Revisiting the carbon isotope discrimination and water use efficiency relation: the influence of mesophyll conductance

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Summary

- The carbon isotope discrimination (Δ) has been used widely to infer intrinsic water-use efficiency (iWUE) of C_3 plants, a key parameter linking carbon and water fluxes. Despite the essential role of mesophyll conductance (g_m) in photosynthesis and Δ , its effect on Δ -based predictions of iWUE has generally been neglected.
- Here, we derive a mathematical expression of iWUE as a function of Δ that includes g_m (iWUE_{mes}) and exploits the g_m -stomatal conductance (g_{sc}) relationship across drought-stress levels and plant functional groups (deciduous or semi-deciduous woody, evergreen woody and herbaceous species) in a global database. iWUE_{mes} was further validated with an independent dataset of online- Δ and CO_2 and H_2O gas exchange measurements with seven species.
- Drought stress reduced g_{sc} by 52% and g_m by 45% averaged over all plant functional groups, but had no significant effect on the g_{sc}/g_m ratio, suggesting a well-constrained g_{sc}/g_m ratio of 0.79 ± 0.07 (95%CI, $n=198$) across plant functional groups and drought-stress treatments. Due in part to the synchronous behavior of g_{sc} and g_m , g_m was negatively correlated to iWUE. Incorporating the g_{sc}/g_m ratio in the iWUE_{mes} model significantly improved the estimation of iWUE compared to the simple model.
- The inclusion of g_m effects, even using a fixed g_{sc}/g_m ratio of 0.79 when g_m is unknown, proved desirable to eliminate significant bias in estimating iWUE from Δ across various C_3 vegetation types.

Key words: stable isotope; water use efficiency; transpiration; drought stress; stomatal conductance; mesophyll conductance; ^{13}C discrimination; photosynthesis.

INTRODUCTION

Water use efficiency (WUE) is a capital parameter of leaf physiology to assess photosynthetic performance and the trade-off between CO₂ fixation and water loss. In addition to photosynthetic capacity, the key actor of WUE is stomatal conductance, which effectively plays a pivotal role in terrestrial carbon and water cycles because it constrains the diffusion of CO₂ (g_{sc}) and water vapor (g_{sw} , with $g_{sw}=1.6g_{sc}$) between leaf photosynthetic tissues and the atmosphere. Leaf intrinsic water use efficiency (iWUE; see definitions in [Table 1](#)) is thus defined as the net photosynthetic rate (A_n) per unit g_{sw} and is given by (disregarding boundary layer resistance):

$$iWUE = \frac{A_n}{g_{sw}} = \frac{c_a}{1.6} \cdot \left(1 - \frac{c_i}{c_a}\right) \quad (1)$$

where c_a is the concentration of CO₂ in the atmosphere and c_i the intercellular CO₂ concentration. iWUE is useful to both describe plant physiological responses to environmental parameters (such as temperature and water availability) and understand the role of vegetation in hydrological cycles via its influence on water vapor and thus rainfall and runoff at regional to continental scales ([Betts *et al.*, 2007](#); [Kooperman *et al.*, 2018](#)). As such, some researchers have called for more quantitative data on iWUE values in response to climate change to improve climate predictions ([Adams *et al.*, 2019, 2020](#)). However, our ability to predict the response of carbon and water cycles in a changing climate depends ultimately on our understanding of mechanisms that control variation in iWUE.

Our current knowledge of iWUE is mostly based on its relationship with the photosynthetic ¹²C/¹³C isotope discrimination (Δ , [Farquhar *et al.*, 1982, 1989](#)), which explicitly describes the isotopic fractionation associated with physical and biochemical processes during photosynthetic gas exchange. Using the simplified, linear relationship of [Farquhar *et al.* \(1982\)](#), neglecting mesophyll resistance (i.e. assuming an infinite internal conductance), the intrinsic water use efficiency (here denoted as iWUE_{sim}, see [Table 1](#) for definitions) is obtained from c_i/c_a as:

$$iWUE_{sim} = \frac{c_a}{1.6} \cdot \frac{b' - \Delta}{b' - a} \quad (2)$$

where a (4.4‰) is the ¹²C/¹³C discrimination during CO₂ diffusion through stomata, b' (27‰) is the net discrimination during carboxylation (includes the potential contribution of effects other than Rubisco-catalysed reactions), and Δ is the net fractionation during photosynthesis that can be calculated from the isotope composition ($\delta^{13}C$) of atmospheric CO₂ and plant fixed carbon ([Farquhar & Richards, 1984](#); [Ubierna *et al.*, 2018](#)). Δ can be estimated from on-line ¹³CO₂/¹²CO₂

gas exchange during photosynthesis or the $\delta^{13}\text{C}$ of photosynthetic products (e.g. sugars and bulk leaf organic carbon). The iWUE_{sim} model (Eqn 2) has been widely used to infer iWUE from $\delta^{13}\text{C}$ values in climate archives like herbage (Koehler *et al.*, 2012), tree rings (Frank *et al.*, 2013; van der Sleen *et al.*, 2015), or animal tissues (Barbosa *et al.*, 2010). Compared to instantaneous iWUE derived from gas exchange measurements, biomass-based Δ provides a time-integrated iWUE and enables time-series analysis spanning from days to millennia (Soh *et al.*, 2019; Adams *et al.*, 2020). This time-integrated iWUE is particularly useful for screening of high water-use efficiency crops thus has been used in breeding programs (for a review, see Flexas *et al.*, 2013). Similarly, genetic determinants of iWUE have been searched for nearly 25 years using biomass-based Δ as a quantitative trait in QTL analyses (Rebetzke *et al.* 2008; Yang *et al.* 2016). Also, using surveys with many plant samples of diverse origins, spatial variation in vegetation iWUE could be assessed or integrated at a regional (Wittmer *et al.*, 2010) or global scale (Cornwell *et al.*, 2018; Adams *et al.*, 2020). More recently, $\delta^{13}\text{C}$ -derived iWUE , together with transpiration inferred from sap flow, have been used to estimate canopy photosynthesis (Klein *et al.*, 2016). However, it has been argued that the iWUE_{sim} model gives biased predictions of iWUE , limiting its application to qualitative assessments (Seibt *et al.*, 2008; Barbosa *et al.*, 2010).

In fact, the main limitation of the use of iWUE_{sim} is the simplifying assumption that mesophyll conductance (g_m , the diffusive conductance of CO_2 from intercellular space to the site of carboxylation) is infinite (Seibt *et al.*, 2008; Franks *et al.*, 2013; Stangl *et al.*, 2019). This assumption, theoretically, leads to an overestimation of iWUE (see Theory below) because g_m is not conservatively high (for reviews see Flexas *et al.*, 2008, 2012) and thus this causes an increased CO_2 drawdown from the atmosphere to chloroplasts and thereby decreases the actual isotope fractionation Δ . Furthermore, responses of g_m to environmental stimuli have been reported, including water stress (Galmes *et al.*, 2007; Warren *et al.*, 2011; Barbour & Kaiser, 2016), temperature (Yamori *et al.*, 2006; Evans & Von Caemmerer, 2013), and CO_2 mole fraction (Flexas *et al.*, 2007; Tazoe *et al.*, 2011). In other words, applying either an infinite or a species-specific, constant g_m in iWUE models is perhaps improper. Water stress is particularly relevant in the context of water utilization by plant leaves, because it strongly influences g_{sw} and thus iWUE (Lin *et al.*, 2015). However, its impact on g_m is currently uncertain. Some studies have reported that both g_m and g_{sw} decreased under edaphic drought, i.e. low soil water content (Galmes *et al.*, 2007; Cano *et al.*, 2014). In addition, while stomatal limitation of photosynthetic gas exchange has been

found to be the dominant factor in the early stage of drought, the decrease in g_m further restricts photosynthesis later on (Perez-Martin *et al.*, 2014). Vapor pressure deficit (VPD), a key factor that influences both plant water potential and stomatal conductance, has been found to decrease (Loucos *et al.*, 2017) or have no influence on g_m (Warren, 2008; Stangl *et al.*, 2019). In principle, a low sensitivity of g_m to water deficit is beneficial for maintaining a high CO₂ assimilation rate (Cano *et al.*, 2014). That is, a combination of a low g_{sw} and a high g_m should lead to a high iWUE if photosynthetic capacity is maintained (Barbour *et al.*, 2010; Flexas *et al.*, 2013; Barbour & Kaiser, 2016). Accordingly, g_m has been suggested to be an important target for crop improvement (Cano *et al.*, 2014; Barbour & Kaiser, 2016), although this hypothesis was not supported by experimental results of wheat genotypes (Barbour & Kaiser, 2016). Therefore, the responses of g_m and g_{sw} to long-term drought stress is not well understood, and the implication for iWUE estimation is not clear.

