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PLOS ONE

Understanding the Low Photosynthetic Rates of Sun and Shade Coffee Leaves: Bridging the Gap on the Relative Roles of Hydraulic, Diffusive and Biochemical Constraints to Photosynthesis

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Abstract

It has long been held that the low photosynthetic rates (*A*) of coffee leaves are largely associated with diffusive constraints to photosynthesis. However, the relative limitations of the stomata and mesophyll to the overall diffusional constraints to photosynthesis, as well as the coordination of leaf hydraulics with photosynthetic limitations, remain to be fully elucidated in coffee. Whether the low actual *A* under ambient CO_2 concentrations is associated with the kinetic properties of Rubisco and high (photo)respiration rates also remains elusive. Here, we provide a holistic analysis to understand the causes associated with low *A* by measuring a variety of key anatomical/hydraulic and photosynthetic traits in sun- and shade-grown coffee plants. We demonstrate that leaf hydraulic architecture imposes a major constraint on the maximisation of the photosynthetic gas exchange of coffee leaves. Regardless of the light treatments, *A* was mainly limited by stomatal factors followed by similar limitations associated with the mesophyll and biochemical constraints. No evidence of an inefficient Rubisco was found; rather, we propose that coffee Rubisco is well tuned for operating at low chloroplastic CO_2 concentrations. Finally, we contend that large diffusive resistance should lead to large CO_2 drawdown from the intercellular airspaces to the sites of carboxylation, thus favouring the occurrence of relatively high photorespiration rates, which ultimately leads to further limitations to *A*.

Citation: Martins SCV, Galmés J, Cavatte PC, Pereira LF, Ventrella MC, et al. (2014) Understanding the Low Photosynthetic Rates of Sun and Shade Coffee Leaves: Bridging the Gap on the Relative Roles of Hydraulic, Diffusive and Biochemical Constraints to Photosynthesis. PLoS ONE 9(4): e95571. doi:10.1371/journal.pone.0095571

Editor: Gerrit T.S. Beemster, University of Antwerp, Belgium

Received September 7, 2013; **Accepted** March 28, 2014; **Published** April 17, 2014

Funding: This research was supported by the Foundation for Research Assistance of the Minas Gerais State, Brazil (Fapemig, Grant APQ-01138-12), by the National Council for Scientific and Technological Development (CNPq) (Grants 302605/2010-0 and 475780/2012-4) to FMD, and by Plan Nacional (Spain) (GrantAGL2009-07999) to JG. A PhD scholarship granted by CNPq to SCVM is also gratefully acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Because CO_2 influx and water vapour efflux share a common pathway through the stomatal pores on leaf surfaces, a trade-off between transpirational costs and CO_2 assimilation is implicitly unavoidable. The coupling between stomatal conductance (g_s) to CO_2 and water vapour (and the need to maintain a proper leaf water balance) has often been evidenced by the strong positive scaling between g_s and the leaf hydraulic conductance per unit area, K_L [1–3]. In turn, the significance of K_L as a potentially limiting component of the vascular system has been further emphasised by the strong hydraulic-photosynthetic coordination observed across a large sample of diverse species [4]. Furthermore, K_L is closely related to the anatomy of the leaf: K_L has been shown to be positively related to both the theoretical axial conductivity of the midrib (determined from xylem conduit numbers and dimensions) and the venation density, D_v [3,5]. A unified control of hydraulic and photosynthetic traits may also be further highlighted by comparing shade and sun leaves: the former have lower rates of net CO_2 assimilation (*A*) and g_s, therefore leading to lower demand for water and correspondingly lower K_L and D_v [4,6].

In addition to stomatal limitations, *A* is currently known to be constrained by mesophyll conductance (g_m), which is defined as the conductance for the transfer of CO_2 from the intercellular airspaces (C_i) to the sites of carboxylation in the chloroplastic stroma (C_c) [7]. According to Flexas et al. [8], g_m limitations to photosynthesis are of similar magnitude as stomatal constraints and generally greater than biochemical limitations. Increasing evidence has shown that g_m is often intrinsically co-regulated with g_s [8]. More recently, Ferrio et al. [9] showed a positive scaling between g_m and K_L and proposed that water and CO_2 share an important portion of their respective diffusion pathways through the mesophyll; thus, any downregulation of leaf hydraulics may reduce not only g_s but also g_m, both of which contribute to reducing *A*. Quantification of g_m has become important in predicting leaf photosynthetic parameters using the Farquhar-von Caemmerer-Berry (FvCB) model of leaf photosynthesis [10]

because such a model underestimates the maximum Rubisco carboxylation rate (V_{cmax}) by considering g_m as being infinite [11,12]. Furthermore, changes in $C_i - C_c$ because of variations in g_m among species result in different A values in plants with the same biochemical activity and g_s [7].

At lower C_c, A is limited by RuBP carboxylase activity, which in turn depends on the concentration, activation and kinetic properties of Rubisco [13]. For example, Rubisco with a higher specificity factor, $S_{c/o}$ (which determines the relative rates of carboxylation and oxygenation by Rubisco at given CO_2 and O_2 concentrations), could be regarded as conferring an advantage in minimising photorespiration rates, R_P [14]. Interestingly, Rubisco seems to have evolved towards higher $S_{c/o}$ under stressful conditions leading to low C_c, such as drought, salinity and high temperature [14]. Additionally, species evolved under stressful conditions with sclerophyll leaves tend to display a higher $S_{c/o}$, which may again be related to lower C_c [14,15]. However, trade-offs between $S_{c/o}$ (or particularly the affinity for CO_2 ($1/K_C$)) and the carboxylase turnover rate (k_{cat}^c) have been described, so that there is no Rubisco in nature with both high affinity for CO_2 and fast activity [16]. At saturating C_c, A becomes limited by the capacity for the regeneration of RuBP (often dominated by the electron transport capacity) but also by $S_{c/o}$ [17]. In any case, when studying the ecophysiology of plants, Rubisco kinetic parameters should not be considered a constant but rather as an active part determining the biochemical potential of plants to assimilate CO_2 under optimal and suboptimal conditions.

