



RESEARCH PAPER

Sustained enhancement of photosynthesis in coffee trees grown under free-air CO₂ enrichment conditions: disentangling the contributions of stomatal, mesophyll, and biochemical limitations

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Abstract

Coffee (*Coffea* spp.), a globally traded commodity, is a slow-growing tropical tree species that displays an improved photosynthetic performance when grown under elevated atmospheric CO₂ concentrations ([CO₂]). To investigate the mechanisms underlying this response, two commercial coffee cultivars (Catuaí and Obatã) were grown using the first free-air CO₂ enrichment (FACE) facility in Latin America. Measurements were conducted in two contrasting growth seasons, which were characterized by the high (February) and low (August) sink demand. Elevated [CO₂] led to increases in net photosynthetic rates (*A*) in parallel with decreased photorespiration rates, with no photochemical limitations to *A*. The stimulation of *A* by elevated CO₂ supply was more prominent in August (56% on average) than in February (40% on average). Overall, the stomatal and mesophyll conductances, as well as the leaf nitrogen and phosphorus concentrations, were unresponsive to the treatments. Photosynthesis was strongly limited by diffusional constraints, particularly at the stomata level, and this pattern was little, if at all, affected by elevated [CO₂]. Relative to February, starch pools (but not soluble sugars) increased remarkably (>500%) in August, with no detectable alteration in the maximum carboxylation capacity estimated on a chloroplast [CO₂] basis. Upregulation of *A* by elevated [CO₂] took place with no signs of photosynthetic downregulation, even during the period of low sink demand, when acclimation would be expected to be greatest.

Key words: Carbohydrates, *Coffea arabica* L., FACE, nitrogen, photosynthetic limitations, photosynthetic acclimation, starch.

Introduction

The actual increase in atmospheric CO₂ concentration ([CO₂]) is one of the most well-documented aspects of global climatic change. In 2014, [CO₂] exceeded, for the first time in at least the past 650 000 years, 400 µmol mol⁻¹ of air, as

recorded at the Mauna Loa Observatory in Hawaii. Indeed, over the last decade, atmospheric [CO₂] has increased at a rate of approximately 2 µmol mol⁻¹ of air year⁻¹, and projections suggest that atmospheric [CO₂] will exceed 936 µmol mol⁻¹ of

air (Representative Concentration Pathway 8.5) by the end of this century (IPCC, 2013). Given that photosynthesis is limited by the CO₂ supply at the current atmospheric [CO₂] (Long *et al.*, 2004), an increase in [CO₂] is expected to increase the net photosynthetic rate (*A*) of plants (Ainsworth and Long, 2005). In fact, theoretical analyses suggest that *A* values might increase by 38% as atmospheric [CO₂] increases from 380 to 550 μmol mol⁻¹ of air (von Caemmerer and Furbank, 2003). Nevertheless, the results from free-air CO₂ enrichment (FACE) experiments reveal that the photosynthesis of crop plants fails to match the theoretical increase that could be obtained under elevated [CO₂] and that increases in *A* do not always translate into concordant increases in biomass production and crop yield (Long *et al.*, 2006; Leakey *et al.*, 2009). In any case, increased *A* under elevated [CO₂] is accompanied by consistent, although not universal, reductions in stomatal conductance (*g_s*) (Medlyn *et al.*, 2001). Meta-analyses of FACE experiments have reported mean reductions in *g_s* of 16–19% (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007). The dichotomous responses of *A* and *g_s* to elevated atmospheric [CO₂] ultimately lead to improvements in the water-use efficiency of the plant, reduced soil moisture depletion, and ameliorated stress during periods of drought (DaMatta *et al.*, 2010a).

In addition to *g_s*, an increasing body of evidence suggests that the mesophyll conductance (*g_m*), which is defined as the conductance for the transfer of CO₂ from the intercellular airspaces (*C_i*) to the sites of carboxylation in the chloroplastic stroma (*C_c*), may also be a key constraint to photosynthesis (Flexas *et al.*, 2012; Sun *et al.*, 2014). However, the potential responses of *g_m* to climate changes, including elevated atmospheric [CO₂], have largely been neglected up to now (Flexas *et al.*, 2012, 2014). In contrast to the well-known overall pattern of decreased *g_s* under long-term [CO₂] exposure, the available information shows no general trend for *g_m* in plants grown under elevated [CO₂] (i.e. 500–600 μmol mol⁻¹ of air); indeed, no change, either decreased or increased *g_m*, has been reported, possibly depending on the species and time (Flexas *et al.*, 2012, and references therein). In any case, not considering *g_m* in photosynthesis calculations can significantly alter our interpretation of the effects of environmental factors, including CO₂ supply, on photosynthesis (Sun *et al.*, 2014). For example, mis-interpretations of photosynthetic downregulation (see below) based on decreases in the maximum apparent carboxylation capacity (*V_{cm}*) on a *C_i* basis may occur when a decreased *g_m* under elevated [CO₂] is not considered.

In some FACE studies that were conducted with temperate tree species, the initial stimulation of *A* was preserved during the long-term exposure to elevated [CO₂] (Liberloo *et al.*, 2007; Bader *et al.*, 2010; Streit *et al.*, 2014). Frequently, however, plants that were exposed to elevated [CO₂] in FACE trials show reductions in photosynthetic capacity (Rogers and Ellsworth, 2002; Hyvönen *et al.*, 2007; Norby *et al.*, 2010), termed photosynthetic downregulation (acclimation), associated with end-product accumulation (Ainsworth and Rogers, 2007; Leakey *et al.*, 2009) that is usually linked to nutrient limitations, particularly nitrogen (N) (Ainsworth and Long,

2005). A reduced or acclimated stimulation of photosynthesis has been mechanistically and quantitatively attributed to decreased *V_{cm}* and investment in ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Ainsworth and Long, 2005), but may also be linked to a reduced ribulose-1,5-bisphosphate (RuBP) regeneration, decreasing the *in vivo* maximum rate of carboxylation that is limited by electron transport (*J_{max}*) due to either a lowered electron transport capacity or inorganic phosphate availability in the chloroplast for ATP synthesis (Ainsworth and Rogers, 2007; Kirschbaum, 2011; Zhu *et al.*, 2012). As a rule, limited sink strength predisposes plants to a greater acclimation of photosynthetic capacity and decreases the stimulation of photosynthesis by growth under elevated [CO₂] (Long *et al.*, 2004; Ainsworth and Rogers, 2007). Overall, trees, particularly fast-growing individuals, display a large sink capacity (root–trunk system) compared with that of annuals, which, to a great extent, explains their higher stimulation of photosynthesis when grown under elevated atmospheric [CO₂] compared with shrubs and annual crops (Ainsworth and Long, 2005).

