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# The slope of assimilation rate against stomatal conductance should not be used as a measure of water use efficiency or stomatal control over assimilation

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## Abstract

Quantifying water use efficiency, and the impact of stomata on CO<sub>2</sub> uptake are pivotal in physiology and efforts to improve crop yields. Although tempting, relying on regression slopes from assimilation-stomatal conductance plots to estimate water use efficiency or stomatal control over assimilation is erroneous. Through numerical simulations, I substantiate this assertion. I propose the term ‘instantaneous transpiration efficiency’ for the assimilation-to-transpiration ratio to avoid confusion with ‘intrinsic water use efficiency’ which refers to the assimilation-to-stomatal conductance ratio, and recommend to compute both metrics for each gas exchange data point.

**Keywords** Photosynthesis · Analysis · Response · Limitation · Optimality

Stomata regulate the rate of water vapour escaping leaves (transpiration,  $E$ ) by adjusting stomatal aperture, inevitably restricting the rate of CO<sub>2</sub> uptake, called assimilation ( $A$ ). This results in a difference in CO<sub>2</sub> concentration from higher atmospheric ( $C_a$ ), to lower internal concentration ( $C_i$ ). The ease with which water vapour permeates stomata is referred to as stomatal conductance to water ( $g_{sw}$ ), which is higher than that for CO<sub>2</sub> due to the smaller size of the H<sub>2</sub>O molecule ( $g_{sw} \approx 1.6g_{sc}$ ). The theoretical basis for identifying the extent to which stomata restrict assimilation was laid by Gaastra (1959) and further developed by Jones (1985). Stomatal restriction was associated to sensitivity: how much  $A$  varies in response to a change in  $g_{sc}$ . Mathematically, the latter is achieved by expressing  $A$  as a function of multiple variables and calculating the change in  $A$  resulting from a small alteration of  $g_{sc}$ , while keeping all other variables

unchanged. In formal notation, this is the partial derivative,  $\partial A / \partial g_{sc}$ .

The trade-off between assimilation and the rate of water loss can be represented by a similar metric, the marginal carbon revenue of water,  $\partial A / \partial E$  (Buckley et al. 2017), or, alternatively, by water use efficiency. The notion of water use efficiency had its origins in agronomy, and measured the production (grain harvested, above ground biomass, total plant or biomass) per unit of water used (water supplied through irrigation, rain, water transpired, or evapotranspiration) (Hatfield and Dold 2019). At the leaf level, two formulations of water use efficiency are commonly used. The ratio between assimilation and  $g_{sw}$  is referred to as intrinsic water use efficiency ( $iWUE$ ), while the ratio between assimilation and transpiration is often termed instantaneous water use efficiency. However, this terminology can lead to confusion. To mitigate this ambiguity, I encourage using the term ‘instantaneous transpiration efficiency’ (ITE) for the ratio between  $A$  and  $E$ , as suggested by Duursma et al. (2013). While  $iWUE$  is dependent solely on leaf properties, ITE increases without bound as air humidity rises, irrespective of the plant. This is because transpiration is the product of  $g_{sw}$  and the air-to-leaf water mole fraction gradient,  $D_s$  (ITE =  $iWUE / D_s$ ). Consequently,  $iWUE$  is typically favoured over ITE when comparing plants (Table 1).

Regression analysis, a widely employed statistical technique, offers a easily accessible approach for exploring

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**Table 1** Acronyms, definitions, variables, and units used