Coffee is an evergreen perennial shrub with hypostomatous leaves that evolved in the African forest understoreys and is thus considered a shade-demanding species. However, in many situations, modern coffee cultivars grow well without shade and even out-yield shaded coffee [18,19]. At atmospheric CO_2 concentrations and saturating light, coffee displays low A, typically in the range of 4–11 μmol CO_2 m^{-2} s^{-1} [20,21], which is in the lower range recorded for trees [22]. However, the maximum A values obtained in common A/C_i curves can surpass 20 μmol CO_2 m^{-2} s^{-1} [23], whereas the photosynthetic capacity, determined under true CO_2 saturation (~50 mmol CO_2 mol^{-1} air), reaches values exceeding 30 μmol O_2 m^{-2} s^{-1} [21]. Taking the above information together, the low A in coffee might largely result from diffusive constraints to photosynthesis [24]. However, the relative contributions of the stomata and mesophyll to the overall diffusional limitations to photosynthesis, as well as the coordination of leaf hydraulics with photosynthetic limitations, remain to be fully elucidated in coffee. Furthermore, the anticipated low g_s and g_m would compromise the transference of CO_2 through stomata and leaf mesophyll to Rubisco sites, therefore decreasing C_c and ultimately favouring RuBP oxygenation in relation to carboxylation [25]. Under these conditions, Rubisco kinetic properties with a high affinity for CO_2 may be critical to secure optimal carbon balance and yield.

Although the general descriptors of the ecophysiology of sun-shade plants are well-known, an integrative analysis of these descriptors has rarely been performed in conjunction (hydraulics, stomata, mesophyll, Rubisco, etc). Here, we provide a holistic analysis to understand the causes associated with low A in coffee by measuring a variety of key anatomical/hydraulic and photosynthetic traits, including D_v, K_L, the actual and maximum theoretical g_s (g_{wmax}) and several parameters from A/C_i and A/C_c curves. We have centred our attention on coffee given that, in addition to being a highly important commodity [18], it could also be considered as a model plant for other important crops which evolved as understorey trees, such as cacao, citrus and tea; these crops are traditionally considered to have low A (seldom above 10 μmol CO_2 m^{-2} s^{-1}, even in the field under favourable conditions) likely as consequence of large diffusive, rather than biochemical, limitations to photosynthesis [26]. Our main goals were (i) to examine the role of leaf hydraulics as a prime limiting factor for photosynthetic gas exchange, (ii) to calculate g_m to properly parameterise the responses of A to C_c, and (iii) to disentangle the relative contributions of stomatal, mesophyll and biochemical limitations to photosynthesis, and how all of these facts may be affected by the light supply. A further goal was to analyse how the kinetic properties of coffee's Rubisco [27] may affect the actual A and evaluate if Rubisco in coffee is well tuned for operating at low C_c by comparing it with the highly efficient Rubisco of *Limonium gibertii*; this shade-intolerant species is particularly adapted to highly stressing environments, whose Rubisco has evolved under extremely low C_c ranges [14]. We demonstrate that leaf hydraulic architecture imposes a major constraint on the maximisation of the photosynthetic gas exchange of coffee leaves and that A was mainly limited by stomatal factors followed by similar limitations associated with mesophyll and biochemical constraints, regardless of the light supply. We also found that, despite the relatively high $S_{c/o}$ of coffee Rubisco, the large diffusive resistances favour the occurrence of relatively high R_P, which ultimately leads to further limitations to A.

Materials and Methods

Plant Material, Growth Conditions and Experimental Design

The experiment was conducted in Viçosa (20°45′S, 42°54′W, altitude 650 m) in southeastern Brazil. Uniform seedlings of *Coffea arabica* L. cv 'Catuaí Vermelho IAC 44′ obtained from seeds were grown in 12 L pots containing a mixture of soil, sand and composted manure (4:1:1, v/v/v). Plants were grown either under full sunlight conditions (100% light) or under low light in a shade environment (10% full sunlight). The shade enclosure was constructed using neutral-density black nylon netting, and the plants were kept in these conditions for 12 months before measurements. Throughout the experiment, the plants were grown under naturally fluctuating conditions of temperature and relative air humidity and were fertilised and irrigated as necessary. The pots were randomised periodically to minimise any variation within each light environment. For all samplings and measurements, the youngest fully expanded leaves, corresponding to the third or fourth pair from the apex of the plagiotropic branches, were used. The experiment was arranged in a completely randomised design, with six plants in individual pots per treatment as replicates. The experimental plot included one plant per container.

Morpho-anatomical Features and Related Hydraulic and Diffusive Traits

The specific leaf area (SLA) was computed using the dry mass of 20 leaf discs (1.13 cm diameter each). For anatomical measurements, leaves were collected and fixed in FAA_{70} for 48 h, followed by storage in 70% (v/v) aqueous ethanol. Samples of the mean region of each leaf blade were embedded in methacrylate (Historesin–Leica Microsystems Nussloch, Heidelberg, Germany) according to the manufacturer. Transverse sections (7 μm thickness), obtained using a rotary microtome (model RM2155, Leica Microsystems Inc., Deerfield, USA), were stained with toluidine blue at pH 4.0 and mounted in synthetic resin (Permount). Anatomical data were quantified using an image analysis program (Image Pro-Plus, version 4.5, Media Cybernetics, Silver Spring, USA). Video images were acquired using a video

camera attached to an Olympus microscope (AX70TRF, Olympus Optical, Tokyo, Japan). The following anatomical data were then assessed: (i) total leaf thickness; (ii) palisade and spongy mesophyll thickness; (iii) upper and lower epidermis thickness; (iv) guard cell length (L); (v) vertical distance from the vein to stomatal epidermis, $D_{v\text{-}e}$; (vi) stomatal density (SD, according to DaMatta et al. [28]); (vii) stomatal index that was estimated as $(S/(E+S))\times 100$, where S and E are the number of stomata and epidermal cells per unit leaf area, respectively [29]; and (viii) stomatal pore index based on guard cell length (SPI_{gcl}) that was estimated as $SPI_{gcl} = SD \times L^2$. For determining venation traits, samples of leaf lamina were cut from two leaves per plant and cleared as described by Scoffoni et al. [30]. Regions of approximately 6 mm^2 were imaged at 30X, and D_v was calculated as the sum of the vein lengths divided by the total image area using the image analysis program described above. Leaf size was estimated, using the maximum leaf widths and lengths, with the equations described by Antunes et al. [31]. Stomatal size and epidermal cell size, as well as the level of coordination between anatomical traits and leaf size (quantified as the deviation from the expected proportional relationship to each other), were all determined following Carins Murphy et al. [6].

