Superimposed Impacts of Enhanced [CO_2] and High Temperature on the Photosynthetic Metabolism of *C. arabica* and *C. canephora* Genotypes

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SUMMARY

Coffee crop has been predicted to become threatened by future climate changes and global warming conditions. Yet, the long-term effects of elevated [CO_2] on this plant remain to be fully elucidated. In this context, this work aims at linking coffee biochemical responses to environmental changes of [CO_2] and temperature on genotypes from the two major producing species, using the photosynthetic metabolism as probe to evaluate the plant acclimation ability. Potted plants from *C. arabica* cv. IPR 108 and of *C. canephora* cv. Conilon Clone 153 were grown under environmental controlled conditions, either at 380 or 700 μL CO_2 L^{-1} air, for 1 year, without water, nutrient or root development restrictions. After that the temperature was gradually increased from 25/20 ºC (day/night) up to 42/34 ºC. The effects of elevated [CO_2] and enhanced temperature on the photosynthetic structures were assessed through the characterization of the lipid components of chloroplast membranes, whereas the leaf metabolic performance was evaluated through the thylakoid electron transport rates (involving both photosystem (PS) I and II), and the activities of enzymes (ribulose 1,5-biphosphate carboxylase/oxygenase and ribulose 5-phosphate kinase), as well as through stable isotopes of C and N. The activities of respiratory enzymes (NADH-dependent malate dehydrogenase and pyruvate kinase) were also analyzed. The results pointed for a higher functional status along the experiment in the plants grown under elevated [CO_2], with special relevance at 37 and 42ºC in IPR108. These results could be related to the qualitative changes of the membrane lipid matrix that might have helped to preserve suitable membrane fluidity

for the membrane bound events (*e.g.*, thylakoid electron transport). The PSs and enzyme data reflect an enhancement of the energetic metabolism (both photosynthesis and respiration), mostly, until 31 ºC for IPR108 and 37 ºC for CL153 at normal [CO_2]. Yet, under enhanced [CO_2] it was found an increase in the temperature (to 37 ºC) at which maximal values of some parameters in IPR108 (MDH, PSs activities, RuBisCO) were observed, concomitantly with the maintenance of high performance in other parameters when compared to the 380 plants. Under the highest temperature (42 ºC) the enzymes were the most sensitive point, displaying the strongest reductions, irrespective of genotype and [CO_2] treatments. The temperature promoted changes in leaf $\delta^{13}C$, irrespective of genotype and [CO_2], reflecting a decrease in WUE with heat. The changes in $\delta^{15}N$ values may indicate different limitation steps of N assimilation, requiring further investigation. It was concluded that the coffee plants grown under elevated [CO_2] apparently showed a better endurance to high temperatures, what is quite relevant in a context of predicted climate changes and global warming scenarios.

INTRODUCTION

Coffee is one of the world's most traded agricultural products, currently growing in ca. 80 countries, and modeling studies have foreseen that climate change and global warming will strongly impact the suitability of current cultivation areas and coffee biodiversity, especially of C. arabica. Yet, these studies have not considered possible mitigating effects of the increasing atmospheric [CO2], as no biological information is available regarding the long-term effects of [CO2] and temperature enhancements on the coffee plant.

The photosynthetic pathway is a key metabolism as regards plant survival and acclimation to environmental variations. Under enhanced growth [CO2], C3 plants often present net photosynthesis (Pn) increases above 50% and changes in stomatal conductance (gs), as also recently reported for Coffea spp.. This Pn stimulation results from a simultaneous higher RuBisCO carboxylation rate, due to increased substrate availability, and a competitive inhibition to O2, reducing the oxygenation rate and, subsequently, decreasing the CO2 loss and energy costs associated with the photorespiratory pathway. In C3 plants the impact on photorespiration under CO2 enrichment is expected to enhance Pn to a greater degree at high than at low temperatures, thereby, at least partially, offsetting the negative effects of supra-optimal temperatures on yield. Even so, a partial down-regulation (negative acclimation) of the photosynthetic apparatus might occur, often related to limitations on sink strength that prevents the plant from fully utilizing the higher photosynthate production. That may lead to non-structural carbohydrates (NSC) accumulation, which could in turn depress the gene expression and the amount/activity of photosynthetic enzymes, including that of RuBisCO, and may decrease the components of the photosynthetic apparatus. None of them were found in coffee plants. Such high [CO2] impact would also depend on the interactions with other environmental limitations, namely water availability and enhanced temperature, changing as well with the plant developmental stage.

