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Cuticular conductance of adaxial and abaxial leaf surfaces and its relation to minimum leaf surface conductance

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Summary

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Key words: abaxial cuticular conductance, adaxial cuticular conductance, amphistomatous leaf, cuticular conductance to water, leaf gas exchange, nocturnal conductance, photosynthetic induction.

- Cuticular conductance to water (g_{cw}) is difficult to quantify for stomatous surfaces due to the complexity of separating cuticular and stomatal transpiration, and additional complications arise for determining adaxial and abaxial g_{cw} . This has led to the neglect of g_{cw} as a separate parameter in most common gas exchange measurements. Here, we describe a simple technique to simultaneously estimate adaxial and abaxial values of g_{cw} , tested in two amphistomatous plant species.
- What we term the 'Red-Light method' is used to estimate g_{cw} from gas exchange measurements and a known CO₂ concentration inside the leaf during photosynthetic induction under red light. We provide an easy-to-use web application to assist with the calculation of g_{cw} .
- While adaxial and abaxial g_{cw} varies significantly between leaves of the same species we found that the ratio of adaxial/abaxial g_{cw} (γ_n) is stable within a plant species. This has implications for use of generic values of g_{cw} when analysing gas exchange data.
- The Red-Light method can be used to estimate total cuticular conductance (g_{cw-T}) accurately with the most common setup of gas exchange instruments, i.e. a chamber mixing the adaxial and abaxial gases, allowing for a wide application of this technique.

Introduction

Leaf transpiration is the composite of water lost through the stomata and water transpired from the leaf cuticle, and the rate with which water is lost through them is determined by the stomatal and cuticular conductances to water (g_{sw} and g_{cw} ; abbreviations are summarized in Table 1). Cuticular conductance impacts estimations of gas exchange on different scales. It forms part of modelling water loss of developing leaves (Kane *et al.*, 2020), and affects the estimation of water-use efficiency and other gas exchange parameters from the leaf scale (Mizokami *et al.*, 2015; Tominaga & Kawamitsu, 2015; Hanson *et al.*, 2016; Márquez *et al.*, 2021) to the ecological scale (Barnard & Bauerle, 2013; Laning *et al.*, 2020). Even though cuticular conductance is important at different scales, g_{cw} is routinely neglected in gas exchange calculations due to the inherent difficulties of estimating it separately from g_{sw} , which is usually the main factor determining gas diffusion through the leaf surfaces under illuminated conditions. Including g_{cw} can provide an accurate assessment of the intercellular CO₂ concentration (c_i) and thus of modelled photosynthetic CO₂ uptake (Boyer, 2015a). This is true in particular when plants are exposed to stressful conditions, such as drought or low light, and stomata are largely closed. Accounting for g_{cw} when modelling photosynthesis can therefore broaden the range of environmental conditions under which reliable estimates of photosynthetic parameters can be obtained.

The most common methods for estimating cuticular conductance *in planta* are (1) to measure the 'minimum conductance' (Duursma *et al.*, 2018) or (2) to measure the conductance of an astomatous surface (Boyer *et al.*, 1997). The first approach, determining g_{cw} from the leaf surface conductance (g_{lw}), brings with it the assumption that the stomata are completely closed while the second assumes that the adaxial and abaxial g_{cw} are equal. Though separating cuticular transpiration from stomatal transpiration has been regarded as almost impossible when both fluxes are present (Sheriff, 1984), another group of approaches (3) estimates cuticular conductance from independent measurements of adaxial and abaxial leaf gas exchange (Boyer, 2015a; Tominaga & Kawamitsu, 2015; Márquez *et al.*, 2021). The methods vary in the mathematics and equipment used but are based on the impact of cuticular conductance on the balance of water and CO₂ crossing through the leaf surface. They estimate g_{cw} for a single surface of the leaf, and so to obtain results on a projected leaf area basis some previous knowledge of the g_{cw} ratio of the two surfaces is required.

Additional complications arise when trying to determine adaxial and abaxial cuticular conductances independently. Measurements of weight loss in detached hypostomatous leaves treated to avoid stomatal opening have shown differences in adaxial and abaxial cuticular transpiration (Zhang *et al.*, 2020). However, these results must be assessed carefully as the leaf dehydrates during the measurements, which has been reported to cause cuticular conductance to vary (Boyer, 2015b). Nevertheless, studies on isolated

Table 1 List of abbreviations and subscripts used in the text, including units of parameters.

Abbreviation	Name	Unit
A	Net CO ₂ assimilation rate	mol m ⁻² s ⁻¹
β	Ratio g_{cc}/g_{cw}	—
c_a	CO ₂ concentration in the atmosphere	mol mol ⁻¹
c_i	CO ₂ concentration in the intercellular air space of a leaf	mol mol ⁻¹
c_s	CO ₂ concentration at the surface of a leaf	mol mol ⁻¹
E_c	Rate of transpiration through the cuticle	mol m ⁻² s ⁻¹
E_s	Rate of transpiration through the stomata	mol m ⁻² s ⁻¹
E	Transpiration rate	mol m ⁻² s ⁻¹
g_{bc}	Boundary layer conductance to CO ₂	mol m ⁻² s ⁻¹
g_{bw}	Boundary layer conductance to water	mol m ⁻² s ⁻¹
g_{cc}	Cuticular conductance to CO ₂	mol m ⁻² s ⁻¹
g_{cw}	Cuticular conductance to water	mol m ⁻² s ⁻¹
g_{lc}	Conductance to CO ₂ through the surface of a leaf (g_{sc} and g_{cc})	mol m ⁻² s ⁻¹
g_{lw}	Conductance to water through the surface of a leaf (g_{sw} and g_{cw})	mol m ⁻² s ⁻¹
$g_{lw-dark}$	Dark-acclimated minimum leaf surfaces conductance to water	mol m ⁻² s ⁻¹
g_{lw-min}	Minimum leaf surfaces conductance to water (dark-acclimated + abscisic acid)	mol m ⁻² s ⁻¹
g_{mc}	Mesophyll conductance to CO ₂	mol m ⁻² s ⁻¹
g_{sc}	Stomatal conductance to CO ₂	mol m ⁻² s ⁻¹
g_{sw}	Stomatal conductance to water	mol m ⁻² s ⁻¹
g_{tc}	Total conductance to CO ₂ (g_{bc} , g_{sc} and g_{cc})	mol m ⁻² s ⁻¹
g_{tw}	Total conductance to water (g_{bw} , g_{sw} and g_{cw})	mol m ⁻² s ⁻¹
Γ	CO ₂ compensation point	mol mol ⁻¹
Γ^*	CO ₂ compensation point in absence of mitochondrial respiration	mol mol ⁻¹
γ_n	Ratio g_{cw-ad}/g_{cw-ab}	—
κ	Ratio c_{i-ad}/c_{i-ab}	—
w_a	Water vapour concentration in the atmosphere	mol mol ⁻¹
w_i	Water vapour concentration in the intercellular air space of a leaf	mol mol ⁻¹
w_s	Water vapour concentration at the surface of a leaf	mol mol ⁻¹
Subscript ab	The abaxial face of the leaf	
Subscript ad	The adaxial face of the leaf	
Subscript T	Total leaf, adaxial plus abaxial faces of the leaf	

cuticles indirectly support the hypothesis of different surfaces having different g_{cw} , showing different physical and chemical properties for adaxial and abaxial sides (Onoda *et al.*, 2012; Li *et al.*, 2020) as well as different permeabilities for adaxial and abaxial isolated cuticles (Karbalková *et al.*, 2008). However, there is no clear relationship between cuticular conductance and cuticle composition and thickness (Yeats & Rose, 2013; Duursma *et al.*, 2018), nor for isolated cuticle permeability and *in planta* cuticular conductance (Kerstiens, 2006; Zhang *et al.*, 2020). Thus, still little is known about adaxial and abaxial cuticular conductance differences in single leaves or between leaves of different species.

