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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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  from the atmosphere to photosynthesise organic carbon in the chloroplast of the mesophyll cells, the location of the primary carboxylating enzyme Rubisco.  $\text{CO}_2$  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  $\text{CO}_2$

crosses the stomatal pore and the mesophyll space is referred to as stomatal conductance to  $\text{CO}_2$  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  $\text{CO}_2$  and water vapour must cross in the exchange between the atmosphere and the leaf, thereby impacting photosynthesis rates.

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Efficiently easing  $\text{CO}_2$  diffusion to the chloroplast is crucial for plants because, for a given amount of Rubisco active sites, Rubisco's activity and thus net  $\text{CO}_2$  assimilation rate ( $A$ ) is largely dependent on the  $\text{CO}_2$  concentration inside the chloroplast ( $c_c$ ) (Farquhar et al., 1980). Plants open stomatal pores to increase  $g_{sc}$ , allowing  $\text{CO}_2$  to diffuse into the leaf and increasing the  $\text{CO}_2$  concentration in the substomatal cavity ( $c_i$ ), while Rubisco activity fixing  $\text{CO}_2$  decreases  $c_c$ . Mesophyll conductance to  $\text{CO}_2$  ( $g_m$ ) bridges these two concentrations, facilitating the diffusion of  $\text{CO}_2$  from the substomatal cavity to the chloroplast.

Consequently, environmental factors and plant properties that affect  $c_c$  and Rubisco activity, such as light intensity,  $\text{CO}_2$  concentration, Rubisco content,  $g_{sc}$  and  $g_m$ , determine the value of  $A$ . For instance, stomatal closure in response to environmental stress can limit  $\text{CO}_2$  availability in the mesophyll, decreasing the photosynthetic rate. Conversely, when stomata are open, mesophyll conductance must be sufficiently high to facilitate the movement of  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  is a composite of different biochemical and anatomical properties of the

leaf that, in combination, ultimately determine the diffusion of  $\text{CO}_2$  inside the mesophyll (Evans & von Caemmerer, 1996). Given that  $g_m$  represents the ease of  $\text{CO}_2$  diffusion through the mesophyll, it can be understood analogously as its inverse: mesophyll resistance to  $\text{CO}_2$  diffusion ( $r_m = 1/g_m$ ) is the sum of leaf properties that impede  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\Gamma^*$  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  $\Phi_{PSII}$  is the photochemical quantum yield of photosystem II (PSII) obtained from fluorescence,  $\alpha$  is the leaf absorptance,  $\beta$  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 ( $\Phi_{PSII}$ ), fluorescence emissions from Photosystem I (PSI) and the strength of other electron sinks (see Pons et al., 2009).

While  $\alpha$  can be measured or estimated reasonably accurately (Evans & Poorter, 2001),  $\beta$  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  $\Gamma^*$ , which is usually assumed constant and taken from the literature. In  $C_3$  plants,  $\Gamma^*$  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  $\Gamma^*$  near that temperature. However, the temperature response of  $\Gamma^*$  varies significantly between species (Hermida-Carrera et al., 2016; Orr et al., 2016), and, potentially, growth environments. Thus, it can be risky to obtain  $\Gamma^*$  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  $\Gamma^*$  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  $\Gamma^*$  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  $\text{CO}_2$  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  $g_m$  ( $\Delta_i$ ) against the observed isotopic discrimination ( $\Delta_{\text{obs}}$ ) (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  $\text{CO}_2$  diffusion in air,  $a_m$ ,  $b$  and  $e'$  are the isotopic fractionations associated with diffusion through water, Rubisco carboxylation and respiration, respectively.  $\alpha_b$  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  $\text{CO}_2$  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  roux-Rancourt & Gilbert, 2017); however, they are considered the most trustworthy methods for estimating  $g_m$  (Pons et al., 2009).

