the g_m variation occurs only in the cell membrane or by adding a source of CO₂ along the diffusion path, but could be explained by a facilitated transfer process in the liquid volume. However, direct evidence of measured c_i - c_w gradients along with measured CA activity is still pending in support of this hypothesis and has not been tested under other variable environmental conditions such as light intensity or temperature.

6 | MODELLING THE SHORT-TERM RESPONSE OF g_m

Due to the lack of a mechanistic explanation for the short-term responses of g_m , there are still difficulties in generating effective models of short-term responses of g_m to environmental changes (e.g., Thérroux-Rancourt & Gilbert, 2017; Tholen et al., 2012; Ubierna et al., 2019; Yin & Struik, 2009, 2017). The existing semi-mechanistic and empirical models have inherent limitations, resulting in an incomplete explanation of the observed variabilities of g_m in response to environmental changes and the underlying mechanisms. Notably, while empirical models have a role in predicting g_m under certain situations, they do not allow the results to be linked with mechanisms that regulate the variability of g_m .

The semi-mechanistic model presented by Tholen et al. (2012) proposes to split the mesophyll resistance into two components, r_{wp} (consisting of cell wall and plasma membrane resistances) and r_{ch} (consisting of chloroplast envelope and stroma resistances), resulting in an overall mesophyll conductance that varies apparently with the amount of CO₂ entering the pathway from (photo) respiration (Tholen et al., 2012). This approach is semi-mechanistic, as it relies on physical diffusion properties and recreates some observed phenomena, such as decreasing g_m with decreasing CO₂ concentrations and a g_m that tends towards zero when approaching the light or CO₂ compensation points (Busch, Holloway-Phillips, et al., 2020). However, it fails to correctly predict the drop in g_m observed at high CO₂ concentrations or towards low light intensities.

The model from Thérroux-Rancourt and Gilbert (2017) attributes the light distribution within the leaf and mesophyll

structures as the mechanism behind g_m variability at different light intensities. This approach suggests that at a low light intensity, the layers of the leaf mesophyll that are farther from the light source contribute less or do not contribute to the total photosynthesis, increasing c_c on those cells and generating an apparent decline of the total g_m . The theory seems conflicted with the evidence that light intensity and photosynthetic capacity distribute in similar proportion in the leaf profile regardless of the light intensity, as the c_c differences in the leaf profile would tend to be also proportional (Evans & Vogelmann, 2003; Vogelmann & Evans, 2002) and the same could be expected for g_m . Still, the model from Thérroux-Rancourt and Gilbert (2017) fits with the observed light response of g_m but does not explain other g_m variations observed in other environmental conditions such as CO₂ concentration and temperature.

The uncertainty about the mechanisms behind the short-term responses of g_m and the consequent lack of a mechanistic model able to explain g_m behaviour has brought large uncertainty from the physiology underneath g_m (Evans & von Caemmerer, 1996; Flexas et al., 2012), field photosynthesis measurements (Niinemets et al., 2009) to the global carbon balance projections scale (Knauer et al., 2019; Rogers et al., 2017). Overall, we still lack a reliable mechanistic model to predict the short-term responses of g_m to environmental changes.

7 | g_{sc} AS A PROXY FOR g_m

Given the lack of mechanistic models for g_m able to be accurately applied under a wide range of conditions, empirical ways to describe g_m may be the best choice for the time being. One such empirical relationship is using g_{sc} as a proxy for g_m . During growth, plants adapt their structures and resources to the environmental conditions of the growing site, generating what is usually referred to as long-term adaptation to environmental factors. There is evidence of long-term adaptation between maximum g_{sc} and g_m to the environmental conditions during growth (Flexas et al., 2013), such as light intensity (Xiong et al., 2018; Yamori et al., 2010), atmospheric CO₂ concentration (Mizokami, Sugiura, et al., 2019), water stress (Du et al., 2018; Galmés et al., 2013), and temperature (Warren, 2008a; Yamori et al., 2006).

Modelling stomatal conductance behaviour has been a long-standing challenge, taking different approaches and combinations of known correlated effects on stomatal response, such as light, drought and CO₂ concentration (Buckley, 2017). Many models have been suggested to predict stomatal conductance response to environmental conditions, such as those presented by Farquhar and Wong (1984), Ball et al. (1987), Medlyn et al. (2011), Potkay and Feng (2023), among others. Significant advances have been made in understanding stomatal behaviour, helping advance those models (Damour et al., 2010; Potkay & Feng, 2023). Thus, it is tempting to capitalise on this knowledge and the ease with which stomatal response can be measured and use them as a proxy for

g_m . Here, we explore possible relationships between the g_{sc} and g_m short-term responses and some correlations present in the literature.

Studies have found empirical correlations between the short-term responses of g_m and g_{sc} to light under stable gas exchange conditions in the form of g_{sc}/g_m ratio (e.g., Ma et al., 2021; Sun et al., 2023). In this correlation, both conductances increase when

the light intensity rises, following a similar pattern as that shown in Figure 2. Such findings invite speculation about potential coordination between these responses. However, it has been confirmed that there is a small variation of g_m during light induction (Sakoda et al., 2020), which suggests that the time response to adjust g_m by light variations must be significantly faster than g_{sc} response. Thus, the apparent coordination of