Currently, stable isotope analysis is a powerful tool in physiological and ecological studies to trace, record, source, and integrate environmental parameters. With regard to carbon isotopes, the basis for much of the observed variation in δ13C of organic samples derives from two metabolic processes, photosynthesis and respiration. In the case of nitrogen, variation in the δ15N in its cycle processes has been increasingly studied. Knowledge of how the isotopes of N fractionate during catabolic reactions in soils and in plants in relation to N utilization, transformation, and fixation elucidate the pathways and interactions that many times result from not only particular symbiotic associations at root level (i.e., mycorrhizae and bacteria), but also N-fluxes and sources as well land-use and agricultural practices.

As modifications in atmospheric [CO2] affect fundamental plant processes and may alter plant growth, agronomic yields and quality, it is of crucial importance to gather information concerning the biological extent of the impact on the coffee plant and their capability to respond and cope with new environmental conditions. Therefore, to our knowledge, we report the first results concerning the underlying biochemical responses to enhanced [CO2] and high temperature in two genotypes of the major coffee producing species.

MATERIALS AND METHODS

Plant material and experimental design

Plants with ca. 1.5 years from *C. arabica* L. cv. IPR 108 (IPR108) and *C. canephora* Pierre ex A. Froehner cv. Conilon Clone 153 (CL153), were transferred into walk-in growth chambers (EHHF 10000, ARALAB, Portugal) and grown in 28 L pots under controlled conditions of temperature (25/20 ºC, day/night), irradiance (ca. 650-800 μmol m-2 s-1), RH (75%), photoperiod (12 h), and either 380 μL CO2 L-1 (380) or 700 μL CO2 L-1 (700) air for 1 year, without water, nutrient or root development restrictions. Thereafter the temperature was increased from 25/20 ºC up to 42/34 ºC, at a rate of 0.5 ºC day-1, with a 7 days temperature stabilization at 31/25, 37/30 and 42/34 ºC to allow analysis. The temperature was then set to 37/30 ºC for 2 months (37/30 Long-Term, LT) and the plants further analyzed. Analyses were performed on newly matured leaves.

Thylakoid electron transport rates

To obtain sub-chloroplast fractions and determine the in vivo electron transport rates associated with both PSI (DCPIPH2→MV) and PSII, including (H2O→DCPIP) or excluding (DPC→DCPIP) the oxygen evolving complex (OEC), measured polarographically using an LW2 O2 electrode (Hansatech, UK) at 25 °C, optimized methods for coffee leaves were followed.

Carbon and nitrogen isotopes

Carbon and nitrogen stable isotope ratios were determined on an Isoprime (Micromass, UK) isotope ratio mass spectrometer coupled to an elemental analyzer (EuroVector, Italy), accordingly to standard methods. Isotope ratios were calibrated against international standards (IAEA CH6 and IAEA CH7 for carbon isotope ratio, IAEA N1 for nitrogen isotope ratios. Precision (standard deviation of the set of standards analyzed in each batch) was 0.06‰ for carbon and 0.08‰ for nitrogen isotope ratio determinations.