Here we describe a new technique that allows the estimation of adaxial and abaxial cuticular conductance inferring the CO₂ concentration inside the leaf (c_i) from measurements of leaf gas exchange and taking advantage of the physiological response of

stomata to red light (henceforth referred to as the Red-Light method). The Red-Light method is based on the observation that the activation of photosynthetic biochemistry generally occurs faster than stomatal opening during the initial phase of photosynthetic induction (Deans *et al.*, 2019a; Deans *et al.*, 2019b) and on the well-known stomatal responses to different light intensities and wavelengths, where stomata open more slowly at low intensities of red than of blue light (Shimazaki *et al.*, 2007). Broadly, the method consists of generating a stable gas exchange condition where stomata are noticeably limiting the diffusion of CO₂ into the leaf, while at the same time ensuring photosynthetic biochemistry is fully active. This leads to the net CO₂ assimilation rate (A) being largely limited by CO₂ diffusion through the stomata. We calculate cuticular conductance applying the equation for g_{cw} derived by Márquez *et al.* (2021), which depends on knowing c_i as the only critical input parameter. As A under the experimental conditions is close to zero, c_i must be close to the CO₂ compensation point (Γ), allowing us to infer a value for c_i . This approximation, as we show here, is sufficiently precise to yield an accurate estimate of g_{cw} .

To evaluate our results using the Red-Light method for estimating g_{cw} , a separate set of experiments was performed using an independent method. This second method was presented and tested in Márquez *et al.* (2021) (henceforth MSF method) and consists of measuring adaxial and abaxial gas exchange independently when stomata are open. Therefore, the Red-Light and MSF methods have opposite approaches to estimating g_{cw} : the Red-Light method relies on knowing c_i and holds when the stomata remain nearly closed, while the MSF method relies on stomata staying open and therefore can be used to infer a value of the ratio of adaxial to abaxial c_i (κ).

Finally, the values of g_{cw} obtained using the Red-Light method for each leaf surface are contrasted with values of leaf surface conductances (g_{lw}) measured in the dark with and without application of the phytohormone abscisic acid (ABA), which causes stomatal closure (Mittelheuser & Van Steveninck, 1969; Raschke, 1975; Munemasa *et al.*, 2015). These two kinds of measurements, along with others, are often called ‘minimum conductance’ (Duursma *et al.*, 2018). Strictly speaking, the minimum leaf surface conductance is the cuticular conductance under the current leaf conditions plus the stomatal conductance when the stomata are minimally open, such as found in the dark after forcing maximum stomatal closure with ABA application. Here, the term minimum leaf surface conductance (g_{lw-min}) is used when the leaf is dark-acclimated and ABA is applied, and the dark-acclimated minimum leaf surface conductance ($g_{lw-dark}$) is adopted for the leaf surface conductance achieved after a long dark acclimation period without ABA application. We assess the contribution of the g_{cw} component in g_{lw-min} and $g_{lw-dark}$, and the implications of its inclusion in the estimations.

Materials and Methods

Theory

Gas exchange parameters are usually calculated relying on the assumption that total transpiration (E) is occurring through the

stomata only, neglecting cuticular transpiration. Then, stomatal conductance to water (g_{sw}) is estimated from E and used to calculate stomatal conductance to CO_2 (g_{sc}) and subsequently the CO_2 concentration inside the leaf. As the measured E consists of water transpired through stomata (E_s) as well as through the cuticle (E_c), this assumption carries different levels of error depending on the gas exchange conditions. For instance, neglecting g_{cw} introduces large errors into the calculations when leaf surface conductance is low ($g_{lw} < 160 \text{ mmol m}^{-2} \text{ s}^{-1}$; Márquez *et al.* (2021)) such as under high vapour pressure deficit, soil water stress, or low light (see Mizokami *et al.*, 2015; Tominaga & Kawamitsu, 2015; Hanson *et al.*, 2016). Here, we compare two methods for estimating g_{cw} , the Red-Light method and the MSF method.

Red-Light method Instead of calculating the internal CO_2 concentration c_i from E via g_{sw} , for the Red-Light method we take the opposite approach by calculating the gas exchange parameters from the rate of net CO_2 uptake (A), with known concentrations of CO_2 in the air surrounding the leaf (c_a) and inside the leaf (c_i), the latter being estimated under some assumptions (detailed later). Then, g_{sw} can be derived from g_{sc} , which allows the separation of g_{cw} and g_{sw} from the total leaf surface conductance to water (g_{lw}). For the calculations, the equations for leaf gas exchange proposed by Márquez *et al.* (2021) were used. These equations include g_{cw} from the outset of the derivation, so we can solve for g_{cw} and obtain (see Supporting Information Notes S1):

$$g_{cw} = \frac{(c_s - c_i)G_i - (A + c_s E)}{(c_s - c_i)\alpha - c_s D}, \quad \text{Eqn 1}$$

where

$$\begin{aligned} D &= w_i - w_s, \\ G_i &= \frac{E}{1.6(w_i - w_s)}(1 - \bar{w}_s) + \frac{E}{2}, \\ \bar{w}_s &= \frac{w_i + w_s}{2}, \\ w_s &= \frac{w_a + \left(1 - \frac{w}{2}\right) \frac{E}{g_{bw}}}{1 + \frac{E}{2g_{bw}}}, \\ \alpha &= \frac{1 - \bar{w}_s}{1.6} + \frac{D}{2} - \beta, \\ \beta &= \frac{g_{cc}}{g_{cw}} \end{aligned}$$

where w_i represents the vapour concentration in the intercellular air space of the leaf, w_s is the vapour concentration at the surface of the leaf, w_a is the vapour concentration in the atmosphere, g_{bw} is the boundary layer conductance to water and g_{cc} is the cuticular conductance to CO_2 . Equation 1 accounts for the impacts of cuticular flux on the gas exchange, e.g. interaction with stomatal transpiration, with A and with gaseous concentration near the leaf surface. To help with calculations using Eqn 1, we provide a

web-application (www.plantphysiologist.com/gcwCi), which requires data such as net assimilation rate, transpiration rate, atmospheric CO_2 concentration, etc. as provided from standard gas exchange devices (see details in Notes S2).

Another approach to approximating cuticular conductance when c_i is known is using von Caemmerer & Farquhar (1981) equations including cuticular conductance as a correction factor. The cuticular conductance to water, g_{cw} , is derived from the mismatch between the total conductance to CO_2 (g_{tc}) estimated as usual from von Caemmerer & Farquhar (1981) equations (g_{tc-vCF}), which incorrectly assume that E occurs through the stomata solely, and the estimate of g_{tc-ci} from a known c_i . Then, asserting that boundary layer conductance is great enough to be neglected, g_{cw} is approximated by the difference between the two estimates, calculated as (Notes S3):

$$g_{cw} \approx 1.6[g_{tc-vCF} - g_{tc-ci}] = 1.6 \left[g_{tc-vCF} - \frac{A}{(c_a - c_i)} \right]. \quad \text{Eqn 2}$$

This simplification is identical to the equation presented by Mizokami *et al.* (2015), Boyer (2015a) and Tominaga & Kawamitsu (2015), where it is used to separate stomatal and cuticular conductance from leaf surface conductance assuming that the whole mismatch is due to cuticular conductance. Equation 2 differs from Eqn 1 in ignoring the effects of g_{bw} and g_{cc} , the interaction between stomatal and cuticular fluxes as well as the impact of ternary effects. All these factors are considered to have an unimportant impact on the outcome when Eqn 2 is used. Here, we tested how precise this approach is for general gas exchange measurements and calculations.