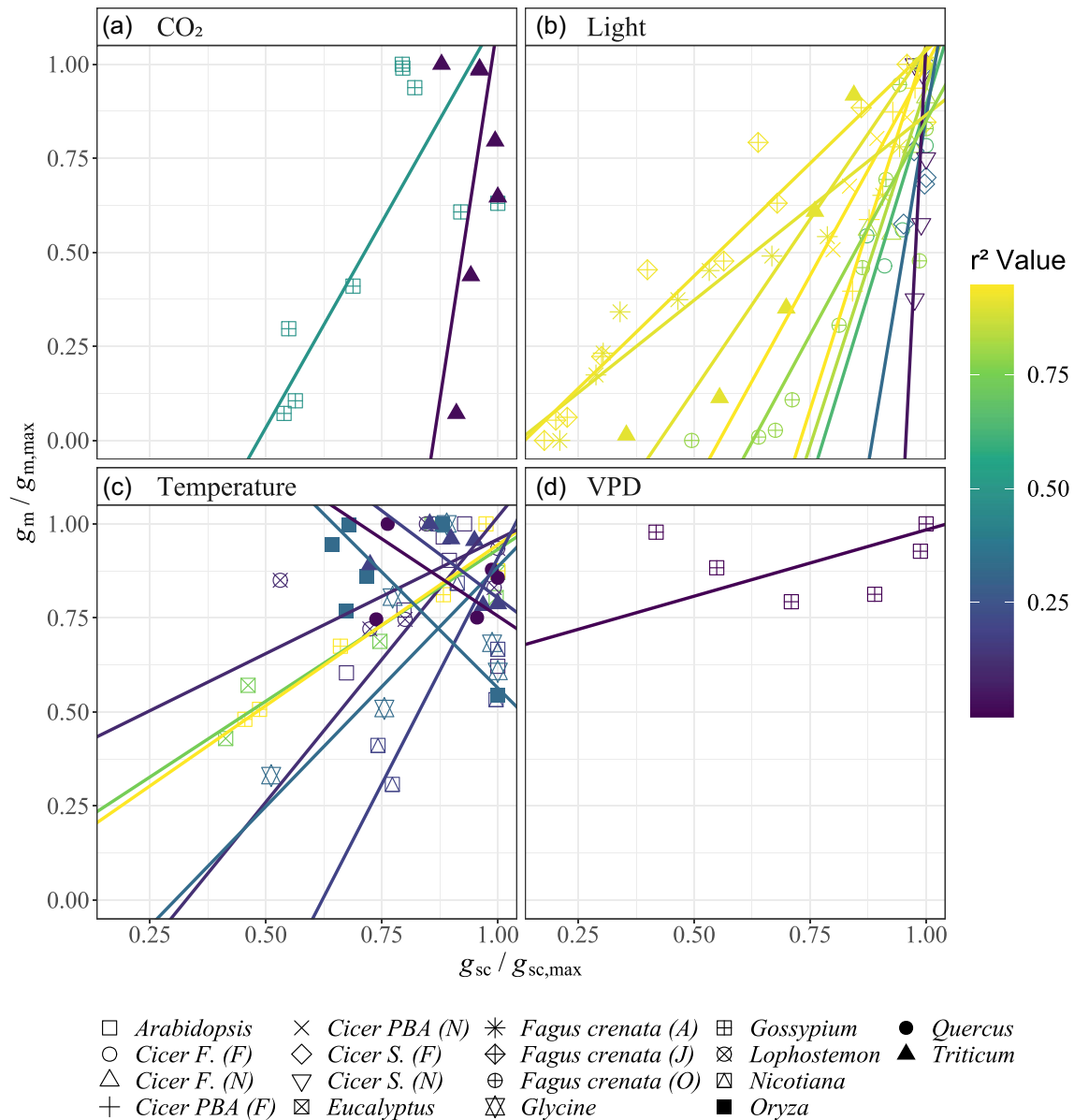


FIGURE 5 Relation between g_m and g_{sc} . Lines are the orthogonal regressions for each data set. Line colours represent the squared Pearson correlation coefficient (r^2) of each data set. The data on each panel is from the following sources and methods: (a) Busch, Holloway-Phillips, et al. (2020) used the isotope method in *Triticum aestivum* and Márquez et al. (2023) used the fluorescence method in *Gossypium hirsutum*; (b) Busch, Holloway-Phillips, et al. (2020) used the isotope method in *Triticum aestivum*, Shrestha, Buckley, et al. (2019) used the isotope method in *Cicer arietinum* and Hoshika et al. (2020) used the fluorescence method in *Fagus crenata*. *Cicer F, S and PBA* are genotypes Flip079C, Sonali and Pulse Breeding Australia, respectively; N and F in parenthesis stand for nodule N Rhizobium inoculated and fertilised, respectively (see Shrestha, Buckley, et al. (2019)). Letters in parenthesis next to *Fagus crenata* stand for the time of the measurements, A: August, J: June and O: October; (c) von Caemmerer and Evans (2015) used the isotope method in *Eucalyptus pauciflora*, *Quercus engelmannii*, *Lophostemon confertus*, *Nicotiana tabacum*, *Oryza sativa*, *Triticum aestivum*, *Gossypium hirsutum*, *Glycine max* and *Arabidopsis thaliana*. (d) Wong et al. (2022) used the isotope method in *Gossypium hirsutum*.

short-term responses to light intensity changes between g_{sc} and g_m is only present at steady-state. It can be seen in Figure 5b that the pattern between g_{sc} and g_m is not consistent among measurements either, supporting the idea that they are not coordinated. This suggests that the g_{sc} and g_m pattern sometimes observed is a consequence of the processes adjusting the light and CO₂ supply more than a coordinated response for increasing g_{sc} and g_m together. Therefore, such empirical approximation to obtain g_m under variable light conditions, while simple if no other method is available, can induce significant estimation errors.

Under natural conditions, c_a can be considered constant and short-term variations of g_m to CO₂ concentration are mostly in response to stomatal activity and associated changes in c_i . For instance, Th eroux-Rancourt et al. (2014) showed that g_m was responsive to c_i rather than to c_a . Experimentally varying c_a and c_i to trigger short-term responses presents characteristic behaviour patterns for g_{sc} and g_m . An increase in c_a and c_i above ambient induces a decrease in g_{sc} and g_m ; on the other hand, decreasing c_a and c_i causes g_{sc} and g_m to depart, as g_{sc} tends to increase and g_m tends to decrease. Thus, these variations are independent of the g_{sc} response (Mizokami, Noguchi, et al., 2019). Figure 5a shows that g_m does not follow g_{sc} , presenting an almost vertical orthogonal regression and indicating that g_m response to CO₂ changes is independent of g_{sc} .

It can be seen in Figure 5c that short-term responses of g_m to changes in temperature on different species showed no consistent trend between g_{sc} and g_m responses, where different species show different trajectories to the orthogonal regression. This suggests that there is no direct causal correlation between g_m and g_{sc} response to temperature, even though some lines seem to have a 1:1 relation. Other studies have also shown that variations in g_m in response to temperature shifts occur independently of g_{sc} (Huang et al., 2022; Li et al., 2020). Diao et al. (2024) provided further evidence of the complexity of the g_{sc} and g_m behaviour response to changes in leaf temperature, showing that in plants exposed to temperatures above the optimum for photosynthesis, g_{sc} tends to increase and g_m to decrease, even at constant VPD. The authors argue that this indicates a notable decoupling in the short-term responses of g_{sc} and g_m at higher temperatures, implying a shift in stomatal behaviour towards facilitating leaf cooling through increased transpiration at the expense of water use efficiency. Such decoupling also implies that factors other than CO₂ availability, potentially including temperature-sensitive enzymatic activities, inversely affect g_m . The findings by Diao et al. (2024) further support that any parallel trends observed during specific assessments of short-term response of g_m and g_{sc} are coincidental rather than indicative of mechanistic coordination.

The stomatal response to increases in VPD is well-documented and the decrease in g_{sc} is expected to impact the short-term response of g_m directly by decreasing c_i . However, it has been shown that VPD and water stress do not impact the

short-term response of g_m during the day (Stangl et al., 2019), which suggests that the short-term response of g_m is insensitive to changes in VPD or drought other than those carried by the stomata closure (see Wang et al., 2018; Warren, 2008b). Compelling evidence of g_m insensitivity to VPD changes was presented by Wong et al. (2022) (Figure 5d) by inducing unsaturation in the substomatal cavity and observing almost constant g_m while g_{sc} declined.

8 | CONCLUSIONS

There is enough evidence to argue that there is no direct relation between short-term responses to environmental changes of g_{sc} and g_m ; thus, it is misguided to use g_{sc} or g_{sc}/g_m ratio as a proxy to predict the g_m behaviour. Considering the variations found in g_m as a response to environmental changes, it is risky to assume constant g_m when evaluating photosynthetic variations to external inputs. While the mechanisms for the short-term response of g_m are not completely decrypted, most trends in g_m response to environmental changes seem to agree in the literature.

There is still conflicting evidence regarding the responses of g_m to drought and VPD, which may suggest that g_m respond differently to leaf water potential and VPD stress. More research is needed regarding water stress and short-term g_m responses. In this regard, it is crucial to consider the complications that arise by measuring g_m under water stress where stomatal conductance tends to be low, affecting the reliability of the measurements if small fluxes, unsaturation and patchiness are not attended.

