Perspective

Linking structure to function: the connection between mesophyll structure and intrinsic water use efficiency

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Supplement: S1 References used in Fig. 2

S2 Fig. S1 and references used in Fig. S1

Short summary

Leaf mesophyll cells are often approximated by capsules and spheres to discuss structure-function relationships. These assumptions allow an easy assessments based on widely available 2D datasets of foliar tissue. However, this is a rough approximation of often irregularly shaped spongy mesophyll cells. We suggest to use more rare 3D assessments to provide corrections and functions to be used in 2D assessments, rather than scaling 2D analysis to 3D structures based on the assumption of ideal shapes.

Abstract

Climate change-driven drought events are becoming unescapable in an increasing number of areas worldwide. Understanding how plants are able to adapt to these changing environmental conditions is a non-trivial challenge. Physiologically, improving a plant's intrinsic water use efficiency (WUE_i) will be essential for plant survival in dry conditions. Physically, plant adaptation and acclimatisation are constrained by a plant's anatomy. In other words, there is a strong link between anatomical structure and physiological function. Former research predominantly focussed on using 2D anatomical measurements to approximate 3D structures based on the assumption of ideal shapes, such as spherical spongy mesophyll cells. As a result of increasing progress in 3D imaging technology, the validity of these assumptions is being assessed, and recent research has indicated that these approximations can contain significant errors. We suggest to invert the workflow and use the less common 3D assessments to provide corrections and functions for the more widely available 2D assessments. By combining these 3D and corrected 2D anatomical assessments with physiological measurements of WUE_i, our understanding of how a plant's physical adaptation affects its function will increase and greatly improve our ability to assess plant survival.

Keywords:

functional plant anatomy, intercellular airspace, leaf anatomy, leaf functional traits, leaf structure, mesophyll, structure-function relations, water use efficiency

Introduction

An increasing number of climate change-driven drought events (IPCC, 2018) is pushing plant species to the limits of their climatic tolerance (Fitzpatrick *et al.*, 2008; Feeley *et al.*, 2020). This abiotic stressor is a major constraint for crop production, greatly affecting food security (Fahad *et al.*, 2017). Agriculture can attempt to breed more drought-tolerant cultivars, which can be classified as a guided form of adaptation. Natural migration of wild plants to areas with more favourable conditions might partially alleviate the impact of these abiotic stressors. Still, numerous species are unable to keep pace with these imposed environmental changes (e.g., Corlett & Westcott, 2013). Alternatively, plants under abiotic stress can try to acclimate or adapt (Corlett & Westcott, 2013) to deal with these new hydrological conditions.

Plant adaptation and acclimatisation can occur on different levels: genetic (Lauteri *et al.*, 1997; Roddy *et al.*, 2020), anatomy (Théroux-Rancourt *et al.*, 2021) and physiology (Shao *et al.*, 2008) (Fig. 1). These different levels can interact, with changes in genetics leading to anatomical and physiological changes. If these anatomical and physiological changes are favourable, they will result in higher survival rates (Maggio *et al.*, 2001), relatively enriching the genetic pool with these modifications, and so forth. If plants are unable to adjust or migrate, they are pushed to extinction because of newly imposed climatic conditions (Corlett & Westcott, 2013). Knowing which species are able to adjust and how these modifications manifest is essential to assess plant survival rates under climate change.

Physically, these changes are constrained by a plant's anatomy. In other words, there is a strong link between structure and function or anatomy and physiology, respectively, which amalgamates in the field of functional plant anatomy (Fig. 1). Mesophyll structure can affect multiple important traits, e.g., leaf hydraulics and light perception (Théroux-Rancourt & Gilbert, 2017; Théroux-Rancourt *et al.*, 2023). This Perspective article focuses on the construction of a leaf's mesophyll tissue and how this structure affects mesophyll conductance for water vapour and carbon dioxide out and into the leaf, respectively (Evans *et al.*, 2009; Earles *et al.*, 2018). While plants have some capacity for anatomical adaptation, the limits of these physical changes to improve carbon gain and reduce water loss during dry conditions remain poorly understood.