Having said that, incorporating mesophyll conductance is still challenging because measuring g_m involves technically demanding methods, such as online carbon isotope discrimination (Evans *et al.*, 1986), chlorophyll fluorescence (Harley *et al.*, 1992), or ¹⁸O mass balance (Gauthier *et al.*, 2018). This explains why g_m measurements cannot be carried out at high-throughput and therefore, our knowledge of mechanisms driving variation in g_m is still limited. In practice, there is presently no available method to incorporate g_m effects in iWUE and carbon cycle models derived therefrom (Flexas *et al.*, 2012; Sun *et al.*, 2014a). Alternatively, empirical estimates can be used to scale g_m to other photosynthetic parameters, since g_m has been shown to be positively related to net assimilation rate or g_{sc} (Flexas *et al.*, 2008; Gong *et al.*, 2018). Such relationships could be useful – providing they are well-constrained – for improving estimates of iWUE. However, such relationships have not been tested with a global compiled dataset of a diverse range of functionally distinct species.

Taken as a whole, the broad application of ¹²C/¹³C isotope fractionation to compute intrinsic water use efficiency generally neglects the impact of mesophyll conductance, either because of technical reasons (difficult access to mesophyll conductance measurements) or because its impact is assumed to be relatively modest (assumption of infinite g_m in iWUE_{sim}). However, the reliability of iWUE estimates omitting mesophyll conductance is questionable, in particular when the photosynthetic limitation associated with mesophyll resistance is large. Here, we derive an expression of iWUE that includes the effect of g_m (denoted as iWUE_{mes}), and test the numerical

impact of g_m using independent experimental datasets. We address the following questions: 1) can we substantially improve the prediction of iWUE (compared with iWUE_{sim}) by including a term accounting for g_m ? and, 2) could we take advantage of a g_m - g_{sc} relationship and if so, can it be generalized to compute iWUE? To answer these questions, we collated a database of more than 300 pairs of g_{sc} and g_m measurements of more than 84 species from >40 publications. We analyzed the response of g_m and g_{sc} to long-term drought stress in different plant functional groups to parameterize iWUE_{mes}. To test whether iWUE_{mes} provides reliable estimates of intrinsic water use efficiency, we used an independent dataset of iWUE_{obs} values (obtained directly from gas exchange), and on-line photosynthetic Δ corrected for side effects of respiratory isotopic imbalance (Gong *et al.*, 2015). The latter dataset compiled measurements obtained with seven species grown in controlled-environment conditions.

MATERIALS AND METHODS

Theory

Farquhar *et al.* (1982) and Farquhar & Cernusak (2012) established a comprehensive theoretical model to describe the photosynthetic ^{13}C discrimination of C_3 leaves (Δ) as:

$$\Delta = \frac{1}{1-t} \left[a_{ac} \frac{c_a - c_i}{c_a} \right] + \frac{1+t}{1-t} \left[a_m \frac{c_i - c_c}{c_a} + b \frac{c_c}{c_a} + \frac{\alpha_b}{\alpha_e} e \frac{R_d(c_c - \Gamma^*)}{(A_n + R_d)c_a} - \frac{\alpha_b}{\alpha_f} f \frac{\Gamma^*}{c_a} \right] \quad (3)$$

where c_a , c_s , c_i and c_c are CO_2 mole fractions in the atmosphere, at the leaf surface, and in the substomatal cavity and chloroplast, respectively. a_m (1.8‰) is the fractionation associated with CO_2 dissolution and diffusion in the mesophyll; R_d represents the day respiration rate; Γ^* is the CO_2 compensation point in the absence of mitochondrial respiration.; b (29‰), e (-6-0‰) and f (11‰) represent the fractionations due to carboxylation, daytime mitochondrial respiration and photorespiration, respectively. Also, $\alpha_b=1+b$, $\alpha_e=1+e$, $\alpha_f=1+f$. t is the ternary correction factor and a_{ac} is the weighted fractionation for diffusion across the boundary layer and stomata. They can be calculated as:

$$t = \frac{(1+a_{ac})E}{2g_{ac}} \quad (4)$$

$$a_{ac} = \frac{a_b(c_a - c_s) + a_s(c_s - c_i)}{c_a - c_i} \quad (5)$$

where a_b (2.9‰) and a_s (4.4‰) are the $^{12}\text{C}/^{13}\text{C}$ fractionations during CO_2 diffusion through the leaf boundary layer and stomata, respectively. E is the transpiration rate. c_s is the CO_2 concentration at the leaf surface; g_{ac} is the combined boundary layer and stomatal conductance to CO_2 .

From this point, we can use Δ to calculate an exact value of intrinsic water use efficiency that accounts for both internal conductance and ternary effects. It will be denoted as $iWUE_{com}$. We recall here that accounting for ternary effects is so that:

$$A_n = g_{ac}(c_a - c_i) - E\bar{c} \text{ where } \bar{c} = \frac{c_a + c_i}{2}$$

At this stage, there are two possibilities to compute intrinsic water use efficiency: accounting for boundary layer conductance, or disregarding boundary layer resistance. As per [Eqn 3 to 5](#), the observed isotope fractionation accounts for boundary layer effects and the CO_2 drawdown from c_a to c_i is the result of both boundary layer and stomatal resistance.

• If the definition of intrinsic water use efficiency encapsulates both boundary layer and stomatal effects (we denote this $iWUE$ as $iWUE_{com}^*$), we have:

$$iWUE_{com}^* = \frac{A_n}{kg_{ac}} = \frac{g_{ac}(c_a - c_i) - E\bar{c}}{kg_{ac}} \quad (6)$$

Where k is the coefficient between CO_2 and H_2O conductances (it equals 1.6 in [Eqn 1](#)). Using the expression $E = kg_{ac}W$ (where W is the water vapor drawdown from intercellular spaces to air), we have:

$$iWUE_{com}^* = c_a \cdot \left(\frac{1}{k} - \frac{W}{2}\right) - c_i \cdot \left(\frac{1}{k} + \frac{W}{2}\right) \quad (7)$$

Substituting [Eqns 6 and 7](#) into [3](#), we have (for intermediate calculations, see [Note S1](#)):

$$iWUE_{com}^* = c_a \frac{a_{ac}(1-\varepsilon) + (1+t) \left[\frac{r^*}{c_a} \left(\frac{e'R_d}{A_n + R_d} - f' \right) + \varepsilon \left(b - \frac{e'R_d}{A_n + R_d} \right) \right] - (1-t)\Delta}{\frac{(1+t) \left(b - \frac{e'R_d}{A_n + R_d} \right) - a_{ac}}{\frac{1}{k} + \frac{W}{2}} - (1+t)k \frac{g_{ac}}{g_m} \left(a_m - b + \frac{e'R_d}{A_n + R_d} \right)} \quad (8)$$

where $e' = e\alpha_b/\alpha_e$, $f' = f\alpha_b/\alpha_f$ and

$$\varepsilon = \frac{\frac{1}{k} - \frac{W}{2}}{\frac{1}{k} + \frac{W}{2}} \quad (9)$$

Here, it is useful to indicate the order of magnitude of terms found in [Eqn 8](#) but not found in simplified expressions commonly used (such as [Eqn 2](#)). Assuming that E is typically about $2 \text{ mmol m}^{-2} \text{ s}^{-1}$ and stomatal conductance for CO_2 is $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$ gives t in the order of $1/100^{\text{th}}$ thus its impact is small. W is homogenous to a mole fraction and thus about 0.01 (i.e. typically in the order of $10 \text{ mmol H}_2\text{O mol}^{-1}$) and thus $\varepsilon \approx 0.98$ and its impact is rather small. Taken as a whole, the ternary correction affects $iWUE$ by about 1%.

