Received: 7 December 2020 | Accepted: 1 February 2021

DOI: 10.1111/gcbb.12819

GCB-BIOENERGY BIOPRODUCTS FOR A SUSTAINABLE BIOECONOMY Open Access WILEY

ORIGINAL RESEARCH

Elevated efficiency of C_3 photosynthesis in bamboo grasses: A possible consequence of enhanced refixation of photorespired CO_2

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico : 310070/2015-5 to D. Graciano-Ribeiro; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior to M. Peixoto; Natural Sciences and Engineering Research Council of Canada grant RGPIN-2017-06476 to RF Sage.

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_*, C_3 photosynthesis, CO_2 refixation, *Dendrocalamus*, *Guadua*, photorespiration, water use efficiency

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1 | INTRODUCTION

Bamboos are widespread C_3 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., 2008; 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 C_4 plant *Miscanthus* × *giganteus*, while in Amazonia, bamboos grow fast enough to shade out C_4 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 C_3 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 C_3 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 C_4 pathway are reduced.

As a warm climate C_3 species, bamboos should experience high rates of Rubisco oxygenase activity and the subsequent loss of previously fixed CO_2 during photorespiration. For example, C_3 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 C_3 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 C_4 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 CO_2 relative to O_2, which results in a lower value of the biochemical CO_2 compensation point of Rubisco, termed Γ_*; however, reduction in Γ_* may come at the cost of other kinetic properties of Rubisco, for example, a decrease in the catalytic turnover rate (k_{cat}; Sage, 2002). Alternatively, C_3 plants can trap and refix photorespired CO_2, either in single cells or internal compartments within leaves (Busch et al., 2013; Sage & Khoshravesh, 2016). Trapping of photorespired CO_2 within single mesophyll (M) cells of C_3 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 stromules 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 CO_2 to exit the cell via the chloroplast, allowing for refixation of the CO_2 (Busch et al., 2013). In rice, trapping and refixation of photorespired CO_2 can boost net CO_2 assimilation rate (A) by over 10% at current CO_2 levels, and by over 30% at the reduced CO_2 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 CO_2, 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 C_4 grasses is high. Similarly, bamboos are also highly successful C_3 plants where C_4 species thrive. In rice, an ability to trap and refix photorespired CO_2 may help explain the success of this crop in warm environments (Busch et al., 2013). Similarly, we hypothesize that efficient refixation of photorespired CO_2 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 CO_2 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 CO_2 enhancement (Sage et al., 2013). When nearly all GDC is expressed in the BS cells, CO_2 concentrations around BS Rubisco can be threefold greater than that in M chloroplasts (Keerberg et al., 2014), and the physiology resulting in this modest CO_2 enrichment is termed C_2 photosynthesis (Sage et al., 2012). C_2 photosynthesis is recognized as a major intermediate state in C_4 evolution (Monson & Rawsthorne, 2000), but is also present in numerous species with no close relationship to C_4 taxa, for example in the Brassicaceae genus *Moricandia* (Schlüter et al., 2017). Numerous C_3-like species express a weak version of C_2 photosynthesis, indicating glycine shuttles between M and BS-like compartments enhance the performance of C_3 photosynthesis in photorespiratory environments (Sage et al., 2014; Yorimitsu et al., 2019). Evidence of such C_2-like physiologies include enlarged BS cells enriched with organelles, centripetal positioning of organelles, particularly mitochondria, and reduced CO_2 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 C_2-like mechanism.

