Importance of leaf anatomy in determining mesophyll diffusion conductance to CO₂ across species: quantitative limitations and scaling up by models

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Abstract

Foliage photosynthetic and structural traits were studied in 15 species with a wide range of foliage anatomies to gain insight into the importance of key anatomical traits in the limitation of diffusion of CO₂ from substomatal cavities to chloroplasts. The relative importance of different anatomical traits in constraining CO₂ diffusion was evaluated using a quantitative model. Mesophyll conductance (gₘ) was most strongly correlated with chloroplast exposed surface to leaf area ratio (Sᶜ/S) and cell wall thickness (Tcw), but, depending on foliage structure, the overall importance of gₘ in constraining photosynthesis and the importance of different anatomical traits in the restriction of CO₂ diffusion varied. In species with mesophytic leaves, membrane permeabilities and cytosol and stromal conductance dominated the variation in gₘ. However, in species with sclerophytic leaves, gₘ was mostly limited by Tcw. These results demonstrate the major role of anatomy in constraining mesophyll diffusion conductance and, consequently, in determining the variability in photosynthetic capacity among species.

Key words: cell wall thickness, diffusion model, leaf anatomy, leaf structure, photosynthesis, quantitative photosynthetic limitations.

Abbreviations: α, leaf absorptance; ρ, fraction of absorbed light that reaches photosystem II; Γ°C, CO₂ compensation point in the absence of mitochondrial respiration; ÐPSII, effective quantum efficiency of the PSII photochemistry; ΔLₕₑₓ, effective diffusion path length in the gas phase; εPSII, fraction of electrons absorbed by PSII; ζ, diffusion path tortuosity; Aₐₐₓ, photosynthetic capacity per dry mass; ΔAₚ, net CO₂ assimilation rate; C₀, atmospheric CO₂ concentration; Cc, chloroplastic CO₂ concentration; Cₛ, substomatal CO₂ concentration; C-Cₓ, CO₂ drawdown from intercellular airspace to chloroplasts; Dₘ, diffusion coefficient for CO₂ in the gas phase; Dₛ, leaf density; Dₚₐ, aqueous phase diffusion coefficient for CO₂; Lₛ, volume fraction of intercellular air spaces; Fₘₚₑ, maximum fluorescence in light-adapted state; Fₛ, steady-state fluorescence emission; gₚₐ, partial liquid phase conductance for different portions along cell walls; gₛₛ, cytosol conductance; gₑₚₑ, chloroplast envelope conductance; gₛₑₑ, intercellular air space conductance to CO₂ (gas phase conductance); gₚₛₚ, sum of liquid and lipid phase conductances; gₛₚₑ, mesophyll diffusion conductance; gₛₚₛ, plasma membrane conductance; gₛₑₑ, stomatal conductance to CO₂; gₛₚₕₖ, total conductance to CO₂ from ambient air to chloroplasts; H/Φ(Tₓ), dimensionless form of Henry’s law constant; Jₑ, linear electron transport rate from chlorophyll fluorescence; Jₘₚₑ, maximum photosynthetic electron transport rate; Kₛ, Michaelis–Menten constant for the carboxylation activity of Rubisco; Kₛₚₑ, Michaelis–Menten constant for the oxygenation activity of Rubisco; iₑ, biochemical limitation; Lₛₑₑₑ, length of chloroplasts exposed to intercellular air spaces; Lₛₑₑₑₑ, diffusion pathway length in the cytoplasm; iₚₑ, gas-phase limitation; iₛₚₑ, mesophyll limitation; iₛₑₑₑ, stomatal limitation; Mₛₑₑₑ, leaf mass per area; O, leaf intercellular oxygen concentration; Pₑₓ, effective porosity in the given part of the diffusion pathway; Qₑₓ, incident quantum flux density; Rₑₑₑ, gas constant; Rₛₑₑₑ, leaf respiration in the dark; Rₛₑₑₑₑ, proportional reduction of Dₛₑₑₑ in the cytosol and in the stroma compared with free diffusion in water; Rₛₑₑₑₑₑ, leaf respiration in the light; Sᶜ, chloroplastic surface area exposed to intercellular air spaces per unit of leaf area; Sᶜ/Sₑₑₑₑₑ, ratio of exposed chloroplasts to mesophyll surface areas; Sₛₑₑₑₑₑ, mesophyll surface area exposed to intercellular air spaces per unit of leaf area; Sₑₑₑₑₑ, cross-sectional area of mesophyll cells in micrograph; SE, standard error; Tₛₑₑₑₑₑ, chloroplast thickness; Tₛₑₑₑₑₑₑ, cell wall thickness; Tₛₑₑₑₑₑₑₑ, cyttoplasm thickness; Tₑₑₑₑₑₑₑ, absolute temperature; Tₛₑₑₑₑₑₑₑₖ, leaf thickness; Tₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑئة; W, width of the leaf anatomical section.

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Introduction

Leaf anatomical characteristics are key functional and adaptive traits determining plant capacity to thrive in specific environments, in particular, because these traits also have important implications for foliage potential photosynthesis (Niinemets et al., 2009a; Scafaro et al., 2011; Terashima et al., 2011). Analysis of global variations in leaf functional traits—the leaf economics spectrum—has established that the variation in leaf dry mass per area ($M_A$) is strongly associated with other key leaf traits such as maximum photosynthetic capacity per dry mass ($A_{max}$), leaf life span, nitrogen and phosphorous contents per dry mass, and respiration (Wright et al., 2004). Species with lower $M_A$ present short leaf life spans, high photosynthetic capacities and nutrient contents, and low leaf area construction costs, resulting in fast growth in environments with high availability of resources. In contrast, species with higher $M_A$ and lower $A_{max}$ present the opposite suite of traits and have higher cost for leaf area formation, particularly due to investment in vasculature and cell walls (Niinemets et al., 2007; Hikosaka & Shigeno, 2009) and overall improved resistance to low fertility and drought, but low growth rates (Niinemets, 2001; Wright et al., 2004). It has been hypothesized that the negative relationship between $M_A$ and photosynthetic capacity is partly because of greater biomass investment in support tissues and cell wall thickening involving stronger $CO_2$ diffusion limitations to photosynthesis (Niinemets, 1999; Wright et al., 2004; Niinemets et al., 2007).

Mesophyll conductance to $CO_2$ ($g_m$) is the measure of the $CO_2$ diffusion facility from substomatal cavities to the sites of carboxylation in the chloroplasts (Flexas et al., 2008, 2012) Mesophyll conductance is finite and variable and plays a major role in constraining photosynthetic productivity (Niinemets et al., 2009a). Large differences in $g_m$ have been shown between and within species with different leaf forms and habits (Flexas et al., 2008; Warren, 2008; Niinemets et al., 2009a, 2011). Whilst rapid changes of $g_m$ in response to environmental drivers probably depend on biochemical factors such as changes in the permeability of membranes to $CO_2$ facilitated by coooperators (Hanba et al., 2004; Flexas et al., 2006, 2012), maximum values of $g_m$ for a given species or genotype are suggested to be related to leaf anatomical properties (Niinemets et al., 2009a; Tosens et al., 2012a).

