The strategies of water-carbon regulation of plants in a subtropical primary forest on Karst soils in China

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Abstract:
Coexisting plant species in a Karst ecosystem may use diversity strategies of trade off between carbon gain and water loss to adopt to the low soil nutrient and water availability conditions. Understanding of the impact of CO2 diffusion and maximum carboxylase activity of Rubisco (V_{cmax}) on the light-saturated net photosynthesis (A) and intrinsic water use efficiency (iWUE) can provide insight into physiological strategies of water-carbon regulation of coexisting plant species used in adaptation to Karst environments at the leaf scale. We selected 63 dominant species (across 6 life forms) in a subtropical Karst primary forest in southwestern China, measured their CO2 response curves, and calculated the corresponding stomatal conductance to CO2 (g_s), mesophyll conductance to CO2 (g_m), and V_{cmax}. The results showed that g_s and g_m varied about 7.6- and 34.5-fold, respectively, and g_s was positively related to g_m. The contribution of g_m to leaf CO2 gradient was similar to that of g_s, g_s/A, g_m/A and g_s/A was negative related to V_{cmax}/A. The relative limitations of g_s (l_s), g_m (l_m) and V_{cmax} (l_b) to A for the whole group (combined 6 life forms) were significantly different from each other (P<0.05). l_m was the largest (0.38±0.12), followed by l_b (0.34±0.14) and l_s (0.28±0.07). No significant difference was found between l_s, l_m, and l_b for Trees and Tree/shrubs, while l_m was the largest, followed by l_b and l_s for Shrubs, Grasses, Viens and Ferns (P<0.05). iWUE varied about 3-fold (from 29.52 to 88.92 μmol CO2 mol^{-1} H2O) across all species, and was significantly correlated with g_s, V_{cmax}, g_m/g_s, and V_{cmax}/g_s. These results indicated that Karst plants maintained relatively high A and low iWUE through the co-variation of g_s, g_m, and V_{cmax} as adaptation to Karst environment.

Key words: iWUE; mesophyll conductance; stomatal conductance; Karst critical zone; V_{cmax}
1 Introduction

Diversity strategies of trade off between carbon gain and water loss are critical for the survival of coexisting plant species. In order to adapt to the harsh environment, coexisting plant species develop distinct patterns of strategies of carbon-water regulation (light-saturated net photosynthesis (A) and intrinsic water use efficiency (iWUE)) (Sullivan et al., 2017). iWUE is the ratio of A to stomatal conductance to H₂O (g_sw) (Moreno-Gutierrez et al., 2012). Plants with high iWUE are better able to adapt to the nutrient- and water-limited environment (Flexas et al., 2016). Due to the greater hydraulic erosion and complex underground drainage network (Nie et al., 2014; Chen et al., 2015), Karst soils cannot retain enough nutrients and water for plant growth even though precipitation is high (1000-2000 mm) (Liu et al., 2011; Fu et al., 2012; Chen et al., 2015). Understanding of the impact of CO₂ diffusion and maximum carboxylase activity of Rubisco (V_cmax) on A and iWUE in Karst plants can provide insight into physiological strategies of water-carbon regulation of plants used in adaptation to Karst environments at the leaf scale. Until now, variability in A and iWUE has been reported only in 13 co-occurring trees and 12 vines (Chen et al., 2015), and 12 co-occurring tree species (Fu et al., 2012) in two tropical Karst forests in southwestern China.

Based on Fick's first law, A has been shown to be limited only by leaf stomatal conductance to CO₂ (g_s = g_sw/1.6) and V_cmax (Flexas et al., 2012; Buckley and Warren, 2014); originally, mesophyll conductance to CO₂ (g_m) was proposed to be infinite, i.e. CO₂ concentration in chloroplast (C_c) was equal to the CO₂ concentration in intercellular air space (C_i). However, g_m varies greatly among species (Warren and Adams, 2006; Flexas et al., 2013). Recent studies have confirmed that A was constrained jointly by g_s, g_m, and V_cmax, and their relative contribution to A was species-dependent and site-specific (Carriqui et al., 2015; Tosens et al., 2016; Galmes et al., 2017; Peguero-Pina et al., 2017a; Peguero-Pina et al., 2017b; Veromann-Jurgenson et al., 2017).
Variation in iWUE \( (=A/g_{sw}) \) depends on the relative changes in \( A \) \( (g_s, g_m, V_{cmax}) \) and \( g_{sw} \) \( (g_{sw}=1.6g_s) \) (Flexas et al., 2013; Gago et al., 2014). Theoretical relationships between iWUE and \( g_s, g_m, \) and \( V_{cmax} \) have been deduced using two approaches. Based on Fick’s first law of CO\(_2\) diffusion, Flexas et al. (2013) deduced that iWUE was a function of \( g_{m}/g_s \) and CO\(_2\) gradients \( (C_a-C_c) \) within a leaf. On the other hand, combining Fick’s first law of CO\(_2\) diffusion and Farquhar biochemical model (Farquhar and Sharkey, 1982), Flexas et al. (2016) deduced that iWUE was a function of \( V_{cmax}/g_s, C_c, \) CO\(_2\) compensation point of photosynthesis \( (I^s) \), and the effective Michaelis–Menten constant of Rubisco for CO\(_2\) \( (K_m) \). Until now, most previous studies focused on the role of CO\(_2\) diffusion in limiting iWUE, and suggested that iWUE was negatively related to \( g_s \), and positively related to \( g_{m}/g_s \) (Flexas et al., 2013).