MSF method Using the MSF method, another set of experiments was performed on nonstressed plants with open stomata, for which κ (= ratio of adaxial to abaxial c_i) often has a value of 1.0 (Mott & O'Leary, 1984; Parkhurst *et al.*, 1988; Syvertsen *et al.*, 1995; Márquez *et al.*, 2021). These measurements were conducted to estimate g_{cw} of one surface when c_i is unknown, using the experimental setup ($\kappa \approx 1$) and model presented by Márquez *et al.* (2021):

$$ag_{cw-ab}^2 + bg_{cw-ab} + c = 0, \quad \text{Eqn 3}$$

where the subscripts 'ad' and 'ab' refer to adaxial and abaxial surfaces of the leaf; and

$$\begin{aligned} a &= (c_{s-ad} - c_{s-ab}\kappa)\alpha_{ad}\alpha_{ab}\gamma_n + \kappa c_{s-ab}D_{ab}\alpha_{ad}\gamma_n \\ &\quad - c_{s-ad}D_{ad}\alpha_{ab}\gamma_n \\ b &= (c_{s-ad}D_{ad}G_{i-ab}\gamma_n + (A_{ad} + c_{s-ad}E_{T-ad})\alpha_{ab}) \\ &\quad - (c_{s-ad} - c_{s-ab}\kappa)(\alpha_{ad}G_{i-ab}\gamma_n + G_{i-ad}\alpha_{ab}) \\ &\quad - \kappa(c_{s-ab}D_{ab}G_{i-ad} + (A_{ab} + c_{s-ab}E_{ab})\alpha_{ad}\gamma_n) \\ c &= (c_{s-ad} - c_{s-ab}\kappa)G_{i-ad}G_{i-ab} - (A_{ad} + c_{s-ad}E_{ad})G_{i-ab} \\ &\quad + \kappa(A_{ab} + c_{s-ab}E_{ab})G_{i-ad} \end{aligned}$$

The MSF method requires an experimental setup that allows independent measurement of adaxial and abaxial gas exchange. To parameterize γ_n ($= g_{cw-ad}/g_{cw-ab}$) we estimated values using Eqn 1. The lesser positive root of Eqn 3 was taken. Notice that in Márquez *et al.* (2021) the variable γ is defined as the ratio of abaxial over adaxial g_{cw} (g_{cw-ab}/g_{cw-ad}); here, we are redefining γ_n as the ratio of adaxial over abaxial ($\gamma_n = \gamma^{-1} = g_{cw-ad}/g_{cw-ab}$), which is more consistent with other parameters used in this analysis and makes it easier to recall the definition (upper/lower).

From now on, when the Red-Light induction method is used and the calculations are made with Eqns 1 or 2, they will be referred to as Red-Light-Full and Red-Light-Simple equations, respectively; calculations made with adaxial and abaxial steady-state measurements using Eqn 3 will be referred to as the MSF method. The β value (g_{cc}/g_{cw}) for Eqns 1 and 3 was tested within the range reported for different species (Boyer *et al.*, 1997; Boyer, 2015a,b) from 0.025 to 0.05.

As noted earlier, g_{w-dark} and g_{w-min} are the sums of the cuticular conductance plus the stomatal conductance from partially open stomata. The latter contribution was calculated from leaves with known g_{cw} (see calculations in Notes S4). The total cuticular conductance of a leaf (g_{cw-T}) is estimated as the sum of the adaxial and abaxial cuticular conductances,

$$g_{cw-T} = g_{cw-ad} + g_{cw-ab} \quad \text{Eqn 4}$$

Plant material

Two amphistomatous species were used in our experiments, *Capsicum annuum* L. and *Helianthus annuus* L., which were chosen for their large difference in stomatal density ratio (adaxial over abaxial stomatal density). All plants were grown from seeds in 3 l pots filled with Martins Potting Mix (Martins Fertilizers, Yass, NSW, Australia); 5 g of slow-release Osmocote Exact fertiliser (Scotts Australia, Bella Vista, NSW, Australia) were applied at the time of sowing and another 5 g eight weeks later. After sowing, all plants were kept in a glasshouse under natural light, temperature of 28°C during the day, 20°C at night and watered once a day. Measurements were made on fully expanded leaves of 6- to 9-wk-old *H. annuus* plants, and on 8- to 12-wk-old *Capsicum annuum* plants.

Fully expanded leaves of *H. annuus* present a stomatal density ratio between 0.84 and 0.85 growing in natural conditions (Furukawa, 1992; Nascimento *et al.*, 2016). For *Capsicum annuum*, the stomatal density ratio was measured on nine fully expanded leaves from different individuals, where nine samples of 5 mm by 5 mm of lamina were taken from each leaf (81 samples). In each sample, the adaxial and abaxial surfaces were imaged in three different places on each surface using a scanning electron microscope (243 images per surface), each image covering an area of 1.47 mm². The average stomatal density was 19 ± 9 mm⁻² on the adaxial and 145.8 ± 22.7 mm⁻² on the abaxial surface, resulting in a stomatal density ratio between 0.1 and 0.15.

Gas exchange measurements

A custom-built gas exchange analysis system (Wong *et al.*, 1978; Wong *et al.*, 1985) was used to evaluate the adaxial and abaxial leaf gas exchange independently (from now on Double Chamber system). The Double Chamber gas exchange analysis system uses a Vaisala HMP50 to analyse [H₂O] (Vaisala, Finland) and two LI-6251 gas analysers (Li-Cor, Lincoln, NE, USA) to measure the [CO₂] of the upper and lower cuvettes (adaxial and abaxial leaf surfaces) independently. The Double Chamber system was used to determine the leaf surface conductance (g_{w}) in the dark and cuticular conductance to water by the Red-Light and MSF methods. During the measurements, c_a and air vapour pressure deficit were matched in the upper and lower chambers, at approximately 400 $\mu\text{mol mol}^{-1}$ and 1 kPa, respectively.

The leaf surface conductance in the dark was measured under two conditions: one forcing the stomata to close using ABA (g_{w-min}) and a second measuring the natural stomatal closure in the dark to obtain the dark-acclimated minimum leaf surface conductance (g_{w-dark}). Both were measured after exposing the leaf to 12 h of darkness. The g_{w-dark} was measured at the end of the dark period without applying ABA at any stage. For g_{w-min} measurements, the ABA solution was prepared at 0.1 mM in water with < 0.5% ethanol content to pre-solubilize the ABA and applied to both surfaces of the leaf with a spray bottle. There were two applications of ABA in the dark, one the night before and a second 30 min before starting the gas exchange measurements.

Red-Light method The red-light intensity must be higher than the light compensation point but also low enough to avoid fast stomatal aperture changes. The light intensity where these conditions are found might vary between species and growth conditions. In our experiments, and on the chosen plants, we found that 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ allowed us to achieve the desired conditions. Plants were dark-adapted for 12 h before exposure to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of red light and measurements were taken every 7 s during the light induction until the maximum A was achieved.

The main set of experiments was carried out using ABA application to help establish photosynthetic induction without promoting stomatal opening. The effect of ABA in our measurements was tested by re-measuring the adaxial and abaxial cuticular conductance of some leaves one week later, following the same procedure but without applying ABA. To test whether Rubisco activation is a limitation during the experiment, a subset of leaves was tested under the same initial dark conditions but using 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ red-blue light to promote fast stomatal opening (without ABA application).

For the A/c_i curves made with an LI-6800 (Li-Cor) leaf temperature was set to 25°C, air saturation deficit to 1 kPa, light intensity to either 100 or 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue) and readings were taken at reference gas CO₂ concentrations of 400, 350, 300, 250, 200, 150, 125, 100, 75, 50, 25, 10, 400, 400, 600, 800, 1200, 1600 and 2000 $\mu\text{mol mol}^{-1}$. To determine the maximum A at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of red light, stomata were first induced to open at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of red-

blue light, $400 \mu\text{mol mol}^{-1}$ of atmospheric CO_2 (c_a) and 1 kPa of air saturation deficit. Then, the light intensity was changed to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light and the leaf was left until A was stable before stomatal limitation appears in the reading.

The Red-Light method was designed to evaluate adaxial and abaxial cuticular conductance independently using a Double Chamber system; however, the method was also evaluated in the normal setup of the LI-6800 (from now on Single Chamber system).

MSF method The MSF method relies on data that is fully independent of the Red-Light induction technique. Data for the MSF method were obtained under steady-state conditions with $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red-blue light illumination and without ABA application. Adaxial and abaxial leaf gas exchange were measured separately using the Double Chamber system. In the MSF method, the leaf is under conditions that will promote a large stomatal aperture, opposite to the desired conditions during the Red-Light method. The same leaves used for the Red-Light method were measured with the MSF method, and the calculations from the MSF method were subsequently used to determine the accuracy of the Red-Light-Full and Red-Light-Simple equations.