Coffee is one of the most heavily globally traded commodities, with retail sales worldwide estimated at US\$90 billion (DaMatta *et al.*, 2010b). The coffee tree is a slow-growing evergreen species that displays relatively low *A*, with maximum values typically around or below 10 μmol m⁻² s⁻¹ with current atmospheric [CO₂] and saturating light (DaMatta, 2004). Such low values have largely been associated with diffusive rather than biochemical limitations to photosynthesis (Batista *et al.*, 2012), particularly diffusional constraints at the stomatal level (Martins *et al.*, 2014). Consequently, coffee trees are expected to largely benefit, in terms of photosynthetic performance, by the elevated atmospheric [CO₂], considering that increasing atmospheric [CO₂] increases the gradient that ensures an adequate diffusion of CO₂ from the atmosphere to the chloroplasts. Indeed, the recent efforts of Ramalho *et al.* (2013), who studied potted coffee plants growing in an enclosure system under different [CO₂] over 1 year, revealed greater *A* (ranging from 34 to 49%) under elevated (700 μmol CO₂ mol⁻¹ of air) than under normal (380 μmol CO₂ mol⁻¹ of air) [CO₂]. This positive effect was later confirmed by Ghini *et al.* (2015) in coffee plants that were grown using the first FACE facility in Latin America (ClimapestFACE). Interestingly, *g_s* responds little, if at all, to elevated [CO₂] in coffee; taken together, these results imply that substantial increases in water-use efficiency occur in coffee grown under elevated [CO₂] conditions (Ramalho *et al.*, 2013; Ghini *et al.*, 2015).

In this study, two commercial coffee cultivars with contrasting crop yields were grown under current ambient and elevated [CO₂] at the ClimapestFACE facility (Ghini *et al.*, 2015). We demonstrated previously that, integrated over the course of the day, the *A* values that were measured *in situ* were ≥40% higher for the plants that were grown under elevated atmospheric [CO₂], which may in part explain the enhanced crop yields under elevated [CO₂] (Ghini *et al.*, 2015). Given these previous results and those of enclosure studies (Ramalho *et al.*, 2013), our main goal was to examine the mechanisms underlying this response. To achieve this goal, we conducted

an in-depth analysis of the photosynthetic performance by calculating g_m to properly parameterize the responses of A to C_c in addition to disentangling the relative contributions of the stomatal, mesophyll, photochemical, and biochemical limitations of photosynthesis. This analysis was conducted in two contrasting growth seasons, which were characterized by strong and weak sink demand, when photosynthetic down-regulation, if occurring, would be expected to be minimal and maximal, respectively.

Materials and methods

Site description, CO₂ treatments, plant materials, and samplings

We carried out the experiment using the ClimapestFACE facility located in Jaguariúna (22°43'S, 47°01'W, 570 m above sea level), south-eastern Brazil. The soil at the experimental area is a typical dystroferic red latosol. The climate is humid subtropical, a Cfa type according to the Köppen classification, with hot rainy summers and cold dry winters. Mean monthly air temperature and precipitation were recorded during the experiment (see [Supplementary Fig. S1 at JBX online](#)).

To mimic coffee agrosystems, the FACE system increased the ambient [CO₂] in six 10 m diameter ring plots (elevated CO₂) within a continuous 7 ha coffee field. Six additional 10 m diameter ring plots served as controls, i.e. were left under ambient [CO₂]. Elevated- and ambient-CO₂ plots were at least 70 m apart to minimize cross-plot contamination.

Fumigation with CO₂ began on 25 August 2011. The average atmospheric [CO₂] at the beginning of the experiment was approximately 390 µmol mol⁻¹. The performance of the FACE system was adjusted so that the [CO₂] as measured at the centre of the ring achieved target levels of 550 µmol mol⁻¹ of air. The plots were not enriched with CO₂ at night. Further details regarding the experimental site set-up and CO₂ control performance can be found in [Ghini et al. \(2015\)](#).

Two coffee (*Coffea arabica* L.) cultivars, cv. Catuaí Vermelho IAC 144 and cv. Obatã IAC 1669-20, were assessed. The latter displays significantly greater (>30%) crop yields than the former, as found in test trials under common agronomic practices. Plantlets with three to four pairs of leaves were transplanted into the plots in March 2011. The cultivars were interspersed in rows that were 1.75 m apart, with 0.60 m between plants in the rows. The plots were located in a continuous 7 ha coffee field of cv. Catuaí Vermelho IAC 144, into which plantlets were transplanted on March 2010; the rows were 3.5 m apart, with 0.60 m between plants in the rows. Fertilization per hectare at planting was accomplished with 2300 kg of single super phosphate, 2300 kg of dolomitic limestone, 285 kg of chloride potassium and 570 kg of Yoorin Master 2S® (a granulated fertilizer with fast and slow release of P, Ca, Mg and S). The plants were submitted to routine agricultural practices for commercial coffee bean production, including several applications of herbicides, fungicides, and insecticides. Each tree was fertilized annually with 46 g of N, 9 g of P and 23 g of K plus micronutrients. The fertilizer was applied three times during the growing season, coinciding approximately with periods of supplemental fertilization for most commercial coffee crops in Brazilian Arabica coffee-producing regions. The crop was grown without supplemental irrigation.

Sampling and measurements were carried out in two contrasting periods of the coffee growth cycle: February and August (2013). In February, the vegetative growth rates are relatively low due to competition with reproductive growth (bean-filling phase), and thus assimilate demand is at its maximum; in August, the plants are fruitless (crop harvests are usually completed in June–July), and vegetative growth is negligible. Thus, assimilate demand by the sinks is at its minimum ([Silva et al., 2004](#)).

Gas-exchange and chlorophyll *a* fluorescence measurements

The net rate of carbon assimilation (A), stomatal conductance [which was subsequently converted into stomatal conductance to CO₂ (g_s)] and internal CO₂ concentration (C_i) were measured simultaneously with chlorophyll *a* fluorescence parameters in the youngest fully expanded leaves [the third or fourth leaf pair from the apex of the plagiotropic (lateral) branches in the upper third of the plants] using an open system under ambient temperature and a [CO₂] of 390 or 550 µmol mol⁻¹ of air, depending on the treatment. All of the measurements, which were conducted using three cross-calibrated infrared gas analysers (LI-6400XT; Li-Cor, Lincoln, NE, USA) using an integrated fluorescence chamber head (LI-6400-40; Li-Cor), were made on clear-sky days during four time periods: 08:00–09:00, 10:00–11:00, 13:00–14:00, and 16:00–17:00 (solar time). To improve the uniformity over the course of the day, the measurements were conducted at the leaf level at an artificial photosynthetically active radiation (PAR) level of 1000 µmol of photons m⁻² s⁻¹. This PAR intensity is sufficiently high to saturate the photosynthetic machinery without causing photoinhibition ([Cavatte et al., 2012](#)); in addition, these PAR values approximated the ambient irradiance that was intercepted by the sampled leaves (in their natural angles) in most measurements at each time point. After the leaf tissue was clamped in the leaf chamber, the gas-exchange rates usually stabilized within approximately 3 min. In both February and August 2013, the measurements were repeated once using different plants within a given ring of the FACE. Even without supplemental irrigation, the pre-dawn xylem water potential, as measured with a Scholander chamber, was greater than –0.1 MPa in both periods, suggesting that our gas-exchange data were not constrained by the water supply.