Gago et al. (2014) used a meta-analysis with 239 species, and were the first to confirm that iWUE was positively related to \( V_{cmax}/g_s \). Although both \( g_{m}/g_s \) and \( V_{cmax}/g_s \) were positively correlated with iWUE, there was only a weak correlation between \( g_{m}/g_s \) and \( V_{cmax}/g_s \), which indicates that iWUE can be improved by increasing \( V_{cmax} \) or \( g_m \) (proportionally higher than \( g_s \)), not both (Gago et al., 2014).

It is noteworthy that Flexas et al. (2016) and Gago et al. (2014) found that most of the previous work on constraints of \( g_s, g_m, \) and \( V_{cmax} \) on \( A \) were conducted in crops or saplings, and only a few studies were in natural ecosystems. For example, \( g_m \) was the main factor limiting \( A \) in two Antarctic vascular grasses (Saez et al., 2017), and in 35 Australian sclerophylls (Niinemets et al., 2009b) in different habitats. The \( A \) of two closely-related Mediterranean Abies species growing in two different habitats was mainly constrained by \( g_m \) in one, and by \( g_s \) in the other habitat (Peguero-Pina et al., 2012). Beyond that, it still remains unknown how \( g_{sw}, g_m, \) and \( V_{cmax} \) regulate \( A \) and iWUE across species in natural ecosystems.

In this study, we selected 63 dominant plant species, including six life forms (Tree \( (n=29) \), Tree/Shrub \( (n=11) \), Shrub \( (n=11) \), Grass \( (n=11) \), Vine \( (n=5) \), and Fern \( (n=3) \)),
from a subtropical primary forest in the Karst critical zone of southwestern China, and measured their A and CO₂ response curves. \( g_m \) was calculated using the curve-fitting method (Ethier and Livingston, 2004). The obtained \( g_m \) was used to transform the \( A-C_i \) into \( A-C_c \) response curves, and then to calculate the A and \( V_{cmax} \). Our objective was to determine and distinguish the limitations of CO₂ diffusion (\( g_s \) and \( g_m \)) and \( V_{cmax} \) on A and iWUE in different life forms in this Karst primary forest, and to understanding the patterns of strategies of carbon-water regulation of Karst plants.

2 Materials and Methods

2.1 Site information

This study was conducted in a subtropical primary forest (26°14′48″N, 105°45′51″E; elevation, 1460 m), located in the Karst CZ of southwestern China. This region has a typical subtropical monsoon climate, with a mean annual precipitation of 1255 mm, and mean annual air temperature of 15.1°C (Zeng et al., 2016). The soils are characterized by a high ratio of exposed rock, shallow and nonhomogeneous soil cover, and complex underground drainage networks, e.g. grooves, channels and depressions (Chen et al., 2010; Zhang et al., 2011; Wen et al., 2016). Soils and soil water are easily leached into underground drainage networks. Soil texture is silt-clay loam, and soil PH is 6.80±0.16 (Chang et al., 2018). The total nitrogen and phosphorus content in soil is 7.30±0.66 and 1.18±0.35 g Kg⁻¹, respectively, which was similar with that of non-Karst CZs (Wang et al., in review). However, the soil quantities (16.04~61.89 Kg m⁻²) and nitrogen and phosphorus storage (12.04 and 1.68 t hm⁻²) is much lower than that of non-Karst CZs, due to the thin and heterogeneous soil layer (He et al., 2008; Jobbagy et al., 2000; Lu et al., 2010; Li et al., 2008). The typical vegetation type is mixed evergreen and broadleaf deciduous primary forest, dominated by Itea yunnanensis Franch, Carpinus pubescens Burk., and Lithocarpus confinis Huang, etc. (Wang et al., 2018).

2.2 Leaf gas-exchange measurements
In July and August 2016, 63 species (Table S1) were selected for measurements of the A and CO$_2$ response curves. The species sampled were selected according to their abundance in the study site. They are the main component of this forest, including 55 woody species (46 deciduous and 10 evergreen species) and 5 herb species. To distinguish the strategies of water-carbon regulation of plants among different life forms, those species were grouped into 6 life forms, including (1) Tree (n=29), (2) Tree/Shrub (n=11), (3) Shrub (n=11), (4) Grass (n=11), (5) Vine (n=5), and (6) Fern (n=3). “Tree/Shrub” is a kind of low wood plant between Tree and Shrub. Fern grow in understory. Vine climb up to the shrub canopy to get light.

Details of leaf sampling and measurements of the CO$_2$ response curve were briefly described as follows. Branches exposed to the sun were excised from the upper part of the crown (Trees, Tree/Shrubs, Shrubs and Vines) or aboveground portion (Grasses, Ferns), and immediately re-cut under water to maintain xylem water continuity. Back into the laboratory, branches and aboveground portions were kept at 25°C for 30 min. Fully-expanded and mature leaves were induced for 30 minutes at a saturating light density (1500 μmol m$^{-2}$ s$^{-1}$). CO$_2$ response curves measurements were performed when $A$ and $g_s$ was stable. Three leaves per species were collected and measured. A total of 189 leaves were collected from adult individuals of 63 species.

