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## Commentary

# A step forward in the study of photosynthetic limitation by CO<sub>2</sub> diffusion into the mesophyll

Photosynthesis drives crop yield and the land carbon sink. Investigating how CO<sub>2</sub> 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, CO<sub>2</sub> molecules still have to reach the site of carboxylation, and CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sup>18</sup>O 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 CO<sub>2</sub> and leaf water ( $\theta$ ). 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 CO<sub>2</sub> hydration and CO<sub>2</sub>-water isotope exchange in mesophyll cells, is nonlimiting such that CO<sub>2</sub> and water are always in isotopic equilibrium at the site of CO<sub>2</sub> hydration ( $\theta = 1$ ). This assumption has often been questioned, especially in C<sub>4</sub> plants because of their known lower CA activity, compared to C<sub>3</sub> 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  $\theta$  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 CO<sub>2</sub> and leaf water ( $\theta$ ).’

Among the different methods proposed to study mesophyll conductance, the  $\Delta^{18}\text{O}$ -based method is the only one that is applicable to C<sub>4</sub> plants, as well as C<sub>3</sub> plants. This is because  $\Delta^{18}\text{O}$ -based  $g_m$  characterises CO<sub>2</sub> diffusion to the site of CO<sub>2</sub> hydration, a reaction catalysed by CA that works in tandem with phosphoenolpyruvate carboxylase (PEPC) CO<sub>2</sub> fixation in the cytosol of C<sub>4</sub> plants. The results of Rao *et al.* confirm *a posteriori* that setting  $\theta = 1$  is a fair assumption for well-watered C<sub>3</sub> plants but it did not hold for any of the C<sub>4</sub> plants tested (*Zea mays*, *Sorghum bicolor*, *Setaria italica*). In these plants,  $\theta$  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  $\theta$  with temperature may also apply to C<sub>3</sub> plants, as already suggested by a modelling analysis based on known temperature responses of carbonic anhydrase extracts and *in vivo* CO<sub>2</sub> 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 C<sub>3</sub> 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 C<sub>4</sub> plants, and potentially also C<sub>3</sub> 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 CO<sub>2</sub> recycling and for the competition between CO<sub>2</sub> 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 ( $k_{\text{CA}}$ ), 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  $k_{\text{CA}}$ , Rao *et al.* compared the results of this comprehensive  $\Delta^{18}\text{O}$  model with their estimates of  $g_m$  and  $\theta$ . 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*  $k_{\text{CA}}$  in the model, by a factor of 1.7 (C<sub>4</sub> plants) to 4 (C<sub>3</sub> plants) (compare their figs 4, S4). Rao *et al.* did not specify how the *in vitro* CA assays were performed, nor whether their estimated  $k_{\text{CA}}$  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  $28.5^\circ\text{C}$  compared to a value of  $14 \text{ mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$  at  $25^\circ\text{C}$  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  $\theta$  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  $\theta$  (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  $\text{CO}_2$  at the hydration site ( $\delta^{18}\text{O}_j$ ): one corresponding to the situation where  $\text{CO}_2$  and water are in full equilibrium ( $\delta^{18}\text{O}_c = \delta^{18}\text{O}_{c_e}$ ), and another one where  $\text{CO}_2$  would not have undergone any equilibration with water ( $\delta^{18}\text{O}_c = \delta^{18}\text{O}_{c_0}$ ). Finding an expression for  $\delta^{18}\text{O}_{c_0}$  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  $\delta^{18}\text{O}_{c_0}$  was small. An expression for  $\delta^{18}\text{O}_{c_e}$  requires knowledge of the isotopic composition of leaf water at the site of  $\text{CO}_2$  hydration ( $\delta^{18}\text{O}_l$ ). Provided that  $\text{CO}_2$  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:  $\delta^{18}\text{O}_c = \theta\delta^{18}\text{O}_{c_e}(h_i) + (1 - \theta)\delta^{18}\text{O}_{c_0}$ . 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  $h_i$ ,  $\theta$  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  $\text{CO}_2$  uptake during drought (Niinemets *et al.*, 2009), or can lead to significantly larger estimates of the land  $\text{CO}_2$  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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