In light-adapted leaves, the steady-state fluorescence yield (F_s) was measured after registering the gas-exchange parameters. A saturating white light pulse (8000 µmol m⁻² s⁻¹; 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off, and far-red illumination was applied (2 µmol m⁻² s⁻¹) to measure the light-adapted initial fluorescence (F_0'). Using the values of these parameters, the photochemical quenching coefficient (q_p) and the capture efficiency of excitation energy by open photosystem (PS) II reaction centres (F_v'/F_m') were estimated ([Logan et al., 2007](#)). The actual PSII photochemical efficiency (ϕ_{PSII}) was determined as $\phi_{PSII} = (F_m' - F_s)/F_m'$ following the procedures of [Genty et al. \(1989\)](#). The electron transport rate (ETR) was then calculated from the equation $ETR = \phi_{PSII} \times \beta \times \alpha \times \text{photosynthetic photon flux density (PPFD)}$, where α is leaf absorptance, and β reflects the partitioning of absorbed quanta between PSII and PSI ([Genty et al., 1989](#)). The product of β and α was determined according to [Valentini et al. \(1995\)](#) from the relationship between ϕ_{PSII} and ϕ_{CO_2} as obtained by varying the light intensity under non-photorespiratory conditions.

The rate of mitochondrial respiration in darkness (R_D) was measured early in the morning in dark-adapted leaves and used to estimate mitochondrial respiration in the light (R_L) according to [Lloyd et al. \(1995\)](#) as $R_L = [0.5 - 0.05 \ln(\text{PPFD})] \times R_D$.

The photorespiratory rate of Rubisco (R_p) was calculated as $R_p = 1/12[ETR - 4(A + R_L)]$ according to [Valentini et al. \(1995\)](#). The R_p/A_{gross} ratio was obtained throughout the day by computing the values of R_p , A , and R_L . The values of R_L were corrected for leaf temperature (assessed with temperature sensors coupled to infrared gas analysers over the course of the gas-exchange measurements) using the temperature response for R_L as described by [Bernacchi et al. \(2001\)](#).

Four to six A/C_i curves were obtained *in situ* (from approximately 07:00 to 10:30 in February, and from approximately 08:30 to 12:00 in August, when g_s values were relatively elevated) from different plants per treatment. These curves were initiated at an ambient [CO₂] (C_a) of 400 µmol mol⁻¹ under a saturating PPFD of 1000 µmol m⁻² s⁻¹. Once a steady state was reached, C_a was gradually decreased to 50 µmol mol⁻¹ of air. Upon the completion of the measurements at low C_a , C_a was returned to 400 µmol mol⁻¹ of air to restore the original A . Next, C_a was increased stepwise to 1600 µmol mol⁻¹ of

air. The A/C_i curves consisted of 13 different C_a values. This procedure was successfully applied to the measurements that were conducted in February. In August, however, due to the intrinsically low g_s for the season (coupled to the negative g_s response to increases in C_a), we were unable to reliably estimate C_i at C_a values greater than $600 \mu\text{mol mol}^{-1}$. Therefore, A/C_i curves only consisted of seven different C_a values ($<600 \mu\text{mol mol}^{-1}$). Regardless of the season, corrections for the leakage of CO_2 into and out of the leaf chamber of the LI-6400 were applied to all gas-exchange data as described by Rodeghiero *et al.* (2007).

Estimations of the mesophyll conductance to CO_2 (g_m), the maximum rate of carboxylation (V_{cmax}), the maximum rate of carboxylation limited by electron transport (J_{max}), and the chloroplastic $[\text{CO}_2]$ of transition ($C_{\text{c,trans}}$)

C_c was estimated according to Harley *et al.* (1992) as follows:

$$C_c = \{\Gamma^*[\text{ETR} + 8(A + \text{RL})]/[\text{ETR} - 4(A + \text{RL})]\}$$

where ETR and A were obtained from the gas-exchange and chlorophyll a fluorescence measurements as conducted under saturating light; R_L was estimated as described above; and Γ^* is the CO_2 compensation point in the absence of mitochondrial respiration (the conservative value of Γ^* for coffee was obtained from Martins *et al.*, 2013). We next estimated g_m as the slope of the A versus $C_i - C_c$ relationship as $A = g_m(C_i - C_c)$ so the estimated g_m was an averaged value over the points used in the relationship ($C_i < 350 \mu\text{mol mol}^{-1}$ of air).

Because all of the available methods for estimating g_m rely on models that include several assumptions as well as technical limitations and sources of error that need to be considered to obtain reliable estimates of this parameter (Pons *et al.*, 2009), g_m was also estimated using an alternative approach: the exhaustive dual optimization (EDO) curve-fitting technique of Gu *et al.* (2010). For this purpose, the data from the A/C_i curves were uploaded to the Oak Ridge National Laboratory (USA) website (<http://leafweb.ornl.gov>), which uses EDO to parameterize A/C_i curves based on the Farquhar–von Caemmerer–Berry model (Gu *et al.*, 2010).

The g_m values were used to convert $A - C_i$ into $A - C_c$ curves. From these curves, V_{cmax} and J_{max} were calculated by fitting the mechanistic model of CO_2 assimilation as proposed by Farquhar *et al.* (1980) using the C_c -based temperature dependence of the kinetic parameters of Rubisco (Bernacchi *et al.*, 2002). The curve-fitting procedures have been detailed elsewhere (Martins *et al.*, 2014). Afterwards, the photosynthetic parameters V_{cmax} , J_{max} , and g_m were normalized to 25°C using the temperature response equations from Sharkey *et al.* (2007).

The chloroplastic $[\text{CO}_2]$ of transition ($C_{\text{c,trans}}$), which denotes the transition between the Rubisco- and RuBP regeneration-limited states, was estimated as described by Gu *et al.* (2010):

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

where K_m , the effective Michaelis–Menten constant for CO_2 that considers the competitive inhibition by O_2 , was taken from Martins *et al.* (2013). $C_{\text{c,trans}}$ was calculated using V_{cmax} , J_{max} , K_m , and Γ^* at the ambient temperatures realized during the gas-exchange measurements at 08:00–09:00 h in order to allow proper comparisons with the measured C_c at this time.

Quantitative analysis of the limitations of photosynthesis

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 that was proposed by Grassi and Magnani (2005) as follows:

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

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

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

where g_s is the stomatal conductance to CO_2 and g_m is the mesophyll conductance to CO_2 according to Harley *et al.* (1992), and g_{tot} is the total conductance to CO_2 from ambient air to chloroplasts ($g_{\text{tot}} = 1 / [(1/g_s) + (1/g_m)]$). $\partial A / \partial C_c$ was calculated as

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

Biochemical, N and P, analyses

Leaf discs were collected on clear-sky days prior to flash freezing in liquid nitrogen and subsequent storage at -80°C until analysis. For carbohydrate analyses, a 10 mg sample of ground tissue was added to pure methanol and the mixture was incubated at 70°C for 30 min. After centrifugation (13 000g, 5 min), the hexoses (glucose and fructose) and sucrose in the supernatant were quantified; the concentration of starch was determined from the methanol-insoluble pellet as detailed previously (Praxedes *et al.*, 2006; Ronchi *et al.*, 2006). The levels of malate and fumarate were determined exactly as reported elsewhere (Nunes-Nesi *et al.*, 2007).