The CO$_2$ response curves were measured with 11 CO$_2$ concentration gradients in chamber following the procedural guidelines described by Long and Bernacchi (2003). The photosynthetic photon flux density was 1500 μmol m$^{-2}$ s$^{-1}$. The leaf temperature was 25°C, controlled by the block temperature. The humidity in the leaf chamber was maintained at ambient condition. Leaf area, thickness (LT) and dry mass were measured after the CO$_2$ response measurements. Leaf mass per area (LMA) was calculated by dividing the corresponding dry mass by leaf area. And leaf density (LD) was calculated by dividing the corresponding LMA by LT. More details were described in Wang et al. (2018).
2.3 Response curve analyses

A and the corresponding \( g_{sw} \) (\( g_s = g_{sw}/1.6 \)), \( C_a \), and \( C_i \) were extracted from the CO\(_2\) response curve under saturating light (1500 \( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \)) conditions, with CO\(_2\) concentration inside the cuvette set to 400 \( \mu \text{mol mol}^{-1} \) (Domingues et al., 2010). \( V_{\text{cmax}} \) was estimated by fitting \( A-C_c \) curves (Ethier and Livingston, 2004). The obtained values of \( g_m \) were used to transform the \( A-C_i \) into \( A-C_c \) response curves as \( C_c = C_i - \frac{A}{g_m} \).

Three methods are most commonly used for \( g_m \) estimation. Those methods have been reviewed by Warren (2006) and Pons et al. (2009). Briefly, \( g_m \) can be calculated by the stable isotope method (Evans, 1983; Sharkey et al., 1991; Loreto et al., 1992), \( J \) method (Bongi and Loreto, 1989; Dimarco et al., 1990; Harley et al., 1992; Epron et al., 1995; Laisk et al., 2005), and ‘curve-fitting’ method (Ethier and Livingston, 2004; Sharkey et al., 2007). All of these methods are based on gas exchange measurements (Pons et al., 2009), and some common assumptions (Warren, 2006). Thus, the accuracy of each method is to some extent unknown (Warren, 2006).

\( g_m \) was estimated by the ‘curve-fitting’ method in this study. Although the ‘curve-fitting’ method is less precise than the stable isotope method, the ‘curve-fitting’ method is much more readily available and has been used for several decades (Warren, 2006; Sharkey, 2012). Accurate measurements of \( A \) and \( C_i \) is a prerequisite for estimating \( g_m \) using the ‘curve-fitting’ method (Pons et al., 2009). Warren (2006) pointed out that highly-accurate measurements need small leaf area and low flow rates. We confirmed that the calculated \( C_c \) and the initial slope of \( A-C_c \) curves were positive, suggesting that the measured \( g_m \) was reliable (Warren, 2006).

2.4 Theory of trade-off between carbon and water at leaf scale

The exchange of H\(_2\)O and CO\(_2\) between the leaf and the atmosphere is regulated by stomata (Gago et al., 2014). According to Fick’s first law of diffusion, \( A \) and \( g_s \) are
related as:

\[ A = g_c(C_a - C_i) \]  

(1)

where \( A \) is the photosynthetic rate (\( \mu \text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1} \)); \( C_a \) is the ambient CO2 concentration (\( \mu \text{mol mol}^{-1} \)); \( C_i \) is the intercellular CO2 concentration (\( \mu \text{mol mol}^{-1} \)).

Mesophyll is the barrier for CO2 inside the leaf. \( A \) and mesophyll conductance to CO2 (\( g_m \)) are related as:

\[ A = g_m(C_i - C_c) \]  

(2)

where \( C_c \) is the CO2 concentration at the sites of carboxylation (\( \mu \text{mol mol}^{-1} \)). \( C_c \) not only depends on CO2 supply by \( g_m \), but also on CO2 demand (the maximum carboxylase activity of Rubisco, \( V_{\text{cmax}} \)).

\[ \text{(1) The relationship between \( iWUE \) and \( \frac{g_m}{g_s} \)} \]

\( iWUE \) is a function of CO2 diffusion conductances (e.g. \( g_s \) and \( g_m \)) and leaf CO2 concentration gradients. We can express \( A \) as the product of the total CO2 diffusion conductance (\( g_t \)) from ambient air to chloroplasts, and the corresponding CO2 concentration gradients by combining Eq. (1) and (2) (Flexas et al., 2013):

\[ A = g_t [(C_a - C_i) + (C_i - C_c)] \]  

(3)

where \( g_t = 1/(1/g_s + 1/g_m) \). This equation demonstrates that CO2 concentration gradients in leaves are constrained by stomatal and mesophyll resistance to CO2. Therefore, \( iWUE \) can be expressed as:

\[ \frac{A}{g_{sw}} = \frac{1}{1.6} \left( \frac{g_m/g_s}{1+g_m/g_s} \right) [(C_a - C_i) + (C_i - C_c)] \]  

(4)

Eq. (4) means that \( iWUE \) is positively related to \( \frac{g_m}{g_s} \), but not to \( g_m \) itself (Warren and Adams, 2006; Flexas et al., 2013; Buckley and Warren, 2014; Cano et al., 2014).

\[ \text{(2) The relationship between \( iWUE \) and \( \frac{V_{\text{cmax}}}{g_s} \)} \]

When Fick’s first law and the Farquhar biochemical model (Farquhar and Sharkey, 1982) are combined, \( iWUE \) is also a function of \( V_{\text{cmax}} \). Based on the Farquhar biochemical model (Farquhar and Sharkey, 1982), when \( A \) is limited by Rubisco, it
can be expressed by the following equation (Sharkey et al., 2007):

\[ A = \frac{V_{\text{max}}(C_r \Gamma^*)}{(C_r + K_m)} - R_d \]  

(5)

where \( \Gamma^* \) is the CO\(_2\) compensation point of photosynthesis in the absence of non-photorespiratory respiration in light \( (R_d) \), and \( K_m \) is the effective Michaelis–Menten constant of Rubisco for CO\(_2\). Combining Eq. (1) and (5) (Flexas et al., 2016), we obtain:

\[ \frac{V_{\text{max}}}{g_s} = \frac{(C_r + K_m)(C_r - C_i)(A + R_d)}{(C_r - \Gamma^*)A} \]

