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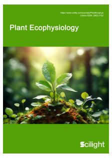

Review

Navigating Challenges in Interpreting Plant Physiology Responses through Gas Exchange Results in Stressed Plants

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Abstract: This paper explores the challenges that arise when performing and interpreting leaf gas exchange measurements in plants subjected to abiotic stress. It highlights how factors such as cuticular fluxes, stomatal closure, and common assumptions about gas exchange can lead to errors, especially under stress conditions. Key phenomena such as substomatal cavity unsaturation and stomatal patchiness during water stress are discussed in detail, as they significantly complicate the calculation of gas exchange parameters under stress. The paper also addresses the importance of other factors, including steady-state conditions, the differences between adaxial and abaxial surface responses, and boundary layer effects, all of which play critical roles in influencing the accuracy of measurements. Important physiological indicators—such as intrinsic water-use efficiency, minimum leaf conductance, substomatal CO_2 concentration, and mesophyll conductance—are analysed in the context of how stress-induced discrepancies in data often result from measurement artefacts rather than true physiological differences. To address these challenges, the paper outlines practical approaches to improving measurement accuracy, offering insights on standardising experimental conditions and minimising errors. By recognising these issues, gaps in current knowledge are identified, providing a comprehensive overview of the challenges in interpreting leaf gas exchange data under stress conditions and suggesting areas for further study.

Keywords: plant stress; cuticular conductance; stomatal patchiness; unsaturation; abaxial; adaxial; leaf gas exchange

1. Introduction

Leaf gas exchange measurements have been instrumental in plant physiology research, underpinning critical aspects of photosynthesis and transpiration (Long & Bernacchi, 2003; Sharkey, 2016). Gas exchange involves the movement of carbon dioxide (CO_2), oxygen (O_2), and water vapour (H_2O) into and out of the leaf. A significant area of scientific interest has been understanding the mechanisms governing the ratio of CO_2 uptake to water loss (Cowan & Farquhar, 1977; Deans et al., 2020), i.e., assimilation (A) and transpiration (E) rates. Over the past century, these measurements have been used to explore plant-environment interactions at the leaf level, with methods evolving from early porometer techniques to today's sophisticated systems such as the LI-6800 (LI-COR, Lincoln, NE, USA), Walz GFS-3000 (Walz, Effeltrich, Germany), CIRAS-4 (PP Systems, Amesbury, MA, USA), among others. From the early stages of using these techniques, one of the aims was to estimate the internal leaf microenvironmental conditions that would allow us to better scrutinise the plant's physiological responses related to photosynthesis. Over time, reliable models were developed to estimate internal leaf conditions from external measurements, leading to the definition of key physiological parameters that encapsulate biological meaning (Gaastra, 1959; Moss & Rawlins, 1963; von Caemmerer & Farquhar, 1981; Márquez, Stuart-Williams, & Farquhar, 2021). Physiological parameters such as stomatal conductance to water (g_{sw}) and internal CO_2 concentration (c_i) have become standard and common language in plant sciences, as they serve as the cornerstone for comparing trends, checking for improvement, or evaluating performance *in planta*.

External measurements, combined with modelling, enable us to explore *in planta* physiological responses to varying environments, growth conditions, and broader ecophysiological trends (Wong, Cowan, & Farquhar, 1979; Farquhar, von Caemmerer, & Berry, 1980; Farquhar & Richards, 1984). These approaches have provided a wealth of information linking biochemical and biophysical knowledge in plant sciences with leaf- and plant-scale phenomena. Thus, leaf gas exchange measurements have been pivotal in numerous areas of plant science research (see, for example Wong, Cowan and Farquhar (1979); von Caemmerer and Farquhar (1981) and Long and Bernacchi (2003)) and are often the benchmark for assessing treatment effects and evaluating genetically modified organisms *in planta* (von Caemmerer, 2000; Long & Bernacchi, 2003; von Caemmerer, 2013).

The physical principles—including diffusion theory, Fick's laws of diffusion, and mass conservation—underlying the estimation of gas exchange parameters and the interpretation of these calculated values are well-established and scientifically sound. These principles are based on assumptions that have been developed from studies conducted on healthy, well-watered plants, which typically exhibit high assimilation rates and stomatal conductances (Gaastra, 1959; Scholander et al., 1965; von Caemmerer & Farquhar, 1981). While these assumptions work reliably for unstressed plants, they were not initially designed for application to stressed plants. As a result, when transitioning to stress conditions, such as drought or extreme temperatures, the robustness of these assumptions is called into question (Turner, Schulze, & Gollan, 1984; Boyer, Wong, & Farquhar, 1997; Boyer, 2015a; Yan, Zhong, & Shangguan, 2016; Cernusak et al., 2018; Buckley & Sack, 2019; Cernusak et al., 2019). This raises important concerns about the reliability of gas exchange data collected under stress conditions and whether the interpretations drawn from these measurements remain valid.

Some notable examples of these assumptions during gas exchange measurements include the assumption that leaf cuticle conductance to water (g_{cw}) is negligible. This assumption is critical for calculating g_{sw}, assuming that transpiration passes only through the stomatal pores (von Caemmerer & Farquhar, 1981). However, under water stress conditions, the cuticle can become a more significant pathway for water loss, invalidating this assumption and potentially leading to errors in the estimation of gas exchange parameters (Boyer, Wong, & Farquhar, 1997; Boyer, 2015a; Tominaga & Kawamitsu, 2015; Márquez, Stuart-Williams, & Farquhar, 2021). Another key assumption is that the internal leaf gas space is saturated with water vapour (Gaastra, 1959), which is a crucial assumption for determining the driving force for water vapour diffusion from the leaf to the atmosphere. However, evidence suggests that the saturation assumption may no longer hold under mild to severe water stress conditions, resulting in inaccuracies in gas exchange measurements and interpretations (Cernusak et al., 2018; Wong et al., 2022; Márquez et al., 2024). Additionally, there is the assumption that leaf surface properties, such as stomatal aperture and stomatal conductance, are uniform across the leaf (Moss & Rawlins, 1963). While this assumption may be valid for unstressed plants, it does not always hold for stressed plants (Laisk, 1983; Downton, Loveys, & Grant, 1988). Stressed plants frequently exhibit substantial heterogeneity in stomatal behaviour within a single leaf and among different leaves on the same plant (Mott, Cardon, & Berry, 1993; Cardon, Mott, & Berry, 1994; Mott & Buckley, 2000). These inconsistencies can introduce significant errors in gas exchange calculations and complicate the interpretation of the data, potentially undermining our calculations or even making them essentially wrong.

> "*Lo que por sabido se calla, por callado se olvida*"
> — Spanish Proverb
> (Translation: "What is assumed to be known and left unspoken, by remaining unspoken, falls into oblivion.")

Over the past century, scientists have extensively documented the weakening of fundamental assumptions in gas exchange measurements when the conditions required to uphold these assumptions are unmet. It has been noted that the robustness of these assumptions is closely linked to the presence of an unstressed plant during measurement. This is especially important considering the increasing interest in assessing how stressful conditions affect plant physiology, particularly in light of the anticipated challenges posed by climate change (Grossiord et al., 2020; Fu et al., 2024). Addressing these uncertainties in a practical manner, rather than merely theoretically, is essential, but they are often overlooked in many studies. This mirrors the old Spanish proverb, "*Lo que por sabido se calla, por callado se olvida*:" despite general awareness of these uncertainties, they often remain implicit, theoretical, and largely unspoken in the context of plant physiology. However, we must critically examine and challenge these underlying assumptions to fully capitalise on our understanding of gas exchange in stressed plants. This requires investigating when, why, and how these assumptions fail and determining how we can account for or bypass these weaknesses to improve the reliability of our measurements.

Importantly, we are not starting from scratch. Numerous researchers have already explored the weakening of these assumptions in stressed plants, offering potential solutions, alternative methods, and strategies to address various aspects of the problem. Building on this foundation, our aim here is to identify the challenges encountered during gas exchange measurements of stressed plants, discuss the causes of these challenges, and highlight the remaining gaps that still need to be addressed for a better understanding of leaf gas exchange under stressed conditions. Additionally, when possible, we seek to provide practical solutions to mitigate or avoid these problems to ensure that results extracted from experiments under stressed conditions are robust and reliable.

2. Gas Exchange Measurements

Let us briefly address the calculations involved in common leaf gas exchange measurements. Gas exchange systems typically include a chamber that encloses an area of leaf in an open gas system, where a reference gas is introduced (Gaastra, 1959). The entering gas (reference) and the gas exiting the chamber (sample) are analysed for water vapour and CO_2 concentrations (Figure 1a). Additional measurements are taken from the chamber and leaf, including leaf and air temperature, flow rate into the chamber, and light intensity.

The mass balance obtained from the flow rate, and the reference and sample concentrations, permit to derive the following expression for the transpiration rate (E):

$$E = \frac{\mu_{\mathrm{out}} w_{\mathrm{a}} - \mu_0 w_0}{s} \tag{1}$$

where μ_{out} is the flow rate exiting the chamber, μ_0 is the flow rate entering the chamber, w_0 is the water vapour mole fraction entering the chamber, w_{a} is the water vapour mole fraction exiting the chamber, and s is the projected leaf surface area within the chamber. The flow rate exiting the chamber is approximated as $\mu_{\mathrm{out}} \approx \mu_0 + sE$, neglecting CO_2 uptake due to its significantly lower magnitude compared to water release. Consequently, the equations for E become:

$$E = \frac{\mu_0 \left(w_{\mathrm{a}} - w_0 \right)}{s \left(1 - w_{\mathrm{a}} \right)} \tag{2}$$

Analogous for CO_2 assimilation rate (A)

$$A = \frac{\mu_0}{s} \left[c_0 - c_{\mathrm{a}} \left(\frac{1 - w_0}{1 - w_{\mathrm{a}}} \right) \right] \tag{3}$$

where c_0 is the CO_2 mole fraction entering the chamber and c_{a} is the CO_2 mole fraction exiting the chamber.

These equations allow us to estimate A and E based on the known leaf surface area within the chamber. While the degree of control over gases entering or exiting the chamber, temperatures, light intensity, and other factors varies across different commercial and in-house developed gas exchange systems, the fundamental objective remains the same. These systems aim to estimate the same gas exchange parameters, the ease with which water escapes the leaf and the CO_2 concentration within the leaf, from the above mass flow balances.

Gas exchange parameters are defined using an analogy to electrical resistances, with the boundary layer, stomata, and cuticle acting as resistors for water vapour and CO_2 diffusion (Figure 1b) or their inverses, conductances. The most recent theory for estimating these parameters was presented by Márquez, Stuart-Williams and Farquhar (2021), representing water vapour diffusion from the leaf surface and substomatal cavity to the atmosphere as:

$$r_{\mathrm{tw}} = \frac{1}{\frac{1}{r_{\mathrm{cw}}} + \frac{1}{r_{\mathrm{sw}}}} + r_{\mathrm{bw}} = \frac{1}{\frac{1}{\left[\frac{w_{\mathrm{c}} - w_{\mathrm{s}}}{E_{\mathrm{c}}} \right]} + \frac{1}{\left[\frac{w_{\mathrm{i}} - w_{\mathrm{s}}}{E_{\mathrm{s}} - E_{\mathrm{s}} \bar{w}_{\mathrm{s}}} \right]}} + \left[\frac{w_{\mathrm{s}} - w_{\mathrm{a}}}{E - E\bar{w}_{\mathrm{b}}} \right]$$

and (4)

$$\bar{w}_{\mathrm{s}} = \frac{w_{\mathrm{i}} + w_{\mathrm{s}}}{2}$$

$$\bar{w}_{\mathrm{b}} = \frac{w_{\mathrm{s}} + w_{\mathrm{a}}}{2}$$

where r_{tw}, r_{cw}, r_{sw}, and r_{bw} are the total, cuticular, stomatal, and boundary layer resistances to water vapour diffusion, respectively. E_{c}, and E_{s} are the cuticular and stomatal transpiration rates such that $E = E_{\mathrm{s}} + E_{\mathrm{c}}$, and w_{c}, w_{s}, and w_{i} are the cuticular, leaf surface, and substomatal cavity mole fractions of water vapour, respectively. Parameters $\bar{w}_s$ and $\bar{w}_b$ are associated with ternary corrections through the stomata and boundary layer. The aim of water measurements is usually to obtain r_{sw}, which from Equation (4) is

$$r_{\mathrm{sw}} = \frac{w_{\mathrm{i}} - w_{\mathrm{s}}}{E_{\mathrm{s}} - E_{\mathrm{s}} \bar{w}_{\mathrm{s}}} \tag{5}$$

To solve Equation (5) the values for r_{bw}, w_{s}, r_{cw}, and E_{c} ($E_s = E - E_c$) are needed.

Empirical values of r_{bw} for the gas mixing system in the chamber are typically embedded in the instrument's calculations. Given r_{bw}, w_{s} can be estimated as,

$$w_{\mathrm{s}} = \frac{r_{\mathrm{bw}} E \left(1 - \frac{w_{\mathrm{a}}}{2} \right) + w_{\mathrm{a}}}{1 + r_{\mathrm{bw}} \frac{E}{2}} \tag{6}$$

There are independent methods that allow for the estimation of r_{cw}, which should be conducted either before or after the experiment at hand. These estimates assume r_{cw} remains constant as long as the leaf is turgid, with w_{c} generally considered equal to w_{i}, thereby allowing for the estimation of E_{c} (from Equation (4): $E_{\mathrm{c}} = \left(w_{\mathrm{i}} - w_{\mathrm{s}} \right) / r_{\mathrm{cw}}$). Details of the available techniques and the assumption of constant r_{cw} will be discussed later in the text.

