

RESEARCH PAPER

# Sustained enhancement of photosynthesis in coffee trees grown under free-air CO<sub>2</sub> 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 CO<sub>2</sub> concentrations ([CO<sub>2</sub>]). To investigate the mechanisms underlying this response, two commercial coffee cultivars (Catuá and Obatá) were grown using the first free-air CO<sub>2</sub> 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 [CO<sub>2</sub>] 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 CO<sub>2</sub> 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 [CO<sub>2</sub>]. 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 [CO<sub>2</sub>] basis. Upregulation of A by elevated [CO<sub>2</sub>] 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 CO<sub>2</sub> concentration ([CO<sub>2</sub>]) is one of the most well-documented aspects of global climatic change. In 2014, [CO<sub>2</sub>] exceeded, for the first time in at least the past 650 000 years, 400 μmol mol<sup>-1</sup> of air, as

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

air (Representative Concentration Pathway 8.5) by the end of this century (IPCC, 2013). Given that photosynthesis is limited by the CO<sub>2</sub> supply at the current atmospheric [CO<sub>2</sub>] (Long *et al.*, 2004), an increase in [CO<sub>2</sub>] 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 [CO<sub>2</sub>] increases from 380 to 550 μmol mol<sup>-1</sup> of air (von Caemmerer and Furbank, 2003). Nevertheless, the results from free-air CO<sub>2</sub> enrichment (FACE) experiments reveal that the photosynthesis of crop plants fails to match the theoretical increase that could be obtained under elevated [CO<sub>2</sub>] 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 [CO<sub>2</sub>] is accompanied by consistent, although not universal, reductions in stomatal conductance (*g<sub>s</sub>*) (Medlyn *et al.*, 2001). Meta-analyses of FACE experiments have reported mean reductions in *g<sub>s</sub>* of 16–19% (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007). The dichotomous responses of *A* and *g<sub>s</sub>* to elevated atmospheric [CO<sub>2</sub>] 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<sub>s</sub>*, an increasing body of evidence suggests that the mesophyll conductance (*g<sub>m</sub>*), which is defined as the conductance for the transfer of CO<sub>2</sub> from the intercellular airspaces (*C<sub>i</sub>*) to the sites of carboxylation in the chloroplastic stroma (*C<sub>c</sub>*), may also be a key constraint to photosynthesis (Flexas *et al.*, 2012; Sun *et al.*, 2014). However, the potential responses of *g<sub>m</sub>* to climate changes, including elevated atmospheric [CO<sub>2</sub>], have largely been neglected up to now (Flexas *et al.*, 2012, 2014). In contrast to the well-known overall pattern of decreased *g<sub>s</sub>* under long-term [CO<sub>2</sub>] exposure, the available information shows no general trend for *g<sub>m</sub>* in plants grown under elevated [CO<sub>2</sub>] (i.e. 500–600 μmol mol<sup>-1</sup> of air); indeed, no change, either decreased or increased *g<sub>m</sub>*, has been reported, possibly depending on the species and time (Flexas *et al.*, 2012, and references therein). In any case, not considering *g<sub>m</sub>* in photosynthesis calculations can significantly alter our interpretation of the effects of environmental factors, including CO<sub>2</sub> 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<sub>cmax</sub>*) on a *C<sub>i</sub>* basis may occur when a decreased *g<sub>m</sub>* under elevated [CO<sub>2</sub>] 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 [CO<sub>2</sub>] (Liberloo *et al.*, 2007; Bader *et al.*, 2010; Streit *et al.*, 2014). Frequently, however, plants that were exposed to elevated [CO<sub>2</sub>] 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<sub>cmax</sub>* 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<sub>max</sub>*) 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 [CO<sub>2</sub>] (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 [CO<sub>2</sub>] 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<sup>-2</sup> s<sup>-1</sup> with current atmospheric [CO<sub>2</sub>] 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 [CO<sub>2</sub>], considering that increasing atmospheric [CO<sub>2</sub>] increases the gradient that ensures an adequate diffusion of CO<sub>2</sub> 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 [CO<sub>2</sub>] over 1 year, revealed greater *A* (ranging from 34 to 49%) under elevated (700 μmol CO<sub>2</sub> mol<sup>-1</sup> of air) than under normal (380 μmol CO<sub>2</sub> mol<sup>-1</sup> of air) [CO<sub>2</sub>]. 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<sub>s</sub>* responds little, if at all, to elevated [CO<sub>2</sub>] in coffee; taken together, these results imply that substantial increases in water-use efficiency occur in coffee grown under elevated [CO<sub>2</sub>] 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 [CO<sub>2</sub>] 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 [CO<sub>2</sub>], which may in part explain the enhanced crop yields under elevated [CO<sub>2</sub>] (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, CO<sub>2</sub> 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 dystroferric 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 JXB online).