(6)

Because \( R_d \) is much smaller than \( A \) in actively photosynthesizing leaves, \( V_{\text{max}}/g_s \) can be approximated as:

\[ \frac{V_{\text{max}}}{g_s} \approx \frac{(C_r + K_m)(C_r - C_i)}{(C_r - \Gamma^*)A} = \frac{(C_r + K_m)}{(C_r - \Gamma^*)} \frac{A}{g_s} \]

(7)

Consequently, \( iWUE \) can be expressed as:

\[ \frac{A}{g_{\text{so}}} = \frac{1}{1.6} \frac{V_{\text{max}}}{g_s} (C_r - \Gamma^*) \]

(8)

### 2.5 Statistical analysis

(1) Quantitative analysis of limitations on \( A \)

The relative contribution of \( g_s \) \( (l_s) \), \( g_m \) \( (l_m) \) and \( V_{\text{max}} \) \( (l_b) \) to \( A \) can be separated by a quantitative limitation model introduced by Jones (Jones, 1985) and further developed by Grassi & Magnani (2005). The sum of \( l_s \), \( l_m \), and \( l_b \) is 1. \( l_s \), \( l_m \) and \( l_b \) can be calculated as:

\[ l_s = \frac{g_s/g_i \partial A/\partial C_c}{g_i + \partial A/\partial C_c} \]

(9)

\[ l_m = \frac{g_m/g_i \partial A/\partial C_c}{g_i + \partial A/\partial C_c} \]

(10)

\[ l_b = \frac{g_i}{g_i + \partial A/\partial C_c} \]

(11)
where $\frac{\partial A}{\partial C_c}$ was calculated as the slope of $A-C_c$ response curves over a $C_c$ range of 50–100 $\mu$mol mol$^{-1}$. $l_s$, $l_m$ and $l_b$ have no units. $A$ is co-limited by the three factors when $l_s=0.3$, $l_m=0.3$ and $l_b=0.4$ (Galmes, J. et al., 2017).

(2) Data analysis

Data were analyzed either as a whole group (six life forms combined) or by individual life forms. The bivariate linear regressions of leaf gas exchange parameters were performed using the standardized major axis (SMA) regression fits, and all of the data were made on log$_e$-transformed data (Table S2).

To test for the differences among life forms, SMA regression fits were used to compare the slope of regression lines which significant relationships had already been obtained. Note that Grass, Vine and Fern were not considered due to the small sample size. A similar trend was obtained, and no significant difference was found between life forms although significant relationships were not obtained for some bivariate linear regressions. Accordingly, six life forms were grouped together to analyze the strategy of water-carbon regulation of plants in the whole text.

The difference of relative limitation of $g_s$, $g_m$ and $V_{cmax}$ to $A$ for life forms or as a whole group were performed using one-way ANOVA and Duncan multiple comparison. The probability of significance was defined at $p<0.05$.

3 Results

3.1 Interrelation among $g_s$, $g_m$, $g_t$ and $V_{cmax}$

CO$_2$ concentration gradients in leaf were controlled by CO$_2$ diffusion conductance and $V_{cmax}$. Fig. 1 shows the relationship between CO$_2$ gradients ($C_a$-$C_i$, $C_i$-$C_c$ and $C_a$-$C_c$) in leaf and the corresponding CO$_2$ diffusion conductance ($g_s$, $g_m$ and $g_t$) (Fig. 1a-c), and between $C_a$-$C_c$ and $V_{cmax}$ (Fig. 1d). CO$_2$ concentration gradients ($C_a$-$C_i$, $C_i$-$C_c$ and $C_a$-$C_c$) in leaf and the corresponding CO$_2$ diffusion conductance ($g_s$, $g_m$ and $g_t$) (Fig. 1a-c), and between $C_a$-$C_c$ and $V_{cmax}$ (Fig. 1d).
\[ C_r-C_s \text{ and } C_s-C_c \] were significantly negatively associated with the corresponding CO₂ diffusion conductance \((g_s, g_m \text{ and } g_t)\) \((P<0.001)\). \(V_{cmax}\) was positively associated with \(C_s-C_c\) \((P<0.001)\).

\(g_s, g_m, \text{ and } g_t\) were significantly positively related to each other \((P<0.001)\) (Fig. S1). The contribution of \(g_m\) to leaf CO₂ gradient was similar to that of \(g_s\). The contribution of \(g_s\) \((57.51–155.13 \text{ μmol mol}^{-1})\) to \(C_s-C_c\) \((98.50–282.94 \text{ μmol mol}^{-1})\) varied from 28% to 86%, and the contribution of \(g_m\) \((18.15–179.36 \text{ μmol mol}^{-1})\) to \(C_s-C_c\) varied from 14% to 72%. But the variation range of \(g_m\) \((0.02 –0.69 \text{ mol CO₂ m}^{-2} \text{ s}^{-1})\) was 4.5 times that of \(g_s\) \((0.05–0.38 \text{ mol CO₂ m}^{-2} \text{ s}^{-1})\).