Midrib xylem conduits were measured to determine the theoretical midrib axial hydraulic conductance (K_t), where the conduits were treated as ellipses to calculate K_t as

$$K_t = \sum [\pi a^3 b^3 / 64\eta(a^2 b^2)]$$

where a and b are the long and short internal vessel diameters, and η is the viscosity of water at 25°C [32], further normalising by leaf length and area.

The maximum stomatal conductance to water vapour (g_{wmax}) was calculated according to Franks et al. [33] as

$$g_{w\ max} = SD\ d_w\ a/[v(l+\pi/2\sqrt{(a/\pi)}]$$

where SD is the stomatal density, d_W is the diffusivity of water vapour in air, a is the maximum area of the open stomatal pore, v is the molar volume of air, and l is the stomatal pore depth for fully open stomata. Values for standard constants d_W and v were those for 25°C (24.9×10^{-6} m^2 s^{-1} and 24.4×10^{-3} m^3 mol^{-1}, respectively). a was calculated as $\pi(p/2)^2$, where p is the stomatal pore length, which was approximated as $L/2$ according to Franks and Farquhar [34]. l for fully open stomata was taken as $L/4$, assuming guard cells inflate to a circular cross section [33].

Leaf Hydraulic Conductance

The leaf hydraulic conductance (K_L) was estimated according to Brodribb and Holbrook [35] by following the kinetics of water potential (Ψ_l) relaxation in rehydrated leave as:

$$K_L = C\ \ln(\Psi_o/\Psi_f)/t$$

where C is leaf capacitance, estimated using pressure-volume curves [36], Ψ_o is Ψ_l before rehydration, and Ψ_f is Ψ_l after rehydration for t seconds. Leaf Ψ_l was measured using a Scholander-type pressure chamber (model 1000, PMS Instruments, Albany, NY, USA).

Gas Exchange and Fluorescence Measurements

Leaf gas exchange and chlorophyll a fluorescence were measured simultaneously using an open-flow infrared gas-exchange analyser system equipped with a leaf chamber fluorometer (LI-6400XT, Li-Cor, Lincoln, NE, USA). Environmental conditions in the leaf chamber consisted of a leaf-to-air vapour pressure deficit between 1.2 and 2.0 kPa and a leaf temperature of 25°C.

In light-adapted leaves, the actual quantum yield of PSII (Φ_{PSII}) was determined by measuring steady-state fluorescence (F_s) and maximum fluorescence during a light-saturating pulse of $c.$ 8,000 μmol m^{-2} s^{-1} (F_m'), following the procedures of Genty et al. [37]:

$$\Phi_{PSII} = (F_m' - F_s)/F_m'$$

The electron transport rate (J_F) was then calculated as

$$J_F = \alpha\beta PPFD\Phi_{PSII}$$

where PPFD is the photosynthetically active photon flux density, α is the leaf absorptance and β is the PSII optical cross section. The product $\alpha\ \beta$ was herein determined from the relationship between Φ_{PSII} and Φ_{CO2}, obtained by varying the CO_2 concentration under non-photorespiratory conditions in an atmosphere containing less than 1% O_2, as described by Valentini et al. [38].

The light respiration rate (R_L) was determined according to the 'Laisk-method' [39], using the y axis intersection of A/C_i (internal CO_2 concentration) curves performed at three different PPFD intensities (750, 250 and 75 μmol m^{-2} s^{-1}). The rate of mitochondrial respiration at darkness (R_D) was measured early in the morning in dark-adapted leaves.

The photorespiratory rate of Rubisco (R_P) was obtained according to Valentini et al. [38] using the following equation:

$$R_P = 1/12[J_F - 4(A+R_L)]$$

Six A/C_i curves were obtained from different plants per treatment. In light-adapted leaves, A/C_i curves were initiated at an ambient CO_2 concentration (C_a) of 400 μmol mol^{-1} under a saturating PPFD of 1000 μmol m^{-2} s^{-1}. Once steady state was reached, C_a was decreased stepwise to 50 μmol mol^{-1} air. Upon completion of the measurements at low C_a, C_a was returned to 400 μmol mol^{-1} air to restore the original A. Next, C_a was increased stepwise to 2,000 μmol mol^{-1} air. A/C_i curves consisted of 13 different C_a values.

C_c was calculated after Harley et al. [40] as

$$C_c = (\Gamma^*(J_F + 8(A+R_L)))/(J_F - 4(A+R_L))$$

where Γ^* was determined from the *in vitro* Rubisco specificity factor ($S_{c/o}$) as

$$\Gamma^* = O/S_{C/O}$$

A was taken from gas-exchange measurements, and the J_F values were obtained from chlorophyll a fluorescence yield. After estimating C_c, g_m was calculated following Harley et al. [40]:

$$g_m = A/(C_i - C_c)$$

From the A/C_i and A/C_c curves, the maximum carboxylation capacity (V_{cmax}) and maximum capacity for electron transport rate (J_{max}) were calculated on a C_i and C_c basis using the kinetic parameters for coffee described in Martins et al. [27]. The FvCB model was fitted to the data by applying iterative curve fitting (minimum least square difference) using the Microsoft Excel Solver tool (Microsoft Corporation, Redmond, WA, USA). Additionally, g_m, V_{cmax} and J_{max} were estimated using the Ethier and Livingston [11] method, which is based on fitting A/C_i curves with a non-rectangular hyperbola version of the FvCB model, relying on the hypothesis that g_m reduces the curvature of the Rubisco-limited portion of an A/C_i response curve. Again, the kinetic parameters of Rubisco measured on coffee were used. Corrections for the leakage of CO_2 and water vapour into and out of the leaf chamber of the Li-6400-40 have been applied to all gas-exchange data, as described by Rodeghiero et al. [41].