Leaf samples were used to measure the N (using an elemental analyser; Carlo Erba, Milan, Italy) and P (using routine spectrophotometric methods) contents.

Statistical analyses

The experiment was designed as a split-plot design with CO_2 level as the whole-plot factor and cultivar as the subplot factor with six replicates per treatment (ambient and elevated $[\text{CO}_2]$), and the data were subjected to ANOVA. The treatment differences were also tested using ANOVA. Throughout the text, mean differences were considered significant at $P \leq 0.05$ (see Supplementary Table S1 at JBX online).

Results

Most of the significant treatment effects on the variables analysed were observed for the $[\text{CO}_2]$ factor rather than for the cultivar factor; overall, the traits showed no significant responses to the $[\text{CO}_2] \times$ cultivar interaction (see Supplementary Table S1).

During the growing season, A was significantly greater under elevated than under ambient $[\text{CO}_2]$ at two of the four time points in both cultivars (Fig. 1). g_s did not respond to the CO_2 enrichment in cv. Catuaí; in cv. Obatã, the g_s values at two time points were lower under elevated than under ambient $[\text{CO}_2]$ (Fig. 1). The R_p/A_{gross} ratio was clearly lower in the morning in both cultivars that were treated with CO_2 , with a less clear pattern in the afternoon (Fig. 1). Notably, diurnally integrated A was 40% greater and the R_p/A_{gross} ratio was 20% lower under elevated than under ambient $[\text{CO}_2]$, with no significant change in g_s in either cultivar (Fig. 1). During the period of limited growth (winter), both A and g_s values were, as expected, much lower than during the growing season, whereas an opposite pattern was noted for the R_p/A_{gross}

ratio (Fig. 1). In all of the winter measurements, both of the cultivars responded to CO₂ fertilization by increasing A significantly, with little, if any, alteration in g_s . The R_p/A_{gross} ratio was consistently lower in cv. Obatã under elevated than under ambient [CO₂], whereas in cv. Catuaí, this ratio was unresponsive to the CO₂ supply. The diurnally mean A values in winter averaged 56% higher in the plants that were treated with CO₂ than in their untreated counterparts. In cv. Obatã, g_s values integrated over the course of the day were significantly lower under elevated [CO₂] (Fig. 1).

Overall, diurnally integrated gas exchange was essentially similar in both cultivars during the growing season (Fig. 1). In winter, A was significantly greater in cv. Obatã (irrespective of CO₂ fertilization), and R_p/A_{gross} was lower in this cultivar (only at elevated [CO₂]) than in cv. Catuaí (Fig. 1).

From the chlorophyll a fluorescence analysis, we estimated the ETR, q_p , and F_v'/F_m' over the course of the day (Fig. 2). In both the growing season and winter, all of these traits were virtually unaltered by the CO₂ treatments. However, we noted that, irrespective of cultivar, the overall values of ETR and q_p were higher in the growing season than in the phase of restrained growth, whereas F_v'/F_m' varied little, if at all, between seasons.

We analysed g_m using two independent methods. Overall, similar values and trends for g_m were observed regardless of the experimental approach that was used to estimate g_m (Table 1). Therefore, only the g_m values that were obtained using the method of Harley *et al.* (1992) are discussed below and were used to parameterize the responses of A to C_c . In both the growing season and winter, g_m did not respond to the CO₂ treatments (Table 1). Both V_{cmax} and J_{max} , estimated on a C_c and C_i basis, were unresponsive to the CO₂ supply in the growing season, as was V_{cmax} during the winter (Table 1 and Supplementary Table S2 at JBX online). In fact, the absolute V_{cmax} values on a C_i basis were essentially similar regardless of the season (57 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ on average), as can be deduced from Supplementary Table S2. Notably, had the results been calculated only on a C_i basis, V_{cmax} would be underestimated by approximately 40%, thus highlighting the importance of estimating g_m to properly describe the responses of A to the CO₂ supply.

We also determined data for C_c and $C_{c,\text{trans}}$ (Table 1) as estimated in the early morning (08:00–09:00 h) when A is at its maximum. Overall, C_c was significantly higher under elevated than ambient [CO₂], and $C_{c,\text{trans}}$ (estimated only in the growing season due to the available J_{max} data) decreased under elevated [CO₂]. Regardless of cultivars, C_c was lower than $C_{c,\text{trans}}$ under ambient [CO₂]. Under elevated [CO₂], the values of C_c did not differ significantly from those of $C_{c,\text{trans}}$ irrespective of cultivar ($P > 0.05$).

The functional components (l_s , l_m , and l_b) of the overall photosynthetic limitations did not respond to [CO₂] in cv. Catuaí in the growing season (Fig. 3). In cv. Obatã, l_b was unresponsive to the [CO₂] supply, regardless of the season; however, elevated [CO₂] decreased l_s (37%) significantly in parallel with an increase in l_m (73%) in the growing season. In winter, regardless of cultivar, significant changes were only noticeable in l_m , which decreased by 50% under elevated [CO₂]

compared with under ambient [CO₂]. Importantly, our results indicate that diffusional limitation ($l_s + l_m$) accounted for the most prominent constraints to photosynthesis ($\geq 60\%$ in the growing season and $\geq 80\%$ in winter), and these responses were not altered by the CO₂ supply in either cultivar (Fig. 3). By comparing the two growth periods, the greater diffusional limitations in winter were chiefly associated with increased l_s (~60% vs ~37% in the growing season), whereas the magnitude of changes in l_m was much narrower when comparing both seasons. Increased diffusional constraints in winter resulted in a lower l_b that was approximately half relative to that obtained in the growing season (Fig. 3). We also assessed intrinsic physiological changes as a function of growth CO₂ by calculating the limitations using different ambient [CO₂] (390 $\mu\text{mol mol}^{-1}$ of air for the plants grown at 550 $\mu\text{mol mol}^{-1}$ of air and vice versa); the results were nearly identical to those shown previously (Fig. 3) with the exception that l_s was higher in cv. Obatã even in the winter season (see Supplementary Fig. S2 at JBX online).

In the growing season, the pools of carbohydrates (and malate/fumarate) were measured at the beginning, middle, and end of the photoperiod. Regardless of the cultivar, the concentrations of glucose, fructose, and sucrose were similar or slightly higher in the plants that were treated with CO₂ compared with their untreated counterparts, but only in a few measurements did these differences reach statistical significance (Fig. 4). The concentrations of starch were not significantly affected by the treatments in cv. Catuaí, whereas in Obatã, the starch levels were consistently higher (64% on average) under elevated than under ambient [CO₂]. In winter, measurements were conducted only at midday for both cultivars. The glucose and sucrose pools were unaffected by the CO₂ supply independently of cultivar, whereas the starch pools averaged 38% higher under elevated than under ambient [CO₂]. Fructose pools increased significantly in response to the CO₂ supply but only in cv. Catuaí. It should be emphasized that there was an almost invariant total soluble sugar concentration [represented by the sum of glucose, fructose, and sucrose (on average, the total soluble sugars totalled 6.6 and 6.8% of leaf dry weight in the growing season and winter, respectively; data not shown)], whereas the levels of starch were remarkably higher (522% on average) in winter than in the growing season.