Online isotopic discrimination and chlorophyll fluorescence methods to estimate variability in g_m generally seem to agree with the short-term trends of g_m at different conditions. Still, some research has shown mixed results concerning the absolute values obtained with each method. Further evaluation of the robustness of the estimation with both methods in comparison is necessary, along with an investigation of the source of the differences found sometimes when used together. Transversal agreement exists on the major components that play a role in g_m , and even though it is still challenging to evaluate them independently, progress has been made with some methods to separate mesophyll air space, liquid volume and cell wall.

Pressing issues are to explore the mechanism behind the short-term responses of g_m and develop reliable models to predict such responses. There is a set of theories to be explored further to unravel what physiological mechanisms are involved in the short-term response of g_m to environmental changes. Exploring the underlying physiological processes is essential for advancing mechanistic models that bridge laboratory insights with broader ecological implications.

Box 1 summarises pressing issues, key challenges and cautions addressed in this review.

BOX 1: Pressing issues, key challenges and best practice.**Pressing Issues**

- **Implement the most updated model when using the isotopic method to measure g_m .** Failing to employ the most current model limits the validity and interpretability of results, especially under conditions where the assimilation rate is decreased due to changed environmental factors.
- **Implement calibration in chlorophyll fluorescence methods for g_m estimation.** Failing to calibrate the chlorophyll fluorescence method limits its validity and the interpretability of results, especially under conditions where common assumptions for J estimation are known to fail or possibly fail.
- **Explore the mechanism behind short-term g_m responses.** Various mechanisms have been suggested; however, direct proof for these has not yet been established. Therefore, further efforts should be concentrated on this matter.

Key Challenges

- **Investigate the accuracy of methods to approximate the 'true' value of g_m .** Besides employing the most current approaches for the isotope and fluorescence methods, further assessments of the values obtained with each method are needed.
- **Develop reliable and robust models of g_m for short-term response to environmental changes.** Current models fail to reliably describe g_m behaviour under various environmental changes, forcing reliance on direct measurements for detailed analysis. Efforts are needed to create a robust model linking laboratory insights with broader ecological and production-related scenarios.

Cautions and best practices

- **Importance of correct c_i estimation in g_m calculation, especially under water stress.** Accounting for cuticular conductance, the unsaturation of the substomatal cavity, and patchiness during experiments involving drought or vapour pressure deficit (VPD) stress is vital.
- **Caution against potential errors in using g_{sc} as a proxy for g_m .** Evidence presented here indicates that using g_{sc} as a proxy for g_m is inadvisable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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REFERENCES

- Ball, J.T., Woodrow, I.E. & Berry, J.A. (1987) A model predicting stomatal conductance and its contribution to the control of photosynthesis under different environmental conditions. In: Biggins, J., (Ed.) *Progress in Photosynthesis Research: Volume 4 Proceedings of the Vllth International Congress on Photosynthesis Providence, Rhode Island, USA, August 10–15, 1986*. Dordrecht: Springer Netherlands. pp. 221–224.
- Bloom, A.J., Caldwell, R.M., Finazzo, J., Warner, R.L. & Weissbart, J. (1989) Oxygen and carbon dioxide fluxes from barley shoots depend on nitrate assimilation. *Plant Physiology*, 91(1), 352–356. Available from: <https://doi.org/10.1104/pp.91.1.352>
- Boyer, J.S. (2015) Impact of cuticle on calculations of the CO_2 concentration inside leaves. *Planta*, 242, 1405–1412.
- Boyer, J.S., Wong, S.C. & Farquhar, G.D. (1997) CO_2 and water vapor exchange across leaf cuticle (Epidermis) at various water potentials. *Plant Physiology*, 114, 185–191.
- Buckley, T.N. (2005) The control of stomata by water balance. *New Phytologist*, 168(2), 275–292. Available from: <https://doi.org/10.1111/j.1469-8137.2005.01543.x>
- Buckley, T.N. (2017) Modeling stomatal conductance. *Plant Physiology*, 174(2), 572–582. Available from: <https://doi.org/10.1104/pp.16.01772>
- Buckley, T.N. (2023) Is carbon, not water, the resource that limits stomatal opening? *New Phytologist*, 238(2), 457–460. Available from: <https://doi.org/10.1111/nph.18818>
- Buckley, T.N. & Mott, K.A. (2013) Modelling stomatal conductance in response to environmental factors. *Plant, Cell & Environment*, 36(9), 1691–1699. Available from: <https://doi.org/10.1111/pce.12140>
- Buckley, T.N. & Warren, C.R. (2014) The role of mesophyll conductance in the economics of nitrogen and water use in photosynthesis. *Photosynthesis Research*, 119(1), 77–88. Available from: <https://doi.org/10.1007/s11120-013-9825-2>
- Busch, F.A. (2020) Photorespiration in the context of Rubisco biochemistry, CO_2 diffusion and metabolism. *The Plant Journal*, 101(4), 919–939. Available from: <https://doi.org/10.1111/tbj.14674>
- Busch, F.A., Holloway-Phillips, M., Stuart-Williams, H. & Farquhar, G.D. (2020) Revisiting carbon isotope discrimination in C_3 plants shows respiration rules when photosynthesis is low. *Nature Plants*, 6(3), 245–258. Available from: <https://doi.org/10.1038/s41477-020-0606-6>
- Busch, F.A., Sage, R.F. & Farquhar, G.D. (2018) Plants increase CO_2 uptake by assimilating nitrogen via the photorespiratory pathway. *Nature Plants*, 4(1), 46–54. Available from: <https://doi.org/10.1038/s41477-017-0065-x>

