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Elevated efficiency of $C_3$ photosynthesis in bamboo grasses: A possible consequence of enhanced refixation of photorespired CO$_2$

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Abstract

Bamboos are productive grasses that currently yield a high-quality wood and potentially an abundance of lignocellulose for bioenergy. All are $C_3$ grasses of warm habitats, where they are prone to significant photorespiratory inhibition and competitive suppression by $C_4$ grasses. Here, we investigate whether three bamboo species from the Brazilian Cerrado (Dendrocalamus asper, Guadua angustifolia, and Guadua magna) exhibit unique adaptations that suppress photorespiratory costs and enhance photosynthetic efficiency. We evaluated photosynthetic efficiency of the bamboos and rice (Oryza sativa) by measuring $C_*$, the CO$_2$ compensation point in the absence of mitochondrial respiration. At 25°C, $C_*$ averaged 2.81 Pa in each of the bamboo species, which is closer to a $C_2$ plant (2.71 Pa) than the $C_3$ plant rice (3.31 Pa). Assuming a chloroplast CO$_2$ concentration of 200 µmol mol$^{-1}$, this represents an 18% lower cost of apparent photorespiration in bamboo than rice. Light and transmission electronic microscopy of the bamboo leaves exhibited few organelles in the bundle and mestome sheath cells, and mesophyll (M) cells are deeply lobed with 99% of the cell periphery adjacent to intercellular air space covered by chloroplast and stromules. The chloroplast layer in bamboo M cells is thick, with mitochondria adjacent to or engulfed by chloroplasts. This arrangement slows CO$_2$ efflux and facilitates refixation of photorespired CO$_2$, which could explain the low $C_*$ in the bamboos. The bamboos also had higher water use efficiency than rice, which may reflect efficient refixation of photorespired CO$_2$.

KEYWORDS

Bambuseae, $C_3$, $C_4$ photosynthesis, CO$_2$ refixation, Dendrocalamus, Guadua, photorespiration, water use efficiency
1 | INTRODUCTION

Bamboos are widespread C₃ grasses from the tropics to warm-temperate latitudes, where they have been widely exploited for fiber, construction materials, food, and environmental restoration, particularly in Asia (Kelchner & Bamboo Phylogeny Group, 2013; Scurlock et al., 2000). The Bambusoideae subfamily represents one of the most successful grass subfamilies, with 16 genera comprising more than 1400 species, and can form large monocultures in tropical and subtropical woodlands (Kellogg, 2015). The long fibers of bamboos are valued in paper and textile production, and due to rapid growth and high energy content of the biomass, bamboos are a promising biofuel feedstock (Gratani et al., 2014; Kleinhenz & Midmore, 2001; Li et al., 1998; Nandy et al., 2004; Scurlock et al., 2000). Bamboos of the genus *Phyllostachys* grow up to 20 m tall and produce a culm that reaches 11 cm in diameter. They have important characteristics for a promising bioenergy crop, such as cold resistance (down to −20°C in some species), low ash content and a low alkali index (Scurlock et al., 2000). Bamboos also produce a large amount of belowground biomass, increasing its potential for carbon sequestration (Casler et al., 2011). In the Brazilian Cerrado, species such as *Actinocladum verticillatum* (Taquari), *Dendrocalamus asper*, *Phyllostachys aurea*, *Bambusa vulgaris*, and *Bambusa textilis* grow well in acidic soil with frequent fire, and are noted for their ability to arrest erosion and facilitate regeneration of woody ecosystems (Elias et al., 2015; Filgueiras & Pereira, 1984; França, 2011; Mews et al., 2013). Internodes of some bamboos grow up to 50 cm per week after shoot emergence (Scurlock et al., 2000). In China, the bamboo *Phyllostachys pubescens* exhibits photosynthetic potential similar to that of the C₄ plant *Miscanthus × giganteus*, while in Amazonia, bamboos grow fast enough to shade out C₄ competitors (Cao et al., 2011; Collatz et al., 1998). Other bamboo species are noted to have high efficiency of water, nutrient, and light use relative to other C₃ species (Grombone-Guaratini et al., 2013; Kleinhenz & Midmore, 2001; Li et al., 1998; Mulkey, 1986). These features raise the question of whether bamboos have evolved mechanisms to boost photosynthetic performance of the C₃ pathway, for example, through the suppression of photorespiration. If so, bamboos may be well positioned to serve as bioenergy feedstocks in wetter and often cloudier environments where the advantages of the C₄ pathway are reduced.