Symbol	Definition	Units
$A, A_{op}$	Net assimilation, unspecified or measured under ordinary, operational conditions	$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$
$E$	Leaf level water transpiration rate $E = g_S D_S$	$\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$
$A_{SAT}, A_{SAT}'$	$\text{CO}_2$ -saturated $A$ in absence or in presence of $L_{NS}$ , respectively,	$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$
$C_a$	$\text{CO}_2$ concentration outside the leaf	$\mu\text{mol CO}_2 \text{ mol air}^{-1}$
$CE, CE'$	Initial slope of the $A/C_i$ curve in absence or in presence of $L_{NS}$ , respectively	$\text{mol air m}^{-2} \text{ s}^{-1}$
$C_i, C_{iop}$	$\text{CO}_2$ concentration in the substomatal cavity as calculated by the infra-red gas exchange analyser, unspecified, or under ordinary operational conditions	$\mu\text{mol CO}_2 \text{ mol air}^{-1}$
$iWUE$	Intrinsic water use efficiency $iWUE = A/g_S$	$\mu\text{mol CO}_2 \text{ mol air}^{-1}$
ITE	Instantaneous transpiration efficiency; $ITE = A/E = iWUE/D_S$	$\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$
$D_S$	Water vapour mole fraction difference between the leaf and air	$\text{mmol H}_2\text{O mol air}^{-1}$
$g_S, g_{SC}, g_{SW}$	Stomatal conductance, in general, to $\text{CO}_2$ , or to water vapour, respectively	$\text{mol air m}^{-2} \text{ s}^{-1}$
$L_S$	Stomatal limitation to photosynthesis	Dimensionless
$L_{NS}$	Stomatal limitation to photosynthesis	Dimensionless
$\Gamma$	$C_i$ - $A$ compensation point, <i>i.e.</i> , $C_i$ at which $A=0$	$\mu\text{mol CO}_2 \text{ mol air}^{-1}$
$\omega$	Curvature of the non-rectangular hyperbola describing the $C_i$ dependence of $A$	Dimensionless

relationships between variables. The output of regression analysis is the slope and the intercept of the line that best fits this relationship, and, optionally, the associated statistics. Unfortunately, confusion between the partial derivative, the slope of the regression, and the average value of the datapoints used for the regression has led to the incorrect utilization of regression analysis as a substitute for sensitivity analysis or water use efficiency. I will present two instances of these concepts being confounded.

### The slope of the regression of $A$ on $g_{SC}$ does not measure stomatal control over assimilation

The simple argument that if plants with a steeper slope in the regression of  $A$  against  $g_{SC}$  were to increase  $g_{SC}$ , they would enhance  $A$  more than plants with a lower slope has been frequently put forth (Yan et al. 2016; Kawamitsu et al. 1993; Carriqui et al. 2015), but is actually incorrect. Assimilation typically responds to multiple factors, therefore  $\partial A/\partial g_{SC}$  captures the incremental change in  $\text{CO}_2$  assimilation resulting from a slight alteration in stomatal conductance to  $\text{CO}_2$ , while maintaining other factors constant. However, in experimental conditions, any change observed in  $g_{SC}$  is typically not independent of other variables affecting  $A$ . Instead,  $g_{SC}$  may respond to environmental drivers that typically also affect other processes involved in photosynthesis. For instance, the imposition of drought leads to alterations in the plant water relations, stomatal closure, reduced biochemical activities, and decreased efficiency in energy conversion processes (Bellasio et al. 2023). These changes can impact intercellular and intracellular  $\text{CO}_2$  and bicarbonate diffusion,

light interception, structural integrity, and nutrient uptake, all of which have a depressive effect on assimilation (Lalor and Cornic 2002). When a population of experimental datapoints of  $A$  are plotted over  $g_{SC}$ , the response of  $A$  will therefore aggregate both the impact of  $g_S$  on  $A$  and the influence of other drivers on  $A$ , hence include both stomatal and non-stomatal effects. As a result, the slope of a regression fitted through experimental data points of  $A$  against  $g_{SC}$  is typically not indicative of  $\partial A/\partial g_{SC}$ . As Wong (1979) wrote, "linear relationships between  $A$  and  $g_S$  do not necessarily indicate that stomata control the rate of assimilation". Postulating a link between stomatal control and the slope of the regression fitted to a set of empirical  $A$  and  $g_{SC}$  data pairs becomes particularly problematic when comparing  $C_3$  and  $C_4$  plants. This is due to the fact that regression lines of  $A$  to  $g_{SC}$  are typically steeper in  $C_4$  plants than in  $C_3$  plants (Quirk et al. 2019); however,  $C_4$  plants usually have less advantage than  $C_3$  plants when they open their stomata (Farquhar and Sharkey 1982). I will now illustrate this numerically.

To calculate operational values of  $A$  and  $g_S$  starting from input values of stomatal and non-stomatal limitations ( $L_S$  and  $L_{NS}$ ), I start from describing the dependence of  $A$  upon  $C_i$  with a function after Prioul and Chartier (1977) in the formulation of Bellasio et al. (2016b) as:

$$A = \frac{CE(C_i - \Gamma) + A_{SAT} - \sqrt{(CE[C_i - \Gamma] + A_{SAT})^2 - (4\omega A_{SAT} CE[C_i - \Gamma])}}{2\omega}, \quad (1)$$

where  $A_{SAT}$  is the  $\text{CO}_2$ -saturated rate,  $CE$  is the initial slope,  $\Gamma$  is the  $x$ -intercept,  $\omega$  is defining curvature. Typical  $A/C_i$  curves (Fig. 1A) were obtained using a generic  $C_3$  and  $C_4$  parameterisation (inset in Fig. 1B).