Carbon gained and water lost

As drought events increase in frequency and intensity (IPCC, 2018), improving a plant's intrinsic water use efficiency (WUE_i; µmol CO_2.(mol H_2O)$^{-1}$) will be essential to avoid harmful water shortages (Hentschel *et al.*, 2016). WUE_i is defined as the ratio of photosynthetic rate (A; µmol CO_2.m^{-2}.s^{-1}) to stomatal conductance (g_s; mol H_2O.m^{-2}.s^{-1}) (Eq. 1) (Osmond *et al.*, 1980; Seibt *et al.*, 2008), thus combining the carbon and water cycles through stomatal conductance and the gaseous component of mesophyll conductance. In recent decades, the potential of mesophyll conductance (g_m) to significantly affect carbon fixation and water loss has been recognized (Warren, 2008; Evans *et al.*, 2009; Bunce, 2016; Earles *et al.*, 2019). Furthermore, g_m is implicitly included in WUE_i as A is a function of g_s and g_m (Eq. 1). While g_s determines how fast CO_2 can enter the leaf through stomata, g_m determines how fast CO_2 can move from stomata to the chloroplast where it can be fixed as sugars during photosynthesis.

$$WUE_i = \frac{A}{g_s} \quad \frac{f(g_s, g_m, \ldots)}{f(g_s)} \qquad \text{Eq. 1}$$

g_m consists of the algebraic sum of its gaseous (g_{IAS}; Eq. 2) and liquid (g_{liq}; Eq. 3) components, the former denoting diffusion in the intercellular airspace (IAS) (Earles *et al.*, 2018). The effect of the IAS structure on gas diffusion in the IAS is approximated by diffusion in a porous medium (Eq. 2) (Earles *et al.*, 2018).

$$g_{IAS} = \frac{\theta_{IAS} D_m}{0.5 L_{mes} \tau_{leaf} \lambda_{leaf}} \qquad \text{Eq. 2}$$

where θ_{IAS} is mesophyll porosity (m^3.m^{-3}), D_m is the diffusion coefficient of CO_2 in air (m^2.s^{-1}), $0.5L_{mes}$ is half the mesophyll thickness (m), τ_{leaf} is the tortuosity factor (m^2.m^{-2}) and λ_{leaf} is lateral path lengthening (m.m^{-1}).

Eq. 2 assumes that g_{IAS} is a function of foliar structure, yet each element of the equation does not depend on the same tissue or cell type. While θ_{IAS} and τ_{leaf} are a function of mesophyll tissue, λ_{leaf} is highly dependent on stomatal density (e.g. Earles *et al.*, 2018; Théroux-Rancourt *et al.*, 2023), clearly linking the spatial organization of both stomata and mesophyll cells to g_{IAS} and WUE_i.

In terms of functional plant anatomy, maximizing WUE_i entails an optimization problem where both a reduction in g_{IAS} for water vapor diffusion and an increase in g_{IAS} for

CO_2 diffusion are beneficial. As both pathways obviously overlap, it is unclear how a plant will physically optimise at the tissue level for dry conditions. Based on the diffusion coefficient of CO_2 and water vapor in air of 0.158 and 0.247 $cm^2.s^{-1}$ (at 20°C), respectively, it can be hypothesized that reducing g_{IAS} would be more beneficial as the diffusion coefficient for water vapour is larger. This should result in a reduction of water loss that is more pronounced than the reduction in carbon gain, thus increasing WUE_i. However, if g_{IAS} does decrease, it is still unclear how different plant species would accomplish this. Is it more beneficial to increase τ_{leaf} or decrease stomatal density, thus increasing λ_{leaf}? Should θ_{IAS} increase or L_{mes} decrease? These are non-trivial questions which rely on the interplay of multiple variables. For example, sun leaves of *Fagus sylvatica* have been observed to have a higher g_{IAS} compared to shade leaves (Janová *et al.*, 2024), while sun leaves of *Vitis vinifera* indicated lower θ_{IAS} and higher L_{mes} values compared to shade leaves, resulting in a lower g_{IAS} (Théroux-Rancourt *et al.*, 2023). Different tendencies could result from differences in species but could just as likely vary within the same species due to the interplay of multiple variables such as light intensity and hydration.

