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Research paper

Intrinsic non-stomatal resilience to drought of the photosynthetic apparatus in *Coffea* spp. is strengthened by elevated air [CO_2]

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Growing water restrictions associated with climate changes constitute daunting challenges to crop performance. This study unveils the impacts of moderate (MWD) or severe (SWD) water deficit, and their interaction with air [CO_2], on the photosynthetic apparatus of *Coffea canephora* Pierre ex A. Froehner cv. Conilon Clone 153 (CL153) and *Coffea arabica* L. cv. Icatu. Seven year-old potted plants grown under 380 (aCO_2) or 700 μl l $^{-1}$ (eCO_2) [CO_2] gradually reached predawn water potentials between −1.6 and −2.1 MPa (MWD), and below −3.5 MPa (SWD). Under drought, stomata closure was chiefly related to abscisic acid (ABA) rise. Increasing drought severity progressively affected gas exchange and fluorescence parameters in both genotypes, with non-stomatal limitations becoming gradually dominating, especially regarding the photochemical and biochemical components of CL153 SWD plants. In contrast, Icatu plants were highly tolerant to SWD, with minor, if any, negative impacts on the potential photosynthetic functioning and components (e.g., A_{max}, F_v/F_m, electron carriers, photosystems (PSs) and ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) activities). Besides, drought-stressed Icatu plants displayed increased abundance of a large set of proteins associated with the photosynthetic apparatus (PSs, light-harvesting complexes, cyclic electron flow, RuBisCO activase) regardless of [CO_2]. Single eCO_2 did not promote stomatal and photosynthetic down-regulation in both genotypes. Instead, eCO_2 increased photosynthetic performance, moderately reinforced photochemical (PSs activity, electron carriers) and biochemical (RuBisCO, ribulose-5-phosphate kinase) components, whereas photoprotective mechanisms and protein abundance remained mostly unaffected. In both genotypes, under MWD, eCO_2 superimposition delayed stress severity and promoted photosynthetic functioning with lower energy dissipation and PSII impacts, whereas stomatal closure was decoupled from increases in ABA. In SWD plants, most impacts on the photosynthetic performance were reduced by eCO_2,

especially in the moderately drought affected CL153 genotype, although maintaining RuBisCO as the most sensitive component, deserving special breeder's attention to improve coffee sustainability under future climate scenarios.

Keywords: acclimation, C-assimilation, climate change, CO_2 mitigation, coffee tree, drought.

Introduction

Current knowledge regarding global climate has pointed to important weather shifts, especially associated with rising temperature and altered rainfall patterns. In this context, prolonged droughts intercalated with extreme precipitation events are expected to be aggravated, particularly in the tropical regions (IPCC 2014, 2018). These changes are predicted to be accompanied by a rising air [CO_2]. Depending on upcoming anthropogenic greenhouse gas emission scenarios, air [CO_2] might reach 936 µl CO_2 l^{-1} by 2100, accompanied by a global warming up to between 2.6 and 4.8 °C relative to 1986–2005 (IPCC 2013, 2014).

Drought, a major bottleneck to agriculture production, constrains a number of morphological, physiological and biochemical processes, with impacts on growth, nutrient uptake, carbon (C)-assimilation and partitioning (Chaves et al. 2009, Fahad et al. 2017, Lamaoui et al. 2018, Lang et al. 2018). However, plants display a number of responses that allow them to cope with drought events, involving adjustments from the gene to the whole-plant level (Chaves et al. 2003, Xiong et al. 2006, Hummel et al. 2010). Therefore, it is crucial to better understand such acclimation mechanisms to assist selection and improvement of tolerant cultivars to drought (Chaves et al. 2003, Hasan et al. 2018).

Under moderate drought, stomatal closure is crucial for reducing water loss through transpiration (Matos et al. 2002, Brodribb and McAdam 2017), but, at the same time, it also constrains the CO_2 diffusion into the leaf. This can limit photosynthesis through a low CO_2 supply to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), even under moderate drought conditions, when few, if any, impairments on photosystems (PSs) efficiency and photosynthetic capacity (A_{max}) are observed (Chaves et al. 2009, Wang et al. 2016, Zargar et al. 2017). However, with increasing drought severity the photosynthetic performance is also impaired by photochemical and biochemical limitations, including impacts in photosynthetic pigment pools, PSs performance, enzyme activities (e.g., RuBisCO) and membrane integrity (Chaves et al. 2003, Muller et al. 2011, Ramalho et al. 2014, 2018*b*, Fahad et al. 2017). The consequent reduction of photochemical energy use usually imposes a secondary stress related to an uncontrolled generation of reactive species of oxygen (ROS) and chlorophyll, which can aggravate the impairments on chloroplast components (Reddy et al. 2004, Chaves et al. 2009). Therefore, a greater ability to cope with drought is often associated with the triggering of thermal dissipation, photoprotective and antioxidative mechanisms, and cyclic electron flow (CEF) involving PSs (Miyake and Okamura 2003, Chaves and Oliveira 2004, Reddy et al. 2004, Ramalho et al. 2018*b*).

Increasing air [CO_2] affects fundamental plant processes such as photosynthesis, plant growth, crop yield and quality (Idso and Kimball 1997, Bader et al. 2010), altering biomass partitioning (Ainsworth et al. 2004, Yang et al. 2006). Net photosynthesis rates frequently increase by 30–60% at 600–700 µl CO_2 l^{-1}, as compared with their respective values at 370–390 µl CO_2 l^{-1} (Ainsworth and Rogers 2007, Kirschbaum 2011). These increases arise from an enhanced CO_2 availability to RuBisCO, which in parallel reduces RuBisCO oxygenase activity with concordant decreases in photorespiration rates and ROS production (Ainsworth and Rogers 2007, Leakey et al. 2009). This CO_2 fertilization effect can potentially increase crop yields (Long et al. 2004, Norby et al. 2005), although these positive effects can be strongly attenuated under drought conditions, depending on stress severity and duration (Tausz-Posch et al. 2020).

Coffee, one of the most important agricultural commodities worldwide, supports the livelihoods of ~25 million smallholder farmers, while involving about 100–125 million people worldwide in its chain of value (Osorio 2002, DaMatta et al. 2019). Several studies have claimed that we are already in the midst of a climate crisis, estimating that future climate changes will further constrain the coffee crop, promoting vast agricultural, social and economic impacts associated with huge losses of suitable cultivation areas, aggravated incidence of pests and diseases (Magrach and Ghazoul 2015), reduced yields (van der Vossen et al. 2015) and the extinction of at least 60% of all coffee species (Davis et al. 2019). However, recent studies have demonstrated that an elevated air [CO_2] (eCO_2) can improve C-assimilation (Ramalho et al. 2013, Ghini et al. 2015) and promote a higher C-investment in reproductive structures (Rakocevic et al. 2020), thus ultimately increasing productivity at least under adequate water supply (DaMatta et al. 2019). In fact, under unrestricted water availability eCO_2 has been demonstrated to strengthen coffee's plant physiological performance (Ramalho et al. 2013). Furthermore, eCO_2 also increased leaf coffee resilience to heat stress, as supported by reinforced photochemical energy use, protective mechanisms (Martins et al. 2016, Rodrigues et al. 2016) and higher membrane lipid dynamics (Scotti-Campos et al. 2019), while preserving leaf mineral balance (Martins et al. 2014) and bean quality (Ramalho et al. 2018*a*). These findings

underpin a new view, pointing to a less grim impact on coffee crop sustainability than earlier forecasted largely based on temperature drifts (DaMatta et al. 2019). Nonetheless, another growing concern is associated with water scarcity (DaMatta et al. 2018, Ramalho et al. 2018*b*) given that coffee is cultivated in tropical areas, which are expected to be strongly impacted by climate change (IPCC 2018). Drought (and heat) impacts are additionally expected to be aggravated, particularly in coffee plantations under full sunlight exposure, which will impose new management challenges to afford sustainability for the coffee crop (Dubberstein et al. 2018, Semedo et al. 2018).

We recently demonstrated that eCO_2 mitigates the impairments of moderate drought stress on coffee growth and photosynthetic performance by improving plant water status upon drought imposition (Avila et al. 2020*a*, 2020*b*). Here, we expanded the underlying mechanisms by which the photosynthetic apparatus adjusts to increasing drought severity and how eCO_2 could modify these adjustments. We hypothesized that eCO_2 improves resilience of the photosynthetic functioning to drought stress at the biochemical and molecular levels, and that these improvements are dependent on the magnitude of drought severity. To test these hypotheses, we in-depth assessed the plant impacts and responses through physiological (thermal imaging, gas exchanges, chlorophyll *a* fluorescence), biochemical (thylakoid electron transport and carriers, enzyme activities) and molecular (abundance of proteins associated with PSs, RuBisCO and CEF) evaluations. For that, plants from two genotypes, representing the two main coffee producing species, grown under normal (aCO_2) or elevated (eCO_2) air [CO_2] were subjected to moderate water deficit (MWD) or severe water deficit (SWD) conditions. Our findings provide important and timely evidence regarding the role of eCO_2 to mitigate the harmful effects of water deficit and reveal prominent drought tolerance/sensitivity points, therefore advancing our comprehension of coffee performance under future climate scenarios.

Materials and methods

Plant material and growth conditions

Plants of two cropped genotypes (in Brazil) from the two main producing coffee species, *Coffea canephora* Pierre ex A. Froehner cv. Conilon Clone 153 (CL153) and *Coffea arabica* L. cv. Icatu Vermelho (an introgressed variety resulting from a cross of *C. canephora* and *Coffea arabica* cv. Bourbon Vermelho, then further crossed with *C. arabica* cv. Mundo Novo) were used. A total of 36 plants were grown since the seedling stage, during 7 years in 80 l pots, divided in two walk-in growth chambers (EHHF 10,000, ARALAB, Portugal), each one supplied with ambient (aCO_2, 380 ± 5 µl l^{-1}) or elevated (eCO_2, 700 ± 5 µl l^{-1}) air [CO_2]. In both growth chambers, plants were maintained under controlled temperature (25/20 °C, day/night, ±1 °C), irradiance (max. ~750 µmol m^{-2} s^{-1} at the upper part of the plant), relative humidity (70 ± 2%) and photoperiod (12 h). Plants were grown without restrictions of nutrients (provided as in Ramalho et al. 2013), root growth or water (until applying the water treatments), maintaining adequate soil moisture by watering the plants every 2 days. According to current definition (Hurlbert 1984, 2004), our experiments used pseudoreplicates given that all the plants (per each CO_2 treatment) were grown in a single growth chamber. To minimize any 'growth chamber effect', the walk-in growth chambers were regularly and accurately calibrated by the manufacturer, in order to guarantee that the environmental conditions (air humidity, temperature, light intensity and quality) provided to all plants in both chambers were exactly the same, with the exception of air [CO_2], and a weekly chamber swapping was performed so that we minimize as much as possible potential pseudoreplication implications (Johnson et al. 2016).

Determinations were performed on newly matured leaves from the upper third part (well illuminated) from the six plants per treatment. Whenever possible the same leaf (or similar leaves from the same plant) was used for all evaluations. Unless stated otherwise, sample collection and evaluations were performed under photosynthetic steady-state after ~2 h of illumination. For biochemical evaluations, the collected leaf material was flash frozen in liquid nitrogen and stored at −80 °C, being finely powdered in liquid N_2 prior to analysis. Leaf tissue extractions were performed using an ice-cold mortar and pestle, as well as cold homogenizing solutions.

Water deficit imposition and leaf water status

Plants were divided into three groups. In the first one, individuals were maintained well irrigated (WW) along the experiment, displaying leaf predawn water potential (Ψ_{pd}) above −0.35 MPa. In the other two groups, water deficit was gradually imposed along 2 weeks by partially withholding irrigation (with a partial water replacement of the amount lost, individually analyzed in each pot) until stability of Ψ_{pd} to values between −1.5 and −2.5 MPa (MWD) or below −3.5 MPa (SWD). Leaf Ψ_{pd} was determined immediately after leaf excision in five to six true replicates per treatment, using a pressure chamber (Model 1000, PMS Instrument Co., Albany, OR, USA). These watering conditions represented ~80% (WW), 25% (MWD) or 10% (SWD) of maximal pot water availability (Ramalho et al. 2018*b*). When the desired Ψ_{pd} was reached (MWD or SWD), pot moisture was maintained thereafter for another 2 weeks by adding adequate water amounts according to each watering treatment before measurements and samplings. Exceptionally, the Icatu 700-plants under MWD conditions were exposed to total water withholding in the last 5 days of the 4 week period, in order to further force the reduction of Ψ_{pd} values, which, even so, did not shift below −0.6 MPa.