The common assumption is that there are vapour water-saturated conditions within the leaf ($w_{\mathrm{i}} = w_{\mathrm{sat}}$), so that w_{i} can be estimated from the dew point at leaf temperature:

$$w_{\mathrm{sat}} = \frac{0.611121 e^{\frac{17.502 T_{\mathrm{l}}}{240.97 + T_{\mathrm{l}}}}}{P_{\mathrm{atm}}} \tag{7}$$

where T_{l} is leaf temperature in Celsius, and P_{atm} is atmospheric pressure (kPa). Then, all the parameters to obtain r_{sw} (Equation (5)) are estimated.

Analogous to Equation (4), for CO_2 the resistance becomes:

$$r_{\mathrm{tc}} = \frac{1}{\frac{1}{r_{\mathrm{cc}}} + \frac{1}{r_{\mathrm{sc}}}} + r_{\mathrm{bc}} = \frac{1}{\frac{1}{\left[\frac{c_{\mathrm{cut}} - c_{\mathrm{s}}}{A_{\mathrm{c}}} \right]} + \frac{1}{\left[\frac{c_{\mathrm{i}} - c_{\mathrm{s}}}{A_{\mathrm{s}} + E_{\mathrm{s}} \bar{c}_{\mathrm{s}}} \right]}} + \left[\frac{c_{\mathrm{s}} - c_{\mathrm{a}}}{A + E\bar{c}_{\mathrm{b}}} \right]$$

and (8)

$$\bar{c}_{\mathrm{s}} = \frac{c_{\mathrm{i}} + c_{\mathrm{s}}}{2}$$

$$\bar{c}_{\mathrm{b}} = \frac{c_{\mathrm{s}} + c_{\mathrm{a}}}{2}$$

where r_{tc}, r_{cc}, r_{sc}, and r_{bc} are the total, cuticular, stomatal, and boundary layer resistances to CO_2 diffusion, respectively. A_{c} and A_{s} are the cuticular and stomatal assimilation rates, and c_{cut}, c_{s}, and c_{i} are the cuticular, leaf surface, and substomatal. cavity mole fractions of CO_2, respectively. The aim of CO_2 measurements is usually to obtain c_{i}.

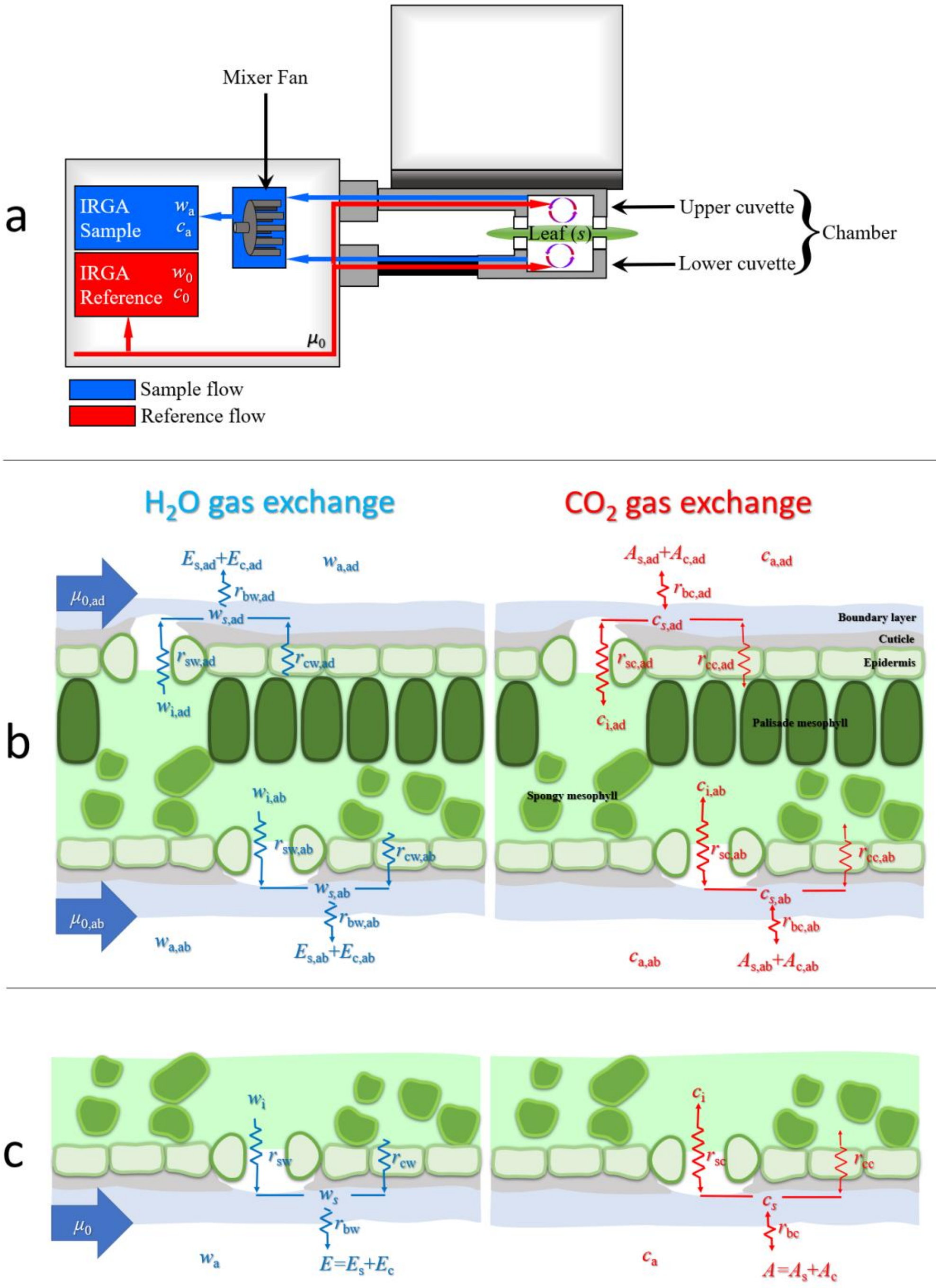

Figure 1. Schematic of gas exchange system setup and illustration of H_2O and CO_2 gas exchange. (**a**) Diagram of a typical gas exchange chamber system, illustrating the flow of the sample (blue lines) and reference (red lines) air through the chamber, which houses the leaf. Infrared gas analysers (IRGA) measure water vapour and CO_2 concentrations in the sample and reference flows to estimate bulk transpiration (E) and assimilation (A) rates. The upper and lower cuvette fluxes are typically mixed in standard gas exchange systems, resulting in bulk measurements of gas exchange parameters. (**b**) Detailed representation of H_2O gas exchange (**left**) and CO_2 gas exchange (**right**) at the adaxial (upper) and abaxial (lower) leaf surfaces in an amphistomatous leaf. This panel illustrates the separate gas exchange processes for each leaf surface, which can be measured individually if the upper and lower cuvettes are analysed separately—though this setup is not common in commercial gas exchange systems. Stomatal, cuticle, and boundary layer resistances are depicted for both surfaces, along with the mole fractions of water vapour and CO_2. (**c**) Simplified depiction of overall H_2O and CO_2 gas exchange as in most commercial systems. A bulk measurement of transpiration (E) and CO_2 assimilation (A) rate is obtained, representing the combined contributions of stomatal and cuticular components from adaxial and abaxial surfaces alongside a bulk boundary layer conductance. Parameters: w_i is the water vapour mole fraction in the substomatal cavity, w_s is the water vapour mole fraction at the leaf surface, w_a is water vapour mole fraction exiting the chamber (i.e., atmospheric), w_0 is the water vapour mole fraction in the reference gas, c_i is the CO_2 mole fraction in the substomatal cavity, c_s is the CO_2 mole fraction at the leaf surface, c_a is the CO_2 mole fraction exiting the chamber (i.e., atmospheric), c_0 is the CO_2 mole fraction in the reference gas, r_{sw} is the stomatal resistance to water vapour, r_{bw} is the boundary layer resistance to water vapour, r_{sc} is the stomatal resistance to CO_2, r_{bc} is the boundary layer resistance to CO_2, r_{cw} is the cuticular resistance to water vapour, r_{cc} is the cuticular resistance to CO_2, μ_0 is the flow rate of the reference gas, ad and ab subscripts refer to the adaxial and abaxial leaf surfaces, respectively.

Table 1. Summary of the key challenges in gas exchange measurements under stress conditions.

Category	Key Challenges
Small fluxes (Leaf cuticle)	**Neglecting g_{cw}:** Ignoring cuticular conductance when stomatal conductance is low can result in significant overestimations of g_{sw} and c_i. **Variability in g_{cw}:** Differences in g_{cw} across species and under stress conditions complicate the universal application of a value for the parameter.
Unsaturation in the substomatal cavity	**Saturation assumption ($w_i = w_{sat}$):** Assumption of water vapour saturation fails under moderate to high VPD, resulting in underestimation of g_{sw} and c_i. **Non-stomatal control of transpiration:** Often overlooked, it introduces errors in physiological interpretations, including g_{sw} and c_i.
Patchiness	**Stomatal spatial and temporal heterogeneity:** Uneven stomatal behaviour across the leaf surface distorts gas exchange parameters such as c_i and g_{sw}. **Unpredictability:** Limited understanding of the drivers of patchiness and lack of methods to account for its effects in gas exchange measurements.
Adaxial and abaxial flux differences	**Combining fluxes:** Mixing fluxes from both leaf surfaces may obscure important stress responses and result in misinterpretations.
Steady-state conditions	**Complexity of defining:** Fluctuations and transient responses under stress make it challenging to define and evaluate steady states in gas exchange measurements.
Technical and calibration errors	**Unreliable raw data:** Sensor drift, condensation, lack of equipment maintenance, and unstable environmental conditions can compromise the accuracy of gas exchange measurements (not discussed in detail here, but a key challenge overall).
Flow rate adjustments	**Signal-to-noise ratio:** Balancing the signal-to-noise ratio with maintaining environmental conditions is challenging, particularly for low gas exchange rates under stress.
Practical constraints in fieldwork	**Complexity in measurements:** Translating lab insights, such as the implementation of advanced measurement techniques or specific conditions, to field settings is challenging due to environmental variability and logistical constraints.

To obtain r_{sc} and r_{bc}, the estimates of r_{sw} and r_{bw} are used with the ratio of water over CO_2 diffusivity coefficients ($D_{H_2O,air}/D_{CO_2,air}$), which range around 1.58 ± 0.04, (Massman, 1998), usually taken as 1.6. Then, $r_{sc} = 1.6 r_{sw}$ and from Cowan (1972) $r_{bc} = 1.6^{2/3} r_{bw} = 1.37 r_{bw}$. Then, c_s can be estimated,

$$c_s = \frac{c_a\left(\frac{1}{r_{bc}} - \frac{E}{2}\right) - A}{\frac{1}{r_{bc}} + \frac{E}{2}} \tag{9}$$

Unlike r_{cw}, reliable techniques to estimate r_{cc} are scarce due to the difficulty of obtaining accurate A_c measurements. Currently, r_{cw} is used as a reference with a diffusion coefficient ratio for CO_2 and water through the cuticle between 20 and 40 (Boyer, 2015b; Márquez, Stuart-Williams, & Farquhar, 2021). Regardless, assuming A_c equals 0 and neglecting r_{cc} introduces negligible error in gas exchange calculations (Márquez, Stuart-Williams, & Farquhar, 2021).

Finally, c_i can be estimated as,

$$c_i = \frac{c_s\left(\frac{1}{r_{sc}} + \frac{1}{r_{cc}} - \frac{E_s}{2}\right) - A}{\frac{1}{r_{sc}} + \frac{1}{r_{cc}} + \frac{E_s}{2}} \approx \frac{c_s\left(\frac{1}{r_{sc}} - \frac{E_s}{2}\right) - A}{\frac{1}{r_{sc}} + \frac{E_s}{2}} \tag{10}$$

From the equation presented above, it is evident that the assumptions discussed earlier significantly influence our estimations and propagate through subsequent calculations if incorrectly applied (see Table 1). This highlights how errors in parameter assumptions can undermine the reliability of measurements under stress conditions, ultimately leading to misinterpretations of physiological responses.

3. Risk of Misreading Physiological Trends in Stressed Plants

It is essential to recognise that technical and calibration issues are a significant source of error in gas exchange measurements. Common problems include erroneous calibrations leading to physically impossible values, such as negative c_i, and technical issues related to temperature fluctuations, condensation, or measurements taken during transition states. These errors can compromise the accuracy of raw data, making proper instrument calibration and operation critical before proceeding to parameter estimations. While technical and calibration issues are fundamental and warrant detailed exploration, they are not the focus of this manuscript. Other publications, manuals and reviews, such as Flexas et al. (2007a), Kitao, Harayama and Uemura (2017), LICOR (2020), Garen et al. (2022), Busch et al. (2024) among others, have addressed these topics comprehensively.

Leaving aside possible technical errors and calibration issues that could arise from the instruments and sensors during gas exchange measurements, the main source of error is usually the erroneous estimation of r_{sw} or its inverse stomatal conductance to water ($g_{sw} = 1/r_{sw}$) (Laisk, 1983; Mott, 1995; Boyer, 2015a; Márquez, Stuart-Williams, & Farquhar, 2021; Wong et al., 2022; Márquez et al., 2023a; Hussain et al., 2024). This error can emerge from neglecting some relevant fluxes, such as cuticular transpiration, erroneously assigning values to parameters indirectly estimated, such as w_i, or a more general problem with the used model, such as assuming even stomatal behaviour on the leaf surface (Table 1). Regardless of the source, any parameters derived from r_{sw} and the physiological trends based on those parameters will be compromised, with the likelihood of these errors increasing when dealing with stressed plants.

In this section we explore the potential for misinterpretations of commonly used parameters and physiological trends derived from leaf gas exchange measurements in plant stress research. These parameters are often employed to compare treatments in experiments and to draw conclusions in studies. Thus, misinterpretations can result in inaccurate assessments and potentially flawed conclusions.

