EFFECT OF HIGH TEMPERATURES AND CO_2 CONCENTRATION ON PHYSIOLOGICAL, BIOCHEMICAL AND GROWTH TRAITS IN *Coffea* sp.: ASPECTS RELATED TO THE SINGLE LEAF AND WHOLE CANOPY

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"Thesis presented to Centro de Ciências e Tecnologias Agropecuárias da Universidade Estadual do Norte Fluminense Darcy Ribeiro, as part of the requirements for obtaining a PhD degree in Plant Production".

Advisor: Prof. Eliemar Campostrini

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LIST OF ABBREVIATIONS

A: net photosynthesis;

A_c : Whole-canopy photosynthesis rate;

A_{ca}: Daily accumulated whole-canopy photosynthesis rate;

ANOVA: Analysis of Variance;

Chl: Chlorophyll;

E_c: Whole-canopy transpiration;

E_{ca}: daily accumulated whole-canopy transpiration;

F_o: minimal fluorescence from the antennae;

F_v/F_m: maximal photochemical efficiency of PS II;

F_v'/F_m': actual PSII efficiency of energy conversion under light;

g_c: Whole-canopy conductance;

g_s: stomatal conductance;

iWUE: instantaneous water use efficiency;

MDH: NADH-dependent malate dehydrogenase;

OEC: oxygen evolving complex;

ϕ: estimated apparent quantum yield;

PAR: photosynthetic active radiation;

PI: photosynthetic index;

PI_{Chr}, PI_{Dyn} and PI_{Tot}: chronic photoinhibition, dynamic photoinhibition and total photoinhibition;

PK: pyruvate kinase;

PS: photosystem;

q_N: non-photochemical quenching;

q_P and q_L: photochemical quenching, based on the concept of separated or interconnected PSII antennae, respectively;

Rd_c: dark respiration;

RuB5PK: ribulose 5:phosphate kinase;

RuBisCO: ribulose:1,5:bisphosphate carboxylase/oxygenase;

SD: stomatal density;

SI: stomatal index;

SS: stomatal size;

VPD: Vapor pressure deficit;

WUE: water use efficiency;

WUE_c: Whole-canopy water use efficiency;

$Y_{(II)}$ (= ϕ_e): estimate of the quantum yield of photosynthetic non-cyclic electron transport;

$Y_{(NO)}$: estimate of the quantum yield of non-regulated energy (heat and fluorescence) dissipation of PSII;

$Y_{(NPQ)}$: estimate of the quantum yield of regulated energy dissipation of PSII.

ABSTRACT

RODRIGUES, Weverton Pereira; D.Sc; Universidade Estadual do Norte Fluminense Darcy Ribeiro; February 2017; Effect of high temperatures and CO_2 concentration on physiological, biochemical and growth traits in *Coffea* sp.: aspects related to single leaf and whole canopy; Professor Advisor: Eliemar Campostrini; Co-Advisor: Professor José Cochicho Ramalho

The tropical coffee crop has been predicted to be threatened by future climate changes and global warming. However, the real biological effects of such changes remained unknown at both leaf and whole-canopy level. Therefore, we designed a set of experiments in *Coffea* sp*.* under both controlled and non-controlled (seasonal fluctuations) conditions. The experiments were related to single and combined effects of the increase in the atmospheric CO_2 concentration and temperature on photosynthesis at leaf–scale, as well as related to impacts of rising temperatures on gas exchanges at whole-canopy scale. The first experiment aimed at to evaluate changes at stomatal and photochemical levels in *Coffea arabica (*cv*.* Catuaí Amarelo 65*)* and *C. canephora (*cv. Emcapa 8111 Clone 02) under mild temperature (spring) and high temperature (summer) exposure. Potted plants were maintained in a greenhouse, watered to field capacity and subject to the natural variations of light, temperature and relative humidity (Chapter 1). In the second experiment, cropped genotypes of *C. arabica* L. (cv. Icatú and IPR108) and *C. canephora* cv. Conilon Clone 153 (CL153) were grown for *ca.* 10 months at 25/20 °C (day/night) and 380 or 700 µL CO_2 L^{-1}. After that they were subjected to a