To mimic coffee agrosystems, the FACE system increased the ambient [CO<sub>2</sub>] in six 10 m diameter ring plots (elevated CO<sub>2</sub>) within a continuous 7 ha coffee field. Six additional 10 m diameter ring plots served as controls, i.e. were left under ambient [CO<sub>2</sub>]. Elevated- and ambient-CO<sub>2</sub> plots were at least 70 m apart to minimize cross-plot contamination.

Fumigation with CO<sub>2</sub> began on 25 August 2011. The average atmospheric [CO<sub>2</sub>] at the beginning of the experiment was approximately 390 μmol mol<sup>-1</sup>. The performance of the FACE system was adjusted so that the [CO<sub>2</sub>] as measured at the centre of the ring achieved target levels of 550 μmol mol<sup>-1</sup> of air. The plots were not enriched with CO<sub>2</sub> at night. Further details regarding the experimental site set-up and CO<sub>2</sub> 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 CO<sub>2</sub> ( $g_s$ )] and internal CO<sub>2</sub> 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 plagioprotropic (lateral) branches in the upper third of the plants] using an open system under ambient temperature and a [CO<sub>2</sub>] of 390 or 550 μmol mol<sup>-1</sup> 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<sup>-2</sup> s<sup>-1</sup>. 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<sup>-2</sup> s<sup>-1</sup>; 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<sup>-2</sup> s<sup>-1</sup>) 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 ( $F_s'/F_m'$ ) were estimated ([Logan et al., 2007](#)). The actual PSII photochemical efficiency ( $\varphi_{PSII}$ ) was determined as  $\varphi_{PSII} = (F_m' - F_s)/F_m'$  following the procedures of [Genty et al. \(1989\)](#). The electron transport rate (ETR) was then calculated from the equation  $ETR = \varphi_{PSII} \times \beta \times \alpha \times \text{photosynthetic photon flux density (PPFD)}$ , where  $\alpha$  is leaf absorptance, and  $\beta$  reflects the partitioning of absorbed quanta between PSII and PSI ([Genty et al., 1989](#)). The product of  $\beta$  and  $\alpha$  was determined according to [Valentini et al. \(1995\)](#) from the relationship between  $\varphi_{PSII}$  and  $\varphi_{CO_2}$  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 [CO<sub>2</sub>] ( $C_a$ ) of 400 μmol mol<sup>-1</sup> under a saturating PPFD of 1000 μmol m<sup>-2</sup> s<sup>-1</sup>. Once a steady state was reached,  $C_a$  was gradually decreased to 50 μmol mol<sup>-1</sup> of air. Upon the completion of the measurements at low  $C_a$ ,  $C_a$  was returned to 400 μmol mol<sup>-1</sup> of air to restore the original  $A$ . Next,  $C_a$  was increased stepwise to 1600 μmol mol<sup>-1</sup> of air.