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- von Caemmerer, S. & Evans, J.R. (2015) Temperature responses of mesophyll conductance differ greatly between species. *Plant, Cell & Environment*, 38(4), 629–637. Available from: <https://doi.org/10.1111/pce.12449>
- Cano, F.J., Sánchez-Gómez, D., Rodríguez-Calcerrada, J., Warren, C.R., Gil, L. & Aranda, I. (2013) Effects of drought on mesophyll conductance and photosynthetic limitations at different tree canopy layers. *Plant, Cell & Environment*, 36(11), 1961–1980. Available from: <https://doi.org/10.1111/pce.12103>
- Carriqui, M., Douthe, C., Molins, A. & Flexas, J. (2019) Leaf anatomy does not explain apparent short-term responses of mesophyll conductance to light and CO₂ in tobacco. *Physiologia Plantarum*, 165(3), 604–618. Available from: <https://doi.org/10.1111/ppl.12755>
- Cernusak, L.A., Ubierna, N., Jenkins, M.W., Garrity, S.R., Rahn, T., Powers, H.H. et al. (2018) Unsaturation of vapour pressure inside leaves of two conifer species. *Scientific Reports*, 8(7667), 7667.
- Clarke, V.C., De Rosa, A., Massey, B., George, A.M., Evans, J.R., von Caemmerer, S. et al. (2022) Mesophyll conductance is unaffected by expression of arabidopsis *PIP1* aquaporins in the plasmalemma of *Nicotiana*. *Journal of Experimental Botany*, 73(11), 3625–3636. Available from: <https://doi.org/10.1093/jxb/erac065>
- Cowan, I.R. (1986) *Economics of carbon fixation in higher plants*. Cambridge
- Cowan, I.R. (1978) Stomatal behaviour and environment. In: Preston, R.D. & Woolhouse, H.W., (Eds.) *Advances in Botanical Research*, Vol. 4. Academic Press. pp. 117–228.
- Crous, K.Y., Quentin, A.G., Lin, Y.-S., Medlyn, B.E., Williams, D.G., Barton, C.V.M. et al. (2013) Photosynthesis of temperate *Eucalyptus globulus* trees outside their native range has limited adjustment to elevated CO₂ and climate warming. *Global Change Biology*, 19(12), 3790–3807. Available from: <https://doi.org/10.1111/gcb.12314>
- Damour, G., Simonneau, T., Cochard, H. & Urban, L. (2010) An overview of models of stomatal conductance at the leaf level. *Plant, Cell & Environment*, 33(9), 1419–1438. Available from: <https://doi.org/10.1111/j.1365-3040.2010.02181.x>
- Diao, H., Cernusak, L.A., Saurer, M., Gessler, A., Siegwolf, R.T.W. & Lehmann, M.M. (2024) Uncoupling of stomatal conductance and photosynthesis at high temperatures: mechanistic insights from online stable isotope techniques. *New Phytologist*, 241(6), 2366–2378. Available from: <https://doi.org/10.1111/nph.19558>
- Du, Q., Xing, G., Jiao, X., Song, X. & Li, J. (2018) Stomatal responses to long-term high vapor pressure deficits mediated most limitation of photosynthesis in tomatoes. *Acta Physiologiae Plantarum*, 40(8), 149. Available from: <https://doi.org/10.1007/s11738-018-2723-7>
- Ellsworth, P.V., Ellsworth, P.Z., Koteyeva, N.K. & Cousins, A.B. (2018) Cell wall properties in *Oryza sativa* influence mesophyll CO₂ conductance. *New Phytologist*, 219(1), 66–76. Available from: <https://doi.org/10.1111/nph.15173>
- Enns, T. (1967) Facilitation by carbonic anhydrase of carbon dioxide transport. *Science*, 155(3758), 44–47. Available from: <https://doi.org/10.1126/science.155.3758.44>
- Ermakova, M., Osborn, H., Groszmann, M., Bala, S., Bowerman, A., McGaughey, S. et al. (2021) Expression of a CO₂-permeable aquaporin enhances mesophyll conductance in the C₄ species *Setaria viridis*. *eLife*, 10, e70095. Available from: <https://doi.org/10.7554/eLife.70095>
- Evans, J., Sharkey, T., Berry, J. & Farquhar, G. (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO₂ diffusion in leaves of higher plants. *Functional Plant Biology*, 13(2), 281–292. Available from: <https://doi.org/10.1071/PP9860281>
- Evans, J.R. (2021) Mesophyll conductance: walls, membranes and spatial complexity. *New Phytologist*, 229(4), 1864–1876. Available from: <https://doi.org/10.1111/nph.16968>
- Evans, J.R. & von Caemmerer, S. (1996) Carbon dioxide diffusion inside leaves. *Plant Physiology*, 110(2), 339–346. Available from: <https://doi.org/10.1104/pp.110.2.339>
- Evans, J.R. & von Caemmerer, S. (2013) Temperature response of carbon isotope discrimination and mesophyll conductance in tobacco. *Plant, Cell & Environment*, 36(4), 745–756. Available from: <https://doi.org/10.1111/j.1365-3040.2012.02591.x>
- Evans, J.R., von Caemmerer, S., Setchell, B.A. & Hudson, G.S. (1994) The relationship between CO₂ transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. *Australian Journal of Plant Physiology*, 21(4), 475–495.
- Evans, J.R., Kaldenhoff, R., Genty, B. & Terashima, I. (2009) Resistances along the CO₂ diffusion pathway inside leaves. *Journal of Experimental Botany*, 60(8), 2235–2248. Available from: <https://doi.org/10.1093/jxb/erp117>
- Evans, J.R. & Poorter, H. (2001) Photosynthetic acclimation of plants to growth irradiance: the relative importance of specific leaf area and nitrogen partitioning in maximizing carbon gain. *Plant, Cell & Environment*, 24(8), 755–767. Available from: <https://doi.org/10.1046/j.1365-3040.2001.00724.x>
- Evans, J.R. & Vogelmann, T.C. (2003) Profiles of ¹⁴C fixation through spinach leaves in relation to light absorption and photosynthetic capacity. *Plant, Cell & Environment*, 26(4), 547–560. Available from: <https://doi.org/10.1046/j.1365-3040.2003.00985.x>
- Farquhar, G., O'Leary, M. & Berry, J. (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Functional Plant Biology*, 9(2), 121–137. Available from: <https://doi.org/10.1071/PP9820121>
- Farquhar, G. & Wong, S. (1984) An empirical model of stomatal conductance. *Functional Plant Biology*, 11(3), 191–210. Available from: <https://doi.org/10.1071/PP9840191>
- Farquhar, G.D., von Caemmerer, S. & Berry, J.A. (1980) A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species. *Planta*, 149(1), 78–90. Available from: <https://doi.org/10.1007/BF00386231>
- Farquhar, G.D. & Cernusak, L.A. (2012) Ternary effects on the gas exchange of isotopologues of carbon dioxide. *Plant, Cell & Environment*, 35(7), 1221–1231. Available from: <https://doi.org/10.1111/j.1365-3040.2012.02484.x>
- Farquhar, G.D. & Sharkey, T.D. (1982) Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology*, 33, 317–345.
- Fick, A. (1855) Ueber diffusion. *Annalen der Physik*, 170(1), 59–86. Available from: <https://doi.org/10.1002/andp.18551700105>
- Fick, A. (1995) On liquid diffusion. *Journal of Membrane Science*, 100(1), 33–38. Available from: [https://doi.org/10.1016/0376-7388\(94\)00230-V](https://doi.org/10.1016/0376-7388(94)00230-V)
- Flexas, J., Barbour, M.M., Brendel, O., Cabrera, H.M., Carriqui, M., Díaz-Espejo, A. et al. (2012) Mesophyll diffusion conductance to CO₂: an unappreciated central player in photosynthesis. *Plant Science*, 193–194, 70–84. Available from: <https://doi.org/10.1016/j.plantsci.2012.05.009>
- Flexas, J., Bota, J., Escalona, J.M., Sampol, B. & Medrano, H. (2002) Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. *Functional Plant Biology*, 29(4), 461–471. Available from: <https://doi.org/10.1071/PP01119>
- Flexas, J. & Diaz-Espejo, A. (2015) Interspecific differences in temperature response of mesophyll conductance: food for thought on its origin and regulation. *Plant, Cell & Environment*, 38(4), 625–628. Available from: <https://doi.org/10.1111/pce.12476>
- Flexas, J., Diaz-Espejo, A., Galmés, J., Kaldenhoff, R., Medrano, H. & Ribas-Carbo, M. (2007) Rapid variations of mesophyll conductance in response to changes in CO₂ concentration around leaves. *Plant, Cell & Environment*, 30(10), 1284–1298. Available from: <https://doi.org/10.1111/j.1365-3040.2007.01700.x>