No relationship was found between the CO₂ diffusion conductance \((g_s, g_m, \text{ and } g_t)\) and \(V_{cmax}\) (Fig. S2). However, after normalization of \(g_s, g_m, g_t, \text{ and } V_{cmax}\) for \(A\) (normalized parameters are hereafter called \(G_S=g_S/A, G_m=g_m/A, G_t=g_t/A, \text{ and } V=V_{cmax}/A\)), \(V\) was significantly positively correlated with \(G_m\) and \(G_t\) \((P<0.001)\) (Fig. 2b and c), and was slightly positively correlated with \(G_s\) \((P<0.05)\) (Fig. 2a), which represented the trade-off between CO₂ supply and demand.

### 3.2 Contribution of \(g_s, g_m\) and \(V_{cmax}\) to \(A\)

The variation in \(A\) was attributed to variation in \(g_s, g_m, g_t, \text{ and } V_{cmax}\). \(A\) was positively correlated with \(g_s\) (Fig. 3a), \(g_m\) (Fig. 3b), and \(V_{cmax}\) (Fig. 3c). We used the quantitative limitation model (Eqs. (9), (10) and (11)) to separate \(g_s\) \((l_s)\), \(g_m\) \((l_m)\), and \(V_{cmax}\) \((l_b)\) limitations to \(A\). \(l_s, l_m, \text{ and } l_b\) were negatively associated with \(g_s, g_m, \text{ and } V_{cmax},\) respectively (Fig. 4). The contributions by \(g_s, g_m, \text{ and } V_{cmax}\) to limiting \(A\) were different for each species (Fig. S3). \(l_s\) varied 2.6-fold \((0.17 \text{ to } 0.45)\), \(l_m\) varied 10.5-fold \((0.05 \text{ to } 0.55)\), and \(l_b\) varied 6.2-fold \((0.11 \text{ to } 0.68)\) across species. Overall, \(l_m\) \((0.38±0.12)\) was significantly larger than \(l_b\) \((0.34±0.14)\), and \(l_s\) \((0.28±0.07)\) \((P<0.05)\).
To further understand how $A$ was limited by $g_s, g_m$, and $V_{cmax}$ among life forms, we grouped the 63 species into 6 life forms: Tree, Tree/Shrub, Shrub, Grass, Vine, and Fern. The results showed that there was no significantly difference between $l_s, l_m$ and $l_b$ for Trees and Tree/shrubs. $l_m$ of Shrubs and Grasses was significantly higher than that of $l_s$ and $l_b$ (P<0.05). $l_m$ of Vines and Ferns was significantly higher than that of $l_s$ (P<0.05) (Fig. 5).

### 3.3 Effect of $g_s$, $g_m$ and $V_{cmax}$ on iWUE

iWUE varied from 29.52 to 88.92 μmol CO$_2$ mol$^{-1}$ H$_2$O. In theory, iWUE is regulated by $g_s$ ($g_{sw}=1.6g_s$), $g_m$, and $V_{cmax}$. However, a simple correlation analysis showed that iWUE was negatively related to $g_s$ (Fig. 6b), and not related to $A$ (Fig. 6a), $g_m$ (Fig. 6c), and $V_{cmax}$ (Fig. 6d).

A correlation analysis was used to test how $g_m/g_s$ and $V_{cmax}/g_s$ affected iWUE. The results showed that iWUE was positively correlated with $g_m/g_s$ (Fig. 7a) and $V_{cmax}/g_s$ (Fig. 7b). However, there was no significant relationship between $g_m/g_s$ and $V_{cmax}/g_s$.

iWUE was regulated by co-variation between $g_s$, $g_m$ and $V_{cmax}$.

### 4 Discussion

#### 4.1 Co-variation in $g_s$, $g_m$ and $V_{cmax}$ in regulating $A$

$A$ was constrained by $g_s$, $g_m$, and $V_{cmax}$ acting together, however, variability in the relative contribution of these three factors depended on species and habitats (Tosens et al., 2016; Galmes et al., 2017; Peguero-Pina et al., 2017a; Veromann-Jurgenson et al., 2017). $A$ was significantly correlated with $g_s$, $g_m$, and $V_{cmax}$ (Fig. 3a-c). $g_s$ was positively related to $g_m$ (Fig. S1c), while no relationship was found between the CO$_2$ diffusion conductance ($g_s$ and $g_m$) and $V_{cmax}$ (Fig. S2). The relative limitations of $g_s$, $g_m$, and $V_{cmax}$ were separated by a quantitative limitation model (Jones, 1985; Grassi & Magnani, 2005). The results showed that $l_s, l_m$ and $l_b$ of 63 species varied in a large range (Fig. S3), indicating plants have a diverse strategies to co-ordinate the CO$_2$
diffusion ($g_s$ and $g_m$) and $V_{\text{cmax}}$ to maintain relative high $A$. The order of factors limitations to $A$ was $l_m > l_b > l_s$ ($P<0.05$) (Fig.S3). Furthermore, we tested the relationship between the relative limitations and the corresponding limitation factors. The results showed that $l_s$, $l_m$, and $l_b$ were negatively associated with $g_s$, $g_m$, and $V_{\text{cmax}}$ respectively (Fig. 4). And the relationship was stronger for $g_m$- $l_m$ ($r^2=0.65$) than $V_{\text{cmax}}$- $l_b$ ($r^2=0.27$) and $g_s$- $l_s$ ($r^2=0.19$).

$g_s$ was better correlated with $A$, while the results showed that $A$ was more limited by $g_m$. That could be explained by two possible reasons. Firstly, compare to the linear relationship between $A$ and $g_s$, a nonlinear trend has been found between $A$ and $g_m$ when $g_m>0.4$ (Fig. 3a, b). Secondly, leaf structure plays an important role in regulating $g_m$ and $V_{\text{cmax}}$, consequently, in determining $A$ (Veromann-Jurgenson et al., 2017). Negative relationships between $A$/LMA and LT ($r^2=0.16$, $p=0.002$), and $A$/LMA and LT ($r^2=0.3$, $p<0.001$) have been observed (Fig. S4c,d), while $A$ was not correlated to LT and LD (Fig. S4a,b).