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  $\text{CO}_2$  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  $\text{CO}_2$  ( $g_m$ ), the maximum rate of carboxylation ( $V_{\text{cmax}}$ ), the maximum rate of carboxylation limited by electron transport ( $J_{\text{max}}$ ), and the chloroplastic [ $\text{CO}_2$ ] of transition ( $C_{\text{c\_trans}}$ )*

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

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

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  $\Gamma^*$  is the  $\text{CO}_2$  compensation point in the absence of mitochondrial respiration (the conservative value of  $\Gamma^*$  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,  $V_{\text{cmax}}$  and  $J_{\text{max}}$  were calculated by fitting the mechanistic model of  $\text{CO}_2$  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  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ , 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  $\text{CO}_2$  that considers the competitive inhibition by  $\text{O}_2$ , was taken from Martins *et al.* (2013).  $C_{\text{c\_trans}}$  was calculated using  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ ,  $K_m$ , and  $\Gamma^*$  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  $g_s$ ,  $g_m$ ,  $V_{\text{cmax}}$ ,  $\Gamma^*$ ,  $K_m$ , 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  $\text{CO}_2$  and  $g_m$  is the mesophyll conductance to  $\text{CO}_2$  according to Harley *et al.* (1992), and  $g_{\text{tot}}$  is the total conductance to  $\text{CO}_2$  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  $\text{CO}_2$  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 *JXB* 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  $\text{CO}_2$  enrichment in cv. Catuai; in cv. Obatã, the  $g_s$  values at two time points were lower under elevated than under ambient  $[\text{CO}_2]$  (Fig. 1). The  $R_p/A_{\text{gross}}$  ratio was clearly lower in the morning in both cultivars that were treated with  $\text{CO}_2$ , with a less clear pattern in the afternoon (Fig. 1). Notably, diurnally integrated  $A$  was 40% greater and the  $R_p/A_{\text{gross}}$  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  $R_p/A_{\text{gross}}$

ratio (Fig. 1). In all of the winter measurements, both of the cultivars responded to CO<sub>2</sub> 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 [CO<sub>2</sub>], whereas in cv. Catuai, this ratio was unresponsive to the CO<sub>2</sub> supply. The diurnally mean  $A$  values in winter averaged 56% higher in the plants that were treated with CO<sub>2</sub> than in their untreated counterparts. In cv. Obatã,  $g_s$  values integrated over the course of the day were significantly lower under elevated [CO<sub>2</sub>] (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 CO<sub>2</sub> fertilization), and  $R_p/A_{gross}$  was lower in this cultivar (only at elevated [CO<sub>2</sub>]) than in cv. Catuai (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 CO<sub>2</sub> 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 CO<sub>2</sub> treatments (Table 1). Both  $V_{cmax}$  and  $J_{max}$ , estimated on a  $C_c$  and  $C_i$  basis, were unresponsive to the CO<sub>2</sub> 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 µmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> 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 CO<sub>2</sub> supply.

We also determined data for  $C_c$  and  $C_{c\_trans}$  (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 [CO<sub>2</sub>], and  $C_{c\_trans}$  (estimated only in the growing season due to the available  $J_{max}$  data) decreased under elevated [CO<sub>2</sub>]. Regardless of cultivars,  $C_c$  was lower than  $C_{c\_trans}$  under ambient [CO<sub>2</sub>]. Under elevated [CO<sub>2</sub>], the values of  $C_c$  did not differ significantly from those of  $C_{c\_trans}$  irrespective of cultivar ( $P>0.05$ ).