3.1. Intrinsic water-use efficiency interpretation

Intrinsic water-use efficiency (*i*WUE = A/g_{sw}) provides a simple yet effective means for evaluating plant water-use efficiency by combining the rate of carbon assimilation with stomatal conductance to water into a single, easily comparable ratio (Flexas et al., 2013; Leakey et al., 2019). This approach is often preferred over direct flux measurements (A/E) as it accounts for the influence of atmospheric vapour pressure deficit (VPD), normalising the data in relation to atmospheric demand. Such normalisation is particularly advantageous in drought and stress research, where it is believed to assist in identifying species or genotypes better adapted to water-limited environments.

Moreover, *i*WUE, combined with techniques like carbon isotope discrimination, enables comparative analyses across plant species and environmental conditions. This makes it valuable for breeding programs, ecological studies, and agronomic research focused on optimising water use (Condon et al., 2004). In this regard, recent studies have gone even further by correlating long-term *i*WUE with nonphotosynthetic $^{12}C/^{13}C$ fractionation in carbon isotope discrimination data from plant biomass. Yu et al. (2024) showed that correcting *i*WUE estimates for this fractionation helps to reconcile discrepancies between isotope-based *i*WUE and those derived from gas exchange measurements, giving a practical approach for long-term *i*WUE estimations.

It is important to note that the strengths of *i*WUE can also be a limitation. While *i*WUE is closely linked to stomatal conductance, it provides a limited snapshot that may not fully capture the plant's overall water use strategy (Liang et al., 2023). Factors such as VPD can influence stomatal conductance and transpiration, creating feedback loops that complicate the assumption of a steady-state relationship among these variables. Additionally, other factors beyond stomatal behaviour can influence transpiration at any given time, potentially leading to incorrectly estimating g_{sw} and biasing *i*WUE analysis, for instance, if factors such as patchiness or unsaturation in the substomatal cavity are neglected.

In this regard, another important consideration beyond the calculation of *i*WUE is its interpretation, which inherently assumes that stomatal aperture is the sole regulator of plant transpiration (Farquhar & Raschke, 1978). However, even if g_{sw} is accurately estimated while accounting for non-stomatal control of transpiration, the interpretation of *i*WUE becomes problematic. Non-stomatal control of transpiration allows for reductions in transpiration rate without impacting carbon gain, meaning transpiration can decrease without stomatal closure. This decoupling makes the traditional definition of *i*WUE (*i*WUE = A/g_{sw}) less straightforward in conditions of substomatal cavity unsaturation (Márquez et al., 2024). Further research is necessary to fully understand the role of non-stomatal factors in regulating transpiration and how these factors may influence the interpretation of *i*WUE measurements.

3.2. Minimum leaf conductance

Minimum leaf conductance to water ($g_{lw,min}$) is a measurement that reflects the lowest conductance to water loss through the leaf surface, typically observed in the dark when stomata are likely closed (Duursma et al., 2018). This conductance is a composite value that reflects both cuticular and minimum stomatal conductance and should not be attributed as a proxy of either (Márquez et al., 2021). The importance of studying $g_{lw,min}$ lies in the fact that plants continue to lose water at night without any corresponding carbon gain, and nocturnal respiration further contributes to carbon depletion (Caird, Richards, & Donovan, 2007; Resco de Dios et al., 2019). In stressed plants, such as those under soil moisture depletion, where water use efficiency becomes even more critical, $g_{lw,min}$ can account for a significant portion of total water use and influence the plant's overall carbon balance.

Under stress conditions, minimum leaf conductance has been observed to vary in response to environmental factors such as low relative humidity and variation in temperature (Duursma et al., 2018; Wang et al., 2024). This raises important questions about the respective contributions of stomatal and cuticular conductance under these conditions and how each responds to environmental stress. Research has shown that the proportion of water transpired through the cuticle relative to that lost through the stomata can vary widely among species and is likely influenced by growth conditions (Caird, Richards, & Donovan, 2007; Márquez et al., 2021). However, detailed information on these responses remains limited and warrants further investigation.

Cuticular conductance can fluctuate when leaves lose turgor (Boyer, 2015b), and there is speculation that it may also change under extreme heat, based on isolated cuticle temperature permeability evaluation (Burghardt & Riederer, 2006). However, existing *in planta* measurements (Márquez, Stuart-Williams, & Farquhar, 2021) do not support this proposition. It is important to note that these measurements have not been extensively conducted across a broad range of species and temperature ranges, highlighting the need for further research on the topic. On the other hand, stomatal conductance is known to vary even in the dark, but the physiological significance of this response is not yet fully understood (Caird, Richards, & Donovan, 2007; Resco de Dios et al., 2019). The physiological role of minimum stomatal conductance also requires more attention to fully understand its impact on plant water and carbon dynamics. Still, in this context, cuticular conductance remains the most elusive and least understood parameter for studying $g_{\mathrm{lw,min}}$.

3.3. CO_2 concentration in the substomatal cavity (c_i)

The concentration of CO_2 within the leaf's air spaces, particularly in the substomatal cavity (c_i), serves as a crucial gateway for understanding the intricacies of photosynthesis and CO_2 diffusion within the leaf (von Caemmerer & Farquhar, 1981; Long & Bernacchi, 2003). Accurately estimating c_i is paramount for plant stress research, as it directly influences the interpretation of physiological responses and the calculation of critical parameters such as photosynthetic capacity (Busch et al., 2024) and mesophyll conductance (Márquez & Busch, 2024).

One of the primary tools for assessing the photosynthesis response under varying conditions is the A-c_i curve, which plots the rate of photosynthesis (A) against the intercellular CO_2 concentration (c_i). This curve is instrumental in diagnosing limitations to photosynthesis, whether they are biochemical (e.g., limitations in the Calvin cycle) or physical (e.g., limitations due to stomatal conductance) (Farquhar, von Caemmerer, & Berry, 1980; von Caemmerer & Farquhar, 1981; Farquhar & Sharkey, 1982; Busch & Sage, 2017). Additionally, the ratio of c_i to ambient CO_2 concentration (c_a) is frequently used to infer the efficiency of CO_2 uptake relative to the external environment, providing a window into the plant's physiological state under stress conditions. Typically, a c_i/c_a ratio of 0.6–0.7 for C_3 plants and 0.3–0.4 for C_4 plants is regarded as optimal or indicative of unstressed conditions (Wong, Cowan, & Farquhar, 1979).

However, the accurate estimation of c_i is fraught with challenges, particularly under stress conditions. The standard approach to estimating c_i involves the calculation of g_{sw} and assumes that this is the sole pathway for gas exchange. This approach, however, overlooks other potential factors, such as cuticular conductance to water (g_{cw}) or the uneven closure of stomata across the leaf surface (stomatal patchiness) (von Caemmerer & Farquhar, 1981). During stress conditions, failing to account for g_{cw} or stomatal patchiness can lead to an overestimation of c_i (Mott, 1995; Boyer, 2015a). Conversely, c_i can be underestimated if the assumption of saturated water vapour in the substomatal cavity, which is typically assumed to be saturated at leaf temperature, does not hold. Under certain stress conditions, such as high temperature or low humidity, the water vapour in the substomatal cavity may not be fully saturated, which can lead to an overestimation of the diffusion gradient and, consequently, an underestimation of c_i (Wong et al., 2022; Cernusak et al., 2024).

Moreover, the internal CO_2 concentration is not uniform throughout the leaf. Gradients of CO_2 are likely to form, particularly in amphistomatous leaves, which have stomata on both the adaxial (upper) and abaxial (lower) surfaces. In such leaves, each surface may have a distinct c_i, influenced by different rates of stomatal conductance and photosynthetic activity (Wong, Cowan, & Farquhar, 1985c; Wong, Cowan, & Farquhar, 1985b; Parkhurst et al., 1988; Wall et al., 2022; Márquez et al., 2023a). Most gas exchange measurements, however, combine signals from both leaf surfaces, resulting in a single, averaged c_i value (Figure 1c). This average c_i is a weighted value, reflecting the relative contributions of the adaxial and abaxial surfaces to the overall assimilation rate (Márquez et al., 2023a), which can obscure the underlying physiological differences between the two surfaces.

Given the critical role of c_i in understanding plant physiology, particularly under stress conditions, it is imperative to ensure that its estimation is accurate and precise. Inaccuracies in c_i estimation can lead to the misinterpretation of physiological trends and erroneous conclusions about a plant's response to stress. Therefore, researchers must carefully consider the potential sources of error, including the effects of cuticular conductance, stomatal patchiness, and unsaturation of water vapour in the substomatal cavity, when interpreting c_i data from experiments in plants under stress.

3.4. Mesophyll conductance

Mesophyll conductance (g_m) refers to the ease with which CO_2 moves from the substomatal cavity to the sites of carboxylation in the chloroplasts (Evans et al., 1986). It plays a crucial role in determining the efficiency of photosynthesis and overall plant productivity. In studies of plants under stress, g_m has gained importance due to the suggested interaction between mesophyll cell wall thickness and composition with water stress tolerance and the potential trade-off that could occur with a reduction in g_m (Clemente-Moreno et al., 2019; Roig-Oliver et al., 2020).

The common calculations of g_m rely on c_i measurements, meaning that any errors in c_i can lead to inaccurate g_m values. Even when c_i is accurately estimated, measuring and comparing g_m under stress conditions presents additional challenges. A key issue arises when measurements are conducted under constant atmospheric CO_2 concentration (c_a) rather than constant c_i (Márquez & Busch, 2024). This approach can introduce inconsistencies when comparing g_m across treatments, as g_m is known to respond to variations of c_i (Flexas et al., 2007b; Vrábl et al., 2009; Márquez & Busch, 2024), potentially compromising the robustness of the analysis and leading to misleading conclusions.

There is substantial evidence that CO_2 concentration affects g_m measurements, although the underlying mechanism is still debated (Flexas et al., 2007b; Hassiotou et al., 2009; Tazoe et al., 2011; Xiong et al., 2015; Busch et al., 2020; Márquez & Busch, 2024). Typically, g_m reaches a maximum at specific c_i levels and decreases with either increasing or decreasing c_i. This variability highlights the difficulty of accurately correlating a single g_m measurement with treatment effects without additional data. Given the impact of CO_2 concentration on g_m estimations, the practice of using constant c_a in experiments should be approached with caution, as it risks leading to incorrect conclusions. Instead, methodologies that account for varying c_i should be employed to ensure more accurate and reliable assessments of g_m variations associated with specific treatments, especially under stress conditions.

4. Small Fluxes (Leaf Cuticle)

Accounting for small fluxes in leaf gas exchange measurements is crucial for accurately calculating gas exchange parameters such as c_i and stomatal conductance (Boyer, 2015a; Márquez et al., 2021). This accuracy becomes particularly important under conditions of low stomatal conductance, such as during drought or low light conditions when stomata are mostly closed. The small fluxes in question primarily refer to the fluxes of water vapour and CO_2 through the cuticle of the leaf (Hanson, Stutz, & Boyer, 2016). Although these fluxes are typically much smaller than those through the stomata, they can become significant when stomatal conductance is low. Neglecting these small fluxes can lead to significant errors in gas exchange calculations, such as miscalculation of stomatal water and CO_2 fluxes, thereby leading to erroneous estimates of c_i (Márquez, Stuart-Williams, & Farquhar, 2021) (Figure 2). Figure 2 presents the differences between two approaches for estimating c_i—the commonly used vCF theory (von Caemmerer & Farquhar, 1981), neglecting cuticular conductance, and the Márquez, Stuart-Williams and Farquhar (MSF) theory (Márquez, Stuart-Williams, & Farquhar, 2021), which includes it. The data show that in conditions of high total leaf surface conductance to water ($g_{lw} = g_{sw} + g_{cw}$), dominated by stomatal conductance, the vCF theory approaches the estimations provided by the MSF theory. The larger the g_{lw}, the closer the results from both theories become, but the MSF theory continues to provide more accurate estimations of c_i, with differences still observed, particularly at moderate g_{lw} values.

The key issue with neglecting g_{cw} arises when g_{sw} is not largely dominating transpiration, specifically when cuticular conductance represents 8% or more of the total conductance in these calculations (Márquez, Stuart-Williams, & Farquhar, 2021; Hussain et al., 2024). In these cases (Figure 2), the vCF theory fails to produce accurate c_i values, with errors reaching up to 100 μmol mol^{-1}. This discrepancy is significant enough to interfere with measurements that rely on precise c_i values, such as A-c_i curves and g_m calculations. Studies have shown that cuticular conductance can vary between species, generally ranging from 5 to 20 mmol m^{-2} s^{-1} (Holmgren, Jarvis, & Jarvis, 1965; Kerstiens, 1996; Boyer, Wong, & Farquhar, 1997; Márquez et al., 2021; Slot et al., 2021). This means that depending on the species, cuticular conductance can become a critical factor when g_{lw} falls below 160 mmol m^{-2} s^{-1}, and in some cases, even when g_{lw} is as high as 250 mmol m^{-2} s^{-1}. Thus, incorporating cuticular conductance into calculations is essential for more accurate c_i estimations, particularly in species with lower stomatal conductance under natural (uncontrolled) or stress conditions where the contribution of the cuticle to gas exchange is substantial.

Additionally, there is the importance of $g_{lw,min}$ and nighttime transpiration leading to water loss without carbon gain (Coupel-Ledru et al., 2016; Resco de Dios et al., 2019; Yu et al., 2019). For accurate analysis, it is essential to separate residual stomatal conductance from cuticular conductance (Márquez et al., 2021), especially in studies focused on plant water relations under stress conditions such as drought. Distinguishing g_{sw} and g_{cw} from $g_{lw,min}$ is crucial for understanding plant behaviour under varying environmental conditions (Duursma et al., 2018). Also, it is important to note that $g_{lw,min}$ is neither equivalent to minimum stomatal conductance nor cuticular conductance, and should not be used as a proxy for either (Márquez et al., 2021). This distinction is critical for assessing how plants regulate water loss by balancing cuticular permeability and stomatal closure, particularly under stress conditions, and provides valuable insights into their adaptive responses to environmental challenges.