In particular, it has been shown that leaves with a more robust structure and higher $M_A$ exhibit lower photosynthetic rates due to large $CO_2$ drawdown from substomatal cavities ($C_i$) to chloroplasts ($C_c$), $C_i$-$C_c$, demonstrating that the photosynthetic capacity is limited by $g_m$ (Flexas et al., 2008, Niinemets et al., 2009a). Therefore, understanding the structural and physiological basis of variation in $g_m$ is crucial for understanding photosynthetic controls in natural ecosystems and for breeding of plant cultivars with improved photosynthetic characteristics.

At the leaf level, two components of $M_A$—leaf thickness and density—have been proposed to exert opposite effects on setting the maximum $g_m$ with increases in thickness increasing $g_m$ and increases in density reducing it (Niinemets et al., 2009b, Hassiotou et al., 2010). Inside leaves, the $CO_2$ diffusion pathway consists of two phases, an intercellular $CO_2$ phase and a cellular liquid phase, the latter consisting of aqueous and lipid components (Niinemets and Reichstein, 2003b; Evans et al., 2009). The gas phase pathway through intercellular air spaces is assumed to have a smaller effect on the overall diffusion limitations than the components of the liquid phase (Evans et al., 2009). This was confirmed in several studies comparing $CO_2$ diffusion in air and helox—air where helium replaces nitrogen to increase diffusivity—showing that the diffusion in the intercellular gas phase had little effect on photosynthesis (Parkhurst and Mott, 1990) The cellular phase is composed of the cell wall, plasma membrane, cytosol, and chloroplast envelopes and stroma. Among these components, the cell walls and chloroplast envelope have been suggested to limit $g_m$ most severely (Terashima et al., 2011). Accordingly, several reports have shown positive correlations between $g_m$ and the surface of chloroplasts adjacent to intercellular air spaces ($S/S$), which is sometimes considered as the most important anatomical trait affecting $g_m$ (Evans et al., 1994; Terashima et al., 2006; Tholen et al., 2008). However, some estimates suggest that differences in cell wall thickness ($T_{cw}$) can explain as much as 25–50% of the variability in $g_m$ (Evans et al., 2009; Terashima et al., 2011; Tosens et al., 2012b). Negative correlations between $g_m$ and $T_{cw}$ have been shown when comparing Australian Banksia species (Hassiotou et al., 2010), rice relatives (Scafaro et al., 2011), Eastern Australian species with varying anatomy (Tosens et al., 2012b), and Mediterranean Abies species (Peguero-Pina et al., 2012). Recently, Terashima et al. (2011) showed that $g_m/(S/S)$ decreases with increasing $T_{cw}$, i.e. the relative influence of the exposed chloroplast surface in setting the maximum $g_m$ is variable, and that this variation can potentially be explained by variations in $T_{cw}$.

Few previous studies have quantitatively addressed the influence of leaf anatomical traits on the diffusion of $CO_2$, and these studies have focused only on a few species and specific parts of the $CO_2$ diffusion pathway (Evans et al., 1994; Terashima et al., 2006; Hassiotou et al., 2010; Scafaro et al., 2011; Peguero-Pina et al., 2012; Tosens et al., 2012b). Hence, the whole diffusion pathway of $CO_2$ from the substomatal cavities to the chloroplasts has not been quantitatively linked to $g_m$ in plants with widely varying leaf structures and photosynthetic capacities. Furthermore, the overall importance of $g_m$ in plants with different foliage architecture has not been characterized. To fill this gap, we aimed with the present study: (i) to analyse the role of different components of the diffusion pathway across a wide range of foliage architectures and leaf photosynthetic capacities; (ii) to associate the interspecific differences in leaf anatomy with the integrated leaf architectural traits such as $M_A$ and $g_m$; (iii) to quantify the distribution of overall photosynthetic limitation among biochemistry, mesophyll diffusion, and stomata; and (iv) to quantify the resistance that each anatomical component exerts on the diffusion of $CO_2$ inside the leaf.

Material and methods

Plant material

Fifteen taxa of different growth form and leaf longevity were selected for the study to obtain an extensive range of variation in leaf morphology and anatomy (Supplementary Table S1 at JXB online). Five
species were annual herbs (Capsicum annuum, Helianthus annuus, Phaseolus vulgaris, Spinacea oleracea, Ocimum basilicum) and the rest were broad-leaved trees: four deciduous (Acer negundo, Alnus subcordata, Betula pubescens, Catalpa speciosa), one semi-deciduous (Quercus brantii) and five evergreens (Quercus ilex, Ficus elastica, Pittosporum tobira, Washingtonia filifera). All species were dicots, except for the palm, Washingtonia filifera.

All plants were grown either from commercial seed or from seeds collected in the field, except for F. elastica where rooted cuttings of a single mother plant were used. The plants were grown in a growth room with a 10 h photoperiod, a day/night temperature of 24/18 °C, 60% air humidity, and a constant photon flux density of 350 µmol m−2 s−1 at plant level provided by Philips HPI-T Plus 400 W metal halide lamps. The daily integrated incident quantum flux density was 12.6 mol m−2 d−1. The growth substrate was a 1/1 mix of quartz sand and standard potting soil (Biolan Oy, Finland) including slow-release NPK (3/1/2 ratio) fertilizer with microelements, and the plants were irrigated daily to soil field capacity. The size of the pots varied between 1 and 5 l depending on plant age and size. In all cases, fully developed young (current-season leaves in evergreens) leaves were used for the measurements. In herbs, the plants were measured 1 month after seed germination, whilst woody species were measured on the second growing year. All physiological and structural analyses were replicated with at least three independent plants per taxa.

Foliage gas exchange and fluorescence measurements

Attached leaves were used for simultaneous leaf gas-exchange and chlorophyll-fluorescence measurements using a portable gas exchange fluorescence system GFS-3000 (Walz, Effeltrich, Germany) equipped with a leaf chamber fluorometer with an 8 cm² cuvette window area. Light was provided by the LED light source of the leaf chamber fluorometer (10% blue and 90% red light) and the humidity was controlled by a built-in GFS-3000 humidifier. Use of a certain fraction of blue light is routinely used in portable photosynthesis devices to induce stomatal opening. Although blue light is absorbed more strongly by the upper leaf layers and may lead to discrepancies among photosynthesis and fluorescence profiles (Evans and Vogelmann, 2006), thereby altering ΦPSII estimations by the combined gas-exchange/fluorescence techniques (Loreto et al., 2009), the amount of blue light used in our study was small and the expected effect minor.