Thermal imaging analysis

Thermal images were acquired with a thermal imager (GF300, FLIR Systems, Wilsonville, OR, USA) and processed using a Thermal Cam Explorer software (FLIR Systems), following the procedures of Grant et al. (2007). Images were corrected for spatial calibration drift by subtracting corresponding reference images of an isothermal surface. The canopy was imaged using reference leaves to simulate fully closed and fully open stomata. Reference leaves with fully closed stomata had both sides covered with petroleum jelly (Vaseline) to obtain the dry temperature (T_{dry}). Their counterparts with fully open stomata were sprayed with water using a hand spray bottle to maintain their moisture level and to obtain the wet temperature (T_{wet}). The temperatures of the reference leaves (T_{wet} and T_{dry}) together with the actual leaf temperature (T_{leaf}) were used to obtain the stomatal conductance index [$I_G = (T_{dry} - T_{leaf})/(T_{leaf} - T_{wet})$], which is theoretically proportional to stomatal conductance to water vapor (g_s), and the crop water stress index [$CWSI = (T_{dry} - T_{leaf})/(T_{dry} - T_{wet})$] (Grant et al. 2007). For CWSI, values close to 0 indicate a fully transpiring leaf/crop (i.e., with no stress), and close to 1 indicate a non-transpiring leaf/crop (i.e., under maximum stress).

Leaf gas exchanges measurement

Net photosynthesis rate (P_n), g_s and internal [CO_2] (C_i) were obtained using a portable open-system infra-red gas analyzer (Li-Cor 6400, LiCor, Lincoln, NE, USA), under 25 °C, with an external CO_2 supply of ~380 or 700 µl CO_2 l^{-1}, and ~650 µmol m^{-2} s^{-1} of irradiance. This irradiance level is close to the maximal ambient photosynthetic photon flux density (PPFD) in the growth chambers, and high enough to saturate P_n under the [CO_2] used in this study, as found in preliminary experiments.

Photosynthetic capacity (A_{max}), reflecting the potential photosynthetic rate obtained under saturating light and [CO_2], was measured in 1.86 cm^2 leaf disks through the evolution of O_2 detected by a Clark-type O_2 electrode (LD2/2, Hansatech, King's Lynn, Norfolk, UK) (Ramalho et al. 1997). A_{max} was obtained at 25 °C, ~7% [CO_2] (supplied by 400 µl 2 M $KHCO_3$), and by exposing the leaf samples to increasing irradiance up to 1200 µmol m^{-2} s^{-1} using a Björkman lamp (Hansatech) and neutral filters.

Chlorophyll a fluorescence analysis

Chlorophyll (Chl) *a* fluorescence parameters were determined on the same leaves and conditions used for gas exchange measurements using a PAM-2000 system (H. Walz, Effeltrich, Germany), exactly as previously described (Rodrigues et al. 2016). Measurements in dark-adapted leaves included the F_0 (minimum fluorescence from excited Chl *a* molecules from the antennae), and F_v/F_m (maximal PSII photochemical efficiency). A second set of parameters, evaluated under photosynthetic steady-state conditions (650 µmol m^{-2} s^{-1} of actinic light) and superimposed saturating flashes (~7500 µmol m^{-2} s^{-1}), included the F_v'/F_m' (PSII photochemical efficiency of energy conversion under light exposure), q_L (photochemical quenching based on the concept of interconnected PSII antennae, representing the proportion of energy captured by open PSII centers and driven to photochemical events), and F_s/F_m' (predictor of the rate constant of PSII inactivation). Additionally, estimates of photosynthetic quantum yields of non-cyclic electron transfer [$Y_{(II)}$], photoprotective regulated energy dissipation of PSII [$Y_{(NPQ)}$], and non-regulated energy dissipation of PSII as heat and fluorescence [$Y_{(NO)}$], where [$Y_{(II)} + Y_{(NPQ)} + Y_{(NO)} = 1$], were also calculated.

Thylakoid electron transport rates

Pools of leaves [~5 g fresh weight (FW)] from six plants were used to obtain sub-chloroplast membrane fractions, as described for coffee (Ramalho et al. 1999). The in vivo electron transport rates associated with PSI ($DCPIPH_2 \rightarrow MV$) and PSII, including ($H_2O \rightarrow DCPIP$) or excluding ($DPC \rightarrow DCPIP$) the oxygen-evolving complex (OEC) were obtained with an O_2 electrode (LW2, Hansatech), using 1 ml of reaction mixture containing ~100 mg Chl, at 25 °C, under ~3000 µmol m^{-2} s^{-1} irradiance supplied by a Björkman lamp.

Thylakoid electron carriers

Pools of leaves to obtain sub-chloroplast fractions for plastoquinone (PQ-9) (~5 g FW) and cytochrome (Cyt) (~7 g FW) evaluation were collected from six plants. Spectrophotometric measurements were carried out as previously described (Dubberstein et al. 2020). Briefly, PQ-9 content was determined by measuring the absorption difference between the oxidized and reduced forms of PQ-9 at 255 nm, relative to isosbest wavelengths of 276 and 308 nm, and assuming an extinction coefficient of 14.8 mmol l^{-1} cm^{-1}. The content of Cyt b_{559LP}, b_{559HP}, b_{563} and *f* were obtained with readings at 545 nm, and isosbest wavelengths at 528 and 568 nm for Cyt b_{559}, and 552 and 572 nm for Cyt b_{563}. An extinction coefficient of 20 mmol l^{-1} cm^{-1} was assumed. For Cyt *f*, readings were performed at 554 nm, and an extinction coefficient of 19.7 mmol l^{-1} cm^{-1} was assumed.

Photosynthetic enzymes

Samples of 100 mg FW of powdered frozen leaf material were used to evaluate the initial and total activities of RuBisCO (EC 4.1.1.39) (Tazoe et al. 2008), and ribulose-5-phosphate kinase (Ru5PK; EC 2.7.1.19) (Souza et al. 2005), with some modifications for coffee leaves (Ramalho et al. 2013).

The homogenization was done in 1 ml extraction buffer of 100 mM Tris-HCl, (pH 8), containing 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 2 mM DTT, 1% (v/v) Triton X-100, 10% (v/v) glycerol and 2% (v/v) 'Complete-protease

inhibitor cocktail' (Roche, ref. 04693159001), together with 100 mg insoluble PVPP per homogenate, and with the absence of $NaHCO_3$. The extracts were then centrifuged (16,000*g*, 15 min, 4 °C) and the obtained clean supernatant was used for RuBisCO and Ru5PK spectrophotometric assays). Briefly, RuBisCO activity evaluation was performed by using an assay medium containing 50 mM Tris-HCl buffer (pH 8.0), 15 mM $MgCl_2$, 20 mM $NaHCO_3$, 100 mM phosphocreatine, 10 mM ATP, 0.2 mM NAPH, 20 U ml^{-1} creatine kinase, 15 U ml^{-1}, 3-phosphoglycerate kinase and 15 U ml^{-1} glyceraldehyde-3-phosphate dehydrogenase.

For the initial RuBisCO activity, 15 µl of 667 mM RuBP (10 mM as final concentration) were added to the assay medium, and then 20 µl of the clean supernatant, followed by immediate reading. For the total RuBisCO activity, to the assay medium 20 µl of the clean supernatant were added, followed by a 20 min incubation period. The reaction was then started with addition of 10 mM RuBP (as final concentration). In both cases, measurements followed the 3-PGA-dependent NADH oxidation at 340 nm.

For Ru5PK activity, 20 µl of clean supernatant were added to the spectrophotometer cell with 100 mM Tris-HCl pH 8.0 buffer assay, containing 8 mM $MgCl_2$, 40 mM KCl, 20 mM phosphoenolpyruvate, 5 mM ATP, 1 mM NADH, 20 mM DTT, 8 U pyruvate kinase, 10 U ml^{-1} lactate dehydrogenase and 5 U ml^{-1} phosphoriboisomerase. After a 15-min incubation period, the reaction was started by adding 10 µl of 500 mM ribose-5-phosphate, and NADH oxidation was monitored at 340 nm.

For both enzymes, spectrophotometric measurements were done in a final volume of 1 ml, at 25°C.

Leaf abscisic acid

Samples of ~100 mg FW of powdered frozen leaf material were used for abscisic acid (ABA) analysis, according to Rodrigues et al. (2008). Extraction was performed in 1.0 ml of 200 mM Tris–HCl (pH 8.0), containing 2% Triton X-100, 10% PVPP and 10% glycerol, and centrifuged (5000*g*, 5 min, 4 °C). Abscisic acid was then quantified by an ELISA assay using a monoclonal antibody for ABA (kit-Phyto Detek, Agdia, Elkhart, IN, USA).

Proteins associated with the photosynthetic apparatus

All procedures, including protein extraction (from ~200 mg FW samples of powdered frozen coffee leaves), liquid chromatography and high-resolution mass spectrometry (NanoLC-MS/MS) analysis, and protein identification and quantification were performed as previously described in detail (Dubberstein et al. 2020). A reference database from *C. canephora* (Denoeud et al. 2014) of 25,574 polypeptide sequences totalling 10,251,572 residues was downloaded from Genoscope (http://coffee-genome.org/sites/coffee-genome.org/files/download/coffea_cds.fna.gz) on 1 July 2019, and used for peptide and protein inference by MASCOT Daemon 2.6.1 search algorithm (Matrix Science). For this study, we followed a targeted approach associated with the photosynthetic apparatus, by selecting and presenting the abundance changes of a set of 26 proteins, aiming to relate their results with physiological and biochemical data to improve our understanding regarding plant response to drought and/or eCO_2 conditions. These proteins comprise PSI and PSII, including, the OEC (related to PSII), and light-harvesting complexes, LHC (from both PSs), CEF involving PSI, and RuBisCO and RuBisCO activase (Table 3). Protein annotation was obtained at The UniProt Knowledgebase (UniProtKB) (https://www.uniprot.org/uniprot/?query=&sort=score). The original mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD019830 and Project DOI: 10.6019/PXD019830 for *C. arabica*, and the data set identifier PXD019831 and Project DOI: 10.6019/PXD019831 for *C. canephora.* Data set identifiers PXD019474 and PXD019541 were also used in the present study.

Experimental design and statistical analysis

Plants from each coffee genotype were subjected to six treatment combinations, forming a 2 × 3 factorial (two [CO_2], aCO_2 or eCO_2; and three levels of available water, WW, MWD or SWD) following a completely randomized design, with six plants in individual pots per treatment. Physiological and biochemical data were analyzed using a three-way ANOVA to evaluate the differences between genotypes (CL153 or Icatu), air [CO_2] conditions (aCO_2 or eCO_2), between watering treatments (WW, MWD or SWD), and their interaction (see Tables S1 and S2 available as Supplementary data at *Tree Physiology* Online). Given that a significant genotype effect was only observed in very few cases (except in protein abundance), and our main focus was to compare the impact of air [CO_2] conditions and watering treatments (and their interaction) in each genotype, an a posteriori Tukey's HSD test for mean comparisons was performed separately for each genotype (as shown in figures and tables). Data analysis was performed using STATISTICA v7.0 (StatSoft, Hamburg, Germany).

Results

Leaf water status

Leaf Ψ_{pd} values evidenced a progressive transition from well-watered status (WW: ~−0.30 MPa) to moderate (MWD: between −1.6 and −2.1 MPa, except the 700-Icatu plants) and severe (SWD: between −3.7 and −4.5 MPa) water deficit in both genotypes (Figure 1). Notably, MWD plants displayed higher Ψ_{pd} at eCO_2 than at aCO_2 (significant in Icatu). The higher Ψ_{pd} (−0.6 MPa) in MWD Icatu plants was even maintained under a harsher water restriction by total irrigation withholding for 5 days prior to data collection.

