



RESEARCH PAPER

Photosynthetic and metabolic acclimation to repeated drought events play key roles in drought tolerance in coffee

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Abstract

Over the last decades, most information on the mechanisms underlying tolerance to drought has been gained by considering this stress as a single event that happens just once in the life of a plant, in contrast to what occurs under natural conditions where recurrent drought episodes are the rule. Here we explored mechanisms of drought tolerance in coffee (*Coffea canephora*) plants from a broader perspective, integrating key aspects of plant physiology and biochemistry. We show that plants exposed to multiple drought events displayed higher photosynthetic rates, which were largely accounted for by biochemical rather than diffusive or hydraulic factors, than those submitted to drought for the first time. Indeed, these plants displayed higher activities of RuBisCO and other enzymes associated with carbon and antioxidant metabolism. Acclimation to multiple drought events involved the expression of trainable genes related to drought tolerance and was also associated with a deep metabolite reprogramming with concordant alterations in central metabolic processes such as respiration and photorespiration. Our results demonstrate that plants exposed to multiple drought cycles can develop a differential acclimation that potentiates their defence mechanisms, allowing them to be kept in an 'alert state' to successfully cope with further drought events.

Key words: Carbon metabolism, differential acclimation, drought memory, metabolic acclimation, oxidative stress, photosynthesis, water deficit.

Introduction

Drought is, by far, the single most stressful environmental factor affecting plant growth and productivity (Bray *et al.*, 2000). Furthermore, the negative impacts of drought are expected to

become even greater due to the occurrence of longer, more frequent and more intense drought episodes associated with the present and ongoing climate changes (DaMatta *et al.*,

2010; IPCC 2014). A proper understanding of the mechanisms that govern drought tolerance in plants is therefore of the utmost importance in producing cultivars with improved crop yields under water scarcity conditions.

Plants have evolved a number of acclimative responses to face drought stress, encompassing alterations at the molecular, biochemical, and physiological levels (Passioura, 1997). From the physiological perspective partial stomatal closure is one of the earlier responses to water deficit. Given that CO₂ influx and water vapor efflux share a common pathway through the stomatal pores on leaf surfaces, a trade-off between transpirational water loss and CO₂ assimilation is inextricably hard-wired into the system. In fact, it has been demonstrated that decreases in photosynthetic rate under mild drought are fundamentally associated with diffusive limitations, and only under severe drought conditions is the photosynthetic process expected to be constrained by biochemical limitations (Chaves and Oliveira, 2004; Flexas *et al.*, 2004). In addition to physiological alterations, plants can undergo several metabolic adjustments to cope with reductions in water availability (Obata and Fernie, 2012; Silvente *et al.*, 2012). These alterations must involve a tight coordination between central physiological processes, including photosynthesis, respiration and photorespiration, in order to avoid an imbalance between absorption and use of light energy, which often occurs under drought. In addition, production of key protective and structural metabolites (e.g. sugars, amino acids, polyols, amides, and secondary metabolites) is also common under drought and can help plants to maintain their homeostasis under drought conditions (Krasensky and Jonak, 2012; Silvente *et al.*, 2012; Barnaby *et al.*, 2013). Nonetheless, if excess excitation energy arises, it can potentially lead to the production of reactive oxygen species (ROS) that can promptly oxidize lipids, proteins and nucleic acids (Asada, 2006; Noctor *et al.*, 2014). In order to avoid photooxidative damage, plants have evolved complex photoprotective mechanisms, encompassing both enzymatic (e.g. superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR)) and non-enzymatic (e.g. ascorbate, glutathione, α -tocopherol, and flavonols) mechanisms to scavenge excess ROS (Asada, 2006). Taken together, these facts highlight the importance of proper metabolic integration to successfully allow plants to acclimate to drought.

Knowledge of the mechanisms underlying drought tolerance has grown considerably over the last decades. Nonetheless, the vast majority of this information has been gained by considering drought as a single event that happens just once in the life of a plant, in contrast to what occurs under natural conditions where recurrent drought episodes, with distinct severity and duration, are the rule (Galle *et al.*, 2011). In the light of these facts, it is difficult to extrapolate information related to drought responses obtained upon a single drought episode without great caution. Whereas some findings suggest that a continuous exposure to a certain type of stress results in a reduced performance when compared with a single stress event (Scheffer *et al.*, 2001), other findings suggest that plants are able to acclimatize to recurrent stress episodes by displaying faster and stronger responses

when submitted to a cyclic stress (Bruce *et al.*, 2007; Hu *et al.*, 2015). In this sense, plants that are previously exposed to a certain stress can develop a kind of differential acclimation, sometimes called stress memory, which would potentiate their defense responses in order to prime them to a subsequent exposure to that same stress (Bruce *et al.*, 2007; Iwasaki and Paszkowski, 2014; Fleita-Soriano and Munné-Bosch, 2016). Indeed a growing body of evidence has highlighted differential acclimation to a range of stresses including cold (Byun *et al.*, 2014), high temperature (Hu *et al.*, 2015), salinity (Hu *et al.*, 2016) and drought (Ding *et al.*, 2012).

Our understanding of the mechanisms associated with drought tolerance in coffee has been gained upon submitting plants to a single drought event (e.g. Lima *et al.*, 2002; Pinheiro *et al.*, 2004, 2005; Marraccini *et al.*, 2012; Silva *et al.*, 2013; Menezes-Silva *et al.*, 2015). Furthermore, most studies concerning drought effects have been performed using plants grown in small containers that limit root growth, an approach that does not allow the phenomenon of acclimation to occur, with observations largely dependent on the rate of stress progression. Given the facts described above, here we assessed the possible existence of a differential acclimation to single or repeated long-term drought episodes in coffee (*Coffea canephora* Pierre ex Froehner), a slow-growing evergreen tropical woody species that is one of the most important commodities of international agriculture. To this end, we examined differential acclimative mechanisms associated with drought tolerance from a broader perspective, integrating key aspects of the physiology and biochemistry of plants exposed to just one or multiple drought events. Two coffee clones with contrasting abilities to tolerate drought stress were compared to test the following hypotheses: (i) plants submitted to multiple drought cycles are able to develop a differential acclimation which might help them to tolerate drought stress better as compared with plants suffering from drought for the first time; and (ii) the differential acclimation to drought involves concordant physiological and biochemical alterations that should be more evident in drought-tolerant plants than in their sensitive counterparts. We demonstrated here that coffee plants exposed to multiple drought events are better able to cope with water scarcity than were plants that experienced drought for the first time. The data obtained are discussed from an integrated physiological and metabolic perspective in which we suggest that drought tolerance in coffee might be linked to a stress memory that, in turn, would allow plants to keep themselves in an 'alert state' to successfully cope with recurrent episodes of drought.

Material and methods

Plant growth and experimental design

The experiment was conducted in Viçosa (20°45'S, 42°51'W, 650 m altitude), south-eastern Brazil. Plants of two clones of *C. canephora* Pierre ex Froehner with contrasting tolerance to drought (clones 109 and 120 that were previously characterized as drought-sensitive and drought-tolerant, respectively; Pinheiro *et al.*, 2004, 2005; Silva *et al.*, 2013; Menezes-Silva

et al., 2015) were grown in a greenhouse with semi-controlled conditions (maximum photosynthetically active radiation (PAR) of $c. 1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), with a mean temperature of $30 \pm 2 \text{ }^\circ\text{C}$.