The standard conditions for leaf stabilization in the cuvette were: leaf temperature of 25 °C, saturating quantum flux density of 1500 µmol m−2 s−1, and CO₂ concentration in the cuvette (Cᵣ) of 385 µmol CO₂ mol air−1. Once the steady-state conditions were reached, typically 15–20 min after clamping the leaf in the cuvette, CO₂ response curves of net assimilation (A) were measured. First, Cᵣ was lowered stepwise from 385 to 50 µmol CO₂ mol air−1 and then raised again to 385 µmol CO₂ mol air−1, and the leaf was kept at this Cᵣ until the original Aₕ value was achieved. Next, Cᵣ was increased stepwise from 385 to 1500 µmol CO₂ mol air−1 and returned again to 385 µmol CO₂ mol air−1. In all cases, measurements of Aₕ and steady-state fluorescence yield (Fₒ) were recorded after the gas-exchange rates stabilized at the given Cᵣ. After recording the Aₕ value, a flash of saturating white light was given to determine the maximum fluorescence yield in light-adapted state (Fₚmax). After completion of the CO₂ response curves, the light was switched off and respiration rate in the dark (Rᵡ) was determined. In calculations of Aₖ, Rᵡ, and intercellular CO₂ concentration (Cᵣ), corrections for the diffusion leakage of CO₂ into and out of the leaf chamber were included as described by Flexas et al. (2007).

Measurements of leaf optical properties

Leaf transmittance and reflectance measurements were conducted with a spectrometer ( AvaSpec-2048-2; Avantes, Apeldoorn, The Netherlands) using an integrating sphere (ISP-80-S-R; Ocean Optics, Dunedin, FL, USA). Leaf absorptance (α) was calculated as 1 minus the sum of reflectance and transmittance. Three leaves of each species were measured, and within each leaf, three replicate measurements were made. Average absorptance across the 400–700 nm region was used to characterize the fraction of incident photosynthetically active radiation absorbed by the leaf.

Anatomical measurements

After the gas-exchange measurements, 1 × 1 mm pieces were cut between the main veins from the same leaves for anatomical measurements. Leaf material was quickly fixed under vacuum with 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Afterwards, the samples were fixed in 1% osmium tetroxide for 1 h and dehydrated in a graded ethanol series, followed by washing three times in propylene oxide. The dehydrated segments were embedded in Spurr’s resin (Monocomp Instrumentacion, Madrid, Spain) and cured in an oven at 60 °C for 48 h. Semi-thin (0.8 µm) and ultrathin (90 nm) cross-sections were cut with an ultramicrotome (Reichert & Jung model Ultracut E). Semi-thin cross-sections were stained with 1% toluidine blue and viewed under an Olympus BX60light microscope. Photos were taken at 2000× magnification (Supplementary Fig. S1D–F) to measure the size of mesophyll cells and chloroplasts adjacent to intercellular air spaces and chloroplast width and thickness, and the volume fraction of intercellular air space calculated as:

$$\text{f}_{\text{ias}} = 1 - \frac{\sum S_i}{t_{\text{mes}} \cdot W} \tag{1}$$

where $\Sigma S_i$ is the total cross-sectional area of mesophyll cells, $W$ is the width of the section, and $t_{\text{mes}}$ is the mesophyll thickness between the two epidermises. Mesophyll ($S_i/S$) and chloroplast ($S/S$) surface area exposed to intercellular air spaces per leaf area were calculated separately for spongy and palisade tissues as described by Evans et al. (1994) and Syvertsen et al. (1995). The curvature correction factor was measured and calculated for each species according to Thain (1983) for palisade and spongy cells by measuring their width and height and calculating an average width/height ratio. The curvature factor correction ranged from 1.16 to 1.4 for spongy cells and from 1.4 to 1.5 for palisade cells. All parameters were analysed at least in four different fields of view and at three different sections. Weighted averages based on tissue volume fractions were calculated for $S_i/S$ and $S/S$.

$T_{w}$ and cytoplasm thickness ($T_{cyt}$) were measured at 20 000–40 000× magnification depending on the species (Supplementary Fig. S1–I). Three different sections per species and four to six different fields of view were measured for each anatomical characteristic. Micrographs were selected randomly in each section and $T_{w}$ was measured for two to three cells per micrograph. Ten measurements for spongy tissue and ten for palisade parenchyma cells were made for each anatomical trait, and weighted averages based on tissue volume fractions were calculated. All images were analysed with Image analysis software (ImageJ, Wayne Rasband/NIH, Bethesda, MD, USA).

$M_A$ and leaf density

The leaves were scanned at 300 dpi, and then oven dried at 70 C for 48 h and their dry mass was estimated. Leaf area was determined from the images with Image J. From these measurements, $M_A$ was calculated. Using the estimates of leaf thickness from anatomical
Estimation of $g_m$ and model parameters Farquhar et al. (1980) by combined gas-exchange/fluorescence method

The chlorophyll hypothesis CO$_2$ compensation point ($\Gamma^*$) in the absence of $R_d$ was calculated from the Rubisco specificity factor ($S_{CO2}$) as:

$$\Gamma^* = 0.5 \text{ O}/\text{SC/O}$$ (2)

using the average values for $S_{CO2}$ reported by Galmés et al. (2005) for each different leaf habit (Supplementary Table S2 at JXB online). A sensitivity analysis showed that the precise value of $\Gamma^*$ within the reported range did not significantly affect the $g_m$ estimates (Supplementary Table S3A at JXB online).

From chlorophyll fluorescence measurements, the actual photo-chemical efficiency of photosystem II ($\Phi_{PSII}$) was determined from $F_s$ and the maximum fluorescence yield during a light-saturating pulse of 4500 μmol m$^{-2}$ s$^{-1}$ ($F_{m}'$) following the method of Genty et al. (1989):

$$\Phi_{PSII} = (F_{m}' - F_s)/F_{m}'$$ (3)

The linear electron transport rate on the basis of chlorophyll fluorescence ($J_F$) was then calculated as:

$$J_F = \Phi_{PSII}Q\alpha\varepsilon_{PSII}$$ (4)

where $Q$ is the photosynthetically active quantum flux density, $\alpha$ is the leaf absorptance, and $\varepsilon_{PSII}$ is the fraction of light absorbed by PSII. As routinely assumed, $\varepsilon_{PSII}$ was taken as 0.5 (Loreto et al., 1994; Niinemets et al., 2005).

Furthermore, the $g_m$ to CO$_2$ was estimated according to Harley et al. (1992) as:

$$g_m = \frac{A_{N} - 4\left(\frac{R_{d}}{R_{s}} + R_{e}\right)}{C_{i} - \frac{\Gamma^* \left(\frac{1}{J_F} + 8\left(\frac{A_{N}}{R_{d}} + R_{e}\right)\right)}{J_F - 4\left(\frac{A_{N}}{R_{d}} + R_{e}\right)}}$$ (5)

where $R_e$ is the respiration rate in the light. In this study, $R_d$ was used as a proxy for $R_e$ (Gallé et al., 2009). In other studies, half $R_d$ has been used (Piel et al., 2002; Niinemets et al., 2005). However, as shown in Supplementary Table S3B, no significant differences in $g_m$ were found when using the proxy for $R_e$, and hence we concluded that selection of the appropriate value for $R_e$ is not a critical issue for our $g_m$ estimates, confirming a previous sensitivity analysis (Niinemets et al., 2006).