• If intrinsic water use efficiency does not encapsulate boundary layer effects (it is then denoted as $iWUE_{com}$), then:

$$iWUE_{com} = \frac{A_n}{k g_{sc}} = \frac{A_n}{k g_{ac}} \cdot \frac{g_{ac}}{g_{sc}} = iWUE_{com}^* \frac{g_{ac}}{g_{sc}} \quad (10)$$

Note that Eqn 10 means that boundary layer effects are so that $iWUE_{com} < iWUE_{com}^*$ since $g_{ac} < g_{sc}$. When ternary corrections are ignored and boundary layer conductance is presumed to be infinite, then $t=0$, $\varepsilon=1$, $c_a=c_s$ and thus $a_{ac}=a_s$ and $g_{ac}=g_{sc}$. Eqn 8 simplifies to:

$$iWUE_{mes} = \frac{c_a}{k} \frac{b - \Delta - f' \frac{\Gamma^*}{c_a} - e' \frac{R_d}{A_n + R_d} \left(1 - \frac{\Gamma^*}{c_a}\right)}{b - a_s + \frac{g_{sc}}{g_m} \left(b - a_m + e' \frac{R_d}{A_n + R_d}\right) - e' \frac{R_d}{A_n + R_d}} \quad (11)$$

Here, the subscript “mes” indicates that this expression accounts for mesophyll conductance effects but not ternary correction or boundary layer effects. If the impact of day respiration is assumed to be negligible ($e = 0$), or if the photosynthetic fractionation has been corrected for R_d (see below), Eqn 11 gives the proxy:

$$iWUE_{mes} = \frac{c_a}{k} \frac{b - \Delta - f' \frac{\Gamma^*}{c_a}}{b - a_s + \frac{g_{sc}}{g_m} (b - a_m)} \quad (12)$$

The simplifying assumptions used here to obtain Eqn 12, which have been commonly applied (Ubierna *et al.*, 2018), are supported by sensitivity tests (see below). Compared with $iWUE_{sim}$ (Eqn 2), $iWUE_{mes}$ (Eqn 12) takes into account the effects of mesophyll conductance and photorespiration. Combining Eqn 12 and Eqn 2, $iWUE_{mes}$ is related to $iWUE_{sim}$ as:

$$iWUE_{mes} = \frac{c_a (b - b') + k iWUE_{sim} (b' - a_s) - f' \Gamma^*}{k \left(b - a_s + (b - a_m) \frac{g_{sc}}{g_m} \right)} \quad (13)$$

Eqn 12 can also be used to examine the sensitivity of $iWUE_{mes}$ with respect to the g_{sc} -to- g_m ratio. Using the partial derivative of $iWUE_{mes}$ gives the elasticity (relative change in $iWUE_{mes}$ for a unit variation in g_{sc}/g_m):

$$\frac{\partial \ln(iWUE_{mes})}{\partial \ln\left(\frac{g_{sc}}{g_m}\right)} = \frac{1}{1 + \frac{b - a_s}{b - a_m} \frac{g_m}{g_{sc}}} \quad (14)$$

To assess the sensitivity of $iWUE$ models (Eqn 8, 10 and 12) to different simplifications, we used theoretical data and varied the photosynthetic discrimination within 18 and 22‰ at different g_{sc}/g_m ratios: $g_{sc}/g_m=0$ ($iWUE_{sim}$ model), $g_{sc}/g_m=0.4$, 0.8 and 1.6 ($iWUE_{com}$ and $iWUE_{mes}$ models) (for details on calculations, see Table S1).

Collection of literature data of g_m and g_{sc}

To parameterize g_{sc}/g_m in Eqn 12, we performed an extensive literature review of leaf-level g_m and g_{sc} measurements by searching the keywords ‘mesophyll conductance’ and ‘internal conductance’ in ISI Web of Science and checking the cited references in recent review articles of this topic. Data published before 2020 that met the following criteria were included in our analyses: (i) leaf-level g_m and g_{sc} data were reported; (ii) g_m was measured with either the online carbon isotope discrimination (Evans *et al.* 1986) or fluorescence (Harley *et al.*, 1992) methods; and (iii) both control and edaphic drought treatments were included. We neglected results with short-term drought treatment (i.e. exposure time shorter than 2 days) and results of re-watering experiments, since acclimated parameters are more representative for the steady-state physiological status of plants compared with dynamically changing parameters. Gas exchange parameters (e.g. net assimilation rate, transpiration rate) were also collected if presented. Furthermore, we eliminated outliers which were defined by the criteria: $A_n/g_{sc} > 300$, $g_{sc}/g_m < 0.2$, or $g_m > 0.8 \text{ mol m}^{-2} \text{ s}^{-1}$ (cf. Flexas *et al.*, 2013). Average values for of each species in each treatment combination were compiled, and the compiled dataset included 198 pairs of g_m and g_{sc} measurements of 32 species under manipulated soil water availability or VPD from 15 studies (‘drought-stress dataset’). Additionally, published data reporting long-term treatments other than edaphic drought (e.g. temperature, salinity, soil nutrients, light, etc.) were collected and screened with the same criteria to detect outliers. We thus obtained a more comprehensive dataset (‘the main dataset’) that included 364 pairs of g_m and g_{sc} measurements of 84 species from 50 studies. We used the ‘drought-stress dataset’ to analyze the effects of water stress treatments and plant functional groups, and ‘the main dataset’ was used to examine the g_{sc} - g_m relation in a larger dataset.

Statistical analyses on the compiled drought-stress dataset of g_m , g_{sc} , A_n , and g_{sc}/g_m were performed by using the general linear model of SPSS Statistics 19 (IBM Corp). The general linear model included water stress and plant functional groups (PFGs) and their interaction as the main factors. The factor of drought stress had two levels: control (non-stressed plants) and stressed. Plant species were classified into three PFGs: deciduous or semi-deciduous woody (DW), evergreen woody (EW), and herbaceous (HB, including annual, bi-annual and perennial) species. The parameters were logarithmic and square-root transformed to improve the normality before ANOVA tests or linear regressions, and both methods had no effect on the statistical results. We

present here square-root transformed results because it improved the normality to a greater extent compared to the logarithmic transformation, as detected from histograms.

Experimental data and photosynthetic ^{13}C discrimination

To validate the iWUE_{mes} model (Eqn 12), we used independent datasets from two experiments (Gong *et al.* 2015, 2018) which included simultaneous measurements of photosynthetic gas exchange and online ^{13}C discrimination on fully expanded young leaves of seven species (Table 2). Most of the data have been published with the exception of *Vicia faba* and *Glycine max* grown and measured in the same way as the other species (Gong *et al.*, 2018). These datasets were particularly suitable for evaluating the relation between Δ and iWUE because advanced protocols were applied to minimize any effect of diffusive leaks in the leaf cuvette and the isotopic disequilibrium between photosynthesis and day respiration. That system used a $^{13}\text{CO}_2/^{12}\text{CO}_2$ gas exchange and labelling system connected with a portable leaf gas exchange apparatus (LI-6400, LICOR Inc., Lincoln, USA) and gas exchange mesocosms, with the air supply to the LI-6400 and mesocosms provided by mixing CO_2 -free, dry air and CO_2 of known $\delta^{13}\text{C}$ (Schnyder *et al.*, 2003). This setup enabled the quantification of diffusive leak coefficients during measurements with intact leaves by measuring and manipulating $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$ gradients between leaf cuvette and the surrounding air in the dark, thus effectively correcting leak artefacts (Gong *et al.* 2015, 2018).

The iWUE_{mes} model (Eqn 12) excludes the fractionation component associated with R_d , therefore, to precisely compare the modeled and the observed iWUE , the isotopic fractionation of R_d (Δ_{R_d}) should be subtracted from the observed net discrimination (Δ_{AN}) as $\Delta_P = \Delta_{\text{AN}} + \Delta_{R_d}$, where Δ_P is the photosynthetic discrimination corrected for day respiration. Alternatively, our experimental data can be used to parameterize Eqn 11, and in that case Δ_{R_d} is not subtracted. Subtracting the effect of R_d is particularly useful when there is an isotopic disequilibrium whereby respiratory substrates have an isotope composition that is substantially different from that of current assimilates.