The clones, which were grown from rooted stem cuttings, were obtained from the Institute for Research and Rural Assistance of the Espírito Santo State (INCAPER) in Brazil. A total of 21 uniform seedlings per clone (with four leaf pairs) were planted in January 2013 and grown in 24-litre pots containing a mixture of soil, sand and composted manure (4:1:1, v/v/v). The plants were irrigated and fertilized as needed, and no restriction of root development was observed at the end of the experiment. When 9 months old, plants (averaging 1.0 m in height) of each coffee clone were separated into three groups of seven individuals. One group received irrigation during the entire experiment so that the soil moisture was the same as the field capacity (FC) (control plants). The second group was subjected to just one drought event (D1 plants), while a third group was submitted to three drought events intercalated with drought recovery periods (D3 plants) (Fig. 1). Each drought event was imposed by suspending irrigation until the soil water content reached approximately 25% of the FC; the plants were kept at this soil moisture

conditions for *ca* 14 days (to allow acclimation), when physiological traits were assessed and leaf samples were collected for further analyses (D1 plants). In the case of D3 plants, the pots containing the drought-stressed individuals were rewatered so that the soil moisture reached the FC (recovery phase). The recovery phase lasted *ca* 10 days, i.e. the time that was required for the values of predawn water potential (Ψ_{pd}), net CO_2 assimilation rate (A), and stomatal conductance to water vapour (g_s) of the previously drought-stressed plants to recover to the levels displayed by control plants. After full recovery, additional drought–recovery cycles were imposed on the D3 plants, exactly as described above. Samplings and measurements in these plants were performed at the end of the dehydration phase of the third drought cycle (at 14 days after the soil moisture reached 25% of FC). The duration of these long-term drought cycles was established so that the sampled leaves from D3 plants could initiate and complete their expansion by the beginning of the drought treatments. This approach was expected to allow the acclimative potential of D3 leaves to drought including alterations, if any, in structural components such as hydraulic architecture, which could ultimately impact leaf hydraulic conductance (K_{leaf}) and gas exchange. It is important to mention that (i) at the

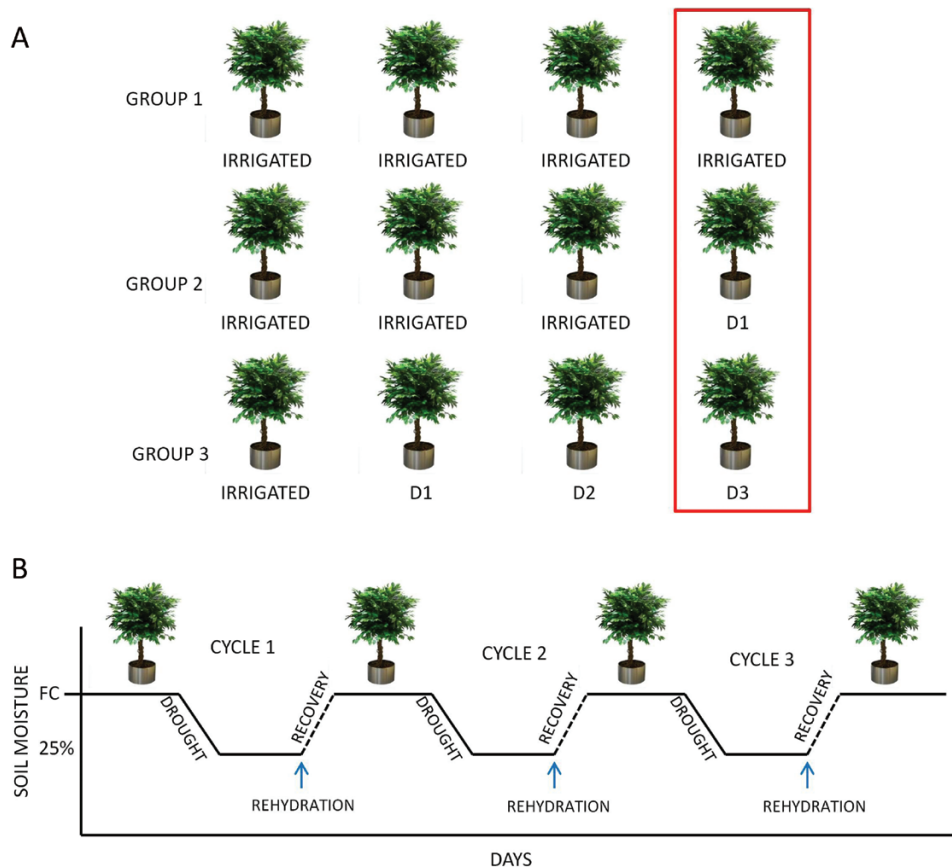


Fig. 1. (A) Schematic representation of the experiment design: group 1, plants that were kept at field capacity over the entire experiment (CT); group 2, plants that were submitted to just one drought event (D1); and group 3, plants that were submitted to three drought events (D3). All of the CT, D1 and D3 plants were evaluated at the same time, as represented by the rectangle. (B) Schematic representation of the cycles of dehydration and rehydration. Each drought cycle consisted of a dehydration phase followed by a recovery period. Dehydration was imposed by suspending irrigation until the soil water content reached approximately 25% of the field capacity (FC) (*ca* 7 days); the plants were kept at this soil water status for *ca* 14 days, after which the pots were rewatered until the soil moisture reached the FC. The plants were then maintained during for *ca* 10 days at FC (recovery phase). See further details in 'Material and methods'. (This figure is available in color at *JXB* online.)

end of the first or third drought cycles, visual symptoms of leaf wilting were evident, especially in clone 109; (ii) all replicates of the control, D1 and D3 individuals were evaluated at the same time (Fig. 1A); and (ii) values of Ψ_{pd} , A and g_s were essentially the same among these individuals, regardless of clone, when starting to impose the drought treatments.

The available water was calculated through the values of soil volumetric moisture at both field capacities (-0.010 MPa) and permanent wilting points (-1.5 MPa) following a soil moisture retention curve (Cavatte *et al.*, 2012). Previously, the weights of all pots were standardized, using the same substrate amounts. Subsequently, pot weights at field capacity and 25% available water were gravimetrically established using a balance (0.1 g precision). Moisture levels were controlled by monitoring the weights of the pots. Adjustments in pot weights due to increased biomass over time were carried out fortnightly. The plants were irrigated every 2 days.

All of the samplings and measurements were conducted using the youngest, fully expanded leaves, which correspond to the third or fourth leaf pair from the apex of the plagiotropic (lateral) branches. Sampled leaves were immediately frozen in liquid nitrogen and then stored at -80 °C until further analysis. Overall, the coffee plants were submitted to six treatments in the form of a 2×3 factorial experimental design (two clones and three watering regimes, i.e. plants that were fully irrigated (controls) or to just one or three drought cycles). This design was completely randomized: seven coffee plants in individual pots per treatment combination were used as replicates, and the experimental plot consisted of one plant per container.

Water relations

Leaf water potentials (Ψ_L) were measured at predawn (04:30–05:30 h) (Ψ_{pd}) and midday (Ψ_{md}) using a Scholander-type pressure chamber (model 1000, PMS Instruments, Albany, NY, USA). The K_{leaf} was estimated according to Brodribb and Holbrook (2003) by following the kinetics of Ψ_L relaxation in rehydrated leaves as:

$$K_{leaf} = C \ln(\Psi_0 / \Psi_f) / t$$

where C is leaf capacitance, estimated using pressure–volume curves (Pinheiro *et al.*, 2005; Cavatte *et al.*, 2012), Ψ_0 is Ψ_L before rehydration, and Ψ_f is the Ψ_L after rehydration for t seconds.

Gas exchange measurements

The leaf gas exchange parameters (net CO_2 assimilation rate (A), stomatal conductance to water vapour (g_s), transpiration rate (E) and internal CO_2 concentration (C_i)) were determined simultaneously by conducting measurements of chlorophyll (Chl) a fluorescence using two cross-calibrated portable open-flow gas exchange systems (LI-6400XT; LI-COR Inc., Lincoln, NE, USA) equipped with integrated fluorescence chamber heads (LI-6400-40; LI-COR Inc.). Measurements were performed on attached, fully expanded leaves from

09:00 to 11:00 h, which is when A was at its maximum, under $400 \mu\text{mol } CO_2 \text{ mol}^{-1}$ air and artificial, saturating PAR, i.e. $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$. This PAR intensity (approximately the ambient irradiance that was intercepted by the sampled leaves at their natural angles) was sufficiently high to saturate the photosynthetic machinery (as previously assessed via A –PAR curves) but without causing photoinhibition (Cavatte *et al.*, 2012). All measurements were performed by fixing the block temperature at 25 °C; the vapour pressure deficit was maintained at *ca* 1.0 kPa while the amount of blue light was set to 10% of PAR to optimize the stomatal aperture.

Previously dark-adapted (30 min) leaf tissues were illuminated with weak modulated measuring beams ($0.03 \mu\text{mol m}^{-2} \text{ s}^{-1}$) to obtain the initial fluorescence (F_0). Saturating white light pulses of $8000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ were applied for 0.8 s to ensure maximum fluorescence emissions (F_m), from which the variable-to-maximum Chl fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m]$, was calculated. In light-adapted leaves, the steady-state fluorescence yield (F_s) was measured after registering the gas exchange parameters. Then, a saturating white light pulse ($8000 \mu\text{mol m}^{-2} \text{ s}^{-1}$; 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off, and far-red illumination was applied ($2 \mu\text{mol m}^{-2} \text{ s}^{-1}$) to measure the light-adapted initial fluorescence (F_0'). Using the values of these parameters, the photochemical (q_p) and non-photochemical (NPQ) quenching coefficients, the actual quantum yield of the PSII electron transport (Φ_{PSII}) and apparent electron transport rate (ETR) were calculated using the equations described by Maxwell and Johnson (2000).