Photosynthetic and respiratory enzymes

The activities of enzymes were determined in four freshly cut leaf discs (0.5 cm2 each). The homogenization procedure and the evaluation of the total activities of ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO; EC 4.1.1.39), ribulose 5-phosphate kinase (Ru5PK; EC 2.7.1.19), both related to the photosynthetic pathway, and of NADH-dependent malate dehydrogenase (MDH: EC 1.1.1.37) and pyruvate kinase (PK: EC 2.7.1.40), both related to the respiratory pathway, were performed as described in Ramalho, J.C. et al. (2013).

Chloroplast membranes lipids

The homogenization (ca. 4 g of freshly cut leaf tissue), extraction, separation, identification and quantification of fatty acids (FAs) from chloroplast membranes were performed as

previously optimized for coffee leaves [18]. The double bond index (DBI) was calculated as DBI = [(% monoenes + 2 x % dienes + 3 x % trienes / (% saturated FAs)].

RESULTS AND DISCUSSION

Thylakoid electron transport rates

Concerning the functioning of the photosynthetic apparatus, the rate of thylakoid electron transport at PSI and PSII level was promoted under high growth [CO2] at 25 ºC in both genotypes, suggesting a higher investment in the photochemistry structures (Fig. 1). With the increase in temperature up to 37 ºC, a stimulation of thylakoid electron transport occurred in both photosystems and genotypes, but the [CO2] effect was noted only in CL153. Upon 42 ºC exposure the potential activity of PSI and PSII decreased roughly to control levels. Yet, in IPR108 a lower impact was observed in the high [CO2] plants. Notably, the CL153 plants were the only ones to show significant negative after-effects (at 37 ºC LT).

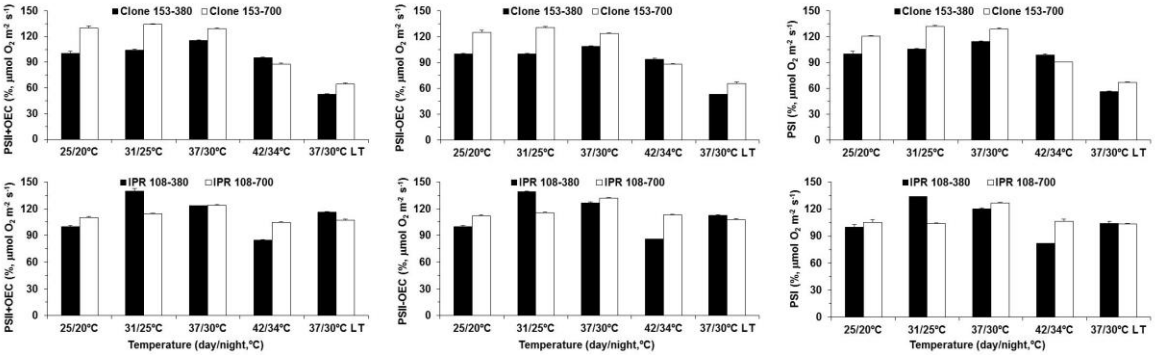

Figure 1. - Changes (in %, from results expressed in µmolO_2 m^{-2} s^{-1}, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the *in vivo* electron transport rates associated with PSII, including (PSII+OEC) or excluding (PSII-OEC) the oxygen evolving complex (OEC), and PSI. Each value represents the mean ± SE (n=4-5).

C-isotope discrimination and N isotope values

The carbon and nitrogen isotope ratios in plants were linked to the prevalent climatic conditions during growth, mainly water and nutrient availability along with light intensity and temperature, which is in agreement with earlier studies that showed the potential use of the stable isotopes as tracers of coffee geographic origin. This study also points to the value of the application of stable isotopes for medium to long-term adaptation to both temperature and elevated CO2 conditions. During photosynthesis there are two major processes that lead to changes in δ13C in plant material: RuBisCO carbon fixation discriminates against 13CO2 of up to 29‰, and a lower diffusion of 13CO2 through stomata. Thus, it is expected that the δ13C in plant material reflects differences in WUE; more enriched 13C (less negative δ13C) leaf tissue is associated with a higher WUE, linked, e.g., to prolonged stomatal closure periods with CO2 restriction to the carboxylation sites, but also with lower H2O consumption rates.