As a warm climate C₃ species, bamboos should experience high rates of Rubisco oxygenase activity and the subsequent loss of previously fixed CO₂ during photorespiration. For example, C₃ photosynthesis is estimated to be suppressed by over 25% above 30°C due to the cost of photorespiratory metabolism (Jordan & Ogren, 1984; Sage, 2013). However, numerous C₃ species from warm climates have evolved mechanisms to compensate for high rates of photorespiration, and in doing so may be able to maintain fitness in the presence of aggressive C₄ plants, where photorespiration is largely suppressed (Sage, 2013). One important means to compensate for high photorespiratory potentials is to increase the specificity of Rubisco for CO₂ relative to O₂, which results in a lower value of the biochemical CO₂ compensation point of Rubisco, termed Γₛ; however, reduction in Γₛ may come at the cost of other kinetic properties of Rubisco, for example, a decrease in the catalytic turnover rate (*kₗ*: Sage, 2002). Alternatively, C₃ plants can trap and refix photorespired CO₂, either in single cells or internal compartments within leaves (Busch et al., 2013; Sage & Khoshravesh, 2016). Trapping of photorespired CO₂ within single mesophyll (M) cells of C₃ species is facilitated by a near continuous sheath of chloroplasts around the periphery of the M cell facing the intercellular air spaces, coupled with localization of mitochondria to the interior of the cell (Busch et al., 2013; Sage & Sage, 2009; Tholen et al., 2012). Rice (*Oryza sativa* and relatives) has deeply lobed M cells, with large chloroplasts filling the lobes and mitochondria that are largely restricted to the interior of the cells (Sage & Sage, 2009). Chloroplasts extensions termed stomules fill the gaps between chloroplasts, such that a near continuous sheath of Rubisco-filled stroma is present between mitochondria and the exterior of the cell (Hanson & Sattarzadeh, 2011; Kwok & Hanson, 2004). This arrangement forces photorespired CO₂ to exit the cell via the chloroplast, allowing for refixation of the CO₂ (Busch et al., 2013). In rice, trapping and refixation of photorespired CO₂ can boost net CO₂ assimilation rate (A) by over 10% at current CO₂ levels, and by over 30% at the reduced CO₂ levels of recent geological time (Busch et al., 2013). The deeply lobed M cells of rice are proposed to be an adaptation to maximize the refixation of photorespired CO₂, which may explain the success of these species in warm, low latitude environments (Sage & Sage, 2009). Rice is the world’s leading crop, which is surprising given it is cultivated in warm to hot environments where competition from C₄ grasses is high. Similarly, bamboos are also highly successful C₃ plants where C₄ species thrive. In rice, an ability to trap and refix photorespired CO₂ may help explain the success of this crop in warm environments (Busch et al., 2013). Similarly, we hypothesize that efficient refixation of photorespired CO₂ in bamboos may explain their success in tropical and subtropical climates.

Another means of enhancing photosynthetic efficiency is through the photosynthetic activation of bundle sheath (BS) cells by boosting chloroplasts and mitochondria content in the BS (Sage et al., 2014). If the organelles are centripetally positioned, this can establish shuttles where excess photorespiratory glycine diffuses from M to BS cells, allowing for decarboxylation by BS mitochondria and subsequent refixation by BS chloroplasts. In warm conditions, when
oxygenase activity is high, the glycine flux to the BS can be great enough to significantly enhance CO₂ around Rubisco in centripetal chloroplasts, thus improving photosynthetic efficiency (Sage et al., 2014). Glycine shuttles can be coupled with a reduction of glycine decarboxylase (GDC) expression in the M cells, thus enhancing the glycine flux to the BS and the degree of CO₂ enhancement (Sage et al., 2013). When nearly all GDC is expressed in the BS cells, CO₂ concentrations around Rubisco can be threefold greater than that in M chloroplasts (Keerberg et al., 2014), and the physiology resulting in this modest CO₂ enrichment is termed C₂ photosynthesis (Sage et al., 2012). C₂ photosynthesis is recognized as a major intermediate state in C₄ evolution (Monson & Rawsthorne, 2000), but is also present in numerous species with no close relationship to C₄ taxa, for example in the Brassicaceae genus *Moricandia* (Schlüter et al., 2017). Numerous C₃-like species express a weak version of C₂ photosynthesis, indicating glycine shuttles between M and BS-like compartments enhance the performance of C₃ photosynthesis in photorespiratory environments (Sage et al., 2014; Yorimitsu et al., 2019). Evidence of such C₂-like physiologies include enlarged BS cells enriched with organelles, centripetal positioning of organelles, particularly mitochondria, and reduced CO₂ compensation points (Γ; Khoshravesh et al., 2016; Sage et al., 2013). Bamboos exhibit pronounced BS cells in light micrographs (Dengler et al., 1994), but it is unknown whether these cells are also enriched with organelles, or whether bamboos exhibit low Γ values that would be indicative of a C₂-like mechanism.