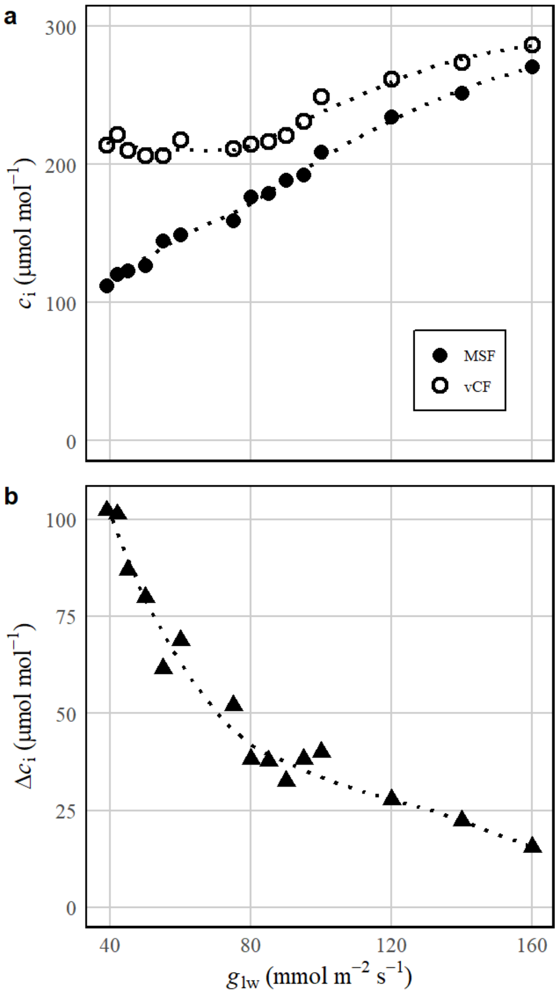

Figure 2. Estimation of substomatal CO_2 concentration (c_i) as a function of total leaf surface conductance to water (g_{lw} = stomatal conductance + cuticular conductance). (**a**) Substomatal CO_2 concentration (c_i) plotted against changes in g_{lw}, which follow the leaf's circadian rhythm. The comparison includes two models to estimate c_i: one that accounts for small fluxes (MFS, solid circles) and one that does not (vCF, open circles). (**b**) The difference in c_i (Δc_i = vCF-MSF, solid triangles) between the two models is plotted against g_{lw}. Measurements were conducted under constant light, vapour pressure deficit, and ambient CO_2 concentration. The estimated cuticular conductance for this leaf is 10 mmol m^{-2} s^{-1}. Data sourced from Márquez, Stuart-Williams and Farquhar (2021). Each point represents a single measurement, calculated independently using the vCF and MFS models.

4.1. Practical approaches

To account for small fluxes and avoid the issues discussed earlier, it is essential to use a model that includes cuticular conductance. The most updated approach is the MSF theory (Márquez, Stuart-Williams, & Farquhar, 2021), which applies ternary corrections to stomatal and boundary layer fluxes while recognising the independence of cuticular fluxes. Although the MSF model is not yet integrated into most commercial gas exchange systems, an add-on script is available for the LI-6800 system (https://github.com/PlantPhysiologist/Add-on-MSF-calculations-for-LI6800 (accessed on 6 of January of 2025)), allowing easy integration with a LI-6800 to obtain real-time parameter recalculation. Additionally, a post-analysis tool recently published (Tholen, 2024) includes the MSF model and can be adapted to various instruments, enabling an analysis of small fluxes from the raw data.

To effectively account for g_{cw} in models, it is essential to accurately estimate it. This estimation is not straightforward; however, a few techniques have been developed to facilitate this measurement. A non-destructive method, the Red-Light method (Márquez et al., 2021), offers a practical solution by measuring gas exchange as the leaf transitions from darkness to red light. This technique enables the estimation of g_{cw} on an attached leaf, and it is particularly useful for continuous *in planta* experiments, as it does not interfere with ongoing measurements (Hussain et al., 2024). Alternatively, traditional methods can be employed (Kerstiens, 1996), such as measuring leaf transpiration in the dark after detaching the leaf and estimating minimum leaf surface conductance ($g_{lw,min}$). However, these methods usually include residual stomatal and cuticular conductance, so they should be used with caution and only when no other options are available.

In field experiments, performing specific analyses, such as the red-light method, can be challenging due to the requirement for dark-acclimated leaves, which complicates measurements when experiments are conducted outdoors during the day. As a result, full quantification of g_{cw} may not always be feasible under field conditions. However, the method introduced by Laisk (1983), which involves comparing the behaviour of the A-c_i curve under different conditions, provides a practical alternative for detecting issues in gas exchange measurements (hereafter referred to as the Laisk method). This approach enables researchers to identify problems such as excessively small fluxes, stomatal patchiness, or general inconsistencies in measurements by comparing observed A-c_i curves based on previously measured under non-stressful conditions to the relation of A and c_i observed during the experiments.

While the Laisk method is highlighted here and in the following sections as a diagnostic tool tailored to field conditions, it is important to note that, although effective in detecting the presence of issues, it does not pinpoint or account for the specific cause of the problem. Despite this limitation, the Laisk approach has been successfully employed to verify the reliability of gas exchange measurements under stressful field conditions. For example, Grassi and Magnani (2005) used the method to ensure measurement reliability while identifying various limitations to photosynthesis in field settings. Thus, the Laisk method provides a valuable strategy for validating gas exchange data in challenging experimental setups.

Finally, if direct measurements of cuticular conductance are not feasible, a tentative correction or estimate of uncertainty can be incorporated by assuming a value of 5 to 10 mmol m^{-2} s^{-1}, which provides a reasonable estimate of uncertainty. However, this should be used with caution, as g_{cw} can vary significantly across species. While g_{cw} seems to remain stable under a range of conditions, including temperatures between 15 °C to 30 °C, g_{cw} may change if the leaf loses turgor or is exposed to extreme temperatures (Schreiber, 2001; Boyer, 2015b). While more research is needed to explore variability across species and conditions further, this practical approach provides a reliable means of accounting for small fluxes and enhancing the accuracy of gas exchange measurements.

5. Unsaturation in the Substomatal Cavity

Unsaturation in the substomatal cavity is the consequence of a recently recognised plant adaptation non-stomatal control of transpiration (Wong et al., 2022), allowing for more nuanced water regulation under high evaporative conditions. Research has shown that non-stomatal control of transpiration plays a vital role in maintaining a favourable microclimate with higher CO_2 concentrations in the mesophyll air space, supporting high carbon fixation under vapour pressure deficit (VPD) stress (Márquez et al., 2024). This mechanism, previously overlooked, causes the water vapour concentration of the substomatal cavity (w_i) to decline below saturation (w_{sat}), contrary to traditional assumptions used in gas exchange measurements (Cernusak et al., 2024).

Traditionally, during gas exchange measurements, it is assumed that the internal leaf air space remained fully saturated with water vapour due to rapid evaporation from the mesophyll surface, with stomatal conductance considered the main regulator of water loss (Gaastra, 1959). This translated into the leaf temperature used as a proxy to estimate w_i, assuming $w_i = w_{sat}$. However, recent studies have shown that unsaturation is a common phenomenon in plants (Cernusak et al., 2018; Wong et al., 2022; Márquez et al., 2024), particularly under moderate to high VPD. The assumption of full saturation has long been a fundamental premise in plant physiology research, but it leads to significant errors when unsaturation is not accounted for.

The error arises from assuming $w_i = w_{sat}$ when, in reality, w_i is below the saturation point. When this is an incorrect assumption, it leads to an underestimation of the vapour gradient that drives transpiration, which in turn results in an underestimation of g_{sw} and c_i (Figure 3). The errors become especially pronounced at higher VPD, where w_i deviates significantly from w_{sat}. This discrepancy can result in underestimating g_{sw} by as much as 20% to 30% and c_i by 50 μmol mol^{-1} or more, being particularly significant in C_4 plants (Márquez et al., 2024). Cotton is used as an example in Figure

3, but studies have shown that substomatal cavity unsaturation can vary between species, with the degree of unsaturation typically increasing under moderate to severe VPD stress (Cernusak et al., 2018; Wong et al., 2022; Márquez et al., 2024).

This variation underscores the importance of incorporating a more reliable estimation w_i into the gas exchange calculations, particularly in water-stressed environments, where plants may experience significant deviations from saturation. These deviations can impact physiological interpretations, such as A-c_i curves, *i*WUE, and g_m, among many other estimations, making accurate accounting of w_i essential for reliable results in the contexts of stressed plants.

Further research is necessary to fully understand the non-stomatal control of transpiration and the role of unsaturation. Current gas exchange models do not account for this process, leaving a gap in our ability to accurately predict or estimate the effects of unsaturation during routine measurements.

5.1. Practical approaches

There are currently four methods to evaluate actual w_i during gas exchange measurements. Three of these methods require a dual chamber setup, which enables independent control and measurement of gas exchange on the upper and lower surfaces of the leaf. (1) The dual chamber using the CO_2 Gradient Method, which creates a CO_2 concentration difference between the two leaf surfaces to assess unsaturation (Wong et al., 2022); (2) the dual chamber using the c_w Correction Method, which focuses on the assimilation rate in response to changes in the minimum CO_2 concentration (c_w) in the leaf airspace (Márquez et al., 2023a); (3) the Inert Gas Method that uses inert gases like nitrous oxide or neon to refine assessments of w_i (Jarvis & Slatyer, 1970; Wong et al., 2022); and (4) the Stable Isotope Method that leverages the equilibrium reached by the exchange of ^{18}O between CO_2 and H_2O in the liquid volume of mesophyll cells to infer w_i (Cernusak et al., 2018). Lastly, a fifth promising technique worth mentioning is the AquaDust Method (Jain et al., 2021), which offers a potential real-time measurement alternative using a fluorescent reporter, making it independent of gas exchange measurements. However, further validation of external measurements with AquaDust is required to ensure consistency compared to more direct techniques. Below is a discussion of each method in more detail.

The dual chamber using the CO_2 Gradient Method leverages reducing the CO_2 concentration on one side of the leaf until the assimilation rate on that side of the leaf is zeroed, creating a CO_2 gradient between the adaxial and abaxial substomatal cavities under benign conditions (assuming saturation). This gradient serves as a baseline to study unsaturation under stress conditions to compute the expected CO_2 gradient during increased stress or to compute the expected resistance to CO_2 diffusion within the leaf (Wong et al., 2022; Márquez et al., 2024). Even though this CO_2 gradient is an artificial setup, it encourages stomatal opening, allowing us an easier study of the non-stomatal control of transpiration and the effects of unsaturation. This trade-off is valuable for studies aimed at isolating non-stomatal mechanisms, but it may not be suitable for more standard measurements that require natural conditions.

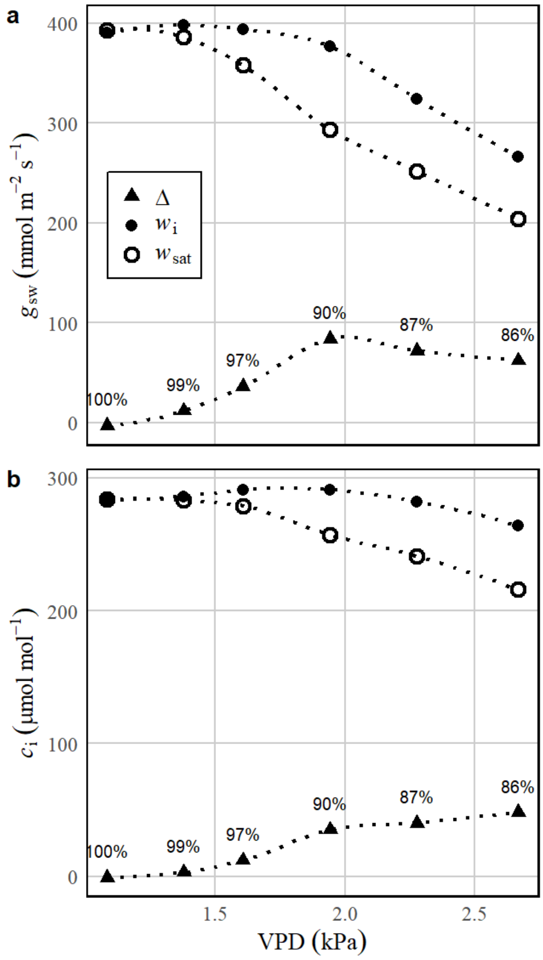

Figure 3. Computation of stomatal conductance (g_{sw}) and substomatal CO_2 concentration (c_i) assuming saturation in the substomatal cavity (w_{sat}) and accounting for actual vapour concentration (w_i) as a function of atmospheric vapour pressure deficit (VPD). (**a**) g_{sw} plotted against VPD, comparing the calculations for w_{sat} (open circles) and w_i (filled circles). The triangles represent the difference (Δ) between these calculations. (**b**) c_i plotted against VPD, with the same model comparisons as in panel (**a**). The percentages at the top of the triangles indicate the relative humidity (RH) in the substomatal cavity calculated as the ratio of w_i/w_{sat}. The species used in this example is cotton. Data sourced from Wong et al. (2022).

The dual chamber using c_w Correction Method monitors the assimilation rate as it responds to changes in the minimum CO_2 concentration (c_w) within the leaf airspace. This approach requires performing a CO_2 response curve under benign conditions (assuming saturation), relating c_w to the measured assimilation rate, and then the rest of the measurements can be taken under any desired atmospheric condition (Márquez et al., 2023a). It does not require zeroing the assimilation rate on either leaf side, allowing measurements to be conducted under standard conditions (i.e., both cuvettes present the same atmospheric conditions). This method also helps identify other stress responses, such as patchy stomatal closure, which might otherwise obscure the effects of unsaturation.