The importance of $g_m$ in constraining $A$ was variable, and depended on leaf structural traits, only LMA, LT, and LD were analyzed in this study. Large variability in $g_m$ has been shown both between and within species with different life forms and habits (Gago et al., 2014; Flexas et al., 2016). Variability in $g_m$ in this study is similar to that in global datasets (Gago et al., 2014; Flexas et al., 2016). There was no significantly difference among life forms ($P>0.05$). Previous studies have confirmed that LMA (Tomas et al., 2013), thickness of leaf cell wall (Peguero-Pina et al., 2017b), liquid phase of mesophyll (Veromann-Jurgenson et al., 2017), cell wall thickness of mesophyll (Terashima et al., 2011; Tosens et al., 2016), and surface area of mesophyll and chloroplast exposed to intercellular space (Veromann-Jurgenson et al., 2017) were the main limitations for $g_m$. The wide variability of $g_m$ between different species and life forms in the same ecosystem seems to be related to the diversity of leaf anatomical traits.
No significant difference of LMA, LT, and LD was found among life forms (P<0.05).

The negative correlation of $g_m$ (Terashima et al., 2005) or $g_m$/LMA (Ninemets et al., 2009; Veromann-Jurgenson et al., 2017) with LMA have been reported. In this study, there was a significant relationship between $g_m$/LMA with LMA (P<0.01), however, no relationship was found between $g_m$ with LMA. $g_m$/LMA was significantly negative related to LD (p<0.01) (Fig. S5c), and weak negative related to LT (p=0.06) (Fig. S5d), demonstrating that the negative role of cell wall thickness on $g_m$ (Terashima et al., 2006; Ninemets et al., 2009). The strong investment in supportive structures was the main reason for the limitation of $g_m$ on $A$ (Veromann-Jurgenson et al., 2017). However, it is still unknown how leaf anatomical traits affect $g_m$ and $A$, and this should be further explored.

$g_s$ is responsible for CO$_2$ exchange between atmosphere and leaf, and regulate the CO$_2$ fixation ($A$) and water loss (Lawson and Blatt, 2014). The variability of $g_s$ was controlled by stomatal anatomy, i.e. stomata density and size, and mesophyll demands for CO$_2$ (Lawson and Blatt, 2014). However, the stomatal anatomy was not analyzed in this study. We only focused on how the relationship between $g_s$ and $g_m$ regulate $A$. Positive relationship between $g_s$ and $g_m$ has been observed (Flexas et al., 2013). For example, the restricted CO$_2$ diffusion from the ambient air to chloroplast is the main reason for a decreased $A$ under water stress conditions due to both the stomatal and mesophyll limitations (Olsovska et al., 2016). $g_s$ was significantly positive related to $g_m$ for 63 species (P<0.001, Fig. S1) in this study, and no difference of the slopes of regression lines between $g_s$ and $g_m$ was found among life forms, demonstrating that $A$ was regulated by the co-variation of $g_s$ and $g_m$. However, the variability of $g_m$ and $l_m$ was larger than $g_s$ and $l_s$, respectively (Fig.1 and Fig.S3).

The wide variation range of $l_b$ (0.11-0.68) highlighted the importance role of $V_{cmax}$ in regulating $A$. $V_{cmax}$ was used to represent the CO$_2$ demand in photosynthetic process in this study. The relative contribution of $V_{cmax}$ to $A$ not only depends on $C_a$-$C_c$, but also on leaf nutrient levels. Positive relationship was found between $C_a$-$C_c$ and $V_{cmax}$ (Fig.
1d). And the $V_{cmax}$/LMA was co-regulated by leaf N, P and Mg content (Jing et al. 2018). In addition, $V_{cmax}$/LMA was negatively related to LT ($p<0.05$) (Fig. S6c) and LD ($p<0.05$) (Fig. S6d), while $V_{cmax}$ was not correlated to LT and LD (Fig. S6a,b), demonstrating that leaf structure plays an important role in regulating $V_{cmax}$.

The trade-off between CO$_2$ supply ($g_s$ and $g_m$) and demand (carboxylation capacity of Rubisco) can help maintain relative high $A$ (Galmes et al., 2017; Saez et al., 2017). In this study, we used $V_{cmax}$ as a proxy for the carboxylation capacity of Rubisco, and the normalized $V_{cmax}$ by $A$ ($V$=$V_{cmax}$/A) was significantly negatively correlated with the normalized $g_t$ by $A$ ($G_t$ =$g_t$/A) ($P<0.001$) (Fig. 2c), indicating that the trade-off between CO$_2$ supply and demand also existed among different species in the same ecosystems. For genus Limonium (flowering plants) (Galmes et al., 2017), $g_t$ was significantly positively related to Rubisco carboxylase specific activity, and significantly negatively related to Rubisco specificity factor to CO$_2$. In case of Antarctic vascular (Saez et al., 2017) and Mediterranean plants (Flexas et al., 2014), $A$ was mainly limited by low $g_m$, but it could be partially counterbalanced by a highly efficient Rubisco through high specificity for CO$_2$. This highlights the importance of the trade-off between CO$_2$ supply and demand in plant adaptation to Karst environment. However, it is still unknown how leaf anatomical traits affect $g_m$, $V_{cmax}$ and $A$, and this should be further explored.