The rate of mitochondrial respiration in darkness (R_D) was measured using the same gas exchange system described above in dark-adapted leaves and used to estimate light respiration (R_L) according to Niinemets *et al.* (2006, 2009) as $R_L = R_D/2$. The photorespiratory rate of Rubisco (R_p) was estimated as $R_p = 1/12[ETR - 4(A + R_L)]$ according to Valentini *et al.* (1995).

Enzymatic assays and malondialdehyde pools

Enzymes associated with carbon metabolism were extracted from leaf samples as described by Nunes-Nesi *et al.* (2007). The activities of the following enzymes were determined: NAD⁺-malate dehydrogenase, sucrose synthase, triose-phosphate isomerase, NADP⁺-glyeraldehyde-3-phosphate dehydrogenase, RuBisCO (exactly as described in Sulpice *et al.* 2009), and aldolase, phosphofructokinase and pyruvate kinase (according to Gibon *et al.*, 2004). In addition, key antioxidant enzymes, including SOD, APX, CAT and GR, as well as malondialdehyde (MDA; a marker for lipid peroxidation) pools, were extracted and assayed exactly as described in Pinheiro *et al.* (2004).

Metabolites

Metabolite extraction was performed by grinding the lyophilized leaf tissues (approximately 75 mg) with liquid nitrogen and immediate addition of the appropriate extraction buffers.

The concentration of glucose, fructose, sucrose, starch and total soluble amino acids was determined exactly as described by Fernie *et al.* (2001).

To obtain a broad overview of the major pathways of central metabolism, an established gas chromatography–mass spectrometry (GC-MS)-based metabolite profiling method (Fernie *et al.*, 2004) was used to quantify the relative metabolite levels in response to the imposed treatments. The extraction, derivatization, standard addition, and sample injection were performed exactly as described previously (Lisec *et al.*, 2006). The GC-MS system comprised a CTC CombiPAL auto sampler, an Agilent 6890N gas chromatograph and a LECO Pegasus III TOF-MS running in EI+ mode. Chromatograms and mass spectra were evaluated by using Chroma TOF 1.0 (Leco, <http://www.leco.com/>) and TagFinder 4.0 software (Lisec *et al.*, 2006). Metabolite identification was manually supervised using the mass spectral and retention index collection of the Golm Metabolome Database in comparison with database entries of authentic standards (Kopka *et al.*, 2005; Hummel *et al.*, 2010). Peak heights of the mass fragments were normalized on the basis of the dry weight of the sample and the added amount of an internal standard (ribitol). Data were normalized with respect to the mean response calculated for the control plants (to allow statistical assessment, individual plants from this set were normalized in the same way).

Real-time PCR analysis

Total RNA was isolated from leaves using PureLink® Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA quantification was performed using the NanoDrop™ Spectrophotometer ND-1000 (Life Technologies), and the integrity of RNA was assessed by 1% agarose gel electrophoresis and ethidium bromide staining. Aliquots of 10 µg of total RNA were treated with Ambion® TURBO DNA-free™ DNase (Life Technologies). cDNAs were synthesized by adding 50 µM of Oligo (dT24V) primer and 10 mM of each dNTP to 1 µg of total RNA. This mixture was incubated at 65 °C for 5 min and then briefly chilled on ice. First Strand Buffer (Invitrogen), 20 mM of dithiothreitol and 200 units of Superscript III (Invitrogen) were added to the prior mixture and the total volume (20 µl) was incubated at 50 °C for 1 h following the manufacturer's instructions. For clone 109, three pools of three independent plants were used representing the biological samples. For clone 120, four independent plants were used as biological samples. Each biological sample was qPCR analysed with three technical replicates.

Two genes (*RD29B* and *RAB18*) that are categorized as trainable and one gene (*RD22*) that is considered as non-trainable after a genome-wide analysis of response of Arabidopsis genes to dehydration stress (Ding *et al.*, 2012, 2013) were isolated from *C. canephora*. The primers *RD22* (F: 5' CCTCCAAACTGGGGAAGAAC 3'; R: 5' TTTTGCTGG TGGCACACTAC 3'), *RD29B* (F: 5' AAAACCGGCCAGG AGAAG 3'; R: 5' GGATTGGATCAGTGGGGTTA 3') and *RAB18* (F: 5' AACTGGAGCACATGGGACTT 3'; R: 5' TTCATCCCCTTCTTCCTCCT 3') were designed in this

study. The expression of *RD22*, *RD29B* and *RAB18* was normalized using the reference genes *UBQ10* and *GAPDH*, which were previously tested by our group (Cruz *et al.*, 2009).

Polymerase chain reactions were carried out in an optical 96-well plate with 7500 Fast Real Time PCR (Applied Biosystems) sequence detection system, using SYBR Green to monitor dsDNA. Reaction mixtures contained 10 µl of diluted cDNA (1:50), 0.2 µM of each primer, 50 µM of each dNTP, 1× PCR Buffer (Invitrogen), 3 mM MgCl₂, 2 µl of SYBR Green I (Molecular Probes) water diluted (1:10 000), and 0.25 U of Platinum Taq DNA polymerase (Invitrogen), in a total volume of 20 µl. Reaction mixtures were incubated for 5 min at 94 °C, followed by 40 amplification cycles of 15 s at 94 °C, 10 s at 60 °C and 15 s at 72 °C. PCR efficiencies and optimal quantification cycle threshold were estimated using the online real time PCR Miner tool (Zhao, 2005). C_q values were converted in qBase software v1.3.5 (Hellemans *et al.*, 2007) into non-normalized relative quantities, and corrected by PCR efficiency, using the formula $Q = E^{\Delta C_q}$, where E is the efficiency of the gene amplification and ΔC_q is the C_q value of the sample with the lowest expression in the data set minus the C_q value of the sample in question.

Statistical analyses

Unless otherwise stated, data were obtained from seven plants in individual pots per treatment combination that served as conditional replicates, and analysed by two-way ANOVA, and the means were compared using the Newman–Keuls test at $P \leq 0.05$. To study the relationships between the variables, Pearson's linear correlation was employed. All of the statistical analyses were performed using SAEG software, version 9.1 (SAEG, 2007).

Results

Water relations

Under control conditions, Ψ_{pd} was always above -0.01 MPa regardless of the clone studied thus indicating that control plants were fully hydrated over the course of the experiment (Fig. 2A). Drought stress led to significant reductions in Ψ_{pd} in the plants that were subjected to just one or three drought cycles (hereafter referred to as D1 or D3 plants, respectively) of both clones, but to a greater extent in the drought-sensitive clone 109 (Fig. 2A). Interestingly, D3 plants of the drought-tolerant clone 120 displayed higher Ψ_{pd} (-0.32 MPa) than their D1 counterparts (-0.41 MPa), whilst the same tendency was not observed in clone 109 (Fig. 2A). Within each watering treatment, Ψ_{md} did not differ significantly between clones that displayed, as expected, higher values under control conditions (~ -0.60 MPa) than under D1/D3 conditions (~ -2.24 MPa) (Fig. 2B).

In irrigated plants, K_{leaf} values were similar between the two clones (~ 13.2 mmol m⁻² s⁻¹ MPa) (Fig. 2C). In sharp contrast, clonal differences in K_{leaf} were evident upon drought imposition, with greater decreases in clone 109 (79%) than in clone 120 (29%), although K_{leaf} did not differ significantly between D1 and D3 plants irrespective of clone (Fig. 2C).