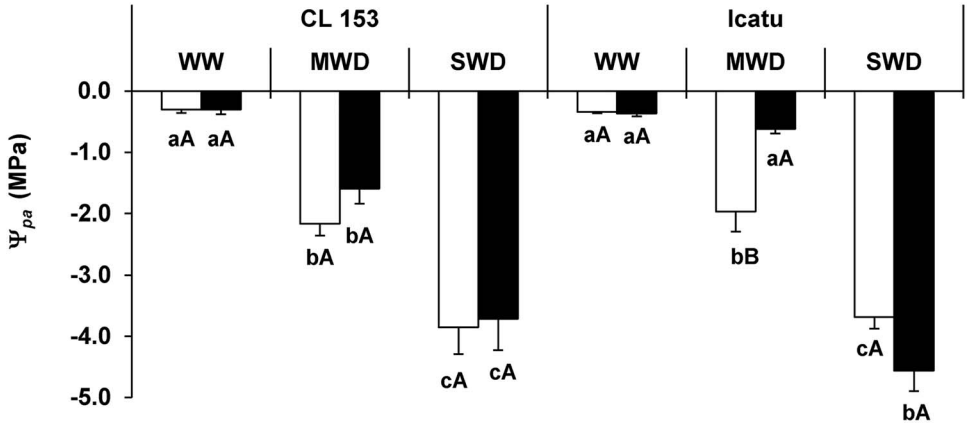

Figure 1. Pre-dawn leaf water potential (Ψ_{pd}) in *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 µl l^{-1} –, white bars) or elevated (700 µl l^{-1} –, black bars) CO_2 conditions, and submitted to WW, MWD or SWD. For each parameter, different letters after the mean values ± SE (n = 5–6) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

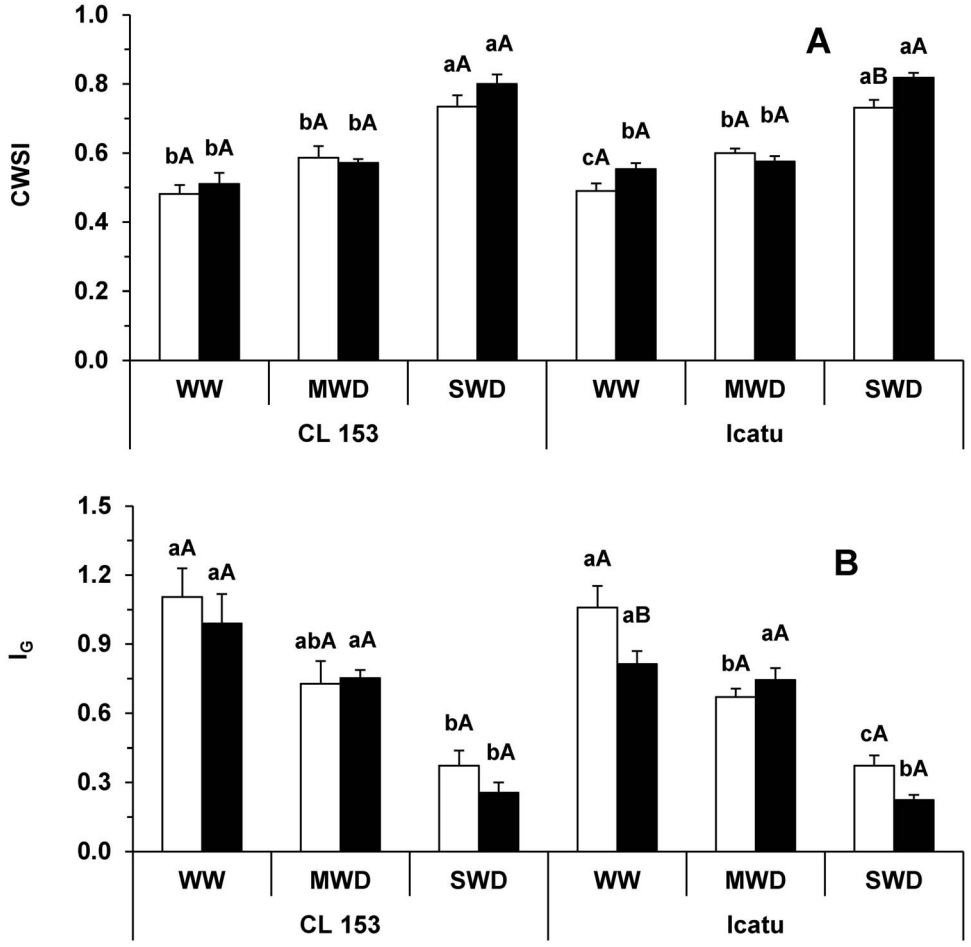

Figure 2. Water stress index (CWSI) (A) and stomatal conductance index (I_G) (B), calculated from leaves of *C. canephora* cv. Conilon clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 µl l^{-1}, white bars) and elevated (700 µl l^{-1}, black bars) CO_2 conditions, and submitted to WW, MWD and SWD. For each parameter, different letters after the mean values ± SE (n = 5) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

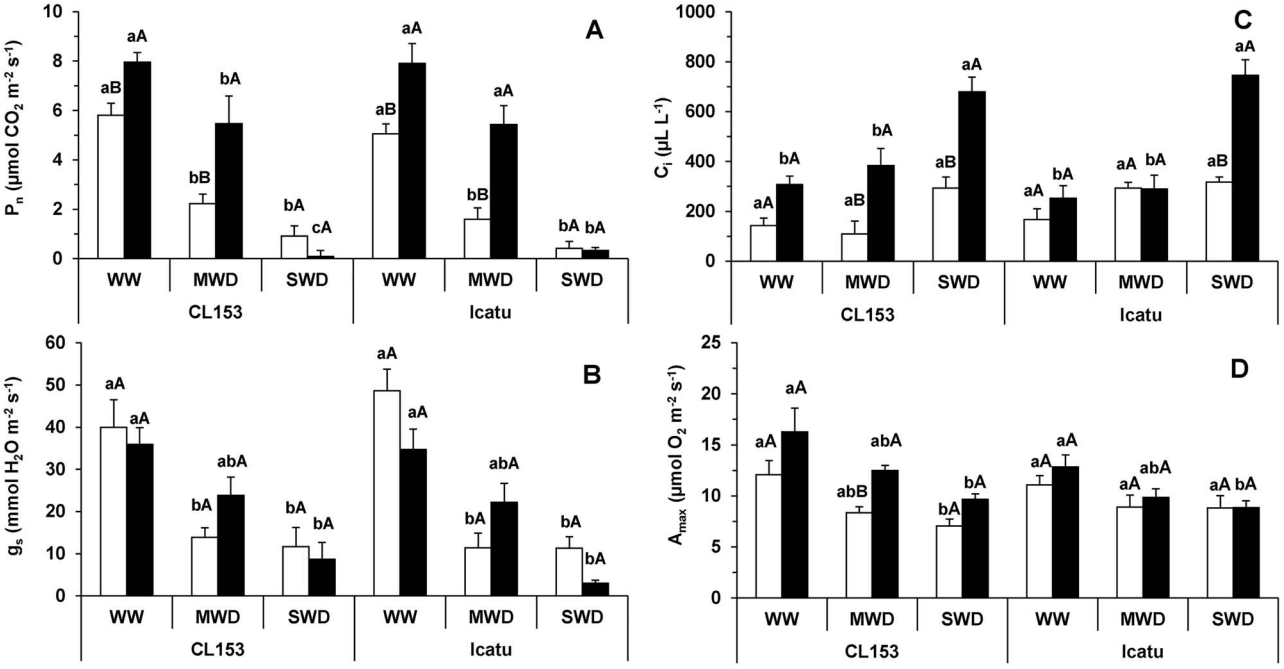

Figure 3. Leaf gas exchange parameters (A) net photosynthesis rate (P_n), (B) stomatal conductance to water vapor (g_s), (C) internal concentration of CO_2 (C_i) and (D) photosynthetic capacity (A_{max}) in *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 μl l^{-1}, white bars) or elevated (700 μl l^{-1}, black bars) CO_2 conditions, and submitted to WW, MWD or SWD. For each parameter, different letters after the mean values ± SE (n = 5–6) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

Thermal imaging analysis

The gradual drift of thermal indexes for both crop water stress (CWSI) and stomatal conductance (I_G) (Figure 2) showed that different drought degrees were progressively reached until maximal severity in SWD plants, in line with Ψ_{pd} variation (Figure 1). Greater stress severity was always observed in SWD plants, as judged from the maximal CWSI paralleling minimal I_G values irrespective of genotype or [CO_2]. Although no differences were observed between [CO_2] treatments within each water condition, under eCO_2 these indexes barely changed from WW to MWD conditions (somewhat clear in Icatu), in somewhat contrast to 380-plants, as compared with their respective WW plants.

Leaf gas exchanges

Single drought exposure depressed the net photosynthetic rate (P_n) by 62 and 68% in MWD plants, and by 84 and 92% in SWD plants, in CL153 and Icatu plants, respectively, as compared with their WW controls (Figure 3). Additionally, stomatal conductance (g_s) was decreased by 65 and 77% in MWD plants, and by 69 and 77% in SWD individuals, in the same genotype order. Under SWD conditions, internal [CO_2] (C_i) ca doubled the values in both genotypes, whereas the photosynthetic capacity (A_{max}) declined by 32% (CL153) and 20% (Icatu), always as compared with their WW controls.

Long-term eCO_2 exposure significantly increased P_n values in WW plants of CL153 (37%) and Icatu (56%) as compared with their 380-plants, concomitantly with a relevant (although non-significant) increase in A_{max} values by 35% (CL153) and 16% (Icatu).

The eCO_2 greatly attenuated the decreases in P_n, g_s and A_{max} imposed by MWD, but had not effect under the hasher SWD conditions. In fact, the 700-plants of both genotypes showed some P_n reduction under MWD, but maintained values close to their respective WW 380-plants, as well as displayed higher P_n values (146% for CL153, and 240% for Icatu) than in their MWD 380-counterparts. This was accompanied by non-significant changes of g_s and C_i when comparing WW and MWD plants under eCO_2. A_{max} showed a similar pattern to that of P_n in the MWD 700-plants of both genotypes, that is, although showing some decrease when compared with the WW 700-plants, the MWD 700-plants still maintained higher A_{max} values (50% in CL153 or 11% in Icatu) than those of the 380-plants under MWD.

Under SWD conditions P_n and g_s were severely reduced regardless of [CO_2] or genotype. However, under such drought conditions a relevant potential for C-assimilation was preserved, with A_{max} still showing values close to 60% (CL153), or even higher than 70% (Icatu) relative to those displayed by their respective WW controls.