Adding the third dimension

3D structure and WUE_i

As WUE_i is a function of g_m, structural features in mesophyll anatomy are expected to correlate with a plant's WUE_i. In coniferous species this resulted in a significant correlation between WUE_i and the number of stomata per unit of mesophyll volume (Trueba *et al.*, 2022) (Fig. 2a), the number of stomata per unit of mesophyll surface area (Fig. 2b) and the number of stomata per unit of mesophyll intercellular airspace (Fig. 2c), but not the mesophyll surface area exposed to IAS per unit of total leaf area (S_m; Fig. 2d). While also significant correlations between WUE_i and vein-based variables were found (Trueba *et al.*, 2022), these observations suggest that stomatal density and variables based on mesophyll area, volume and porosity could be some of the major drivers for WUE_i.

Even though stomatal density is the major component of g_s, stomatal size also has a significant impact on g_s, and inversely on a plant's estimated water use efficiency (Liu *et al.*, 2016). As stomatal density and stomatal size covary (Jordan *et al.*, 2020), a simplified assessment can be performed by investigating one of these parameters, but a stronger

relationship is expected when using stomatal area fraction (Liu *et al.*, 2016), defined as the product of stomatal density and stomatal size, divided by leaf area. In terms of adaptation to aridity, stomatal density appears to be the dominant factor over stomatal size (Liu *et al.*, 2016). When stomatal densities scaled by mesophyll variables decrease, WUE_i increases (Fig. 2a-c). This could be the result of a decreased g_{IAS}, combined with a larger diffusion coefficient for water vapor compared to CO_2 in air. However, based on these data it is unclear whether stomatal density or mesophyll structure has the strongest influence on WUE_i. Stomatal density predominantly affects λ_{leaf}, with a decrease in stomatal density leading to an increase in λ_{leaf} (Earles *et al.*, 2018). However, mesophyll cell size has been linked to CO_2 diffusion inside leaves (Théroux-Rancourt *et al.*, 2021), suggesting that the interaction between stomatal density and mesophyll structure is driving WUE_i, as has been indicated by Lundgren *et al.* (2019), and not one or the other.

Significant changes in mesophyll structure as a result of varying environmental conditions, such as light and water availability (Théroux-Rancourt & Gilbert, 2017; Momayyezi *et al.*, 2022; Théroux-Rancourt *et al.*, 2023), have been observed. Drought can result in a decreased mesophyll cell volume, in turn causing an increase in θ_{IAS} and g_{IAS} (Momayyezi *et al.*, 2022). However, whether the effect of drought amplifies or reduces the effect of increased light interception through interactive effects, such as reduced light absorption due to drought (Momayyezi *et al.*, 2022), remains unclear. Furthermore, as structural variables are interlinked, different anatomical changes might result in the same physiological optimum, e.g., (i) a decrease in θ_{IAS} could alleviate the effect of a decrease in L_{mes} and (ii) an increase in τ or λ can have similar effects with respect to g_{IAS}. To add to this complexity, some of these changes might rely on 3D directional structures (Harwood *et al.*, 2021) rather than single-leaf variables.

Errors based on 2D assessments

Palisade and spongy mesophyll cells have often been approximated by capsules and spheres, respectively, to discuss structure-function relationships (Nobel, 2020). However, this is a rough approximation as spongy cells are often irregularly shaped (Haberlandt, 1904; Théroux-Rancourt *et al.*, 2020b; Borsuk *et al.*, 2022). These assumptions have been advantageous as they allowed easy assessments based on widely available 2D datasets of

foliar tissue and required low processing power. However, as we are entering a new era where 3D observations are becoming more common and processing power is rarely limiting for this type of research, the validity of 2D approximations for 3D traits such as θ_{IAS}, τ_{leaf} and λ_{leaf} should be investigated, especially with respect to the irregular cell shape of spongy mesophyll. Furthermore, these traits can exhibit high spatial heterogeneity (Earles *et al.*, 2018), making τ_{leaf} directional rather than encompassing (Harwood *et al.*, 2021), and can strongly influence g_{IAS} (Earles *et al.*, 2018).