gradual temperature increase (0.5 °C day^{-1}) up to 42/34 °C. Leaf impacts related to stomatal traits, gas exchanges, C-isotope composition, chlorophyll *a* fluorescence parameters, thylakoid electron transport rates and enzyme activities were assessed at 25/20, 31/25, 37/30 and 42/34 °C (Chapter 2). The third experiment evaluated whole-canopy gas exchanges on genotypes from the two main coffee producing species (*C.arabica* cv. Catuaí Amarelo 65 and *C. canephora* cv. Emcapa 8111 Clone 02) during two different seasons varying in temperature. Six plants with *ca.* 1-year-old of each species were grown in a greenhouse and kept well-watered. Data were continuously collected for 10 days during spring (September 2014 - moderate temperature) and summer (February 2015 -high temperature) and micrometeorological variables were monitored inside the greenhouse (Chapter 3). Overall, our results showed under controlled conditions, both coffee genotypes were tolerant up to 37/30 ºC, whereas declines in photosynthetic rates were observed at 42/34 ºC mainly associated with higher heat sensibility of the photosynthetic enzymes, namely ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and ribulose 5-phosphate kinase (RuB5PK). However, enhanced [CO_2] strongly alleviated the impacts of high temperatures, particularly at 42/34ºC, modifying the response of coffee plants to supra-optimal temperatures. Additionally, coffee genotypes grown under elevated [CO_2] did not show an acclimation of photosynthesis so that photosynthetic rates and photochemical and biochemical activities were all improved at all temperatures. On the other hand, under a fluctuating environment conditions, supra-optimal temperatures lead to increases in air DPV affecting both leaf and whole-canopy photosynthetic rates in *C. arabica* plants. Decreases in photosynthetic rates in this specie during summer were linked to declines in both stomatal and canopy conductance, however without an apparent damage to the photochemical pathway. Finally, although *C.canephora* showed higher heat tolerance than *C. arabica,* maintaining similar both whole-canopy and leaf CO_2 assimilation values in both seasons, its canopy architecture limited whole-canopy gas exchange due to poor light distribution within the canopy.

RESUMO

RODRIGUES, Weverton Pereira; D.Sc; Universidade Estadual do Norte Fluminense Darcy Ribeiro; February 2017; Efeito de altas temperaturas e concentração de CO_2 nas características fisiológicas, bioquímicas e de crescimento em *Coffea* sp.: aspectos relacionados à folha e à planta inteira; Orientador: Professor Eliemar Campostrini; Co-Orientador: Professor José Cochicho Ramalho

É previsto que a cultura do café esteja ameaçada pelas mudanças climáticas futuras e pelo aquecimento global. Entretanto, os efeitos biológicos reais de tais mudanças permanecem desconhecidos tanto em nível de folha quanto em nível de planta inteira. Assim, um conjunto de experimentos foi proposto em *Coffea* sp. sob condições controladas e não controladas (flutuações sazonais). Os experimentos foram relacionados aos efeitos combinados ou não de aumentos na concentração de CO_2 e temperatura nas taxas fotossintéticas em escala de folha, bem como relacionado aos impactos do aumento da temperatura nas trocas gasosas em escala de planta inteira. O primeiro experimento objetivou avaliar mudanças em nível estomático e fotoquímico em *Coffea arabica* (cv. Catuaí Amarelo 65) and *C. Canephora* (cv. Emcapa 8111 Clone 02) expostas à temperaturas medianas (primavera) e altas temperaturas (verão). As plantas foram cultivadas em casa de vegetação utilizando vasos, os quais foram mantidos na capacidade de campo. As plantas foram submetidas às variações sasonais de luz, temperatura e umidade relativa (Capítulo 1). No segundo experimento,