Leaf abscisic acid

Single drought prompted gradual ABA increases of ~46% in MWD plants in both genotypes, and 100% (CL 153) and 184%

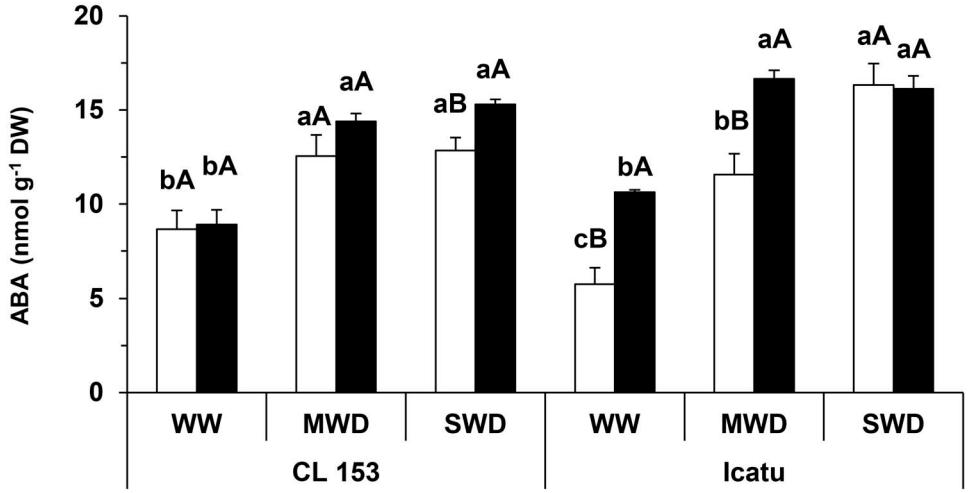

Figure 4. Abscisic acid content from leaves of *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 µl l^{-1}, white bars) or elevated (700 µl l^{-1}, black bars) CO_2 conditions, and submitted to WW, MWD or SWD. For each parameter, different letters after the mean values ± SE (n = 4) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

Table 1. Leaf chlorophyll *a* fluorescence parameters in *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 µl l^{-1}) or elevated (700 µl l^{-1}) CO_2 conditions, and submitted to WW, MWD or SWD. Parameters include: initial fluorescence (F_0), maximum *PSII* photochemical efficiency (F_v/F_m), photochemical quenching coefficient (q_L), actual PSII photochemical efficiency of energy conversion (F_v'/F_m') and the rate constant of PSII inactivation (F_s/F_m'), as well as the estimate of quantum yields of non-cyclic electron transport [$Y_{(II)}$], of regulated energy dissipation in PSII [$Y_{(NPQ)}$], and of non-regulated energy dissipation in PSII [$Y_{(NO)}$]. For each parameter, different letters after the mean values ± SE (n = 5) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

Genotype	[CO_2] (µl l^{-1})	Water treatment	F_0	F_v/F_m	F_v'/F_m'	$Y_{(II)}$	$Y_{(NPQ)}$	$Y_{(NO)}$	q_L	F_s/F_m'
CL153	380	WW	0.210 ± 0.004 aB	0.770 ± 0.008 aA	0.595 ± 0.023 aA	0.358 ± 0.044 aA	0.245 ± 0.043 bA	0.397 ± 0.015 aA	0.448 ± 0.066 aA	0.642 ± 0.044 bA
		MWD	0.224 ± 0.007 aA	0.771 ± 0.010 aA	0.550 ± 0.015 aA	0.130 ± 0.034 bB	0.552 ± 0.032 aA	0.319 ± 0.024 aA	0.132 ± 0.038 bA	0.870 ± 0.034 aA
		SWD	0.233 ± 0.008 aA	0.705 ± 0.025 bA	0.355 ± 0.037 bA	0.065 ± 0.010 bA	0.615 ± 0.021 aA	0.320 ± 0.026 aA	0.156 ± 0.030 bA	0.935 ± 0.010 aA
	700	WW	0.285 ± 0.014 aA	0.774 ± 0.006 aA	0.642 ± 0.013 aA	0.396 ± 0.023 aA	0.303 ± 0.021 bA	0.301 ± 0.022 aB	0.375 ± 0.032 aA	0.604 ± 0.023 bA
		MWD	0.235 ± 0.007 bA	0.777 ± 0.010 aA	0.649 ± 0.025 aA	0.354 ± 0.021 aA	0.272 ± 0.041 bB	0.373 ± 0.035 aA	0.304 ± 0.031 aA	0.646 ± 0.021 bB
		SWD	0.236 ± 0.009 bA	0.747 ± 0.023 aA	0.423 ± 0.039 bA	0.153 ± 0.032 bA	0.564 ± 0.039 aA	0.283 ± 0.015 aA	0.247 ± 0.044 aA	0.847 ± 0.032 aA
Icatu	380	WW	0.251 ± 0.007 aB	0.753 ± 0.005 aA	0.593 ± 0.022 aA	0.356 ± 0.029 aA	0.295 ± 0.034 bA	0.349 ± 0.018 aA	0.380 ± 0.029 aA	0.644 ± 0.029 bA
		MWD	0.247 ± 0.009 aA	0.755 ± 0.011 aA	0.416 ± 0.026 bA	0.191 ± 0.032 bA	0.564 ± 0.039 aA	0.246 ± 0.020 aA	0.345 ± 0.063 abA	0.809 ± 0.032 aA
		SWD	0.244 ± 0.005 aA	0.761 ± 0.008 aA	0.449 ± 0.020 bA	0.136 ± 0.013 bA	0.585 ± 0.026 aA	0.280 ± 0.024 aA	0.205 ± 0.028 bA	0.864 ± 0.013 aA
	700	WW	0.308 ± 0.011 aA	0.734 ± 0.004 aA	0.588 ± 0.019 aA	0.351 ± 0.029 aA	0.314 ± 0.019 bA	0.335 ± 0.024 aA	0.385 ± 0.033 aA	0.649 ± 0.029 bA
		MWD	0.254 ± 0.007 bA	0.744 ± 0.012 aA	0.529 ± 0.024 aA	0.265 ± 0.019 abA	0.361 ± 0.043 abB	0.374 ± 0.035 aA	0.320 ± 0.021 abA	0.735 ± 0.019 abA
		SWD	0.242 ± 0.005 bA	0.757 ± 0.008 aA	0.521 ± 0.032 aA	0.199 ± 0.017 bA	0.486 ± 0.028 aA	0.315 ± 0.024 aA	0.236 ± 0.024 bA	0.801 ± 0.017 aA

(Icatu) under SWD conditions, whereas single eCO_2 increased ABA levels (by 85%) only in Icatu (Figure 4).

Under water restriction, the eCO_2 tended to increase ABA content in both genotypes (except in Icatu SWD plants), and under stimulated an earlier response in Icatu given that ABA levels peaked at MWD conditions and were so maintained afterwards, whereas in the 380-plants maximal ABA values were precisely observed in SWD conditions.

Chlorophyll a fluorescence analysis

Single drought (380-plants) did not affect F_0 (even under SWD conditions) regardless of genotype. In contrast, single eCO_2 (WW plants) promoted significant F_0 rises, but upon MWD and SWD exposure F_0 was unaltered by eCO_2 (Table 1). In turn, single eCO_2 did not affect F_v/F_m in both genotypes, whereas single drought significantly reduced F_v/F_m only in CL153 SWD plants, an effect that was largely attenuated by eCO_2.

photosynthetic steady-state functioning, the actual PSII photochemical efficiency (F_v'/F_m') remained unaffected under single eCO_2 exposure, but it was reduced by single drought in MWD (Icatu—30%) and SWD (CL153—40%; Icatu—24%) plants. Also, eCO_2 clearly attenuated drought impacts on F_v'/F_m', particularly in Icatu, which showed no significant reductions in either MWD or SWD 700-plants, in contrast with the impact found in CL153 SWD 700-plants. In turn, the PSII inactivation estimate (F_s/F_m') greatly increased due to single drought exposure (MWD and SWD) in either genotype, although eCO_2 mitigated these impacts, particularly in MWD plants.

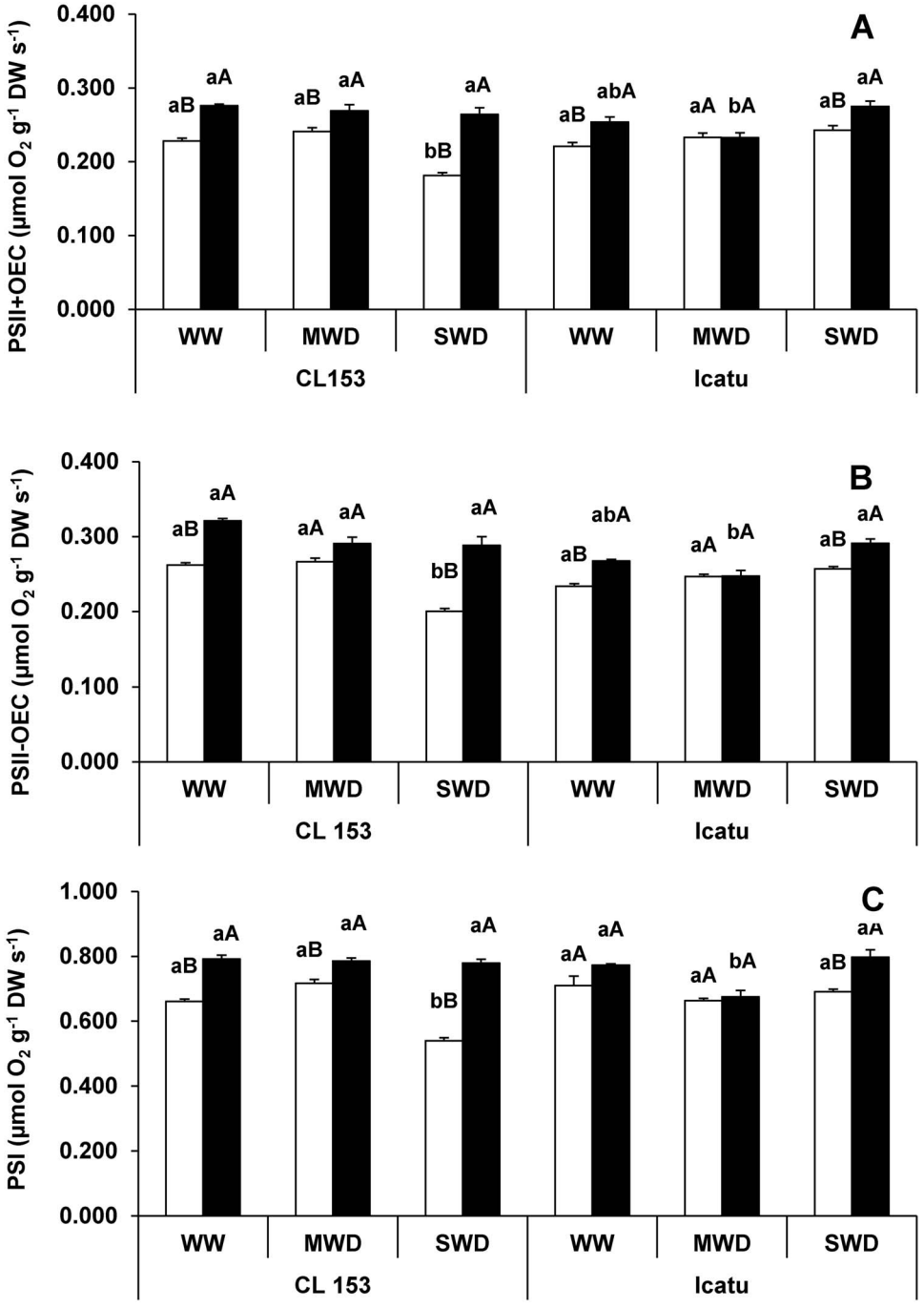

Figure 5. Potential thylakoid electron transport rates of PSII, (A) with (+OEC) or (B) without (−OEC) the OEC participation, and of (C) PSI in *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 µl l^{-1}, white bars) or elevated (700 µl l^{-1}, black bars) CO_2 conditions, and submitted to WW, MWD or SWD. For each parameter, different letters after the mean values ± SE (n = 3) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

The photochemical energy use, assessed by $Y_{(II)}$ and q_L, was not significantly modified by single eCO_2 exposure, but was markedly impacted by MWD and, especially, SWD conditions, the latter reducing $Y_{(II)}$ by 82 and 62%, and q_L by 65 and 46%, in CL153 and Icatu plants, respectively. Yet, eCO_2 clearly reduced the MWD and SWD impacts on $Y_{(II)}$ and q_L in both genotypes, particularly in MWD plants, which showed values not significantly different from those of their WW counterparts.

The photochemical energy use is balanced with dissipation mechanisms under conditions of excessive available energy. The $Y_{(NPQ)}$ remained unaffected by single eCO_2, although increasing

Table 2. Contents of the thylakoid electron carriers plastoquinone (PQ-9), and cytochromes (Cyt) b_{559LP}, b_{559HP}, b_{563} and *f* in *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 µl l^{-1}) or elevated (700 µl l^{-1}) CO_2 conditions, and submitted to WW, MWD or SWD. For each parameter, different letters after the mean values ± SE (n = 3) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype. DW, dry weight.