During recent years, advances in methods for high-resolution 3D anatomical observations, such as, confocal microscopy, multiphoton laser scanning microscopy (Wuyts *et al.*, 2010), serial block-face scanning electron microscopy (SBF-SEM) (Harwood *et al.*, 2020), nuclear magnetic resonance (NMR) and X-ray computed microtomography (microCT) (Brodersen & Roddy, 2016; Earles *et al.*, 2018, 2019; Mathers *et al.*, 2018) combined with full-stack tissue segmentations based on machine learning (Théroux-Rancourt *et al.*, 2020a; Rippner *et al.*, 2022), specialized software (Barbier de Reuille *et al.*, 2015) and other pipelines (Wuyts *et al.*, 2010), have bloomed leading to new insights and the unravelling of errors based on former 2D approaches. This type of research has indicated that including 3D data of τ_{leaf} and λ_{leaf} reduced the estimates of g_{IAS} based on anatomy, on average, by 37% in bromeliad species (Earles *et al.*, 2018). Furthermore, 2D leaf sections underestimated the mesophyll surface area exposed to IAS per unit of total leaf area (S_m), in some cases leading to errors of almost 50% compared to 3D microCT images (Théroux-Rancourt *et al.*, 2017; Mathers *et al.*, 2018). This error can affect the assessment of other variables as S_m closely correlates with A (Théroux-Rancourt *et al.*, 2017) and g_{liq} (Eq. 3) (Théroux-Rancourt *et al.*, 2021). Evans *et al.* (2009), for example, indicated that leaves with a large photosynthetic capacity (P_c ~ f(A) ~ f(WUE_i)) tend to increase g_m by increasing the surface area of chloroplast exposed to IAS (S_c ~ f(S_m)), clearly denoting the interlinkage between S_m and other variables and the possibility of cascading errors due to 2D assessments.

$$g_{liq} = \frac{\frac{SA_{mes}}{V_{mes}} \times \frac{S_c}{S_m}}{R} \qquad \text{Eq. 3}$$

where SA_{mes} is the mesophyll surface are exposed to IAS (m^2), V_{mes} is the mesophyll volume (m^3) and R is a combination of different resistance components of the liquid diffusion path through mesophyll cells (m^2 chlorplast.s.mol^{-1}), including diffusion through cell walls.

While a lot of variation exists in the relation between S_c and S_m (Fig. S1), an error in S_m will not directly affect S_c S_m^{-1} as this term is generally estimated as a ratio, e.g. 0.73 ± 0.01 (mean ± SE; Fig. S1). However, S_m is SA_{mes} scaled per unit of leaf area (LA). As a consequence, errors in S_m will result in errors of SA_{mes}/V_{mes}. Based on the 3D dataset available in Trueba *et al.*, (2022), a significant ($p < 0.001$) logarithmic relation between S_m and SA_{mes}/V_{mes} can be observed (Fig. 3). This makes sense as $SA_{mes}/LA \sim SA_{mes}/V_{mes}$ corresponds to $1/LA \sim 1/V_{mes}$. However, when using 2D data from Hogan *et al.* (1994) and Vyas *et al.* (2007), no significant trend could be found. It is important to note that sample sizes are small and that the 2D dataset is based on broadleaved species, while the 3D dataset is based on coniferous species. As such, part of the observed difference could thus be ascribed to statistical errors and species diversity; however, it is expected that a significant amount of difference originated from errors in 2D anatomical assessments of 3D structures.