The obtained values of $g_m$ were used to transform the $A_{N}$-$C_{i}$ response curves into $A_{N}$ versus $C_{i}$ response curves as $C_{i} = C_{i} - A_{N}/g_m$. Finally, Farquhar et al. (1980) model parameters, the maximum velocity of carboxylation ($V_{cmax}$) and the capacity for photosynthetic electron transport ($J_{max}$) on the basis of $C_{i}$ were calculated according to Bernacchi et al. (2002). Three replicates estimates of $g_m$ were available for every species.

Estimation of $g_m$ from gas exchange measurements only: the curve-fitting method

The curve-fitting method introduced by Ether and Livingston (2004) as applied by Niinemets et al. (2005) was used to obtain an alternative estimate of $g_m$. This method is based on changes in the curvature of $A_{N}$ versus $C_{i}$ response curves due to finite $g_m$ such that the Farquhar et al. (1980) model based on $C_{i}$ imperfectly fits the data (Ether and Livingston 2004). Thus, including $g_m$ as a fitted parameter significantly improves the model fit. Estimates of $J_{max}$, $V_{cmax}$, and $g_m$ were derived from fitting $A_{N}$-$C_{i}$ curves as previously described. Values of the Michaelis–Menten constant for CO$_2$ ($K_c$), and oxygen ($K_o$) and their temperature responses used for these estimations were from Bernacchi et al. (2002). $\Gamma^*$ was calculated according to Eqn 2, and $R_d$ by gas exchange measurements at 385 μmol CO$_2$ mol$^{-1}$. At least three plants per species were used to estimate $g_m$. The same leaves were used for estimation of $g_m$ by the Ether and Livingston (2004) and Harley et al. (1992) methods.

$g_m$ modelled from anatomical characteristics

The one-dimensional gas diffusion model of Niinemets and Reichstein (2003a) as applied by Tosens et al. (2012a) was employed to estimate the share of different leaf anatomical characteristics in determining $g_m$. $g_m$ as a composite conductance for within-leaf gas and liquid components is given as:

$$g_m = \frac{1}{k_{gas} + \frac{RT}{H \cdot g_{liq}}}$$ (6)

where $g_{gas}$ is the gas phase conductance inside the leaf from substomatal cavities to outer surface of cell walls, $g_{liq}$ is the conductance in liquid and lipid phases from outer surface of cell walls to chloroplasts, $R$ is the gas constant (Pa m$^3$ K$^{-1}$ mol$^{-1}$), $T$ is the absolute temperature (K), and $H$ is the Henry’s law constant (Pa m$^3$ mol$^{-1}$). $g_m$ is defined as a gas-phase conductance, and thus $H(RT)$, the dimensionless form of Henry’s law constant, is needed to convert $g_{gas}$ to corresponding gas-phase equivalent conductance (Niinemets and Reichstein, 2003a). In the model, the gas-phase conductance (and the reciprocal term, $r_{gas}$) is determined by average gas-phase thickness, $\Delta L_{gas}$, and gas-phase porosity, $f_{gas}$ (fraction of leaf air space):

$$g_{gas} = \frac{1}{r_{gas}} = \frac{D_{l}}{\Delta L_{gas}} \cdot c$$ (7)

where $D_{l}$ is the diffusion path tortuosity (m$^{-1}$) and $c_i$ is the diffusion coefficient for CO$_2$ in the gas phase ($1.51 \times 10^{-5}$ at 25°C). $\Delta L_{gas}$ was taken as half the mesophyll thickness. The partial determination of the liquid-phase diffusion pathway (the reciprocal term $r_{liq}$, where $j$ stands either for cell wall, cytosol, or stroma conductance) were calculated as:

$$g_{liq} = \frac{1}{r_{liq}} = \frac{f_{gas} \cdot D_{gas} \cdot p_i}{\Delta L_{liq}}$$ (8)

where $\Delta L_{liq}$ (m) is the diffusion path length in the corresponding component of the diffusion pathway, $p_i$ (m$^3$ m$^{-1}$) is its effective porosity, and $D_{gas}$ is the aqueous phase diffusion coefficient for CO$_2$ ($1.79 \times 10^{-5}$ m$^2$ s$^{-1}$ at 25°C). The dimensionless factor $r_{f,i}$ accounts for the reduction of $D_{gas}$ compared with free diffusion in water, and was taken as 1.0 for cell walls (Rondeau-Mouro et al., 2008) and 0.3 for cytosol and stroma (Niinemets and Reichstein, 2003b). In addition, $r_{f,i}$ values for cytosol and stroma were estimated using a least-squares iterative analysis to assess the sensitivity of $g_m$ to values of $r_{f,i}$ (Supplementary Figs S2 and S3 at JXB online). In this analysis, $r_{f,i}$ was allowed to vary between 1 and 0.05, and the values of $r_{f,i}$ were varied within this range to minimize the difference between measured and modelled $g_m$. Whilst this approach improved the agreement between modelled and measured $g_m$, the extreme values obtained for $r_{f,i}$ seemed unrealistic (Supplementary Figs S2 and S3). $p_i$ was set to 1.0 for cytosol and stroma. There are no direct measurements of cell wall porosity, but it has been suggested that this parameter might vary with $T_{cw}$ among species (Terashima et al., 2006; Evans et al., 2009; Tosens et al., 2012b). Therefore, given the heterogeneous
series of species used in this experiment, $p_r$ was estimated using a least-squares iterative analysis assuming a hypothetical relationship between porosity and $T_m$, as described by Tosens et al. (2012b). Again, a least-squares iterative approach was employed to get the best fit between measured and modelled $g_m$. The $p_r$ range in this analysis was fixed at 0.028 (Tosens et al., 2012b) for the thickest to 0.3 (Nobel, 1991) for the thinnest cell walls (Supplementary Table S5 at JXB online). We used an estimate of 0.0035 m s$^{-1}$ for both plasma membrane conductance ($g_{pl}$) and chloroplast envelope conductance ($g_{env}$) as previously suggested (Evans et al., 1994; Tosens et al., 2012a).

Carbonic anhydrase in cytosol and chloroplasts could facilitate the diffusion of CO$_2$ through the liquid phase. However, there is little evidence for the involvement of carbonic anhydrase in $g_m$ and $A_N$ (reviewed by Flexas et al., 2008, 2012). Therefore, following Tosens et al. (2012a), we did not include the potential effect of carbonic anhydrase in our analysis.