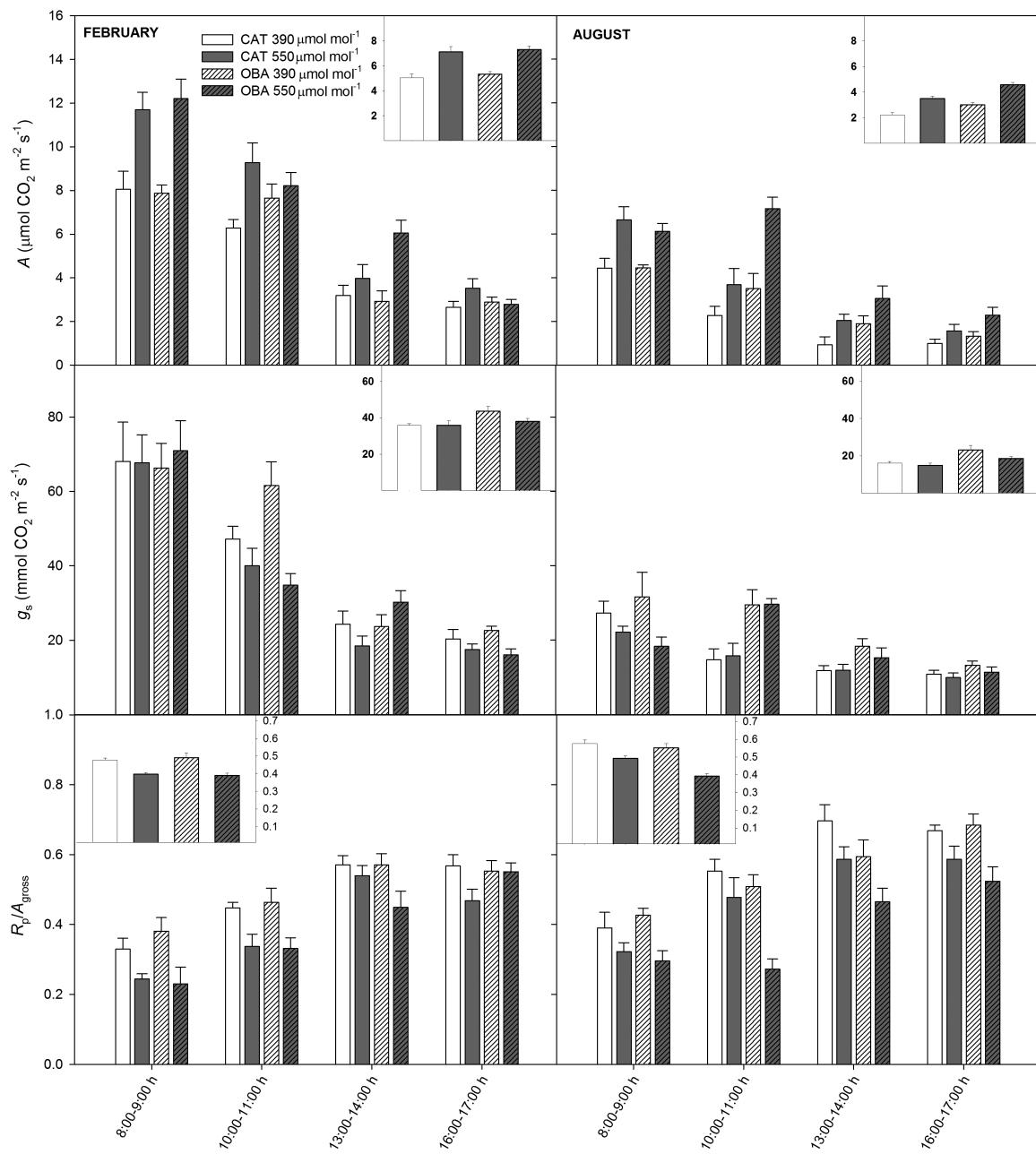
The functional components ( $l_s$ ,  $l_m$ , and  $l_b$ ) of the overall photosynthetic limitations did not respond to [CO<sub>2</sub>] in cv. Catuai in the growing season (Fig. 3). In cv. Obatã,  $l_b$  was unresponsive to the [CO<sub>2</sub>] supply, regardless of the season; however, elevated [CO<sub>2</sub>] 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 [CO<sub>2</sub>]