A way forward

Despite its increasing use in publications, 3D methods are still not universally available. The main goal of 3D methods should be to provide corrections and adaptations for the more widely available 2D methods. One such adaptation is determining the number of 2D slices needed to make an acceptable approximation of S_m (Théroux-Rancourt *et al.*, 2017), or providing an equation that encompasses the 3D reality of leaves but uses readily available 2D data (Earles *et al.*, 2018). Furthermore, knowledge gained from 3D anatomical assessments must eventually be shaped in a way that allows the use of lower dimensional data for upscaling, e.g., from 2D leaves to canopy-level models (Earles *et al.*, 2019).

We suggest to use 3D assessments to provide corrections and functions to be used in 2D assessments rather than scaling 2D analysis to 3D structures based on the assumption of ideal shapes such as spherical spongy mesophyll cells. Furthermore, by improvements in computational power and advancements in 3D structural assessment methods, the potential for new avenues in the field of functional plant anatomy is increasing drastically. By combining corrected 3D anatomical assessment with physiological measurements, our

understanding of how a plant's physical adaptation affects its function increases and will greatly improve our ability to assess plant survival.

Data Availability Statement

No original data are used in this perspective.

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Figure legends

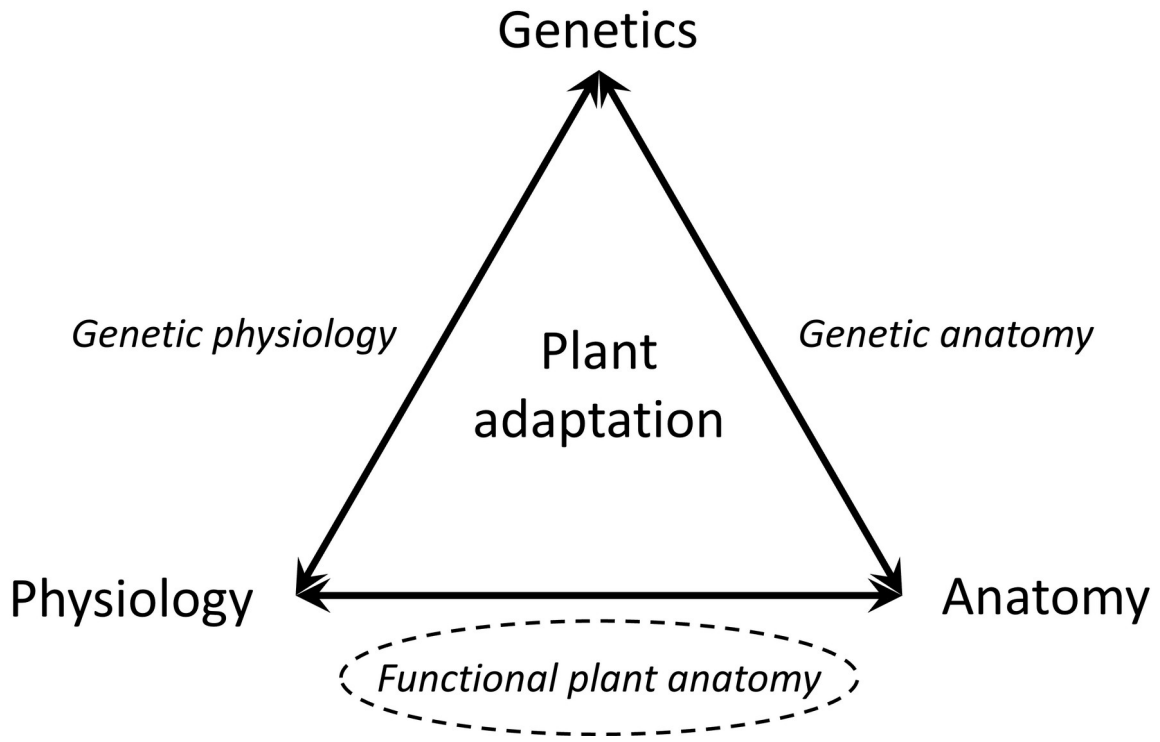

Fig. 1 Three main fields of plant sciences that can assess different aspects of plant adaptation: genetics, anatomy and physiology. The interaction of these fields creates subfields. In this Perspective, the subfield of functional plant anatomy is being proposed as a promising way forward to better understand plant adaptations.