In previous studies, we scaled the total liquid-phase diffusion conductance by $S/S$ ratio (Tosens et al., 2012a) that determines the number of parallel diffusion pathways from outer surfaces of cell walls to chloroplasts.

$$g_{liq} = \frac{S_c}{(\tau_{cw} + \tau_{pl} + \tau_{cyt1} + \tau_{cyt2} + \tau_{st1})} S \quad (9)$$

Although, cell wall and plasmalemma resistances actually scale with the $S_c/S$ ratio, use of $S_c/S$ has been deemed to be appropriate, as $S_c/S$ is generally close to the $S_p/S$ ratio (Scalfaro et al., 2011; Peguero-Pina et al., 2012), i.e. there is little cell wall area free of chloroplasts. Even if $S_c/S$ is significantly smaller than $S_p/S$, the cytosolic distance between the neighbouring chloroplasts is generally large and this can still constrain the diffusion flux in interchloroplastial areas of cell wall (locally large cytosol conductance; $g_{cyt1}$ in Fig. 1). However, the significance of the $\tau_{cyt}$ depends on the other parts of the diffusion pathway as well.

To explicitly assess the importance of diffusion of CO$_2$ through interchloroplastial areas, we considered two different pathways of CO$_2$ inside the cell, one for cell wall parts with chloroplasts and the other for interchloroplastial areas as described by Tholen et al. (2012). For exposed cell wall portions lined with chloroplasts, the partial liquid phase conductance, $g_{cel,1}$, inside the cell is given as:

$$g_{cel,1} = \frac{1}{\tau_{cyt1} + \tau_{env} + \tau_{st1}} \quad (10)$$

where $\tau_{cyt1}$ and $\tau_{st1}$ are cytosolic resistance from the plasmalemma inner surface to the outer surface of chloroplasts and the stromal resistance in the direction perpendicular to cell wall (Fig. 1), respectively, both calculated by Eqn 8. For $\tau_{cyt1}$, the diffusion pathway length, $\Delta L_{cyt1}$, is given as the average distance between the chloroplasts and cell wall in cell wall areas lined by chloroplasts (Fig. 1), whilst for $\tau_{st1}$, $\Delta L_{st}$ was taken as half of the chloroplast thickness, $\Delta L_{2}/2$.

For the cell wall portions without chloroplasts, the partial conductance, $g_{cel,2}$, is given analogously as:

$$g_{cel,2} = \frac{1}{\tau_{cyt2} + \tau_{env} + \tau_{st2}} \quad (11)$$

where $\tau_{cyt2}$ is the cytosolic resistance from interchloroplastic cell wall portions towards the chloroplast and $\tau_{st2}$ is the stromal conductance in a direction parallel with the cell wall (Fig. 1). The diffusion path length for $\tau_{cyt2}$ (Eqn 1), $\Delta L_{cyt2}$, is driven both by the distance between the neighbouring chloroplasts, chloroplast thickness, and chloroplast distance from the cell wall and was approximated as:

$$\Delta L_{cyt2} = \sqrt{\left(\frac{\Delta L_{ch1}}{2} + \left(\frac{\Delta L_{cyt1}}{2}\right)^2\right) + \left(\frac{\Delta L_{ch2}}{2}\right)^2} \quad (12)$$

where $\Delta L_{ch}$ is the distance between the neighbouring chloroplasts. $\Delta L_{cyt2}$ was calculated as a harmonic average, which more correctly represents the diffusion pathway of $\tau_{cyt2}$. Finally, the diffusion pathway length for $\tau_{st2}$ was taken as a quarter of the chloroplast length.

Considering further that the fraction of exposed cell wall area lined with chloroplasts is given by $S_c/S_m$ and the fraction free of chloroplasts as $1 - S_c/S_m$, the total cellular conductance (sum of parallel conductances) is given as:

$$g_{cel,tot} = \frac{S_c}{S_m} g_{cel,1} + \left(1 - \frac{S_c}{S_m}\right) g_{cel,2} \quad (13)$$

Total liquid phase conductance from the outer surface of cell walls to carboxylation sites in the chloroplasts is the sum of serial conductances in the cell wall, plasmalemma, and inside the cell:

$$g_{liq} = \frac{S_m}{(\tau_{cw} + \tau_{pl} + \tau_{cel,tot})} S \quad (14)$$

Alternatively, the total cellular diffusion pathway can be considered to consist of two parallel pathways from the outer surface of the cell walls to the chloroplasts, one pathway corresponding to the diffusion flux through cell wall areas lines with chloroplasts and the other without chloroplasts:

Fig. 1. Illustration of the diffusion pathway in exposed cell wall areas lined with chloroplasts (path 1) and interchloroplastial areas (path 2). The diffusion pathway in leaf lipid and liquid phases includes cell wall, plasmalemma, cytosol (shown by red arrows), chloroplast envelope membranes, and chloroplast stroma (shown by dark green arrows). The effective diffusion path length in cytosol along path 1 is taken as the average distance of chloroplasts from the cell wall, $\Delta L_{cyt1}$, whilst the diffusion pathway length in interplastidial areas is determined by the distance between the chloroplasts and $\Delta L_{cyt1}$ (Eqn 12).
\[ g_{\text{eq}} = \frac{S_c}{(e_w + r_{l1} + r_{c1,1})} S + \frac{S_m - S_c}{(e_w + r_{l2} + r_{c1,2})} S \]  

(15)

Although Eqns 14 and 15 are conceptually different, the values of conductances calculated by both equations were very similar, differing at most by 4%. In the current study, we have used Eqn 14.

Analysis of quantitative limitations on \( A_N \)

To separate the relative controls on \( A_N \) resulting from limited stomatal conductance (\( l_s \)), mesophyll diffusion (\( l_m \)), and limited biochemical capacity (\( l_b \) (\( l_s + l_m + l_b = 1 \)), we used the quantitative limitation analysis of Jones (1985) and implemented by Grassi and Magnani, (2005). The limitations of the different components were calculated as:

\[ l_s = \frac{g_{\text{tot}}/g_s \cdot \partial A_N/\partial C_c}{g_{\text{tot}} + \partial A_N/\partial C_c}, \]  

(16)

\[ l_m = \frac{g_{\text{tot}}/g_m \cdot \partial A_N/\partial C_c}{g_{\text{tot}} + \partial A_N/\partial C_c}, \]  

(17)

\[ l_b = \frac{g_{\text{tot}}/g_b \cdot \partial A_N/\partial C_c}{g_{\text{tot}} + \partial A_N/\partial C_c}, \]  

(18)

where \( g_s \) is the stomatal conductance to \( \text{CO}_2 \), \( g_m \) was according to Harley et al. (1992, Eqn 5), and \( g_{\text{tot}} \) is the total conductance to \( \text{CO}_2 \) from ambient air to chloroplasts (sum of the inverse serial conductances \( g_s \) and \( g_m \)). \( \partial A_N/\partial C_c \) was calculated as the slope of \( A_N - C_c \) response curves over a \( C_c \) range of 50–100 \( \mu \text{mol} \cdot \text{mol}^{-1} \). At least three curves per species were used, and average estimates of the limitations were calculated.