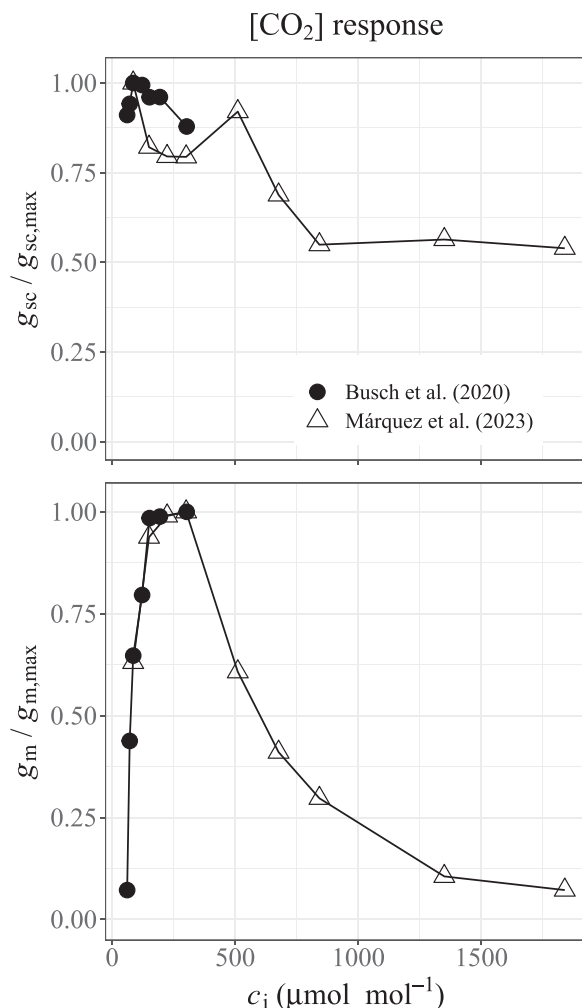
### 3 | SHORT-TERM RESPONSE OF $g_m$

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  roux-Rancourt & Gilbert, 2017; Tosens et al., 2012; Veromann-J  rgenson et al., 2020). In general, they have demonstrated consistent trends of  $g_m$  responses for  $\text{CO}_2$  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  $g_{\text{sc}}$  obtained from experiments where both sets of data are available for paring  $g_m$  and  $g_{\text{sc}}$  responses to  $\text{CO}_2$  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  $g_{\text{sc}}$  was not reported. We analyse each response in detail below.

#### 3.1 | Response to $\text{CO}_2$ concentrations

Many studies have shown short-term  $g_m$  responses to changes in  $\text{CO}_2$  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  rquez et al., 2023; Tazoe et al., 2011; V  r  bl 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

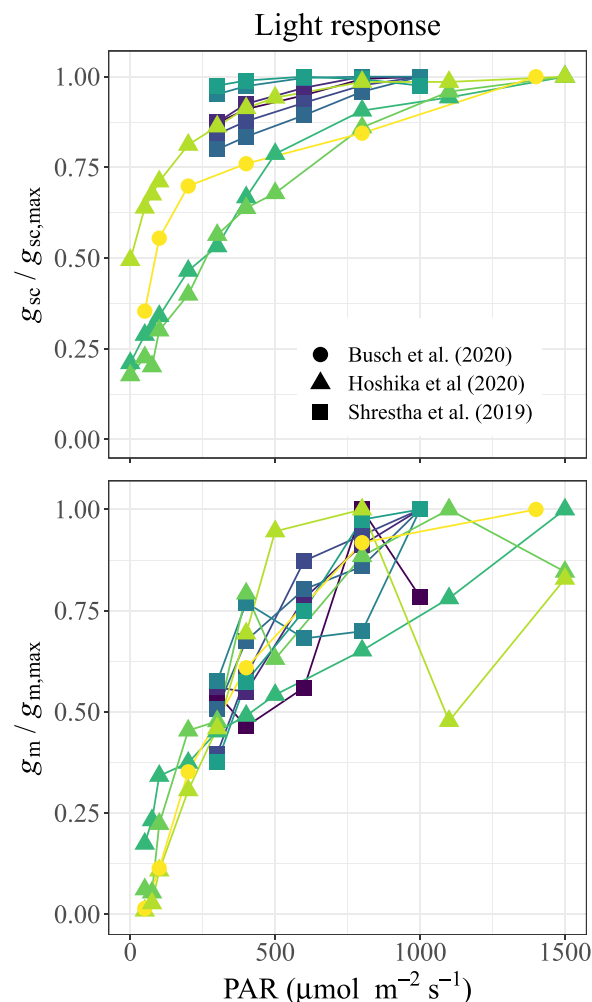


**FIGURE 1** Proportional  $g_{sc}$  and  $g_m$  responses to changes in  $CO_2$  concentration. Data from Busch, Holloway-Phillips, et al. (2020) used the isotope method in *Triticum aestivum* and Márquez et al. (2023) used the fluorescence method in *Gossypium hirsutum*.