The slight but consistent decrease in leaf δ13C, irrespective of genotype and [CO2], accompanied the temperature rise (including the 37 ºC LT) (Fig. 2) do reflects a reduction in WUE, agreeing with a larger increase in gs than in Pn (data not shown). Yet, the plants under high [CO2] displayed a much more negative δ13C than those at 380 µL CO2 L-1 (close to -37 and -27‰, respectively), at all temperatures, what could point to a lower WUE linked to an

enhanced growth [CO2]. However, this was not confirmed through gas exchanges assessments, both at 25ºC or at higher temperatures, where the 700 plants denoted higher iWUE than the 380 ones. In fact, the direct comparison [CO2] treatments can be misleading and should be avoided in the present case, since the 700 plants received more CO2 from a bottled gas mixture less enriched in 13CO2 (δ13C = -37.6‰) than the 380 ones (that received a mix of the same bottled air with common air, the latter with an approximate composition of δ13C = -8‰).

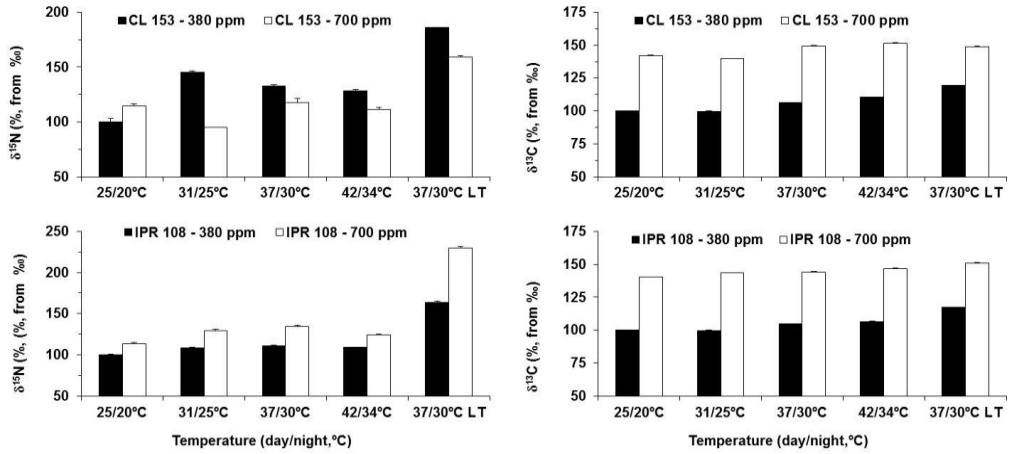

Figure 2. Changes (in %, from results expressed in ‰, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the leaf isotope composition of C ($\delta^{13}C$ in negative values) and N ($\delta^{15}N$) Each value represents the mean ± SE (n=4).

On the other hand, more enriched δ15N values were observed at 25 ºC for both genotypes in the plants grown at higher [CO2] (Fig. 2). With the temperature rise, that difference was maintained in IPR108, but was inverted in CL153, which maybe related with different limiting metabolic assimilatory steps. Furthermore, both genotypes irrespective of [CO2], tended to slight higher values of δ15N with higher temperature, which paralleled an N increase in both [CO2] and genotypes with the temperature rise (data not shown). The stronger δ15N increase was observed in the 37 ºC LT. All that δ15N changes upon heat exposure, further suggests different impacts on the N-assimilation of both genotypes, as the δ15N composition reflects nitrogen sources, as well as plant 15N fractionation, and depends on assimilatory steps, such as enzymatic ammonium or nitrate assimilation. It has been shown that such processes discriminate against the isotopically heavier N substrates, resulting in 15N -depleted products and 15N-enriched residual substrates. Efflux of these 15N-enriched residual substrates renders the plant 15N depleted, contrary to what was found here with temperature increase. Plant 15N fractionation has been shown to depend strongly on the ratio between plant N demand and N supply, or on external N concentration relative to assimilatory capacity. Further studies are needed to clarify the results.