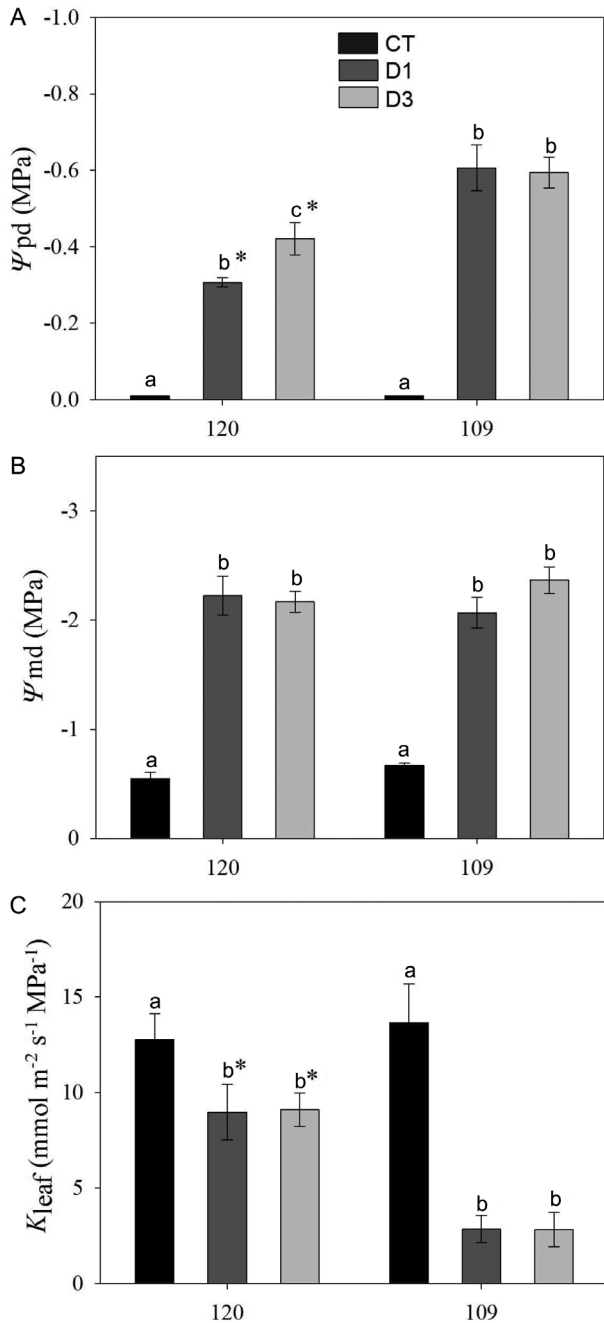


Fig. 2. The predawn leaf water potential (Ψ_{pd} ; A), midday leaf water potential (Ψ_{md} ; B) and leaf hydraulic conductivity (K_{leaf} ; C) in two clones of *C. canephora* (120, drought tolerant; and 109, drought sensitive) under ample irrigation (black bars) or subjected to one (dark gray bars) or three drought events (light grey bars). The values followed by the same letter do not differ significantly between the irrigation treatments within the same clone ($P \leq 0.05$; Newman-Keuls test). The asterisks indicate significant differences between the clones within the same irrigation treatment ($P \leq 0.05$; *t*-test). $n=7$, \pm SE.

Gas exchange and chlorophyll fluorescence parameters

In control plants, both clones displayed similar values of A ($\sim 10.7 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), g_s ($\sim 0.181 \text{ mol m}^{-2} \text{ s}^{-1}$), E ($\sim 2.9 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and C_i ($\sim 282 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$) (Fig. 3A–D). Drought, however, provoked remarkable alterations in gas exchange traits, especially in clone 109, which

displayed (considering both D1 and D3 plants together) average decreases of 74% in A , 85% in g_s , 80% in E and 24% in C_i (Fig. 3A–D), when compared with their respective control plants. Notably, irrespective of the genotype, the D3 plants showed higher A values than those of D1 plants, and this pattern was not associated with significant alterations in g_s , E or C_i (Fig. 3A–D). Indeed, compared with control plants, A decreased by 54 and 84% in D1 plants against 33 and 66% in D3 plants, in clones 120 and 109, respectively. Values of R_L and R_p were also higher in D3 plants (approximately 30%) than in their D1 counterparts regardless of clone (Fig. 3E, F). Noticeably, for both clones, leaf mass ratio was unresponsive to the applied treatments (see Supplementary Fig. S1 at *JXB* online) and as such the same pattern of A , R_L or R_p is expected regardless of expressing them on a per area or per mass basis.

Differences in chlorophyll fluorescence traits were not observed between clones, but drought led to some alteration in these traits. Reductions in ETR (Fig. 3G) and increases in NPQ (Fig. 3H) were observed in D1 plants of both clones, whereas reduction in q_p was only observed in clone 109 (Fig. 3I). Conversely, in D3 plants the values of ETR, NPQ and q_p were essentially the same as those of control plants (clone 120) (Fig. 3E–G), or displayed significantly less alterations when compared with D1 plants (clone 109). Regardless of treatments, F_v/F_m remained invariant (≥ 0.80) over the course of the experiment (Fig. 3J).

Enzyme activities and malondialdehyde pools

In general, the activities of all of the enzymes we analysed were similar between the clones under control conditions (Table 1). Under drought, the activities of phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, aldolase, RuBisCO, SOD and APX increased consistently in both clones (Table 1 and Fig. 4A, B), whereas the activities of the other enzymes varied in a clone-dependent manner. Notably some of these response were differentially seen in D3 plants of both clones, for example, the activities of the phosphofructokinase, aldolase, RuBisCO, GR and catalase (Table 1 and Fig. 4C, D), whereas for the other enzymes, these differential responses were clone-specific, as were the cases of the higher activities of pyruvate kinase and isocitrate dehydrogenase in clone 120, and glyceraldehyde-3-phosphate-dehydrogenase in clone 109 (Table 1).

The concentration of MDA was unresponsive to the applied treatments in both clones (Fig. 4E).

Metabolite levels

Under control conditions, although glucose and fructose pools were higher in clone 120 (Fig. 5A, B), and starch pools were higher in clone 109 (Fig. 5E), the levels of sucrose and total soluble sugars (as well as total soluble amino acids pools) did not differ between clones (Fig. 5C, D, F). However, drought stress led to remarkable alterations in the levels of carbohydrates and amino acids, as illustrated by a consistent decrease in the levels of sucrose