- Flexas, J., Ribas-Carbó, M., Diaz-Espejo, A., Galmés, J. & Medrano, H. (2008) Mesophyll conductance to CO_2 : current knowledge and future prospects. *Plant, Cell & Environment*, 31(5), 602–621. Available from: <https://doi.org/10.1111/j.1365-3040.2007.01757.x>
- Flexas, J., Ribas-Carbó, M., Hanson, D.T., Bota, J., Otto, B., Cifre, J. et al. (2006) Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 in vivo. *The Plant Journal*, 48(3), 427–439. Available from: <https://doi.org/10.1111/j.1365-313X.2006.02879.x>
- Flexas, J., Scoffoni, C., Gago, J. & Sack, L. (2013) Leaf mesophyll conductance and leaf hydraulic conductance: an introduction to their measurement and coordination. *Journal of Experimental Botany*, 64(13), 3965–3981. Available from: <https://doi.org/10.1093/jxb/ert319>
- Franck, F., Juneau, P. & Popovic, R. (2002) Resolution of the photosystem I and photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1556(2), 239–246. Available from: [https://doi.org/10.1016/S0005-2728\(02\)00366-3](https://doi.org/10.1016/S0005-2728(02)00366-3)
- Gaastera, P. (1959) *Photosynthesis of crop plants as influenced by light, carbon dioxide, temperature, and stomatal diffusion resistance*. Wageningen, Netherlands: Meded. Landbouwhogeschool, Wageningen.
- Gago, J., Daloso, D.M., Figueroa, C.M., Flexas, J., Fernie, A.R. & Nikolowski, Z. (2016) Relationships of leaf net photosynthesis, stomatal conductance, and mesophyll conductance to primary metabolism: a multispecies meta-analysis approach. *Plant Physiology*, 171(1), 265–279. Available from: <https://doi.org/10.1104/pp.15.01660>
- Galmés, J., Ochogavía, J.M., Gago, J., Roldán, E.J., Cifre, J. & Conesa, M.À. (2013) Leaf responses to drought stress in Mediterranean accessions of *Solanum lycopersicum*: anatomical adaptations in relation to gas exchange parameters. *Plant, Cell & Environment*, 36(5), 920–935. Available from: <https://doi.org/10.1111/pce.12022>
- Gauthier, P.P.G., Battle, M.O., Griffin, K.L. & Bender, M.L. (2018) Measurement of gross photosynthesis, respiration in the light, and mesophyll conductance using $H_2^{18}O$ labeling. *Plant Physiology*, 177(1), 62–74. Available from: <https://doi.org/10.1104/pp.16.00741>
- Gong, X.Y., Ma, W.T., Yu, Y.Z., Fang, K., Yang, Y., Tcherkez, G. et al. (2022) Overestimated gains in water-use efficiency by global forests. *Global Change Biology*, 28(16), 4923–4934. Available from: <https://doi.org/10.1111/gcb.16221>
- Gu, L. & Sun, Y. (2014) Artefactual responses of mesophyll conductance to CO_2 and irradiance estimated with the variable J and online isotope discrimination methods. *Plant, Cell & Environment*, 37(5), 1231–1249. Available from: <https://doi.org/10.1111/pce.12232>
- Harley, P.C., Loreto, F., Di Marco, G. & Sharkey, T.D. (1992) Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2 . *Plant Physiology*, 98(4), 1429–1436. Available from: <https://doi.org/10.1104/pp.98.4.1429>
- Hassiotou, F., Ludwig, M., Renton, M., Veneklaas, E.J. & Evans, J.R. (2009) Influence of leaf dry mass per area, CO_2 , and irradiance on mesophyll conductance in sclerophylls. *Journal of Experimental Botany*, 60(8), 2303–2314. Available from: <https://doi.org/10.1093/jxb/erp021>
- Heckwolf, M., Pater, D., Hanson, D.T. & Kaldenhoff, R. (2011) The *Arabidopsis thaliana* aquaporin AtPIP1;2 is a physiologically relevant CO_2 transport facilitator. *The Plant Journal*, 67(5), 795–804. Available from: <https://doi.org/10.1111/j.1365-313X.2011.04634.x>
- Hermida-Carrera, C., Kapralov, M.V. & Galmés, J. (2016) Rubisco catalytic properties and temperature response in crops. *Plant Physiology*, 171(4), 2549–2561. Available from: <https://doi.org/10.1104/pp.16.01846>
- Holloway-Phillips, M., Cernusak, L.A., Stuart-Williams, H., Ubierna, N. & Farquhar, G.D. (2019) Two-Source $\delta^{18}O$ method to validate the $CO^{18}O$ -photosynthetic discrimination model: implications for mesophyll conductance. *Plant Physiology*, 181(3), 1175–1190. Available from: <https://doi.org/10.1104/pp.19.00633>
- Hoshika, Y., Haworth, M., Watanabe, M. & Koike, T. (2020) Interactive effect of leaf age and ozone on mesophyll conductance in Siebold's beech. *Physiologia Plantarum*, 170(2), 172–186. Available from: <https://doi.org/10.1111/ppi.13121>
- Hu, H., Rappel, W.-J., Occhipinti, R., Ries, A., Böhmer, M., You, L. et al. (2015) Distinct cellular locations of carbonic anhydrases mediate carbon dioxide control of stomatal movements. *Plant Physiology*, 169(2), 1168–1178. Available from: <https://doi.org/10.1104/pp.15.00646>
- Huang, G., Zhang, Q., Yang, Y., Shu, Y., Ren, X., Peng, S. et al. (2022) Interspecific variation in the temperature response of mesophyll conductance is related to leaf anatomy. *The Plant Journal*, 112(1), 221–234. Available from: <https://doi.org/10.1111/tpj.15942>
- Israel, D., Khan, S., Warren, C.R., Zwiazek, J.J. & Robson, T.M. (2021) The contribution of PIP2-type aquaporins to photosynthetic response to increased vapour pressure deficit. *Journal of Experimental Botany*, 72(13), 5066–5078. Available from: <https://doi.org/10.1093/jxb/erab187>
- Kai, L. & Kaldenhoff, R. (2014) A refined model of water and CO_2 membrane diffusion: effects and contribution of sterols and proteins. *Scientific Reports*, 4(1), 6665. Available from: <https://doi.org/10.1038/srep06665>
- Knauer, J., Cuntz, M., Evans, J.R., Niinemets, Ü., Tosens, T., Veromann-Jürgenson, L.-L. et al. (2022) Contrasting anatomical and biochemical controls on mesophyll conductance across plant functional types. *New Phytologist*, 236(2), 357–368. Available from: <https://doi.org/10.1111/nph.18363>
- Knauer, J., Zaehle, S., De Kauwe, M.G., Bahar, N.H.A., Evans, J.R., Medlyn, B.E. et al. (2019) Effects of mesophyll conductance on vegetation responses to elevated CO_2 concentrations in a land surface model. *Global Change Biology*, 25(5), 1820–1838. Available from: <https://doi.org/10.1111/gcb.14604>
- Kromdijk, J., Glowacka, K. & Long, S.P. (2019) Photosynthetic efficiency and mesophyll conductance are unaffected in *Arabidopsis thaliana* aquaporin knock-out lines. *Journal of Experimental Botany*, 71(1), 318–329. Available from: <https://doi.org/10.1093/jxb/erz442>
- Laisk, A. (1983) Calculation of leaf photosynthetic parameters considering the statistical distribution of stomatal apertures. *Journal of Experimental Botany*, 34(12), 1627–1635. <https://doi.org/10.1093/jxb/34.12.1627>
- Laisk, A., Oja, V., Rasulov, B., Rämama, H., Eichelmann, H., Kasparova, I. et al. (2002) A computer-operated routine of gas exchange and optical measurements to diagnose photosynthetic apparatus in leaves. *Plant, Cell & Environment*, 25(7), 923–943. Available from: <https://doi.org/10.1046/j.1365-3040.2002.00873.x>
- Lawson, T. & Matthews, J. (2020) Guard cell metabolism and stomatal function. *Annual review of plant biology*, 71(1), 273–302. Available from: <https://doi.org/10.1146/annurev-arplant-050718-100251>
- Li, Y., Song, X., Li, S., Salter, W.T. & Barbour, M.M. (2020) The role of leaf water potential in the temperature response of mesophyll conductance. *New Phytologist*, 225(3), 1193–1205. Available from: <https://doi.org/10.1111/nph.16214>
- Loucos, K.E., Simonin, K.A. & Barbour, M.M. (2017) Leaf hydraulic conductance and mesophyll conductance are not closely related within a single species. *Plant, Cell & Environment*, 40(2), 203–215. Available from: <https://doi.org/10.1111/pce.12865>
- Ma, W.T., Tcherkez, G., Wang, X.M., Schäufele, R., Schnyder, H., Yang, Y. et al. (2021) Accounting for mesophyll conductance substantially improves ^{13}C -based estimates of intrinsic water-use efficiency. *New*