genótipos de *C. arabica* L. (cv. Icatú e IPR108) e *C. canephora* (cv. Conilon CL153) foram cultivados durante aproximadamente 10 meses a 25/20 °C (dia/noite) e 380 ou 700 µL de CO_2 L^{-1}. Após isto, as plantas foram submetidas à um aumento gradual na temperatura (0,5 ºC por dia) até 42/34 ºC. Os impactos foliares relacionados às características dos estômatos, trocas gasosas, composição isotópica de carbono, parâmetros de fluorescência da clorofla *a*, transporte de elétrons nos tilacóides e atividades enzimáticas foram avaliadas a 25/20, 31/25, 37/30 e 42/34 (Capítulo 2). O terceiro experimento avaliou as trocas gasosas de cafeeiros pertencendo a duas espécies de café (*C. arabica* cv. Catuaí Amarelo 65 e *C. canephora* cv. Emcapa 8111 Clone 02) durante duas diferentes estações com diferentes temperaturas. Seis plantas com mais de um ano de idade de cada espécie foram cultivadas em estufa, com o solo mantido na capacidade de campo. Os dados foram coletados continuamente por 10 dias durante a primavera (setembro de 2014 - temperaturas moderadas) e verão (fevereiro de 2015 - com alta temperatura) e as variáveis micro meteorológicas foram monitoradas dentro da estufa (Capítulo 3). De maneira geral os resultados mostraram que sob condiçoes controladas, ambos os genótipos de café foram tolerantes até 37/30 ºC, porém declínios nas taxas fotossinteticas foram observados a 42/34 ºC, principalmente associado com a maior sensibilidade das enzimas fotossintéticas, nomeadamente ribulose-1,5 bisfosfato carboxilase/oxigenase (RuBisCO) e ribulose-1,5 fosfato quinase (RuB5PK). No entanto, elevada concentração de CO_2 fortemente aliviou os impactos de elevadas temperaturas, particulamente a 42/34 ºC, modificando as respostas de plantas de café para supra-ótimas temperaturas. Adicionalmente, genótpos de café crescidos sob elevada concentração de CO_2 não mostraram uma aclimatação da fotossíntese de maneira que as taxas fotossinteticas e as atividades fotosquímicas e bioquímicas foram melhoradas em todas as temperaturas. Por outro lado, sob condições de flutuações ambiente, supra-ótimas temperaturas levou para aumentos no DPV do ar afetando as taxas fotossinteticas das folhas e da planta interia em *C. arabica.* Decréscimos nas taxas fotossínteticas nesta espécie durante o verão foram relacionados com declínios na condutânica dos estomatos e da copa, no entanto, sem danos aparente à via fotoquímica. Por fim, embora *C. canephora* mostrou maior tolerância às supra-ótimas temperaturas do que *C arabica*, sua arquitetura de

copa limitou as trocas gasosas da planta interia devido à uma pobre distribuição de luz dentro do dossel.

1. INTRODUCTION

In recent years, the impact of global climate changes is a central issue in scientific events, in particular those from biology, ecology and agriculture areas. This is due to the foreseeable effects of climate changes on plants metabolism, namely on plants growth and productivity, as well as on agricultural products quality. In fact, global climate changes are usually associated with the increase in air CO_2 concentration ($[CO_2]$) and temperature, and reduced amount of water available for agriculture (Drake et al., 1997; Idso and Kimball, 1997; Luo et al, 1999), with negative impacts for the food and feed supply, regarding a growing world population (DaMatta et al., 2010).

Presently, mostly related to anthropogenic action air $[CO_2]$ is increasing at a rate close to 2 µL L^{-1}per year, and depending on future emissions, it is estimated to reach values exceeding 700 µL CO_2 L^{-1} along the second half of the present century, probably accompanied by global warming up to 3.7 to 4.8 °C by 2100 (IPCC, 2014). Since the optimal annual mean temperatures for plant development are in the range of 18-23 ºC for *C. arabica* and of 22-26 °C for *C. canephora* (DaMatta and Ramalho, 2006), it has been argued that coffee, particularly *C. arabica*, will be highly sensitive to climate changes and that global warming will threaten the coffee supply in a near future (Davis et al., 2012; Bunn et al., 2015).

Coffee is a tropical crop that is grown in approximately 80 countries, making it one of the world's most traded agricultural products. The world coffee trade is supported by *C. arabica* L. (Arabica type of coffee) and *C. canephora*

Pierre ex A. Froehner (Robusta type of coffee) species, which are responsible for nearly 99% of coffee production (Davis et al., 2012). Global annual yield is currently above 8.5 million tons of green coffee beans (ICO, 2016), and is estimated that the chain of value of coffee generates an income of *ca*. US$ 173,000 million, and involves *ca*. 100 million people worldwide (ICO, 2014).