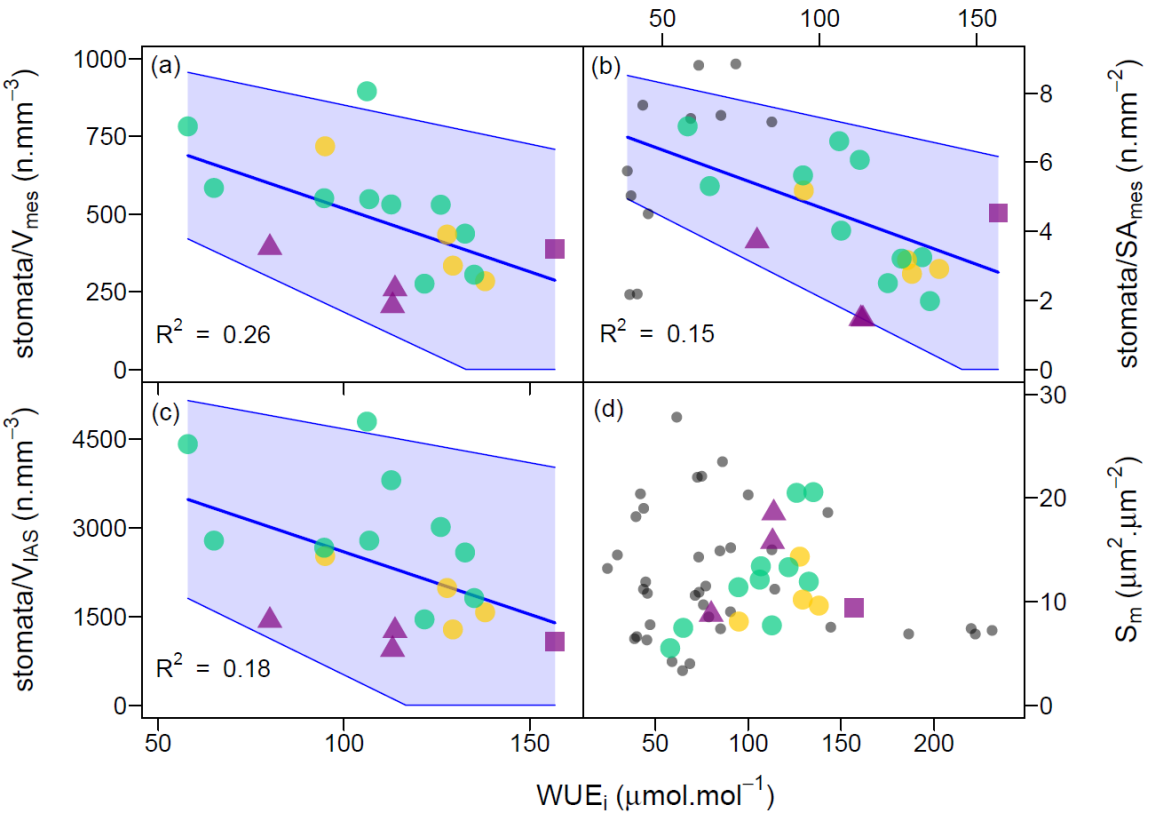

Fig. 2 Physiological leaf traits as a function of intrinsic water use efficiency (WUE_i). (a) Number of stomata per unit of mesophyll volume (stomata/V_{mes}); (b) Number of stomata per unit of mesophyll surface area (stomata/SA_{mes}); (c) Number of stomata per unit of mesophyll intercellular airspace volume (stomata/V_{IAS}); (d) Mesophyll surface area per unit of total leaf area (S_m). Solid blue regression lines and SE (shaded areas) are included. Coefficients of determination are included. All fits have a p-value < 0.05. *Pinus* species from the subgenera *Pinus* (green) and *Strobus* (yellow), along with other conifer species (purple), are indicated. Species bearing flat leaves (square), flattened needle leaves (triangles), and needle-like leaves (circles) are also identified (data from the supplement of Trueba *et al.* (2022)). Grey circles represent data from other references (see supplementary information).