In the grass phylogeny, the subfamily Bambusoideae branches in a sister position to the largely tropical subfamily Ehrhartioideae, which includes rice (Kellogg, 2015). This relatively close relationship, and the presence of deeply lobed M cells indicate bamboos may also have similar photosynthetic strategies as rice (Li et al., 2013; Vieira et al., 2002). Alternatively, the pronounced BS cells indicate bamboos may exploit a C₂-like M to BS glycine shuttle. Gas exchange studies in bamboo have not evaluated the degree to which photorespiratory CO₂ is trapped and refixed, for example, through the determination of the apparent CO₂ compensation point of net CO₂ assimilation rate in the absence of day respiration, a parameter known as Cᵣ (Busch et al., 2013). Cᵣ values approximate Γ, if there is no refixation of photorespired or respired CO₂ in a C₃ leaf; however, if trapping mechanisms such as chloroplast sheaths or M to BS glycine shuttles allow a significant fraction of Rubisco to function under elevated CO₂ conditions, then oxygenase activity is slowed and Cᵣ declines below Γ, in a pattern that can be easily detected using whole leaf gas exchange (Busch et al., 2013; Tholen et al., 2012). For example, the decrease in Cᵣ in rice below Γ₂ can exceed 10 μmol mol⁻¹ above 30°C, while species operating a glycine shuttle between M and BS cells reduce Cᵣ below Γ₂ by 15 μmol mol⁻¹ (where there is little loss of GDC expression in M tissue) to over 30 μmol mol⁻¹ (where GDC is largely localized in BS cells, as observed in C₂ species; Busch et al., 2013; Ku et al., 1991; Sage et al., 2013; Vogan et al., 2007).

In this study, we examined the photosynthetic efficiency of three bamboo species present in the Cerrado of Brazil (*Guadua magna*, native; *D. asper* and *Guadua angustifolia*, exotic). These species are valuable for building materials and ecosystem restoration. Given their potential production rates, they are also candidates to become important lignocellulose feedstocks in a biofuel industry. We first measured their gas exchange responses to variable CO₂ at different light intensities, which allowed us to estimate Cᵣ using a modified version of the Laisk (1977) method (Walker & Ort, 2015; Walker et al., 2016). We then examined the leaf anatomy and ultrastructure with light and transmission electron microscopy (TEM) to evaluate the potential for photorespiratory CO₂ trapping in M versus BS cells. Chloroplast sheaths with high coverage of the M periphery would indicate a strong potential for trapping and refixation of photorespired CO₂, as seen in rice and wheat, while an abundance of organelles in the inner, centripetal region of the BS cells would indicate the potential for trapping of photorespired CO₂ in BS cells (Sage, 2013).

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material

For the gas exchange portion of the study, we examined the bamboo species *G. magna*, *G. angustifolia*, and *D. asper*, rice (*O. sativa* cv. BRS Sertaneja), and sugar cane *Saccharum* sp. variety RB867515. Rice served as a representative C₃ species known to trap and refix photorespired CO₂ within the M cell, while sugarcane is a representative C₄ species to demonstrate precision in our gas exchange estimates of CO₂ compensation points and carboxylation efficiencies. On well-calibrated equipment with a good seal of the leaf chamber, photosynthetic rate measured on a C₄ plant should approach 0 μmol m⁻² s⁻¹ at a Cᵣ of 0 μmol CO₂ mol⁻¹ air (Vogan et al., 2007). For the anatomy and ultrastructure evaluation, only the three bamboo species were imaged.

The plants were grown in a naturally illuminated greenhouse at the Institute of Biological Sciences of the Universidade Federal de Goiás (UFG), Goiânia—GO, Brazil. Six plants of each species were grown in 20-L plastic pots (one plant per pot) filled with equal parts of a dark red latosol soil, sand, and manure. Bamboos and sugarcane were grown from culms and rice was grown from seeds. Temperature in the greenhouse ranged between 35 ± 4°C day and 25 ± 4°C night. Plants were grown under natural light, which exceeded...
1500 μmol photons m$^{-2}$ s$^{-1}$ photosynthetic photon flux density (PPFD) on clear days. To avoid drought in the pots, plants were watered three times daily, and fertilized monthly with a slow release 20:20:20 NPK fertilizer, and weekly with Johnson-Hoagland’s solution (Sage & Pearcy, 1987).

### 2.2 Gas exchange measurements

At 8 weeks after transplanting, five plants of each species were randomly chosen for leaf gas exchange assessments using a LiCor Li-6400 portable gas exchange system (LiCor, Lincoln-NE; www.licor.com). All measurements were conducted at 30°C. The atmospheric pressure in Goiânia-GO at the time of the measurements was 92 ± 0.5 kPa (value measured by the LiCor 6400). A small drop of vacuum grease sealed the gasket of the LiCor 6400 chamber and leaf margin, to minimize leaks across the gasket and through the leaf. For the analysis of the CO$_2$ response of A (the A-C$_i$ response), a leaf was initially exposed to a PPFD of 1000 μmol photons m$^{-2}$ s$^{-1}$, with 400 μmol of CO$_2$ mol$^{-1}$ of air (ambient concentration) and allowed to come to steady state. The PPFD was then increased to 2000 μmol m$^{-2}$ s$^{-1}$ and after stabilization and remeasurement, the CO$_2$ concentration was reduced in the following sequence: 300, 200, 100, 85, 75, 65, 55, and 45 μmol CO$_2$ mol$^{-1}$ air, with gas exchange measurements conducted at each CO$_2$ level after a 1- to 3-min equilibration period. The CO$_2$ concentration was then returned to 400 μmol mol$^{-1}$ and after steady-state measurements, it was raised to 550, 750, and 1100 μmol mol$^{-1}$ with measurements at each step. The intrinsic water use efficiency (WUE) was measured and then CO$_2$ was decreased to 100, 85, 75, 65, and 55 μmol mol$^{-1}$, with measurements at each CO$_2$ level following a 1- to 3-min equilibration period. The CO$_2$ concentration was then returned to 400 μmol mol$^{-1}$ and the light was reduced to 450 μmol m$^{-2}$ s$^{-1}$ and the measurement process repeated for this new light level.