Results

Red light induction

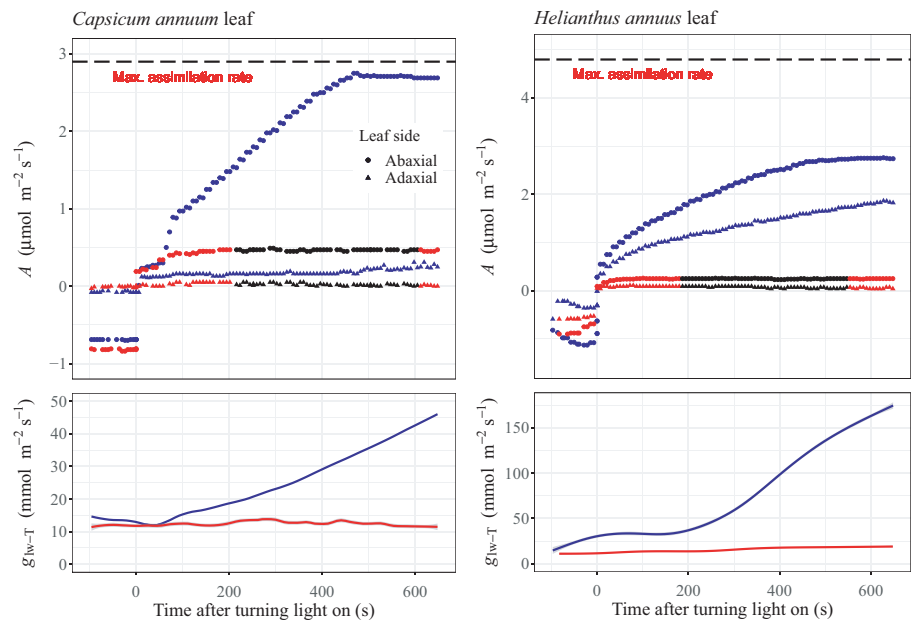
After achieving stable conditions of gas exchange in the dark, the leaves were illuminated with $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of either red light or red-blue light. Under red light illumination, A increased at both surfaces until it reached a stable plateau after about 50 to 100 s (Fig. 1; red symbols), while the leaf surface conductance, g_{w-T} , remained almost unchanged upon turning on the light (Fig. 1; red lines). In contrast, the exposure to red-blue light of the same intensity, which triggers faster stomatal opening, showed that A

continued to increase beyond the stable plateau found under red light (Fig. 1; blue symbols). The increase in A under red-blue light tracked the increase of g_{w-T} (Fig. 1; blue lines), indicating that stomatal opening facilitates CO_2 diffusion into the leaf to sustain the observed A . This demonstrates that insufficient activation of photosynthetic biochemistry, which has been shown to be insensitive to the ratio of red/blue light, is not the reason behind the low A under red light. Thus, stomata were the main limitation to increasing A after about 50 to 100 s into photosynthetic induction under red light. We can infer that by then Rubisco activity exceeds the capacity needed for the observed A because A would increase if Rubisco activation were to continue to increase after the initial $c. 100$ s, even when c_i is constant (Deans *et al.*, 2019b). A Rubisco-limitation of A is thus being ruled out as the underlying biochemical limitation during the stable period. Instead, we observed that A increases when the CO_2 supply improves, which is expected if the biochemical limitation is related to photosynthetic electron transport (Busch & Sage, 2017).

Using adaxial and abaxial gas exchange measurements, the only unknowns in Eqns 1 and 2 (Red-Light-Full and Red-Light-Simple equations) for calculating g_{cw-ad} and g_{cw-ab} are c_{i-ad} and c_{i-ab} . When stomatal aperture limits CO_2 diffusion into the leaf and A is substantially lower (close to zero) than its maximum at that light intensity, the adaxial and abaxial $[\text{CO}_2]$ inside the leaf must correspond to values in the order of the CO_2 compensation point (Γ), at which net CO_2 assimilation rate is zero (Farquhar *et al.*, 1980; Jones, 1985). At this c_i , the CO_2 taken up by the carboxylation reaction equals the rate of CO_2 released by respiration and photorespiration.

To estimate c_i under the red-light induction conditions we can consult a steady-state A/c_i curve performed under the same conditions as during the red-light induction, with the exception that stomatal opening is promoted using red-blue light. Open stomata minimize inaccuracies of c_i calculations due to cuticular

Fig. 1 Example of net assimilation rate (A) measured after turning on either red or red-blue light at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. The black dashed line denotes the maximum assimilation rate at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light when stomata were fully opened. Red and black symbols represent the response of the leaf to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light with abscisic acid (ABA) application. Black circles and triangles mark the section where the assimilation rate was deemed stable for cuticular conductance calculations. Blue symbols represent the response of the same leaf to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red-blue light without ABA application. The bottom panels show the total leaf surface conductance when red light was used (red line) and when red-blue light was used (blue line).



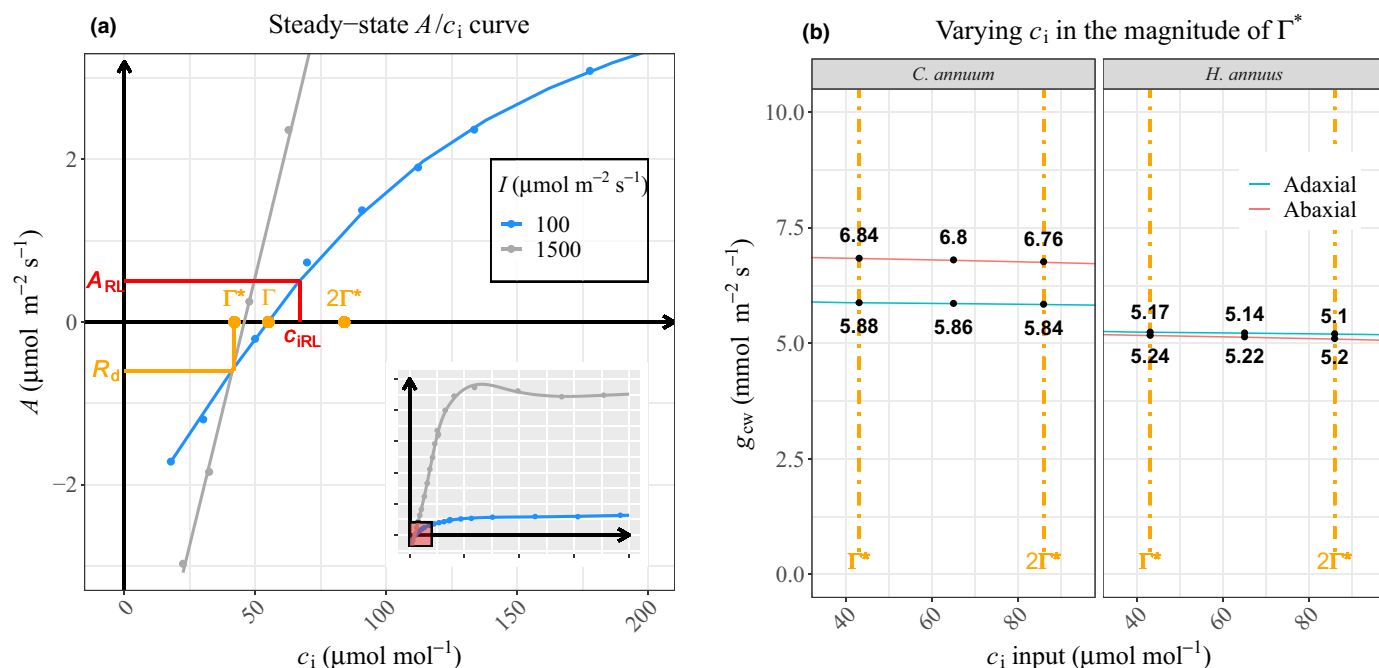


Fig. 2 Example of typical A/c_i curves taken at different light intensities and the results of our calculations varying c_i in the magnitude of Γ^* . (a) Close-up of A/c_i curves (full curves shown in insert) taken with light intensity (I) of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ (blue dots and line) and of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (grey dots and line); orange marks represent the CO_2 compensation points with (Γ) and without (Γ^*) mitochondrial respiration (R_d); red marks represent the estimated values of c_i (c_{iRL}) for a given A (A_{RL}) during the Red-Light induction method. (b) Impact of varying the c_i input within the magnitude of Γ^* for the calculation of g_{cw} using the Red-Light-Full method (Eqn 1). Black dots represent the calculated g_{cw} values at $c_i = \Gamma^*$, $1.5\Gamma^*$ and $2\Gamma^*$.

conductance ($g_{lw} > 160 \text{ mmol m}^{-2} \text{s}^{-1}$; Márquez *et al.* (2021)). Keeping environmental conditions the same between the red-light induction and A/c_i measurements is important, because mesophyll conductance to CO_2 (g_{mc}) has been shown to vary with light intensity and CO_2 concentration (Busch *et al.*, 2020). As all environmental conditions are the same for both sets of measurements other than light quality, we can assume that all photosynthetic parameters that we cannot directly measure, such as Rubisco activation state or g_{mc} , also remain the same. We therefore do not explicitly need to know them but can use a fitted curve for the A/c_i measurements that span the red-light A (A_{RL}) to get a close approximation of the c_i during red-light induction (c_{iRL}) (Fig. 2a).