Genotype	[CO_2] (µl l^{-1})	Water treatment	PQ-9 (nmol g^{-1} DW)	Cyt b_{559LP} (nmol g^{-1} DW)	Cyt b_{559HP} (nmol g^{-1} DW)	Cyt *f* (nmol g^{-1} DW)	Cyt b_{563} (nmol g^{-1} DW)
CL153	380	WW	318 ± 60 aA	14.5 ± 0.4 aA	17.0 ± 0.4 aB	16.7 ± 0.5 aA	26.2 ± 1.5 aA
		MWD	329 ± 29 aA	8.7 ± 0.2 bB	10.2 ± 0.1 bB	11.5 ± 0.1 bB	16.0 ± 0.1 bB
		SWD	381 ± 33 aB	10.1 ± 0.2 bB	11.8 ± 0.2 bB	13.3 ± 0.1 bB	18.5 ± 0.1 bB
	700	WW	383 ± 57 bA	15.9 ± 0.6 aA	18.4 ± 0.4 aA	17.5 ± 0.8 abA	28.2 ± 1.6 aA
		MWD	530 ± 12 abA	11.3 ± 0.2 bA	13.5 ± 0.2 bA	14.6 ± 0.1 bA	20.4 ± 0.2 bA
		SWD	775 ± 59 aA	14.6 ± 0.3 aA	17.4 ± 0.2 aA	18.9 ± 0.1 aA	26.3 ± 0.3 abA
Icatu	380	WW	315 ± 89 bA	13.5 ± 0.5 aA	15.3 ± 0.4 aA	16.4 ± 0.5 bA	26.7 ± 1.4 aB
		MWD	585 ± 111 abA	13.4 ± 0.2 aA	15.3 ± 0.2 aA	19.2 ± 0.2 abA	25.8 ± 0.2 aA
		SWD	638 ± 121 aA	14.6 ± 0.3 aA	16.7 ± 0.2 aA	20.9 ± 0.2 aA	28.1 ± 0.2 aB
	700	WW	460 ± 53 aA	15.3 ± 0.5 aA	16.3 ± 0.3 aA	18.2 ± 0.4 abA	31.7 ± 0.5 aA
		MWD	461 ± 18 aA	10.4 ± 0.3 bB	11.0 ± 0.2 bB	15.7 ± 1.3 bB	19.3 ± 0.1 bB
		SWD	585 ± 69 aA	15.9 ± 0.3 aA	18.3 ± 0.5 aA	22.0 ± 0.2 aA	31.5 ± 0.9 aA

strikingly upon single MWD or SWD exposure regardless of genotype. Under SWD the 380-plants showed increases of 151% (CL153) and 98% (Icatu) in $Y_{(NPQ)}$. Notably, in both genotypes such dissipation capabilities were maintained at a lower level in the 700-plants under MWD and SWD than in their respective 380-plants, which agrees with their higher photochemical energy use under eCO_2.

Finally, $Y_{(NO)}$ was only marginally impacted by the single or combined drought and eCO_2 exposure, reflecting and absence of aggravated status regarding non-regulated energy dissipation processes.

Thylakoid electron transport rates

The potential rates of electron transport involving both PSs were assessed to provide clues regarding potential drought sensitivity points in coffee plants. Drought reduced the activities of PSII (with or without OEC), and PSI by ~20% in CL153 only under SWD, while Icatu plants remained unaffected by drought irrespective of [CO_2] (Figure 5).

Additionally, within each genotype the WW 700-plants displayed improved PSI and II activities, reaching ~20% (CL153) and 15% (Icatu) higher values than in their WW 380-plants. The eCO_2 usually maintained such positive impact under drought, and even reversed the loss of PSs performance observed in CL153 380-plants under SWD.

Thylakoid electron carriers

Single drought exposure promoted different changes among electron carriers and genotypes (Table 2). In Icatu, the Cyt b_{559} and b_{563} contents were not significantly modified, whereas significant increases in Cyt *f* (28%) and PQ-9 (the redox form of plastoquinone, PQ) (102%) were observed under SWD. In sharp contrast, in CL153 significant reductions were found for all Cyts under both MWD and SWD, while PQ-9 did not vary significantly.

The eCO_2 alone did not significantly alter these carrier contents (except for Cyt b_{559HP} in CL153, and Cyt b_{563} in Icatu). Yet, it is noteworthy that a systematic tendency to higher values was observed for all carriers in both genotypes, justifying the observed significant global CO_2 effect (see Table S1 available as Supplementary data at *Tree Physiology* Online).

Under drought and eCO_2, despite some variations between MWD and SWD plants, eCO_2 globally increased these photosynthetic components under SWD conditions. In fact, while CL153 380-plants were clearly affected by single SWD exposure, their 700-plants counterparts showed no impact on Cyt contents (as compared with WW plants, regardless of [CO_2]), and a large PQ-9 increase. In Icatu, eCO_2 did not reverse the single SWD effect given that no significant impact was observed in the 380-plants, but the 700-plants exposed to SWD still showed a tendency to higher contents in all Cyts.

Interestingly, the 700-plants of both genotypes under MWD usually showed lower contents than those of their respective 700-plants under SWD, but without impact on the electron transport rates (Figure 5).

Photosynthetic enzymes

RuBisCO activities were gradually reduced in CL153 plants by single drought, reaching declines of 40% (initial) and 30% (total) under SWD conditions (Figure 6A and B). This contrasted with Icatu plants in which RuBisCO was not negatively affected by drought. RuBisCO activation presented some fluctuations (with a reducing tendency in CL153), and Ru5PK tended to a higher activity at MWD, and with no declines in SWD

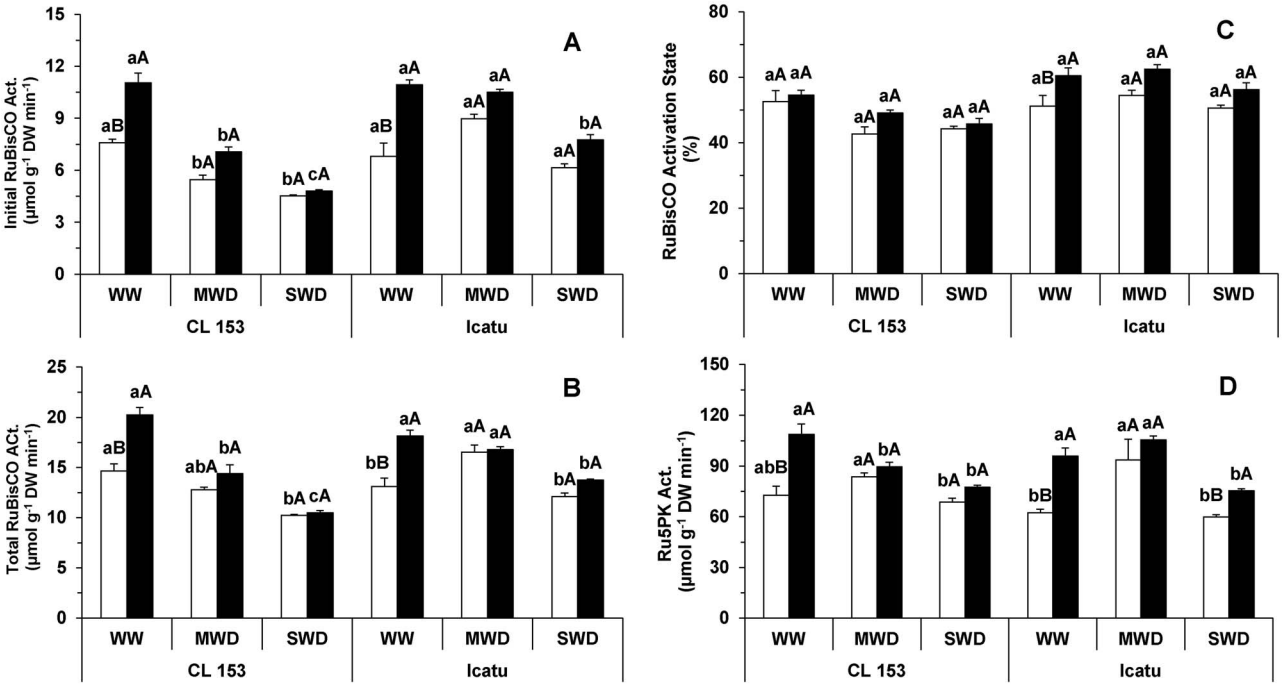

Figure 6. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (A) initial activity, (B) total activity and (C) activation sate, and (D) ribulose-5-phosphate kinase (Ru5PK) maximal activity in *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 μl l^{-1}, white bars) or elevated (700 μl l^{-1}, black bars) CO_2 conditions, and submitted to WW, MWD or SWD. For each parameter, different letters after the mean values ± SE (n = 4) express significant differences between water treatments within each [CO_2] (a, b, c), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

conditions, as compared with their respective WW plants, always for both genotypes.

Single eCO_2 significantly reinforced the initial (45–61%) and total (~38%) activities of RuBisCO, as well as that of Ru5PK (~50%) in WW plants from both genotypes. RuBisCO activation also increased in Icatu.

Under MWD, the 700-plants from both genotypes showed a consistent trend to higher Ru5PK and RuBisCO activities (and activation state for the latter), although non-significantly in most cases. Under SWD conditions, this tendency was only preserved in Icatu.

Proteins from the photosynthetic apparatus

Regarding the altered environmental conditions, drought alone was globally the main driver for abundance increase of most proteins associated with the photosynthetic apparatus (PSI and II, OEC, LHCI and II, RuBisCO, RuBisCO activase and CEF-PSI) (Table 3), different for the genotype factor for most proteins (see Table S2 available as Supplementary data at *Tree Physiology* Online). In fact, under SWD conditions a systematic increase trend was observed in all 26 proteins in both genotypes, but only Icatu showed significant increases (in 15 of them).

In contrast, eCO_2 did not significantly modify the abundance of any of these 26 proteins in WW plants from both genotypes, in line with the absence of significance for the large majority of proteins as regard the CO_2 factor or their interaction with genotype (see Table S2 available as Supplementary data at *Tree Physiology* Online). Still, a closer look revealed a tendency to lower abundance of 15 proteins in 700-plants, as compared with their 380-counterparts, especially in CL153.

Under SWD conditions, eCO_2 did not significantly alter protein abundance in the 700-plants (with the unique exception for the minor represented LHCII 21 kDa protein in CL153), as compared with the respective 380-plants of each genotype.

A more detailed analysis of each protein group revealed that, regardless of CO_2, the proteins associated with PSII and LHCII were more abundant under drought, significantly under SWD only in Icatu for some of them. These proteins included the PsbP (extrinsic subunit of PSII) and the PsbS (PSII 22 kDa) proteins (as noted by their significant interaction of genotype vs water availability—see Table S2 available as Supplementary data at *Tree Physiology* Online), which are associated with O_2 evolution and non-photochemical quenching mechanism, respectively. Greater abundance under drought in Icatu was also observed for seven (aCO_2) and four (eCO_2) proteins (out of eight) from LHCII. As regards CL153 plants, only the abundance of LHCII 21 kDa protein increased significantly, exclusively under eCO_2.

A similar pattern to that of PSII was also found for 10 proteins associated with PSI, their LHC, and with CEF-PSI [two NADH dehydrogenase-like (NDH) complex proteins, and one proton gradient regulation protein (PGR5)]. Overall, abundance

Table 3. Changes in the relative abundance of proteins from the photosynthetic apparatus, regarding PSI and II, OEC (related to PSII), LHCI and II, RuBisCO and RuBisCO activase, as well as associated with CEF involving both PSs, in *C. canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants grown under ambient (380 µl l^{-1}) or elevated (700 µl l^{-1}) CO_2 conditions, and submitted to WW, MWD or SWD. For each protein, different letters after the mean values ± SE (n = 3) express significant differences between water treatments within each [CO_2] (a, b), or between [CO_2] within each water treatment (A, B), always separately for each genotype.