In the grass phylogeny, the subfamily Bambusoideae branches in a sister position to the largely tropical subfamily Ehrhartoideae, 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 C_2-like M to BS glycine shuttle. Gas exchange studies in bamboo have not evaluated the degree to which photorespiratory CO_2 is trapped and refixed, for example, through the determination of the apparent CO_2 compensation point of net CO_2 assimilation rate in the absence of day respiration, a parameter known as C_* (Busch et al., 2013). C_* values approximate Γ_* if there is no refixation of photorespirated or respired CO_2 in a C_3 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 CO_2 conditions, then oxygenase activity is slowed and C_* 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 C_* in rice below Γ_* can exceed 10 μmol mol^{-1} above 30°C, while species operating a glycine shuttle between M and BS cells reduce C_* below Γ_* 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 C_2 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 CO_2 at different light intensities, which allowed us to estimate C_* 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 CO_2 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 CO_2, 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 CO_2 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 C_3 species known to trap and refix photorespired CO_2 within the M cell, while sugarcane is a representative C_4 species to demonstrate precision in our gas exchange estimates of CO_2 compensation points and carboxylation efficiencies. On well-calibrated equipment with a good seal of the leaf chamber, photosynthetic rate measured on a C_4 plant should approach 0 μmol m^{-2} s^{-1} at a C_i of 0 μmol CO_2 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 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 calculated using the ratio of net CO_2 exchange rate (A) and stomata conductance (g_s) multiplied by the VPD at ambient CO_2 [$A/(g_s \times \text{VPD})$]. Carboxylation efficiency (CE) was calculated by the initial slope of the A-C_i curve below 90 μmol mol^{-1} of C_i. The CO_2 compensation point at saturating light (Γ) was calculated from the x-intercept of the A-C_i response.

Values of C_* and R_d were estimated according to Walker et al. (2016) by measurements of leaf gas exchange at low CO_2 concentrations and variable, but low PPFD. C_* is the apparent intercellular CO_2 compensation point of net CO_2 assimilation rate in the absence of day respiration. C_* values approximate Γ_* (biochemical CO_2 compensation point inside the chloroplast) if there is no refixation of photorespired or respired CO_2 in a C_3 leaf. Initially, the leaf was placed in the chamber at a PPFD of 800 μmol m^{-2} s^{-1}, 400 μmol CO_2 mol^{-1} air, and 30°C. After equilibration, gas exchange 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. This process was repeated for PPFD settings of 300, 210, and 140 μmol m^{-2} s^{-1}. Following each set of measurements, leaves were harvested, oven dried at 60°C for 48 h and then milled to a fine powder, and leaf nitrogen (N) was assayed by the Kjeldahl method (Galvani & Gaertner, 2006) at the UFG School of Agronomy.

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_t). 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 CO_2 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_t), 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 CO_2 assimilation rate (A) to increasing intercellular CO_2 concentration (C_i) had the same shape for both rice and the three bamboo species, and distinct from the C_4 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 CO_2 compensation point (Γ) at 30°C was a typical C_3 value of 53 μmol mol^{-1} for rice, while the sugarcane exhibited a typical C_4 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

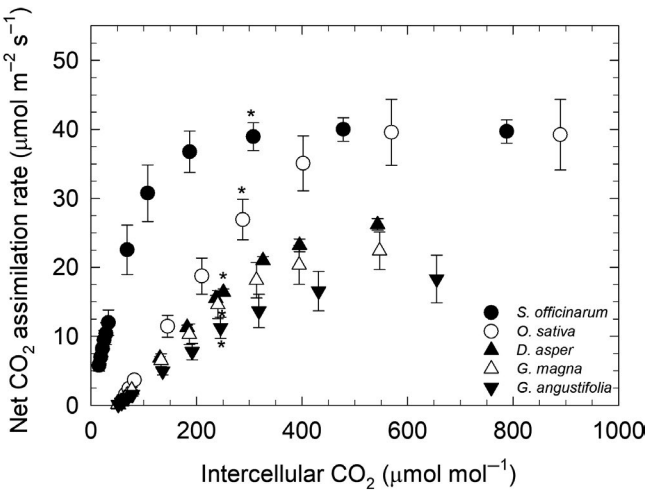

FIGURE 1 Photosynthetic responses to intercellular CO_2 concentration in three bamboo species (*Guadua angustifolia*, *Guadua magna*, and *Dendrocalamus asper*), rice (*Oryza sativa*), and sugarcane (*Saccharum* sp.). Asterisks indicate measurements at an ambient CO_2 concentration of 400 μmol mol^{-1}. Mean ± SE, $N = 5$