As we have determined that A during the Red-Light induction method is electron transport limited, we can fit the A/c_i curve using the Farquhar–von Caemmerer–Berry photosynthesis model (Farquhar *et al.*, 1980) and calculate the electron transport-limited CO_2 assimilation rate A_j as:

$$A = A_j = J_a \frac{c_i - \Gamma^*}{4(c_i + 2\Gamma^*)} - R_d, \quad \text{Eqn 5}$$

where J_a is the potential rate of electron transport and Γ^* the CO_2 compensation point in the absence of mitochondrial respiration. Even though we do not know the exact value of g_{mc} and therefore have to assume mesophyll conductance is infinite (i.e. the chloroplastic CO_2 concentration equals c_i), the model has been long proven to describe the A/c_i response well.

Solving Eqn 5 for c_i yields a description to highlight some general principles about the c_i we may expect during the red-light induction:

$$c_i = \frac{J_a + 8(A + R_d)}{J_a - 4(A + R_d)} \Gamma^*. \quad \text{Eqn 6}$$

Under the light-limited conditions during the measurements we can relate J_a to the incident light intensity (I) by $J_a = 0.5I(1 - f)$, where f denotes the fraction of light not absorbed by the chloroplast (approximately 0.15; Farquhar *et al.* (1980)). The experimental conditions for the red-light induction are chosen to maximize J_a (*c.* $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ at $I = 100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) while keeping $A + R_d$ low ($< 1.5 \mu\text{mol m}^{-2} \text{s}^{-1}$), which results in c_i to be in the order of $1.5\Gamma^*$. More generally, based on Eqn 6 one can assume that $c_i < 2\Gamma^*$, if $A + R_d$ during the red-light induction is smaller than *c.* $2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Furthermore, Γ^* is related to Rubisco specificity, which depends on leaf temperature and somewhat varies by species; however, for C_3 plants Γ^* is on average close to 43 ppm at 25°C (Hermida-Carrera *et al.*, 2016), which matches our observations and was therefore adopted for our calculations. In practice the impact of varying c_i in the order of Γ , i.e. between Γ^* and $2\Gamma^*$, has a negligible effect on our g_{cw} calculations with Eqns 1 and 2 when c_a is $400 \mu\text{mol mol}^{-1}$ (Fig. 2b) and only becomes substantial for a c_i above $150 \mu\text{mol mol}^{-1}$ (*c.* $3.5\Gamma^*$; Supporting Information Fig. S1). During our experiments we kept $A + R_d$

between 1.1 and 1.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ ($A < 0.6 \mu\text{mol m}^{-2} \text{s}^{-1}$), resulting in a c_i between 58 and 68 $\mu\text{mol mol}^{-1}$ ($c_i \approx 1.5\Gamma^*$), and so for simplicity we adopted an adaxial and abaxial c_i equal to $1.5\Gamma^*$ for all the calculations (64.5 $\mu\text{mol mol}^{-1}$).

For the use of Eqns 1 and 3 a β value (g_{cc}/g_{cw}) is needed. Assigning β within the reported range of 0.025 to 0.05 affects the g_{cw} calculation by less than 0.2 $\text{mmol m}^{-2} \text{s}^{-1}$ in our experiments (Notes S5), which has a negligible impact on the estimation of other gas exchange parameters. The estimated β value used for each leaf in this study is presented in Notes S5.

Calculation approaches

We used the same leaves for collecting the data required for the Red-Light-Full and Red-Light-Simple calculations as well as the MSF method, and compared the values estimated from each approach. Estimates of g_{cw} from the Red-Light-Simple calculation were generally 7% to 12% (0.5 to 1 $\text{mmol m}^{-2} \text{s}^{-1}$) lower than those estimated with the Red-Light-Full calculation (Fig. S2). However, Red-Light-Full and Red-Light-Simple calculations resulted in the same adaxial to abaxial g_{cw} ratio ($\gamma_n = g_{cw\text{-ad}}/g_{cw\text{-ab}}$). Using the known γ_n value of the leaves, the calculations by the MSF method and Red-Light-Full calculation estimate the same cuticular conductance for the adaxial and abaxial surfaces (Fig. 3). As MSF and Red-Light methods have different experimental setups and opposite approaches to estimating g_{cw} but still yield the same results, the Red-Light-Full calculations (Eqn 1) prove to be reliable for estimating g_{cw} . Therefore, subsequent analyses are focused on the Red-Light-Full calculations, which are also implemented in the web-app presented here.

Adaxial and abaxial cuticular conductance

We observed that different leaves of the same species and even of the same plant often exhibited different cuticular conductance values (compare Fig. 4a with Fig. 4b; and within Fig. 4d). In contrast, estimates of adaxial and abaxial g_{cw} on the same leaf 6 d after the first measurement showed no variation in the

results for *Capsicum annuum* or *H. annuus* (Fig. 4a–c), and the ABA application had no effect on the calculation of cuticular conductance to water (Fig. 4c; Supporting Information Table S1). However, the rate of failure in keeping the stomata closed during red-light induction increased to approximately 80% of the attempts when ABA was not applied, where a stretch of stable readings lasting less than 1 min was considered a failed attempt. The period of stable assimilation rate for cuticular conductance calculations during the experiment ranged between 1 and 3 min without ABA application and 3–8 min with ABA application.

While leaves of *Capsicum annuum* consistently had different adaxial and abaxial cuticular conductances, the adaxial and abaxial conductances in *H. annuus* leaves were almost identical. Measurements of different *H. annuus* and *Capsicum annuum* leaves showed species trends for the adaxial and abaxial g_{cw} (Fig. 5): the ratios of adaxial to abaxial g_{cw} ($\gamma_n = g_{cw\text{-ad}}/g_{cw\text{-ab}}$) were $\gamma_n = 0.98 \pm 0.02$ for *H. annuus* and $\gamma_n = 0.89 \pm 0.03$ for *Capsicum annuum* leaves, while the stomatal density ratio (S_p , adaxial over abaxial stomatal density) was 0.85 ± 0.1 and 0.13 ± 0.03 , respectively. In both species a positive relationship was observed between g_{cw} and stomatal density, where the higher g_{cw} was found in the surface with higher stomatal density.

Minimum leaf surface conductance

The minimum conductance of each leaf surface ($g_{lw\text{-min}}$) was generally about 5–10% greater than the value of g_{cw} . However, in some cases, $g_{lw\text{-min}}$ was up to 30% higher than g_{cw} (see Notes S5). The dark-acclimated minimum leaf surface conductances ($g_{lw\text{-dark}}$) of the leaves were consistently greater at the abaxial surface of both species. The contribution of stomatal conductance (g_{sw}) to $g_{lw\text{-dark}}$ during our measurements varies between species and even between leaves of the same species (Fig. 6). This highlights that because the g_{sw} component of $g_{lw\text{-min}}$ and $g_{lw\text{-dark}}$ is both variable and nonnegligible, minimum conductance measurements are not a good approximation of cuticular conductance g_{cw} . Equally, $g_{lw\text{-dark}}$ is not a good approximation of nocturnal stomatal conductance, as g_{cw} is not accounted for.