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



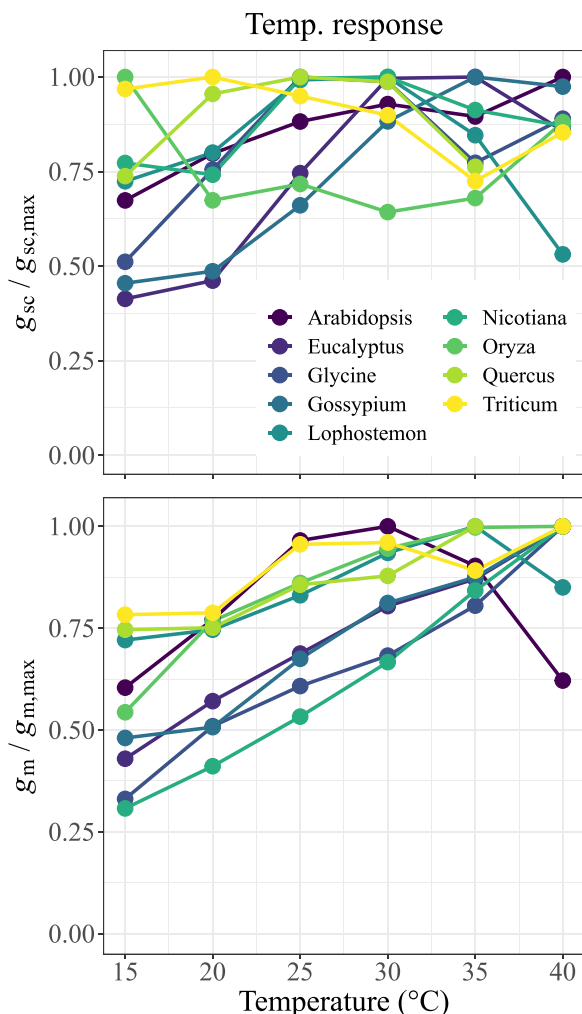
**FIGURE 2** Proportional  $g_{sc}$  and  $g_m$  responses to changes in light intensity. Data from Busch, Holloway-Phillips, et al. (2020) used the isotope method in *Triticum aestivum*, Shrestha, Buckley, et al. (2019) used the isotope method in *Cicer arietinum* and Hoshika et al. (2020) used the fluorescence method in *Fagus crenata*. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.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](http://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; Th  roux-Rancourt et al., 2014).

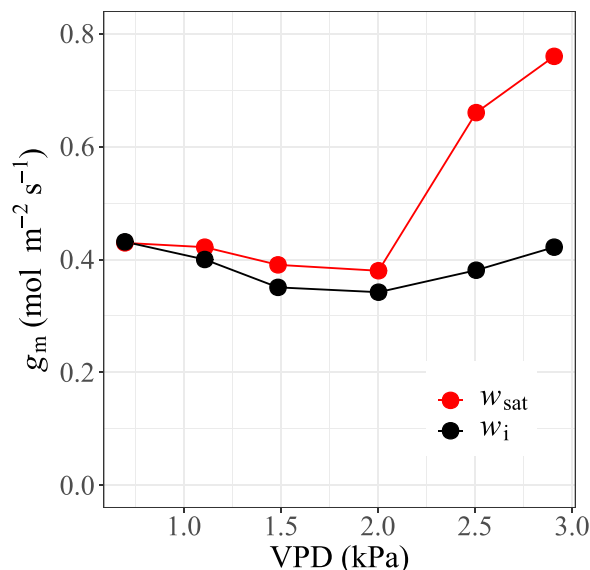
In terms of water relations, there are reports of  $g_m$  sometimes correlating with leaf water potential (Loucos et al., 2017; Th  roux-Rancourt et al., 2014). Leaf water potential has even been suggested as a possible proxy for  $g_m$ , but the mechanisms behind the correlation are still unknown (see 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  $\text{CO}_2$  ( $g_m$ ) 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](http://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 ( $g_{sw} < 160 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) (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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  (Uehlein et al., 2003). They have been shown to help maintain the permeability to  $\text{CO}_2$  of membranes by creating paths in areas of the membrane typically hindered by structures that obstruct  $\text{CO}_2$  movement (Kai & Kaldenhoff, 2014). Thus, some long-term effects in  $g_m$  may be expected by altering their natural abundance in the  $\text{CO}_2$  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 CO<sub>2</sub> concentration, light intensity and leaf temperature.