For some crops considered as primary foodstuffs such as rice, wheat, cotton and soybeans (Baker, 2004; Caldwell et al., 2005; Luo et al., 2005; Ainsworth and Rogers, 2007; Yoon et al., 2009), as well as shrub species such as coffee (Ramalho et al., 2013a; Ghini et al., 2015, DaMatta et al., 2016) several research have been designed to study the effects of the changes in air [CO_2] on plant metabolism, growth and yield. However, only a relatively small percentage of such studies combine the other environmental disturbances that are predicted to accompany such air [CO_2] rise, such as increased temperature and decreased water availability (either by scarcity or by changing rainfall patterns), and their interactive impacts on plant productivity. In this context, studies that integrate these different variables can constitute a more realistic view of how productivity and quality of products obtained from cultivated plants will be affected by global climate changes (DaMatta et al., 2010; Silva et al., 2017). With respect to coffee, a plant with economic and social importance, until very recently (Ramalho et al., 2013a) nothing was known about the potential effects of [CO_2] increase and its interaction with other environmental changes at physiological and biochemical level and grain yield and quality. Recent efforts have addressed the combined effect of increased [CO_2] and supra-optimal temperatures, namely on the dynamic of mineral nutrients (Martins et al., 2014), C-assimilation metabolism (Rodrigues et al., 2016a, which constitutes part of this thesis), and protective mechanisms and gene expression patterns (Martins et al., 2016) in coffee genotypes.

The use of tools to identify the effects of changes in air temperature and/or [CO_2] on both plant metabolism and adaptation/tolerance mechanisms is crucial to future crop yields. Among these useful tools, gas exchanges, chlorophyll *a* fluorescence, electrons transport rates involving photosystems I and II, enzymes activities (*e.g.* related to antioxidant, photosynthetic and respiratory pathway), photosynthetic, pigments, dynamics of assimilate partitioning and membrane lipid composition, growth rates and genes expression measurements have been used as probe to the global functioning of the plant (Falcone et al., 2004; Ramalho et

al., 2013a; Ghini et al., 2015, Martins et al., 2016, Silva et al., 2017). Regarding the gas exchange measurements, many ones have been reported widely at single leaf level and it cannot be extrapolated to the whole plant. In this context, measuring whole-canopy gas exchange will integrate net photosynthesis and transpired water values at the global plant level, and can becomes an important tool to improve the accuracy of data interpretation and, consequently, our knowledge about plants responses to these environmental modifications (Glenn et al., 2003; Poni et al., 2014; Ferraz et al., 2011; Ferraz et al., 2016).

Although several reports have predicted negative impacts of elevated temperature on coffee crop in the most important coffee production areas around the World, including Brazil (Assad et al., 2004; Davis et al., 2012; Bunn et al., 2015), the real impacts of supra-optimal temperature on a long term basis on coffee physiology remains to be studied. This is because, under gradual exposure, several reports have showed coffee plants might cope with some stress such as cold, high irradiance and drought (Ramalho et al., 1998; Lima et al., 2002; Campos et al., 2003; Partelli et al., 2009; Ramalho et al., 2014). The coffee ability to cope with such stress is mainly due to increases in both enzymatic and non-enzymatic antioxidants system as well as changes in lipid classes and degree of saturation of fatty acids in the chloroplast membrane (Ramalho et al., 1998; Lima et al., 2002; Campos et al., 2003). Additionally, global warming is associated with rising atmospheric [CO_2] which, in turn, can stimulate photosynthetic rates in coffee plants (Ramalho et al., 2013a). Therefore, our pioneering work focuses on analyzing the physiological and biochemical performance of coffee genotypes from two main cropped species (*C. arabica* and *C. canephora*) exposed to enhanced air [CO_2] and/or supra-optimal temperature on a long term basis.

2. LITERATURE REVIEW

2.1. Taxonomic classification and favorable climatic conditions

Coffee plant belongs to the Rubiaceae family, and to the genus *Coffea* (Charrier and Berthaud, 1985), the latter including at least 124 species (Davis et al., 2011). However, the world coffee trade is largely supported only by two of those species *Coffea arabica* L. (Arabica type of coffee) and *Coffea canephora* Pierre ex A. Froehner (Robusta type of coffee), which are responsible for nearly 99% of coffee production (Partelli et al., 2011; Davis et al., 2012).

C. arabica is an allotetraploid (2n = 4x = 44 chromosomes) showing predominantly self-pollination, since only *ca.* 10% of cross-pollination can occur (Carvalho and Monaco, 1964). On the other hand, *C. canephora* is diploid (2n = 22 chromosomes) and self-incompatible, showing, therefore, cross-pollination. Such incompatibility is the gametophytic type, and is linked to S1, S2, S3 and S4 alleles (Conagin and Mendes, 1961; Berthaud, 1980; Mishra and Slater, 2012).