Genotype	CL153						Icatu					
[CO_2] (µl l^{-1})	380			700			380			700		
Water treatment	WW	MWD	SWD	WW	MWD	SWD	WW	MWD	SWD	WW	MWD	SWD
Photosystem II and oxygen evolving complex												
Cc07_g05350—Oxygen-evolving enhancer protein 1, chloroplastic	227.4 ± 12.0 aA	226.7 ± 52.9 aA	247.7 ± 47.0 aA	189.7 ± 43.2 aA	221.7 ± 6.7 aA	197.7 ± 22.4 aA	184.7 ± 59.9 aA	260.3 ± 2.9 aA	271.0 ± 14.9 aA	162.3 ± 30.2 abA	101.3 ± 25.8 bB	217.7 ± 9.2 aA
Cc05_g00840—Oxygen-evolving enhancer protein 2, chloroplastic	109.3 ± 2.3 aA	98.7 ± 27.5 aA	115.7 ± 21.8 aA	98.7 ± 22.7 aA	117.7 ± 17.3 aA	98.7 ± 7.4 aA	102.0 ± 45.7 aA	145.3 ± 3.8 aA	140.7 ± 10.7 aA	99.7 ± 28.3 aA	45.3 ± 15.4 aB	133.7 ± 11.5 aA
Cc02_g11770—Oxygen-evolving enhancer protein 3–2, chloroplastic	41.3 ± 8.1 aA	41.3 ± 6.7 aA	61.7 ± 3.5 aA	28.0 ± 4.0 bA	49.0 ± 11.4 abA	56.0 ± 6.7 aA	56.0 ± 15.2 aA	58.0 ± 2.9 aA	63.7 ± 5.5 aA	53.0 ± 4.0 abA	22.3 ± 8.1 bB	71.7 ± 7.3 aA
Cc10_g11890—Photosystem II 22 kDa protein, chloroplastic	42.0 ± 7.0 aA	42.3 ± 1.8 aA	48.3 ± 6.0 aA	38.3 ± 1.9 aA	37.7 ± 2.4 aA	44.7 ± 3.7 aA	54.3 ± 12.3 bA	66.7 ± 5.4 abA	85.0 ± 9.3 aA	44.7 ± 3.5 bA	32.3 ± 4.1 bB	76.0 ± 4.5 aA
Cc02_g35130—PsbP domain-containing protein 1, chloroplastic	12.7 ± 5.7 aA	14.7 ± 2.2 aA	18.0 ± 2.0 aA	9.3 ± 5.5 aA	8.3 ± 0.3 aA	10.3 ± 0.9 aA	10.3 ± 3.5 bA	15.7 ± 2.2 abA	19.3 ± 3.0 aA	9.3 ± 0.3 abA	6.3 ± 2.3 bB	17.0 ± 0.6 aA
Cc06_g20190—PsbP domain-containing protein 6, chloroplastic	2.3 ± 0.3 aA	2.0 ± 0.0 aA	4.7 ± 2.7 aA	2.0 ± 0.0 aA	1.7 ± 0.3 aA	4.7 ± 0.9 aA	5.0 ± 2.6 aA	9.0 ± 2.0 abA	14.7 ± 0.3 bA	4.3 ± 1.9 bA	1.7 ± 0.7 bB	15.3 ± 0.7 aA
Light-harvesting complex proteins from photosystems II												
Cc10_g16210—Chlorophyll a-b binding protein CP26, chloroplastic	28.0 ± 6.0 aA	28.3 ± 8.0 aA	54.0 ± 15.9 aA	30.3 ± 9.1 aA	36.3 ± 7.6 aA	54.3 ± 8.1 aA	39.7 ± 18.2 bA	75.7 ± 6.7 abA	96.0 ± 14.7 aA	46.0 ± 13.7 abA	18.7 ± 7.7 bB	78.3 ± 4.3 aA
Cc09_g09500—Chlorophyll a-b binding protein 36, chloroplastic	15.7 ± 4.3 aA	18.0 ± 6.7 aA	30.7 ± 9.8 aA	19.7 ± 6.4 aA	18.3 ± 6.1 aA	35.7 ± 3.8 aA	27.0 ± 15.8 bA	43.3 ± 0.9 abA	69.7 ± 3.8 aA	25.0 ± 9.5 bA	8.7 ± 3.2 bB	64.0 ± 7.2 aA
Cc09_g09030—Chlorophyll a-b binding protein 21, chloroplastic	2.3 ± 2.3 aA	4.3 ± 4.3 aA	14.7 ± 9.8 aA	1.7 ± 1.2 aA	4.7 ± 2.9 aA	20.7 ± 5.2 aA	14.3 ± 13.8 aA	48.9 ± 17.9 aA	49.0 ± 17.9 aA	13.3 ± 7.3 aA	0.3 ± 0.3 aA	44.7 ± 8.4 aA
Cc02_g21720—Chlorophyll a-b binding protein CP24 10A, chloroplastic	5.7 ± 2.7 aA	7.3 ± 3.9 aA	17.7 ± 8.2 aA	7.7 ± 2.3 aA	8.0 ± 3.5 aA	17.0 ± 4.4 aA	8.3 ± 5.0 bA	12.7 ± 0.9 abA	21.0 ± 3.5 aA	7.0 ± 3.2 abA	1.3 ± 0.9 bB	17.3 ± 0.3 aA
Cc05_g12720—Chlorophyll a-b binding protein 13, chloroplastic	4.7 ± 1.2 abA	3.7 ± 0.7 bA	9.0 ± 2.1 aA	4.0 ± 1.2 bA	3.3 ± 0.3 bA	8.7 ± 1.2 aA	4.7 ± 2.9 bA	6.0 ± 1.2 bA	16.0 ± 2.3 aA	3.0 ± 0.6 bA	1.7 ± 0.9 bA	15.3 ± 2.2 aA
Cc11_g16910—Chlorophyll a-b binding protein, chloroplastic	1.0 ± 1.0 aA	0.0 ± 0.0 aA	2.7 ± 1.7 aA	0.0 ± 0.0 bA	0.7 ± 0.7 abA	4.0 ± 1.0 aA	2.7 ± 2.7 bA	1.3 ± 0.3 bA	11.7 ± 3.3 aA	3.7 ± 2.0 bA	0.0 ± 0.0 bA	13.0 ± 1.5 aA
Cc09_g09020—Chlorophyll a-b binding protein 21, chloroplastic	0.0 ± 0.0 aA	0.0 ± 0.0 aA	0.3 ± 0.3 aA	0.3 ± 0.3 bA	0.7 ± 0.7 bA	2.3 ± 0.3 aB	0.7 ± 0.7 bA	1.3 ± 0.9 abA	3.7 ± 0.7 aA	0.3 ± 0.3 bA	0.0 ± 0.0 bA	4.0 ± 1.0 aA
Photosystem I												
Cc03_g03590—Photosystem I reaction center subunit II, chloroplastic	109.0 ± 10.5 aA	96.0 ± 24.0 aA	128.0 ± 17.1 aA	78.3 ± 12.5 aA	94.7 ± 4.3 aA	103.0 ± 13.7 aA	106.0 ± 47.4 aA	147.0 ± 5.3 aA	187.3 ± 20.2 aA	94.7 ± 15.6 abA	37.0 ± 16.5 bB	128.3 ± 8.5 aA
Cc04_g03050—Photosystem I reaction center subunit VI, chloroplastic	14.7 ± 6.6 aA	12.0 ± 6.4 aA	39.3 ± 12.3 aA	9.7 ± 5.9 aA	20.3 ± 7.7 aA	36.3 ± 6.9 aA	26.7 ± 12.6 aA	31.3 ± 3.8 aA	46.3 ± 5.4 aA	19.7 ± 6.4 bA	2.7 ± 1.2 bB	44.3 ± 0.3 aA
Cc09_g08490—Photosystem I reaction center subunit psaK, chloroplastic	10.3 ± 2.4 aA	10.3 ± 3.4 aA	18.3 ± 3.9 aA	11.3 ± 2.3 aA	11.0 ± 0.0 aA	16.0 ± 2.5 aA	11.7 ± 4.3 aA	18.3 ± 0.9 aA	13.0 ± 4.7 aA	10.0 ± 2.1 aA	5.3 ± 0.9 aB	14.0 ± 1.5 aA
Cc01_g15890—Photosystem I reaction center subunit XI, chloroplastic	5.3 ± 1.9 aA	8.0 ± 3.5 aA	12.7 ± 5.7 aA	7.3 ± 1.7 aA	5.7 ± 0.3 aA	12.3 ± 2.4 aA	8.3 ± 3.5 bA	12.3 ± 1.3 abA	19.7 ± 3.5 aA	7.7 ± 2.6 bA	2.7 ± 0.3 bB	17.7 ± 1.2 aA
Light-harvesting complex proteins from photosystems I												
Cc05_g09930—Chlorophyll a-b binding protein 8, chloroplastic	16.3 ± 2.8 aA	15.7 ± 5.0 aA	29.7 ± 7.9 aA	19.7 ± 3.0 aA	20.7 ± 2.2 aA	25.0 ± 4.7 aA	18.7 ± 8.7 bA	34.0 ± 0.6 abA	40.0 ± 6.1 aA	23.3 ± 5.2 abA	10.0 ± 5.5 bB	36.3 ± 3.8 aA
Cc09_g02010—Chlorophyll a-b binding protein 6A, chloroplastic	7.0 ± 2.1 aA	6.7 ± 2.2 aA	15.0 ± 5.0 aA	5.0 ± 0.0 aA	7.0 ± 1.2 aA	13.7 ± 1.5 aA	8.3 ± 3.9 bA	11.3 ± 2.0 bA	31.7 ± 6.7 aA	9.3 ± 2.7 bA	3.7 ± 2.2 bA	32.0 ± 4.6 aA
Cc04_g16410—Chlorophyll a-b binding protein 4, chloroplastic	5.0 ± 2.0 aA	3.7 ± 3.2 aA	10.3 ± 2.4 aA	4.0 ± 2.1 aA	6.3 ± 2.0 aA	10.0 ± 2.0 aA	6.7 ± 3.8 bA	15.3 ± 1.9 abA	26.0 ± 3.1 aA	10.7 ± 3.0 bA	3.0 ± 2.5 bB	29.3 ± 2.6 aA
Cyclic electron flow												
Cc06_g22890—NDH-dependent cyclic electron flow 1	2.0 ± 1.2 aA	3.0 ± 1.5 aA	3.3 ± 1.3 aA	3.0 ± 2.5 aA	4.7 ± 1.2 aA	3.7 ± 1.9 aA	3.7 ± 1.9 aA	6.0 ± 0.6 aA	9.0 ± 1.5 aA	4.3 ± 2.2 aA	1.3 ± 1.3 aA	6.3 ± 0.9 aA
Cc04_g05100—NDH-dependent cyclic electron flow 1	0.3 ± 0.3 aA	0.0 ± 0.0 aA	1.3 ± 0.9 aA	1.0 ± 0.6 aA	1.3 ± 0.9 aA	1.3 ± 0.3 aA	1.0 ± 0.6 bA	1.7 ± 0.3 abA	3.3 ± 0.3 aA	1.7 ± 0.9 abA	0.0 ± 0.0 aA	2.7 ± 0.7 bA
Cc08_g13730—PGR5-like protein 1A, chloroplastic	0.0 ± 0.0 aA	0.7 ± 0.7 aA	0.3 ± 0.3 aA	0.7 ± 0.7 aA	4.7 ± 3.7 aA	2.3 ± 1.2 aA	3.3 ± 2.8 aA	8.3 ± 0.9 aA	9.3 ± 3.0 aA	6.3 ± 3.2 abA	1.0 ± 1.0 bA	11.3 ± 1.9 aA
RuBisCO and RuBisCO activase												
Cc00_g15710—Ribulose bisphosphate carboxylase small chain SSU11A, chloroplastic	46.3 ± 7.4 aA	49.0 ± 8.2 aA	58.0 ± 8.1 aA	51.0 ± 10.0 aA	62.7 ± 27.4 aA	53.0 ± 5.7 aA	51.0 ± 17.9 aA	78.7 ± 20.3 aA	79.7 ± 5.4 aA	47.7 ± 11.6 abA	25.3 ± 5.2 bB	82.7 ± 3.2 aA
Cc02_g07500—Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic	62.3 ± 16.8 aA	63.0 ± 15.5 aA	76.7 ± 22.1 aA	69.3 ± 14.9 aA	112.3 ± 42.4 aA	112.7 ± 17.5 aA	73.7 ± 43.2 aA	143.0 ± 9.3 aA	153.0 ± 13.7 aA	103.3 ± 44.4 abA	43.7 ± 22.9 bA	154.0 ± 18.5 aA
Cc04_g14500—Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic	1.7 ± 1.7 aA	2.3 ± 1.5 aA	3.0 ± 1.2 aA	3.0 ± 1.7 aA	5.7 ± 1.3 aA	4.7 ± 0.7 aA	1.7 ± 0.9 bA	13.0 ± 3.1 aA	21.0 ± 0.6 aA	3.7 ± 2.0 bA	1.3 ± 1.3 bB	18.0 ± 4.6 aA

of these proteins was also gradually increased by drought, regardless of [CO_2], but significant higher values were observed only in Icatu SWD plants (five in aCO_2; four in eCO_2).