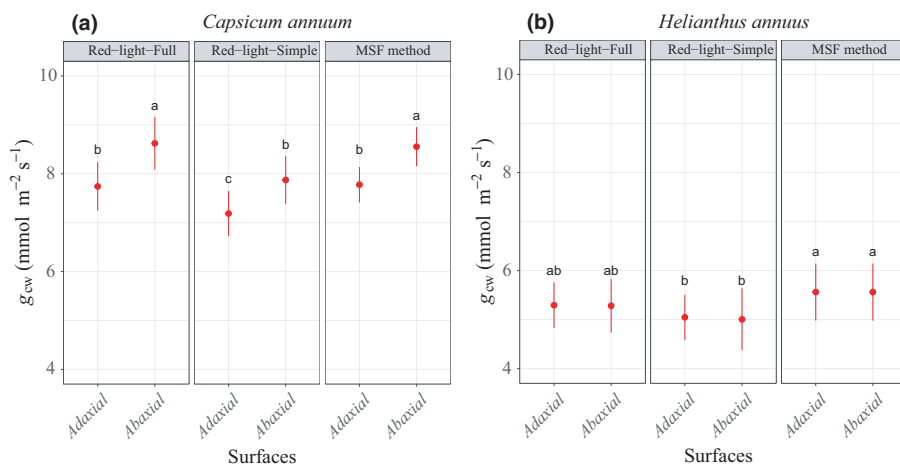


Fig. 3 Comparison of the calculation of g_{cw} from $c_i = 1.5\Gamma^*$ using the Red-Light-Full and Red-Light-Simple calculations and that using the MSF method where c_i is unknown. (a) Calculations for an individual *Capsicum annuum* leaf; (b) calculations for an individual *Helianthus annuus* leaf. Red circles are the mean values of the data set and the lines represent the standard deviation of the mean value during the measurements. For both (a) and (b), different letters denote statistical differences at $P < 0.001$. Number of gas exchange measurements under stable gas exchange conditions $n_{(a)} = 46, 46, 46, 46, 63$ and 63 ; $n_{(b)} = 27, 27, 27, 27, 56$ and 56 .

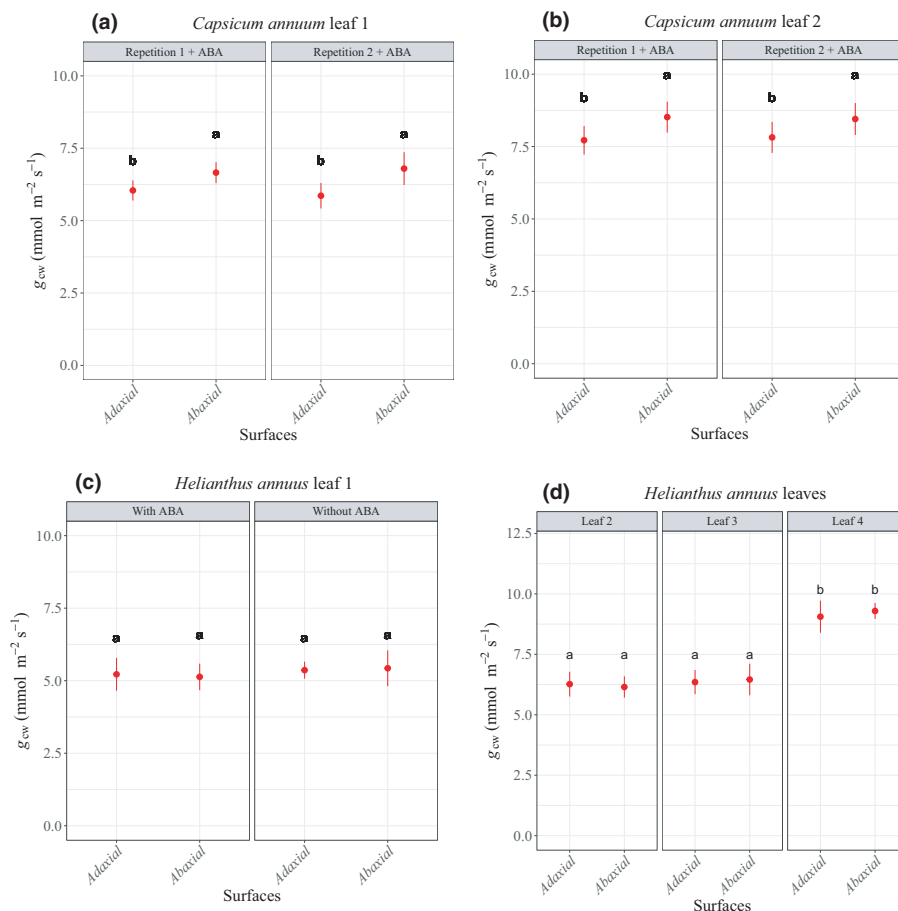


Fig. 4 Adaxial and abaxial cuticular conductances of two different leaves of *Capsicum annuum* and four leaves of *Helianthus annuus* calculated with Eqn 1 and $c_i = 1.5\Gamma^*$. Panels (a), (b) and (c) each show two measurements of the same leaf with 6 d between measurements. *Capsicum annuum* leaves were treated both times with abscisic acid (ABA) and *H. annuus* leaf 1 was treated with ABA for the first measurement only. Panel (d) shows three different leaves of *H. annuus* measured after applying ABA. Red circles are the mean values of the data set and the lines represent the standard deviation of the mean value during the measurements. For each panel, different letters denote statistical differences at $P < 0.001$. Number of gas exchange measurements under stable gas exchange conditions: $n_{(a)} = 42, 42, 51$ and 51 ; $n_{(b)} = 46, 46, 23$ and 23 ; $n_{(c)} = 27, 27, 21$ and 21 ; $n_{(d)} = 44, 44, 51, 51, 14$ and 14 .

Total cuticular conductance

In terms of projected leaf area, the leaf total cuticular conductance to water (g_{cw-T}) is the sum of the adaxial and abaxial cuticular conductances (Eqn 4), which can be estimated from independent measurements of both surfaces using a Double Chamber setup as mentioned earlier. However, in normal gas exchange measurements, the adaxial and abaxial fluxes are mixed and analysed as one. For comparison purposes, we analysed the accuracy of our calculations using the Red-Light method and ABA in a typical Single Chamber setup. In *H. annuus* and *Capsicum annuum* leaves, the g_{cw-T} estimated using a Single Chamber system was statistically not different from g_{cw-T} estimated from independent measurements of adaxial and abaxial g_{cw} recorded with a Double Chamber system (Fig. 7).

Discussion

Suitability of assumptions for the Red-Light technique

The Red-Light induction technique produced consistent and reproducible results for estimating adaxial and abaxial cuticular conductance to water. The comparison with steady-state A/c_i measurements yield reliable and fairly precise c_i estimates during the red-light induction (Fig. 2a), but the more general assumption of adaxial and abaxial c_i being close to Γ is not insignificant

and requires further discussion. The argument for an expected c_i being of the same magnitude as Γ is supported by the calculations of the same estimated g_{cw} using the Red-Light-Full and MSF methods. Nevertheless, it can be argued that c_i is not necessarily a single value and differs depending on the leaf structure and atmospheric conditions (Farquhar & Raschke, 1978; Parkhurst *et al.*, 1988; Parkhurst, 1994). However, the calculation of g_{cw} using the Red-Light-Full method (Eqn 1) is unaffected if c_i is of the magnitude of Γ and c_s is of the order of hundreds of $\mu\text{mol mol}^{-1}$ (*c.* $400 \mu\text{mol mol}^{-1}$ in our experiments, see Fig. 2b) even if we vary the estimated Γ^* . This apparent insensitivity in the calculation to changes in c_i is due to the large difference between c_s and c_i (Δc). Thus, in Eqn 1, $\Delta c G_i$ is two orders of magnitude larger than $A + c_s E$ when c_i approaches Γ^* and the same holds between the components $\Delta \alpha$ and $c_s D$, which minimizes the error caused by imprecision in the assumed $c_i = 1.5\Gamma^*$. In this scenario, variations of c_i in the range $40\text{--}90 \mu\text{mol mol}^{-1}$ affect the g_{cw} calculation by $< 0.09 \text{ mmol m}^{-2} \text{ s}^{-1}$ (Fig. 2b), which can be considered negligible. The same analysis can be made for the simplified Red-Light-Simple calculation using Eqn 2, with a similar outcome. Care should be taken if $A + R_d > 2.5 \mu\text{mol m}^{-2} \text{ s}^{-1}$, as the actual c_i is likely to exceed $2\Gamma^*$. In this case it is advisable to directly measure c_i with a steady-state A/c_i curve as outlined in Fig. 2a.

We evaluated the performance of the induction process from dark to low red-blue light intensity in our experiments, as this is

fundamental to assessing the induction timing of our plants under the experimental conditions. Different species may perform differently during the induction process for reasons such as accumulation of the Rubisco inhibitor 2-carboxy-D-arabitol 1-phosphate during the dark period before induction (Seemann *et al.*, 1985). Thus, it is advisable to perform a red-blue light induction test to assess the Rubisco activation timing in the species being evaluated.