The potential assimilation that would occur if intercellular spaces were directly exposed to  $C_a$  is calculated by substituting  $C_a$  for  $C_i$  in Eq. 1 ( $A_{Pot}$  Fig. 1A, point 1). Ordinarily, non-stomatal limitation ( $L_{NS}$ ) may reduce assimilation to a point (Fig. 1A, point 2) called  $A_{PotCa}$ , which, from the definition of non-stomatal limitation (Bellasio et al. 2018) is:

$$A_{PotCa} = A_{Pot}(1 - L_{NS}). \tag{2}$$

To find the  $A/C_i$  curve passing through  $A_{PotCa}$  one can assume that CE and  $A_{SAT}$  scale linearly to the values of  $CE'$  and  $A_{SAT}'$ . If the ratio  $\mu = A_{SAT}'/CE'$  is invariant, then the standard quadratic form of Eq. 1,

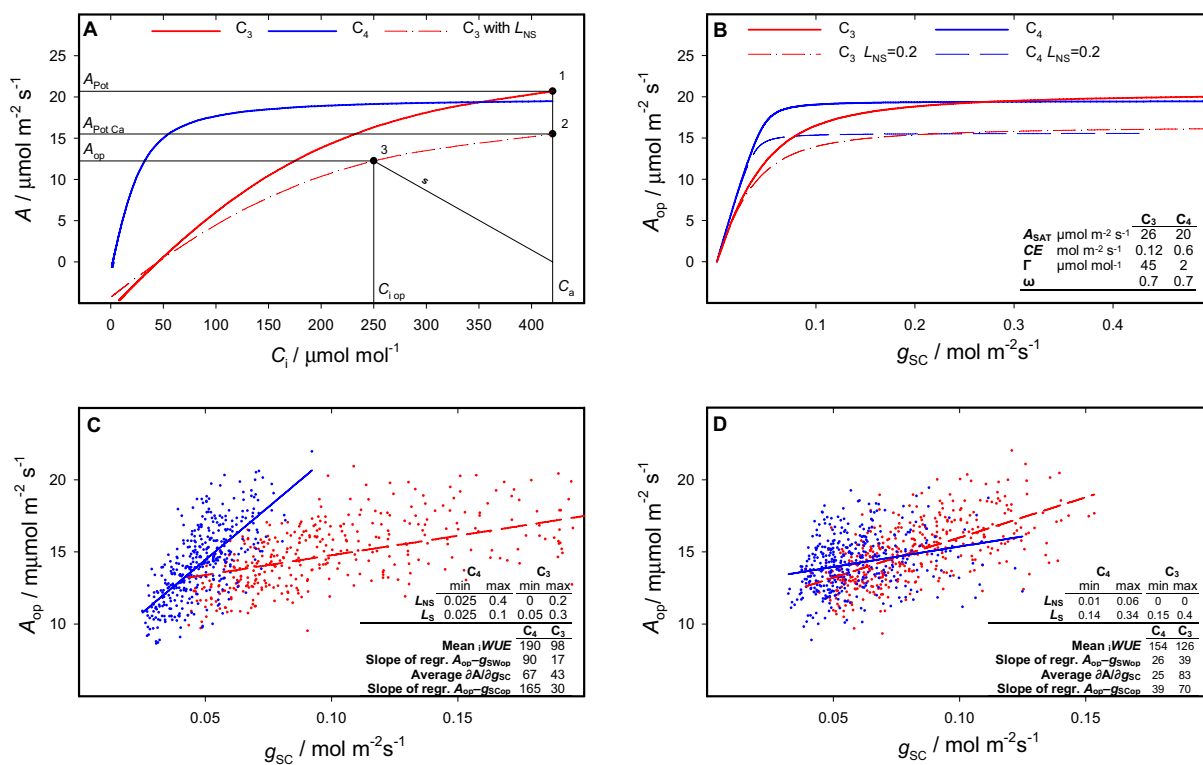
$$\{\omega A^2 - (CE[C_i - \Gamma] + A_{SAT})A + A_{SAT}CE[C_i - \Gamma] = 0\},$$

can be solved for  $CE'$  as:

$$CE' = \frac{A(\mu + C - \Gamma) + \sqrt{[A(\Gamma - C - \mu)]^2 - [4\omega A^2\mu(C - \Gamma)]}}{2\mu(C - \Gamma)} \tag{3}$$

$CE'$ , the estimated value of CE of the leaf in the presence of  $L_{NS}$ , is calculated by inputting  $A_{PotCa}$  (Eq. 2) and  $C_a$  in Eq. 3;  $A_{SAT}'$ , the estimated value of  $A_{SAT}$  in the presence of  $L_{NS}$ , is, by assumption,  $\mu CE'$ .