The chloroplastic CO_2 concentration of transition (C_{c_trans}), where C_c denotes the transition between the Rubisco- and RuBP regeneration-limited states, was estimated as described by Gu et al. [42]:

$$C_{c\_trans} = (J_{max}K_m - 8V_{c\,max}\Gamma^*)/(4V_{c\,max} - J_{max})$$

where K_m is the effective Michaelis–Menten constant for CO_2 that considers the competitive inhibition by O_2 which was taken from Martins et al. [27].

The overall photosynthetic limitations were partitioned into their functional components [stomatal (l_s), mesophyll (l_m) and biochemical (l_b) limitations] using the values of g_s, g_m, V_{cmax}, Γ^*, K_m and C_c following the approach proposed by Grassi and Magnani [43]:

$$l_s = (g_{tot}/g_{s\_CO2} * \partial A/\partial C_c)/(g_{tot} + \partial A/\partial C_c)$$

$$l_m = (g_{tot}/g_m * \partial A/\partial C_c)/(g_{tot} + \partial A/\partial C_c)$$

$$l_b = g_{tot}/(g_{tot} + \partial A/\partial C_c)$$

where g_{s_CO2} is the stomatal conductance to CO_2 ($g_{s\_CO2} = gs/1.6$), g_m is the mesophyll diffusion conductance according to Harley *et al.* [40] and g_{tot} is the total conductance to CO_2 from ambient air to chloroplasts ($g_{tot} = 1/[(1/g_{s\_co2}) + (1/g_m)]$). $\partial A/\partial C_c$ was calculated as:

$$\partial A/\partial C_c = [V_{c\ max}(\Gamma^* + K_m)]/(C_c + K_m)^2$$

Statistical Analyses

Data are expressed as the means ± standard error. Student's *t*-tests were used to compare the parameters between treatments. Additionally, one sample *t*-tests were performed to compare the means for shade plants with the expected values if they were proportional to the sun plants. All statistical analyses were carried out using Microsoft Excel.

Results

Sun- and shade-grown individuals differed reasonably in venation architecture and mesophyll structure (Table 1). The sun leaves, compared with shade leaves, displayed a higher leaf thickness (15%) primarily resulting from a higher thickness of palisade (43%) and spongy (14%) mesophyll, which led to a higher palisade-to-spongy parenchyma ratio (25%) and lower SLA (39%). However, the upper and lower epidermis thickness and the maximum distance from the vein to the epidermis ($D_{v\text{-}e}$) did not change in response to the light treatments. Sun-grown plants had 31% more midrib xylem conduits but with slightly lower mean conduit diameters (7%) than shade-grown plants. The sun plants displayed K_t and K_L values that were 160% and 58% higher, respectively, than in the shade individuals. Adjustments to the light treatments associated with the morphological characteristics of stomata were also observed, as denoted by higher SD (62%), stomatal index (27%) and SPI_{gcl} (62%) in sun leaves compared with the shade leaves; however, both the guard cell length and stomatal size did not change in response to the light treatments. Vein density was proportional to $1/\sqrt{}$ leaf area but slightly higher (17%) than would be expected if it was directly proportional to SD (Figure 1). In line with the differences in the stomatal index, SD and epidermal cell size deviated significantly from the expected proportional relationship to leaf area: SD was 25% higher and epidermal cell size was 31% lower in shade plants than would be expected if these variables were directly proportional (Figure 1). The maximum g_s (g_{wmax}), determined by the stomatal size and density, was larger (61%) in sun leaves than in the shade leaves.

Under saturating PPFD (1,000 μmol photons m^{-2} s^{-1}), both A and g_s values were higher (*c.* 55%) in sun leaves than in the shade leaves (Table 2). The values for several other photosynthetic/respiration parameters were also higher in sun leaves than in the shade leaves (Table 2): 60% for g_m (average of two independent methods), 42% for V_{cmax}, 45% for J_{max} (for V_{cmax} and J_{max}, the values express averages obtained on C_i and C_c bases), 140% for R_D, 200% for R_L, 41% for R_p and 51% for J_F. In contrast, C_i, C_c, C_{c_trans} and the ratios A/g_s, g_m/g_s and J_{max}/V_{cmax} on both C_i and C_c bases did not differ significantly in response to the light treatments. V_{cmax} and J_{max} calculated on a C_c basis were, on average, 101% and 37% higher than V_{cmax} and J_{max}, respectively, calculated on a C_i basis, whereas the J_{max}/V_{cmax} ratio was on average 32% lower when calculated on a C_c basis than on a C_i basis.

The overall photosynthetic limitations were essentially similar when comparing sun- and shade-grown plants (Table 2). We found that A was mainly constrained by stomatal limitations (*c.* 40%), whereas mesophyll and biochemical limitations accounted similarly for the remaining limitations (*c.* 30% each).

The analysis of Rubisco kinetic properties are summarised in Table 3, showing that coffee presents a Rubisco with a relatively high affinity for CO_2 (low K_c) and fast activity (k_{cat}^c), resulting in relatively high values for $S_{c/o}$. From the combination of *in vivo* V_{cmax} and *in vitro* data (k_{cat}^c), the concentration of active Rubisco sites was estimated to be 16 and 20 μmol m^{-2} s^{-1} for shade and sun plants, respectively.