TABLE 1 Net CO_2 assimilation rate (A) at light saturation and the prevailing ambient CO_2 concentration (400 μmol mol^{-1}) at 30°C; dark respiration (R_d) at 30°C; ratio between intercellular [CO_2] and ambient [CO_2] (C_i/C_a); carboxylation efficiency (CE) calculated by the initial slope of the A-C_i curve; water use efficiency (WUE) calculated as $A/(g_s \times$ VPD molar fraction) at light saturation, ambient [CO_2] and 30°C; leaf nitrogen content; apparent intercellular photocompensation point of photorespired CO_2 (C_*); the photosynthetic CO_2 compensation point (Γ); and the percent of the plasma membrane length juxtaposed to chloroplast (S_c) relative to the length of the plasma membrane facing intercellular air spaces (S_m). Mean ± SE; $N = 5$, except for S_c/S_m where $N = 3$. Superscript letters indicate statistical differences between species ($p < 0.05$)

Species	A (μmol m^{-2} s^{-1})	R_d (μmol m^{-2} s^{-1})	C_i/C_a	CE ($\Delta A\ \Delta C_i^{-1}$)	WUE (μmol CO_2 mmol^{-1} H_2O)	Leaf nitrogen (g kg^{-1})	C_* (μmol mol^{-1})	Γ (μmol mol^{-1})	S_c/S_m
Sugarcane (*Saccharum* sp.)	36.8 ± 2.3^a	—	0.55 ± 0.04^c	0.38 ± 0.068^a	6.29 ± 0.39^a	22.7 ± 1.4^c	3.3 ± 1.2^c	0.01 ± 0.2^a	—
Rice (*Oryza sativa*)	26.9 ± 2.9^b	1.0 ± 0.1	0.81 ± 0.01^a	0.13 ± 0.016^b	2.84 ± 0.29^c	38.7 ± 1.3^a	46.1 ± 0.3^a	53.4 ± 0.2^c	—
Dendrocalamus asper	16.4 ± 0.5^c	0.9 ± 0.1	0.66 ± 0.01^b	0.09 ± 0.004^c	3.34 ± 0.22^b	28.6 ± 1.6^c	39.0 ± 0.5^b	50.4 ± 0.5^b	99.1% ± 0.3
Guadua angustifolia	12.8 ± 0.1^c	0.9 ± 0.1	0.65 ± 0.03^b	0.06 ± 0.006^c	3.52 ± 0.26^b	35.1 ± 0.8^b	39.4 ± 0.9^b	49.8 ± 0.2^b	98.5% ± 0.9
Guadua magna	14.6 ± 1.2^c	1.1 ± 0.2	0.64 ± 0.03^b	0.07 ± 0.010^c	3.26 ± 0.14^b	34.2 ± 1.2^b	39.3 ± 1.2^b	48.2 ± 0.9^b	98.6% ± 0.5

initial slope of the A-C_i response versus the intercept, as recommended by Walker et al. (2016). The A-C_i 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 C_* and R_d (Figure 3). From these gas exchange responses, C_* 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). R_d did not vary between rice and the bamboo species (Table 1; $p > 0.05$).

To compare C_* of bamboo species measured here with C_* from other species, we converted the bamboo C_* values from μmol mol^{-1} to Pa, and then estimated the values corresponding to 25°C, using the temperatures responses of C_* for *Nicotiana tabacum* reported by Bernacchi et al. (2001), as proposed by Walker and Ort (2015). At 25°C, it is estimated that the C_* 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 C_* value at 25°C for a C_3 plant is 3.01 for *N. tabacum* (Table S1). Notably, the proto-kranz species *Neurachne annularis*, *Flaveria robusta*, and *Flaveria pringlei* have C_* values greater than the bamboo species. Only C_2 species and C_4 plants (in which C_* approaches zero) have C_* values lower than values we observed in the bamboos.