Quantitative analysis of partial limitations of \( g_m \)

The determinants of \( g_m \) were divided between the component parts of the diffusion pathway (Eqns 6–8). The proportion of \( g_m \) determined by limited gas-phase conductance (\( l_{\text{gas}} \)) was calculated as:

\[ l_{\text{gas}} = \frac{g_m}{g_{\text{gas}}} \]  

(19)

The share of \( g_m \) by different components of the cellular phase conductances (\( l_i \)) was determined as:

\[ l_i = \frac{S_m}{g_i} \cdot \frac{S_m}{S} \]  

(20)

where \( l_i \) is the component limitation in the cell walls, the plasmalemma, and inside the cells, and \( g_i \) refers to the component diffusion conductances of the corresponding diffusion pathways. To determine the limitations derived from the different components inside the cell (cytoplasm, chloroplast envelope, and stroma), weighted limitations of both pathways, the fraction of exposed cell wall area lined with chloroplasts and the fraction free of chloroplasts, were used.

Statistical analyses

Regression and correlation analyses were conducted using the Sigma Plot 10.0 software package (SPSS; Chicago, IL, USA). Univariate analysis of variance was performed to reveal differences between species in the studied characteristics. Differences between means were revealed by Tukey analyses (\( P < 0.05 \)). These analyses were performed with the IBM SPSS statistics 19.0 software package (SPSS).

Results

Leaf structural and anatomical traits

\( M_A \) varied sixfold (20–123 g m\(^{-2}\)) (Supplementary Table S4 at JXB online). The variation in leaf thickness was 3.7-fold with \( \text{Acer negundo} \) having the thinnest (123 \( \mu \text{m} \)) and \( F. \text{elastica} \) the thickest (459 \( \mu \text{m} \)) leaves. Spongy mesophyll thickness varied 5.2-fold, and palisade mesophyll thickness 2.5-fold (Supplementary Table S4). Generally, the palisade tissue comprised approximately 40%, and spongy tissue approximately 60% of total mesophyll, except for some species as \( F. \text{elastica} \) with 75% and \( W. \text{filifera} \) with 100% of spongy tissue. The variation in \( D_t \) was 6.4-fold with \( \text{Phaseolus vulgaris} \) having the least dense (0.11 g cm\(^{-3}\)) and \( Q. \text{ilex} \) the most dense (0.70 g cm\(^{-3}\)) leaves. \( M_A \) exhibited a significant positive correlation with \( D_t \) (Supplementary Fig. S4 at JXB online), but was weakly correlated with leaf thickness (\( r^2=0.27 \), \( P < 0.05 \); data not shown). Therefore, the variation in \( M_A \) was mainly attributed to the leaf density.

Among the leaf ultrastructural characteristics estimated from transmission electron micrographs (Supplementary Tables S4 and S5, and Supplementary Fig. S1D–I), \( S_m/S \) varied 3.3-fold across all species (14.4–40 m\(^2\) m\(^{-2}\)) and \( S_m/S \) varied 2.7-fold (6–19.7 m\(^2\) m\(^{-2}\)). \( S_m/S_m \) varied between 0.31 (\( \text{Citrus reticulata} \)) and 0.74 (\( O. \text{basilicum} \)). For \( T_w \) (Supplementary Fig. S1G–I), 4.8-fold variation was observed between all species (113.6–543.7 nm). Herbaceous species exhibited the thinnest cell walls together with \( \text{Catalpa speciosa} \), whilst evergreens had the thickest cell walls with the maximum value of 543.7 nm observed in \( \text{Pittosporum tobira} \).

Estimation of \( g_m \) with different methods

The values of \( g_m \) calculated according to the methods of Harley et al. (1992) and Ethier and Livingston (2004) were strongly correlated (Supplementary Fig. S5 at JXB online, \( r^2=0.80 \)). However, the Harley et al. -based estimates exhibited the smallest average coefficient of variation for independent estimates within a species and therefore we report the data obtained with this method only.

Mesophyll conductance calculated by the method of Harley et al. (1992) varied 24-fold across all species. \( H. \text{annuus} \) showed the maximum values and \( \text{Citrus reticulata} \) the minimum values of \( g_m \). The minimum value for the coefficient of variation in \( g_m \) was 1.9% (\( \text{Pittosporum tobira} \)), whilst the maximum value was 32.9% (\( Q. \text{ilex} \)). The average of the coefficient of variation for all species was 16.5%.

\( g_m \) in relation to physiological characteristics

Net assimilation rate correlated positively with \( g_s \) and \( g_m \) (Supplementary Fig. S6 at JXB online). \( C_r C_c \) ranged from 240 to 112 \( \mu \text{mol} \cdot \text{mol}^{-1} \) in woody deciduous and evergreen species, and had lower values (40–67 \( \mu \text{mol} \cdot \text{mol}^{-1} \)) in herbs. \( C_r C_c \) decreased with increasing \( g_m \) (Supplementary Fig. S7 at JXB online).
species are represented as: herbs (circles), woody deciduous and semi-deciduous species (triangles), and woody evergreen species (squares). Values are means ±standard error (SE) of three to four replicates per species.

**Overall importance of \( g_m \)**

According to quantitative limitations analysis of \( A_N \), stomatal openness and \( g_m \) restricted the photosynthetic capacity to a similar percentage, 19–65% and 13–64%, respectively. However, the biochemical limitations were lower than the stomatal and mesophyll limitations, being between 6 and 33\% (Fig. 4A–C). Both the stomatal and biochemical components tended to be more important in species with non-sclerophytic leaves (low \( M_A \)), whilst mesophyll diffusion limitation was most significant in species with high \( M_A \) (Fig. 4). Thus, herbaceous plants showed the maximum values for stomatal limitations, whilst the maximum mesophyll limitations were observed in evergreen species with more robust foliage structure.

**Limitation of \( g_m \) due to individual components of the diffusion pathway**

From the different components of the whole diffusion pathway of \( \text{CO}_2 \), the percentage limitations of \( g_m \) were estimated (Fig. 4D–I). Intercellular air spaces represented a smaller resistance to the \( \text{CO}_2 \) diffusion (4–22\%) than the cellular phase, because the rate of \( \text{CO}_2 \) diffusion in air was larger than in water. In the cellular phase, the cell walls appeared to be the most important factor that limited the internal diffusion of \( \text{CO}_2 \) in the species that presented a high \( M_A \). However, the plants with low \( M_A \) that presented a low percentage of limitation of \( g_m \) by the cell wall revealed a higher limitation by the stroma of around 43\%. On the other hand, the plasmalemma and chloroplast envelope accounted for only up to 8\% of the limitation.