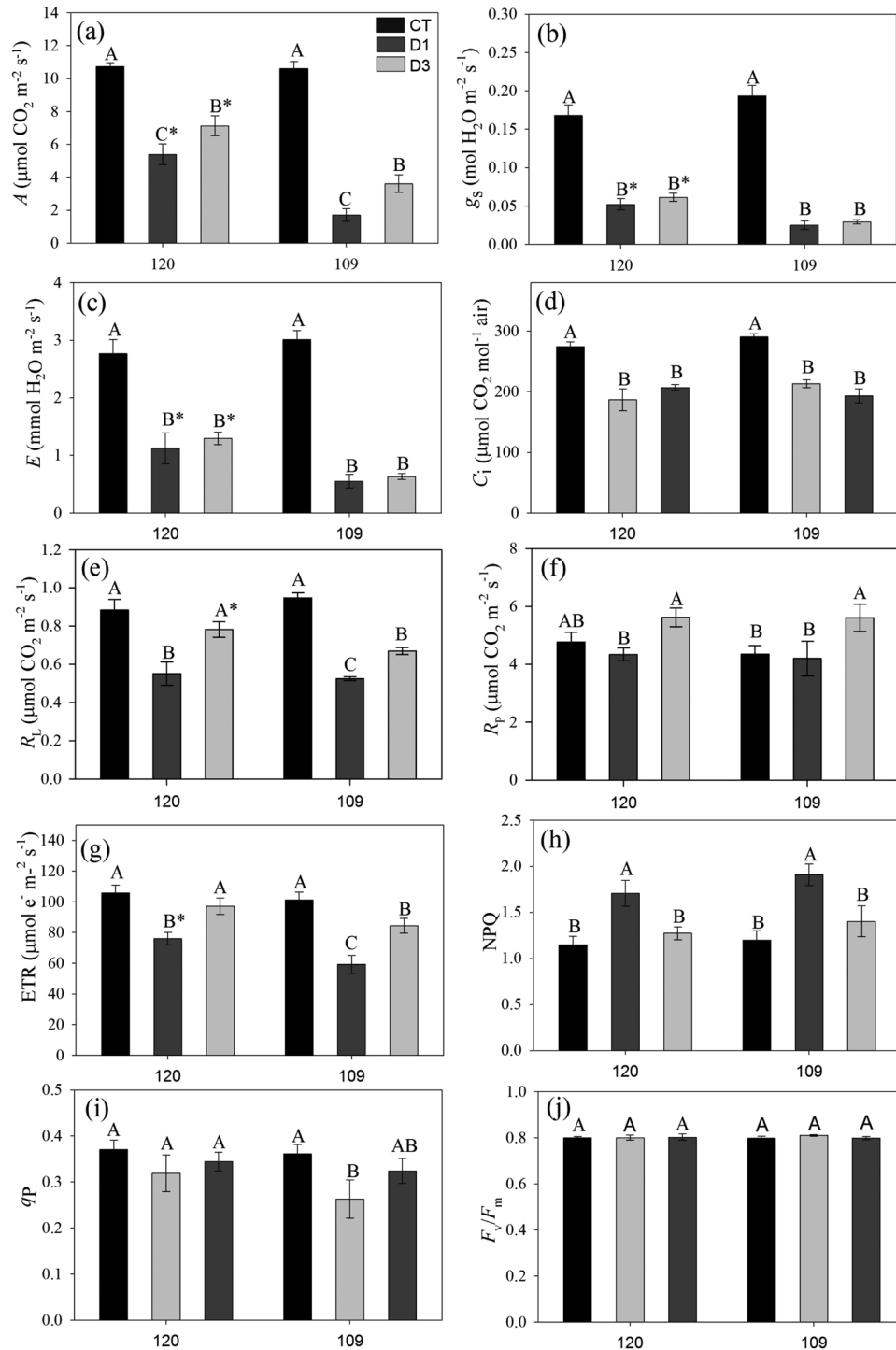


Fig. 3. The net carbon assimilation rate (A; A), stomatal conductance (g_s ; B), internal CO₂ concentration (C_i; C), transpiration rate (E; D), rate of mitochondrial respiration in the light (R_L; E), photorespiration rate (R_p; F), apparent electron transport rate (ETR) (G), non-photochemical quenching (NPQ; H), photochemical quenching (q_P; I) and the variable-to-maximum Chl fluorescence ratio (F_v/F_m; J) in two clones of *C. canephora* (120, drought tolerant; and 109, drought sensitive) under ample irrigation (black bars) or subjected to one (dark gray bars) or three drought events (light grey bars). The values followed by the same letter do not differ significantly between the irrigation treatments within the same clone ($P \leq 0.05$; Newman-Keuls test). The asterisks indicate significant differences between the clones within the same irrigation treatment ($P \leq 0.05$; *t* test). $n=7$, \pm SE.

and starch coupled with an increase in the total soluble amino acid pools regardless of genotype (Fig. 5C–F). Further alterations in sugar concentrations were only noted in clone 120, which displayed decreases in the levels of fructose (D1 and D3 treatments) and glucose (D3 plants) (Fig. 5A, B).

To obtain a broader overview of the major pathways of central metabolism that were altered by the imposed treatments, a GC-MS-based metabolite profiling technique (Fernie *et al.*, 2001) was used (Fig. 6 and Supplementary Table S1). This analysis revealed a remarkable clonal metabolic reprogramming in response to the drought treatments. Among the 38

Table 1. The activity of enzymes of carbon metabolism (expressed as $\mu\text{mol min}^{-1} \text{g}^{-1} \text{DW}$) in two clones of *C. canephora* (120, drought tolerant; and 109, drought sensitive) under ample irrigation (CT) and subjected to one (D1) or three drought events (D3)

The values followed by the same letter do not differ significantly between the irrigation treatments within the same clone ($P \leq 0.05$; Newman-Keuls test). The asterisks indicate significant differences between the clones within the same irrigation treatment ($P \leq 0.05$; *t*-test). $n=7$, $\pm\text{SE}$.

Enzyme	Clone 120			Clone 109		
	CT	D1	D3	CT	D1	D3
Sucrose synthase	1.30 (0.09) a	1.29 (0.11) a*	1.21 (0.15) a	1.13 (0.09) a	1.05 (0.09) a	1.17 (0.10) a
Phosphofructokinase	0.47 (0.02) b	0.44 (0.01) b	0.60 (0.03) a	0.41 (0.01) b	0.45 (0.03) b	0.56 (0.05) a
Triose phosphate isomerase	27.5 (2.2) b	35.5 (2.1) a	33.4 (2.0) ab	31.3 (3.0) a	37.1 (2.7) a	34.7 (2.6) a
Glyceraldehyde 3P-dehydrogenase	3.44 (0.35) b	4.88 (0.19) a	4.61 (0.24) a	4.05 (0.52) b	5.11 (0.30) ab	5.48 (0.57) a
Enolase	1.98 (0.12) ab	1.72 (0.13) b	2.21 (0.07) a*	2.07 (0.07) a	1.66 (0.08) b	1.79 (0.14) ab
Pyruvate kinase	3.94 (0.4) b	3.78 (0.26) b	5.23 (0.21) a*	3.92 (0.5) a	3.77 (0.4) a	3.91 (0.4) a
Isocitrate dehydrogenase	0.17 (0.01) ab	0.16 (0.01) b	0.20 (0.01) a*	0.15 (0.01) a	0.15 (0.01) a	0.17 (0.01) a
Malate dehydrogenase (NAD ⁺)	53.2 (2.59) b	55.5 (3.91) b	71.9 (3.55) a	49.7 (4.37) b	59.9 (1.76) ab	66.5 (6.07) a
Aldolase	5.19 (0.21) c	6.27 (0.24) b	7.33 (0.15) a	5.10 (0.40) b	5.85 (0.23) b	7.46 (0.43) a
RuBisCO total activity	2410 (270) b	2480 (147) b	3915 (218) a	2558 (167) b	3058 (387) b	4582 (526) a
RuBisCO initial activity	1758 (201) b	1809 (72.4) b	2565 (263) a	1785 (125) b	2106 (283) b	2860 (231) a
RuBisCO activation state (%)	72.9 (2.45) a	73.4 (1.86) a	68.2 (3.8) a	69.8 (2.3) a	68.6 (1.33) a	64 (3.28) a

annotated metabolites, increases in the levels of 31 metabolites were noted in at least one of the treatments (D1 or D3). Notably, all of the 17 amino acids annotated (with the exception of alanine and tryptophan) displayed increased levels upon the imposition of drought. In particular, the levels of glycine were higher than those of serine in drought-stressed plants of both clones, thus leading to an increased glycine/serine ratio, and such an increase was more marked in D3 plants when compared with their D1 counterparts (Supplementary Table S1). In addition to amino acids, overall increases in the levels of some intermediates of the tricarboxylic acid (TCA) cycle were also observed under drought conditions, as found for fumarate, malate and isocitrate in both clones; additionally clone-dependent increases were observed for succinate (clone 120) and aspartate (clone 109). Further alterations were noted in response to drought, such as the increases in the levels of polyols such as glycerol, *myo*-inositol and mannitol in both clones; raffinose levels remained invariant in contrast to the significant increase in the levels of galactose in both genotypes. Similarly to what was observed for the compounds of the primary metabolism, the exposure to drought also induced increases in the levels of some secondary metabolites of the metabolism such as cinnamic acid, and caffeate; however, these alterations were only significant in clone 120 (Fig. 6; Supplementary Table 1).

Importantly, several of the above described responses were more marked in D3 plants as compared with those found in D1 plants. In both clones, the D3 plants displayed higher levels of isocitrate, phenylalanine, isoleucine, glycine, glutarate, glycerol, galactose, and *myo*-inositol than their D1 counterparts. Furthermore, the levels of some metabolites varied in a clone-specific manner: for example, compared with D1 plants, D3 plants exhibited differential increases in the levels of aspartate, alanine, asparagine, glutamine, valine, and fumarate, as found in clone 109, whereas in clone 120 these differential increases were broader and involved increases in the levels of lysine, proline, tyrosine, succinate,

glyceraldehyde-3-phosphate, *myo*-inositol, lactate, cinnamic acid, and caffeate (Fig. 6 and Supplementary Table S1).

Gene expression

Regardless of watering treatments, the pattern of gene expression varied significantly between the clones we analysed. In control plants, the relative expression of *RD29B*, *RAB18* and *RD22* genes was higher in clone 109 than in clone 120 (Fig. 7A–C). However, upon drought imposition the relative expression of all of these genes increased in clone 120 and decreased in clone 109 (Fig. 7A–C). Nonetheless, despite the varying clonal patterns of gene expression, the D3 plants of both clones tended to display a higher expression of the *RD29B* and *RD22* genes than their D1 counterparts (Fig. 7A, C).

Discussion

In this long-term study, we compared two coffee clones at the presumably same degree of soil water availability. This approach allows more appropriate assessments of the intrinsic ability of a plant to acclimate to a particular degree of drought without the confounding effects associated with differences in soil water status that are typical of most experiments where genotypes with varying rates of water use are compared. Overall, our results are in good agreement with previous information (Pinheiro *et al.*, 2004, 2005; Silva *et al.*, 2013) that clone 120 is better able to cope with drought stress than clone 109. Despite having values of K_L , g_s and E (traits associated with water use) remarkably higher than those of clone 109 upon drought imposition, clone 120 displayed an improved water status (higher Ψ_{pd}), thus likely denoting an improved ability to take up water from the drying soil and transport it to the leaves. This was ultimately translated into a higher A in this clone than in clone 109.