If we neglect ternary corrections in Eqn 3, the R_d corrected photosynthetic discrimination (Δ_P) can be calculated as:

$$\Delta_P = \Delta_{\text{AN}} + eR_d(c_c - \Gamma^*) / [(A_n + R_d)c_a] \quad (15)$$

This equation is difficult to apply in practice because (i) the term R_d in Eqn 3 is the gross respiration rate (before refixation by photosynthesis and anapleurotic reactions) and is not easily

measurable (Tcherkez *et al.* 2017); (ii) it requires the knowledge of c_c thus we would need to measure g_m first. We thus took advantage of the isotopic disequilibrium approach of Gong *et al.* (2015; 2018) to subtract the term associated with R_d from Δ_{AN} . Assumptions on the isotope effect of R_d are further detailed in Note S2. Online discrimination measurements (Evans *et al.*, 1986) were performed using a ^{13}C -enriched CO_2 source, followed by the measurement using a ^{13}C -depleted CO_2 source, so that the measured net assimilation rate (A_n) could be partitioned into net photosynthesis in the absence of day respiration (P) and day respiration (R'_d) (Gong *et al.*, 2015). Applying isotopic mass balance, the photosynthetic discrimination (Δ_P) was calculated from the measured net discrimination (Δ_{AN}) as (Tcherkez *et al.*, 2011; Gong *et al.*, 2018):

$$\Delta_P = \Delta_{AN} + eR'_d/A_n, \quad (16)$$

where R'_d is day respiration measured by the method of Gong *et al.* (2015; 2018) and e is the measured isotope fractionation of R_d relative to A_n determined as $e = (\delta_{A_n} - \delta_{R_d}) / (1 + \delta_{R_d})$. It is assumed that δ_{R_d} was equal to the measured isotopic signature of leaf respiration in the dark (Gong *et al.* 2015, 2018). Importantly, Eqn 16 assumes that CO_2 evolved by day respiration has a $\delta^{13}\text{C}$ value that is distinct to that of photosynthesis. In other words, the calculation treats R'_d as a flux metabolically disconnected from recent photosynthesis during a short-duration of measurements (Note S2), and the related assumptions have been validated and discussed previously (Gong *et al.* 2015, 2017, 2018) and supported by other work (Barbour *et al.*, 2017; Busch *et al.*, 2020). Furthermore, taking different assumptions concerning the value of respiratory fractionation (Eqn 3 vs. Eqn S1) eventually had very minor influence on iWUE estimates (Note S2, Fig. S4).

Knowing Δ_P , iWUE was calculated from the iWUE_{mes} model (Eqn 12) using a mean g_{sc}/g_m of 0.79 derived from the compiled literature dataset (see below), and Γ^* as estimated from leaf temperature (T) as $\Gamma^* = 42.7 + 1.68(T - 25) + 0.012(T - 25)^2$ following Brooks & Farquhar (1985). In addition, iWUE was also calculated from the simple model (iWUE_{sim} Eqn 2). The estimates of iWUE_{mes} and iWUE_{sim} were compared with the measured iWUE from gas exchange measurements (iWUE_{obs}). The Root Mean Square Error (RMSE) was calculated to evaluate the prediction errors for the simple and the iWUE_{mes} model. The value of g_m of these plants was determined using the improved online Δ method and were published previously (Gong *et al.* 2015; 2018), except for the *Vicia faba* and *Glycine max* data. The detailed description of methods and equations for g_m are presented in Gong *et al.* (2015; 2018).

RESULTS

Sensitivity of iWUE models to g_m and simplifying assumptions

The expression of $iWUE_{mes}$ (Eqn 12) includes the g_{sc}/g_m ratio, implying in principle that $iWUE_{mes}$ varies with g_m . Accordingly, the expression of elasticity (Eqn 14) shows that estimating $iWUE_{mes}$ from Δ using Eqn 12 is sensitive to the g_{sc}/g_m ratio when g_m is varied in relation to g_{sc} . For example, as is shown below, with a very high value of g_{sc}/g_m , e.g. $g_{sc}/g_m = 3$, the elasticity value is 0.77. This implies a 77% change in $iWUE_{mes}$ when g_{sc}/g_m changes by 100%. By contrast, when g_m is large, the denominator in Eqn 14 is large and the elasticity value becomes small. Eqn 14 thus reflects the intuitive result that when g_m is large, its impact on Δ is small and thus numerically, the impact of the g_{sc}/g_m ratio on $iWUE$ is modest (and effectively nil, when g_m is infinite, as assumed by $iWUE_{sim}$).

A sensitivity analysis was performed to quantify the effect of g_{sc}/g_m ratio, dark respiratory fractionation e , photosynthetic fractionation f and ternary corrections on $iWUE$, as predicted by the different $iWUE$ models (Fig. 1). Both $iWUE_{com}$ and $iWUE_{mes}$ were sensitive to the g_{sc}/g_m ratio. The simplified model (that assumed $g_{sc}/g_m=0$) predicted the largest $iWUE$, as expected (Fig. 1a). At all g_{sc}/g_m ratios, $iWUE_{com}$ and $iWUE_{mes}$ predictions were numerically very similar (with an error of generally less than $3 \mu\text{mol mol}^{-1}$, Fig. 1a). Furthermore, assuming an f of 0 or 20‰ led to a variation of predicted $iWUE$ of about $5 \mu\text{mol mol}^{-1}$ (Fig. 1b). Similarly, ignoring respiratory fractionation or ternary correction caused an error of less than $2 \mu\text{mol mol}^{-1}$ (Fig. 1cd). Also, manipulating boundary layer conductance between $1 \text{ mol m}^{-2} \text{ s}^{-1}$ and infinite had very little influence on $iWUE$ estimates (error less than $2 \mu\text{mol mol}^{-1}$, data not shown). Thus, the assumptions we used to derive $iWUE_{mes}$ (Eqn 12) seem to be appropriate.

Overview of g_m and g_{sc} variations

Water availability and PFGs significantly impacted A_n , g_{sc} , and g_m (Table 3). Compared with the non-stressed condition, drought stress decreased A_n , g_{sc} , and g_m for all three PFGs significantly (Fig. 2), but the magnitude of the decrease varied between PFGs. Within a PFG, drought stress reduced A_n by 36% for the DW, 67% for the EW and 42% for the HB (Fig. 2a); and reduced g_{sc} by 37% for the DW, 81% for the EW and 47% for the HB (Fig. 2b), on average. The magnitude of the reduction in g_m was similar to that of g_{sc} : 36% in the DW, 80% in the EW and 35% in the HB PFG (Fig. 2c). The HB had the highest values of A_n , g_{sc} , and g_m among the three PFGs under

both non-stressed and drought-stressed conditions (Fig. 2). That is, the higher A_n , the higher were g_{sc} and g_m within a functional group. This relationship was generally true also for the individual species within a PFG. Accordingly, positive correlations between A_n and g_{sc} or g_m were found for the different species of a PFG across water stress treatments, although the quantitative relations were significantly different between PFGs (Supplementary Data Fig. S1).

The g_{sc}/g_m ratio showed no significant differences between water availability conditions or PFGs ($p>0.05$), while $iWUE_{obs}$ was significantly influenced by drought stress (Table 3). Drought stress improved $iWUE_{obs}$ by 25% compared with non-stress conditions when pooling the data of all plant functional groups (Fig. 3b). The g_{sc}/g_m ratio of drought-stressed plants (0.79 ± 0.11 , 95%CI) was almost identical to that of non-stressed plants (0.79 ± 0.09 , 95%CI) ($p>0.05$) (Fig. 3a). The g_{sc}/g_m ratio of the EW (0.87 ± 0.13 , 95%CI) appeared to be higher, but not significantly so, than that of the DW (0.71 ± 0.11 , 95%CI) and the HB (0.77 ± 0.12 , 95%CI) PFG. The mean g_{sc}/g_m ratio was 0.79 (±0.07 , 95%CI, $n=198$) across drought-stress levels and PFGs. There was a significant correlation between g_m and g_{sc} that was similar for drought-stressed ($r^2=0.58$, $n=106$, $p<0.0001$, Fig. 4) and non-stressed conditions ($r^2=0.20$, $n=92$, $p<0.0001$, Fig. 4). When pooling all data of the main dataset to include other long-term treatments, g_{sc} and g_m were also positively correlated ($r^2=0.47$, $n=364$, $p<0.0001$) with a mean g_{sc}/g_m of 0.88 ± 0.06 (95%CI) (Supplementary Data Fig. S2).