Analysis of some photosynthetic and respiratory enzyme activities

The activities of the enzymes related to the photosynthetic pathway, RuBisCO and Ru5PK, increased until 37 ºC (Fig. 3). At this point, the RuBisCO activity increased 55% (380) and 97% (700) in CL153 and 21% (380) and 85% (700) in IPR108, as compared to 25ºC-380 □L CO2 L-1 of each genotype. Furthermore, Ru5PK activity increased 42% (380) and 55% (700) for CL153 and 13% (380) and 53% (700) in IPR108. Upon 42 ºC a strong impact was observed in the activity of both enzymes, although higher values were usually maintained in the high [CO2] plants. In fact, for both enzymes and genotypes the high [CO2] plants denoted

higher activities along the entire experiment (except in CL153 at 42 ºC). By decreasing the temperature from 42 ºC to a long period at 37 ºC, a recovery in relation to the respective control was usually found. Yet, such recovery was only partial when compared to the previous exposure to 37 ºC.

Concerning the MDH and PK activities, a pattern similar to that of the photosynthetic enzymes was observed (Fig. 4). The higher activity in 700 plants was particularly clear in MDH (IPR108) and PK (CL153), with maximal increases of 2 fold (at 37 ºC) and 1.5 fold (at 31 ºC), respectively, in relation to the 380 plants at those temperatures.

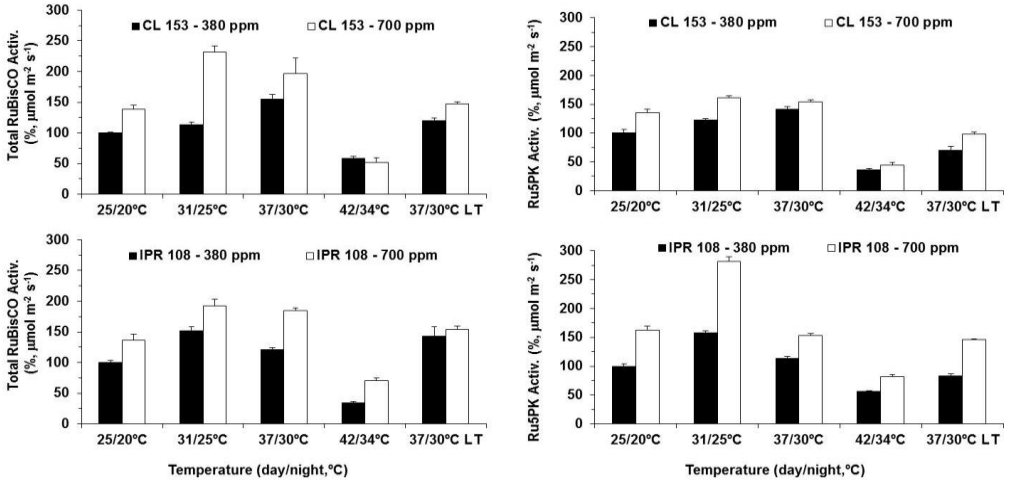

Figure 3. Changes (in %, from results expressed in µmol m^{-2} s^{-1}, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the potential activities of RuBisCO and Ru5PK enzymes. Each value represents the mean ± SE (n=4).