compared with under ambient [CO<sub>2</sub>]. 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 CO<sub>2</sub> supply in either cultivar (Fig. 3). By comparing the two growth periods, the greater diffusional limitations in winter were chiefly associated with increased  $l_s$  ( $\sim 60\%$  vs  $\sim 37\%$  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 CO<sub>2</sub> by calculating the limitations using different ambient [CO<sub>2</sub>] (390 µmol mol<sup>-1</sup> of air for the plants grown at 550 µmol mol<sup>-1</sup> 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 CO<sub>2</sub> 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. Catuai, whereas in Obatã, the starch levels were consistently higher (64% on average) under elevated than under ambient [CO<sub>2</sub>]. In winter, measurements were conducted only at midday for both cultivars. The glucose and sucrose pools were unaffected by the CO<sub>2</sub> supply independently of cultivar, whereas the starch pools averaged 38% higher under elevated than under ambient [CO<sub>2</sub>]. Fructose pools increased significantly in response to the CO<sub>2</sub> supply but only in cv. Catuai. 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. Catuai; in cv. Obatã, malate pools were consistently lower (24% on average) over the course of the day under elevated compared with under ambient [CO<sub>2</sub>]. In winter, malate pools were significantly lower (33%) under elevated than under ambient [CO<sub>2</sub>] in cv. Catuai, whereas these pools were not affected by the CO<sub>2</sub> 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.