### 2.3 Leaf anatomy: Transmission electron microscopy (TEM)

Three leaves of different plants from each of the bamboo species in the study were sampled for analysis by TEM using a modified protocol of Khoshravesh et al. (2017). The middle region of the most recently mature leaf (same leaf used for gas exchange measurements) was cut into ~1 mm$^2$ pieces that were fixed in 2.5% glutaraldehyde for 12 h at room temperature, washed in 0.1 M sodium cacodylate buffer pH 6.9 and subsequently, post-fixed in 1% osmium tetroxide for 2 h at room temperature. Following a final wash in buffer, samples were dehydrated in an ethanol series and embedded in Spurr’s resin. Samples were sectioned using an ultramicrotome mounted on an anti-vibration table (Khoshravesh et al. 2017). Sections (60–70 μm thin) were stained with 2% uranyl acetate and 0.2% lead citrate and imaged with a JEOL, JEM 2100 transmission electron microscope at the LabMic of the UFG. Images were analyzed using the software ImageJ (Schneider et al., 2012). A minimum of 30 cells per sample were used to calculate the ratio of the plasma membrane length juxtaposed to chloroplast (S$_c$) to the length of the plasma membrane facing intercellular air spaces (S$_i$). The thickness of the cell wall adjacent to the intercellular air spaces was measured from the images in 10 cells of each bamboo species, and the cell wall thickness in five cells of O. sativa was measured using TEM images from Figures 3 and 4A from Sage and Sage (2009).

For light microscopy, 4 mm$^{-2}$ leaf samples were preserved in 70% ethanol. Transverse sections were made using free hand. Samples were then clarified using 50% sodium hypochlorite and stained with 1% safranin and 1% aqueous alcian blue (1:4) followed by dehydration in increasing ethanol series. Samples were then cleared using butyl acetate and slides were mounted using verniz vitral incolor 500 (Paiva et al., 2006).

### 2.4 Statistics

For all measurements, five plants were randomly selected for measurements from a population of six plants per species that were growing in the greenhouse. Every week the position of the plants inside the greenhouse was changed to avoid position bias. All statistical analyzes were performed using R software (R-Core-Team, 2017). The photosynthetic...
responses to intracellular CO₂ changes (A-C_i) were analyzed by mixed additive regressions using the “mgcv” package (Wood, 2017). The concentration of leaf nitrogen, C*, R_d, WUE, A_400, Γ, and the cell wall thickness were analyzed by mixed linear regressions using the “lme4” package (Bates et al., 2014). Finally, the relationship between chloroplast cover of the cell periphery opposite the intercellular air spaces (S_c/S_m), C_i/C_a, and CE, which are fractions and do not follow a normal distribution, were analyzed as beta regressions, using the “betareg” package (Cribari-Neto & Zeileis, 2009; Peixoto & Sage, 2017).

3 | RESULTS

3.1 | Photosynthetic responses

The response curve of net CO₂ assimilation rate (A) to increasing intercellular CO₂ concentration (C_i) had the same shape for both rice and the three bamboo species, and distinct from the C₄ species sugar cane (Figure 1). Due in part to lower leaf nitrogen content (N), the bamboo species had lower A than rice at any C_i, and a lower CE (Table 1). Each bamboo exhibited a lower C_i/C_a ratio than rice, which translated into a higher WUE in the bamboos than in rice (Table 1). From the A-C_i curves, the extrapolated CO₂ compensation point (Γ) at 30°C was a typical C₃ value of 53 µmol mol⁻¹ for rice, while the sugarcane exhibited a typical C₄ value near 0 µmol mol⁻¹. With Γ values near 49 µmol mol⁻¹ all the bamboo species exhibited a lower Γ than rice (p < 0.05).