With the C_* estimates, we calculate the relative apparent oxygenase activity of Rubisco in the bamboos and rice assuming a chloroplast CO_2 concentration (C_c) of 200 μmol mol^{-1}, which is a typical C_3 value of C_c that would correspond to an ambient CO_2 of 400 μmol mol^{-1}, assuming no CO_2 refixation (von Caemmerer & Quick, 2000). Because the rate of photorespiratory CO_2 release is half of the oxygenation rate, the Rubisco oxygenase activity relative to carboxylase activity (v_o/v_c) is related to the chloroplastic CO_2 compensation point Γ_* as follows: $v_o/v_c = 2\Gamma_*/C_c$ (von Caemmerer, 2000). To derive this relative apparent oxygenase activity, we assumed there was no CO_2 refixation in the leaf and that $\Gamma_* = C_*$. For the three bamboos, apparent $v_o/v_c = 0.39$, while in rice apparent $v_o/v_c = 0.46$, 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

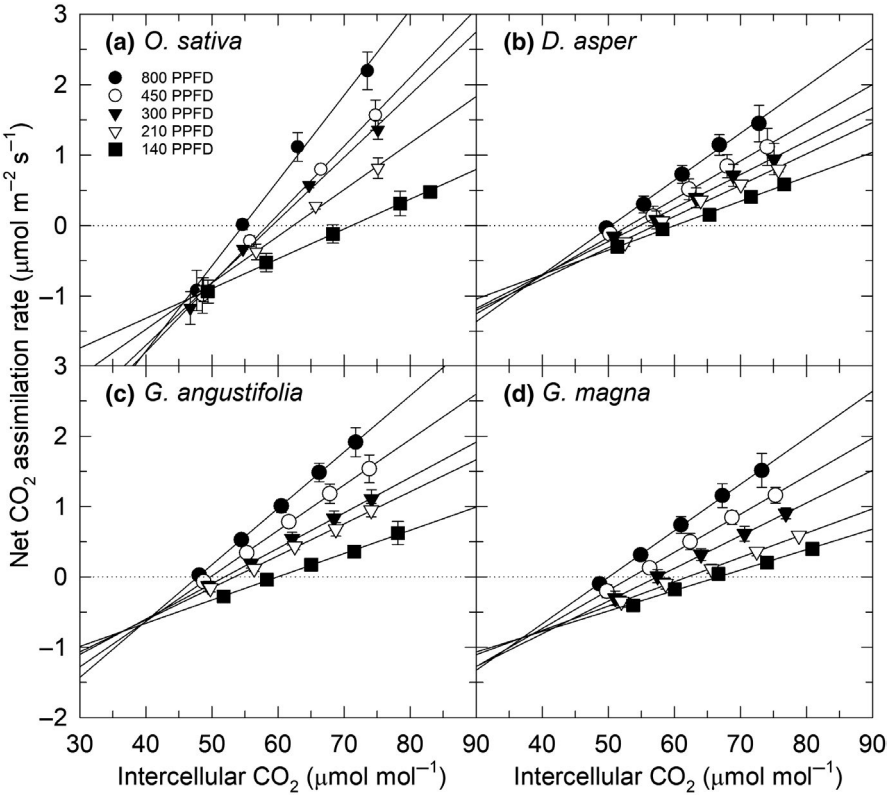

FIGURE 2 Photosynthetic responses to low intercellular CO_2 at five photon flux densities (PPFD, in μmol photons m^{-2} s^{-1}) in (a) rice (*Oryza sativa*) and three bamboo species ((b) *Dendrocalamus asper*, (c) *Guadua angustifolia*, and (d) *Guadua magna*). Due to experimental noise, the curves often will not intercept in one exact point. Although the figure shows the average for the species at each data point, the regressions were calculated individually for each plant. We then used the slope and intercept from each of the regressions at each light level to plot Figure 3. Mean ± SE. $N = 5$