- Phytologist*, 229(3), 1326–1338. Available from: <https://doi.org/10.1111/nph.16958>
- Majeau, N. & Coleman, J.R. (1996) Effect of CO₂ concentration on carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase expression in pea. *Plant Physiology*, 112(2), 569–574. Available from: <https://doi.org/10.1104/pp.112.2.569>
- Di Marco, G., Manes, F., Tricoli, D. & Vitale, E. (1990) Fluorescence parameters measured concurrently with net photosynthesis to investigate chloroplastic CO₂ concentration in leaves of *Quercus ilex* L. *Journal of Plant Physiology*, 136(5), 538–543. Available from: [https://doi.org/10.1016/S0176-1617\(11\)80210-5](https://doi.org/10.1016/S0176-1617(11)80210-5)
- Márquez, D.A., Stuart-Williams, H. & Farquhar, G.D. (2021) An improved theory for calculating leaf gas exchange more precisely accounting for small fluxes. *Nature Plants*, 7(3), 317–326. Available from: <https://doi.org/10.1038/s41477-021-00861-w>
- Márquez, D.A., Stuart-Williams, H., Cernusak, L.A. & Farquhar, G.D. (2023) Assessing the CO₂ concentration at the surface of photosynthetic mesophyll cells. *New Phytologist*, 238(4), 1446–1460. Available from: <https://doi.org/10.1111/nph.18784>
- Márquez, D.A., Stuart-Williams, H., Farquhar, G.D. & Busch, F.A. (2021) Cuticular conductance of adaxial and abaxial leaf surfaces and its relation to minimum leaf surface conductance. *New Phytologist*, 233(1), 156–168. Available from: <https://doi.org/10.1111/nph.17588>
- Medlyn, B.E., Duursma, R.A., Eamus, D., Ellsworth, D.S., Prentice, I.C., Barton, C.V.M. et al. (2011) Reconciling the optimal and empirical approaches to modelling stomatal conductance. *Global Change Biology*, 17(6), 2134–2144. Available from: <https://doi.org/10.1111/j.1365-2486.2010.02375.x>
- Mizokami, Y., Noguchi, K., Kojima, M., Sakakibara, H. & Terashima, I. (2015) Mesophyll conductance decreases in the wild type but not in an ABA-deficient mutant (*aba1*) of *Nicotiana glauca* under drought conditions. *Plant, Cell & Environment*, 38(3), 388–398. Available from: <https://doi.org/10.1111/pce.12394>
- Mizokami, Y., Noguchi, K., Kojima, M., Sakakibara, H. & Terashima, I. (2019) Effects of instantaneous and growth CO₂ levels and abscisic acid on stomatal and mesophyll conductances. *Plant, Cell & Environment*, 42(4), 1257–1269. Available from: <https://doi.org/10.1111/pce.13484>
- Mizokami, Y., Sugiura, D., Watanabe, C.K.A., Betsuyaku, E., Inada, N. & Terashima, I. (2019) Elevated CO₂-induced changes in mesophyll conductance and anatomical traits in wild type and carbohydrate-metabolism mutants of *Arabidopsis*. *Journal of Experimental Botany*, 70(18), 4807–4818. Available from: <https://doi.org/10.1093/jxb/erz208>
- Momayyezi, M., McKown, A.D., Bell, S.C.S. & Guy, R.D. (2020) Emerging roles for carbonic anhydrase in mesophyll conductance and photosynthesis. *The Plant Journal*, 101(4), 831–844. Available from: <https://doi.org/10.1111/tpj.14638>
- Mott, K.A. & Buckley, T.N. (2000) Patchy stomatal conductance: emergent collective behaviour of stomata. *Trends in Plant Science*, 5(6), 258–262. Available from: [https://doi.org/10.1016/S1360-1385\(00\)01648-4](https://doi.org/10.1016/S1360-1385(00)01648-4)
- Murata, Y., Mori, I.C. & Munemasa, S. (2015) Diverse stomatal signaling and the signal integration mechanism. *Annual Review of Plant Biology*, 66(1), 369–392. Available from: <https://doi.org/10.1146/annurev-arplant-043014-114707>
- Niinemets, Ü., Diaz-Espejo, A., Flexas, J., Galmés, J. & Warren, C.R. (2009) Importance of mesophyll diffusion conductance in estimation of plant photosynthesis in the field. *Journal of Experimental Botany*, 60(8), 2271–2282. Available from: <https://doi.org/10.1093/jxb/erp063>
- Ogé, J., Wingate, L. & Genty, B. (2018) Estimating mesophyll conductance from measurements of C₁₈O photosynthetic discrimination and carbonic anhydrase activity. *Plant Physiology*, 178(2), 728–752. Available from: <https://doi.org/10.1104/pp.17.01031>
- Olšovská, K., Kovar, M., Brestic, M., Zivcak, M., Slamka, P. & Shao, H.B. (2016) Genotypically identifying wheat mesophyll conductance regulation under progressive drought stress. *Frontiers in Plant Science*, 7, 1111. Available from: <https://doi.org/10.3389/fpls.2016.01111>
- Orr, D., Alcántara, A., Kapralov, M.V., Andralojc, J., Carmo-Silva, E. & Parry, M.A.J. (2016) Surveying Rubisco diversity and temperature response to improve crop photosynthetic efficiency. *Plant Physiology*, 172, 707–717. Available from: <https://doi.org/10.1104/pp.16.00750>
- Parkhurst, D.F. (1994) Diffusion of CO₂ and other gases inside leaves. *New Phytologist*, 126(3), 449–479.
- Pons, T.L., Flexas, J., von Caemmerer, S., Evans, J.R., Genty, B., Ribas-Carbo, M. et al. (2009) Estimating mesophyll conductance to CO₂: methodology, potential errors, and recommendations. *Journal of Experimental Botany*, 60(8), 2217–2234. Available from: <https://doi.org/10.1093/jxb/erp081>
- Potkay, A. & Feng, X. (2023) Do stomata optimize turgor-driven growth? A new framework for integrating stomata response with whole-plant hydraulics and carbon balance. *New Phytologist*, 238(2), 506–528. Available from: <https://doi.org/10.1111/nph.18620>
- van der Putten, P.E.L., Yin, X. & Struik, P.C. (2018) Calibration matters: on the procedure of using the chlorophyll fluorescence method to estimate mesophyll conductance. *Journal of Plant Physiology*, 220, 167–172. Available from: <https://doi.org/10.1016/j.jplph.2017.11.009>
- Qiu, C., Ethier, G., Pepin, S., Dubé, P., Desjardins, Y. & Gosselin, A. (2017) Persistent negative temperature response of mesophyll conductance in red raspberry (*Rubus idaeus* L.) leaves under both high and low vapour pressure deficits: a role for abscisic acid? *Plant, Cell & Environment*, 40(9), 1940–1959. Available from: <https://doi.org/10.1111/pce.12997>
- Raven, J.A. & Glidewell, S.M. (1981) Processes limiting photosynthetic conductance. In: Johnson, C.B., (Ed.) *Physiological processes limiting plant productivity*. London: Butterworth. pp. 109–136.
- Rockwell, F.E., Holbrook, N.M., Jain, P., Huber, A.E., Sen, S. & Strock, A.D. (2022) Extreme undersaturation in the intercellular airspace of leaves: a failure of Gaastra or Ohm? *Annals of Botany*, 130(3), 301–316. Available from: <https://doi.org/10.1093/aob/mcac094>
- Rogers, A., Medlyn, B.E., Dukes, J.S., Bonan, G., von Caemmerer, S., Dietze, M.C. et al. (2017) A roadmap for improving the representation of photosynthesis in Earth system models. *New Phytologist*, 213(1), 22–42. Available from: <https://doi.org/10.1111/nph.14283>
- Roig-Oliver, M., Fullana-Pericàs, M., Bota, J. & Flexas, J. (2023) Corrigendum for: genotype-dependent changes of cell wall composition influence physiological traits of a long and a non-long shelf-life tomato genotypes under distinct water regimes. *The Plant Journal*, 114(3), 719–721. Available from: <https://doi.org/10.1111/tpj.16187>
- Sakoda, K., Yamori, W., Groszmann, M. & Evans, J.R. (2020) Stomatal, mesophyll conductance, and biochemical limitations to photosynthesis during induction. *Plant Physiology*, 185(1), 146–160. Available from: <https://doi.org/10.1093/plphys/kiaa011>
- Sharkey, T.D., Bernacchi, C.J., Farquhar, G.D. & Singaas, E.L. (2007) Fitting photosynthetic carbon dioxide response curves for C₃ leaves. *Plant, Cell & Environment*, 30(9), 1035–1040. Available from: <https://doi.org/10.1111/j.1365-3040.2007.01710.x>
- Shrestha, A., Buckley, T.N., Lockhart, E.L. & Barbour, M.M. (2019) The response of mesophyll conductance to short- and long-term environmental conditions in chickpea genotypes. *AoB Plants*, 11(1), pty073. Available from: <https://doi.org/10.1093/aobpla/pty073>