To estimate C*, we measured the response of A-C_i at five light intensities below saturation, and then plotted the

![Figure 1](http://example.com/figure1.png)

**FIGURE 1** Photosynthetic responses to intercellular CO₂ concentration in three bamboo species (Guadua angustifolia, Guadua magna, and Dendrocalamus asper), rice (Oryza sativa), and sugarcane (Saccharum sp.). Asterisks indicate measurements at an ambient CO₂ concentration of 400 µmol mol⁻¹. Mean ± SE, N = 5

| Species               | A (µmol m⁻² s⁻¹) | R_d (µmol m⁻² s⁻¹) | CO₂ (µmol mol⁻¹ H₂O⁻¹) | CE (A/ΔA_C_i⁻¹) | WUE (µmol CO₂ mmol⁻¹ H₂O) | Leaf nitrogen (µmol mol⁻¹) | C* (µmol mol⁻¹) | Γ (µmol mol⁻¹) | S_c/S_m | C_i/C_a | CE (ΔA/ΔC_i⁻¹) | ΔA/ΔC_i |
|-----------------------|-----------------|--------------------|------------------------|-----------------|--------------------------|---------------------------|----------------|--------------|---------|---------|----------------|---------|
| Sugarcane (Saccharum sp.) | 36.8 ± 3.2b     | 0.55 ± 0.04a       | 0.38 ± 0.08a           | 0.13 ± 0.01a    | 0.09 ± 0.004b           | 0.66 ± 0.004b             | 0.66 ± 0.004b   | 0.66 ± 0.004b | 0.66 ± 0.004b | 0.66 ± 0.004b | 0.66 ± 0.004b |
| Oryza sativa          | 26.9 ± 2.9b     | 1.0 ± 0.1          | 0.81 ± 0.03a           | 0.13 ± 0.01a    | 0.09 ± 0.004b           | 0.66 ± 0.004b             | 0.66 ± 0.004b   | 0.66 ± 0.004b | 0.66 ± 0.004b | 0.66 ± 0.004b | 0.66 ± 0.004b |
| Guadua angustifolia   | 16.4 ± 0.5      | 0.9 ± 0.1          | 0.65 ± 0.03a           | 0.06 ± 0.006b   | 0.07 ± 0.010a           | 0.64 ± 0.003b             | 0.64 ± 0.003b   | 0.64 ± 0.003b | 0.64 ± 0.003b | 0.64 ± 0.003b | 0.64 ± 0.003b |
| Guadua magna          | 14.6 ± 1.2      | 1.1 ± 0.2          | 1.1 ± 0.2              | 0.64 ± 0.003b   | 0.07 ± 0.010a           | 0.64 ± 0.003b             | 0.64 ± 0.003b   | 0.64 ± 0.003b | 0.64 ± 0.003b | 0.64 ± 0.003b | 0.64 ± 0.003b |

Table 1: Net CO₂ assimilation rate (A) at light saturation and the prevailing ambient CO₂ concentration (400 µmol mol⁻¹) at 30°C, dark respiration (R_d) at 30°C, ratio between intercellular CO₂ concentration and ambient CO₂ (C_i/C_a), leaf nitrogen content, apparent intercellular CO₂ compensation point of photosynthetic CO₂ assimilation, and the initial slope of the A-C_i curve (CE). Statistical differences between species indicate significant differences (p < 0.05).
initial slope of the A–Cᵢ response versus the intercept, as recommended by Walker et al. (2016). The A–Cᵢ plots at each light intensity produced a linear regression with slope and intercept (Figure 2). The individual slope and intercept values for each light level were then plotted in the x and y-axis, respectively. The slope and intercept of this new regression line gave the negative values of Cᵢ and Rₐ (Figure 3). From these gas exchange responses, Cᵢ at 30°C was determined to be 39 µmol mol⁻¹ in the three bamboo species, while in rice it was significantly greater at 46 µmol mol⁻¹ (Table 1). Rₐ did not vary between rice and the bamboo species (Table 1; p > 0.05).

To compare Cᵢ of bamboo species measured here with Cᵢ from other species, we converted the bamboo Cᵢ values from µmol mol⁻¹ to Pa, and then estimated the values corresponding to 25°C, using the temperatures responses of Cᵢ for Nicotiana tabacum reported by Bernacchi et al. (2001), as proposed by Walker and Ort (2015). At 25°C, it is estimated that the Cᵢ for D. asper is 2.80 Pa, for G. magna is 2.81 Pa, and for G. angustifolia is 2.83 Pa. Other than the bamboos, the lowest reported Cᵢ value at 25°C for a C₃ plant is 3.01 for N. tabacum (Table S1). Notably, the proto-kranz species Neurachne annularis, Flaveria robusta, and Flaveria pringlei have Cᵢ values greater than the bamboo species. Only C₂ species and C₄ plants (in which Cᵢ approaches zero) have Cᵢ values lower than values we observed in the bamboos.