The different evolutionary histories of *C arabica* and *C. canephora* justify some differences in the optimal conditions for these species, with particular emphasis on the air temperature. *C. arabica* specie is originated from the tropical forests of Ethiopia, Kenya and Sudan, at altitudes of 1500-2800 meters, average annually air temperature between 18 and 22 °C and annually precipitation ranging from 1600 to more than 2000 millimeters with a well-defined dry season (three to four months) that coincides with the coldest period. Under such environment

conditions, arabica plant became established as a shrub (DaMatta and Ramalho, 2006).

C. canephora is originated from the lowland forests of the Congo River basin, which extend to Lake Victoria in Uganda at altitudes up to 1200 meters, subject to average annually air temperatures between 23 and 26 °C with minor fluctuations, and annually precipitation exceeding 2000 mm spread over 9 to 10 months (Coste, 1992; Davis et al., 2006). So, it is traditionally considered that better plant development is expected occur under an average annual temperature ranging from 19 to 23 °C for *C. arabica* and from 22 to 26 °C for *C. canephora* (DaMatta and Ramalho, 2006; Matiello et al, 2010).

2.2. Predicted environmental changes: atmospheric CO_2 concentration and temperature

The anthropogenic action increased atmospheric CO_2 from *ca*. 280 µL L^{-1} from pre-industrial period, until 400 µL L^{-1} in 2013. CO_2 levels are currently increasing at a rate of *ca.* 2 µL L^{-1} per year and it is estimated that will reach values between 450 and 600 µL L^{-1} by 2050, exceeding 700 µL L^{-1} along the 2nd half of the present century (Collins et al., 2013; Ramalho et al., 2013a). Accompanying this increase in CO_2, the IPCC prediction models estimate a temperature rise that could reach maximal values between 3.7 and 4.8 °C by 2100 (IPCC 2014).

It is known that changes in atmospheric [CO_2] affect plant metabolism and may affect the crops productivity (Drake et al., 1997; Oliveira et al., 2010). Additionally, supra-optimal temperatures also promote changes in the metabolic processes, with impact on photosynthetic carbon assimilation, respiration, water relations, fluidity and stability of membrane systems, modulate hormone levels and primary and secondary metabolism, with implications on the quality of agricultural products (Wise et al., 2004; Wahid et al., 2007, DaMatta et al., 2010). In this context, it will be crucial to assess if the plants will have genetic ability to modify its vital processes functioning at a speed compatible with such environmental changes. Moreover, it will be essential to understand if the selection and classical breeding methods will be able to release genotypes that can better cope with predicted future climate scenarios or whether will be need to use genetic

engineering for obtaining new genotypes in an attempt to maintain or even increase crops yield.

Regarding the coffee plants, several models based on projected increases in atmospheric temperature from IPCC report, have predicted significant impacts on this crop. In fact, those modeling approaches estimated impacts for coffee related to suitability of agro-climatic zoning areas (Assad et al., 2004; Ovalle-Rivera et al., 2015; Magrach and Ghazoul, 2015) decrease productivity (Gay et al., 2006; Craparo et al., 2015), extinction of *C. arabica* wild populations (Davis et al., 2012), and increase crop vulnerability at agricultural, social and economic levels (Baca et al., 2014).

However, although rising air temperature is expected to negatively impact coffee crops as outlined above, it should be noted that studies based on climatic models consider only current cultivars without take into account both mitigation practices (Camargo et al., 2010) and clear potential beneficial effect of increased [CO_2] (Ramalho et al., 2013a, Ghini et al., 2015, Martins et al., 2016; DaMatta et al., 2016).

2.3. Physiological aspects of plants grown under high CO_2 concentrations and supra-optimal temperatures

Coffee is a C_3 metabolism plants, so unlike C_4 plants, is largely limited by the [CO_2] diffusion within the mesophyll until the carboxylation active sites of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), in the chloroplast stroma (Drake et al., 1997). Furthermore, CO_2 and O_2 compete for the same active sites on RuBisCO, with alternative carboxylase function (for CO_2 assimilation) or and oxygenase function (resulting in the CO_2 release through photorespiration) (Ainsworth and Rogers, 2007). Taking into account these considerations, the increase in [CO_2] may act as C-fertilization, promoting net photosynthetic rate and higher photosynthates synthesis. Such increase results from the higher substrate availability (CO_2) for carboxylation, whereas it increases the competition with O_2, for the RuBisCO active sites decreasing the photorespiration rate (Drake et al., 1997; Long et al., 2004; Ainsworth and Rogers, 2007; Kirschbaum, 2011; Ramalho et al., 2013a). However, in the long-term, such increase in photosynthetic rates may result in the accumulation of photoassimilates due to an insufficient sink-

capacity to use them. This will reduce the photosynthetic potential due to a feedback downregulation of photosynthesis, also called negative acclimation (Drake et al., 1999; Ainsworth and Rogers, 2007; Kirschbaum, 2011