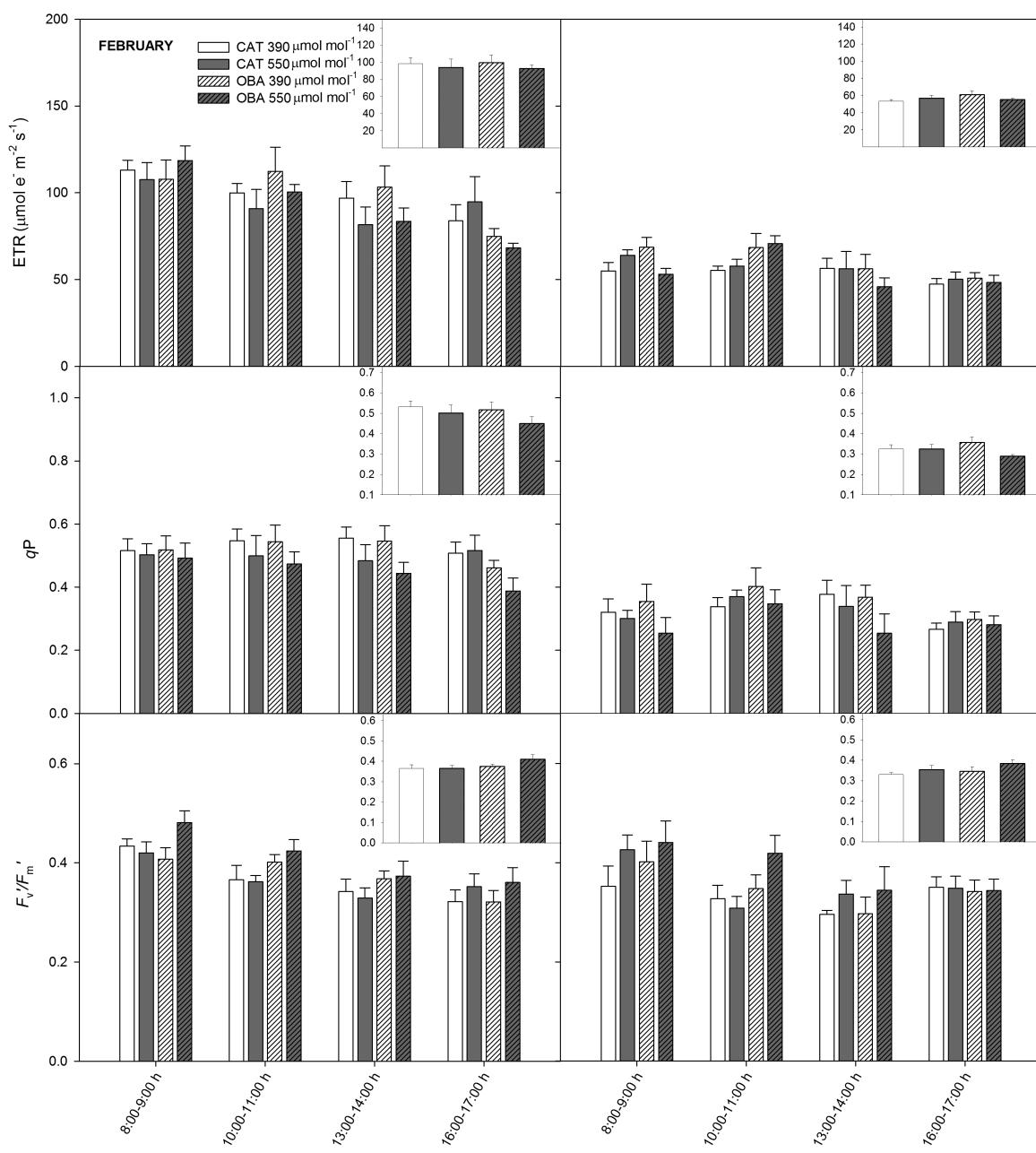


**Fig. 1.** Effect of elevated ( $550 \mu\text{mol mol}^{-1}$ ) and ambient ( $390 \mu\text{mol mol}^{-1}$ ) [ $\text{CO}_2$ ] on the leaf gas exchange of two coffee cultivars (Catuaí and Obatá) growing in a FACE trial during the growing season (February) and winter (August): net  $\text{CO}_2$  assimilation rate ( $A$ ), stomatal conductance to  $\text{CO}_2$  ( $g_s$ ), and ratio of photorespiration-to-gross photosynthetic rate ( $R_p/A_{\text{gross}}$ ). In the insets, the mean diurnal values of  $A$ ,  $g_s$ , and  $R_p/A_{\text{gross}}$  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  $\text{CO}_2$  (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  $\text{CO}_2$  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  $R_p/A_{\text{gross}}$  ratio under elevated [ $\text{CO}_2$ ] (Fig. 1).

Given that the stimulation of  $A$  by  $\text{CO}_2$  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  $\text{CO}_2$  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 Obatá) 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  $\text{CO}_2$  fixation in this study. In any case, seasonal photochemical adjustments (decreased  $q_p$  and ETR in winter) occurred independently of the  $\text{CO}_2$  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  $\text{CO}_2$  (Table 1). Regardless of the  $\text{CO}_2$  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 ( $390 \mu\text{mol mol}^{-1}$ )  $[\text{CO}_2]$  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 ( $V_{c\max}$ ) and the *in vivo* maximum rate of carboxylation as limited by electron transport ( $J_{\max}$ ), both on a chloroplast  $[\text{CO}_2]$  basis; and the chloroplastic  $[\text{CO}_2]$  ( $C_c$ ) and  $C_c$  of transition ( $C_{c\text{-trans}}$ ). Data for  $J_{\max}$  and  $C_{c\text{-trans}}$  were not obtained in August.  $V_{c\max}$ ,  $J_{\max}$  and  $g_m$  were normalized to  $25^\circ\text{C}$  using the temperature response equations from Sharkey *et al.* (2007).  $n=5-6\pm\text{SE}$ .