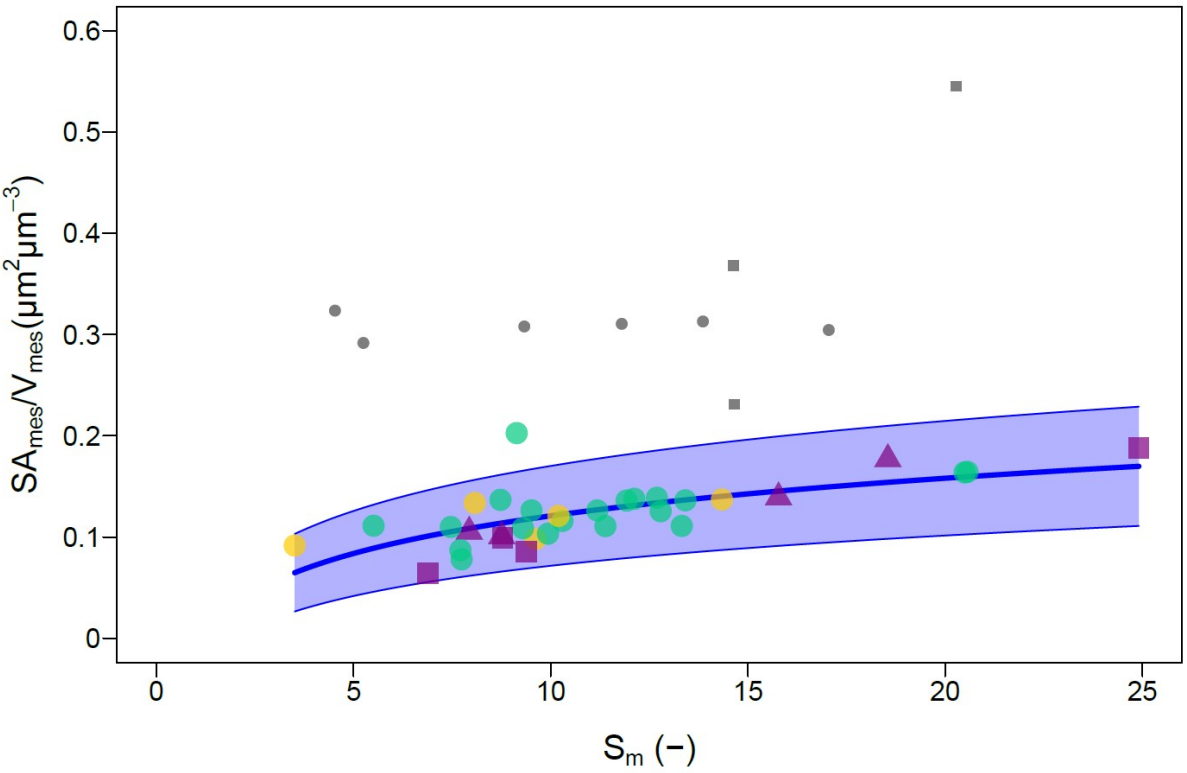

Fig. 3 Ratio of the mesophyll surface area exposed to IAS to the mesophyll volume (SA_{mes}/V_{mes}) as a function of mesophyll surface area exposed to the intercellular air space per unit of total leaf area (S_m). 3D data (in colour) based on X-ray microCT imaging (Trueba *et al.*, 2022), 2D data based on light microscopy (grey circles: Hogan *et al.* (1994); grey squares: Vyas *et al.* (2007)). Blue line indicates a logarithmic fit to 3D colour data with the shaded area visualizing the standard error of the fit ($p < 0.001$).