In fact, photosynthates accumulation triggers signaling pathway which will decrease gene expression and the activity and/or amount of photosynthetic enzymes and other components, including RuBisCO and thylakoid electron carriers. Consequently, reductions in the maximum apparent carboxylation velocity (Vc_{max}) and the maximum apparent rate of electron transport (J_{max}), and therefore on photosynthetic rates are frequently observed (Luo et al., 1999; Long et al., 2004; Zhu et al., 2012.). Indeed, lowered photosynthesis values have been attributed to the decreases in both Vc_{max} and RuBisCO synthesis. Reduction in photosynthesis can also be due to a decrease in ribulose-1,5-bisphosphate (RuBP) regeneration which, in turn, result in decreases in J_{max} (reflecting decreased electron transport in thylakoid membranes), and the inorganic phosphate (Pi) availability to ATP synthesis inside the chloroplasts (Sage, 1994; Drake et al., 1997; Ainsworth and Long, 2005; Ainsworth and Rogers, 2007; Zhu et al., 2012.). It should be underlined that such downregulation of photosynthesis, when occurring, varies among species (even between cultivars of the same species) and depends on the interaction with other environmental variables (Sage, 1994; Long et al., 2004; Ainsworth and Rogers, 2007; Kirschbaum, 2011).

The effect of increased [CO_2] on photosynthetic rates may have positive consequences for plant growth and productivity. Under appropriate environmental conditions, C3 plants often exhibit net photosynthesis rate 50% above that observed under current [CO_2], with increases in both water and nitrogen use efficiency (Drake et al., 1997; Long et al., 2004; Ainsworth and Rogers, 2007; Kirschbaum, 2011). However, such increases in C-assimilation result in much smaller increments (*ca.* 10%) in the growth rates (Kirschbaum, 2011; Ghini et al., 2015). This difference is probably associated with the inability to use the large assimilates availability by sinks (for example, limitations associated with meristematic tissue which can display a standard deterministic growth). Such limitation to use assimilates may, in turn, reduce its export from leaves to other parts of the plant resulting, for example, a lower Pi regeneration, and photosynthesis downregulation of photosynthesis, which involve decreases in apparent maximum carboxylation rates and/or electron transport (Stitt, 1991; Luo

et al., 1999; Kirschbaum, 2011). It is important to underline that, since assimilates partition is also mediated by hormones, which are highly influenced by environmental conditions (Bita and Gerats, 2013), the preservation of hormone balance is also essential to achieve greater crop yield.

Increases in growth and productivity has been observed in some agronomically important crops such as cotton (Yoon et al., 2009), wheat (Luo et al., 2005), soybean (Ainsworth and Rogers, 2007), which has been associated with the rapid growth (exponential growth phase) (Poorter and Navas, 2003). However, considering perennials crop such as coffee, fast initial growth may cause the canopy closure and reduce the benefits of increased [CO_2] (Kirschbaum, 2011). Anyway, under high [CO_2] conditions, trees and shrubs, especially fast-growing species show a higher capacity to use assimilates (root-trunk system) than annual plants (Arp, 1991; Ainsworth and Long, 2005; Ainsworth and Rogers, 2007), if the roots can grow without physical space constraints (Arp, 1991).