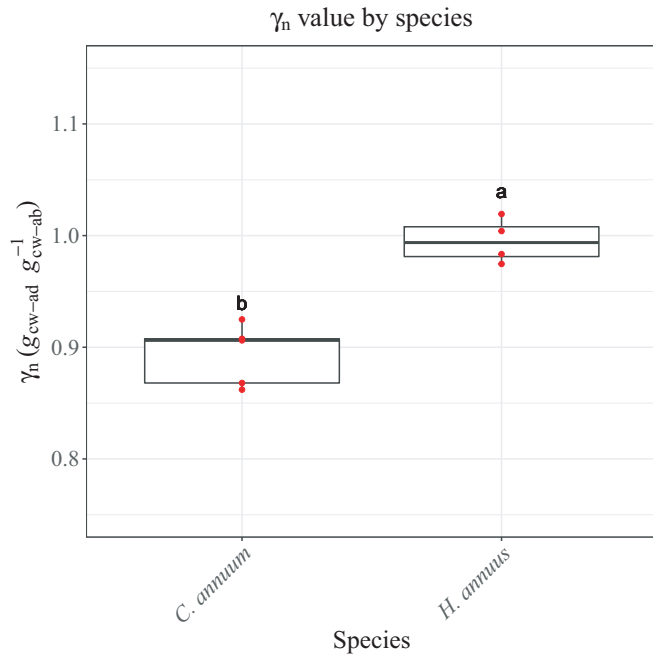


Fig. 5 Adaxial to abaxial ratio (γ_n) values calculated from measurements of adaxial and abaxial g_{cw} of *Capsicum annuum* and *Helianthus annuus* leaves. Estimates were made using the Red-Light-Full calculations (Eqn 1) with $c_i = 1.5\Gamma^*$. Red circles are the mean values of the data set. Boxes represent the range of data between the second-third quartiles (IQ = Q3 – Q2), the horizontal lines in the boxes represent the median of the data set, vertical lines represent the range of maximum and minimum. The average adaxial and abaxial g_{cw} was used to estimate γ_n . Statistical differences are at $P < 0.001$; $n = 5$ and 4 individual leaves for *Capsicum annuum* and *H. annuus*, respectively.

It has previously been shown that ABA does not affect photosynthesis directly and its impact is largely constrained to stomatal physiology (Terashima *et al.*, 1988). In our experiments, ABA application did not affect our estimations in comparison with the non-ABA treatment control. While we did not test the impact of ABA on the cuticular characteristics *per se*, our data suggest that if there is any it must be small and negligible for the propose of our experiment. We observed that when ABA is not applied, stomata quite frequently open too quickly to achieve a stable plateau of A , from which g_{cw} is calculated, even if blue light is not present. So, we treated the leaf with ABA before the measurement, ensuring that the stomatal opening response was minimized. Depending on the species used for experimentation, or the status of the measured plant, measurements of g_{cw} can be successful without applying ABA and yield the same results as measurements performed after ABA treatment. Therefore, the application of ABA on the leaf surfaces is not a requirement for the technique to work but helps to reduce the number of failed attempts and delays stomatal opening, helping to produce stable measurements required for the calculations.

The Red-Light method as an accessible approach for quantifying g_{cw}

The equations for the Red-Light-Full calculations (Eqn 1) and the MSF method (Eqn 3) are derived from the gas exchange equations introduced by Márquez *et al.* (2021) (MSF), which are a structural modification of the widely used von Caemmerer & Farquhar (1981) equations (vCF). An important difference between the MSF and vCF equations is that MSF accounts for cuticular conductance and its interaction with other fluxes while vCF neglects them. This neglect generates a mismatch between the estimations of c_i using vCF and direct measurements of c_i (Tominaga *et al.*, 2018) that has been exploited to derive a correction factor as g_{cw} to rectify the observations of c_i (Boyer, 2015a; Tominaga & Kawamitsu, 2015). These calculations, as with our Red-Light-Simple calculation (Eqn 2; see Notes S3), assume that the mismatch is entirely due to g_{cw} (correction factor = g_{cw}).

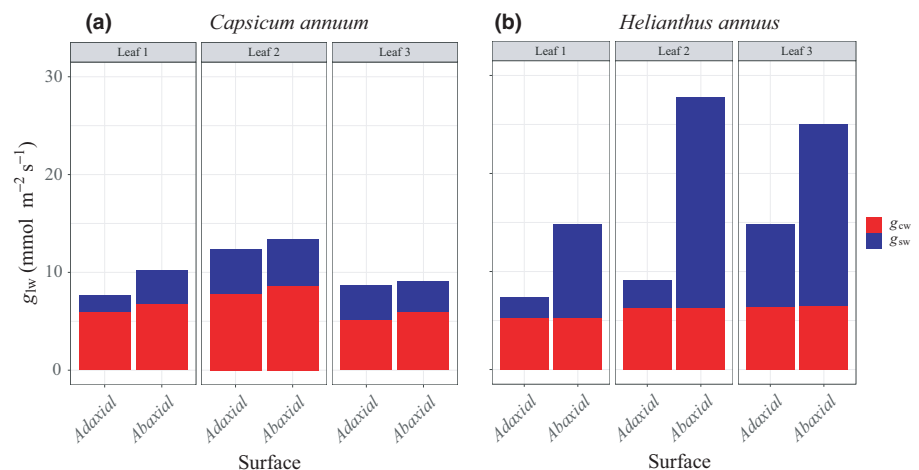


Fig. 6 Adaxial and abaxial dark acclimated minimum leaf surface conductance ($g_{lw-dark}$). The complete bars represent the leaf surface conductance (g_{lw}); the red and blue sections represent the contribution of cuticular (g_{cw}) and stomatal (g_{sw}) conductance, respectively.

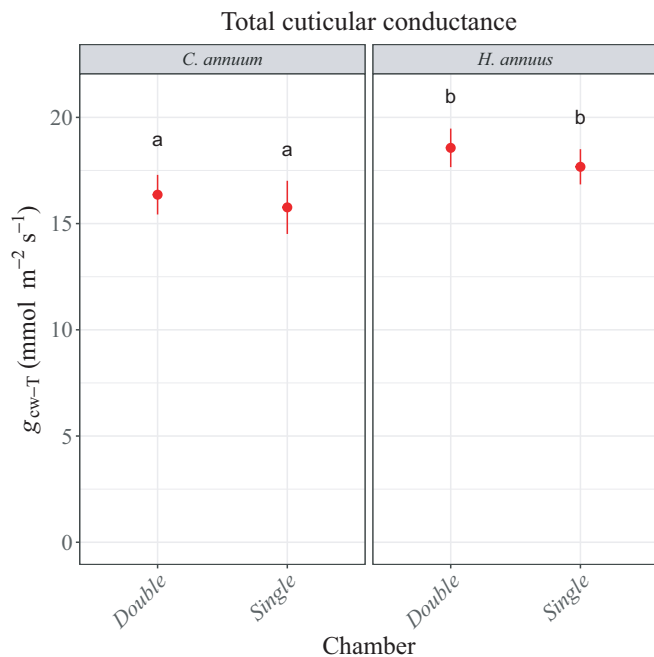


Fig. 7 Estimation of total cuticular conductance to water (g_{cw-T}) for one *Capsicum annuum* leaf and one *Helianthus annuus* leaf. Estimations made using Double Chamber (abaxial and adaxial) and Single Chamber (mixed gases from the adaxial and abaxial surfaces) setups and applying the Red-Light-Full method (Eqn 1) with $c_i = 1.5\Gamma^*$. Red circles are the mean values of the data set and the lines represent the standard deviation of the mean value during the measurements. Statistical differences are at $P < 0.001$. Number of gas exchange measurements under stable gas exchange conditions for *Capsicum annuum* $n_{double} = 46$, $n_{single} = 43$; *H. annuus* $n_{double} = 14$, $n_{single} = 50$.

Here we show that using the approach of taking g_{cw} equal to the vCF correction factor (Red-Light-Simple) leads to an estimate of g_{cw} 7% to 12% (0.5 to 1 mmol m⁻² s⁻¹) lower than results using the Red-Light-Full equation (Fig. S2). The underestimation of g_{cw} is a consequence of the vCF equations neglecting the effect of cuticular CO₂ fluxes and the interaction between cuticular fluxes and stomatal and boundary layer fluxes (Márquez *et al.*, 2021). While this simple approach is an acceptable approximation of g_{cw} for obtaining a rough value to correct gas exchange measurements, we recommend using the Red-Light-Full equation (Eqn 1), as it is the most accurate to date and, most importantly, does not require additional input data. Choosing a value of β within the range of 0.025 to 0.05 for the Red-Light-Full method has no significant impact on other calculations such as γ_n or the statistical comparison between surfaces. However, knowing that $g_{cc} \neq 0$, it is advisable to use a nonzero value of β within that range when Eqn 1 is used, even if the true value is unknown.