Predicting iWUE from photosynthetic ^{13}C discrimination Δ

A value for g_{sc}/g_m of 0.79 was used to compute $iWUE_{mes}$ (Eqn 12) and thus predict $iWUE$ from day-respiration corrected Δ (Δ_P) in seven species in controlled-environment experiments reported in Table 2. $iWUE_{mes}$ agreed much better with observed values of $iWUE_{obs}$ than $iWUE_{sim}$ as shown by the regression analysis (Fig. 5a). This was further supported by the RMSE which was far lower for $iWUE_{mes}$ than for $iWUE_{sim}$ (Fig. 5). The mean error of $iWUE_{mes}$ ($5 \mu\text{mol mol}^{-1}$) was also significantly lower than that of $iWUE_{sim}$ ($53 \mu\text{mol mol}^{-1}$). The difference in RMSE between the two models was largest (about $62 \mu\text{mol mol}^{-1}$) in *G. max* and smallest in *H. lanatus* and *T. aestivum*, at only about one half that of *G. max*. The accuracy of predicted $iWUE$ using $iWUE_{mes}$ thus seemed to vary somewhat between species, likely related to interspecific differences in g_{sc}/g_m .

We analyzed the relationship between the measured g_m or g_{sc}/g_m and $iWUE_{obs}$ also in the drought-stress dataset and the two independent datasets, where g_m was measured using the online

Δ method of Gong *et al.* (2015, 2018). This revealed a weak but statistically significant negative correlation between g_m and $iWUE_{obs}$ across drought-stress levels and species ($p < 0.01$, $r^2 < 0.1$, Fig. 6a), a correlation that was also confirmed in our experimental datasets ($p < 0.0001$, $r^2 = 0.37$, Fig. 6b). We found a significant negative correlation between g_{sc}/g_m and $iWUE_{obs}$ in all datasets (Fig. 6cd). This relation was more robust than the g_m - $iWUE_{obs}$ relationship as was indicated by the higher r^2 , in both the drought-stress dataset ($p < 0.0001$, $r^2 = 0.25$, Fig. 6c) and the independent experimental datasets ($p < 0.0001$, $r^2 = 0.50$, Fig. 6d). Beyond that, relations between g_{sc}/g_m and $iWUE_{obs}$ were statistically identical (i.e. regression parameters were not significantly different) for both datasets.

DISCUSSION

Including mesophyll conductance in iWUE estimation is critical

The importance of accounting for g_m in the Δ - $iWUE$ relationship is supported by our model analysis, which illustrates that omitting mesophyll conductance (or the use of $iWUE_{sim}$ which assumes an infinite g_m) causes a significant overestimation of observed $iWUE$. Of course, the impact of omitting mesophyll conductance is large when mesophyll conductance is small and affecting Δ considerably. At a “common” Δ value of 18‰, the $iWUE_{sim}$ model overestimated $iWUE$ by 65%, clearly showing that $iWUE_{sim}$ did not provide a reasonable estimate of $iWUE$. The overestimation of $iWUE$ resulting from omission of mesophyll conductance was even greater at a lower Δ , showing that the use of $iWUE_{sim}$ is even more problematic under conditions that lead to decreasing Δ . This means, that caution must be taken when comparing $iWUE$ of plants in conditions that lead to divergent Δ , such as contrasting soil water availability, temperature, or transpiration demand when using the $iWUE_{sim}$ model.

Quantitative prediction of $iWUE$ is essential to anticipate plant responses to climate change factors like CO_2 and precipitation regime (Adams *et al.*, 2019; Soh *et al.*, 2019). Recent studies have shown an increased need to quantify the rate at which $iWUE$ has been changing in different geographical regions (Adams *et al.*, 2019). Here, using $iWUE_{mes}$ to account for the g_{sc}/g_m ratio seems necessary. By contrast, the impact of respiratory fractionation, boundary layer conductance, and ternary effect on $iWUE_{mes}$ values is far less critical. This not only supports assumptions we made to derive $iWUE_{mes}$ (in Eqn 12), but also means that comparing crop varieties for $iWUE_{mes}$

should not be compromised significantly by changes in respiratory metabolism or leaf morphology affecting boundary layer.

An accurate estimation of iWUE is also essential to predict relations between water and carbon fluxes at scales higher than the leaf scale. For instance, combining iWUE estimates from the ^{13}C discrimination and transpiration flux inferred from sap flow has been used to quantify photosynthesis of forest canopies (Klein *et al.*, 2016). The isotope fractionation can be estimated using the $\delta^{13}\text{C}$ value of either bulk organic matter or carbohydrates (via compound-specific $\delta^{13}\text{C}$ analysis of sugars). The $\delta^{13}\text{C}$ in sugars is believed to be better proxy for iWUE because it reflects recent photosynthetic assimilation and is less influenced by post-photosynthetic fractionation processes (Hobbie & Werner, 2004; Badeck *et al.*, 2005). Smith *et al.* (2016) showed that using the $\delta^{13}\text{C}$ of leaf and phloem carbohydrates improved the precision of iWUE estimations, as compared to using the $\delta^{13}\text{C}$ value of bulk soluble carbon. However, both methods overestimated iWUE when iWUE_{sim} was used. We used their data (Smith *et al.*, 2016) and calculated iWUE_{mes} using a g_{sc}/g_m of 0.79, with the value of Δ obtained from individual carbohydrates (i.e. sucrose, glucose, and fructose). This significantly reduced the error in predicted iWUE (Fig. S3). That is, neglecting the impact of g_m also overestimates considerably iWUE calculated from the $\delta^{13}\text{C}$ value of individual carbohydrates.

Parameterization of iWUE_{mes} calculation

As shown by Eqn 11 or 12, calculating iWUE_{mes} from Δ requires the knowledge of the g_{sc}/g_m ratio. Our study suggests that a value of g_{sc}/g_m of 0.79 can be applied broadly. In fact, that ratio did not seem to be influenced by drought stress or plant functional groups in the global dataset. Using such a value of the g_{sc}/g_m ratio can therefore be useful to compare iWUE_{mes} values of multiple species grown under varying soil water conditions, particularly if there is no direct information on mesophyll conductance. The use of a value of 0.79 is further supported by our independent experimental data of seven species (Fig. 5). It is worth noting that even the use of a constant value of 0.79 does not simply change the scale of iWUE values by a proportionality constant, because the term associated with g_m in iWUE_{mes} is an additive term in the denominator (Eqn 12). The apparent insensitivity of the g_{sc}/g_m ratio to drought relates to the similar sensitivity of g_{sc} and g_m to long-term water stress, as both were reduced by about 50% across plant functional groups. In other words, our results do not directly support the hypothesis of a lower sensitivity of g_m to drought

stress compared to g_{sc} , to help maintaining leaf carbon gain (Cano *et al.*, 2014). Also, A_n appeared to decline less than g_{sw} , leading to an increased iWUE under sustained drought-stress, as we previously found in the field (Gong *et al.*, 2011). In addition, our compilation of observations on multiple species shows a negative correlation between g_m and iWUE_{obs} across drought-stress levels, challenging the common assumption that a high g_m is beneficial to water-use during photosynthesis (Barbour *et al.*, 2010; Flexas *et al.*, 2013; Barbour & Kaiser, 2016). In other words, our survey suggests that increased iWUE may not necessarily be associated with high g_m .