With respect to coffee plants, either *C. arabica* (cv. Icatú and IPR108.) or *C. canephora* (cv. Conilon Clone 153 -. EMCAPA 8113), grown under environment controlled conditions (using walk in growth chambers) during one year, photosynthesis increases were observed when the atmospheric [CO_2] was enhanced from 380 to 700 μL L^{-1}, without any apparent photosynthetic downregulation (Ramalho et al., 2013a). Such response was linked to the consumption of photosynthates and regeneration of RuBP and Pi (through triose phosphate use), associated with a continuous production of vegetative and reproductive structures during the experiment, therefore reflecting a high sink strength (Ramalho et al., 2013a). Moreover, coffee plants showed no reduction in investment in the components of the photosynthetic pathway, as reflected in maximal activity of enzymes such as RuBisCO and ribulose 5-phosphate kinase (Ru5PK) as well as in the maximum electron transport rates involving both photosystems, justifying the maintenance (or marginal increase) of the increased photosynthetic capacity (Ramalho et al., 2013a). These are in accordance to experiments, where coffee plants grown under field conditions in Free-Air Carbon dioxide Enrichment-FACE system showed higher growth (*ca.* 10%) and yield (*ca.* 13%) at 550 μL L^{-1} of CO_2 (Ghini et al., 2015).

In addition to direct implications in photosynthesis, increased [CO_2] can also affect the stomata, being commonly observed a decrease in stomatal

conductance. This will reduce the transpiration rates, and, together to the photosynthesis increase will promote water use efficiencies (Woodward, 2002; Ainsworth and Long, 2005; Ainsworth and Rogers, 2007; Leakey et al., 2009; Xu et al., 2016). Such decreases have been shown to occur to a lesser extent in shrubs and trees than in herbaceous annuals (Ainsworth and Rogers, 2007).

Photosynthetic activity could efficiently sensitize the guard cells, since such cells have functional chloroplasts (Sage, 2004). Ions concentration and organic solutes mediate turgor pressure into the cells guards, regulating the stomatal opening (Ainsworth and Rogers, 2007). High CO_2 levels could increase the K output channels activity, decrease the K input channels activity, increase the S-type anion channels activity, stimulate the release of Cl from the guard cells and increases the Ca concentration into the guards cells (Webb et al., 1996; Raschke et al., 2003). Together, such changes could depolarize the membrane potential from the guard cells and cause the stomatal closure (Assmann, 1993), resulting in reduced stomatal opening (Ainsworth and Rogers, 2007). However, there are several potential messengers in the stomatal response to [CO_2] so that guard cells are regulated by a complex network of signaling pathways (Ainsworth and Rogers, 2007).

Decreases in stomatal opening, stomatal density (SD) and stomatal index (SI) contributes to reduce stomatal conductance, and have been observed under increased [CO_2] conditions (Lin et al., 2001; Miyazawa et al*.,* 2006; Ainsworth and Rogers, 2007), although in some cases stomatal conductance and not SD determines the long-term reduction in leaf transpiration (Tricker et al., 2005). Even so, the stomatal closure often found under enhanced [CO_2] will lower latent heat loss, therefore increasing leaf temperatures (DaMatta et al., 2010; Ramalho et al., 2013a). Such increase in temperature may result in elevated vapor pressure inside the leaf and raise the vapor pressure difference between leaf and air, thereby promoting the water loss through transpiration. Thus, the transpirations rates may be only slightly reduced under high [CO_2] (Prasad et al., 2005; DaMatta et al., 2010).

Some studied *C. arabica* and *C. canephora* genotypes showed no effect of increased [CO_2], *e.g.*, from 380 to 700 µL L^{-1} on stomata size, leaf specific area, stomatal conductance and photosynthesis (Ramalho et al., 2013a). However, with increased [CO_2] was increased the intrinsic water use efficiency of such genotypes

and a decrease in stomatal density on *C. canephora*. The reduction in stomatal density in plants grown at high [CO_2] is not always associated with a concomitant reduction in leaf transpiration (Tricker et al., 2005).

In coffee leaves, it was observed only a non-significant reducing trend of stomatal conductance in the plants grown at 700 µL L^{-1} (between 4 to 28%), together with a trend towards lower stomatal density (between 5-14%) and increasing the stomata size (between 3-7%). Take into account the marginal reduction (or maintenance) of stomatal conductance and the parallel increase in photosynthetic rates, the instantaneous water use efficiency (iWUE) strongly increased between 56-112% (Ramalho et al., 2013a).