**Discussion**

**Values of \( g_m \) in a range of species exhibiting different foliage morphologies**

The range of \( g_m \) values observed in our study is representative of the whole range of \( g_m \) values described so far in large

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**Fig. 2.** Correlations of mesophyll diffusion conductance (\( g_m \)) determined according to Harley et al. (1992) with the surface area of chloroplasts exposed to intercellular airspaces per unit leaf area (\( S_{/}/S \)) (A), mesophyll diffusion conductance with chloroplast surface area per leaf density (\( S_{/}/S/D_l \)) (B), and mesophyll diffusion conductance per \( S_{/}/S \) (\( g_m/(S_{/}/S) \)) with \( T_{cw} \) (C). In the main panels, the data were fitted by linear (\( B \)) and non-linear (\( C \)) regressions in the form \( y=ae^{bx} \). In the inset, the data were fitted by linear regression. Different species are represented as: herbs (circles), woody deciduous and semi-deciduous species (triangles), and woody evergreen species (squares). Values are means ±standard error (SE) of three to four replicates per species.
The relative contribution of \( g_m \), \( g_m \), and photosynthetic biochemistry to total photosynthesis limitation (following Grassi and Magnani, 2005) was variable and depended on leaf structural characteristics, i.e. \( M_A \) (Fig.4A–C). At a typical operating \( \text{CO}_2 \) concentration, the biochemical limitations of photosynthesis decreased from a maximum of approximately 33% at low \( M_A \) to minimum values as \( M_A \) increased, whilst, in parallel, mesophyll diffusion limitations increased from a minimum of approximately 15% to maximum values up to 65%. Stomatal limitations showed a less clear variation with \( M_A \). Overall, these data demonstrated that species with low \( M_A \) showed a notable coordination of the limiting factors for photosynthesis, i.e. they were similarly co-limited by stomatal, mesophyll, and biochemical limitations. In contrast, species with high \( M_A \) were mostly limited by mesophyll (on average by 57%) and stomatal (30%) diffusion, and were less limited by biochemistry (13%). This is consistent with the idea that species with thicker and denser leaves, e.g. evergreen trees, are more limited by \( g_m \) than species with thinner leaves (Galmés et al., 2007; Niinemets et al., 2011).

**Key structural factors regulating differences in \( g_m \) between distant leaf structures**

The fact that \( g_m \) and mesophyll diffusion limitations were strongly correlated with \( M_A \) suggested that interspecific variations in \( g_m \) are driven by leaf structural characteristics. Among the key structural traits suggested to limit \( \text{CO}_2 \) diffusion the most are the traits that alter effective diffusion path length and area for diffusion, in particular \( T_{cw} \), and chloroplast distribution along the exposed mesophyll cell wall (Fig. 2; Evans et al., 2009), although the role of other variables, such as leaf porosity, and the path lengths for \( \text{CO}_2 \) through the plasmalemma and chloroplast envelope membranes, cytosol, and stroma cannot be ruled out (Evans et al., 2009). In the present study, we modelled \( g_m \) considering all major leaf structural traits as described by Tosens et al. (2012a). A high significant positive correlation between measured and modelled \( g_m \) estimates was found (Fig. 3). This correlation supports the view that at least a significant proportion of the interspecific variations in \( g_m \) is somehow related to differences in the thickness of the structures involved in \( \text{CO}_2 \) diffusion, as well as to the number of parallel \( \text{CO}_2 \) diffusion pathways determined by \( S/S \).

Despite the high correlation, the slope of the relationship was not unity, so that the biggest discrepancies between measured and modelled estimates of \( g_m \) were found at the higher and lower ends of \( g_m \). A similar discrepancy was observed in different Australian sclerophyll species occurring in the field under different soil nutrients and water availabilities, especially at high values of \( g_m \) (Tosens et al., 2012b).

This strong discrepancy between measured and modelled values may arise from the inherent uncertainties associated with both estimates. As for the Harley et al. (1992) approach, besides the small variability in the estimates associated with uncertainties in the exact values of \( R_L \) and \( \Gamma^{*} \) (Supplementary Table S3), it has recently been shown that \( g_m \)
cannot be considered as a purely diffusional component, but instead intrinsically includes a flux-weighted quantity related to the amount of respiratory and photorespiratory CO₂ from the mitochondria diffusing towards the chloroplasts (Tholen et al., 2012). Concerning the anatomically based model used here, the precise outputs largely depend on a number of variables assumed as constants or inferred indirectly. For instance, the reduction in $D_w$ compared with free diffusion in water ($r_f,i$) was considered constant for all species, although different for cell wall and intercellular components. Both $g_{pl}$ and $g_{env}$ were also taken as constant, whilst cell wall porosity ($p_i$) was indirectly estimated from $T_{cw}$ using an empirical equation. There is not sufficient knowledge for the actual values of all these parameters, and they may vary among species, hence contributing to most of the observed slope discrepancy. It can be seen, for instance, that the difference between measured and modelled $g_m$ almost disappeared when the $r_f,i$ values were calculated using a least-squares iterative analysis (Supplementary Fig. S2). However, this ‘perfect correspondence’ is bound to some probably non-realistic $r_f,i$ values as low as 0.05. Moreover, values of $g_m$ have been modelled considering CO₂ diffusivities in the different media involved—assumed to be either ‘pure’ air, lipid, or aqueous phases with fixed thicknesses, whilst, in most cases, determination of the thickness of the given phase is not that straightforward. Also, we assumed no facilitation mechanism that could improve the diffusivities in lipid and aqueous phases. Among these, membrane-bound aquaporins (Uehlein et al., 2003, 2008; Hanba et al., 2004; Flexas et al., 2006) and cytosol and stromal forms of carbonic anhydrases (Price et al., 1994; Gillon and Yakir, 2000) are likely candidates (Terashima et al., 2011). For instance, allowing $g_{pl}$ and/or $g_{env}$ to vary within the range of published values (Evans et al., 2009) also results in a better agreement between the measured and modelled values (Supplementary Fig. S8 at JXB online). In summary, current uncertainties about the actual values of these parameters and
their variability among species preclude the development of a truly predictive anatomically based model for \( g_m \). However, the good correlation, despite the divergent slope, can be taken as strong evidence that a substantial part of \( g_m \) is indeed dependent on a series of leaf anatomical features.

Despite the discussed limitations of the model approach used here, the results suggest that chloroplast distribution and \( T_{cw} \) are the most influential leaf structural characteristics in setting the limits for \( g_m \) (Evans et al., 2009; Terashima et al., 2011). In particular, a significant positive correlation was found between \( g_m \) and \( S/S \) only when species with very large \( T_{cw} \) (\( Q. \) brantii and \( P. \) toitbara) were excluded, highlighting the fact that the impact of chloroplast distribution on \( g_m \) became less important as \( T_{cw} \) increased, in agreement with past suggestions (Terashima et al., 2006, 2011).

In addition, a highly significant negative relationship was observed between the ratio \( g_m/(S/S) \) and \( T_{cw} \) considering all species, similar to that obtained by Terashima et al. (2011) pooling literature data. Using a limitation analysis to separate the contributions of the components of \( g_m \) (Eqns 13 and 14) revealed that, globally, the limitation imposed by \( T_{cw} \) spanned the most, ranging from approximately 4 to 70% (Fig. 4E). This was followed by chloroplast stroma, which ranged from 4 to 46% (Fig. 4E, I). However, the limitations inside the cell (cytosol and stroma) could be underestimated, especially in species with high \( M_A \), as \( g_m \) was modelled assuming that cytosolic and stromal viscosity (\( \tau_1 \)) was constant in all species.