Finally, RuBisCO (small unit) and RuBisCO activase tended to greater abundance under SWD conditions, similarly for both [CO_2], with Icatu SWD plants showing the greater increases, as compared with their WW plants.

Discussion

Firstly, we acknowledge that our experimental design and data collection was based on the use of pseudoreplicates regarding the air [CO_2] treatments (Hurlbert 1984, 2004), given that all the plants per each CO_2 treatment were grown in a single growth chamber. This contrasts with water treatments, in which the implementation of water restriction was performed individually for each plant until the desired level of drought was achieved (as controlled through Ψ_{pd} monitoring and partial water addition). Also, the weekly chamber swapping, although it does not eliminate the potential pseudoreplication effects, is expected to allow us to obtain similar data and conclusions as if we had used true replicates, either by performing one experiment with multiple chambers or using one chamber replicated in multiple experimental runs (Johnson et al. 2016). Still, considering that some statistical bias still can remain, any marginally significant results must be discussed with caution, and it is advisable to interpret effect sizes rather than *P*-values per se (Johnson et al. 2016), which was done herein.Therefore, as long as the pseudoreplicates existence is clearly stated, and readers are aware of the potential problems interpreting such results, we are confident that our study reports solid and useful information despite potential issues associated with pseudoreplication (Newman et al. 2011, Johnson et al. 2016).

Single drought impact on photosynthetic performance and components

A gradual water constraint was imposed until the SWD plants displayed Ψ_{pd} values below −3.7 MPa in both genotypes, a value that reflects an extreme water deficit in coffee (*cf.* Pinheiro et al. 2004, Brum and Melo 2013). Such increasing drought severity was globally reflected in changes in water stress thermal indexes (CWSI and I_G) (Figure 2), which followed the gradual reduction in g_s and Ψ_{pd} values, as also reported in other plant species (Costa et al. 2013, Gómez-Bellot et al. 2015), and are considered useful indicators of microenvironment suitability for the coffee crop (Craparo et al. 2017).

The g_s decline in drought-stressed plants of both genotypes was likely associated with a greater ABA content (Figure 4). Abscisic acid is determinant to stomata responses to increased air evaporative demand and/or reduced soil water availability (Buckley 2019), and a greater ABA synthesis has been implicated in drought tolerance in coffee trees via reductions in g_s, which in turn restrain the transpiration flow and postpone plant dehydration (Silva et al. 2018).

Due to the intrinsically low g_s values of coffee leaves, stomatal limitation, more than mesophyll or biochemical ones, has been shown to constitute the major constraint to photosynthesis in this species (DaMatta et al. 2019, Martins et al. 2019). However, as drought severity increases non-stomatal limitations will gradually become dominating. In fact, under MWD and SWD conditions the g_s reduction was accompanied by a C_i increase (Figure 3C), suggesting that photosynthesis was not limited by stomatal constraints. Additionally, the greater decline of P_n than in A_{max} (the latter assessed under the absence of diffusion-mediated limitations of photosynthesis by using saturating [CO_2]) suggests increased mesophyll diffusional constraints to CO_2 flux towards the carboxylation sites, whereas the A_{max} decline by itself points to photo/biochemical constraints. Collectively, our data indicate that non-stomatal (mesophyll and photo/biochemical) limitations were the major constraints to photosynthesis under drought conditions, which were exacerbated with increasing drought severity, in line with the sharp changes of CWSI and I_G from MWD to SWD conditions (Figure 2).

Non-stomatal limitations of photosynthesis were further confirmed by the negative impacts on the PSII photochemical efficiency (F_v/F_m, F_v'/F_m'), photochemical use of energy [$Y_{(II)}$, q_L] and PSII inactivation (F_s/F_m') (Table 1). These changes were stronger in SWD than in MWD plants, and usually to a higher extent in CL153 than in Icatu, in agreement with their impact on A_{max}. Notably, the lower photochemical use of energy was fully compensated for by the reinforcement of photoprotective thermal dissipation mechanisms [$Y_{(NPQ)}$] that protect the coffee leaves from excessive excitation damages (Pompelli et al. 2010), coupled with the reduction of ROS and chlorophyll (Fortunato et al. 2010, Dalal and Tripathy 2018). It is also remarkable that PSII non-regulated energy dissipation [$Y_{(NO)}$] did not rise in drought-stressed plants of both genotypes. This points that non-photochemical quenching processes attributable to photoinactivation and uncontrolled energy (heat and fluorescence) dissipation in PSII (Kramer et al. 2004, Huang et al. 2011) were not aggravated even under the SWD conditions, implying an intrinsic tolerance of these coffee plants to drought.

Icatu showed a great drought tolerance concerning both PSs activity (Figure 5) and carriers content (Table 2), whereas CL153 was clearly affected, particularly under the harshest drought conditions. In fact, although CL153 presented a consistent tendency to greater abundance of proteins related to PSs, LHCs and CEF-PSI, significant rises for more than half of these photosynthetic related proteins were only observed in Icatu, thus reflecting a greater responsiveness of this genotype (Table 3). Knowing that when C-assimilation is affected by environmental stresses (as was the case in SWD plants), the

resultant generation of ROS can inhibit protein synthesis (Murata et al. 2007), our findings revealed that de novo synthesis was in place to maintain full functioning capabilities, likely associated with the crucial reinforcement of antioxidative mechanisms under drought (Ramalho et al. 2018*b*), similar to this plant's response to cold, high irradiance and heat (Ramalho et al. 1998, Fortunato et al. 2010, Martins et al. 2016). Among the identified proteins associated with PSII, we should highlight the significant increases of PsbS (involved in non-photochemical quenching) and PsbP, an extrinsic subunit of PSII involved in O_2 evolution, in addition to PSII regulation, stabilization (Ifuku et al. 2005), repair and reassembly (Lu 2016) only in SWD Icatu plants, in good agreement with their abilities to maintain PSII activity regardless of [CO_2] (Figure 5). Under a reduced use of energy through photochemistry, the resulting increase of transthylakoid H^+ gradient will promote zeaxanthin synthesis and dimeric PsbS protein interaction with the LHCII antenna, with both promoting a rapid increase of thermal dissipation, thus protecting PSII from photodamage (Niyogi et al. 2005, Ruban 2016). Taken together, our data are consistent with increases in zeaxanthin pools (data not shown) and $Y_{(NPQ)}$ rise (Table 1) in Icatu SWD plants, as also reported in droughted *Arabidopsis thaliana* plants (Chen et al. 2016). From the above, we argue that the reinforcement of both PsbS and PsbP proteins in Icatu SWD 380-plants likely strengthen their photoprotective capabilities and the maintenance of PSII O_2 evolution (Figure 5), supporting their drought resilience. Notably, reductions of potential PSI and PSII activities (Figure 5) and photochemical efficiency (F_v/F_m, Table 1) in CL153 SWD plants under aCO_2 were not associated with reductions in the abundance of PSs-related proteins (Table 3). This suggests that although present these proteins might not be under a fully functional state, which would be associated with a lower efficiency of protective mechanisms as previously reported in *C. canephora* plants under drought (Ramalho et al. 2018*b*). Still regarding PSII, with the exception of PsbP, the abundance of proteins related to O_2 evolution remained mostly unchanged, which, overall, agrees with the similar pattern of PSII with or without the OEC participation in both genotypes (Figure 5). Therefore, we contend that OEC is not a preferential drought-sensitive component in coffee leaves.

Some of the greatest abundance increases, particularly in Icatu, were observed in LHC a/b binding proteins, which are related to the structure and function of both PSs, being associated with antennae pigments and/or with the PSs core reaction centers (Kim et al. 2009, Liu et al. 2013, Pietrzykowska et al. 2014). Given that the expression of the *Lhcb* genes is closely regulated by multiple environmental cues (Liu et al. 2013), and that LHCII functioning plays an important role in preventing PSII photodamage under drought stress (Chen et al. 2016), the higher pools of LHCII and LHCI proteins likely contributed to preserve PSII and PSI activities (Figure 5), the PSII photochemical efficiency (F_v/F_m) (Table 1), and energy capture (F_0) in Icatu SWD plants, thus supporting a high resilience under long-term drought exposure. Additionally, the large and gradual increase of PQ-9 with drought in Icatu is expected to improve the scavenging of singlet oxygen ($^1O_2$) and inhibit lipid membrane oxidation (Ksas et al. 2018), whereas, CEF-PSII (with Cyt b_{559}) and CEF-PSI (with Cyt b_6/f complex, PGR5 and NDH proteins which also increased) were likely stimulated. Overall, these processes should contribute to protect both PSs from photoinhibition by reducing the excess of excitation pressure (Miyake and Okamura 2003, Chu and Chiu 2016, Yamori et al. 2016), with the CEF-PSI further promoting the protective non-photochemical quenching (Sun et al. 2018) and ATP synthesis (Yamori et al. 2016) in Icatu. In contrast, CEF-PSII and CEF-PSI were unlikely to have been stimulated in CL153 since all Cyts content declined, in line with the significant difference in the genotypes response to drought for these electron carriers (see Table S1 available as Supplementary data at *Tree Physiology* Online). In particular, the reduction in the Cyt b_6/f complex components points to a drought sensitivity, as reported in other species (Kohzuma et al. 2009, Sanda et al. 2011). Finally, this might have contributed for decreasing the electron transport ability (PSs activities and F_v/F_m) and photosynthesis (P_n and A_{max}) given that C-assimilation has been reported to be closely related to Cyt b_6/f content under changing environmental conditions (Schöttler and Toth 2014).

Key enzymes from the Calvin-Benson cycle (RuBisCO and Ru5PK) have been used as probes of tolerance of photosynthetic biochemical components to environmental stresses in coffee (Ramalho et al. 1999, 2003, Rodrigues et al. 2016). Here, we demonstrated that the drought sensitivity of CL153 plants was also likely associated with strong impairments on RuBisCO activity (Figure 5), together with the above reported impact in both PSs activity and Cyt contents. These drought-induced impacts on RuBisCO activity have been ascribed to protein denaturation (Hoekstra et al. 2001), decreased synthesis of the small RuBisCO units and increased binding of RuBisCO inhibitors (Vu et al. 1999, Parry et al. 2002, Galmés et al. 2013, Fahad et al. 2017). In contrast, RuBisCO (and Ru5PK) activities were unaffected in Icatu SWD plants, a result consistent with a tendency to higher abundance of RuBisCO small units under drought, and greater RuBisCO activase abundance. This catalytic chaperone modulates RuBisCO activity, and was suggested to constitute a crucial factor in plant response to climate changes (Sage et al. 2008) due to its stress sensitivity, namely to heat and drought (Kumar et al. 2016, Perdomo et al. 2017). Notably, within each genotype, RuBisCO activation state remained mostly unaffected regardless of water and CO_2 conditions, close to previously reported values for coffee (Ramalho et al. 2003, Martins et al. 2013, Dubberstein et al. 2020).