Mechanism/response	[CO <sub>2</sub> ]	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); Théroux-Rancourt and Gilbert (2017)
Variable fluxes along the CO <sub>2</sub> 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); Márquez 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 C<sub>4</sub> species *Setaria viridis* (Ermakova et al., 2021). In contrast,  $g_m$  was unaffected in Arabidopsis aquaporin knockout lines (Kromdijk, Głowacka, & 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> and O<sub>2</sub> 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 CO<sub>2</sub> permeable aquaporins,  $g_m$  would be expected to be relatively insensitive to temperature if the activation energy for CO<sub>2</sub> transport follows that of H<sub>2</sub>O 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  or temperature.

## 5.5 | Variable fluxes along the $\text{CO}_2$ diffusion pathway

Following Fick's First Law assumptions, Parkhurst (1994) pointed out some time ago that conductances become variable when there are  $\text{CO}_2$  sources or sinks along the diffusion pathway, or in

other words when the  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  being released from the mitochondria and mixing with the incoming  $\text{CO}_2$  somewhere inside the cytosol, affecting  $g_m$  by adding another source of  $\text{CO}_2$  between  $c_i$  and  $c_c$  (Tholen et al., 2012). Yin and Struik (2017) later argued that some proportion of the (photo)respired  $\text{CO}_2$  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  $\text{CO}_2$  in the cytosol.

Under both scenarios described above,  $g_m$  is expected to decrease to zero at the  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  gradient from  $c_i$  to the area around the photosynthetically active mesophyll cells ( $c_w$ ) in response to changes in atmospheric  $\text{CO}_2$  levels. Moreover, the study observed that changes in  $c_a$  were accompanied by decreased apparent conductance to  $\text{CO}_2$  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  $\text{CO}_2$  and bicarbonate, facilitating carbon transport, and bicarbonate is converted into  $\text{CO}_2$  near Rubisco (Raven & Glidewell, 1981). It has been shown that plant growth at high

CO<sub>2</sub> concentrations presents lower CA activity and content than growth at ambient or low  $c_a$ , and that CA activity is decreased when CO<sub>2</sub> 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 CO<sub>2</sub> in the chloroplast would already be high, as at high  $c_i$ .

Thus, the observed behaviour in the  $g_m$  short-term response to CO<sub>2</sub> is similar to that expected of the facilitated transfer process to changes in CO<sub>2</sub> 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 CO<sub>2</sub> 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., Thérout-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 CO<sub>2</sub> 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 CO<sub>2</sub> concentrations and a  $g_m$  that tends towards zero when approaching the light or CO<sub>2</sub> compensation points (Busch, Holloway-Phillips, et al., 2020). However, it fails to correctly predict the drop in  $g_m$  observed at high CO<sub>2</sub> concentrations or towards low light intensities.