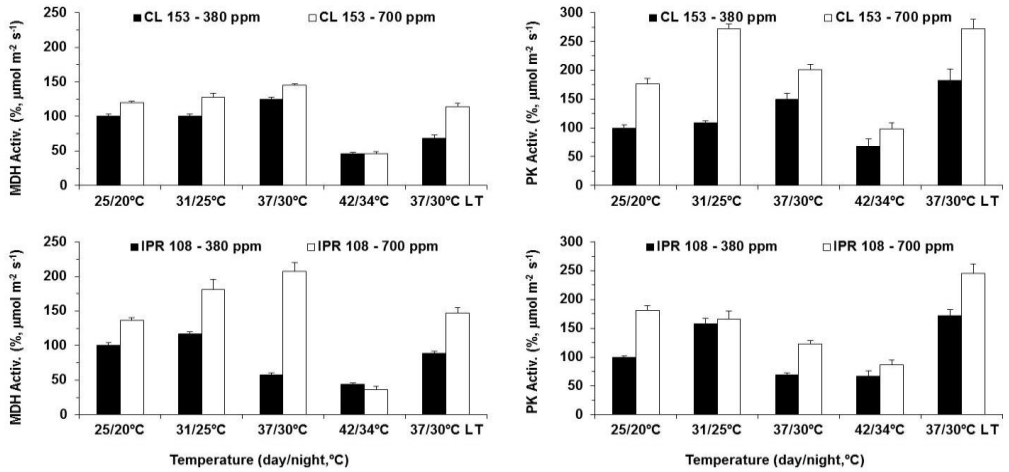

Figure 4. Changes (in %, from results expressed in µmol m^{-2} s^{-1}, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the potential activities of MDH and PK enzymes. Each value represents the mean ± SE (n=4).

As pointed for coffee under enhanced growth [CO2] and 25 ºC [3], the overall reinforced biochemical potential (including enzymes and the electron transport), related to photosynthesis (and with respiration enzymes), might have contributed to prevent photosynthesis down-regulation. In fact, this down-regulation has been linked to reductions in N allocation to RuBisCO, RuBP regeneration and proteins associated with electron transport,

which were absent in the coffee plants. Still concerning these four enzymes, at 380 μL CO2 L-1, the CL153 plants showed their maximal activities at a higher temperature (37 ºC) than the IPR108 plants (31 ºC). Furthermore, IPR108 activities decreased at 37 ºC when compared to 31 ºC. That agrees with the higher temperature optimum of *C. canephora* when compared to *C. arabica*, and the higher heat sensitivity of the latter [23]. Still, for the IPR108-700 the maximal activity values for MDH was at 37 ºC, whereas the total RuBisCO activity at this temperature was similar to that at 31 ºC. Therefore, the higher [CO2] allowed the IPR108 plants to sustain a higher level of activity of these two enzymes under higher temperatures. Yet, at 42 ºC the enzymes were clearly the more sensitive targets with falls higher than 50%, when compared to the respective value at 25 ºC.

Chloroplast membranes lipids

Some alterations in total fatty acid (TFA) content and in the unsaturation level of the chloroplast membranes were noted (Fig. 5). With high temperature, IPR108 showed a TFA increase under both [CO2] until 37 ºC and a decrease at 42 ºC, reflecting a higher lipid dynamics in *C. arabica* than in *C. canephora*, as also found under cold stress for these two species [18,24]. Upon the recovery period at 37 ºC, the TFA content increased again in both [CO2]. Also, the high [CO2] plants tended to lower TFA contents after control, except upon the severe 42 ºC exposure. In CL153-380 plants some TFA decrease was observed up to 37 ºC (and at 37 ºC LT) but not at 42 ºC where a value similar to control was observed. Also, at increased temperatures the 700 plants showed somewhat lower TFA values (ca. 24%) relative to CL153-380 at 25 ºC, except at 37 ºC, when an increase was found. Therefore, CL153 presented lower TFA values in the plants grown in enhanced [CO2] along the experiment, except at 37 ºC and 37 ºC LT.