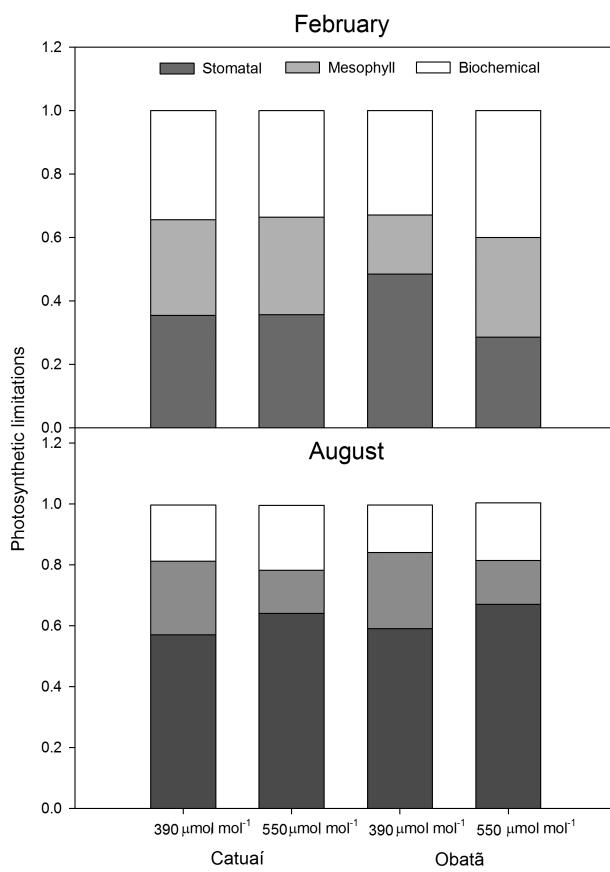
Parameter	Catuaí			
	February		August	
	$390 \mu\text{mol mol}^{-1}$	$550 \mu\text{mol mol}^{-1}$	$390 \mu\text{mol mol}^{-1}$	$550 \mu\text{mol mol}^{-1}$
$g_m$ _EDO	$0.067\pm 0.012$	$0.102\pm 0.015$	$0.072\pm 0.012$	$0.072\pm 0.015$
$g_m$ _Harley	$0.073\pm 0.008$	$0.061\pm 0.007$	$0.071\pm 0.011$	$0.105\pm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

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  $I_s$ . 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  $I_s$  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  $[\text{CO}_2]$  relatively more than other plant species with lower diffusional limitations to photosynthesis (Flexas *et al.*, 2014).

Notably, the absence of  $\text{CO}_2$ -induced alterations in  $I_b$  is consistent with unaltered parameters related to biochemistry ( $J_{\max}$  and  $V_{c\max}$  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  $V_{c\max}$  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  $V_{c\max}$ , in particular, has been shown to be downregulated in response to elevated

$[\text{CO}_2]$  (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  $[\text{CO}_2]$  was independent of the  $\text{CO}_2$  supply because the  $A$  values did not differ significantly between coffee trees that were grown under ambient or elevated  $[\text{CO}_2]$  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  $[\text{CO}_2]$ .