Although a common g_{sc}/g_m value of 0.79 seems to be an acceptable compromise when information on mesophyll conductances is not available, it is certainly desirable to parameterize iWUE_{mes} with proper experimental values in order to compute precise intrinsic water use efficiency values. In fact, we found a significant, negative relationship between iWUE_{obs} and g_{sc}/g_m across a large range of species (Fig. 6). In addition, g_{sc}/g_m explained a higher proportion of variation in iWUE_{obs} compared to g_m alone, as expected from Eqn 12. Therefore, g_{sc}/g_m contributed to explaining better the observed interspecific difference in iWUE_{obs}. That is also probably the case between varieties within the same species, and thus the g_{sc}/g_m ratio should be considered in breeding programs targeting water-use efficiency.

We nevertheless recognize that variations in g_{sc}/g_m amongst functionally similar species or between studies is the main challenge in the use of iWUE_{mes}. Current studies show that responses of g_m to environmental factors are highly complex and such factors include CO₂ (Flexas *et al.*, 2007), temperature (von Caemmerer & Evans, 2013; Shrestha *et al.*, 2019) and VPD (Loucos *et al.*, 2017, Stangl *et al.*, 2019). Up to now, variations in g_m cannot be easily related to anatomical features and it is believed that biochemical processes (e.g. facilitated diffusion by carbonic anhydrase or aquaporins) are involved (Flexas *et al.*, 2008) although controversial results have been obtained (Perez-Martin *et al.*, 2014; Kromdijk *et al.*, 2020). It is also worth noting that observed variations in g_m could be related to the methods used for measurements. Some authors questioned the robustness of current observed values of g_m (Tholen *et al.*, 2012; Sun *et al.*, 2014b), because current methods extract g_m from a residual photosynthetic component after other processes have been accounted for, meaning that all errors (artifacts) accumulate in the estimate of g_m (Pons *et al.*, 2009). For example, Gong *et al.* (2015) demonstrated that g_m estimates can be strongly influenced by diffusive leaks and isotopic disequilibria between respiration and photosynthesis when g_m is computed from non-corrected online Δ values. The contribution of the apparent isotopic

fractionation by day respiration could easily be misquantified if carbon reserves are respired instead of current photosynthates, thus causing errors in g_m estimates (Gong *et al.*, 2015; Tcherkez *et al.*, 2017; Barbour *et al.*, 2017; Busch *et al.*, 2020). The use of $iWUE_{mes}$ effectively provides much more accurate $iWUE$ values than $iWUE_{sim}$, but might be associated with an error (e.g. for species with a different g_{sc}/g_m ratio) if the ‘standard’ 0.79 g_{sc}/g_m value is used. Certainly, in mechanistic studies of the controls of $iWUE$ in a given species it is warranted to examine environmental effects on relationships between g_{sc} and g_m with experimental detail.

Perspectives

Our analysis shows that the expression of $iWUE_{mes}$ that accounts for mesophyll conductance (Eqn 12) can be used to provide better estimates of intrinsic water use efficiency. Together with an observation-based g_{sc}/g_m ratio, it can be used to reanalyze, adjust or correct published $iWUE_{sim}$ data for which measurements of g_m are unavailable. This has far reaching importance, as g_m is a key parameter for most of the models of leaf photosynthesis or carbon cycling, but is often poorly represented in them (Tholen *et al.*, 2012; Sun *et al.*, 2014b). Assuming an infinite g_m leads to an overestimation of global gross primary production by about 16% by earth system models (Sun *et al.*, 2014a). Scaling of g_m to g_{sc} is potentially a useful way to reduce the error related to g_m in such models. Our study also examined potential differences in g_{sc}/g_m ratios across long-term drought-stress treatments and plant functional groups and showed that g_{sc}/g_m has rather similar values such that an average value of 0.79 in the $iWUE_{mes}$ model works well to provide more realistic $iWUE$ values. However, in an effort to compare and select varieties based on water use efficiency, genotypic variation in g_{sc}/g_m should not be overlooked. That is, the effect of mesophyll conductance (relative to that of stomatal conductance) must be accounted for to provide reliable estimates of $iWUE$ based on carbon isotopes. This is particularly true in crop species where mesophyll conductance contributes significantly to photosynthetic limitations such as *V. faba*, *G. max* and *P. vulgaris* in this study.

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AUTHOR CONTRIBUTIONS

X.Y.G. designed and planned the research; G.T. proposed the iWUE_{com} model and the framework of sensitivity analyses; W.T.M. performed the literature survey and data analyses; W.T.M., X.M.W and X.Y.G. wrote the first draft, and all authors discussed the results and implications and contributed to the revision.

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Tables and Figures

Table 1. Definitions of abbreviations used for intrinsic water-use efficiency in this paper.

Abbreviations	Definition
iWUE	Generic term for intrinsic water-use efficiency
iWUE _{obs}	Observed value of intrinsic water use efficiency using gas exchange (A_n/g_{sw})
iWUE _{sim}	Predicted intrinsic water-use efficiency using the simplified model of $^{12}\text{C}/^{13}\text{C}$ discrimination (Eqn 2)
iWUE _{mes}	Predicted intrinsic water-use efficiency using the model of $^{12}\text{C}/^{13}\text{C}$ discrimination accounting for mesophyll conductance but not boundary layer conductance, ternary effects nor day respiration (Eqn 12)
iWUE _{com}	Predicted intrinsic water used efficiency using the model of $^{12}\text{C}/^{13}\text{C}$ discrimination accounting for all effects, including mesophyll conductance, ternary effects and day respiration (Eqn 10)
iWUE* _{com}	Predicted intrinsic water used efficiency using the model of $^{12}\text{C}/^{13}\text{C}$ discrimination accounting for all effects, including mesophyll conductance, ternary effects, boundary layer conductance and day respiration, and assuming that the definition of iWUE also includes boundary layer conductance in the denominator (Eqn 8)

Table 2 Overview of the two experimental datasets of Δ_{obs} in this study

Source	Gong <i>et al.</i> (2015)	Gong <i>et al.</i> (2018)
Plant species	<i>Holcus lanatus</i>	<i>Hordeum vulgare</i> , <i>Triticum aestivum</i> , <i>Ricinus communis</i> , <i>Phaseolus vulgaris</i> , <i>Glycine max</i> , <i>Vicia faba</i>
Leaf types	Fully expanded young leaves	Fully expanded young leaves
Growth condition	Growth chamber, non-stressed condition	Growth chamber, non-stressed condition
Measurement condition	PPFD: 1600 $\mu\text{mol m}^{-2} \text{s}^{-1}$; Leaf temperature: 25 °C; [CO ₂]: 390 ppm; rH: 70%	PPFD: 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$; Leaf temperature, 23 °C [CO ₂]: 390 ppm; rH: 70%
iWUE	Directly measured	Directly measured
Diffusive leak	Quantified and corrected	Quantified and corrected
Contribution of day respiration to Δ_{obs}	Quantified and corrected	Quantified and corrected

1 **Table 3** ANOVA tests for A_n , g_{sc} , g_m , g_{sc}/g_m , and $iWUE_{obs}$ using a general linear model. Data are
2 square root transformed to improve the normality before ANOVA tests. Significant effects are
3 shown in bold ($p < 0.05$)

Source	df	A_n		g_{sc}		g_m		g_{sc}/g_m		$iWUE_{obs}$	
		F	P	F	P	F	P	F	P	F	P
Drought stress	1	101.44	0.000	105.43	0.000	56.13	0.000	0.004	0.949	9.91	0.002
PFG	2	50.85	0.000	31.33	0.000	30.61	0.000	1.530	0.219	0.48	0.617
W*PFG	2	3.85	0.023	6.70	0.002	4.75	0.010	0.199	0.820	0.99	0.374

4

Fig. 1 Sensitivity test of iWUE prediction by the iWUE_{com} model and the iWUE_{mes} model as affected by g_{sc}/g_m ratios (a) and the influence of photorespiratory fractionation (b), mitochondrial respiratory fractionation (c) and leaf-to-air vapor concentration difference (W , mmol mol⁻¹, d) on iWUE_{com} estimations. The black lines in the panels b-d represent the ‘standard scenario’ for data simulation with $g_{sc}/g_m=0.8$, $f=11‰$, $e=-5‰$, and $W=10$ mmol mol⁻¹. The values of parameters for simulations were shown in Table S1.