- Shrestha, A., Song, X. & Barbour, M.M. (2019) The temperature response of mesophyll conductance, and its component conductances, varies between species and genotypes. *Photosynthesis Research*, 141(1), 65–82. Available from: <https://doi.org/10.1007/s11120-019-00622-z>
- Stangl, Z.R., Tarvainen, L., Wallin, G., Ubierna, N., Råntfors, M. & Marshall, J.D. (2019) Diurnal variation in mesophyll conductance and its influence on modelled water-use efficiency in a mature boreal *Pinus sylvestris* stand. *Photosynthesis Research*, 141(1), 53–63. Available from: <https://doi.org/10.1007/s11120-019-00645-6>
- Sugiura, D., Terashima, I. & Evans, J.R. (2020) A decrease in mesophyll conductance by cell-wall thickening contributes to photosynthetic downregulation. *Plant Physiology*, 183(4), 1600–1611. Available from: <https://doi.org/10.1104/pp.20.00328>
- Sun, Y.R., Ma, W.T., Xu, Y.N., Wang, X., Li, L., Tcherkez, G. et al. (2023) Short- and long-term responses of leaf day respiration to elevated atmospheric CO_2 . *Plant Physiology*, 191(4), 2204–2217. Available from: <https://doi.org/10.1093/plphys/kiac582>
- Tazoe, Y., von Caemmerer, S., Estavillo, G.M. & Evans, J.R. (2011) Using tunable diode laser spectroscopy to measure carbon isotope discrimination and mesophyll conductance to CO_2 diffusion dynamically at different CO_2 concentrations. *Plant, Cell & Environment*, 34(4), 580–591. Available from: <https://doi.org/10.1111/j.1365-3040.2010.02264.x>
- Terashima, I., Hanba, Y.T., Tazoe, Y., Vyas, P. & Yano, S. (2006) Irradiance and phenotype: comparative eco-development of sun and shade leaves in relation to photosynthetic CO_2 diffusion. *Journal of Experimental Botany*, 57(2), 343–354. Available from: <https://doi.org/10.1093/jxb/erj014>
- Terashima, I., Wong, S.-C., Osmond, C.B. & Farquhar, G.D. (1988) Characterisation of non-uniform photosynthesis induced by abscisic acid in leaves having different mesophyll anatomies. *Plant and Cell Physiology*, 29(3), 385–394. Available from: <https://doi.org/10.1093/oxfordjournals.pcp.a077506>
- Théroux-Rancourt, G., Éthier, G. & Pepin, S. (2014) Threshold response of mesophyll CO_2 conductance to leaf hydraulics in highly transpiring hybrid poplar clones exposed to soil drying. *Journal of Experimental Botany*, 65(2), 741–753. Available from: <https://doi.org/10.1093/jxb/ert436>
- Théroux-Rancourt, G. & Gilbert, M.E. (2017) The light response of mesophyll conductance is controlled by structure across leaf profiles. *Plant, Cell & Environment*, 40(5), 726–740. Available from: <https://doi.org/10.1111/pce.12890>
- Tholen, D., Boom, C., Noguchi, K., Ueda, S., Katase, T. & Terashima, I. (2008) The chloroplast avoidance response decreases internal conductance to CO_2 diffusion in *Arabidopsis thaliana* leaves. *Plant, Cell & Environment*, 31(11), 1688–1700. Available from: <https://doi.org/10.1111/j.1365-3040.2008.01875.x>
- Tholen, D., Ethier, G., Genty, B., Pepin, S. & Zhu, X.-g. (2012) Variable mesophyll conductance revisited: theoretical background and experimental implications. *Plant, Cell & Environment*, 35(12), 2087–2103. Available from: <https://doi.org/10.1111/j.1365-3040.2012.02538.x>
- Tosens, T., Niinemets, Ü., Westoby, M. & Wright, I.J. (2012) Anatomical basis of variation in mesophyll resistance in eastern Australian sclerophylls: news of a long and winding path. *Journal of Experimental Botany*, 63(14), 5105–5119. Available from: <https://doi.org/10.1093/jxb/ers171>
- Ubierna, N., Cernusak, L.A., Holloway-Phillips, M., Busch, F.A., Cousins, A.B. & Farquhar, G.D. (2019) Critical review: incorporating the arrangement of mitochondria and chloroplasts into models of photosynthesis and carbon isotope discrimination. *Photosynthesis Research*, 141(1), 5–31. Available from: <https://doi.org/10.1007/s11120-019-00635-8>
- Uehlein, N., Lovisolo, C., Siefert, F. & Kaldenhoff, R. (2003) The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. *Nature*, 425(6959), 734–737. Available from: <https://doi.org/10.1038/nature02027>
- Veromann-Jürgenson, L.-L., Brodribb, T.J., Niinemets, Ü. & Tosens, T. (2020) Variability in the chloroplast area lining the intercellular airspace and cell walls drives mesophyll conductance in gymnosperms. *Journal of Experimental Botany*, 71(16), 4958–4971. Available from: <https://doi.org/10.1093/jxb/eraa231>
- Vogelmann, T.C. & Evans, J.R. (2002) Profiles of light absorption and chlorophyll within spinach leaves from chlorophyll fluorescence. *Plant, Cell & Environment*, 25(10), 1313–1323. Available from: <https://doi.org/10.1046/j.1365-3040.2002.00910.x>
- Vrábl, D., Vašková, M., Hronková, M., Flexas, J. & Šantrůček, J. (2009) Mesophyll conductance to CO_2 transport estimated by two independent methods: effect of variable CO_2 concentration and abscisic acid. *Journal of Experimental Botany*, 60(8), 2315–2323. Available from: <https://doi.org/10.1093/jxb/erp115>
- Walker, B., Ariza, L.S., Kaines, S., Badger, M.R. & Cousins, A.B. (2013) Temperature response of *in vivo* Rubisco kinetics and mesophyll conductance in *Arabidopsis thaliana*: comparisons to *Nicotiana tabacum*. *Plant, Cell & Environment*, 36(12), 2108–2119. Available from: <https://doi.org/10.1111/pce.12166>
- Wang, X., Du, T., Huang, J., Peng, S. & Xiong, D. (2018) Leaf hydraulic vulnerability triggers the decline in stomatal and mesophyll conductance during drought in rice. *Journal of Experimental Botany*, 69(16), 4033–4045. Available from: <https://doi.org/10.1093/jxb/ery188>
- Warren, C.R. (2008a) Does growth temperature affect the temperature responses of photosynthesis and internal conductance to CO_2 ? A test with *Eucalyptus regnans*. *Tree Physiology*, 28(1), 11–19. Available from: <https://doi.org/10.1093/treephys/28.1.11>
- Warren, C.R. (2008b) Soil water deficits decrease the internal conductance to CO_2 transfer but atmospheric water deficits do not. *Journal of Experimental Botany*, 59(2), 327–334. Available from: <https://doi.org/10.1093/jxb/erm314>
- Wong, S.C., Canny, M.J., Holloway-Phillips, M., Stuart-Williams, H., Cernusak, L.A., Márquez, D.A. et al. (2022) Humidity gradients in the air spaces of leaves. *Nature Plants*, 8, 971–978. Available from: <https://doi.org/10.1038/s41477-022-01202-1>
- Wong, S.C., Cowan, I.R. & Farquhar, G.D. (1985) Leaf conductance in relation to rate of CO_2 assimilation III. Influences of water stress and photoinhibition. *Plant Physiology*, 78(4), 830–834. Available from: <https://doi.org/10.1104/pp.78.4.830>
- Xiong, D. (2023) Leaf anatomy does not explain the large variability of mesophyll conductance across C_3 crop species. *The Plant Journal*, 113(5), 1035–1048. Available from: <https://doi.org/10.1111/tj.16098>
- Xiong, D., Douthe, C. & Flexas, J. (2018) Differential coordination of stomatal conductance, mesophyll conductance, and leaf hydraulic conductance in response to changing light across species. *Plant, Cell & Environment*, 41(2), 436–450. Available from: <https://doi.org/10.1111/pce.13111>
- Xiong, D., Liu, X., Liu, L., Douthe, C., Li, Y., Peng, S. et al. (2015) Rapid responses of mesophyll conductance to changes of CO_2 concentration, temperature and irradiance are affected by N supplements in rice. *Plant, Cell & Environment*, 38, 2541–2550. Available from: <https://doi.org/10.1111/pce.12558>
- Yamori, W., Evans, J.R. & von Caemmerer, S. (2010) Effects of growth and measurement light intensities on temperature dependence of CO_2 assimilation rate in tobacco leaves. *Plant, Cell & Environment*, 33(3), 332–343. Available from: <https://doi.org/10.1111/j.1365-3040.2009.02067.x>
- Yamori, W., Noguchi, K., Hanba, Y.T. & Terashima, I. (2006) Effects of internal conductance on the temperature dependence of the