Stomatal limitation ( $L_S$ ) would further reduce assimilation to the 'operational'  $A_{op}$  (Fig. 1A point 3), which, from



**Fig. 1** Simulation of  $iWUE$ ,  $\partial A/\partial g_{SC}$ , and regression of  $A$  on  $g_S$ . Panel A shows the modelled dependence of  $C_3$  (thick dashed red line) and  $C_4$  (thick solid blue line) assimilation ( $A$ ) upon  $CO_2$  concentration in the substomatal cavity ( $C_i$ , Eq. 1), parameterised to generically represent the photosynthetic optimum for young leaves under moderate light intensity (inset in Panel B). The rationale of the simulations is exemplified for  $C_3$  assimilation. Point 1 ( $C_a$ ,  $A_{Pot}$ ), lying on the uppermost  $A/C_i$  curve, shows the potential assimilation attained if intercellular spaces were directly exposed to external  $CO_2$  concentration,  $C_a$  (Eq. 1 calculated for  $C_i = C_a$ ). In Point 2 ( $C_a$ ,  $A_{PotCa}$ ) assimilation is only limited by non-stomatal limitations ( $L_{NS}$ , Eq. 2); the thin dashed line is the  $C_3$   $A/C_i$  curve passing through  $A_{PotCa}$ , derived by reducing saturated rate ( $A_{SAT}$ ) and initial slope (CE) proportionally (Eq. 3). Point 3 ( $C_{i,op}$ ,  $A_{op}$ ) is a generic operational point where stomatal limitation ( $L_S$ ) may occur (Eq. 4); the slope of the supply

function  $s$  (Eq. 6) is  $g_{SC}$ . Panel B shows the response of assimilation to  $g_{SC}$  obtained under a  $C_a$  of  $420 \mu\text{mol mol}^{-1}$  for plants operating at their optimum, or with non-stomatal limitation ( $L_{NS}=0.2$ ). The inset shows model inputs, modified from Bellasio et al. (2016a, b) to generically describe  $C_3$  and  $C_4$  assimilation. Panel C shows the plot of  $A_{op}$  and  $g_{SCop}$  obtained using 400 random combinations of  $L_{NS}$  and  $L_S$  between a minimum and maximum shown in the inset. Outputs in the inset are the mean  $iWUE$  ( $A_{op}/g_{SWop}$ ) calculated for 400 ( $g_{SWop}$ ,  $A_{op}$ ) points; the mean of the  $\partial A/\partial g_{SC}$  of the  $A/g_{SC}$  curves passing through each of the 400 ( $g_{SCop}$ ,  $A_{op}$ ) points, and the slope of the regression fitted to the same points after adding normally distributed noise with a standard deviation of 10% of the simulated  $A_{op}$  and  $g_S$ . Panel D shows the plot of  $A_{op}$  and  $g_{SCop}$  obtained using the  $L_{NS}$  and  $L_S$  limits shown in the inset, output as in Panel C. Units of slopes and  $iWUE$  are  $\frac{\mu\text{mol } CO_2}{\text{mol Air}}$

the definition of stomatal limitation (Farquhar and Sharkey 1982) is:

$$A_{op} = A_{PotCa} - A_{Pot}L_S \quad (4)$$

The new corresponding  $C_{iop}$  is found by solving Eq. 1 as:

$$C_{iop} = \frac{\omega A_{op}^2 + CE'\Gamma A_{op} - A_{SAT}^2 A_{op} - A'_{SAT} CE'\Gamma}{CE'A_{op} - A'_{SAT} CE'} \quad (5)$$

Finally,  $g_{SC}$  is:

$$g_{SC} = \frac{A_{op}}{C_a - C_{iop}} \quad (6)$$

The general behaviour of the model is shown in Fig. 1B. When there is only  $L_S$ , at any given  $g_{SC}$  the points  $(C_{iop}, A_{op})$  satisfying Eq. 1 lie on the upper  $A/C_i$  curves. Introducing  $L_{NS}$  lowers  $A_{op}$  mainly when  $g_{SC}$  is high.