The Inert Gas Method also employs dual chambers but introduces inert gases such as neon (Ne) or nitrous oxide (N_2O) into one of the cuvettes and analyses its diffusion through the leaf to the other cuvette of the system (Jarvis & Slatyer, 1970). Then, in a similar approach as the CO_2 Gradient Method, the apparent changes in inert gas diffusion in relation to vapour diffusion are analysed, and the changes are used to compute w_i (Wong et al., 2022). These gases diffuse differently from atmospheric gases, providing additional constraints that improve the accuracy of w_i estimations in the substomatal cavity. Although this method can offer refined measurements, it requires complex calibration and remains resource-intensive, making it less practical for large-scale or routine studies.

The stable isotope method leverages the measurement of stable oxygen isotope (^{18}O) compositions in CO_2 and H_2O vapour entering and exiting the leaf gas exchange chamber. This technique assumes isotopic equilibrium in the liquid volume within mesophyll cells, such as in the cell wall or chloroplasts, which are closely linked to transpiration and assimilation processes (Cernusak et al., 2018). By using the isotopic composition of transpired water and assimilated CO_2, we can independently assess the expected isotopic composition of both gases. In practice, w_i is adjusted in the calculation until the measured isotopic compositions of CO_2 and H_2O reach a calculated equilibrium. This allows for precise estimation of w_i. A key advantage of this method is that, unlike the dual chamber techniques, it does not rely on the estimation of gas diffusion through the leaf. This opens the door to studying hypostomatous species, where stomata are only present on one side of the leaf, or species with challenging leaf shapes, such as conifers (Cernusak et al., 2024). While the stable isotope method requires specialised equipment and technical expertise, often limiting its application to controlled laboratory environments, it presents a valuable alternative for measuring species and leaf types where other methods face practical limitations.

The AquaDust method offers a new approach to monitoring leaf water potential in real time by injecting a fluorescent reporter into the leaf mesophyll airspace and measuring fluorescence from outside the leaf. AquaDust has shown promising results in tracking changes in leaf water potential using an external probe (Jain et al., 2021). In more specialised setups involving confocal microscopy, it has been able to detect changes in the water potential within the liquid of mesophyll cell walls (Jain et al., 2023; Jain et al., 2024), which can be translated into an estimation of w_i. However, there are important distinctions to consider. While confocal microscopy setups show potential for directly measuring w_i, the need for such specialised equipment and the requirement to remove the leaf from its gas exchange conditions make this approach impractical for routine gas exchange measurements. On the other hand, AquaDust's external probe approach, which measures the average water potential across a section of the leaf, seems to be much more compatible with normal gas exchange setups. It should be noted, however, that this method does not specifically target the substomatal cavity and instead provides a more generalised measurement of water potential across the mesophyll of the leaf. To make probe measurements using AquaDust a reliable tool for estimating w_i, further validation is needed using more targeted techniques such as dual-chamber methods or stable isotope analysis. This validation may need to be species-specific, as differences in leaf structure, such as stomatal density, could influence the relationship between AquaDust's averaged water potential measurements and the actual w_i in the substomatal cavity. Consequently, a universal relationship between AquaDust probe readings and w_i may not apply across all plant species.

The techniques described above present varying levels of difficulty for implementation under field conditions. In practice, either new techniques or instrument adaptations, such as an integrated double chamber, are required to meet these demands. However, such techniques or easily integrated solutions are not yet available. There is evidence that the Laisk method may still be effective in C_4 plants for corroborating whether data align with expected A-c_i curves (Márquez et al., 2024). However, Márquez et al. (2023a) tested this method in C_3 plants and found it inconsistent in detecting unsaturation. The physiological reasons why the method appears reliable in C_4 plants but not in C_3 plants remain to be analysed in detail.

If there is no direct way to quantify w_i during gas exchange measurements, it becomes essential to analyse the uncertainty associated with the estimates by imposing ranges of w_i within known or measured values, such as assuming relative humidity (RH) values between 100% and 80%. While this can offer a useful perspective on the uncertainty involved, it is not sufficient to draw definite conclusions. The need for a direct, simple method to measure w_i or a well-established mechanistic model to estimate it remains a priority. Until such measurements become standard for gas exchange measurements for plants under stress, uncertainties in w_i will limit the robustness of gas exchange analyses when w_i is not measured, making it difficult to assess the physiological responses of plants under stress fully.

6. Patchiness

The phenomenon of patchiness in stomatal movements refers to the spatial and temporal heterogeneity in the behaviour of stomata across the leaf surface. Rather than acting uniformly, stomata in discrete areas or "patches" may respond differently to environmental stimuli than those in adjacent regions. This can result in small, localised groups of stomata exhibiting distinct patterns of opening and closing that are not mirrored across the entire leaf.

Patchiness is particularly pronounced in plants under stress, such as during water deficit conditions or fluctuations in light intensity. Under such stresses, stomatal movements become more erratic and less synchronised across the whole leaf. For example, water stress can induce non-uniform stomatal closure, leading to areas with significantly different gas exchange capabilities within the same leaf (Downton, Loveys, & Grant, 1988). It is speculated that this heterogeneity represents an adaptive strategy, allowing parts of the leaf to maintain gas exchange and photosynthesis while other areas are conserving water by closing their stomata (Mott, Cardon, & Berry, 1993).

In gas exchange measurements, it is typically assumed that stomatal conductance is uniform across the leaf surface, allowing a single parameter calculation to represent the leaf surface, such as g_{sw}, c_i, assimilation rate, etc. However, patchy stomatal behaviour can significantly impact gas exchange estimates. Non-uniform stomatal responses may introduce artefacts in the data, complicating the accurate interpretation of whole-leaf gas exchange measurements (Cardon, Mott, & Berry, 1994; Mott & Buckley, 2000). For instance, when patchiness is present, the assumption of uniform conductance can lead to misestimation of c_i and *i*WUE (Laisk, 1983; Mott, 1995). Figure 4 illustrates the distortion of the A-c_i relationship caused by patchy stomatal closure. Panel (a) shows time-lapse data from flowering crabapple (*Malus dolgo*) after ABA treatment, and panel (b) presents steady-state measurements in sunflower (*Helianthus annuus*) under elevated VPD, both highlighting the effects of patchiness on gas exchange dynamics.

A significant challenge in understanding and modelling patchy stomatal behaviour is that the drivers of patchiness are not well understood, and the evidence explaining its occurrence remains limited. Patchiness is highly variable, not only between species and environmental conditions but also between different leaves on the same plant (Kaiser & Kappen, 1997, 2000; Mott & Buckley, 2000). While some models, such as those proposed by Mott and Buckley (2000) and Cardon, Mott and Berry (1994), suggest hydraulic interactions leading to coordinated stomatal opening and closing within patches, this remains speculative. In fact, much of the available evidence suggests a lack of consistent coordination, particularly between the two leaf surfaces.

For example, studies such as Mott, Cardon and Berry (1993) have shown that stomata on the adaxial and abaxial surfaces can behave independently, responding asymmetrically to environmental stimuli such as under stress conditions like low humidity. However, they also found that stomata can respond semi-symmetrically under certain conditions, such as when humidity is decreased on one surface, suggesting some level of communication between the two sides. This inconsistent variability in stomatal responses complicates the modelling of patches responses and raises important questions about how these erratic behaviours can be accounted for in gas exchange studies (Lawson, Weyers, & A'Brook, 1998; Grantz, Karr, & Burkhardt, 2020).

There is even less evidence to support the idea of coordination between stomatal patches. While patches might independently respond to the same environmental cues, potentially resulting in similar overall patterns, the behaviour of one patch does not seem to significantly influence another distant patch (Cardon, Mott, & Berry, 1994). Lawson, Weyers and A'Brook (1998) observed erratic stomatal responses within individual patches, both spatially and temporally, further suggesting that the behaviour of one patch is largely independent of others. This lack of coordination is likely due to the spatial separation and the distinct micro-environments that different patches experience. As Cardon, Mott and Berry (1994) and Kaiser and Kappen (2000) suggested, possibly the physical distance between patches decreases the likelihood of direct hydraulic or chemical signal transference, making it highly unlikely that patches would act in a coordinated fashion.

Overall, the evidence points to more erratic and independent behaviour of stomata both within and between patches. This unpredictability poses a significant challenge for modelling and interpreting gas exchange, as assumptions of uniformity or coordination are unlikely to hold true (Downton, Loveys, & Grant, 1988). Given this complexity, the most reliable approach at present is to use the available techniques that can directly measure patchiness or track its effects on gas exchange, allowing us to detect its occurrence. More research is necessary to better understand the mechanisms behind patchiness, its variability across species, and its implications for physiological measurements, especially in stressed plants.

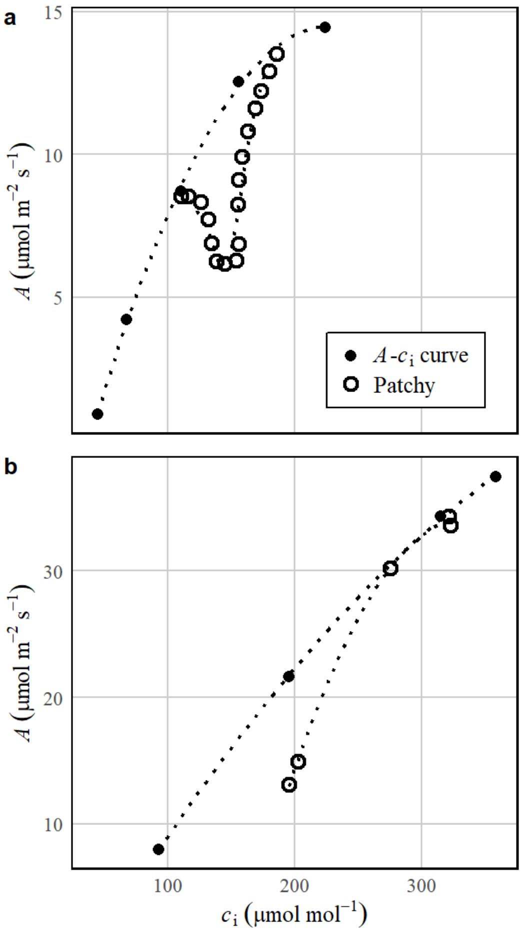

Figure 4. The effect of stomatal patchiness on the assimilation rate (A) vs. CO_2 concentration in the substomatal cavity (c_i) relationship (A-c_i). (**a**) Filled circles represent the A-c_i curve under steady-state and benign conditions. Open circles show how patchy stomatal closure distorts this curve over time after ABA application to induce patchiness in *Malus dolgo*. Time progression in the open circles moves from right to left. Data from Mott (1995). (**b**) Filled circles represent the A-c_i curve under steady-state and benign conditions, while open circles show steady-state measurements at elevated VPDs to induce patchiness in *Helianthus annuus*. Data sourced from Márquez et al. (2023a). The VPD increase imposed for each open circle in panel (b) was 1, 1.2, 1.5, 2, and 2.5 kPa, progressing from right to left as depicted in the figure.

6.1. Practical approaches

Detecting patchiness in stomatal movements during gas exchange measurements requires various sophisticated techniques, each with strengths and limitations.

Chlorophyll fluorescence imaging visualises the spatial distribution of photosynthesis, providing an indirect measure of stomatal patchiness by highlighting areas of varying photosynthetic activity (Mott, 1995). Although informative, this method does not directly quantify the impact of patchiness on gas exchange, limiting its ability to provide comprehensive insights into gas exchange data. Microscopic observation offers direct measurements of individual stomatal apertures, revealing detailed variability in stomatal behaviour (Mott & Buckley, 2000). However, it is labour-intensive, limited to small areas of the leaf, and does not provide information about whole-leaf gas exchange (Lawson, Weyers, & A'Brook, 1998). It is, therefore, not ideal for large-scale measurements or for capturing the full complexity of stomatal responses across the leaf. Similarly, infrared thermography detects temperature variations across the leaf surface, which can be indicative of differences in transpiration rates and thus offer a broader view of patchiness (Mott & Buckley, 2000). While useful for detecting regional differences in water loss, it is sensitive to external temperature fluctuations, which can compromise its accuracy under varying environmental conditions.

Most common techniques, such as chlorophyll fluorescence imaging and infrared thermography, provide insights into the occurrence of patchiness but do not directly inform gas exchange measurements unless specialised (often custom-built) equipment is used to integrate these techniques with gas exchange setups. Therefore, while some methods allow researchers to track the effects of patchiness, others focus on measuring its direct impact on gas exchange, highlighting the need for more advanced and integrative approaches.

Real-time monitoring of stomatal movements using microscope videos, as presented by Sun et al. (2021), provides continuous, high-resolution data that detects dynamic changes in stomatal behaviour. When combined with gas exchange, this method allows for some degree of accounting for the effects of patchiness. However, it requires sophisticated equipment and advanced analytical tools, making it less accessible for routine use.

Márquez et al. (2023a) introduced a technique to evaluate the effects of patchiness and unsaturation by comparing CO_2 response curves under benign and stressful conditions (the previously mentioned c_w Correction Method). While this method helps detect the occurrence of patchiness and account for unsaturation in gas exchange, it does not quantify the level of patchiness or directly observe the spatial behaviour of patches.

In general, current techniques allow us to detect the occurrence of patchiness and quantify its degree in an area but do not provide a way to account for this variability directly in gas exchange measurements. To address patchiness effectively with gas exchange measurements, a model that relates effective surface area and conductances under gas exchange would be needed. One potential approach could involve tracking the mismatch of the A-c_i relation (Mott, 1995), using the Laisk method, giving an apparent conductance reduction, which would allow corrections but will not give information about the patches.

It has been suggested that new models could account for the variability of patches as independent sections of the leaf, with multiple fluxes and resistances in parallel (Laisk, 1983; Rockwell et al., 2022). This would allow for a more nuanced representation of stomatal patchiness by treating different patches as separate entities within the leaf. However, this approach would not be directly compatible with current systems and methods of gas exchange measurements. For now, the best approach remains directly measuring patchiness or tracking its effects on gas exchange, which in practice is detection rather than fully accounting for it in gas exchange measurements. Continued research into methods for more effective modelling and compensating for patchiness will be critical for improving the accuracy of gas exchange studies in plants under stress.