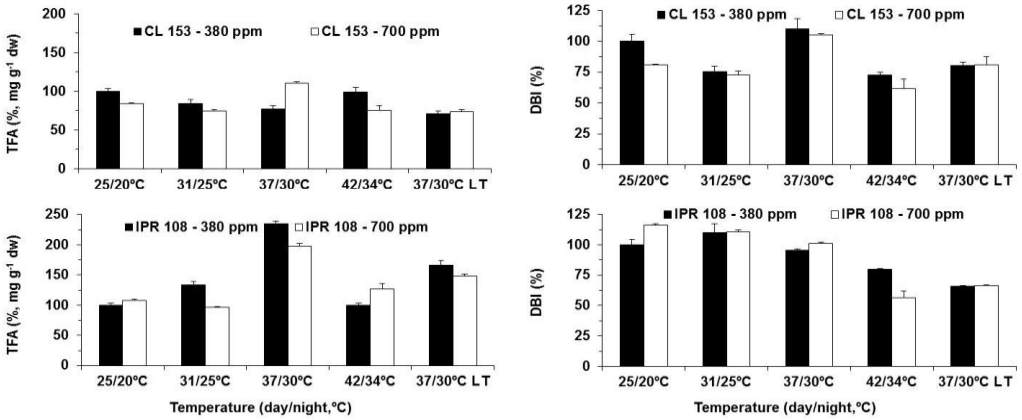

Figure 5. Changes (in %, from results expressed in mg g-1 dry weight for TFA and % for DBI, within each genotype, relative to 380 μL CO2 L-1 at 25/20 ºC) for the potential activities of MDH and PK enzymes. Each value represents the mean ± SE (n=3-4).

Concomitantly to these quantitative variations, some qualitative adjustments were found. Both genotypes displayed a gradual decrease of the unsaturation (DBI) level from 25 to 42 ºC (except at 37 ºC in CL153). This would help to rigidify the chloroplast membranes, counteracting the higher physical fluidity promoted by the increasing temperatures, presumably contributing to preserve appropriate membrane fluidity level and, thus, the membrane-associated functions. Upon 37 ºC LT a reverse trend was observed (except in IPR108-380). Comparing the [CO2] treatments, both CL153-700 and IPR108-700 plants tended to lower the DBI values at 42 ºC, when compared to the respective 380 plants. Therefore, the 700 plants could have benefit from a lower fluidity, what could have

contributed to maintain a higher performance of the photosynthetic apparatus, as reflected, namely, in the higher photosynthetic capacity values (data not shown). Also, the lower absolute DBI value in IPR108-700 (2.3) than in CL153-700 (3.7), could have contributed to maintain higher PSI and PSII activities at 42 ºC (Fig. 1). Such lowered DBI values at 42 ºC were linked to changes in individual FAs, mainly with increases in the more saturated palmitic acid, C16:0 (and a linolenic acid, C18:3, reduction) (data not shown).

In conclusion, the higher photosynthetic metabolic/functional status under elevated [CO2] was related to the up-regulation of several components of the photosynthetic machinery, both under control and under high temperatures (37-42ºC). Such higher performance could also be related to qualitative changes (e.g., higher lipid saturation) in the membrane lipid matrix, which might have preserved an appropriate membrane fluidity level for the membrane-bound events (as electron transport). The PSs and enzymes activity data clearly point to an enhancement of the energetic metabolism (considering both photosynthesis and respiration), mostly, until 31 ºC for IPR108 and 37 ºC for CL153 at normal [CO2]. However, the enhanced growth [CO2] seemed to be implicated in an increase in the temperature (37 ºC) at which maximal values of some parameters were found in IPR108 (MDH, PSs activities, RuBisCO), concomitantly with the maintenance of higher performance in other parameters when compared to the 380 plants. Notably, at 42 ºC the enzymes were the most sensitive point for both genotypes and [CO2] treatments. The changes in leaf δ13C, observed in both genotypes irrespective of growth [CO2], accompanying the temperature rise (including the 37 ºC LT), reflected a decrease in WUE. Although less clear, the δ15N values may indicate different N assimilation processes and limitation steps that requires further investigation. Although a clear distinction was not found between these, IPR108 seemed to have an increased heat tolerance promoted by enhanced [CO2], what is quite relevant in the context of predicted scenarios of increased [CO2] and global warming.

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