With the Cᵢ estimates, we calculate the relative apparent oxygenase activity of Rubisco in the bamboos and rice assuming a chloroplast CO₂ concentration (Cᵢ) of 200 µmol mol⁻¹, which is a typical C₁ value of Cᵢ that would correspond to an ambient CO₂ of 400 µmol mol⁻¹, assuming no CO₂ refixation (von Caemmerer & Quick, 2000). Because the rate of photorespiratory CO₂ release is half of the oxygenation rate, the Rubisco oxygenase activity relative to carboxylase activity (vₒ/vₙ) is related to the chloroplastic CO₂ compensation point Γ* as follows: vₒ/vₙ = 2Γ*/Cᵢ (von Caemmerer, 2000). To derive this relative apparent oxygenase activity, we assumed there was no CO₂ refixation in the leaf and that Γ* = Cᵢ. For the three bamboos, apparent vₒ/vₙ = 0.39, while in rice apparent vₒ/vₙ = 0.46, which is 18% higher than the bamboo values.

3.2 Leaf anatomy and ultrastructure

The three bamboo species exhibited a closely spaced M tissue comprised of deeply lobed M cells situated above and below a layer of fusoid cells (Figure 4; Figure S1). BS cells are prominent and surround a layer of small, thick-walled mestome sheath cells (MS; Figure 4 and Figure S2). The M cells of each bamboo species are densely packed with chloroplasts, particularly in the lobed region (Figure 5). Chloroplasts typically are in contact with an adjacent chloroplast, either directly, or through the extension of stromules that stretch from one chloroplast to another along the cell periphery (Figure 5a,c,f). Quantitative analysis indicates that the periphery of the M cells facing
the intercellular air spaces is almost completely covered by chloroplasts or stromules, such that $S_c/S_t$ estimates are 98.5%–99.1% in the bamboo species (Table 1). Gaps in the chloroplast coverage of the M cell periphery, when observed, typically corresponded to contact points between adjacent M cells. Mitochondria largely occurred in the interior of the M cells, to the inside of chloroplasts (Figure 5c,d,f). Mitochondria were never observed to be in direct contact with the cell periphery; where they approached the cell periphery, for example, in a gap between two adjacent chloroplasts, a stromule extended between the cell periphery and the position of the mitochondria (Figure 5f). In comparison to M cells, BS and MS cells of the bamboo species had relatively few organelles (Figure 6; Figure S2). When present, BS chloroplasts were positioned primarily along the cell wall opposite intercellular air spaces (Figure 6). Chloroplasts never formed aggregates with mitochondria along the centripetal BS cell wall, as observed in C_3 plants (Sage et al., 2014). Plastids in MS cells had weakly developed thylakoids (Figure S2). Plasmodesmata were common between adjacent M cells (Figure 5b,d) and M and BS cells (Figure 6a,c). Compared to rice, only D. asper had thicker cell wall adjacent to intercellular air spaces (Table S2).

To compare our results with previously published relationships, we plot our bamboo and rice $C_*$ values and corresponding $S_c/S_t$ values with the $C_*$ and $S_c/S_t$ data previously published for six C_3 species (Figure 7, using data from figure 5A in Busch et al. 2013). With the exception of the bamboo species, the data from the C_3 plants in Figure 7 fall on a common linear relationship between $C_*$ and $S_c/S_t$. The $C_*$ values measured here for the bamboo species are 6 to 7 µmol mol$^{-1}$ less than observed in either rice at similar $S_c/S_t$ values, indicating bamboos have
an enhanced ability to decrease photorespiratory costs compared to rice and other C₃ species.

4 | DISCUSSION

In the present study we show that three bamboo species found in the Brazilian Cerrado exhibit apparent photocompensation points for CO₂ (C*) that are 7 µmol mol⁻¹ lower than that of rice, and 18 µmol mol⁻¹ lower than the mean of the Rubisco Γ* at 30°C. Previously, rice was hypothesized to have an efficient mechanism to trap and refix photorespired CO₂, thus enhancing its ability to compete in warm environments where photorespiration is pronounced (Busch et al., 2013; Sage & Sage, 2009). The results here indicate bamboo is even more effective at offsetting photorespiratory costs than rice, by an estimated 18%, and thus may be one of the most efficient plants in the world that utilize the C₃ photosynthetic pathway. Indeed, the C₆ we measured in the bamboos is the lowest recorded for any C₃ species at a common temperature (Figure 7; Table S1), indicating bamboos have a most efficient form of C₃ photosynthesis with respect to reducing photorespiration. This greater efficiency would have importance because bamboos produce an abundance of lignocellulose that can become a valuable bioenergy feedstock either directly or as a by-product of forestry applications.

The reduction in C₆ can be explained by the bamboos either having an unusually high specificity of Rubisco for CO₂ relative to O₂, or they employ a mechanism to increase the CO₂ concentration around Rubisco and in doing so suppress photorespiration. A C₄-type CO₂ concentrating mechanism can be ruled out since the bamboos show none of the characteristic features of C₄ photosynthesis, such as large, organelle-filled BS or mestome sheath (MS) cells, a low CO₂...
that facilitate rapid diffusive entry of CO$_2$ into photosynthetic chloroplasts from intercellular air spaces, and would allow for rapid efflux of photorespired CO$_2$ released in BS mitochondria (Evans et al., 2000; von Caemmerer & Evans, 1991). Certain grass clades such as Neurachne co-opt the MS instead of the BS for the site of CO$_2$ concentration in C$_2$ and C$_4$ species (Hattersley et al., 1986; Khoshravesh et al., 2016). In the bamboos, however, we observed no evidence for MS co-option. The MS organelles are few in number, not aggregated, and in the case of chloroplasts, not well developed as they are in M cells.