Discussion

This study provided a holistic examination of the key steps that could limit the photosynthetic capacity to fix CO_2 and demonstrated that leaf hydraulic architecture imposes a major constraint on the maximisation of the photosynthetic gas exchange of coffee leaves by limiting g_s. It is tempting to suggest that these constraints might to some extent explain the photosynthetic behaviour of

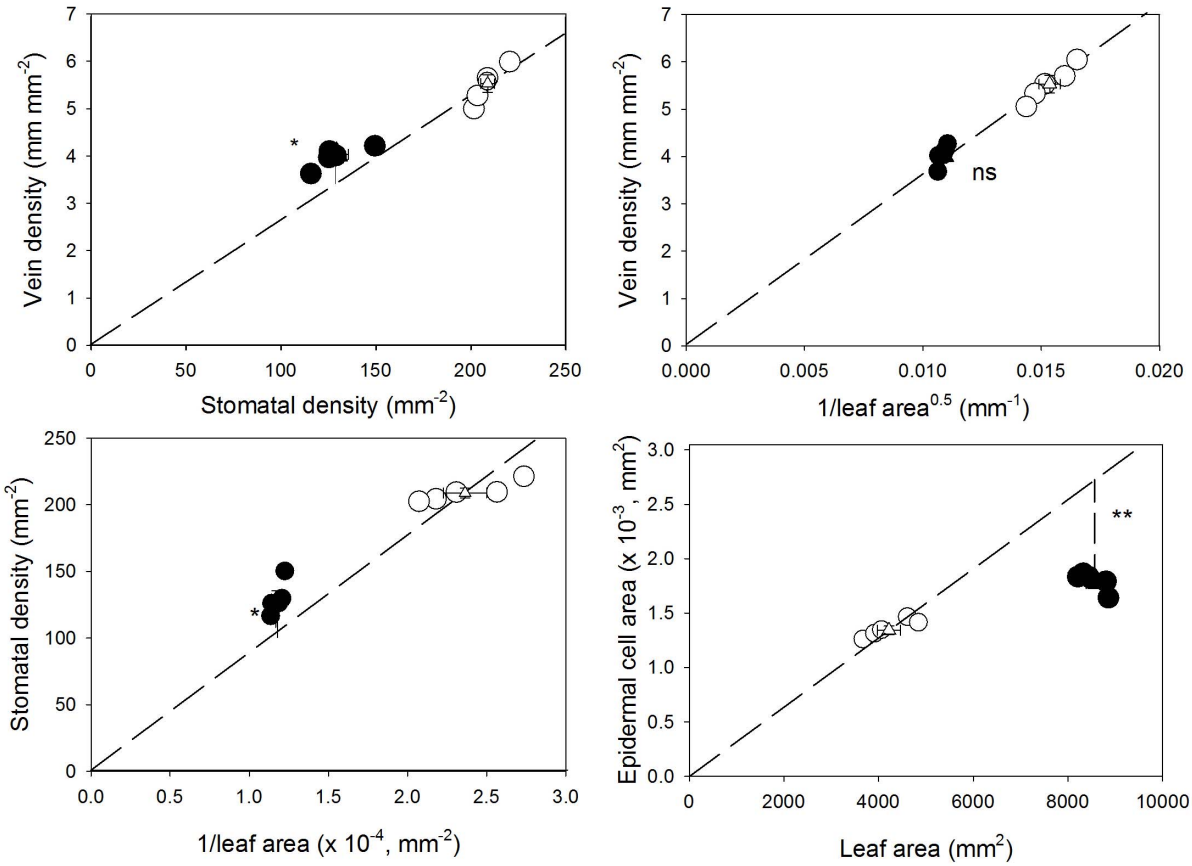

Figure 1. The relationships between vein and stomatal density, vein density and 1/√ leaf area, stomatal density and 1/leaf area, and epidermal cell area and leaf area. Data (± standard error) are shown for coffee plants grown under shade (black circles) or full sun (white circles). Asterisks denote differences (*, $P<0.05$; **, $P<0.01$) between the observed values for the shade plants and the expected values (broken line) if these were proportional to the averages for the sun plants.
doi:10.1371/journal.pone.0095571.g001

other important (sub)tropical crops such as cacao, citrus and tea, which have a photosynthetic performance and a slow-growth behaviour similar to that of coffee [26]. Our results suggest that improvements of the photosynthetic performance of these crops by means of selecting hydraulic traits (e.g., D_v and K_L) that might support higher g_s values (thereby decreasing stomatal limitations of photosynthesis) could be a useful strategy to facilitate the selection of promising genotypes with enhanced crop growth and production.

We have shown that adjustments in leaf hydraulics through increases in D_v and K_L in sun-grown individuals were coordinated with a suite of traits related to water flux and gas exchange per leaf area, such as mesophyll structure (e.g., higher mesophyll thickness and palisade-to-spongy parenchyma ratio) and stomatal attributes (increased SD and stomatal pore index). Importantly, we showed that increases in K_t occurred at the expense of an increased number of midrib conduits with lower lumen in sun plants, suggesting that improvements in the hydraulic safety would not compromise the hydraulic efficiency [44]. In addition, the larger SD coupled with larger stomatal index implies a proportionally greater increase in the number of guard cells than in normal epidermal cells [45]; hence, light availability should directly and positively influence stomatal fate in coffee regardless of passive changes linked by light-induced differences in leaf blade expansion. However, differential leaf expansion was responsible for the adjustment of D_v to SD, which remained nearly proportional to each other. Thus, coffee plants can balance water supply with transpirational demand through a coordination of increased initiation of stomata cells with differential expansion of epidermal cells. Such coordination reflects an optimisation of the trade-off between transpirational costs and CO_2 assimilation, resulting in the higher intrinsic water use efficiency observed in coffee (*c.* 85 μmol CO_2 mol^{-1} H_2O on average), which is markedly high compared with other tropical woody species [46,47].

Our K_L values, determined using the method of relaxation kinetics of Ψ_l, were remarkably lower than those found by Brodribb et al. [1] for other tropical trees using the same method (between 17 and 36 mmol m^{-2} s^{-1} MPa^{-1}). Considerably low values of K_L had already been reported for *C. arabica* (*c.* 4.2 mmol m^{-2} s^{-1} MPa^{-1}) by Gascó et al. [48] using the high-pressure method. Consistent with the close relationship between K_L and D_v [4,5,49], our maximum D_v values were also within the low range recorded for angiosperms [50], suggesting that the hydraulic architecture of coffee leaves imposes strong resistance for water flow which, in turn, should limit the CO_2 diffusion into the leaf [4]. In addition, our K_L and D_v values (Table 1) fit very well in the general relationship of both K_L and D_v with the maximum A proposed by Brodribb et al. [5,51], that is, our estimated K_L and D_v values exactly predict our maximum measured A values. Therefore we contend that the leaf hydraulic architecture ultimately should act as a prime factor limiting photosynthetic gas exchange in coffee leaves.