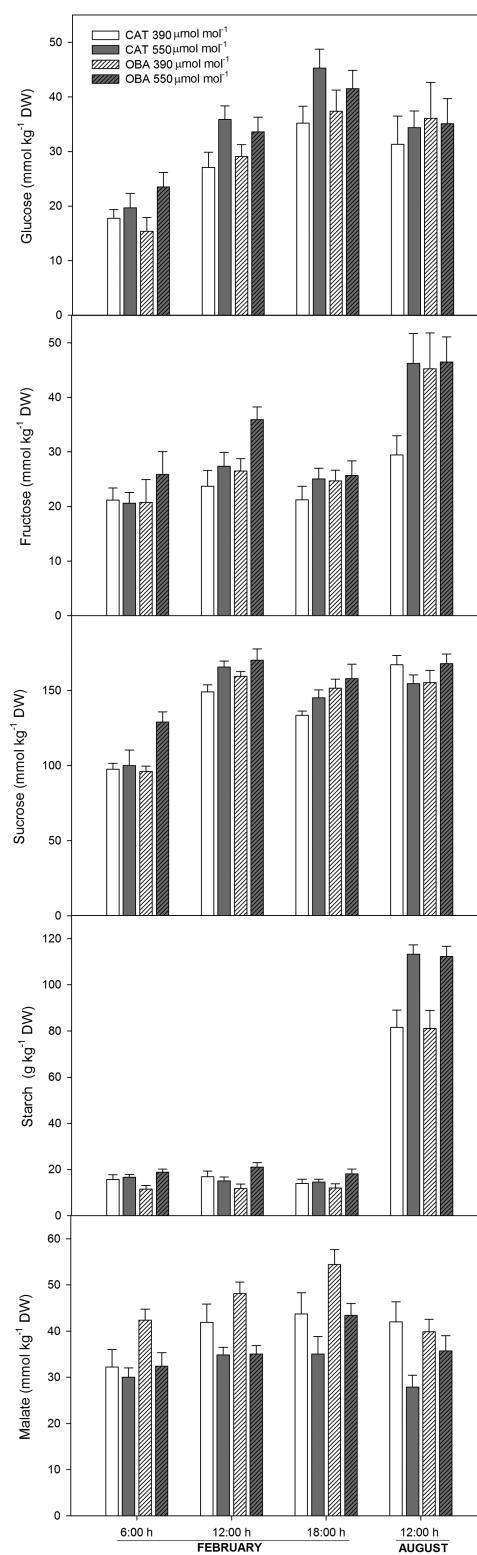
In the early morning, when the  $A$  and  $g_s$  values are at their maxima, the  $A$  in the plants under elevated  $[\text{CO}_2]$  was at the region co-limited by Rubisco and RuBP regeneration, given that  $C_c$  was statistically equal to  $C_{c\text{-trans}}$  regardless of cultivar, as observed 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  $[\text{CO}_2]$  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  $\text{CO}_2$  displayed by coffee's Rubisco (Martins *et al.*, 2013, 2014), which, despite being highly advantageous under low  $\text{CO}_2$  conditions, diminishes the benefit from elevated  $[\text{CO}_2]$  (Martins *et al.*, 2014). In contrast, in winter, the remarkably higher diffusional limitations to  $A$  coupled with unaltered  $V_{c\max}$  (assuming a constant  $J_{\max}/V_{c\max}$



**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 Obatá) growing in a FACE trial during the growing season (February) and winter (August): stomatal ( $I_s$ ), mesophyll ( $I_m$ ) and biochemical ( $I_b$ ) 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  $\text{CO}_2$  diffusion for maximizing  $A$  in coffee.

The remarkable increases in starch levels with no detectable alteration in  $V_{\text{cmax}}$  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  $\text{CO}_2$  (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 parallelizing 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 Obatá) 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 [CO<sub>2</sub>]; 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<sub>2</sub> supply.

In the growing season, there were no cultivar differences in *A*, but cv. Obatā displayed increased starch concentrations, which might support an improved bean-filling capacity; in winter, on the other hand, cv. Obatā 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. Obatā is better able to sustain higher vegetative growth rates and crop yields than cv. Catuaí under elevated [CO<sub>2</sub>] (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 [CO<sub>2</sub>]. Stimulation of *A* by elevated [CO<sub>2</sub>] 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 [CO<sub>2</sub>], as will other species where photosynthesis is largely limited by CO<sub>2</sub> 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 µmol mol<sup>-1</sup> of air) or ambient (390 µmol mol<sup>-1</sup> of air) [CO<sub>2</sub>] on the overall limitations to photosynthesis of two coffee cultivars (Catuaí and Obatā) calculated with different ambient [CO<sub>2</sub>] (390 µmol mol<sup>-1</sup> of air for the plants grown at 550 µmol mol<sup>-1</sup> of air and vice versa).

**Supplementary Table S1.** The results of ANOVA for the effects of cultivar (Cult), CO<sub>2</sub> concentration (CO<sub>2</sub>), and their interaction.

**Supplementary Table S2.** The effect of elevated (550 µmol mol<sup>-1</sup> of air) or ambient (390 µmol mol<sup>-1</sup> of air) [CO<sub>2</sub>] on the maximum carboxylation rate of Rubisco (*V*<sub>cmax</sub>) and the *in vivo* maximum rate of carboxylation as limited by electron transport (*J*<sub>max</sub>), both on an intercellular [CO<sub>2</sub>] basis, of two

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

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