4.2 Co-variation of $g_s$, $g_m$ and $V_{cmax}$ in regulating iWUE

Compared with the global dataset under well-watered conditions (19.27-171.88 μmol CO$_2$ mol$^{-1}$ H$_2$O) (Flexas et al., 2016), iWUE (52.85±13.08 μmol CO$_2$ mol$^{-1}$ H$_2$O) was somewhat lower in this study. iWUE varied from 29.53 to 88.91 μmol CO$_2$ mol$^{-1}$ H$_2$O, and the variability of iWUE was larger than in the Karst tropical primary forest (Fu et al., 2012; Chen et al., 2015). The average iWUE of 12 Vines and 13 Trees in the Karst tropical primary forest was 41.23±13.21 μmol CO$_2$ mol$^{-1}$ H$_2$O (Chen et al., 2015), while that of 6 evergreen and 6 deciduous Trees was 66.7±4.9 and 49.7±2.0
μmol CO$_2$ mol$^{-1}$ H$_2$O, respectively (Fu et al., 2012). The results demonstrated that Karst plants use a diverse strategies of carbon-water regulation to adopt to the harsh Karst environment.

Coexisting species have a diversity strategies of carbon-water regulation, ranging from ‘profligate/opportunistic’ to ‘conservative, that means their ecophysiological niche are separate (Moreno-Gutierrez et al., 2012; Nie et al., 2014; Prentice et al., 2014). Species with high $g_s$, and low iWUE were defined to have ‘profligate/opportunistic’ water use strategy, and species with low $g_s$ and high iWUE were defined to exhibit ‘conservative’ water use strategy (Moreno-Gutierrez et al., 2012). In consistent with previous study (Moreno-Gutierrez et al., 2012), coexisting plant species growing in the Karst ecosystem had a diversity water use strategies. However, Karst plants tended to lose more water to gain more carbon, i.e. Karst plants used ‘profligate/opportunistic’ water use strategy to adopt to the low nutrient availability and water stress conditions.

Prentice et al. (2014) studied the trade-off between carbon gain and water loss of woody species in contrasting climates, and found that species in hot and wet regions tend to lose more water in order to fix more carbon (high $g_s/A$, low $V_{cmax,Ci}/A$), and vice versa. Although Karst soils cannot contain enough water for plant growth, the trade-off between carbon gain and water loss (high $g_s/A$ and low $V_{cmax,Ci}/A$) were similar to the shown for plants growing in hot and wet regions (Prentice et al., 2014).

iWUE is regulated by the co-variation of $g_s$, $g_m$, and $V_{cmax}$. In theory, water loss is regulated by $g_s$ only, while carbon gain ($A$) was regulated by $g_s$, $g_m$, and $V_{cmax}$ (Fig. 3) (Lawson and and Blatt, 2014). However, iWUE in this study was negatively related to $g_s$ ($R^2=0.30$), negatively related to $V_{cmax}$ ($R^2=0.09$), and not related to $A$, $g_m$ (Fig. 6).

CO$_2$ diffusion and Farquhar biochemical model indicated that iWUE is affected by $g_m/g_s$ and $V_{cmax}/g_s$ (Gago et al., 2014; Flexas et al., 2016). There was a hyperbolic
dependency of iWUE on $g_m/g_s$ due to the roles of $g_s$ and $g_m$ in $C_i$ and $C_c$, and of $C_c$ in $A$ (Flexas et al., 2016). In meta-analyses, both Gago et al. (2014) and Flexas et al. (2016) found that iWUE was significantly positively related to $g_m/g_s$ and $V_{cmax}/g_s$. The results of this study are consistent with the meta-analyses (Fig. 7), demonstrating that plant species with relatively high $g_m/g_s$ or $V_{cmax}/g_s$ had relatively high iWUE. The relationship between iWUE and $V_{cmax}/g_s$ ($R^2=0.50$) was stronger than the relationship between iWUE and $g_m/g_s$ ($R^2=0.20$), demonstrating iWUE was mainly regulated by $V_{cmax}/g_s$. The reason maybe that iWUE was correlated to $g_s$ and $V_{cmax}$, and $g_s$ was positive related to $g_m$.

However, plants cannot simultaneously have high $g_m/g_s$ and high $V_{cmax}/g_s$. Similarly to the study of Gago et al. (2014), we found no relationship between $g_m/g_s$ and $V_{cmax}/g_s$. Gago et al. (2014) thought that the poor relationship between $g_m/g_s$ and $V_{cmax}/g_s$ indicated that the iWUE may be improved by $g_m/g_s$ or $V_{cmax}/g_s$ separately; if both of them were simultaneously improved, the enhanced effect on iWUE could be anticipated. In addition, Flexas et al. (2016) showed in a simulation that the increase in iWUE caused by overinvestment in photosynthetic capacity would progressively lead to inefficiency in the trade-off between carbon gain and water use, causing an imbalance between CO$_2$ supply and demand.

5 Conclusions

This study provides information of limitations of $A$ and iWUE by $g_s$, $g_m$, and $V_{cmax}$ in 63 species across 6 life forms in the field. The results showed that plants growing in Karst CZs used a diverse strategies of carbon-water regulation, but no difference was found among life forms. The co-variation of CO$_2$ supply ($g_s$ and $g_m$) and demand ($V_{cmax}$) regulated $A$, indicating that species maintain a relatively high $A$ through co-varying their leaf anatomical structure and $V_{cmax}$. iWUE was relatively low, but ranged widely, indicating that plants used the ‘profligate/opportunistic’ water use strategy to maintain the survival, growth, and structure of the community. iWUE was
regulated by $g_s$, $V_{c_{\text{max}}}$, $g_m/g_s$, and $V_{c_{\text{max}}}/g_s$, indicating that species with high $g_m/g_s$ or $V_{c_{\text{max}}}/g_s$ will have to be much more competitive to respond to the ongoing rapid warming and drought in the Karst CZs.