Table 1. Anatomical and hydraulic traits of coffee plants grown under shade or full sunlight conditions.

Parameters	Treatments	
	Shade	**Full sunlight**
Specific leaf area (m^2 kg^{-1})	22.9±3.5	14.0±2.7*
Total leaf thickness (μm)	333.9±9.1	384.5±17.2*
Palisade thickness (μm)	52.6±1.2	75.24±4.4*
Spongy thickness (μm)	220.9±6.1	253.1±11.5*
Upper epidermis thickness (μm)	38.8±0.2	37.4±1.3
Lower epidermis thickness (μm)	29.6±0.9	26.4±1.2
PP/SP	0.24±0.01	0.30±0.01*
SPI_{gcl}	0.104±0.006	0.168±0.003*
Guard cell length (μm)	28.0±0.7	28.9±0.4
Stomatal density (mm^2)	129.1±7.2	208.8±4.2*
Stomatal index	20.3±1.1	25.7±1.0*
g_{wmax} (mol m^{-2} s^{-1})	1.16±0.05	1.82±0.02*
Venation density (mm mm^{-2})	4.0±0.1	5.5±0.2*
Midrib vessel diameter (μm)	22.8±0.2	21.3±0.4*
Number of midrib conduits	117±7	153±11*
K_t (mmol MPa^{-1} m^{-2} s^{-1})	24.5±2.5	63.8±1.6*
K_L (mmol MPa^{-1} m^{-2} s^{-1})	6.9±0.1	10.9±1.4*
$D_{v\text{-}e}$ (μm)	195.8±8.6	215.4±8.5

$n = 6 \pm$SE. Asterisks indicate statistically significant differences ($P<0.05$) between shade and full sun treatments.
Abbreviations: PP:SP, palisade-to-spongy parenchyma ratio; SPI_{gcl}, stomatal pore index based on guard cell length; g_{wmax}, maximal theoretical stomatal conductance to water vapour; K_t, midrib conductance; K_L, leaf hydraulic conductance; $D_{v\text{-}e}$, vertical distance from the vein to the stomatal epidermis.
doi:10.1371/journal.pone.0095571.t001

Regardless of light regimens, our actual g_s values were relatively similar to the value of 108 mmol H_2O m^{-2} s^{-1} averaged over a range of studies using coffee plants grown under optimal conditions ([10], and references therein). These values are significantly lower than those of the modelled g_{wmax}, resulting in a g_{wmax}/g_s ratio above 13, as can be calculated for sun-grown plants. By comparison, that ratio was *c.* 2.5 in non-water-stressed tomato [52] and 4.5 in *Eucalyptus globulus* [33]. The high hydraulic resistance of the coffee leaves is most likely the cause of the difference between the theoretical g_{wmax} and the recorded g_s. Nevertheless, this large difference raises the following questions: why would the plant invest in a large g_{wmax} if the maximum realisable A is relatively low and constrained by the leaf hydraulic architecture? Furthermore, from an ecological point of view, what would be the advantage of having a large g_{wmax} if, in the humid, shaded understoreys where coffee evolved, A should be more constrained by light limitation than by CO_2 supply? Despite not having immediate responses to these queries, our results suggest a lack of coordination between the maximum capacity for stomatal aperture and carbon fixation, as also noted in saplings of Bornean rainforest tree species grown in the understorey [53]. A large g_{wmax} may not be problematic in terms of water loss in the humid understorey, where transpiration rates are expected to be much more dependent on boundary layer resistance, and thus the importance of the stomata in optimising photosynthetic gas exchange should be reduced. In any case, considering that bigger stomata tend to close slower than smaller stomata [33,54], the relatively large stomata of coffee leaves (combined with low K_L) might result in excessive leaf desiccation if large stomatal apertures are realised. In this sense, the low actual g_s might be a conservative strategy to maintain leaf hydration and minimise the risk of xylem embolism. This observation is in line with recent results obtained for *Toona ciliata*, where transpirational homeostasis to changes in vapour pressure deficit was achieved through dynamic stomatal control rather than modification of the relationship between veins and stomata [55]. Taken together, these findings lead to the interesting question of why long-term adjustments to the parameters that define g_{smax} have not been fixed and regulation of g_s comes predominantly from short-term adjustments to environmental conditions but at the cost of inherently low g_s in some species, such as coffee.

Our maximum g_m value was *c.* two-fold higher than those previously reported for *C. arabica* seedlings [56]. In any case, our g_m values were similar to those obtained for other evergreen woody species (e.g. [57–59]). Greater g_m values for sun leaves, as found here, have been systematically reported and have been often explained by anatomical and morphological differences between shade and sun leaves [60]. Despite the changes in g_m and A between shade- and sun-grown coffee plants, C_i and C_c remained fairly similar. Thus, regardless of the light treatments, when A changed, g_m and g_s scaled accordingly, and, hence, C_c remained constant [61]. This proportional scaling lends support to explain why stomatal, mesophyll and biochemical limitations to photosynthesis were similar between sun and shade leaves. These findings are in agreement with other studies, which show that the approximate scaling of g_s and g_m with A makes the relative limitations to photosynthesis rather conservative between sun and shade leaves, as also noted in *Fagus sylvatica* [62].

Irrespective of the light environment, the mean drawdown from C_i to C_c (*c.* 79 μmol mol^{-1}) was lower than that from C_a to C_i (*c.* 153 μmol mol^{-1}), which is consistent with the fact that the diffusive limitations to CO_2 in mesophyll were lower than those in

Table 2. Mean values for the photosynthetic and respiration parameters of coffee plants grown under shade or full sunlight conditions.