7. Other Important Considerations

7.1. Standardising conditions for valid comparison

Ensuring that environmental conditions are standardised across treatments is critical for valid comparisons in gas exchange studies, particularly in stress conditions where the target variable is often linked to multiple physiological effects. Stomatal conductance, which governs gas exchange, is sensitive to changes in temperature, light intensity, CO_2 availability, and vapour pressure deficit (VPD), among other factors (Cowan & Farquhar, 1977). In parallel, biochemical processes involved in photosynthesis and respiration are primarily influenced by temperature, light intensity, and CO_2 availability but are not directly affected by VPD (Wong, Cowan, & Farquhar, 1985a; Wong, Cowan, & Farquhar, 1985c; Wong, Cowan, & Farquhar, 1985b; Busch et al., 2013). These distinctions are important because gas exchange measurements integrate stomatal and biochemical responses. As a result, isolating their individual effects on the net gas exchange requires careful control of experimental conditions.

Since stomatal conductance and biochemical processes respond to different environmental factors, controlling interrelated variables becomes essential. Such is the case of temperature, RH, and VPD, which are closely interrelated, and while gas exchange systems allow for their control, adjusting one parameter often influences the others. Similarly, leaf temperature, transpiration rate, and light intensity are interdependent, particularly when stomatal conductance is low. In such conditions, reduced transpiration cooling increases the leaf-to-air temperature difference. As light intensity increases, it can further raise leaf temperature, and if stomatal conductance is low, this heat builds up and can increase the leaf-to-air temperature gradient.

A prime example of these interactions is the relationship between temperature and VPD. Stomatal conductance responds to both temperature and VPD, while the biochemical processes in photosynthesis, particularly enzyme activity, are primarily influenced by temperature. As temperature rises, VPD typically increases, which can induce partial stomatal closure and reduce CO_2 diffusion into the leaf. Without careful control of these variables, the effect of temperature on gas exchange can be misinterpreted, as changes in temperature may also involve indirect effects from VPD. It is important to note that maintaining constant RH does not ensure a constant VPD, since VPD is a function of temperature and vapour concentration, not the relative vapour concentration difference. For example, increasing the temperature from 25 °C to 30 °C while holding RH constant at 70% causes the VPD to rise from 0.95 kPa to 1.27 kPa—a 34% increase, even though RH remains unchanged.

Due to this interdependence, it is essential to standardise environmental conditions in order to isolate the variable of interest for the study at hand. Failure to account for the complex interactions between temperature, VPD, RH, light intensity, and CO_2 availability can obscure the physiological processes being studied, potentially leading to misleading conclusions.

7.2. Steady-state conditions

Under stress conditions, fluctuations over time, irregular behaviours, transient responses, and downward trends in gas exchange parameters, such as stomatal conductance, assimilation rate, and transpiration, become more common (Mott, Cardon, & Berry, 1993; Cardon, Mott, & Berry, 1994; Wong et al., 2022). As a result, special care must be taken when defining what constitutes a steady state under these circumstances or how researchers define "steady state" in their work.

Finding a single, universal definition of gas exchange steady-state conditions is challenging, as it often depends on the specific objectives of the experiment and the physiological response being studied. While many publications implicitly assume a shared understanding of steady-state conditions—usually described as "steady for this experiment"—this can be problematic when working with stressed plants, where gas exchange tends to fluctuate more erratically. In such cases, the definition of steady-state needs to be clearly articulated to ensure accurate interpretation of results, particularly when analysing the complex dynamics of stressed plants.

It is essential to define how stability was determined in the study clearly. This includes describing the acceptable range within which the input and output gas concentrations (CO_2 and H_2O) in the gas exchange chamber were allowed to fluctuate. Also, the criteria for evaluating consistency in key physiological parameters, such as stomatal conductance, assimilation rate, and transpiration rate. A practical approach would be to report the stability criteria in terms of:

1. *Duration of the stability period:* The length of time the leaf remained stable to be considered a steady state.
2. *Input/output gas concentration fluctuations:* The allowable variability of CO_2 and H_2O concentrations entering and exiting the chamber during the stability period.
3. *Variability in stomatal conductance:* The peak-to-peak variation in stomatal conductance (g_{sw}) within the defined stability period.
4. *Variability in assimilation rate:* The peak-to-peak variation in the assimilation rate (A) over the same period.
5. *Variability in transpiration rate:* The peak-to-peak variation in the transpiration rate (E) within the stability period.

By providing these specific criteria, researchers can ensure that stability is clearly defined and reproducible across experiments.

7.3. Adaxial and abaxial gas exchange

When analysing gas exchange in plants, it is recommended to consider the adaxial (upper) and abaxial (lower) surfaces independently, especially under stress conditions. While it is common practice to combine adaxial and abaxial fluxes due to the limitations of standard commercial instruments, this approach may overlook important details, particularly in stressed plants.

Under stress conditions, the stomatal responses of the adaxial and abaxial surfaces often behave independently or respond differently to the same stimuli. Studies have shown that the two sides of the leaf can react in distinct ways to environmental stressors, such as water deficit or changes in light intensity (Wong, Cowan, & Farquhar, 1985c; Wong, Cowan, & Farquhar, 1985b; Wall et al., 2022; Márquez et al., 2023a). By mixing fluxes from both surfaces, part of the plant's stress response can be missed, potentially leading to an incomplete understanding of the physiological adaptations.

While combining fluxes might not necessarily result in incorrect conclusions, it could mask the more subtle differences in how each surface adapts to stress (see for example Márquez et al. (2023a) and Collaviti et al. (2024)). Therefore, for a more accurate and detailed analysis of gas exchange, especially under stress conditions, it is recommended to measure the adaxial and abaxial surfaces independently to capture these independent responses. Custom-built or adaptations of commercial instruments exist in the literature to incorporate independent measurements of leaves' adaxial and abaxial surfaces (e.g., Márquez et al. (2023b) for LI-6800 or Wall et al. (2022) for LI-6400).

7.4. Incoming flow rate

Researchers may need to modify the chamber's flow rate depending on their experimental objectives when studying plants under stress, carefully balancing the signal-to-noise ratio with the need to maintain specific environmental conditions. Decreasing the flow rate can help detect smaller changes in gas exchange, particularly when gas exchange rates are low under stress conditions. A lower flow rate increases the concentration differential between incoming and outgoing gases, capturing subtle changes more effectively. However, this also introduces certain limitations. Reducing the flow rate increases the time required to stabilise conditions within the chamber, with this delay being proportional to the chamber volume. Larger chambers and lower flow rates mean that it takes longer to reach desired environmental conditions, which may be a limiting factor (e.g., Farquhar, Griffani and Barbour (2021)). For example, achieving low RH becomes challenging when plant transpiration rate significantly affects humidity levels within the chamber, making it difficult to lower RH at reduced flow rates.

On the other hand, increasing the flow rate may be necessary when the aim is to create conditions like low RH in the chamber, particularly in stress experiments where controlling humidity is critical. Higher flow rates flush the chamber more quickly, preventing transpiration from raising RH to levels that would interfere with the desired conditions. While increasing the flow rate facilitates the creation of such conditions, it can also reduce the signal-to-noise ratio, as smaller changes in gas exchange become harder to detect due to the smaller concentration differential between incoming and outgoing gases.

Thus, researchers must carefully consider the objectives of their experiments and how flow rate adjustments will influence both the environmental conditions they wish to achieve and the precision of gas exchange measurements. Achieving accurate data collection while meeting the experimental goals requires careful management of the chamber's flow rate settings.

7.5. Boundary layer

The mostly unexplored effect of boundary layer conductance on stomatal behaviour adds further uncertainty to the heterogeneity and erratic patterns sometimes observed in stomatal responses, such as patchiness. Bridging insights from laboratory-controlled settings to natural environments requires addressing these interactions to mimic field conditions accurately. In this regard, boundary layer conductance plays an important role in gas exchange measurements, particularly when studying plants under stress. In natural conditions, the boundary layer surrounding a leaf is typically thicker than in the chamber of a gas exchange system and may interact with other stressors, such as water deficit. As a result, the leaf does not directly experience the ambient relative humidity and CO_2 concentrations, as the boundary layer acts as a buffer. This interaction between the boundary layer and stressors can influence how plants respond to their environment (Schuepp, 1993).

Researchers may wish to explore the effects of the boundary layer in their experiments, particularly to understand how a thicker boundary layer in natural settings influences plant stress responses. Adjusting the mixing fan speed in gas exchange chambers can modify boundary layer conductance. Increasing fan speed typically enhances boundary layer conductance, reducing noise in stomatal conductance calculations. On the other hand, decreasing the fan speed or modifying chamber conditions to simulate a thicker boundary layer may help mimic natural conditions more accurately.

It is crucial, however, to be cautious when making such adjustments. Commercial gas exchange instruments are calibrated to operate within specific boundaries, and modifying the boundary layer too drastically—such as by excessively reducing fan speed —can violate these specifications. Doing so may lead to unintended consequences, such as insufficient mixing, which can create unintended gradients within the chamber and result in inaccurate readings. To ensure data quality, researchers must either remain within the specified conditions of the instrument or find a way to account for the effect of altered boundary layer conductance without compromising the integrity of the measurements.

In summary, while exploring the interaction between boundary layer thickness and stress responses can provide valuable insights, it is important to balance these adjustments with the technical specifications of the instruments to maintain the accuracy and reliability of the data.

7.6. Thermodiffusion and its impact on transpiration

Thermodiffusion refers to the movement of water vapour driven by temperature gradients rather than solely by vapour concentration gradients, making it particularly relevant when there is a temperature difference between the leaf and the surrounding air. A recent study by Griffani, Rognon and Farquhar (2024) significantly advanced our understanding of the effects of thermodiffusion on transpiration rates. Thermodiffusion becomes particularly important when stomatal conductance is low, such as during abiotic stress (e.g., drought) or in darkness, where transpiration is already reduced. In extreme cases—such as when there is a significant temperature difference between the leaf and the air, combined with a small water vapour concentration difference across the boundary layer—thermodiffusion can account for more than 30% of total transpiration. This is particularly relevant when boundary layer conductance significantly exceeds stomatal conductance, a common scenario in gas exchange chamber experiments. While boundary layer conductance is typically larger than stomatal conductance in these chambers, the issue becomes problematic under specific, less common conditions, such as when the stomata are nearly closed, and there is a substantial temperature gradient between the leaf and the surrounding air.

Thermodiffusion can also contribute to reverse transpiration, where water vapour is absorbed by the leaf instead of being lost. This phenomenon occurs when external conditions, such as high humidity or cool temperatures, create a reverse water vapour gradient. While this is not common under normal conditions, it becomes relevant when there is a combination of large temperature gradients and high external humidity—conditions that may arise during extreme salt stress or dark periods. Understanding and accounting for thermodiffusion may be crucial in such cases to avoid misinterpreting water loss or gain in the leaf, particularly in experiments involving stressed plants where transpiration dynamics are altered.

8. Conclusions

Gas exchange measurements have long been a cornerstone of plant physiology research, providing critical insights into the interactions between plants and their environment. Their strength lies in the simplicity of the methodology and the flexibility of modern instruments, which enable precise control over experimental conditions. However, the ease with which these measurements are obtained can sometimes lead to overlooking the fundamental assumptions that underlie them—assumptions that often do not hold under stress conditions.

This manuscript has discussed and provided practical approaches to address these limitations, particularly under stress, which is summarised in Table 2. By identifying areas where traditional assumptions may falter, we offer strategies to improve measurement accuracy in stress and non-stress scenarios. Our focus has been on incorporating often overlooked elements such as small fluxes, cuticular conductance, substomatal cavity unsaturation, and stomatal patchiness—factors critical for accurate gas exchange data, especially in stressed plants. These approaches aim to refine the methods, ensuring more reliable results and a deeper understanding of plant physiological responses across diverse environmental conditions.

While this manuscript has addressed key challenges in gas exchange measurements, further research is needed in areas such as cuticular conductance and its relationship to leaf surface composition, the variability and mechanisms behind substomatal cavity unsaturation, and the complexities of non-uniform stomatal behaviour, including patchiness. By incorporating the methods and approaches discussed here, we aim to facilitate addressing these gaps through further research and thus advance our understanding of plant physiology.

Table 2. Overview of methods and techniques for addressing small fluxes, unsaturation, and patchiness in routine gas exchange measurements of stressed plants.