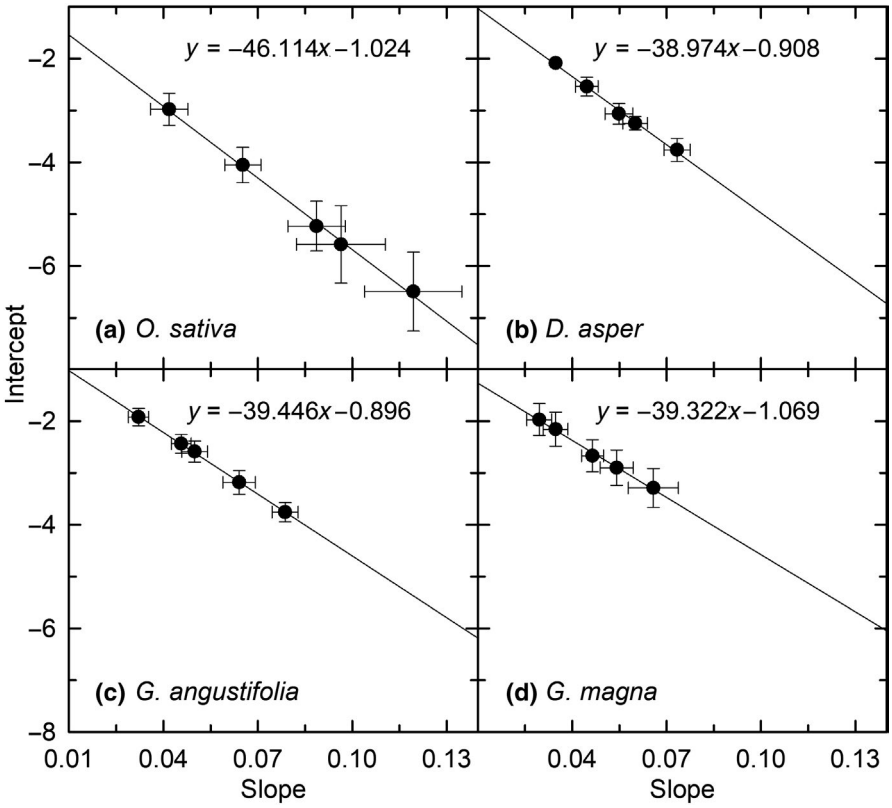

FIGURE 3 Slope and *y*-intercept values from each A-C_i response shown in Figure 2. Symbols are the average slope and *y*-intercept values of each measurement replicate and the lines correspond to the average of regressions separately performed on the data from individual plants. In this method, the slope estimates C_* and the *y*-intercept is the R_d estimation (Walker et al. 2016). (a) *Oryza sativa*, (b) *Dendrocalamus asper*, (c) *Guadua angustifolia*, (d) *Guadua magna*

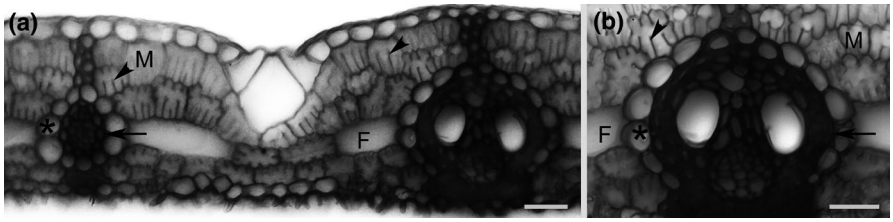

FIGURE 4 Light micrographs of transverse sections of *Dendrocalamus asper* leaves. Deeply lobed mesophyll cells are marked with a black arrowhead. The asterisks mark bundle sheath cells. Black arrows mark mestome sheath cells. See Figure S1 for light micrographs of *Guadua angustifolia* and *Guadua magna*. (a) Panoramic view of the leaf, (b) detail of the Bundle Sheath. Bars, 20 μm. F, fusoid cell; M, mesophyll

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_2 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_* we measured for rice var BRS Sertaneja is identical to that reported for rice var IR64 by Busch et al. (2013). We assume both varieties have similar 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