Instead of a C$_2$ mechanism, the evidence indicates bamboos are utilizing chloroplast sheaths to trap and refix photorespired CO$_2$. As with rice, the bamboo place thick, densely stained chloroplasts into the lobes of the M cells with mitochondria sandwiched between the chloroplasts or localized to the interior of the cell. Stromules are frequently present in gaps between chloroplasts, forming a fence-like barrier between the mitochondria and the cell exterior. Also, we often observed stromules surrounding individual mitochondria. Because stromules contain Rubisco (Hanson & Sattarzadeh, 2011; Kwok & Hanson, 2004), any photorespired CO$_2$ exiting these mitochondria would have to pass through the stromule matrix where it could enhance the CO$_2$ concentration and improve Rubisco efficiency.

The $C_*$ of rice (O. sativa) of 46 µmol mol$^{-1}$ is among the lowest reported in the literature for C$_3$ plants at 30°C (Busch et al., 2013; Walker & Ort, 2015). At a common temperature of 25°C, our estimated $C_*$ values in the bamboos are lower than any other C$_3$ species (Table S1) and approaches values observed in modest C$_2$ species such as Neurachne lanigera, which has reduced (but not eliminated) expression of GDC in M mitochondria (Sage et al., 2013; Schulze et al., 2013). Why are the $C_*$ values lower in bamboo than rice and other C$_3$ taxa? The measurements for both bamboo and rice were conducted on the same days, using the same instrument, so systematic errors are unlikely, particularly given we took extra care to eliminate leaks. The instrument also was working well as indicated by the accurate estimation of a $\Gamma*$ near 0 µmol mol$^{-1}$ in the C$_4$ sugar cane. It is tough to get $\Gamma*$ values near 0 µmol mol$^{-1}$ if the LiCor 6400 is compromised by poor calibration or leaks. One possibility for the difference in $C_*$ is that bamboo has a higher relative specificity of Rubisco ($S_{\phi/\psi}$) than rice, thereby lowering $v_{i/o}/v_{c}$. This possibility requires follow-up study. Rubisco specificity has been noted to vary within C$_3$ plants, with drought-adapted species having higher values than mesic species (Galmés et al., 2005, 2014; Orr et al., 2016). It would be a major finding if bamboo was to shift the relative specificity sufficiently to approach $C_*$ values of C$_2$ species, because this type of Rubisco would have significant benefits in cultivated C$_3$ plants and might be relatively easy to engineer into related species such as rice. However,
in a survey of C₃ grasses, Rubisco specificity varied by approximately 10% and rice species had among the highest specificities measured (Orr et al., 2016). It would thus be surprising if the differences in C₄ between rice and bamboo were due to variation in Rubisco specificity, as bamboo Rubisco would have to have an unusually high value for the C₃ flora, and would exhibit a specificity that is well outside the range reported by Orr et al. (2016) for C₃ grasses.

If $S_{i/o}$ is constant, $C_{i}$ could also vary if the amount of CO₂ released per oxygenation reaction changes. This could happen, for example, if glycine leaves the photosynthetic pathway and is not metabolized by GDC, but is instead metabolized in a reaction that does not release CO₂ (Busch et al., 2018). Use of photorespiratory intermediates as carbon skeletons for de novo nitrogen (N) and sulfur (S) assimilation would lower the amount of CO₂ released per oxygenation, and thus decrease $C_{i}$ (Busch et al. 2018). However, de novo N and sulfur assimilation are unlikely to be sufficient in magnitude to account for the lower $C_{i}$ observed in bamboo, particularly given its lower leaf N content than rice (Table 1; Busch et al. 2018).