We next evaluated the malate and fumarate pools (Fig. 4), given that the concentration of these organic acids often changes when the carbon balance is altered. In the growing season, the levels of malate were not significantly affected by the treatments in cv. Catuaí; in cv. Obatã, malate pools were consistently lower (24% on average) over the course of the day under elevated compared with under ambient [CO₂]. In winter, malate pools were significantly lower (33%) under elevated than under ambient [CO₂] in cv. Catuaí, whereas these pools were not affected by the CO₂ treatment in cv. Obatã. Regardless of the season, fumarate was not detected in this study.

The leaf N and P concentrations were approximately 3.5 and 1.5%, respectively, on a dry-weight basis and remained invariant regardless of treatment (data not shown). These concentrations are within an optimal range for coffee.

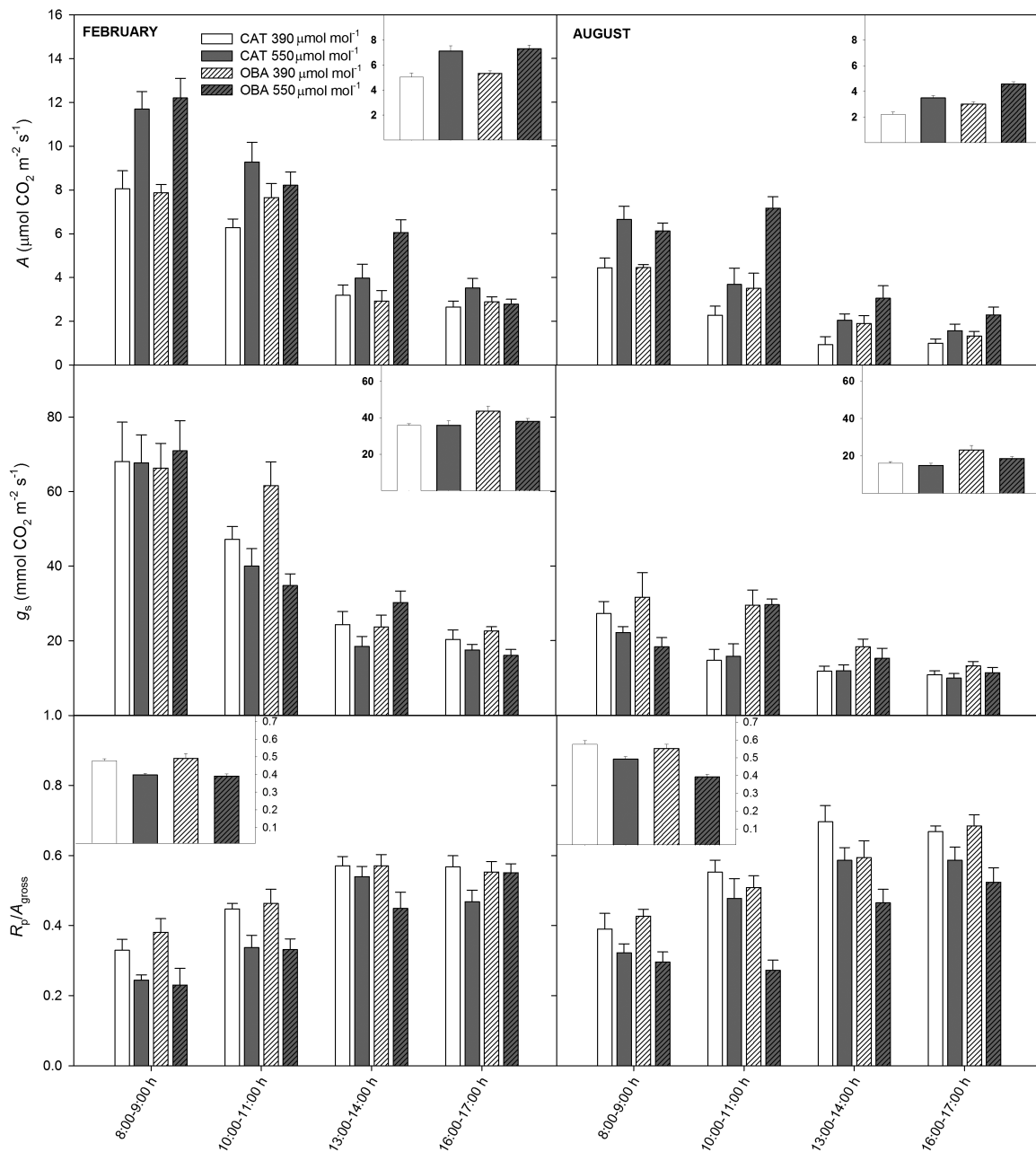


Fig. 1. Effect of elevated ($550 \mu\text{mol mol}^{-1}$) and ambient ($390 \mu\text{mol mol}^{-1}$) $[\text{CO}_2]$ on the leaf gas exchange of two coffee cultivars (Catuaí and Obatã) growing in a FACE trial during the growing season (February) and winter (August): net CO_2 assimilation rate (A), stomatal conductance to CO_2 (g_s), and ratio of photorespiration-to-gross photosynthetic rate (R_p/A_{gross}). In the insets, the mean diurnal values of A , g_s , and R_p/A_{gross} are shown. The $[\text{CO}_2]$ used during the gas-exchange measurements was identical to the growth $[\text{CO}_2]$ (390 or $550 \mu\text{mol mol}^{-1}$). The data for A were taken from Ghini *et al.* (2015). $n=6 \pm \text{SE}$.

Discussion

In this study, we examined in detail the underlying mechanism associated with the photosynthetic enhancement that we demonstrated previously in coffee trees when grown with supplemental CO_2 (Ghini *et al.*, 2015). First (and as expected), a higher A under elevated $[\text{CO}_2]$ was associated not only with improved carboxylation rates coupled with a higher availability of CO_2 as substrate (higher C_c) but also with a relatively higher carboxylation over oxygenation activity of Rubisco, resulting in decreased R_p , here noted through the lower R_p/A_{gross} ratio under elevated $[\text{CO}_2]$ (Fig. 1).

Given that the stimulation of A by CO_2 fumigation would increase the ATP demand that is required for RuBP regeneration and that the control of photosynthesis shifts from Rubisco-limited to RuBP regeneration-limited (Ainsworth and Rogers, 2007), we next analysed whether any impairment in leaf photochemistry could constrain the maximization of A under elevated $[\text{CO}_2]$ (Fig. 2). We found that the CO_2 stimulation of A was not accompanied by concordant alterations in the efficiency of the excitation energy as captured by the open PSII reaction centres (estimated as F_v'/F_m') and in the fraction of absorbed light that is dissipated photochemically (estimated as q_p). Importantly, the ETR values by far exceeded