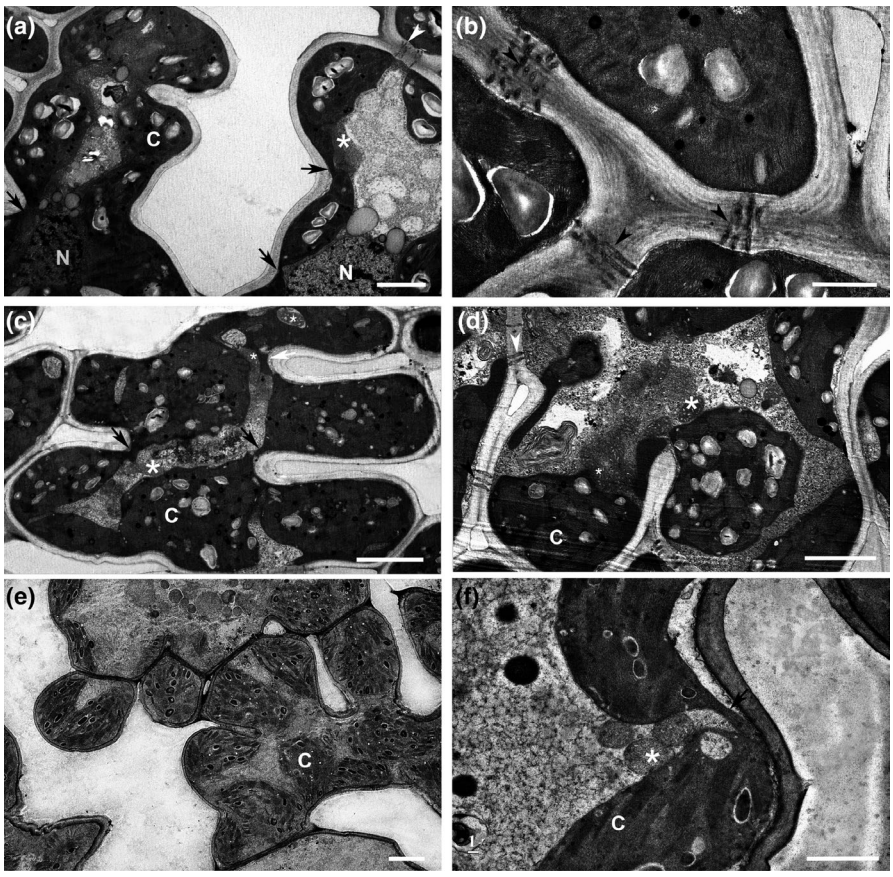

FIGURE 5 Transmission electron micrographs of leaf mesophyll cells from *Dendrocalamus asper* (a, b), *Guadua angustifolia* (c, d), and *Guadua magna* (e, f). Arrowheads (black and white) mark plasmodesmata. Arrows indicate stromules. White asterisks label mitochondria. Bars, 2 μm (a, c–e); 1 μm (b, f). C, chloroplast; N, nucleus

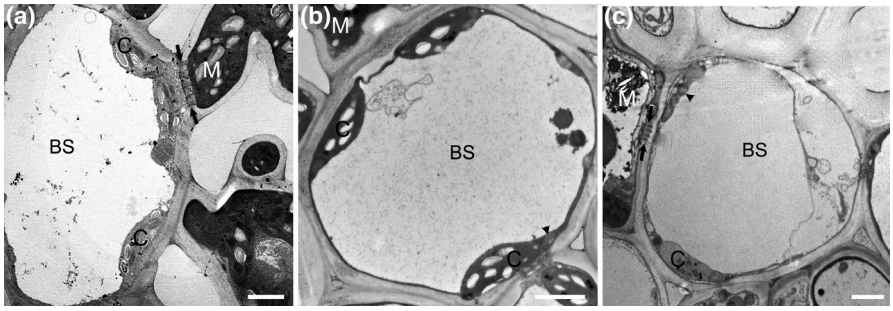

FIGURE 6 Transmission electron micrographs of bundle sheath cells. (a) *Dendrocalamus asper*, (b) *Guadua angustifolia*, and (c) *Guadua magna*. Black arrowheads mark mitochondria. Black arrows label plasmodesmata. Bars, 2 μm. BS, bundle sheath; C, chloroplast; M, mesophyll

an enhanced ability to decrease photorespiratory costs compared to rice and other C_3 species.