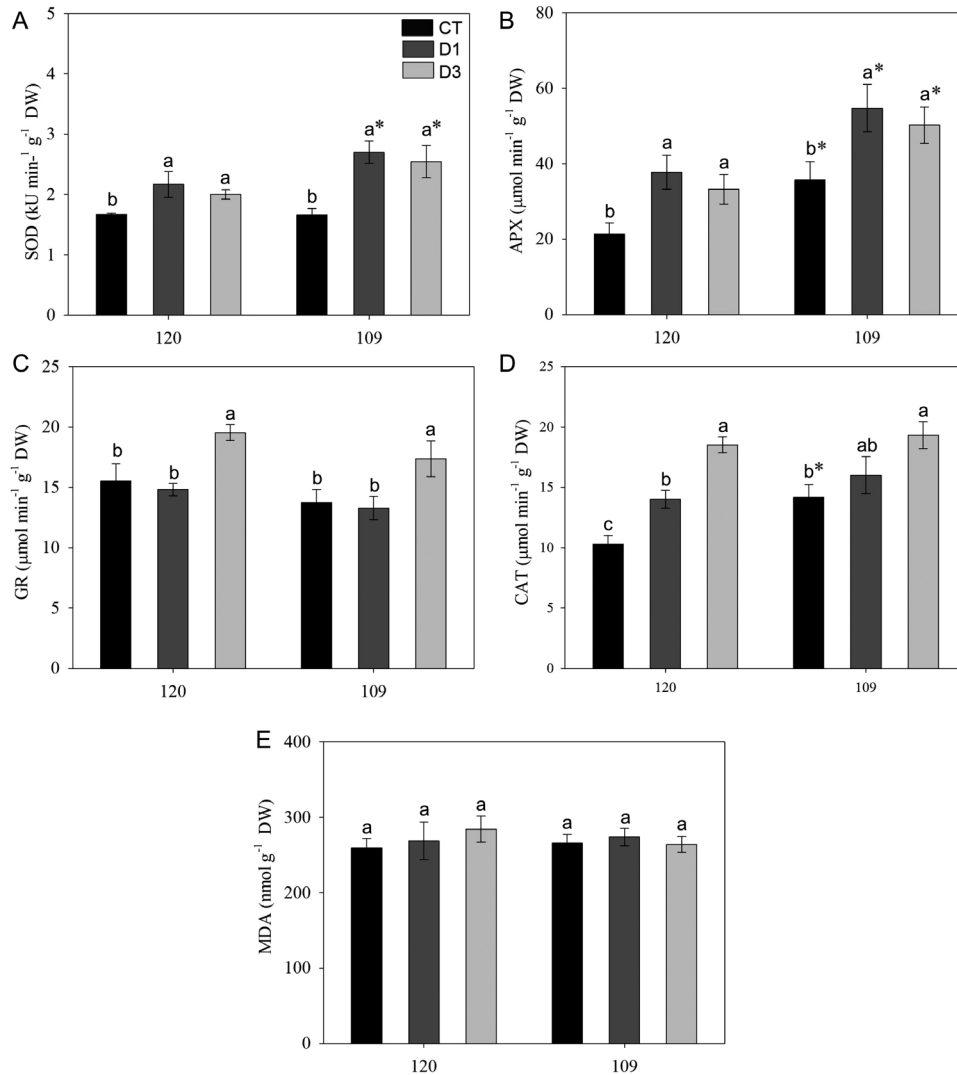


Fig. 4. The activities of antioxidant enzymes superoxide dismutase (SOD; A), ascorbate peroxidase (APX; B), glutathione reductase (GR; C) and catalase (CAT; D) and concentration of malondialdehyde (MDA; E) in two clones of *C. canephora* (120, drought tolerant; and 109, drought sensitive) under ample irrigation (black bars) or subjected to one (dark gray bars) or three drought events (light grey bars). The values followed by the same letter do not differ significantly between the irrigation treatments within the same clone ($P \leq 0.05$; Newman-Keuls test). The asterisks indicate significant differences between the clones within the same irrigation treatment ($P \leq 0.05$; t test). $n=7$, $\pm \text{SE}$.

To the best of our knowledge, the results obtained here are the first demonstration that coffee plants exposed to multiple cycles of drought were better able to cope with water shortage than their counterparts that experienced drought for the first time. As discussed below, our results suggest that this kind of differential acclimation to stress involved not only the expression of trainable genes related to drought tolerance (Fig. 7), but was also associated with an orchestrated reprogramming of metabolic and physiological processes. In this regard, our study offers an integrated view of how a perennial tropical crop species is able to cope with recurrent drought events, a situation that prevails under field conditions. Our data are also in good agreement with our hypothesis that D3 plants of the drought-tolerant clone (120) were able to improve their photosynthetic performance coupled to an accumulation of a larger number of differential responses to drought than were their drought-sensitive counterparts (clone 109). Indeed, from all of the traits we assessed, 46% of them were

differentially expressed in D3 plants (relative to D1 plants) of clone 120 against only 34% in clone 109.

The better photosynthetic performance of plants exposed to multiple drought events was not associated with hydraulic or diffusive factors but rather with biochemical adjustments

Photosynthesis is a key process that integrates the physiological status of a plant. Here we clearly demonstrated that the D3 plants were able to acclimate to recurrent drought episodes by improving their photosynthetic performance (Fig. 3A). We next explored the underlying mechanisms associated with this improved performance. Given that (i) the leaf hydraulic architecture imposes a major constraint on the maximization of the photosynthetic gas exchange of coffee leaves by limiting g_s (Martins *et al.*, 2014b); and (ii) the structural determinants of K_{leaf} (leaf vessel diameter and frequency; Menezes-Silva *et al.*,

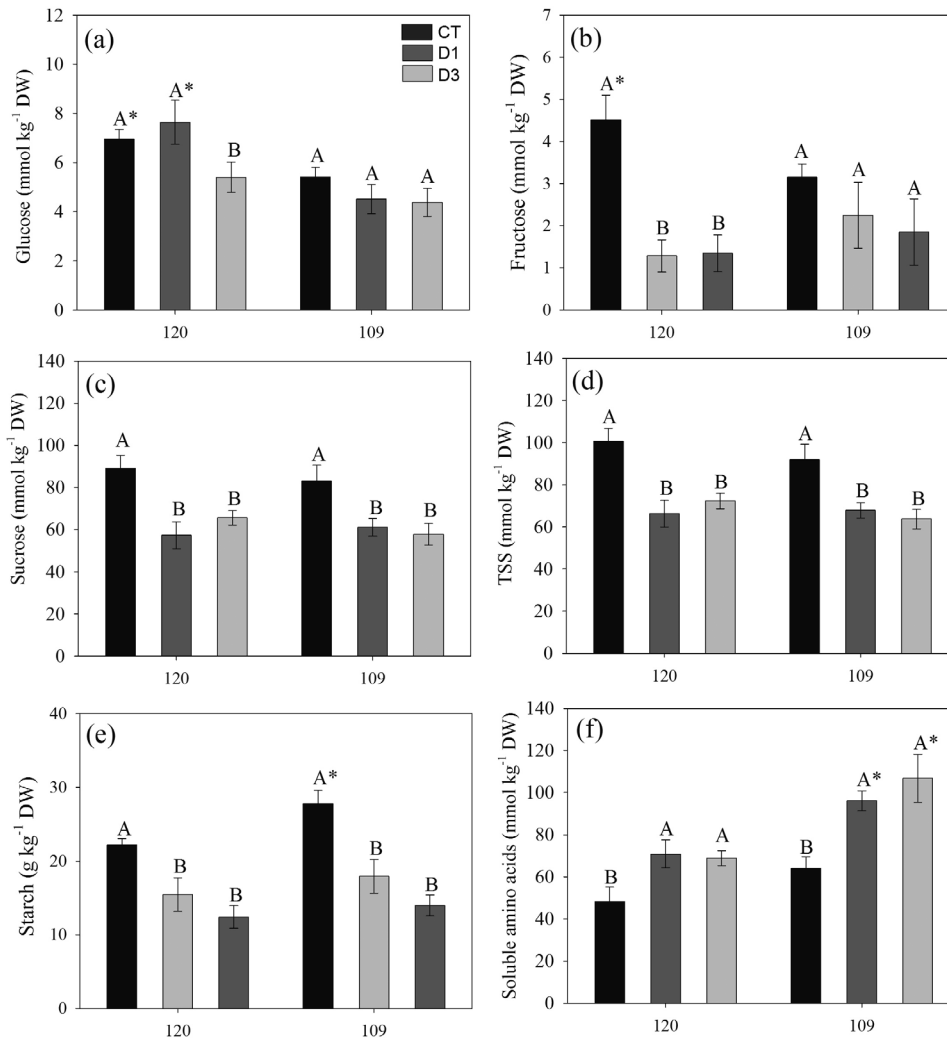


Fig. 5. The concentrations of glucose (A), fructose (B), sucrose (C), total soluble sugars (D), starch (E), and soluble amino acids (E) in two clones of *C. canephora* (120, drought tolerant; and 109, drought sensitive) under ample irrigation (black bars) or subjected to one (dark gray bars) or three drought events (light grey bars). The values followed by the same letter do not differ significantly between the irrigation treatments within the same clone ($P \leq 0.05$; Newman–Keuls test). The asterisks indicate significant differences between the clones within the same irrigation treatment ($P \leq 0.05$; *t* test). $n=7$, \pm SE.