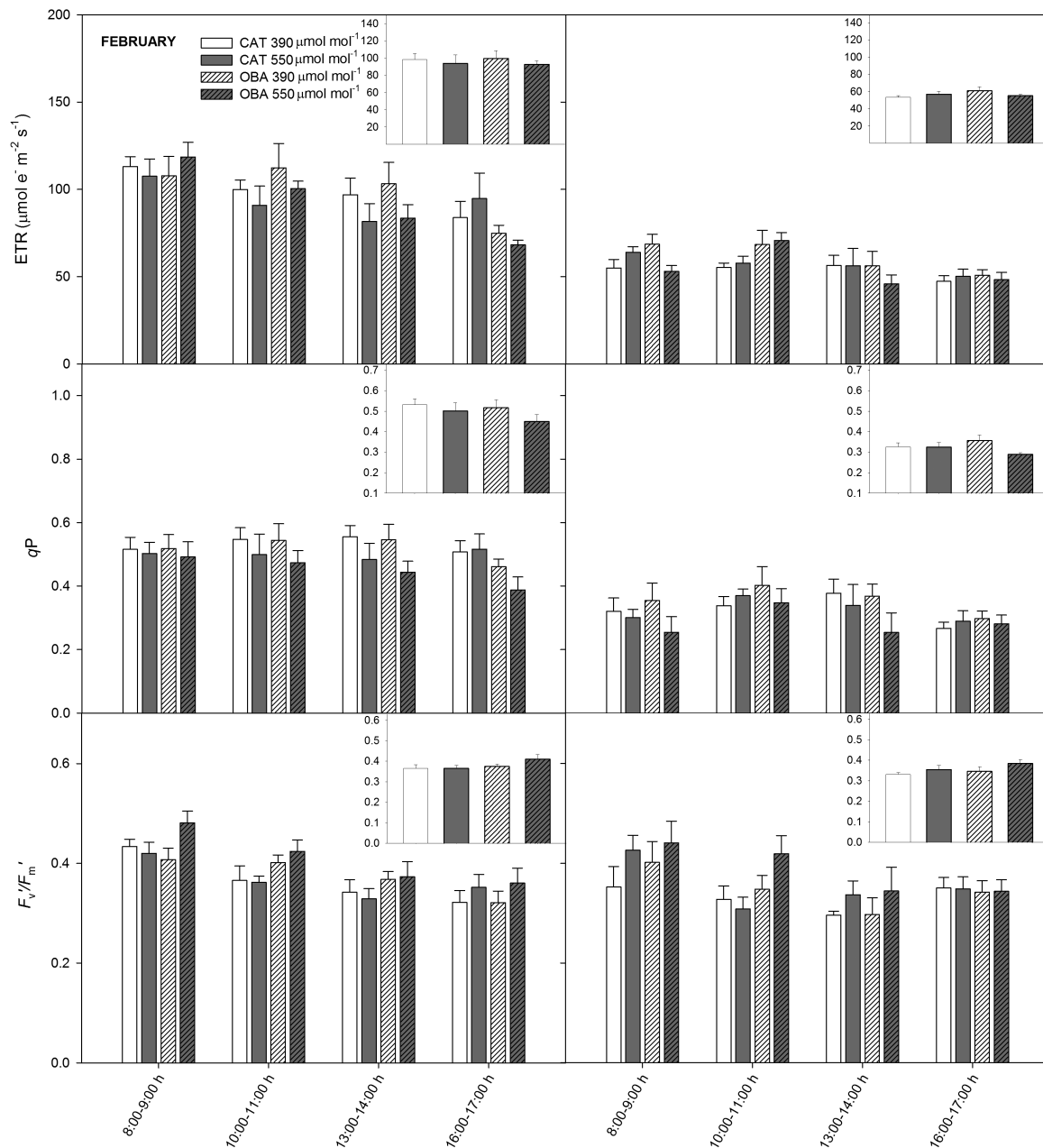


Fig. 2. Effect of elevated ($550 \mu\text{mol mol}^{-1}$) and ambient ($390 \mu\text{mol mol}^{-1}$) $[\text{CO}_2]$ on the photochemical parameters of two coffee cultivars (Catuaí and Obatã) growing in a FACE trial during the growing season (February) and winter (August): electron transport rate (ETR), photochemical quenching coefficient (q_p), and capture efficiency of excitation energy by open PSII reaction centres (F_v'/F_m'). In the insets, the mean diurnal values of ETR, q_p , and F_v'/F_m' are shown. The $[\text{CO}_2]$ used during the chlorophyll a fluorescence measurements was identical to the growth $[\text{CO}_2]$. $n=6 \pm \text{SE}$.

the photochemical needs that were required to support the observed A values (Martins *et al.*, 2013), irrespective of treatments. Therefore, our data indicated that photochemical events are unlikely to have limited CO_2 fixation in this study. In any case, seasonal photochemical adjustments (decreased q_p and ETR in winter) occurred independently of the CO_2 supply, but these adjustments should represent a consequence, rather than a cause, of the decreases in A in winter. In other words, adjustments probably occurred because the light-capture and light-utilization processes are imbalanced in coffee in winter, and thus adjustments in leaf photochemistry may be a proper way of avoiding the occurrence of photoinhibition

in coffee (Chaves *et al.*, 2008; Pompelli *et al.*, 2010), as could also be deduced here by the unchanging variable-to-maximum chlorophyll fluorescence ratio (data not shown).

We subsequently quantified the distribution of the overall photosynthetic limitations between diffusional and biochemical processes. To reach this goal, we first estimated g_m and found no g_m acclimation to elevated CO_2 (Table 1). Regardless of the CO_2 supply, we demonstrated that the major limitations to photosynthesis in coffee are linked to diffusional constraints (Fig. 3), with greater values of l_s than those of l_m , as also reported previously for potted coffee seedlings grown under ambient $[\text{CO}_2]$ (Martins *et al.*, 2014). In addition, we

Table 1. Effect of elevated (550 $\mu\text{mol mol}^{-1}$) and ambient (390 $\mu\text{mol mol}^{-1}$) [CO_2] on some photosynthetic parameters of two coffee cultivars (*Catuaí* and *Obatã*) growing in a FACE trial during the growing season (February) and winter (August)

The following values were determined: mesophyll conductance (g_m), estimated using two independent methods: the EDO curve-fitting technique and the method that was proposed by Harley *et al.* (1992); the maximum apparent carboxylation capacity (V_{cmax}) and the *in vivo* maximum rate of carboxylation as limited by electron transport (J_{max}), both on a chloroplast [CO_2] basis; and the chloroplastic [CO_2] (C_c) and C_c of transition ($C_{c,\text{trans}}$). Data for J_{max} and $C_{c,\text{trans}}$ were not obtained in August. V_{cmax} , J_{max} and g_m were normalized to 25 °C using the temperature response equations from Sharkey *et al.* (2007). $n=5-6\pm\text{SE}$.