The model from Thérout-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 Thérout-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 CO<sub>2</sub> 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 $g_m$

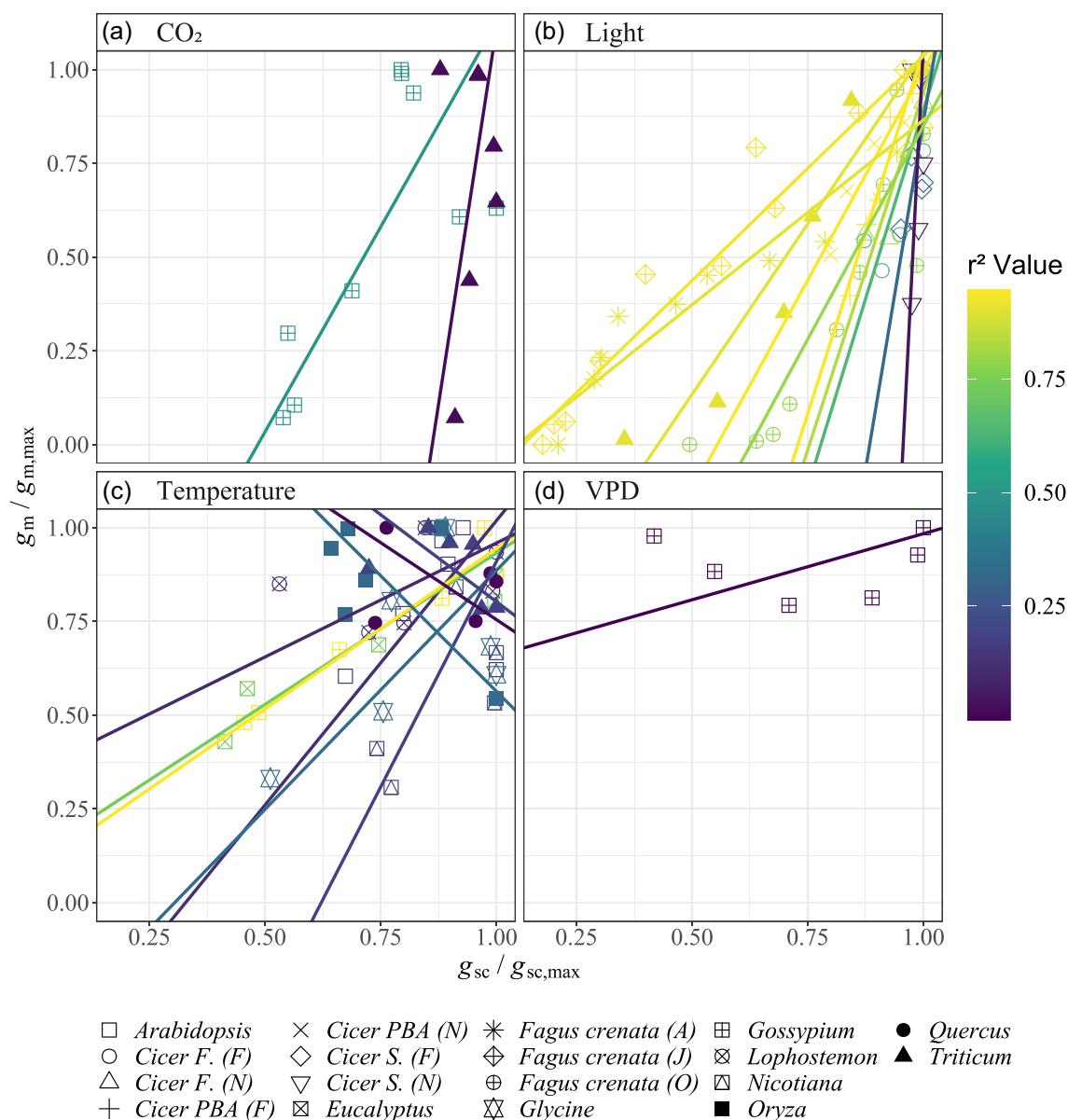
Given the lack of mechanistic models for  $g_m$  able to be accurately applied under a wide range of conditions, empirical ways to describe  $g_m$  may be the best choice for the time being. One such empirical relationship is using  $g_{sc}$  as a proxy for  $g_m$ . During growth, plants adapt their 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> concentration are mostly in response to stomatal activity and associated changes in  $c_i$ . For instance, Th  roux-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 CO<sub>2</sub> 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 CO<sub>2</sub> 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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