2015) are modulated in leaves developed under drought (the case of D3 leaves but not their D1 counterparts), we analysed the behavior of K_{leaf} among treatments and its relationships with other variables related to carbon fixation. Neither K_{leaf} nor g_s differed significantly when comparing the D3 plants with their D1 counterparts irrespective of clone. Although we cannot fully assess the entire contributions of diffusional (stomatal and mesophyll) limitations to photosynthesis given that we did not estimate the mesophyll conductance (g_m), it has been shown that g_m is often intrinsically co-regulated with g_s (Flexas *et al.*, 2012), as seems also to be the case with coffee (Martins *et al.*, 2014b). In this regard, mesophyll and stomatal limitations would act in concert and could play a role in explaining differences between clones, as the clone 109 had lower g_s (and presumably lower g_m) than had the clone 120 under drought. In any case, given that g_s was essentially similar between D1 and D3 plants regardless of clone, we believe that g_m played a minor role, if at all, in explaining the priming effect caused by the drought cycles. Taking all of the above information together it is tempting to postulate,

therefore, that neither hydraulic nor diffusive factors have a major role in explaining the differential ability of D1 and D3 plants to acclimate their photosynthetic apparatus to drought events. We hence contend that biochemical factors associated with higher RuBisCO activity (see below) likely explain the higher *A* in D3 plants. In the light of these results, we postulate that the drought stress faced by D3 plants was able to provoke biochemical and physiological changes that might be more directly related to a ‘memory factor’ (Ding *et al.*, 2013; Fleta-Soriano and Munné-Bosch, 2016) than were anatomical adjustments that would lead to changes in their hydraulic and/or diffusive systems.

The better performance of plants exposed to multiple drought events involved a concerted reprogramming of physiological and metabolic processes

Compared with D1 plants, we demonstrated that D3 plants displayed increased activities of some enzymes coupled with key physiological/biochemical processes, providing further

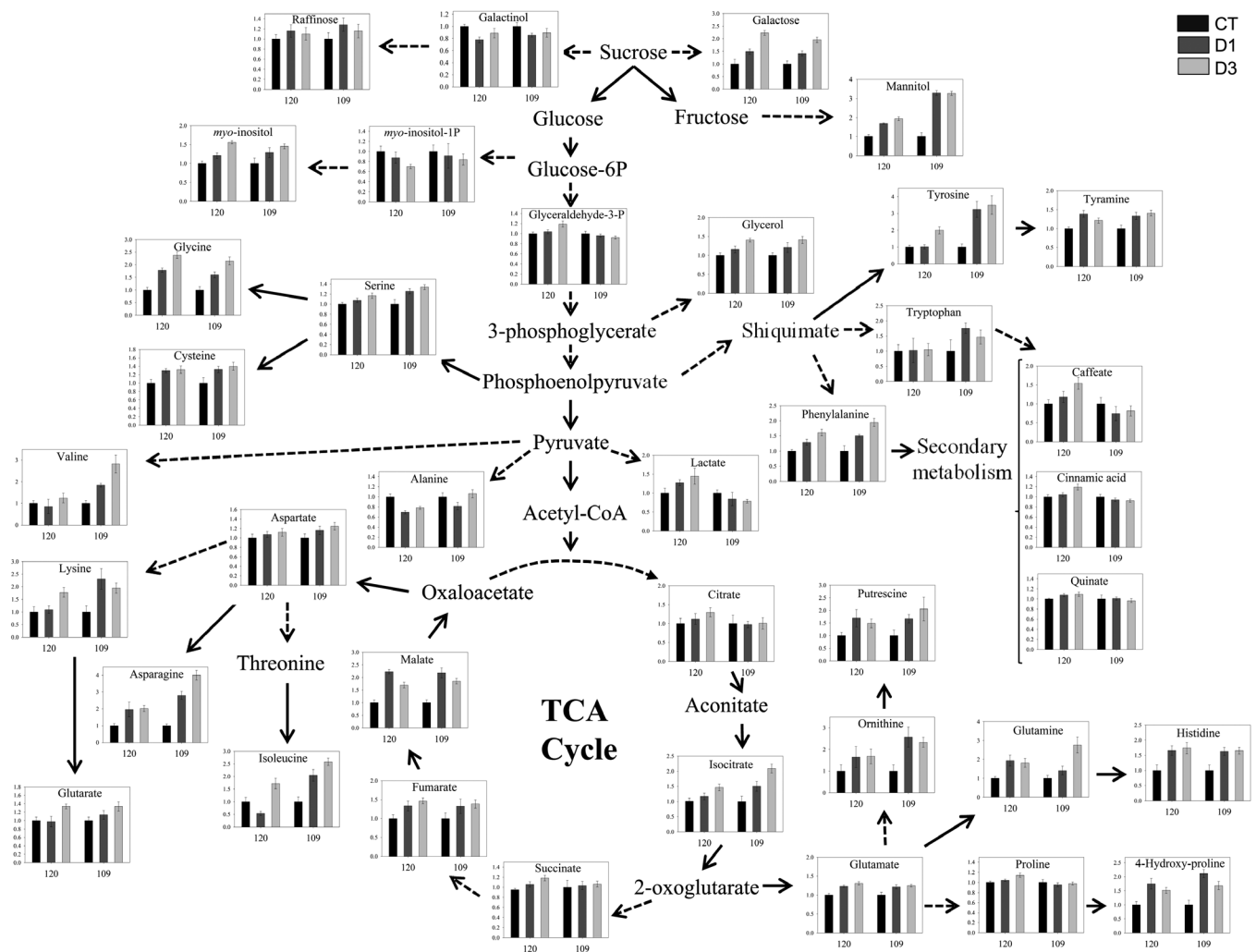


Fig. 6. Changes in metabolic profile in two clones of *C. canephora* (120, drought tolerant; and 109, drought sensitive) under ample irrigation (black bars) and subjected to one (dark gray bars) and three drought events (light grey bars). The schematic summary presents relative levels of the metabolites after normalization against the control plants. $n=7$, \pm SE. The full dataset, including the statistical analysis, is shown in Supplementary Table S1.

evidence that coffee plants are able to acclimate to recurrent drought events. In this regard, an example of biochemical adjustments that could have contributed to the better photosynthetic performance of D3 plants from both clones involves the increases in the activity of RuBisCO (Table 1). Inasmuch as the maximum carboxylation capacity (V_{cmax}) is a function of the product between the RuBisCO concentration, the activation state and the catalytic activity (K_{cat}), that is, $V_{\text{cmax}} = [\text{RuBisCO}] \times \text{activation} \times K_{\text{cat}}$, and considering that the activation state remained unaltered and that K_{cat} is constant for coffee (3.25 s^{-1} ; Martins *et al.*, 2013), an increase in RuBisCO concentration is to be expected in D3 plants, thus leading to increases in the biochemical capacity for CO_2 fixation. This suggestion is consistent with the partial recovery of the ETR (suggesting that a greater fraction of electrons may be diverted to the carboxylation reactions) in D3 plants as compared with the control plants, a fact that was not observed in D1 plants. Noticeably, increases in RuBisCO concentration in response to drought are uncommon (Galmés *et al.*, 2011, 2013), and our results suggest that this might depend on the number of drought cycles experienced by a plant.