Parameters	Treatments	
	Shade	**Full Sun**
A (μmol CO_2 m^{-2} s^{-1})	7.9±0.4	12.0±0.8*
g_s (mmol H_2O m^{-2} s^{-1})	94±9	146±17*
g_{m_Harley} (mmol CO_2 m^{-2} s^{-1})	76±4	116±10*
g_{m_Ethier} (mmol CO_2 m^{-2} s^{-1})	65±12	109±5*
C_i (μmol CO_2 mol^{-1} air)	247±11	246±7
C_{c_Harley} (μmol CO_2 mol^{-1} air)	143±10	142±9
J_F (μmol e^- m^{-2} s^{-1})	70±5	106±5*
V_{cmax_Ci} (μmol CO_2 m^{-2} s^{-1})	26.8±1.6	42.1±2.2*
V_{cmax_Cc} (μmol CO_2 m^{-2} s^{-1})	58.1±3.5	78.5±4.0*
J_{max_Ci} (μmol e^- CO_2 m^{-2} s^{-1})	71.1±4.4	109.5±3.1*
J_{max_Cc} (μmol e^- m^{-2} s^{-1})	102.7±6.3	142.6±7.4*
J_{max}/V_{cmax_Ci}	2.6±0.04	2.7±0.1
J_{max}/V_{cmax_Cc}	1.8±0.1	1.8±0.1
C_{c_trans} (μmol CO_2 mol^{-1} air)	228±17	249±23
R_D (μmol CO_2 m^{-2} s^{-1})	0.5±0.06	1.2±0.05*
R_L (μmol CO_2 m^{-2} s^{-1}	0.1±0.05	0.3±0.08*
A/g_s (μmol CO_2 mol^{-1} H_2O)	86±6	84±4
g_{m_Harley}/g_s (mol CO_2 mol^{-1} CO_2)	1.4±0.1	1.3±0.1
R_p (μmol CO_2 m^{-2} s^{-1})	3.2±0.4	4.5±0.1*
Stomatal limitation	0.41±0.04	0.38±0.02
Mesophyll limitation	0.30±0.02	0.30±0.01
Biochemical limitation	0.29±0.03	0.32±0.03

$n = 6$±SE. The overall photosynthetic limitations associated with stomatal, mesophyll and biochemical factors are also shown. Data for A, g_s, g_{m_Harley}, C_i, C_{c_Harley}, J_F, and R_P were obtained under PPFD of 1000 μmol m^{-2} s^{-1}, C_a of 400 μmol mol^{-1} and leaf temperature of 25°C. Asterisks indicate statistically significant differences ($P<0.05$) between shade and full sun treatments.

Abbreviations: A, net photosynthesis; g_s, stomatal conductance to water vapour; g_m, mesophyll conductance to CO_2; C_i, sub-stomatal CO_2 concentration; C_c, chloroplastic CO_2 concentration; J_F, electron transport rate estimated by chlorophyll fluorescence; V_{cmax}, maximum carboxylation capacity; J_{max}, maximum capacity for electron transport rate; C_{c_trans}, the C_c that denotes the transition between the Rubisco- and RuBP regeneration-limited state; R_D, dark respiration; R_L, light respiration; R_P, photorespiration rate.

doi:10.1371/journal.pone.0095571.t002

stomata, thus ultimately reflecting a high g_m/g_s ratio. Indeed, by analysing the dataset reported by Flexas et al. [63], we noted that species operating at low C_i tended clearly to display an increased g_m/g_s ratio (Figure S1) which contributes to an improved performance in photosynthetic water use [63]. We believe that such feature is essential for keeping a positive carbon balance given that stomatal limitations may even be exacerbated in coffee trees grown under field conditions, particularly because g_s peaks in the early morning and decreases progressively throughout the day, reaching values typically ranging from 10 to 50 mmol H_2O m^{-2} s^{-1} from midday onwards [23,24,64–67] as a consequence of rising vapour pressure deficit.

Under saturating PPFD, A at ambient CO_2 was limited by Rubisco activity regardless of the light treatment given that the estimated C_c was lower than C_{c_trans} (Table 2). However, the realised A and J_F at maximum growth irradiance of shade plants (*c.* 200 μmol PPFD m^{-2} s^{-1}) are *c.* 60% of their saturating values (Figure S2), indicating that, even though these plants operate during most of their development under light limitation, the balance between RuBP regeneration and Rubisco activity (the J_{max}/V_{cmax} ratio) was essentially similar in the shade and sun

Table 3. Rubisco kinetic constants measured for coffee (taken from Martins et al. [27]).

Species	$S_{c/o}$	Γ* (μbar)	K_c (μM)	K_o (μM)	k_{cat}^c (s^{-1})
Coffee	98.4±4.3	39.6±1.7	10.3±1.3	479±113	3.2±0.1

$n = 4$±SE.

Abbreviations: $S_{c/o}$, Rubisco specificity factor; Γ*, CO_2 compensation point in the absence of mitochondrial respiration; K_c and K_o, the Michaelis-Menten kinetics for CO_2 and O_2, respectively; k_{cat}^c, Rubisco catalytic turnover rate for the carboxylase reaction.

doi:10.1371/journal.pone.0095571.t003

plants (Table 2). These data indicate that, in contrast to the optimal distribution principle, which suggests that Rubisco and the regeneration of RuBP should co-limit photosynthesis such that no excess capacities remain [68], the coffee plant does not properly optimise its resource allocation. On the one hand, under light saturation, an excess of electron transport capacity is to be expected, given that the actual C_c operates far from C_{c_trans} (*cf.* Table 2). On the other hand, under light limitation, a great investment in capacity for carbon assimilation and electron transport is retained despite the low realised A and $\mathcal{J}_F$ by the shade leaves.

Coffee Rubisco was characterised as displaying (i) a relatively high $S_{c/o}$, similar to that of woody evergreen species from xeric habitats [14]; (ii) a higher affinity for CO_2 (low K_c), which ranks coffee Rubisco with the third lowest value of K_c among C_3 and C_4 plants recorded to date [69]; and (iii) a relatively high k_{cat}^c, superior to the average of species from warm climates [70]. We next modelled the responses of A as a function of C_c by comparing these Rubisco properties with those of several C_3 species (Table S1) including *Limonium gibertii*, the species with the highest $S_{c/o}$ and lowest K_c reported to date, thus particularly adapted to low C_c [14]. Importantly, we found that, all else being equal, the A values that would be achieved using the Rubisco kinetic properties of coffee or *Limonium* would be quite similar and superior to those from all other species analysed (Figure 2) suggesting that coffee presents an "efficient" Rubisco well-tuned for operating at low C_c. Additionally, the higher k_{cat}^c and lower K_c mean that fewer Rubisco molecules are required to realise a given A. Nevertheless, given that lower K_c leads to a reduction in C_{c_trans}, A of coffee leaves is expected to begin to be limited by RuBP regeneration at relatively low C_c, which could, to some extent, reduce the revenue stream in a scenario of increasing CO_2 atmospheric levels.