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**Author contributions**

JW, XFW, and XYZ planned and designed the research. JW performed experiments and analyzed data. JW prepared the manuscript with contributions from all co-authors.

**Competing interests.**

The authors declare that they have no conflict of interest.

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Figures

Figure 1. Relationships between (a) CO₂ gradient between ambient air and intercellular air space \((C_a-C_i, \mu\text{mol mol}^{-1})\) and stomatal conductance to CO₂ \((g_s, \text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})\); (b) CO₂ gradient between intercellular air space and chloroplasts \((C_i-C_c, \mu\text{mol mol}^{-1})\) and mesophyll conductance to CO₂ \((g_m, \text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})\); (c) CO₂ concentration gradient between ambient air and chloroplasts \((C_a-C_c, \mu\text{mol mol}^{-1})\) and total conductance to CO₂ \((g_t, \text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})\); and (d) \(C_a-C_c\) and the maximum carboxylase activity of Rubisco \((V_{cmax}, \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1})\). Lines refer to regression line for 63 species. T, TS, S, G, V, and F represent Tree, Tree/Shrub, Shrub, Grass, Vine, and Fern, respectively.
Figure 2. Relationships between (a) \(V\) and \(G_s\); (b) \(V\) and \(G_m\); and (c) \(V\) and \(G_t\). \(V\) is the ratio of photosynthetic capacity (\(V_{\text{cmax}}\)) to light-saturated net photosynthesis (\(A\), \(\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}\)); \(G_s\) is the ratio of stomatal conductance to \(CO_2\) (\(g_s\), mol \(CO_2\) m\(^{-2}\) s\(^{-1}\)) to \(A\); \(G_m\) is the ratio of mesophyll conductance to \(CO_2\) (\(g_m\), mol \(CO_2\) m\(^{-2}\) s\(^{-1}\)) to \(A\); \(G_t\) is the ratio of total conductance to \(CO_2\) (\(g_t\), mol \(CO_2\) m\(^{-2}\) s\(^{-1}\)) to \(A\). Lines refer to regression line for 63 species. T, TS, S, G, V, and F represent Tree, Tree/Shrub, Shrub, Grass, Vine, and Fern, respectively.
Figure 3. Relationships between light-saturated net photosynthesis ($A$, $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$) and (a) stomatal conductance to CO$_2$ ($g_s$, mol CO$_2$ m$^{-2}$ s$^{-1}$); (b) mesophyll conductance to CO$_2$ ($g_m$, mol CO$_2$ m$^{-2}$ s$^{-1}$); and (c) the maximum carboxylase activity of Rubisco ($V_{cmax}$, $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$). Lines refer to regression line for 63 species. T, TS, S, G, V, and F represent Tree, Tree/Shrub, Shrub, Grass, Vine, and Fern, respectively.
Figure 4. Relationships between (a) stomatal conductance to CO₂ ($g_s$, mol CO₂ m⁻² s⁻¹) and $l_s$ ($g_s$ limitation on light-saturated net photosynthesis $(A)$); (b) mesophyll conductance to CO₂ ($g_m$, mol CO₂ m⁻² s⁻¹) and $l_m$ ($g_m$ limitation on $A$); and (c) the maximum carboxylase activity of Rubisco ($V_{cmax}$, μmol CO₂ m⁻² s⁻¹) and $l_b$ ($V_{cmax}$ limitation on $A$). Lines refer to regression line for 63 species. T, TS, S, G, V, and F represent Tree, Tree/Shrub, Shrub, Grass, Vine, and Fern, respectively.
Figure 5. Limitation to light-saturated net photosynthesis ($A$) in six life forms by stomatal conductance to CO$_2$ ($l_s$), mesophyll conductance to CO$_2$ ($l_m$), and the maximum carboxylase activity of Rubisco ($l_b$). Error bars denote standard deviation ($1\sigma$).
Figure 6. Relationships between the observed intrinsic water use efficiency (iWUE, μmol CO₂ mol⁻¹ H₂O) and (a) light-saturated net photosynthesis (A, μmol CO₂ m⁻² s⁻¹); (b) stomatal conductance to CO₂ (gₛ, mol CO₂ m⁻² s⁻¹); (c) mesophyll conductance to CO₂ (gₘ, mol CO₂ m⁻² s⁻¹) and (d) the maximum carboxylase activity of Rubisco (Vₖₐₓ, μmol CO₂ m⁻² s⁻¹). Lines refer to regression line for 63 species. T, TS, S, G, V, and F represent Tree, Tree/Shrub, Shrub, Grass, Vine, and Fern, respectively.
Figure 7. The relationships of the intrinsic water use efficiency (iWUE, $\mu$mol CO$_2$ mol$^{-1}$ H$_2$O) and (a) the ratio of mesophyll conductance to CO$_2$ $(g_m/ g_s)$ and (b) the ratio of the maximum carboxylase activity of Rubisco $(V_{cmax})$ to gs $(V_{cmax}/ g_s)$. Lines refer to regression line for 63 species. T, TS, S, G, V, and F represent Tree, Tree/Shrub, Shrub, Grass, Vine, and Fern, respectively.