Approach	Addresses	Consideration	References
Accounting for small fluxes	Small Fluxes	**Pros**: Accurately includes cuticular conductance (g_{cw}) for improved estimates of g_{sw} and c_i. **Cons**: None. **Additional needs**: Requires previous measurement of g_{cw} (some alternative methods for estimating g_{cw}: Márquez et al. (2021) and Kerstiens (1996)).	Márquez, Stuart-Williams and Farquhar (2021)
Chlorophyll Fluorescence Imaging	Patchiness	**Pros**: Visualises photosynthesis distribution. **Cons**: Indirect measure; requires integration with gas exchange setups for complete analysis. **Additional needs**: Requires specialised or homemade setups to combine with gas exchange measurements.	Cardon, Mott and Berry (1994) Lawson, Weyers and A'Brook (1998)
Infrared Thermography	Patchiness	**Pros**: Broad detection of transpiration levels across the leaf. **Cons**: Indirect measure; Sensitive to external temperature changes, limiting reliability in variable environments. **Additional needs**: Integration with gas exchange systems is required to link temperature with gas fluxes.	Downton, Loveys and Grant (1988) Cardon, Mott and Berry (1994)
Microscopic Observation	Patchiness	**Pros**: Direct measurement of stomatal aperture variability. **Cons**: Limited to small leaf areas and labour-intensive. **Additional needs**: Can inform patchiness but lacks direct gas exchange data integration.	Mott, Cardon and Berry (1993) Lawson, Weyers and A'Brook (1998) Mott and Buckley (2000)
Real-time Monitoring via Microscopy	Patchiness	**Pros**: It allows for high-resolution dynamic observation of stomatal movements. **Cons**: It requires advanced equipment and data analysis tools. **Additional needs**: It can be combined with a gas exchange for detailed patchiness tracking.	Mott, Cardon and Berry (1993) Sun et al. (2021)
Dual-chamber CO_2 Gradient Method	Unsaturation	**Pros**: Effective in assessing unsaturation by measuring CO_2 difference between leaf surfaces. **Cons**: Imposes low CO_2 concentration during the measurements. **Additional needs**: Requires dual-chamber setup and careful calibration.	Wong et al. (2022)
Inert Gas Method	Unsaturation	**Pros**: It provides an unbiased analysis of gas flux through the leaf. **Cons**: It requires complex calibration and is resource-intensive. **Additional needs**: It requires a double chamber and instruments capable of measuring the concentration of the inert gas precisely, and it is not easily adaptable for large-scale use.	Jarvis and Slatyer (1970) Wong et al. (2022)
Stable Isotope Method	Unsaturation	**Pros**: it can be applied to any shape or leaf structure. **Cons**: it requires specialised and costly laboratory equipment. **Additional needs**: it requires instruments to measure the isotopic composition of ^{18}O in CO_2 and H_2O, and integration with gas exchange systems requires calibration.	Cernusak et al. (2018)
AquaDust Method	Unsaturation	**Pros:** Real-time, non-invasive tracking of leaf water potential via fluorescence. **Cons:** Provides an averaged water potential, which may not target the substomatal cavity specifically. **Additional needs:** Further validation is needed, especially correlating it with more targeted techniques like gas exchange systems or isotope analysis.	Jain et al. (2021)
Dual-chamber c_w Correction Method	Unsaturation and Patchiness	**Pros**: It is able to account for unsaturation and detect patchiness using the same setup and measurements. **Cons**: It does not directly quantify patchiness and lacks direct spatial analysis of patchiness. **Additional needs**: A double chamber is required.	Márquez et al. (2023a)
Laisk Method for Field Conditions	Checking the reliability of gas exchange data	**Pros:** Allows for evaluation of data reliability in field conditions in a relatively easy and practical way. **Cons:** Does not provide corrections, accountability, or identification of the source of unreliable data if found. **Additional needs:** Requires performing an A-c_i curve under a baseline condition to use as a reference.	Laisk (1983)

Author Contributions

D.A.M. and F.A.B. developed the initial concept of the manuscript. D.A.M. wrote the first draft and A.G. provided practical insights. All authors contributed to writing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

All data discussed in this study are included in the main manuscript or referenced to publicly accessible sources. For further information or requests for additional materials, please contact D.A.M.

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Conflicts of Interest

The authors declare no competing financial interests.

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References

Boyer JS. (2015a). Impact of cuticle on calculations of the CO_2 concentration inside leaves. *Planta*, *242*, 1405–1412.

Boyer JS. (2015b). Turgor and the transport of CO_2 and water across the cuticle (epidermis) of leaves. *Journal of Experimental Botany*, *66*(9), 2625–2633.

Boyer JS, Wong SC, & Farquhar GD. (1997). CO_2 and water vapor exchange across leaf cuticle (Epidermis) at various water potentials. *Plant Physiology*, *114*, 185–191.

Buckley TN, & Sack L. (2019). The humidity inside leaves and why you should care: implications of unsaturation of leaf intercellular airspaces. *American Journal of Botany*, *106*(5), 618–621. https://doi.org/10.1002/ajb2.1282

Burghardt M, & Riederer M. (2006). Cuticular Transpiration. In M Riederer & C Muller (Eds.), *Annual Plant Reviews Volume 23: Biology of the Plant Cuticle* (Vol. 23, pp. 292–311). https://doi.org/10.1002/9780470988718.ch9

Busch FA, Ainsworth EA, Amtmann A, Cavanagh AP, Driever SM, Ferguson JN, Kromdijk J, Lawson T, Leakey ADB, Matthews JSA, Meacham-Hensold K, Vath RL, Vialet-Chabrand S, Walker BJ, & Papanatsiou M. (2024). A guide to photosynthetic gas exchange measurements: Fundamental principles, best practice and potential pitfalls. *Plant, Cell & Environment*, *47*(9), 3344–3364. https://doi.org/10.1111/pce.14815

Busch FA, Holloway-Phillips M, Stuart-Williams H, & Farquhar GD. (2020). Revisiting carbon isotope discrimination in C_3 plants shows respiration rules when photosynthesis is low. *Nature Plants*, *6*(3), 245–258. https://doi.org/10.1038/s41477-020-0606-6

Busch FA, & Sage RF. (2017). The sensitivity of photosynthesis to O_2 and CO_2 concentration identifies strong Rubisco control above the thermal optimum. *New Phytologist*, *213*(3), 1036–1051. https://doi.org/10.1111/nph.14258

Busch FA, Sage TL, Cousins AB, & Sage RF. (2013). C_3 plants enhance rates of photosynthesis by reassimilating photorespired and respired CO_2. *Plant, Cell & Environment*, *36*(1), 200–212. https://doi.org/10.1111/j.1365-3040.2012.02567.x

Caird MA, Richards JH, & Donovan LA. (2007). Nighttime Stomatal Conductance and Transpiration in C_3 and C_4 Plants. *Plant Physiology*, *143*(1), 4–10. https://doi.org/10.1104/pp.106.092940

Cardon ZG, Mott KA, & Berry JA. (1994). Dynamics of patchy stomatal movements, and their contribution to steady-state and oscillating stomatal conductance calculated using gas-exchange techniques. *Plant, Cell & Environment*, *17*(9), 995–1007. https://doi.org/10.1111/j.1365-3040.1994.tb02033.x

Cernusak LA, Goldsmith GR, Arend M, & Siegwolf RTW. (2019). Effect of vapor pressure deficit on gas exchange in wild-type and abscisic acid–insensitive plants. *Plant Physiology*, *181*(4), 1573–1586. https://doi.org/10.1104/pp.19.00436

Cernusak LA, Ubierna N, Jenkins MW, Garrity SR, Rahn T, Powers HH, Hanson DT, Sevanto S, Wong SC, McDowell NG, & Farquhar GD. (2018). Unsaturation of vapour pressure inside leaves of two conifer species. *Scientific Reports*, *8*(7667), 1–7.

Cernusak LA, Wong SC, Stuart-Williams H, Márquez DA, Pontarin N, & Farquhar GD. (2024). Unsaturation in the air spaces of leaves and its implications. *Plant, Cell & Environment*, *47*(10), 3685–3698. https://doi.org/10.1111/pce.15001

Clemente-Moreno MJ, Gago J, Díaz-Vivancos P, Bernal A, Miedes E, Bresta P, Liakopoulos G, Fernie AR, Hernández JA, & Flexas J. (2019). The apoplastic antioxidant system and altered cell wall dynamics influence mesophyll conductance and the rate of photosynthesis. *Plant Journal*, *99*(6), 1031–1046. https://doi.org/10.1111/tpj.14437

Collaviti SE, Stuart-Williams H, Farquhar GD, Cernusak LA, & Márquez DA. (2024). Unsaturation and approximate isotopic homogeneity in leaf air spaces. *bioRxiv*. https://doi.org/10.1101/2024.09.30.610858

Condon AG, Richards RA, Rebetzke GJ, & Farquhar GD. (2004). Breeding for high water-use efficiency. *Journal of Experimental Botany*, *55*(407), 2447–2460. https://doi.org/10.1093/jxb/erh277

Coupel-Ledru A, Lebon E, Christophe A, Gallo A, Gago P, Pantin F, Doligez A, & Simonneau T. (2016). Reduced nighttime transpiration is a relevant breeding target for high water-use efficiency in grapevine. *Proceedings of the National Academy of Sciences*, *113*(32), 8963–8968. https://doi.org/10.1073/pnas.1600826113

Cowan IR. (1972). Mass and heat transfer in laminar boundary layers with particular reference to assimilation and transpiration in leaves. *Agricultural Meteorology*, *10*, 311–329.

Cowan IR, & Farquhar GD. (1977). Stomatal function in relation to leaf metabolism and environment. *Symp Soc Exp Biol*, *31*, 471–505.

Deans RM, Brodribb TJ, Busch FA, & Farquhar GD. (2020). Optimization can provide the fundamental link between leaf photosynthesis, gas exchange and water relations. *Nature Plants*, *6*(9), 1116–1125. https://doi.org/10.1038/s41477-020-00760-6

Downton WJS, Loveys BR, & Grant WJR. (1988). Non-Uniform Stomatal Closure Induced by Water Stress Causes Putative Non-Stomatal Inhibition of Photosynthesis. *The New Phytologist*, *110*(4), 503–509. http://www.jstor.org/stable/2434912

Duursma RA, Blackman CJ, Lopez R, Martin-StPaul NK, Cochard H, & Medlyn BE. (2018). On the minimum leaf conductance: its role in models of plant water use, and ecological and environmental control. *New Phytologist*, (221), 693–705. https://doi.org/10.1111/nph.15395

Evans JR, Sharkey TD, Berry JA, & Farquhar GD. (1986). Carbon isotope discrimination measured concurrently with gas exchange to investigate CO_2 diffusion in leaves of higher plants. *Functional Plant Biology*, *13*(2), 281–292. https://doi.org/10.1071/PP9860281

Farquhar GD, Griffani DS, & Barbour MM. (2021). The effects on isotopic composition of leaf water and transpiration of adding a gas-exchange cuvette. *Plant, Cell & Environment*, *44*(9), 2844–2857. https://doi.org/10.1111/pce.14076

Farquhar GD, & Raschke K. (1978). On the resistance to transpiration of the sites of evaporation within the leaf. *Plant Physiology*, *61*(6), 1000–1005. https://doi.org/10.1104/pp.61.6.1000

Farquhar GD, & Richards RA. (1984). Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes [Article]. *Australian Journal of Plant Physiology*, *11*(6), 539–552. https://doi.org/10.1071/pp9840539

Farquhar GD, & Sharkey TD. (1982). Stomatal conductance and photosynthesis. *Annual Review Plant Physiology*, *33*, 317–345. https://doi.org/10.1146/annurev.pp.33.060182.001533

Farquhar GD, von Caemmerer S, & Berry JA. (1980). A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta*, *149*(1), 78–90. https://doi.org/10.1007/BF00386231

Flexas J, Díaz-Espejo A, Berry J, Cifre J, Galmés J, Kaldenhoff R, Medrano H, & Ribas-Carbó M. (2007a). Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. *Journal of Experimental Botany*, *58*(6), 1533–1543. https://doi.org/10.1093/jxb/erm027

Flexas J, Diaz-Espejo A, Galmes J, Kaldenhoff R, Medrano H, & Ribas-Carbo M. (2007b). Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. *Plant, Cell & Environment*, *30*(10), 1284–1298. https://doi.org/10.1111/j.1365-3040.2007.01700.x

Flexas J, Niinemets Ü, Gallé A, Barbour MM, Centritto M, Diaz-Espejo A, Douthe C, Galmés J, Ribas-Carbo M, Rodriguez PL, Rosselló F, Soolanayakanahally R, Tomas M, Wright IJ, ... Medrano H. (2013). Diffusional conductances to CO_2 as a target for increasing photosynthesis and photosynthetic water-use efficiency. *Photosynthesis Research*, *117*(1), 45–59. https://doi.org/10.1007/s11120-013-9844-z

Fu Z, Ciais P, Wigneron J-P, Gentine P, Feldman AF, Makowski D, Viovy N, Kemanian AR, Goll DS, Stoy PC, Prentice IC, Yakir D, Liu L, Ma H,…Smith WK. (2024). Global critical soil moisture thresholds of plant water stress. *Nature Communications*, *15*(1), 4826. https://doi.org/10.1038/s41467-024-49244-7

Gaastra P. (1959). *Photosynthesis of crop plants as influenced by light, carbon dioxide, temperature, and stomatal diffusion resistance*. Meded. Landbouwhogeschool, Wageningen.

Garen JC, Branch HA, Borrego I, Blonder B, Stinziano JR, & Michaletz ST. (2022). Gas exchange analysers exhibit large measurement error driven by internal thermal gradients. *New Phytologist*, *236*(2), 369–384. https://doi.org/10.1111/nph.18347

Grantz DA, Karr M, & Burkhardt J. (2020). Heterogeneity of Stomatal Pore Area Is Suppressed by Ambient Aerosol in the Homobaric Species, Vicia faba. *Front Plant Sci*, *11*, 897. https://doi.org/10.3389/fpls.2020.00897

Grassi G, & Magnani F. (2005). Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. *Plant, Cell & Environment*, *28*(7), 834–849. https://doi.org/10.1111/j.1365-3040.2005.01333.x

Griffani DS, Rognon P, & Farquhar GD. (2024). The role of thermodiffusion in transpiration. *New Phytologist*, *243*(4), 1301–1311. https://doi.org/10.1111/nph.19642

Grossiord C, Buckley TN, Cernusak LA, Novick KA, Poulter B, Siegwolf RTW, Sperry JS, & McDowell NG. (2020). Plant responses to rising vapor pressure deficit. *New Phytologist*, *226*(6), 1550–1566. https://doi.org/10.1111/nph.16485

Hanson D, Stutz SS, & Boyer JS. (2016). Why small fluxes matter: the case and approaches for improving measurements of photosynthesis and (photo) respiration. *Journal of Experimental Botany*, *67*, 3027–3039.

Hassiotou F, Ludwig M, Renton M, Veneklaas EJ, & Evans JR. (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. https://doi.org/10.1093/jxb/erp021

Holmgren P, Jarvis P, & Jarvis M. (1965). Resistances to carbon dioxide and water vapour transfer in leaves of different plant species. *Physiologia Plantarum*, *18*, 557–573.