Four hundred random combinations of  $L_{NS}$  and  $L_S$  were generated in a manner that they fall within the minimum and the maximum intervals specified in the inset of Fig. 1C, and their sum would be within the range of 0.05 to 0.5. The proportion of  $L_S$  was intentionally set lower for  $C_4$  assimilation than for  $C_3$ , replicating physiological operational conditions (Ghannoum et al. 2003; Bellasio et al. 2018). Equations 4 and 6 were employed to simulate  $A$  and  $g_{SC}$ , respectively, 10% normally distributed-error was added, and a regression line was fitted to the resulting  $(C_{iop}, A_{op})$  pairs (Fig. 1C). Each  $(C_{iop}, A_{op})$  pair corresponds to an  $A/C_i$  curve (Eq. 1) parameterised with  $CE'$ ,  $A_{SAT}'$  (Eq. 3),  $\omega$  and  $\Gamma$  (inset in Fig. 1B). For each of these  $A/C_i$  curves,  $\partial A/\partial g_{SC}$  was calculated numerically at each  $(C_{iop}, A_{op})$  point for small increments of  $L_S$  in Eq. 3.

Despite imposing a physiologically lower proportion of  $L_S$  in  $C_4$  assimilation, the slope of the regression line fitted to the simulated data points was higher for  $C_4$  than  $C_3$  assimilation. The average  $\partial A/\partial g_{SC}$  of the individual  $A/g_{SC}$  curves passing through the  $(g_{SCop}, A_{op})$  pairs differed from the slope of the regression line fitted through the same  $(g_{SCop}, A_{op})$  pairs (insets of Fig. 1C and 1D). This means that the slope of the regression line fitted through operational  $A_{op}$  and  $C_{iop}$  does not serve as a measure of  $L_S$  or of  $\partial A/\partial g_{SC}$ .

## The slope of the regression of $A$ on $g_{SW}$ is not water use efficiency

The slope of the regression for a set of gas exchange points measured in operational conditions  $(g_{SWop}, A_{op})$  has been erroneously employed to measure the average  $iWUE$  of a set of data points, as seen in studies like Vogan and Sage (2011) and Killi et al. (2017). However, the slope of a regression fitted to a set of  $(g_{SWop}, A_{op})$  pairs would equal

their average  $iWUE$  only if the unconstrained regression line passed through the origin, which is typically not the case.

To illustrate this numerically, four hundred random combinations of  $L_{NS}$  and  $L_S$  were generated in a way that they fall within the minimum and the maximum intervals specified in the inset of Fig. 1D. Subsequently, assimilation and  $g_{SC}$  were simulated as described above, and a regression line was fitted to the resulting  $(g_{SCop}, A_{op})$  pairs (Fig. 1D) and  $(g_{SWop}, A_{op})$  pairs. The slope of the regression fitted through the  $(g_{SWop}, A_{op})$  pairs (inset in Fig. 1D) was lower in  $C_4$  than in  $C_3$ , but the ranking of  $iWUE$  was in the opposite order (inset in Fig. 1D). This shows that the slope of the regression of  $A_{op}$  on  $g_{SW}$  may not even scale to  $iWUE$ , and therefore should not be employed as a measure of  $iWUE$ .

Instead of fitting a regression line through the  $(g_{SWop}, A_{op})$  pairs, water use efficiency should be calculated for each individual data point as  $iWUE = A/g_{SW}$ . The implementation is entirely straightforward, because gas exchange measurements automatically provide the necessary information ( $A$  and  $g_{SW}$ ).

## Conclusion

I have provided numerical evidence that inferring water use efficiency or stomatal control over assimilation from the slope of the empirical regression of operational  $CO_2$  assimilation on stomatal conductance is inappropriate. I recommend calculating both metrics: ‘intrinsic water use efficiency’ (representing the assimilation-to-stomatal conductance ratio that is independent of air humidity and therefore preferable for plant comparisons) and ‘instantaneous transpiration efficiency’ (the term I suggest reserving for the assimilation-to-transpiration ratio), for each individual gas exchange data point.

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**Data availability** Code and output available upon request.

## Declarations

**Competing interest** The author declare no competing interest.

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