4 | DISCUSSION

In the present study we show that three bamboo species found in the Brazilian Cerrado exhibit apparent photocompensation points for CO_2 (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 CO_2, 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 C_3 photosynthetic pathway. Indeed, the C_* we measured in the bamboos is the lowest recorded for any C_3 species at a common temperature (Figure 7; Table S1), indicating bamboos have a most efficient form of C_3 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 C_* can be explained by the bamboos either having an unusually high specificity of Rubisco for CO_2 relative to O_2, or they employ a mechanism to increase the CO_2 concentration around Rubisco and in doing so suppress photorespiration. A C_4-type CO_2 concentrating mechanism can be ruled out since the bamboos show none of the characteristic features of C_4 photosynthesis, such as large, organelle-filled BS or mestome sheash (MS) cells, a low CO_2

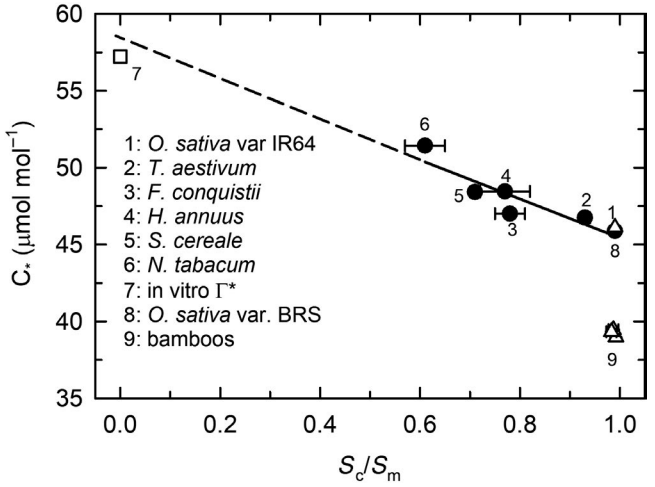

FIGURE 7 The relationship between the C_* at 30°C and the relative chloroplast cover of the mesophyll cell periphery (S_c/S_m) in *Oryza sativa* var BRS Sertaneja and three bamboo species (*Dendrocalamus asper*, *Guadua angustifolia*, and *Guadua magna*) (this study; open triangles); and six C_3 species from Busch et al. (2013; filled circles). Also shown is the biochemical Γ_* (open square) averaged from a range of in vitro estimates, as described by Busch et al. (2013). Data are from Busch et al. (2013) for *O. sativa* var IR64 (rice), *Triticum aestivum* (wheat); *Flaveria cronquistii*; *Helianthus annuus* (sunflower), *Secale cereale* (rye), and *Nicotiana tabacum*. Means for the bamboos are superimposed because their values are similar. Mean ± SE, $N = 5$

compensation point (as we observed in sugar cane), C_4-like carbon isotope ratios, and low interveinal spacing (Dengler et al., 1994; Li et al., 2013; Mulkey, 1986). The other mechanisms by which terrestrial plants can reduce C_* are to trap photorespired CO_2 within BS or M cells and in doing so enrich stromal CO_2 concentrations around Rubisco (Sage & Khoshravesh, 2016). This can occur via a C_2-like mechanism where an M to BS glycine shuttle moves photorespiratory glycine into the BS, where it is metabolized to serine and CO_2 by GDC localized into BS mitochondria. The BS chloroplasts then refix the photorespired CO_2 released from adjacent mitochondria. Alternatively, glycine may be entirely metabolized in M cells, with the resulting photorespired CO_2 being trapped by a sheath of chloroplasts around the M cell periphery. The C_2-type mechanism can be ruled out because our light and TEM images unequivocally demonstrate little organelle mass in the BS cells which would be needed to generate and refix photorespired CO_2. In C_2-like species, mitochondria and chloroplasts are typically enriched in number and clustered next to each other in a centripetal location in BS cells (Rawsthorne, 1992; Sage et al., 2014). This positioning allows the vacuole to act as a diffusive barrier to slow CO_2 efflux following glycine decarboxylation (von Caemmerer, 1989). In the three bamboo species here, the BS chloroplasts are generally positioned along the outer periphery of the cell, opposite intercellular air spaces. These are typical C_3 patterns 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 Γ near 0 μmol mol^{-1} in the C_4 sugar cane. It is tough to get Γ 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 ($S_{c/o}$) than rice, thereby lowering v_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 C_3 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 C_* between rice and bamboo were due to variation in Rubisco specificity, as bamboo Rubisco would have to have an unusually high value for the C_3 flora, and would exhibit a specificity that is well outside the range reported by Orr et al. (2016) for C_3 grasses.