If the relative specificity of Rubisco and glycine export from the photosynthetic pathway are equivalent between rice and bamboo, then the difference in $C_{a}$ would appear to result from different levels of trapping of photorespired CO₂ in the M cells. This is possible given C₃ species show variation in trapping ability as indicated by the range of $C_{i}$ values in Figure 7 (Busch et al., 2013). These differences in Figure 7 are explained by variation in chloroplast coverage and thickness at the M cell periphery (Busch et al., 2013). Both bamboo and rice have high chloroplast coverage, approaching 100%, so variation in the degree of coverage is not the obvious mechanism. An alternative is the thickness of the chloroplast sheath may vary, for example, via differences in chloroplast width, or the position of mitochondria relative to M chloroplasts. Bamboo chloroplasts are thick, and frequently fill the lobes of M cells. Regarding chloroplast and mitochondria associations, the wrapping of stromules around chloroplasts indicates bamboo chloroplasts form intimate associations with mitochondria, which would force photorespired CO₂ directly into the stroma, thereby enabling greater degrees of CO₂ refixation. Another possibility could be reduced M conductance to CO₂ diffusion ($g_{m}$), which would reduce the outward diffusion of (photo)respired CO₂ from the cell, thus allowing for greater refixation and a lower $C_{i}$ (Busch et al., 2013; von Caemmerer, 2000). A lower $g_{m}$ could be due to thicker M cell walls, reduced permeability of the plasmalemma, and/or reduced surface area exposure of the M chloroplasts to intercellular air spaces (Evans et al., 2009). We do not favor a hypothesis of reduced $g_{m}$, because we observed no association between wall thickness and $C_{i}$ in the bamboos and rice. Rice had a similar wall thickness as G. magna and G. angustifolia, yet higher $C_{i}$, while D. asper had thicker walls but the same $C_{i}$ as G. magna and G. angustifolia. In addition, the high degree of lobing of the armed parenchyma in bamboo would facilitate an increase in $g_{m}$, by allowing for higher surface area exposure of chloroplasts to intercellular spaces in their M tissue. Future studies will need to evaluate $g_{m}$ in tandem with detailed quantification of chloroplast exposure to the intercellular spaces, wall thickness, refixation rates, and Rubisco properties to fully quantify the mechanism of photorespiratory CO₂ trapping in bamboo versus other C₄ clades.

Despite the reduced $C_{a}$, rice exhibited a higher photosynthetic capacity than the three bamboo species, which mirrors differences in rates of photosynthesis observed between eudicot crops and woody perennials. Annual crops generally have higher values of $A$, often exceeding 20 µmol m⁻² s⁻¹, while perennials and woody species typically exhibit rates between 10 and 20 µmol m⁻² s⁻¹ (Field & Mooney, 1986; Larcher, 2003). These differences in $A$ reflect different allocation patterns, where allocation to roots, storage, and perennating structures can reduce allocation to photosynthetic capacity per unit leaf area (Fichtner et al., 1995; Mooney, 1983). The three bamboo species exhibited a lower leaf nitrogen content than rice, which is consistent with reduced protein allocation per unit leaf area. Notably, the bamboos had a 20% higher WUE than rice, reflecting their lower $C_{a}/C_{i}$ and rice exhibited a higher photo-WUE than bamboo, particularly given its lower leaf N content than rice (Table 1; Fichtner et al., 1996; Mooney, 1986; Larcher, 2003). These differences in $A$ are commonly above 0.7 (Farquhar & Sharkey, 1982). A higher WUE would be valuable trait for crops grown in marginal habitats where drought is a concern, such as the Cerrado. From an evolutionary perspective, high WUE and the associated reduction in $C_{a}/C_{i}$ in tandem with detailed quantification of chloroplast exposure to the intercellular spaces, wall thickness, refixation rates, and Rubisco properties to fully quantify the mechanism of photorespiratory CO₂ trapping in bamboo versus other C₄ clades.

In conclusion, our results demonstrate that three bamboo species from two distinct Bambusoideae clades have unusually low $C_{i}$ and $Γ$ values for C₃ plants. This is best explained by bamboos expressing a modest carbon concentrating mechanism and/or a Rubisco with an unusually high relative specificity. Our results rule out a $C_{4}$ or $C_{2}$ mechanism
of CO2 concentration, and instead support a hypothesis that bamboos are very effective at trapping and refixing photorespired CO2 in the M tissue. Rice species are also effective at trapping and refixing photorespired CO2, and like bamboo, exhibit a deeply lobed anatomy where mitochondria are isolated from the intercellular air spaces by a sheath of chloroplasts and stromules. Grasses of the Bambusoideae and Oryzeae clades universally exhibit deeply lobed M cells, which is evidence for an widespread ability within these early diverging grass clades to trap and refix photorespired CO2 using thick chloroplast sheaths (Kellogg, 2015). As a result, these grasses appear to be particularly effective at offsetting some of the costs of photorespiration. By doing so, rice and bamboos, and potentially many other warm-season grasses using the C3 pathway realize fitness gains that enable them to not only persist in the face of strong competition from C4 plants, but proliferate as indicated by the high number of bamboo species and their use in agroforestry contexts (Kellogg, 2015). Such traits in bamboo would be valuable in a bioenergy context where losses caused by photorespiration reduce the ability of plants to convert solar energy into usable bioenergy. Because of its low C4, which is the lowest recorded in C3 plants at 25°C, bamboo may exhibit the most efficient form of C3 photosynthesis on earth, at least in terms of suppression of the detrimental effects of photorespiration. As such, its mechanism for reducing photorespiratory costs may be a promising approach to improving C3 photosynthesis in a range of crop and bioenergy plants. Follow-on studies are needed to quantify refixation potentials in bamboo and, in particular, to survey the diversity of bamboos and other tropical species to determine the frequency of mechanisms to trap photorespired CO2 in the low latitude flora.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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