Another promising finding was that the direct estimates of g_{cw-T} were accurate when using the most common setup for gas exchange measurements, i.e. a single chamber mixing the adaxial and abaxial gases. The measurements of g_{cw-T} using the Single Chamber with *H. annuus* and *Capsicum annuum* were the same as those using the Double Chamber (Fig. 7). These results show that the estimation of g_{cw-T} can be made routinely by the Red-Light method using standard gas exchange devices.

We note that the method proposed here for estimating c_i has the practical implication that measurements of g_{cw-T} are not restricted to amphistomatous leaves but can be equally well performed on hypostomatous leaves. The use of Eqn 1, referred to as Red-Light-Full, is also not restricted to the experimental setup presented here, but can be used under any experimental setup where c_i can be considered as known, as, for example, the setup used by Boyer (2015a) and Tominaga & Kawamitsu (2015).

Minimum conductance equals neither cuticular nor nocturnal stomatal conductance

The value of minimum conductance (g_{w-min}) was generally near the value of g_{cw} , which is expected from a leaf that has been dark-adapted and sprayed with an ABA solution twice. However, g_{w-min} was commonly higher and occasionally substantially higher than g_{cw} , especially on the surface with higher stomatal density (the abaxial side in both species tested here). This indicates that g_{w-min} is not a reliable approximation of g_{cw} .

Cuticular conductance has been described as being two orders of magnitude lower than nocturnal stomatal conductance (Duursma *et al.*, 2018) and its contribution to the dark-acclimated minimum conductance ($g_{lw-dark}$) thus considered minor (see for example Resco de Dios *et al.*, 2019). However, taking $g_{lw-dark}$ as a proxy for nocturnal stomatal conductance may be problematic if a large portion of the measured transpiration is cuticular transpiration rather than transpiration through the stomata, as we observed in our *Capsicum annuum* leaves (Fig. 6). Even when the stomatal contribution is proportionally larger, such as in our *H. annuus* measurements, the cuticular component g_{cw} of $g_{lw-dark}$ is likely large enough to cause a substantial and systematic overestimation of nocturnal stomatal conductance. Additionally, cuticular properties were shown to be variable depending on the growth conditions (Kerstiens, 1996; Cameron *et al.*, 2006; Bi *et al.*, 2017), which also affect the proportion of g_{cw} in $g_{lw-dark}$ (Márquez *et al.*, 2021). Uncertainties like these can now be largely avoided by accounting for g_{cw} as described in this study when estimating nocturnal stomatal conductance.

In both species, $g_{lw-dark}$ was greater for the abaxial surface but the proportion of stomatal conductance at each surface was variable among leaves and surfaces. The contribution of stomatal conductance to $g_{lw-dark}$ was positively related to the stomatal density of each surface. However, the relation was not precisely proportional to the stomatal density, with a smaller difference between the adaxial and abaxial stomatal conductance contribution in *Capsicum annuum* than in *H. annuus* leaves.

The measurements presented in Fig. 6 show the minimum value measured during the tests but we observed small oscillations of g_{sw} with a 7- to 12-min period during the measurements in the dark. We briefly evaluated whether the oscillations were only due to stomata following a suppressed expression of their normal diurnal rhythm even though they were in the dark (Holmes & Klein, 1986; Kaiser & Kappen, 1997) but were unable to prove that assertion. The oscillation persisted during measurements performed at night, though the amplitude was lower. Further research should be undertaken to investigate the nature of the

nocturnal stomatal conductance oscillation, which could provide novel information about the leaf surface conductance and water loss at night.

Consistent ratio of adaxial to abaxial g_{cw} within a species

Regarding the adaxial and abaxial cuticular conductance difference, Zhang *et al.* (2020) showed that in detached hypostomatous leaves of *Camellia sinensis* the adaxial to abaxial cuticular transpiration ratio was about 0.71 (Zhang *et al.*, 2020), suggesting a significant cuticular conductance difference between surfaces of these leaves. Another recent study has suggested a large variability in the relation of adaxial to abaxial g_{cw} among hypostomatous leaf species (Machado *et al.*, 2021). In our study on intact leaves, we found that amphistomatous leaves can also present significant differences between the adaxial and abaxial cuticular conductances but that this is species-dependent. Measurements in *H. annuus* leaves showed the same g_{cw} on both surfaces and we found a consistent difference in adaxial and abaxial g_{cw} in *Capsicum annuum* leaves.

The adaxial and abaxial g_{cw} measured for different leaves indicate that despite differences in the total cuticular conductance among leaves, each of the species analysed here has a consistent adaxial to abaxial g_{cw} ratio ($\gamma_n = g_{cw-ad}/g_{cw-ab}$) when grown under the same conditions (Fig. 5). Consistent values of γ_n permit the accurate estimation of adaxial and abaxial g_{cw} using the MSF method (Eqn 3), leading to the same results for g_{cw} as applying the Red-Light-Full method to the same leaf.

The actual mechanistic basis for the difference between adaxial and abaxial g_{cw} remains largely unknown, as is the cause of the differences in g_{cw} between leaves. Schuster *et al.* (2016) and Schuster *et al.* (2017) evaluated the chemical composition of cuticles and were able to explain only part of the variability found in leaf surfaces evaporation of detached leaves and the permeability of isolated cuticles; neither the structure nor the thickness of the cuticle appear directly linked to g_{cw} (Yeats & Rose, 2013; Duursma *et al.*, 2018). It has been suggested that a network of aqueous pores through the cuticle is the main water pathway to the outside (Kerstiens, 2006) with the highest concentration of aqueous pores around the stomata and trichomes (Schlegel *et al.*, 2005). We found a positive correlation between stomatal densities on each surface and the g_{cw} of each surface, which is in agreement with the findings of Schlegel *et al.* (2005) and Kerstiens (2006). Further research combining *in planta* measurements and isolated cuticle characteristics is needed to investigate the relation between stomatal and cuticular properties.

Conclusions

With the Red-Light method, we present a simple and quick approach for estimating cuticular conductance in intact leaves. It is suitable for most commercially available gas exchange equipment but can also be used in Double Chamber systems to obtain independent values for adaxial and abaxial leaf surfaces. As the measurements can be made independently on both sides of the leaf, this method is equally suited for both amphistomatous and

hypostomatous plant species. Here, we found that amphistomatous plants can exhibit different cuticular conductances in the adaxial and abaxial surfaces and that this difference is species dependent. Thus, the Red-Light method should prove useful for establishing the ultimate causes of the adaxial/abaxial difference in g_{cw} and how g_{cw} or g_{cw-T} varies between species and growth conditions.



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Author contribution

DAM, HS-W, GDF and FAB conceived the study. FAB and DAM designed the experiments. DAM undertook the experimental work and data analysis. DAM designed and developed the web-application. DAM and FAB wrote the manuscript with help from all authors.

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Data availability

All generated and analysed data from this study are included in the published article and its Supporting Information. The code of the web-application is available in the GitHub repository https://github.com/PlantPhysiologist/gcwCi_app.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Sensitivity of the calculated g_{cw} to the assumed a_1 input using the Red-Light-Full method (Eqn 1).

Fig. S2 Comparison between g_{cw} calculations with a validated a_1 using the Red-Light-Full equation (Eqn 1) and the Red-Light-Simple equation (Eqn 2).

Notes S1 Derivation of the Red-Light Full equation.

Notes S2 General description of the web-application $g_{cw}a_1$ version 1.0.0.

Notes S3 Derivation of the simplification to calculate g_{cw} as a correction factor to vCF equations used in the Red-Light Simple calculations.

Notes S4 Calculation of stomatal conductance and minimum conductance for a single surface of a leaf.

Notes S5 Impact of variation in β values.

Table S1 Measurements of g_{cw} ($\text{mmol m}^{-2} \text{s}^{-1}$) with and without ABA application on different leaves.

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