Long-term eCO_2 impact on photosynthetic apparatus functioning

The Ψ_{pd} and g_s responsiveness was not modified by long-term eCO_2. This confirmed earlier findings for g_s in coffee

(Ramalho et al. 2013, Ghini et al. 2015, Avila et al. 2020*a*), in contrast to many other species in which g_s is reduced by eCO_2 (Ainsworth and Rogers 2007). Given that coffee trees typically display low g_s, and stomatal limitations are the main constraint to photosynthesis (Martins et al. 2019), the absence of stomatal acclimation to eCO_2 is expected to allow for greater photosynthetic gains associated with eCO_2 (DaMatta et al. 2016, Rodrigues et al. 2016, Avila et al. 2020*a*). Still, it is noteworthy that Icatu plants tended systematically to lowered g_s values at eCO_2, in good agreement with previous reports (Ramalho et al. 2013), which is believed to have been associated with their significantly higher leaf ABA content under well-watered conditions (Figure 4). In fact, even when leaf water potential remained unaffected by eCO_2, the observed increases in leaf and xylem ABA concentrations seem to trigger g_s depression (Fang et al. 2019) via the ABA signaling pathway in guard cells (Chater et al. 2015).

No photosynthetic down-regulation (negative acclimation) to long-term eCO_2 was also observed in both genotypes, as no significant P_n differences were observed between WW 380- and 700-plants when measurements were performed for both at 380 or 700 μl CO_2 l^{-1} (data not shown). Additionally, the marked P_n rises under eCO_2 were likely to have been supported by (i) an enlarged air-to-leaf CO_2 gradient (thus, at least, partially overcoming the diffusional resistances, and then increasing CO_2 availability for RuBisCO assimilation), and (ii) a reduction in photorespiration (associated with the competitive inhibition of RuBisCO oxygenation activity) (DaMatta et al. 2016). This P_n stimulation was in line with the potential increased (~50%) values estimated for C3 trees (Drake et al. 1997, Ainsworth and Rogers 2007), as well as with previous results obtained in field-grown coffee trees (Ghini et al. 2015). Moreover, P_n increases under eCO_2 likely benefited from (i) a consistent trend to higher contents of all electron carriers (Table 2), which likely contributed to the moderate increase in the potential PSs activity (Figure 5), and (ii) the strengthening of the activity of Calvin-Benson cycle enzymes (RuBisCO and Ru5PK). These concomitant activity increases of PSs and RuBisCO agree with the maintenance of a functional balance between carboxylation and electron transport capabilities (J_{max}/V_{cmax}), which seems to be transversally conserved in coffee (Ramalho et al. 2013, DaMatta et al. 2016), as in other species (Possell and Hewitt 2009). Such investments in photo- and biochemical components associated with eCO_2 further support an absence of photosynthetic down-regulation in coffee leaves under long-term eCO_2 (DaMatta et al. 2016, Rodrigues et al. 2016). This clearly contrasts with the reduction of the potential for maximal carboxylation and electron transport due to lowered N-allocation to RuBisCO, RuBP regeneration and proteins associated with electron transport that has been reported in a number of species (Leakey et al. 2009, Bader et al. 2010). Such negative acclimation is commonly associated with low sink strength, leading to an unbalanced C-assimilate synthesis and use (Long et al. 2004, Ainsworth and Rogers 2007, Tausz-Posch et al. 2020). In the case of coffee, adjustments in carbohydrate metabolism via a remarkable ability to accumulate starch has been also shown to allow the plant to avoid photosynthetic acclimation by preventing the cycling and/or accumulation of soluble sugars, especially under conditions of low sink demand (DaMatta et al. 2016, Avila et al. 2020*c*).

Overall, fluorescence parameters reflecting the PSII photochemical efficiency, as well as the photoprotective mechanisms, remained mostly unaffected by eCO_2, as also noted in grapevine (Moutinho-Pereira et al. 2009). These results are in agreement with the maintenance of abundance for most proteins related to the photosynthetic machinery (Table 3). Among fluorescence parameters stand-up one exception related to the significant F_0 rise. This, when coupled to an F_v/F_m reduction (which did not occur), has been taken as an indication of irreversible photoinhibition of PSII reaction centers (Pastenes and Horton 1999), as reported in coffee leaves under excessive irradiance (Ramalho et al. 2000) or heat (Dubberstein et al. 2020). However, in the present case, it is unlikely that such an irreversible damage has occurred. Instead, F_0 rise might have been related to changes in the lipid matrix of chloroplast membranes, associated with increased fluidity (Tovuu et al. 2013), as in CL153, and/or marked shifts in galactolipid and phospholipid classes (as in Icatu) observed under eCO_2 (Scotti-Campos et al. 2019).

Can eCO_2 mitigate the drought impacts at the photosynthetic level in coffee?

The eCO_2 postponed decreases in Ψ_{pd}, as particularly observed in Icatu plants only under MWD, as recently found in coffee (Avila et al. 2020*a*, 2020*b*). This agreed with the absence of an aggravated stress status (assessed by CWSI and I_G) from WW to MWD under eCO_2 (Figure 2).

Stomata opening response was somewhat modified by eCO_2, in line with findings that reported that they become less sensitive to soil drying (Li et al. 2020). Under MWD the 700-plants presented greater ABA levels than their 380-counterparts, especially in Icatu that presented maximal values already under MWD. However, these greater ABA levels had no corresponding impact on g_s since the 700-plants tended to higher g_s values than 380 plants, and g_s did not differ significantly between WW and MWD conditions in either genotype. This is in good agreement with reports of a delayed g_s response to soil drought under eCO_2 in coffee (Avila et al. 2020*b*) and tomato (Liu et al. 2019). Moreover, eCO_2 might have altered the ABA-regulated stomatal control under moderate drought. In fact, eCO_2 was reported to alter the close relation of g_s reduction with increasing xylem ABA content commonly observed under aCO_2. In this case, stomata response can become ABA-independent/insensitive (Liu et al. 2019), and controlled predominantly by turgor pressure (Yan et al. 2017).

There are large uncertainties about the future positive impact of eCO_2 on plants submitted to water deficits, strongly associated to species, responses dependency (Tausz-Posch et al. 2020). Some studies have demonstrated only a modest impact of eCO_2 on plant performance, which usually fades with progressive heat and/or drought conditions (Birami et al. 2020). In contrast, other studies have revealed that eCO_2 can significantly mitigate the drought impairments on crop photosynthesis, growth and yields (Vanaja et al. 2011, Koutavas 2013, Wang et al. 2018), as was the case of coffee (see also Avila et al. 2020*a*). In fact, a large attenuation of drought impacts on P_n was promoted by eCO_2 in MWD plants, in line with a consistent tendency to greater values in all parameters related to the PSII photochemical use of energy [F_v'/F_m', $Y_{(II)}$, q_L] and higher PSs activity in both genotypes. Such photochemical use of energy is, ultimately, the best photoprotective mechanism (Rodrigues et al. 2016), thus resulting in a lower need for dissipation processes [$Y_{(NPQ)}$], and a reduced PSII inhibition status (F_s/F_m') (Table 1). In good agreement, eCO_2 increased P_n, $Y_{(II)}$ and q_P values, as well as crop yield, in soybean plants under severe water deficit, evidencing a greater drought tolerance linked to an improved photosynthetic functioning (Wang et al. 2018).

The harshest drought conditions were reflected in Ψ_{pd}, CWSI, I_G and ABA values, and the maximal impacts on most parameters evaluated under steady-state conditions [e.g., P_n, g_s, F_v'/F_m', $Y_{(II)}$, q_L, $Y_{(NPQ)}$], A_{max} and RuBisCO activity, although the 700-plants of both genotypes tended to be less affected in most parameters. An important relief of SWD impact on the photochemical machinery by eCO_2 was observed in CL153, regarding F_v/F_m, PSs activity and electron carriers, whereas PQ-9 showed its maximal value, what likely promoted CEF-PSII, thus with the capability to reduce the excitation pressure over PSII (Miyake and Okamura 2003). Such better PSs performance was also in line with tendency to increased abundance of proteins related to LHCII under eCO_2, suggesting an improved capability to repair damaged structures (Murata et al. 2007). However, the impact at the biochemical level could have determined the different resilience of these genotypes given that, in contrast to Icatu, CL153 had lowered RuBisCO activity under SWD conditions irrespective of [CO_2].

In Icatu, the potential functioning of the photosynthetic apparatus (considering photochemical and biochemical components) was barely affected by the single SWD; therefore, the exposure to eCO_2 was not evidently translated into a better photosynthetic performance. Still, greater abundance of most proteins at eCO_2 was maintained at SWD conditions, thus keeping the de novo synthesis (and repair) ability as regards the photosynthetic structures (Murata et al. 2007). Additionally, Cyt b_{563} (together with PGR5 protein) involved in CEF-PSI were increased, reinforcing the ability for ATP synthesis, which is the driving force for the highly energy cost of PSII repair processes (Murata and Nishiyama 2018). Finally, Icatu plants exposed to SWD maintained an increased abundance of small RuBisCO subunits and RuBisCO activase, and small impacts on RuBisCO (and Ru5PK) activities, as compared with their WW controls irrespective of [CO_2]. Taking all the above information together, we contend that eCO_2 maintained the intrinsic high resilience of Icatu, and improve that of CL153 to harsh drought stress.

Conclusions

Globally, water restriction was the main environmental driver of coffee responses in terms of photosynthetic functioning. Drought severity, as judged from Ψ_{pd}, CWSI and I_G, progressively affected net photosynthesis rates that were mostly constrained by mesophyll and photo/biochemical rather than stomatal limitations. Under drought, Icatu showed no negative impacts on the potential photosynthetic functioning (e.g., A_{max}, F_v/F_m, PSs and RuBisCO activities) and components (electron carriers), and a great abundance increase of a larger number of proteins related to photosynthetic functioning and protection, irrespective of [CO_2], which altogether supported a high resilience upon drought imposition, in a somewhat contrast to SWD CL153 plants under aCO_2.

Alone, eCO_2 caused no stomatal and photosynthetic acclimation, and the large P_n rises were likely resulted from overcoming diffusive constraints, decreased photorespiration and global reinforcement of photochemical (PSs activity, electron carriers) and biochemical (RuBisCO, Ru5PK) components in both genotypes.

In combination, eCO_2 largely attenuated the MWD impacts on the photosynthetic machinery. For example, in Icatu plants eCO_2 postponed drought imposition, maintaining their stress status (Ψ_{pd}, CWSI, I_G) from WW to MWD. In both genotypes, eCO_2 improved the photosynthetic functioning together with lower energy dissipation and PSII inhibition. Also, eCO_2 might have altered the regulation of stomatal closure given that the lowered g_s in MWD plants was decoupled from the increased ABA levels. Additionally, the marked impacts of SWD condition on most parameters related to energy use (through photochemistry or thermal dissipation) were to a some extent attenuated by eCO_2, or even globally reversed in some cases (e.g., F_v'/F_m' in Icatu). As compared with aCO_2, the eCO_2 canceled the SWD impact on PSII photochemical efficiency, PSs activity, electron carrier contents and the abundance of some proteins related to LHCII in CL153 plants. Still, RuBisCO activity was the most sensitive photosynthetic component to drought in this genotype, regardless of [CO_2], therefore deserving a special attention by breeders in order to promote a future greater sustainability of this crop. Overall, we contend that eCO_2 relieved MWD impact in both genotypes, while maintained the intrinsic high resilience of Icatu, and improved that of CL153, to SWD conditions. In summary, we identified genotype-related responses/impacts associated with the photosynthetic apparatus under the exposure to drought

and/or eCO_2, providing relevant findings in the context of the coffee sustainability under future climate scenarios.

Authors' contributions

J.N.S. performed experiment execution, data collection, data analysis and interpretation, and manuscript writing and revision; A.P.R. was involved in experiment execution, data collection, data analysis and interpretation, and manuscript writing; F.C.L. contributed supervision, methodology implementation, experiment execution, data collection, data analysis and interpretation, and manuscript revision; I.P.P., I.M., D.G., J.A., S.M., M.C.S., and D.D. carried out methodology implementation, experiment execution, data collection, and data analysis; F.L.P. provided supervision, data analysis and interpretation, and manuscript revision; M.J.S., F.H.R., and P.S.-C. performed data analysis and interpretation; A.I.R.-B. was involved in the conception and design, supervision, methodology implementation, data analysis and interpretation, and manuscript writing; F.M.D. was involved in the conception and design, data analysis and interpretation, and manuscript writing and revision; and J.C.R. was involved in the conception and design, supervision, methodology implementation, experiment execution, data collection, data analysis and interpretation, and manuscript writing and revision.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

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