If $S_{c/o}$ is constant, C_* could also vary if the amount of CO_2 released per oxygenation reaction changes. This could happen, for example, if glycine leaves the photorespiratory pathway and is not metabolized by GDC, but is instead metabolized in a reaction that does not release CO_2 (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 CO_2 released per oxygenation, and thus decrease C_* (Busch et al. 2018). However, de novo N and sulfur assimilation are unlikely to be sufficient in magnitude to account for the lower C_* 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 photorespiratory pathway are equivalent between rice and bamboo, then the difference in C_* would appear to result from different levels of trapping of photorespired CO_2 in the M cells. This is possible given C_3 species show variation in trapping ability as indicated by the range of C_* 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 CO_2 directly into the stroma, thereby enabling greater degrees of CO_2 refixation. Another possibility could be reduced M conductance to CO_2 diffusion (g_m), which would reduce the outward diffusion of (photo)respired CO_2 from the cell, thus allowing for greater refixation and a lower C_* (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_* in the bamboos and rice. Rice had a similar wall thickness as *G. magna* and *G. angustifolia*, yet higher C_*, while *D. asper* had thicker walls but the same C_* 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 CO_2 trapping in bamboo versus other C_3 clades.

Despite the reduced C_*, 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_i/C_a. Low C_i/C_a is also reported for other bamboo species, indicating it is a widespread trait in the subfamily (Agnihotri et al., 2009; Bag et al., 2012). The C_i/C_a of 0.65 is lower than typically observed in C_3 crops species, where C_i/C_a values 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_i/C_a would indicate bamboos are prone to even greater rates of photorespiration than would be the case in typical C_3 plants, unless they have a mechanism to offset photorespiratory costs. This would be particularly true in the past few million years, when atmospheric CO_2 levels were 30%–55% less than current values (Gerhart & Ward, 2010). We hypothesize that the mechanism lowering C_* in bamboo is an adaptation that compensates for the high WUE in bamboo, which is caused by a reduced stomatal conductance and C_i in the warm, periodically dry landscapes of the Brazilian Cerrado.

In conclusion, our results demonstrate that three bamboo species from two distinct Bambusoideae clades have unusually low C_* and Γ values for C_3 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 CO_2 concentration, and instead support a hypothesis that bamboos are very effective at trapping and refixing photorespired CO_2 in the M tissue. Rice species are also effective at trapping and refixing photorespired CO_2, 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 CO_2 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 C_3 pathway realize fitness gains that enable them to not only persist in the face of strong competition from C_4 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 C_*, which is the lowest recorded in C_3 plants at 25°C, bamboo may exhibit the most efficient form of C_3 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 C_3 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 CO_2 in the low latitude flora.

ACKNOWLEDGMENTS

This study was funded by CAPES and CNPq grant process no. 310070/2015-5 to DGR, and NSERC grant number RGPIN-2017-06476 to RFS. The authors thank LabMic-UFG personnel for assistance with the transmission electron micrographs, and the Bamboo Goias Network. MMP thanks CAPES for the PNPD funds. The authors also thank Dr. Berkley Walker for providing data presented on Table S1.

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.

How to cite this article: Peixoto MM, Sage TL, Busch FA, et al. Elevated efficiency of C_3 photosynthesis in bamboo grasses: A possible consequence of enhanced refixation of photorespired CO_2. *GCB Bioenergy*. 2021;13:941–954. https://doi.org/10.1111/gcbb.12819