- photosynthetic rate in spinach leaves from contrasting growth temperatures. *Plant and Cell Physiology*, 47(8), 1069–1080. Available from: <https://doi.org/10.1093/pcp/pcj077>
- Yin, X., van der Putten, P.E.L., Belay, D. & Struik, P.C. (2020) Using photorespiratory oxygen response to analyse leaf mesophyll resistance. *Photosynthesis Research*, 144(1), 85–99. Available from: <https://doi.org/10.1007/s11120-020-00716-z>
- Yin, X. & Struik, P.C. (2009) Theoretical reconsiderations when estimating the mesophyll conductance to CO₂ diffusion in leaves of C₃ plants by analysis of combined gas exchange and chlorophyll fluorescence measurements. *Plant, Cell & Environment*, 32(11), 1513–1524. Available from: <https://doi.org/10.1111/j.1365-3040.2009.02016.x>
- Yin, X. & Struik, P.C. (2017) Simple generalisation of a mesophyll resistance model for various intracellular arrangements of chloroplasts and mitochondria in C₃ leaves. *Photosynthesis Research*, 132(2), 211–220. Available from: <https://doi.org/10.1007/s11120-017-0340-8>
- Zhao, M., Tan, H.-T., Scharwies, J., Levin, K., Evans, J.R. & Tyerman, S.D. (2017) Association between water and carbon dioxide transport in leaf plasma membranes: assessing the role of aquaporins. *Plant, Cell & Environment*, 40(6), 789–801. Available from: <https://doi.org/10.1111/pce.12830>
- Zhou, L., Xiang, X., Ji, D., Chen, Q., Ma, T., Wang, J. et al. (2023) A carbonic anhydrase, ZmCA4, contributes to photosynthetic efficiency and modulates CO₂ signaling gene expression by interacting with Aquaporin ZmPIP2;6 in maize. *Plant and Cell Physiology*, 65, 243–258. Available from: <https://doi.org/10.1093/pcp/pcad145>

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