Fig. 2 A_n a), g_{sc} b) and g_m c) across drought-stress levels and plant functional groups (PFGs: DW, deciduous and semi-deciduous woody species; EW evergreen woody species; and HB, herbaceous species). Data collection of different species under non-stressed (blue boxes) or

16 water-stressed conditions (red boxes). Boxplots show median (center line), mean (black dot),
 17 inter-quartile range, 10-90% range (whiskers), and outliers (open circles). Numbers in brackets
 18 are the number of observations. Data are from the following references: Bongi et al. (1989),
 19 Delfine et al. (2001, 2005), Flexas et al. (2002), Galmes et al. (2007), Miyzawa et al. (2008),
 20 Barbour and Kaiser (2016), Brilli et al. (2013), Cano et al. (2013, 2014), Centritto et al. (2009),
 21 Ferrio et al. (2012), Perez-Martin et al. (2009, 2014), Warren et al. (2008, 2011).

Fig. 3 g_{sc}/g_m a) and $iWUE_{obs}$ b) across drought treatments and plant functional groups (PFGs: DW, deciduous and semi-deciduous woody species; EW evergreen woody species; and HB, herbaceous species). Data source and the meaning of symbols as in Fig. 2.

Fig. 4 The relation between g_m and g_{sc} of non-stressed plants (blue solid lines) and drought-stressed plants (red solid line) pooling over plant functional groups. Symbols and species: ∇ , deciduous semi-deciduous woody species (DW); $\square$, evergreen woody species (EW); $\circ$, herbaceous species (HB). The internal panel shows the relation between square-root transformed g_m and g_{sc} . Regressions are linear function through the origin across all genotypes with the slope of 0.78 ± 0.02 (SE) of non-stressed plants ($r^2=0.20$, $p<0.0001$, $n=92$) and 0.72 ± 0.02 (SE) of drought-stressed plants ($r^2=0.58$, $p<0.0001$, $n=106$) for the square-root transformed data. The dashed black line is a 1:1 line.

Fig. 5 iWUE calculated from photosynthetic Δ using the simple model ($iWUE_{sim}$) and the $iWUE_{mes}$ model compared to the measured iWUE ($iWUE_{obs}$) a) and the comparison of RMSE of the two models b). Symbols and species: $\square$, *G. max*; $\circ$, *H. lanatus*; $\triangle$, *H. vulgare*; ∇ , *P. vulgaris*; $\diamond$, *R. communis*; $\hexagon$, *T. aestivum*; $+$, *V. faba*. The solid lines are least-squares linear regressions: (a) $iWUE_{obs} = -22.5(\pm 4.7) + 0.7(\pm 0.05)iWUE_{sim}$, $r^2 = 0.84$, $p < 0.0001$; (b) $iWUE_{obs} = -22(\pm 4.7) + 1.3(\pm 0.09)iWUE_{mes}$, $r^2 = 0.84$, $p < 0.0001$. Values are mean $\pm$ SE ($n = 46$). The dashed black line is a 1:1 line.

Fig. 6 Relation between the measured $iWUE$ ($iWUE_{obs}$) and g_m of a) synthesized drought-stress dataset and b) experimental dataset of Table 2; The lines are least-squares linear regressions across all species: a) $iWUE=80.10(\pm 3.29)-32.56(\pm 12.46)g_m$, $r^2=0.03$, $p<0.01$, $n=198$; b) $iWUE=79.52(\pm 7.48)-147.12(\pm 29.03)g_m$, $r^2=0.37$, $p<0.0001$, $n=46$; c) $iWUE=96.75(\pm 3.43)-29.39(\pm 3.63)g_{sc}/g_m$, $r^2=0.25$, $p<0.0001$, $n=198$; d) $iWUE=81.67(\pm 6.25)-30.77(\pm 4.69)g_{sc}/g_m$, $r^2=0.50$, $p<0.0001$, $n=46$; values are mean $\pm$ SE.

50 Supporting Information

Fig. S1 Relationships between A_n and g_{sc} , or g_m across two water availability levels (control, blue dots and drought stress, red dots) for the DW (a and b), EW (c and d), and HB (e and f). Data were fitted use a function of $y = ax/(b+x)$, $n=58$ for DW, $n=74$ for EW, $n=66$ for HB, and $p < 0.0001$ for all regressions.

Fig. S2 The relation between g_m and g_{sc} across treatments and species in the main dataset compiled from the published papers. The internal panel shows a box plot of g_{sc}/g_m calculated from the same data with a mean $g_{sc}/g_m=0.88(\pm 0.06$ 95%CI). The dashed line is a 1:1 line, and the solid line is the regression line: $r^2=0.47$, $p<0.0001$, $n=364$.

Fig. S3 Comparison of the measured $iWUE_{obs}$ and the modeled $iWUE$ using the simple ($iWUE_{sim}$, blue symbols and lines) and the $iWUE_{mes}$ model (black symbols and lines) calculated from the data of Smith *et al.* (2016) using Eqn 13. Symbols and leaf metabolites: $\square$, sucrose; $\circ$, glucose; $\triangle$, fructose. The dashed black line is a 1:1 line. The solid lines are least-squares linear regressions: $iWUE_{obs}=20.17(\pm 4.63)+0.37(\pm 0.06)iWUE_{sim}$, $r^2=0.30$, $p<0.0001$; $iWUE_{obs}=24.57(\pm 3.97)+0.69(\pm 0.11)iWUE_{mes}$, $r^2=0.30$, $p<0.0001$. Values are mean $\pm$ SE ($n=90$).

Fig. S4 Comparison of the iWUE_{mes} prediction estimated from Eqn 11 and S2 as affected by the influence of mitochondrial respiratory fractionation (e)

Table S1 Variables used for sensitivity tests. g_{sc} and c_i were stepwise increased to create a gradient of photosynthetic discrimination, and iWUE models were used to calculate iWUE_{sim}, iWUE_{mes}, and iWUE_{com}.

Variable (units)	Definition	Fixed value (standard scenario)	Manipulations
g_{sc} (mol m ⁻² s ⁻¹)	Stomatal conductance to CO ₂	step-wise increased from 0.05 to 0.25	step-wise increased from 0.05 to 0.25
c_i (μmol mol ⁻¹)	CO ₂ concentration in the substomatal cavity	step-wise increased from 250 to 350	step-wise increased from 250 to 350
W (mmol mol ⁻¹)	leaf-to-air vapor concentration difference	10	5, 10, 40
c_a (μmol mol ⁻¹)	CO ₂ concentration in the atmosphere	400	
c_c (μmol mol ⁻¹)	CO ₂ concentration in the chloroplast	$c_c = c_i - A_n / g_m$	$c_c = c_i - A_n / g_m$
g_m (mol m ⁻² s ⁻¹)	mesophyll conductance	$g_{sc} / g_m = 0.8$	$g_{sc} / g_m = 0.4, 0.8, 1.6$
g_{bc} (mol m ⁻² s ⁻¹)	boundary layer conductance to CO ₂	5	1, 5, +∞
g_{ac} (mol m ⁻² s ⁻¹)	conductance to CO ₂ of the stomata plus boundary layer	$g_{ac} = 1 / (1 / g_{sc} + 1 / g_{bc})$	$g_{ac} = 1 / (1 / g_{sc} + 1 / g_{bc})$
t	ternary correction factor	$t = (1 + a_{ac})E / 2g_{ac}$	$t = (1 + a_{ac})E / 2g_{ac}$
a_{ac} (‰)	combined fractionation for CO ₂ diffusion across the boundary layer and the stomata	$a_{ac} = a_b g_{ac} / g_{bc} + a_s g_{ac} / g_{sc}$	$a_{ac} = a_b g_{ac} / g_{bc} + a_s g_{ac} / g_{sc}$
a_b (‰)	fractionation during CO ₂ diffusion across the boundary layer	2.9	-
a_s (‰)	fractionation during CO ₂ diffusion through the stomata	4.4	-
a_m (‰)	fractionation associated with CO ₂ dissolution and diffusion in the mesophyll	1.8	-
b (‰)	fractionation by Rubisco carboxylation	29	-
e (‰)	fractionation by mitochondrial respiration	-5	0, -5, -10
R_d (μmol m ⁻² s ⁻¹)	day respiration rate	0.5	-
A_n (μmol m ⁻² s ⁻¹)	Net assimilation rate	$A_n = g_{ac}(C_a - C_i) - 1.6g_{ac}W(C_a - C_i)/2$	$A_n = g_{ac}(C_a - C_i) - 1.6g_{ac}W(C_a - C_i)/2$
Γ^* (μmol mol ⁻¹)	CO ₂ compensation point in the absence of mitochondrial respiration	35	-
f (‰)	fractionation during photorespiration	11‰	0, 11, 20
E	transpiration rate	$E = 1.6g_{ac}W$	$E = 1.6g_{ac}W$

Note S1 Derivation of [Eqn 8](#) from the comprehensive photosynthetic discrimination model.