Parameter	Catuaí			
	February		August	
	390 $\mu\text{mol mol}^{-1}$	550 $\mu\text{mol mol}^{-1}$	390 $\mu\text{mol mol}^{-1}$	550 $\mu\text{mol mol}^{-1}$
g_{m_EDO}	0.067 \pm 0.012	0.102 \pm 0.015	0.072 \pm 0.012	0.072 \pm 0.015
g_{m_Harley}	0.073 \pm 0.008	0.061 \pm 0.007	0.071 \pm 0.011	0.105 \pm 0.024
V_{cmax}	61.0 \pm 1.8	54.8 \pm 2.8	57.3 \pm 5.4	58.3 \pm 3.3
J_{max}	95.6 \pm 3.9	81.1 \pm 4.6	-	-
C_c	152 \pm 15	205 \pm 15	107 \pm 9	182 \pm 19
C_{c_trans}	268 \pm 15	231 \pm 13	-	-
Parameter	Obatã			
	February		August	
	390 $\mu\text{mol mol}^{-1}$	550 $\mu\text{mol mol}^{-1}$	390 $\mu\text{mol mol}^{-1}$	550 $\mu\text{mol mol}^{-1}$
g_{m_EDO}	0.060 \pm 0.015	0.083 \pm 0.017	0.088 \pm 0.021	0.063 \pm 0.010
g_{m_Harley}	0.091 \pm 0.018	0.065 \pm 0.007	0.058 \pm 0.010	0.082 \pm 0.014
V_{cmax}	55.2 \pm 3.3	54.8 \pm 2.7	59.9 \pm 5.6	52.3 \pm 1.9
J_{max}	86.8 \pm 6.2	80.0 \pm 5.3	-	-
C_c	173 \pm 15	253 \pm 31	104 \pm 19	165 \pm 23
C_{c_trans}	303 \pm 10	231 \pm 9	-	-

found, relative to the growing season, an exacerbation of diffusional constraints to coffee's photosynthesis in winter that was almost entirely traceable by further increases in l_s . Such an increase may be a consequence of the stomatal sensitivity to low nocturnal temperatures in *C. arabica* (Barros *et al.*, 1997; Silva *et al.*, 2004). In contrast to other studies suggesting that mesophyll constraints to photosynthesis are of a similar magnitude as stomatal limitations (Warren, 2008; Flexas *et al.*, 2012), we found that l_s was exacerbated in coffee trees in the afternoon, particularly because g_s peaked in the early morning and decreased sharply in the afternoon, reaching values that were typically below 40 $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$ (Chaves *et al.*, 2008; DaMatta *et al.*, 2008) as a consequence of increasing vapour pressure deficit. Therefore, as coffee trees display an inherently low g_s and g_m (see also Martins *et al.*, 2014), it is expected that this species might benefit remarkably from increasing [CO_2] relatively more than other plant species with lower diffusional limitations to photosynthesis (Flexas *et al.*, 2014).

Notably, the absence of CO_2 -induced alterations in l_b is consistent with unaltered parameters related to biochemistry (J_{max} and V_{cmax} on a C_c basis) and N and P concentrations, suggesting that the amount of resources (e.g. N) that were allocated to electron carriers and Calvin cycle enzymes remained unchanged. Additionally, unchanged V_{cmax} has been associated with unaltered Rubisco amounts and/or activation state. Overall, these results, which are consistent with a lack of photosynthetic acclimation, contradict what has been noted in many studies in which V_{cmax} , in particular, has been shown to be downregulated in response to elevated

[CO_2] (Ainsworth and Long, 2005, and references therein). Our results, however, agree with the report of Ramalho *et al.* (2013), who suggested a lack of photosynthetic downregulation in coffee seedlings grown in large pots in enclosure systems. Other evidence demonstrates that photosynthetic downregulation was unlikely to have occurred in this study: (i) the photosynthetic enhancement due to elevated [CO_2] was independent of the CO_2 supply because the A values did not differ significantly between coffee trees that were grown under ambient or elevated [CO_2] when measurements were conducted at 390 or 550 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ of air (Ghini *et al.*, 2015); and (ii) such an enhancement occurred regardless of the higher carbohydrate accumulation under elevated [CO_2].

In the early morning, when the A and g_s values are at their maxima, the A in the plants under elevated [CO_2] was at the region co-limited by Rubisco and RuBP regeneration, given that C_c was statistically equal to $C_{c,\text{trans}}$ regardless of cultivar, as observed in the growing season (Table 1). On the one hand, this response means no investment excess in Rubisco or ETR; on the other hand, it would also imply diminishing returns in A with further increments in [CO_2] and no adjustments in g_s and g_m (Martins *et al.*, 2014). Such a fast transition from the Rubisco-limited to the electron transport-limited phase is due to a higher affinity for CO_2 displayed by coffee's Rubisco (Martins *et al.*, 2013, 2014), which, despite being highly advantageous under low CO_2 conditions, diminishes the benefit from elevated [CO_2] (Martins *et al.*, 2014). In contrast, in winter, the remarkably higher diffusional limitations to A coupled with unaltered V_{cmax} (assuming a constant $J_{\text{max}}/V_{\text{cmax}}$

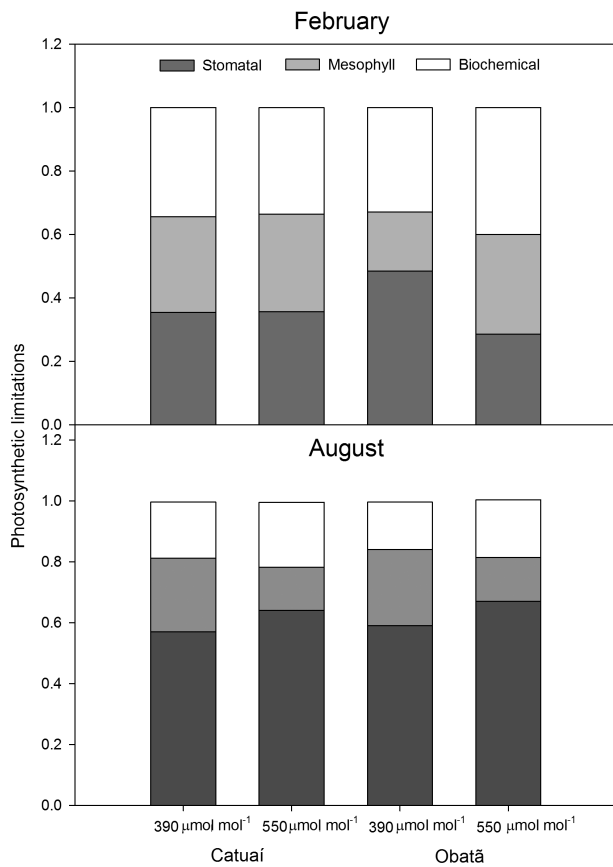


Fig. 3. Effect of elevated ($550 \mu\text{mol mol}^{-1}$) and ambient ($390 \mu\text{mol mol}^{-1}$) $[\text{CO}_2]$ on the overall limitations to photosynthesis of two coffee cultivars (Catuaí and Obatã) growing in a FACE trial during the growing season (February) and winter (August): stomatal (l_s), mesophyll (l_m) and biochemical (l_b) limitations. The required gas-exchange parameters used in the estimations were obtained at an ambient $[\text{CO}_2]$ identical to the growth $[\text{CO}_2]$ (390 or $550 \mu\text{mol mol}^{-1}$). $n=5-6 \pm \text{SE}$.

ratio) would lead the C_c values to be in the Rubisco-limited phase, where the response of A to C_c is more prominent. This pattern, regardless of season, is also expected from midday onwards due to the low g_s values (Fig. 1) and highlights the importance of elevated $[\text{CO}_2]$ for overcoming the high constraints to CO_2 diffusion for maximizing A in coffee.

