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Commentary

A step forward in the study of photosynthetic limitation by CO₂ diffusion into the mesophyll

Photosynthesis drives crop yield and the land carbon sink. Investigating how CO₂ supply to photosynthesis can be maximised is therefore of central importance to food security and climate mitigation policies. Stomata limit this supply but also control plant water loss. Nevertheless, once within the substomatal cavity, CO₂ molecules still have to reach the site of carboxylation, and CO₂ diffusion along this pathway can limit plant photosynthesis to levels comparable to stomatal limitation (e.g. Grassi & Magnani, 2005). This limitation of photosynthesis is characterised by a plant trait called mesophyll conductance (g_m) that varies between species, and with growth and environmental conditions (Flexas *et al.*, 2012). Despite intensive research programmes investigating mesophyll conductance, our knowledge of how g_m varies between plant forms and responds to rapid changes in environmental factors such as temperature or CO₂ is still limited. This is partly because all methods for estimating this trait rely on several assumptions whose validity is not always easy to verify (Cousins *et al.*, 2020). In this context, the new study by Rao *et al.* (2024; doi: [10.1111/nph.19767](https://doi.org/10.1111/nph.19767)), published in *New Phytologist*, is undoubtedly a significant step forward. By recording changes in photosynthetic CO¹⁸O discrimination ($\Delta^{18}\text{O}$) under steady-state gas exchange conditions while operating a step change in the isotopic composition of water vapour, Rao *et al.* propose a new approach to estimate g_m without the need to make assumptions about the degree of oxygen isotope equilibration between CO₂ and leaf water (θ). Indeed, to derive g_m from $\Delta^{18}\text{O}$ measurements, it is often assumed that the *in vivo* activity of carbonic anhydrase (CA), the enzyme that catalyses CO₂ hydration and CO₂-water isotope exchange in mesophyll cells, is nonlimiting such that CO₂ and water are always in isotopic equilibrium at the site of CO₂ hydration ($\theta = 1$). This assumption has often been questioned, especially in C₄ plants because of their known lower CA activity, compared to C₃ plants. The study by Rao *et al.* addresses this issue by exploiting isotopic labelling dynamics during steady-state leaf gas exchange, which allows them to estimate g_m and θ simultaneously.

‘...Rao *et al.* propose a new approach to estimate mesophyll conductance (g_m) without the need to make assumptions about the degree of oxygen isotope equilibration between CO₂ and leaf water (θ).’

Among the different methods proposed to study mesophyll conductance, the $\Delta^{18}\text{O}$ -based method is the only one that is applicable to C₄ plants, as well as C₃ plants. This is because $\Delta^{18}\text{O}$ -based g_m characterises CO₂ diffusion to the site of CO₂ hydration, a reaction catalysed by CA that works in tandem with phosphoenolpyruvate carboxylase (PEPC) CO₂ fixation in the cytosol of C₄ plants. The results of Rao *et al.* confirm *a posteriori* that setting $\theta = 1$ is a fair assumption for well-watered C₃ plants but it did not hold for any of the C₄ plants tested (*Zea mays*, *Sorghum bicolor*, *Setaria italica*). In these plants, θ was always below 0.8 and decreased with increasing temperature to values as low as 0.5 at 40°C. As expected from theory, having $\theta < 1$ resulted in higher g_m values compared to estimates assuming $\theta = 1$, and the discrepancy increased at higher temperatures, reaching *c.* 50% above 30°C. A decreasing trend of θ with temperature may also apply to C₃ plants, as already suggested by a modelling analysis based on known temperature responses of carbonic anhydrase extracts and *in vivo* CO₂ partial pressure at the site of carboxylation (Sonawane & Cousins, 2019). The new method proposed by Rao *et al.* could be used to test if setting $\theta = 1$ for C₃ plants remains valid at high temperature. These results clearly call for a careful re-examination of studies reporting temperature responses of g_m derived from $\Delta^{18}\text{O}$ measurements in C₄ plants, and potentially also C₃ plants.

Rao *et al.* also compared their results with those derived using a comprehensive model of $\Delta^{18}\text{O}$ that explicitly accounts for respired CO₂ recycling and for the competition between CO₂ hydration and carboxylation, potentially leading to an incomplete isotopic equilibration (Ogée *et al.*, 2018). This model relies, however, on *a priori* knowledge of *in vivo* CA activity (k_{CA}), as well as leaf respiration rate, although the latter appears to be much less critical (Ogée *et al.*, 2018; Rao *et al.*, 2024). Using *in vitro* measurements of CA activity as a surrogate for k_{CA} , Rao *et al.* compared the results of this comprehensive $\Delta^{18}\text{O}$ model with their estimates of g_m and θ . They found that the comprehensive $\Delta^{18}\text{O}$ model systematically predicted a lower degree of equilibration and a higher g_m , compared to their isotopic labelling dynamics technique. This result indicates that the *in vitro* CA activity measurements underestimate *in vivo* k_{CA} in the model, by a factor of 1.7 (C₄ plants) to 4 (C₃ plants) (compare their figs 4, S4). Rao *et al.* did not specify how the *in vitro* CA assays were performed, nor whether their estimated k_{CA} values were corrected for differences in pH and temperature between the