Using the notations $e' = ea_b/\alpha_e$, $f' = fa_b/\alpha_f$, and substituting c_c by $c_i - A_n/g_m$, [Eqn 3](#) gives:

$$(1-t)c_a\Delta = a_{ac}c_a + (1+t)\left[\frac{A_n}{g_m}\left(a_m - b + e'\frac{R_d}{A_n + R_d}\right) - f'\Gamma^* + e'\frac{R_d\Gamma^*}{A_n + R_d}\right] \\ + c_i\left[-a_{ac} + (1+t)\left(b - e'\frac{R_d}{A_n + R_d}\right)\right]$$

Rewriting A_n/g_m as $(A_n/kg_{ac})kg_{ac}/g_m = iWUE_{com}^*kg_{ac}/g_m$ and using [Eqn 7](#) leads to:

$$(1-t)c_a\Delta = a_{ac}c_a + (1+t)\left[iWUE_{com}^*\frac{kg_{ac}}{g_m}\left(a_m - b + e'\frac{R_d}{A_n + R_d}\right) - f'\Gamma^* + e'\frac{R_d\Gamma^*}{A_n + R_d}\right] \\ + \frac{c_a\left(\frac{1}{k} - \frac{W}{2}\right) - iWUE_{com}^*}{\frac{1}{k} + \frac{W}{2}}\left[-a_{ac} + (1+t)\left(b - e'\frac{R_d}{A_n + R_d}\right)\right]$$

Rearranging (factorization by $iWUE_{com}^*$) gives:

$$(1-t)c_a\Delta = a_{ac}c_a(1-\varepsilon) + (1+t)\left[c_a\varepsilon\left(b - e'\frac{R_d}{A_n + R_d}\right) + \Gamma^*\left(e'\frac{R_d\Gamma^*}{A_n + R_d} - f'\right)\right] \\ + iWUE_{com}^*\left[k(1+t)\frac{g_{ac}}{g_m}\left(a_m - b + e'\frac{R_d}{A_n + R_d}\right) - \frac{(1+t)\left(b - e'\frac{R_d}{A_n + R_d}\right) - a_{ac}}{\frac{1}{k} + \frac{W}{2}}\right]$$

where ε is defined by [Eqn 9](#). Rearranging to express $iWUE_{com}^*$ gives [Eqn 8](#).

Note S2 The modified carbon isotope discrimination model for partitioning day respiration and its isotopic contribution to photosynthetic discrimination

The comprehensive model of Eqn 3 can be modified as:

$$\Delta = \frac{1}{1-t} \left[a_{ac} \frac{c_a - c_i}{c_a} \right] + \frac{1+t}{1-t} \left[a_m \frac{c_i - c_c}{c_a} + b \frac{c_c}{c_a} + \frac{\alpha_b}{\alpha_e} e \frac{R_d'}{A_n} - \frac{\alpha_b}{\alpha_f} f \frac{\Gamma^*}{c_a} \right] \quad S1$$

by assuming that day respiration is separated from the pool of primary assimilates during a short exposure to an atmosphere with a different $\delta^{13}\text{C}$ value in CO_2 . Furthermore, R_d' is defined as the respired CO_2 that is released into the ambient air, thus is a net flux whereas R_d in the original Farquhar model was defined as a gross respiratory flux (net and gross referring here to refixation) (Tcherkez et al. 2017). That is, R_d' is considered as the flux of CO_2 liberated by the leaf from respiratory substrates with a slow turn-over and thus not influenced by the isotope composition of photosynthetic assimilates in the short term. Using Eqn S1, R_d' can be estimated from measurements of online isotope discrimination in a gas exchange system where inlet CO_2 can be switched between two sources of CO_2 with contrasted $\delta^{13}\text{C}$ values. More details are provided in Gong et al. (2015; 2018).

We can then derive equations for iWUE similarly as in Note S1. Using the notations $e' = e\alpha_b/\alpha_e$, $f' = f\alpha_b/\alpha_f$, and substituting c_c by $c_i - A_n/g_m$, Eqn S1 gives:

$$(1-t)c_a\Delta = a_{ac}c_a + (1+t) \left[\frac{A_n}{g_m} \left((a_m - b) - e' \frac{R_d'c_a}{A_n} - f'\Gamma^* \right) \right] + c_i[-a_{ac} + (1+t)b]$$

Rewriting A_n/g_m as $(A_n/kg_{ac}) \times kg_{ac}/g_m = \text{iWUE}'_{\text{com}} kg_{ac}/g_m$ and using Eqn 7 leads to (the symbol prime for iWUE refers to the fact we now use a “disconnected” respiration term):

$$(1-t)c_a\Delta = a_{ac}c_a + (1+t) \left[\text{iWUE}'_{\text{com}} \frac{kg_{ac}}{g_m} (a_m - b) - e' \frac{R_d'c_a}{A_n} - f'\Gamma^* \right] + \frac{c_a \left(\frac{1}{k} - \frac{W}{2} \right) - \text{iWUE}'_{\text{com}}}{\frac{1}{k} + \frac{W}{2}} [-a_{ac} + (1+t)b]$$

Rearranging (factorization by $\text{iWUE}'_{\text{com}}$) gives:

$$\text{iWUE}'_{\text{com}} = c_a \frac{a_{ac}(1-\varepsilon) + (1+t) \left(\varepsilon b - \frac{e' R_d'}{A_n} - f' \frac{\Gamma^*}{c_a} \right) - (1-t)\Delta}{\frac{(1+t)b - a_{ac}}{\frac{1}{k} + \frac{W}{2}} - (1+t)k \frac{g_{ac}}{g_m} (a_m - b)} \quad S2$$

115 Again, ignoring ternary corrections and boundary layer resistance ($t=0$, $\varepsilon=1$, $c_a=c_s$, $g_{as}=g_{sc}$, $a_{ac}=$
116 a_s), [Eqn S2](#) simplifies to:

$$117 \quad iWUE'_{mes} = \frac{c_a}{k} \frac{b - \Delta - f \frac{\Gamma^*}{c_a} - e \frac{R'd}{A_n}}{b - a_s + \frac{g_{sc}}{g_m}(b - a_m)} \quad S3$$

118 If we further neglect the term associated with day respiration, [Eqn S3](#) simplifies to [Eqn 12](#). Using
119 a series of theoretical data of the standard scenario ([Table S1](#), $g_{sc}/g_m=0.8$), we calculated $iWUE'_{mes}$
120 ([Eqn S3](#)) and compared with $iWUE_{mes}$ ([Eqn 11](#)), showing that the estimates provided by the two
121 equations (which account for the respiratory fractionation term differently) were very similar (the
122 difference being less than $1 \mu\text{mol mol}^{-1}$, see below). Taken as a whole, within a range of intrinsic
123 respiratory fractionation e (far from very high, artificial isotopic disequilibrium), day respiration
124 has little effect on $iWUE_{mes}$ estimates computed from [Eqn 11](#) and [S2](#) ([Fig. S4](#)), and of course have
125 no effect at all on $iWUE_{mes}$ estimates generated by [Eqn 12](#) which neglects the respiratory isotope
126 contribution.