Hussain SB, Stinziano J, Pierre MO, & Vincent C. (2024). Accurate photosynthetic parameter estimation at low stomatal conductance: effects of cuticular conductance and instrumental noise. *Photosynthesis Research*, *160*(2), 111–124. https://doi.org/10.1007/s11120-024-01092-8

Jain P, Huber AE, Rockwell FE, Sen S, Holbrook NM, & Stroock AD. (2023). Localized measurements of water potential reveal large loss of conductance in living tissues of maize leaves. *Plant Physiology*, *194*(4), 2288–2300. https://doi.org/10.1093/plphys/kiad679

Jain P, Huber AE, Rockwell FE, Sen S, Holbrook NM, & Stroock AD. (2024). New approaches to dissect leaf hydraulics reveal large gradients in living tissues of tomato leaves. *New Phytologist*, *242*(2), 453–465. https://doi.org/10.1111/nph.19585

Jain P, Liu W, Zhu S, Chang CY-Y, Melkonian J, Rockwell FE, Pauli D, Sun Y, Zipfel WR, Holbrook NM, Riha SJ, Gore MA, & Stroock AD. (2021). A minimally disruptive method for measuring water potential in planta using hydrogel nanoreporters. *Proceedings of the National Academy of Sciences*, *118*(23), e2008276118. https://doi.org/10.1073/pnas.2008276118

Jarvis PG, & Slatyer RO. (1970). The role of the mesophyll cell wall in leaf transpiration. *Planta*, *90*(4), 303–322. https://doi.org/10.1007/BF00386383

Kaiser H, & Kappen L. (1997). In situ observations of stomatal movements in different light-dark regimes: the influence of endogenous rhythmicity and long-term adjustments1. *Journal of Experimental Botany*, *48*(8), 1583–1589. https://doi.org/10.1093/jxb/48.8.1583

Kaiser H, & Kappen L. (2000). In situ observation of stomatal movements and gas exchange of Aegopodium podagraria L. in the understorey. *Journal of Experimental Botany*, *51*(351), 1741–1749. https://doi.org/10.1093/jexbot/51.351.1741

Kerstiens G. (1996). Cuticular water permeability and its physiological significance. *Journal of Experimental Botany*, *47*(305), 1813–1832.

Kitao M, Harayama H, & Uemura A. (2017). A practical approach to estimate diffusional leakages of leaf chamber of open gas exchange systems using intact leaves. *Plant, Cell & Environment*, *40*(11), 2870–2874. https://doi.org/10.1111/pce.13032

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

Lawson T, Weyers J, & A'Brook R. (1998). The nature of heterogeneity in the stomatal behaviour of *Phaseolus vulgaris* L. primary leaves. *Journal of Experimental Botany*, *49*(325), 1387–1395. https://doi.org/10.1093/jxb/49.325.1387

Leakey ADB, Ferguson JN, Pignon CP, Wu A, Jin Z, Hammer GL, & Lobell DB. (2019). Water Use Efficiency as a Constraint and Target for Improving the Resilience and Productivity of C_3 and C_4 Crops. *Annual Review of Plant Biology*, *70*(Volume 70, 2019), 781–808. https://doi.org/10.1146/annurev-arplant-042817-040305

Liang J, Krauss KW, Finnigan J, Stuart-Williams H, Farquhar GD, & Ball MC. (2023). Linking water use efficiency with water use strategy from leaves to communities. *New Phytologist*, *240*(5), 1735–1742. https://doi.org/10.1111/nph.19308

LICOR B. (2020). *Using the LI-6800 Version 1.4*. LI-COR, Inc. https://licor.app.boxenterprise.net/s/2yrup4d2nq0n07sur6a7xph893tyr284

Long SP, & Bernacchi CJ. (2003). Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *Journal of Experimental Botany*, *54*(392), 2393–2401. https://doi.org/10.1093/jxb/erg262

Márquez DA, & Busch FA. (2024). The interplay of short-term mesophyll and stomatal conductance responses under variable environmental conditions. *Plant, Cell & Environment*, *47*(9), 3393–3410. https://doi.org/10.1111/pce.14880

Márquez DA, Stuart-Williams H, Cernusak LA, & Farquhar GD. (2023a). Assessing the CO_2 concentration at the surface of photosynthetic mesophyll cells. *New Phytologist*, *238*(4), 1446–1460. https://doi.org/10.1111/nph.18784

Márquez DA, Stuart-Williams H, & Farquhar GD. (2021). An improved theory for calculating leaf gas exchange more precisely accounting for small fluxes. *Nature Plants*, *7*, 317–326. https://doi.org/10.1038/s41477-021-00861-w

Márquez DA, Stuart-Williams H, Farquhar GD, & Busch FA. (2021). Cuticular conductance of adaxial and abaxial leaf surfaces and its relation to minimum leaf surface conductance. *New Phytologist*, *233*(1), 156–168. https://doi.org/10.1111/nph.17588

Márquez DA, Stuart-Williams H, Wong SC, & Farquhar GD. (2023b). An improved system to measure leaf gas exchange on adaxial and abaxial surfaces. *Bio-protocol*, *13*(11), e4687. https://doi.org/10.21769/BioProtoc.4687

Márquez DA, Wong SC, Stuart-Williams H, Cernusak LA, & Farquhar GD. (2024). Mesophyll airspace unsaturation drives C_4 plant success under vapour pressure deficit stress. *Proceedings of the National Academy of Sciences*, *121*(39). https://doi.org/10.1073/pnas.2402233121

Massman WJ. (1998). A review of the molecular diffusivities of H_2O, CO_2, CH_4, CO, O_3, SO_2, NH_3, N_2O, NO, and NO_2 in air, O_2 and N_2 near STP. *Atmospheric Environment*, *32*(6), 1111–1127. https://doi.org/10.1016/S1352-2310(97)00391-9

Moss DN, & Rawlins SL. (1963). Concentration of carbon dioxide inside leaves. *Nature*, *197*(4874), 1320–1321. https://doi.org/10.1038/1971320a0

Mott KA. (1995). Effects of patchy stomatal closure on gas exchange measurements following abscisic acid treatment. *Plant, Cell & Environment*, *18*(11), 1291–1300. https://doi.org/10.1111/j.1365-3040.1995.tb00188.x

Mott KA, & Buckley TN. (2000). Patchy stomatal conductance: emergent collective behaviour of stomata. *Trends in Plant Science*, *5*(6), 258–262. https://doi.org/10.1016/S1360-1385(00)01648-4

Mott KA, Cardon ZG, & Berry JA. (1993). Asymmetric patchy stomatal closure for the two surfaces of *Xanthium strumarium* L. leaves at low humidity. *Plant, Cell & Environment*, *16*(1), 25–34. https://doi.org/10.1111/j.1365-3040.1993.tb00841.x

Parkhurst DF, Wong SC, Farquhar GD, & Cowan IR. (1988). Gradients of intercellular CO_2 levels across the leaf mesophyll. *Plant Physiology*, *86*(4), 1032–1037. https://doi.org/10.1104/pp.86.4.1032

Resco de Dios V, Chowdhury FI, Granda E, Yao Y, & Tissue DT. (2019). Assessing the potential functions of nocturnal stomatal conductance in C_3 and C_4 plants. *New Phytologist*, *223*(4), 1696–1706. https://doi.org/10.1111/nph.15881

Rockwell FE, Holbrook NM, Jain P, Huber AE, Sen S, & Stroock AD. (2022). Extreme undersaturation in the intercellular airspace of leaves: a failure of Gaastra or Ohm? *Annals of Botany*, *130*(3), 301–316. https://doi.org/10.1093/aob/mcac094

Roig-Oliver M, Nadal M, Clemente-Moreno MJ, Bota J, & Flexas J. (2020). Cell wall components regulate photosynthesis and leaf water relations of *Vitis vinifera* cv. Grenache acclimated to contrasting environmental conditions. *Journal of Plant Physiology*, *244*, 153084. https://doi.org/10.1016/j.jplph.2019.153084

Scholander PF, Bradstreet ED, Hemmingsen EA, & Hammel HT. (1965). Sap Pressure in Vascular Plants. *Science*, *148*(3668), 339–346. https://doi.org/10.1126/science.148.3668.339

Schreiber L. (2001). Effect of temperature on cuticular transpiration of isolated cuticular membranes and leaf discs. *Journal of Experimental Botany*, *52*(362), 1893–1900. https://doi.org/10.1093/jexbot/52.362.1893

Schuepp PH. (1993). Tansley Review No. 59. Leaf Boundary Layers. *The New Phytologist*, *125*(3), 477-507. http://www.jstor.org/stable/2558258

Sharkey TD. (2016). What gas exchange data can tell us about photosynthesis. *Plant, Cell & Environment*, *39*(6), 1161–1163. https://doi.org/10.1111/pce.12641

Slot M, Nardwattanawong T, Hernández GG, Bueno A, Riederer M, & Winter K. (2021). Large differences in leaf cuticle conductance and its temperature response among 24 tropical tree species from across a rainfall gradient. *New Phytologist*, *232*(4), 1618–1631. https://doi.org/10.1111/nph.17626

Sun Z, Song Y, Li Q, Cai J, Wang X, Zhou Q, Huang M, & Jiang D. (2021). An Integrated Method for Tracking and Monitoring Stomata Dynamics from Microscope Videos. *Plant Phenomics*, *2021*. https://doi.org/10.34133/2021/9835961

Tazoe Y, von Caemmerer S, Estavillo GM, & Evans JR. (2011). Using tunable diode laser spectroscopy to measure carbon isotope discrimination and mesophyll conductance to CO_2 diffusion dynamically at different CO_2 concentrations. *Plant, Cell & Environment*, *34*(4), 580–591. https://doi.org/10.1111/j.1365-3040.2010.02264.x

Tholen D. (2024). GasanalyzeR: advancing reproducible research using a new R package for photosynthesis data workflows. *AoB PLANTS*, *16*(4). https://doi.org/10.1093/aobpla/plae035

Tominaga J, & Kawamitsu Y. (2015). Cuticle affects calculations of internal CO_2 in leaves closing their stomata. *Plant and Cell Physiology*, *56*(10), 1900–1908.

Turner NC, Schulze ED, & Gollan T. (1984). The responses of stomata and leaf gas exchange to vapour pressure deficits and soil water content. *Oecologia*, *63*(3), 338–342. https://doi.org/10.1007/BF00390662

von Caemmerer S. (2000). *Biochemical models of leaf photosynthesis*. CSIRO Publishing. https://doi.org/10.1071/9780643103405

von Caemmerer S. (2013). Steady-state models of photosynthesis. *Plant, Cell & Environment*, *36*(9), 1617–1630. https://doi.org/10.1111/pce.12098

von Caemmerer S, & Farquhar GD. (1981). Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta*, *153*, 376–387.

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. https://doi.org/10.1093/jxb/erp115

Wall S, Vialet-Chabrand S, Davey P, Van Rie J, Galle A, Cockram J, & Lawson T. (2022). Stomata on the abaxial and adaxial leaf surfaces contribute differently to leaf gas exchange and photosynthesis in wheat. *New Phytologist*, *235*(5), 1743–1756. https://doi.org/10.1111/nph.18257

Wang S, Hoch G, Grun G, & Kahmen A. (2024). Water loss after stomatal closure: quantifying leaf minimum conductance and minimal water use in nine temperate European tree species during a severe drought. *Tree Physiology*, *44*(4). https://doi.org/10.1093/treephys/tpae027

Wong SC, Canny MJ, Holloway-Phillips M, Stuart-Williams H, Cernusak LA, Márquez DA, & Farquhar GD. (2022). Humidity gradients in the air spaces of leaves. *Nature Plants*, *8*, 971–978. https://doi.org/10.1038/s41477-022-01202-1

Wong SC, Cowan IR, & Farquhar GD. (1979). Stomatal conductance correlates with photosynthetic capacity. *Nature*, *282*(5737), 424–426. https://doi.org/10.1038/282424a0

Wong SC, Cowan IR, & Farquhar GD. (1985a). Leaf conductance in relation to rate of CO_2 assimilation I. Influence of nitrogen nutrition, phosphorus nutrition, photon flux density, and ambient partial pressure of CO_2 during ontogeny. *Plant Physiology*, *78*(4), 821–825. https://doi.org/10.1104/pp.78.4.821

Wong SC, Cowan IR, & Farquhar GD. (1985b). Leaf conductance in relation to rate of CO_2 assimilation II. Effects of short-term exposures to different photon flux densities assimilation. *Plant Physiology*, *78*(4), 826–829. https://doi.org/10.1104/pp.78.4.826

Wong SC, Cowan IR, & Farquhar GD. (1985c). Leaf conductance in relation to rate of CO_2 assimilation III. Influences of water stress and photoinhibition. *Plant Physiology*, *78*(4), 830–834. https://doi.org/10.1104/pp.78.4.830

Xiong D, Liu XI, Liu L, Douthe C, Li Y, Peng S, & Huang J. (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. https://doi.org/10.1111/pce.12558

Yan W, Zhong Y, & Shangguan Z. (2016). A meta-analysis of leaf gas exchange and water status responses to drought. *Scientific Reports*, *6*(1), 20917. https://doi.org/10.1038/srep20917

Yu K, Goldsmith GR, Wang Y, & Anderegg WRL. (2019). Phylogenetic and biogeographic controls of plant nighttime stomatal conductance. *New Phytologist*, *222*(4), 1778–1788. https://doi.org/10.1111/nph.15755

Yu YZ, Ma WT, Wang X, Tcherkez G, Schnyder H, & Gong XY. (2024). Reconciling water-use efficiency estimates from carbon isotope discrimination of leaf biomass and tree rings: Nonphotosynthetic fractionation matters. *New Phytologist*, *244*(6). 2225–2238. https://doi.org/10.1111/nph.20170