The remarkable increases in starch levels with no detectable alteration in V_{max} in winter suggests, overall, that coffee displays a relatively high ability to accumulate starch in its leaf tissues without compromising its photosynthetic performance. Although there are a number of studies supporting direct couplings between starch accumulation and photosynthetic acclimation in response to elevated CO_2 (e.g. Eguchi *et al.*, 2008; Dawes *et al.*, 2013), our results agree with other studies in which uncoupling between high starch pools and photosynthetic downregulation has not been observed, as reported for fast-growing poplar clones (grown in FACE under elevated $[\text{CO}_2]$) displaying a combination of a high capacity for starch synthesis and a high sink demand; Davey *et al.*, 2006; Liberloo *et al.*, 2009). Given that starch concentrations changed paralleling an almost invariant total soluble sugar concentrations (Fig. 4), it is tempting to suggest that increased starch levels, especially under elevated $[\text{CO}_2]$, instead of feeding back to

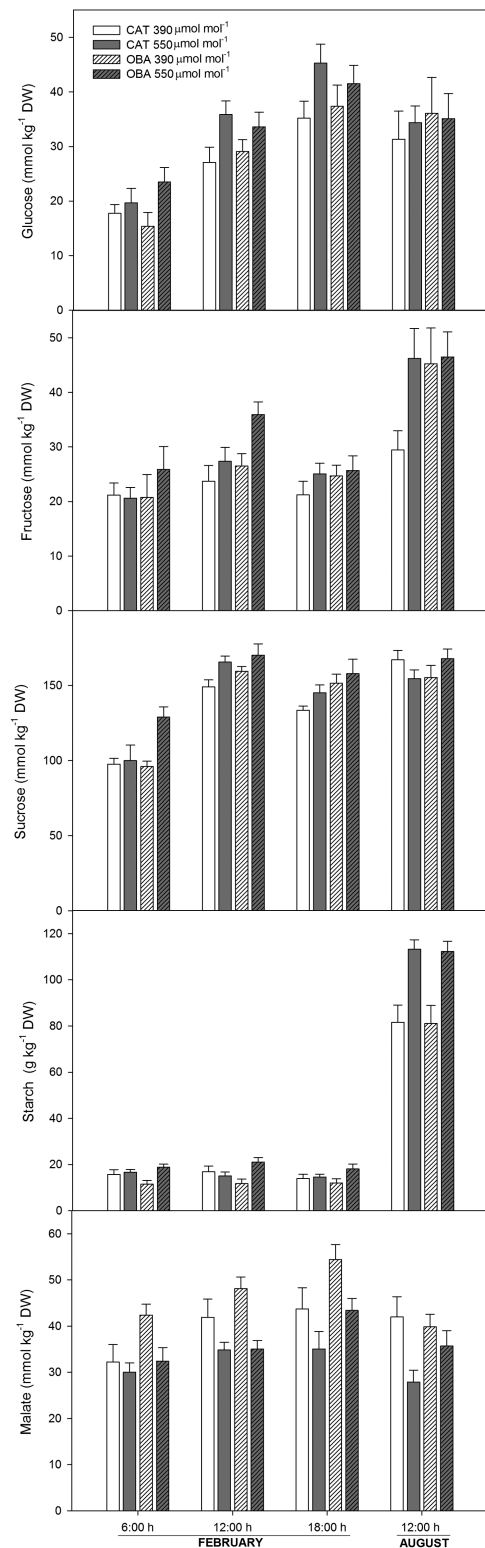


Fig. 4. Effect of elevated ($550 \mu\text{mol mol}^{-1}$) and ambient ($390 \mu\text{mol mol}^{-1}$) $[\text{CO}_2]$ on the leaf concentrations of carbohydrates and malate of two coffee cultivars (Catuaí and Obatã) growing in a FACE trial during the growing season (February) and winter (August). $n=6 \pm \text{SE}$. DW, dry weight.

decrease photosynthetic performance, allowed the coffee trees to avoid photosynthetic acclimation by preventing the cycling and/or accumulation of soluble sugars. When it occurs, soluble sugar accumulation could in turn more directly repress photosynthetic gene expression (Paul and Foyer, 2001; Paul

and Pellny, 2003) and ultimately provoke downregulation. Nevertheless, in sharp contrast to fast-growing poplar clones, we demonstrated here a lack of photosynthetic downregulation in a tropical, slow-growing species, and this fact was observed not only during the growing season but also during the period of the lowest sink demand when acclimation would be expected. This information implies a sustained ability of coffee trees that are rooted freely in the soil to benefit from elevated $[\text{CO}_2]$; it additionally highlights the fact that the remarkable decreases in A in winter (Fig. 1) could not be associated with a starch build-up and concomitant photosynthetic acclimation but rather are chiefly associated with diffusional constraints regardless of the CO_2 supply.

In the growing season, there were no cultivar differences in A , but cv. Obatã displayed increased starch concentrations, which might support an improved bean-filling capacity; in winter, on the other hand, cv. Obatã displayed significantly higher A values than cv. Catuaí with similar leaf carbohydrate pools, which might be translated into a higher photoassimilate production that could be stored in the root–trunk system, guaranteeing improved vegetative growth when the environmental conditions become more conducive for growth. Overall, these facts may help to explain why cv. Obatã is better able to sustain higher vegetative growth rates and crop yields than cv. Catuaí under elevated $[\text{CO}_2]$ (Ghini *et al.*, 2015).

In conclusion, we demonstrated under plantation conditions that coffee photosynthesis is strongly limited by diffusional constraints, particularly at the stomata level, and that this pattern is little, if at all, affected by elevated $[\text{CO}_2]$. Stimulation of A by elevated $[\text{CO}_2]$ occurred without any photosynthetic downregulation, even during the period of lowest sink demand by the crop. Given these facts, we therefore suggest that coffee will greatly benefit from the rise in atmospheric $[\text{CO}_2]$, as will other species where photosynthesis is largely limited by CO_2 diffusion.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. The time course of mean monthly air temperature and precipitation at the experimental site from January 2012 through December 2013.

Supplementary Fig. S2. The effect of elevated (550 $\mu\text{mol mol}^{-1}$ of air) or ambient (390 $\mu\text{mol mol}^{-1}$ of air) $[\text{CO}_2]$ on the overall limitations to photosynthesis of two coffee cultivars (Catuaí and Obatã) calculated with different ambient $[\text{CO}_2]$ (390 $\mu\text{mol mol}^{-1}$ of air for the plants grown at 550 $\mu\text{mol mol}^{-1}$ of air and vice versa).

Supplementary Table S1. The results of ANOVA for the effects of cultivar (Cult), CO_2 concentration (CO_2), and their interaction.

Supplementary Table S2. The effect of elevated (550 $\mu\text{mol mol}^{-1}$ of air) or ambient (390 $\mu\text{mol mol}^{-1}$ of air) $[\text{CO}_2]$ on the maximum carboxylation rate of Rubisco (V_{cmax}) and the *in vivo* maximum rate of carboxylation as limited by electron transport (J_{max}), both on an intercellular $[\text{CO}_2]$ basis, of two

coffee cultivars (Catuaí and Obatã) growing in a FACE trial during the growing season (February) and winter (August).

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