This article is a Commentary on Rao *et al.* (2024), doi: [10.1111/nph.19767](https://doi.org/10.1111/nph.19767).

assay and *in vivo* conditions (Ogée *et al.*, 2018; Sonawane & Cousins, 2019). However, their reported values of k_{CA} (table S2) are unusually low compared to other reported values: for example, for *Nicotina tabacum* (a C_3 plant), Rao *et al.* report a value for k_{CA} of $2.77 \text{ mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ at 28.5°C compared to a value of $14 \text{ mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ at 25°C reported by another study using similar growth conditions (Barbour *et al.*, 2016). This fivefold increase in k_{CA} would be sufficient to reconcile the comprehensive $\Delta^{18}\text{O}$ model with their g_m and θ estimates. This is reassuring, as it shows that our understanding of $\Delta^{18}\text{O}$ discrimination remains valid.

While the approach proposed by Rao *et al.* to estimate g_m has the advantage of not requiring any assumption about θ (or *a priori* knowledge of k_{CA}), some other assumptions remain whose applicability may be questioned, especially under water limitation or high leaf-air vapour pressure deficits (VPD). Indeed, estimating g_m from $\Delta^{18}\text{O}$ measurements following Rao *et al.* requires two theoretical expressions for the isotopic composition of CO_2 at the hydration site ($\delta^{18}\text{O}_o$): one corresponding to the situation where CO_2 and water are in full equilibrium ($\delta^{18}\text{O}_c = \delta^{18}\text{O}_{ce}$), and another one where CO_2 would not have undergone any equilibration with water ($\delta^{18}\text{O}_c = \delta^{18}\text{O}_{c0}$). Finding an expression for $\delta^{18}\text{O}_{c0}$ is not easy and requires additional assumptions that are not trivial for C_4 plants (Ogée *et al.*, 2018; Rao *et al.*, 2024). Rao *et al.* minimised this problem by using strongly labelled water vapour so that the dependence of $\delta^{18}\text{O}_c$ on $\delta^{18}\text{O}_{c0}$ was small. An expression for $\delta^{18}\text{O}_{ce}$ requires knowledge of the isotopic composition of leaf water at the site of CO_2 hydration ($\delta^{18}\text{O}_l$). Provided that CO_2 hydration and leaf water evaporation occur at the same location in the mesophyll (but see Holloway-Phillips *et al.*, 2019), a theoretical expression for $\delta^{18}\text{O}_e$ is available. However, this expression depends strongly on assumptions about the degree of saturation h_i of the intercellular air space where leaf water evaporates, that is: $\delta^{18}\text{O}_c = \theta\delta^{18}\text{O}_{ce}(h_i) + (1 - \theta)\delta^{18}\text{O}_{c0}$. Because most leaves lose turgor at leaf water potentials below -2 to -5 MPa, corresponding to an air relative humidity of 97–99%, it has traditionally been accepted that the intercellular air space is saturated in water vapour (i.e. $h_i = 1$), and Rao *et al.* working on well-watered plants at moderate VPD also made this assumption. However, there is increasing evidence that, at least at high VPD, the assumption $h_i = 1$ does not hold (Wong *et al.*, 2022). In fact, the dependency of $\delta^{18}\text{O}_e$ on h_i has been instrumental in demonstrating in several recent $\Delta^{18}\text{O}$ studies that under drought stress or high VPD, h_i could become less than unity and as low as 0.7 (Cernusak *et al.*, 2018, 2019; Holloway-Phillips *et al.*, 2019; Wong *et al.*, 2022). All of these studies made the *a priori* assumption that CA activity was nonlimiting ($\theta = 1$), and Wong *et al.* showed that this assumption gave results that were consistent with other nonisotopic gas exchange techniques for estimating h_i . While all the studies so far reporting values of $h_i < 1$ have been conducted in C_3 plants, the implicit assumption that $\theta = 1$ holds regardless of the environmental conditions may be questioned (Ogée *et al.*, 2018; Sonawane & Cousins, 2019). Conversely, in C_4 plants where there is evidence that $\theta < 1$, the approach of Rao *et al.*, would need to be modified to retrieve h_i , θ and g_m simultaneously.

These new challenges should not discourage more detailed documentation of how g_m varies across biomes and in response to changing environmental conditions. Currently, the limitation of photosynthesis by mesophyll conductance is often implicitly accounted for in ecosystem and Earth system models, using apparent photosynthetic capacity parameters (Rogers *et al.*, 2017). However, there is increasing evidence that this implicit accounting of g_m is unsatisfactory and leads to biased (underestimated) predictions of photosynthesis. For example, it has been shown that an explicit representation of g_m in photosynthesis models is necessary to explain sustained photosynthetic CO_2 uptake during drought (Niinemets *et al.*, 2009), or can lead to significantly larger estimates of the land CO_2 sink by Earth system models (Sun *et al.*, 2014; Knauer *et al.*, 2023). For sure, the method proposed by Rao *et al.* for investigating g_m has great potential ahead, be it for crop sciences or carbon cycle research.

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