The rates of respiration and photorespiration are other key processes influencing the plant carbon balance. In sun plants, R_D and R_P corresponded to 10% and 38% of A, respectively. However, given that R_L represented only a fraction (25%) of R_D, which is in line with the inhibition of mitochondrial respiration in the presence of light [71,72], the realised constraint of respiration on A is expected to be relatively low. In turn, R_P is expected to significantly affect A at midday, when the stomatal closure in coffee leaves exacerbates the drawdown from C_a to C_i [24], thus favouring the oxygenase activity of Rubisco.

Conclusion

Regardless of the light treatments, A was mainly limited by stomatal factors followed by similar limitations associated with the mesophyll and biochemical constraints. Our data suggest that an increased g_m/g_s ratio coupled with a Rubisco well-tuned for operating at low C_c might be adaptations to a lower C_i resulting from the low g_s; these adaptations might have helped the establishment of coffee plants when they were moved from the understoreys to the more stressful conditions of open fields, where high vapour pressure deficit and elevated temperatures may constrain gas exchange. Our results also suggest that the coffee plant does not properly optimise its resource allocation: on the one hand, there seems to be an over-investment in capacity for carboxylation and electron transport despite limited light availability under shade; on the other hand, an excess of electron transport capacity is to be expected under full sun. Nevertheless, this excess might be useful in attempts to increase A in coffee through breeding aimed at improving hydraulic traits (D_v and K_L)

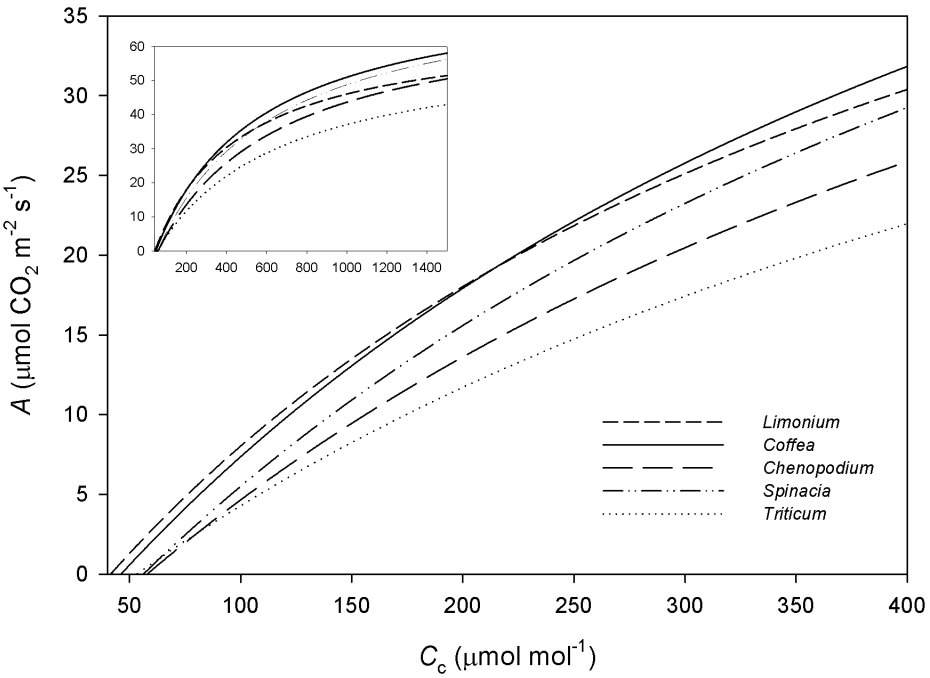

Figure 2. Comparison of the CO_2 assimilation rate as a function of C_c, modelled with the kinetic parameters of Rubisco in coffee and several C_3 species. The RuBP saturated rates of CO_2 assimilation at 25°C were calculated using the following equation [13]: $A=(Cc-\Gamma^*)\cdot(k\text{cat}^c\cdot[\text{Rubisco}])/\{Cc+Kc(1+O/Ko)\}-RL$. C_c, the chloroplastic CO_2 partial pressure; O, the intercellular O_2 partial pressure; Γ^*, the CO_2 compensation point in the absence of day respiration; [Rubisco], the catalytic site content of Rubisco. The Rubisco kinetic properties for coffee (Table 3) and the kinetic properties for the other species were retrieved from Savir et al. [69] and are summarized in Table S1. The R_L and [Rubisco] were set as 1.0 μmol CO_2 m^{-2} s^{-1} and 25 μmol sites m^{-2}, respectively.
doi:10.1371/journal.pone.0095571.g002

and expectedly supporting a high g_s. Finally, we contend that the large diffusive resistance should lead to large drawdown from C_a to C_c, thus favouring the occurrence of relatively high R_p despite the relatively high $S_{c/o}$ of coffee Rubisco, which ultimately leads to further limitations to A.

Supporting Information

Figure S1 The relationship between sub-stomatal CO_2 concentration and mesophyll-to-stomatal conductance ratio in the multi-species dataset from Flexas et al. [63].
(TIF)

Figure S2 The response of net photosynthetic rate and electron transport rate to the photosynthetic photon flux density in coffee plants developed under shade or full sun.
(TIF)

Table S1 The Rubisco kinetic constants for several C_3 species.
(DOCX)

Author Contributions

Conceived and designed the experiments: SCVM JG FMD. Performed the experiments: SCVM PCC LFP MCV. Analyzed the data: SCVM JG FMD. Contributed reagents/materials/analysis tools: FMD. Wrote the paper: SCVM JG FMD.

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