The limitations imposed by intercellular air spaces, the plasmalemma, and the chloroplast envelope were much smaller than the rest of the diffusion pathway components as was observed by Tosens et al. (2012a,b). The fact that the latter two components had only a moderate effect on limiting \( g_m \) is in conflict with the observed larger \( g_m \) changes observed in aquaporin mutant plants without any appreciable differences in \( S/S \) or any other leaf structural characteristic (Flexas et al., 2006). This could be due to the fact that the assumed values for \( g_{pl} \) and \( g_{en}\) are constant among species, which may not necessarily be the case. Differences of up to four orders of magnitude have been reported for \( CO_2 \) permeabilities of biological membranes. For instance, if the permeability for a given species was 0.00002 m s\(^{-1}\), as found for chloroplast envelopes by Uehlein et al. (2008), instead of the 0.0035 m s\(^{-1}\) used in the present simulation, the combined limitation to \( g_m \) imposed could be larger than 40% (data not shown). At the other extreme, if values were closer to the 0.016 m s\(^{-1}\) reported by Missner et al. (2008) for lipid bilayers, the maximum modelled \( g_m \) values will be closer to estimates based by the Harley et al. (1992) approach (data not shown). Clearly, improved knowledge on the actual permeability to \( CO_2 \) of biological membranes is required to fully understand the basis for the regulation of \( g_m \).

Despite these general tendencies, the impact of each specific leaf component on \( g_m \) changed with \( M_A \). Specifically, the limitations imposed by \( T_{cw} \) strongly increased with increasing \( M_A \), whilst the limitations associated with all the other components decreased with increasing \( M_A \). Thus, in species with low \( M_A \), like annual herbs, about 60% of the total limitation to \( g_m \) is imposed by cytoplasm and stroma, whilst another 12% is accounted for by the plasmalemma and chloroplast envelope. Moreover, in species with thinner leaves, the fraction of exposed cell wall lined with chloroplasts (\( g_{col.1} \)) was higher, whilst limitations inside the cell through interchlorenchymatous areas (\( g_{col.2} \)) were more important in species with higher \( M_A \) (Fig. S9 at JXB online). This suggests that it is in such species where facilitating mechanisms (aquaporins, carbonic anhydrases, chloroplast movements, and others) have the strongest influence on \( g_m \). In contrast, in species with high \( M_A \), like evergreen sclerophylls, \( g_m \) is mostly (up to 70%) limited by \( T_{cw} \), which is likely to be less variable in the short term, and may explain the low photosynthetic capacity displayed by these plants even under non-limiting conditions. Possible interspecific variation in the role of aquaporins in limiting \( g_m \) is clearly a topic that deserves high priority in future studies.

In conclusion, the present study showed that mesophyll limitations are crucial in determining the maximum photosynthetic capacity when a large range of leaf types are analyzed collectively. These limitations are variable depending on the leaf structural properties, i.e. \( M_A \) and associated structural traits such as leaf density. The variability in mesophyll diffusion limitations was explained mainly by variations in the rate of \( CO_2 \) diffusion pathways through cell walls, as well as the area for diffusion determined by the chloroplast distribution. However, the impact of each component of the diffusion pathway largely depended on \( M_A \), so that \( CO_2 \) diffusion in species with thin leaves (e.g. herbs) depends more on membranes and aqueous compartments—and is probably more influenced by aquaporins and carbonic anhydrases. In contrast, diffusion in species with thick leaves is almost fully determined by cell wall conductance. Altogether, the variability in \( g_m \) with \( M_A \) helps explain the worldwide leaf economics spectrum showing a negative dependency between photosynthetic capacity and \( M_A \).

Supplementary data can be found at JXB online.

Supplementary Table S1. List of studied species, species origin, life form, and leaf longevity.

Supplementary Table S2. Physiological characteristics measured in all studied species.

Supplementary Table S3. Sensitivity analysis of the influence of uncertainties in chloroplastic hypothetical \( CO_2 \) compensation point (\( \Gamma^* \)) and day respiration on the estimation of mesophyll conductance (\( g_{en} \)).

Supplementary Table S4. Leaf dry mass per unit area (\( M_A \)), leaf thickness (\( T_{pl} \)), leaf density (\( D_{pl} \)), thickness of mesophyll layers, number of palisade cell layers, mesophyll surface area exposed to intercellular airspace (\( S_{c}/S \)), chloroplast surface area exposed to intercellular airspace (\( S_{ct}/S \)), and the ratio \( S_{c}/S_{ct} \) in all studied species.

Supplementary Table S5. Cell wall thickness (\( T_{cw} \)), cytoplasm thickness (\( T_{cyt} \)), chloroplasts length (\( L_{col} \)), chloroplasts thickness (\( T_{col} \)), and effective porosity of the cell wall (\( p_e \)).

Supplementary Fig. S1. Representative light micrographs at 200× magnification for Phaseolus vulgaris, Ficus elastica,
and *Washingtonia filifera*, and representative transmission electron micrographs at 2000× magnification for *Helianthus annuus*, *Acer negundo*, and *Washingtonia filifera* and at 2000× magnification for *H. annuus*, *Alnus subcordata* and *Pittosporum tobira*.

**Supplementary Fig. S2.** The relationship between mesophyll diffusion conductance (g<sub>m</sub>) measured with the Harley Harley et al. (1992) method and g<sub>m</sub> modelled with anatomical parameters using least-squares iterative analysis for the r<sub>i,i</sub> parameter.

**Supplementary Fig. S3.** Effects of the parameter r<sub>i,i</sub> of the cytosol and chloroplast stroma on g<sub>m</sub> modelled from anatomical characteristics.

**Supplementary Fig. S4.** Correlation between leaf density (D<sub>L</sub>) and leaf dry mass per unit area (M<sub>A</sub>).

**Supplementary Fig. S5.** Relationship between g<sub>m</sub> measured according to Harley et al. (1992) method versus the Ethier and Livingston (2004) method.

**Supplementary Fig. S6.** Net photosynthesis rate (A<sub>N</sub>) in relation to stomatal (g<sub>s</sub>) and mesophyll (g<sub>m</sub>) conductance.

**Supplementary Fig. S7.** The relationship between g<sub>m</sub> and CO<sub>2</sub> drawdown (C<sub>i</sub>–C<sub>o</sub>).

**Supplementary Fig. S8.** The relationship between mesophyll diffusion conductance (g<sub>m</sub>) measured with the Harley et al. (1992) method and g<sub>m</sub> modelled with anatomical parameters using different values for the membrane permeabilities of plasmalemma (g<sub>pl</sub>) and chloroplast membrane (g<sub>cp</sub>) conductances.

**Supplementary Fig. S9.** Quantitative limitation analysis of conductance to CO<sub>2</sub> inside the cell (g<sub>cel,tot</sub>) calculated on the basis of leaf anatomical characteristics.

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