In addition to RuBisCO, the activity of some antioxidant enzymes was also differentially altered by the drought treatments, with D3 plants displaying, overall, higher activities than D1 plants. At a first glance, there would not be a need for enhancing the robustness of the antioxidant system of D3 plants relative to that of D1 plants taking into account that D1 individuals had already been able to adequately avoid photoinhibition and photooxidative stress (as judged from the unchanged MDA levels and F_v/F_m ratio), even displaying lower A than the D3 plants. Therefore, in the plants exposed to multiple drought events, a higher use of excitation energy is expected to be dissipated through higher CO_2 fixation rates, thus implying a lower oxidative pressure in the chloroplast electron transport chain and, ultimately, less ROS production. We therefore contend that the increases in the activities of some antioxidant enzymes, coupled with increases in both R_p and pools of a range of protective compounds (see below) might represent the triggering of an alert state in the defense systems of plants exposed to multiple drought events. Such an alert state, by accelerating and maximizing the defense responses, probably would confer a significantly

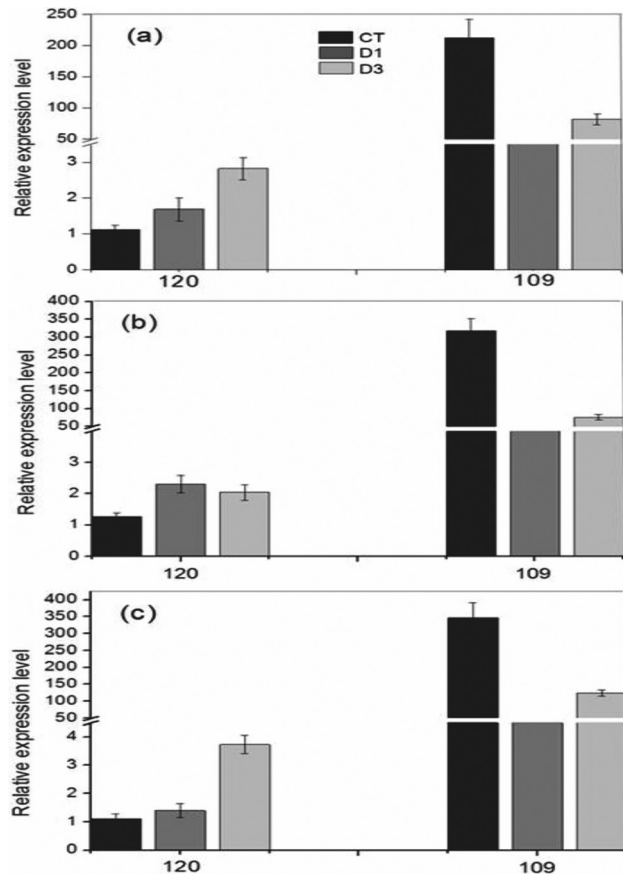


Fig. 7. Relative mRNA level measured by real-time qPCR of trained *RD29B* (A) and *RAB18* (B) and non-trainable *RD22* (C) genes in two clones of *C. canephora* (120, drought tolerant; and 109, drought sensitive) under ample irrigation (black bars) and subjected to one (dark gray bars) or three drought events (light grey bars). The expression level was relative to constitutive *UBQ10* and *GAPDH* genes. Three independent biological replicates were used and bars represent the mean \pm SD of technical triplicates.

higher tolerance of plants to successfully cope with further drought episodes (Kim et al., 2012). Taken together, these results provide additional evidence that the D3 plants were able not only to increase their ability to assimilate carbon, but also to improve their defense mechanisms against the deleterious effects of drought as compared with D1 plants.

Marked alterations in the metabolic profile in response to the drought treatments were also observed. Our results, along with the recent findings by Hu et al. (2016), who studied the responses of rice plants subjected to cycles of salt stress, represent one of the first reports dealing with differential acclimation from a metabolic perspective. Our metabolite profiling clearly suggests that other key physiological processes such as respiration and photorespiration, in addition to photosynthesis, should play important roles in the process of acclimation to multiple drought episodes. In fact, a number of alterations (some of them clone-dependent) such as reductions in concentrations of carbohydrates (e.g. glucose, fructose, sucrose, and starch) coupled with increases in the levels of some amino acids (e.g. glycine, phenylalanine, valine, and isoleucine) and some organic acids (e.g. isocitrate, succinate, fumarate, and malate), associated with increased activity of some enzymes

from glycolysis and the TCA cycle (e.g. phosphofructokinase, pyruvate kinase, aldolase and malate dehydrogenase), suggest the involvement of the respiratory process in directing carbon skeletons for production of metabolites that are essential to allow the D3 plants to successfully face the drought stress. Photorespiration seemed also to play a key role in this context. The higher R_p is consistent with higher glycine/serine ratio (Supplementary Table S1), which often increases with an increased photorespiratory activity (Wingler et al., 1999; Novitskaya et al., 2002), a fact that is further consistent with the positive correlations between glycine pools and CAT activity ($r=0.49$; $P<0.01$). Given that the process of glycine production in the peroxisomes involves the generation of large amounts of H_2O_2 (Novitskaya et al., 2002; Florian et al., 2013), it thus seems reasonable to suggest that D3 plants were able to balance the detrimental effects of increased H_2O_2 production through fine adjustments in the activity of CAT.

The increased production of compatible solutes, such as sugar alcohols and polyols, seems also to be a marked metabolic characteristic of plants exposed to multiple cycles of drought as compared with plants exposed to drought for the first time. Accumulation of these solutes has long been suggested as an important metabolic strategy in plants facing drought conditions (Martin, 1930; Bernstein, 1961; Serraj and Sinclair, 2002; Obata and Fernie, 2012). In fact, compatible solutes, in addition to their role in cell turgor maintenance (Rivero et al., 2014), are also involved in processes such as detoxification of ROS and membrane stabilization (Hoekstra et al., 2001). In this context, it seems likely that the enhanced production of these molecules may have played an important role in determining the differential responses exhibited by the plants exposed to multiple drought cycles.

In addition to changes in primary metabolism, the differential acclimation observed in D3 plants also appears to involve secondary metabolism in coffee plants. Evidence for this comes from the enhanced production of aromatic amino acids such as phenylalanine and tyrosine in conjunction with increased levels of certain secondary metabolites, suggesting an increased flux of carbon skeletons to the shikimate pathway in D3 plants, particularly in clone 120. Some studies have shown that production of secondary metabolites, in addition to being an important source of antioxidants (Grace and Logan, 2000), also involves the use of large amounts of reducing power (Maeda and Dudareva, 2012), thus having a fundamental role in protection against stressful conditions, as has previously been demonstrated in coffee plants under high-light stress (Martins et al., 2014a). In this context, the results presented here provide evidences that the differential adjustments in the secondary metabolism of D3 plants might have contributed to the better performance of these plants under drought conditions.

Concluding remarks

Our results show that plants exposed to multiple drought cycles can develop a differential acclimation that potentiates their defense mechanisms. Such an acclimation was deeper

in the drought-tolerant clone than in its drought-sensitive counterpart, and might help to explain, at least partially, the varying degrees of drought tolerance that exist in coffee. Acclimation to repeated drought episodes seemed to depend on an orchestrated reprogramming of plant metabolism, involving key processes such as photosynthesis, respiration, photorespiration, and the antioxidant system. In addition, the exposure to recurrent stress events also resulted in differential molecular adjustments, as noted in the qPCR data, suggesting the existence of so-called trainable genes in coffee. It is therefore tempting to suggest that the differential acclimation observed in D3 plants might be the results of the generation of a 'drought memory' (Ding *et al.*, 2013; Fleta-Soriano and Munné-Bosch, 2016). Our study provides, therefore, new perspectives to understand the underlying mechanisms of drought tolerance in coffee. For instance, under field conditions where plants are often recurrently exposed to drought episodes, a drought memory involving different levels of complexity should play a pivotal role in helping plants to keep themselves in an alert state to successfully cope with recurrent episodes of drought. However, many questions remain unanswered and additional studies employing more sophisticated combined analysis of the transcriptome, epigenome, proteome, and metabolome will likely be of fundamental importance to provide a holistic understanding of the underlying mechanisms that affect the creation and maintenance of a drought memory. This could ultimately contribute to improved crop growth and productivity in the likely scenario of increasing future water scarcity.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Leaf mass per area (LMA) in two clones of *C. canephora*.

Table S1. The relative metabolite profile of two clones of *C. canephora* under ample irrigation (CT) and subjected to one (D1) or three drought events (D3).

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