In addition to the changes involving the photosynthetic rate and stomatal functioning, it's also important to consider the responses related to the respiratory level, since this pathway directly influences the net carbon balance and also provides carbon skeletons which are needed to both primary and secondary metabolism. Decreases in mitochondrial respiratory rate (*ca.* 20%) at a CO_2 concentration twice the current one have been reported (Drake et al., 1997). Such response can be linked to both direct effects, namely inhibition of key enzymes, in the mitochondrial electron transport (cytochrome oxidase and succinate dehydrogenase), and the indirect effects related to the carbohydrates availability, growth rate and biomass allocation (Drake et al., 1997). However, in plants with large carbohydrate availability, the respiratory rate increases to greater extent in young leaves with rapid growth, indicating that only where the carbohydrates are not consumed due to a demand for growth, respiration is limited by substrate (Drake et al., 1999). This suggests a rate regulation at which the products, particularly adenilate, NAD(P)H and carbonic skeletons are used due to the energy requirement for growth, maintenance and nutrient uptake (Drake et al., 1999). In fact, enhanced [CO_2] during the plant life can promote mitochondrial respiration due to a greater activity and transcription of several enzymes related to glycolysis pathway, Krebs cycle, as well as components of the electron transport chain (Leakey et al., 2009). Since increased respiratory rate is clearly supported by the additional use of carbohydrates, linked to an increase in photosynthetic rate at high [CO_2] (Leakey et al., 2009), net carbon gain may not be affected. In fact, no accumulation of sugars was found under high [CO_2] in coffee leaves, that showed increased A values and somewhat increased respiratory activity (although not

significantly), in line with the rise of the potential activity of enzymes from this pathway (malate dehydrogenase and pyruvate kinase) in all studied coffee genotypes.

Another important aspect is related with the mineral balance of plants gown under increased [CO_2]. It is known that in plants grown under high [CO_2], where photosynthesis is RuBP regeneration-limited, the required amount of RuBisCO is reduced so that, reallocating the excess N could improve nitrogen use efficiency without impacting the carbon acquisition, since 25% of leaf N is the structure of RuBisCO protein (Drake et al., 1997). However, such N reallocation, as well as the utilization of assimilates, will depend on the capacity of utilization by sinks (Ainsworth and. Rogers, 2007). On the other hand, decreases in several nutrients content have been interpreted as a “dilution” effect in the plant tissues due to increased growth rate promoted by high [CO_2] (Brown, 1991; Blank et al., 2011). Decreases in N, P, C and Mg content were found in *Picea abies* and *Quercus rubra* grown at 350 µL L^{-1} of CO_2 higher than ambient air [CO_2] (Thiec et al., 1995), while decreases in K, Ca, Mg, Mn and Fe content were observed in herbaceous and woody plants with increased [CO_2] (Overdieck, 1993). Moreover, as presented above, increased [CO_2] (Ainsworth and Rogers, 2007) and/or supra-optimal temperature (Prasad et al., 2005) may reduce the stomatal conductance and transpiration rate, affecting xylem flow and, therefore nutrients uptake and translocation into the leaves (Marschner, 1995). Such changes in the nutrients balance can influence the fundamental processes such as photosynthetic machinery regulation (*e.g*., N, S and Fe), enzymatic activity (*e.g*., K, P, Mn and Fe) and the maintenance of the chloroplasts structures (*e.g*. B) (Overdieck, 1993; Leakey et al., 2009; Blank et al., 2011), and so affecting the plant growth. In the case of coffee plants, stomatal conductance is barely reduced when coffee plants were grown at 700 µL CO_2 L^{-1} and 25 ºC (Ramalho et al., 2013a). Even so plants showed a moderate dilution effect (between 7% and 25%) in CL 153 (for N, Mg, Ca, Fe) and Icatú (for N, K and Fe), but not in IPR 108 (except for Fe) when compared to the 380 µL CO_2 L^{-1} (Martins et al., 2014a), remaining within an appropriate range (Ramalho et al., 1995; Bragança et al., 2007; Guimarães and Reis, 2010), therefore without any expected impact in the photosynthetic performance.

In conclusion, considering that other factors such as water availability, temperature and mineral nutrition are not limiting, the coffee plant seems to have a considerable ability to adjust the photochemical and biochemical processes of photosynthesis under increased [CO_2]. In fact, coffee plants are able to sustain high photosynthetic rates at increased [CO_2] without sugar accumulation, decrease investment on the photosynthetic components, and, ultimately, photosynthetic downregulation (Ramalho et al., 2013a). Furthermore, although with some dilution, mineral contents were maintained at an adequate level in the leaves (Martins et al., 2014a). However, rapid growth may affect the future productions due to self-shading, since the light is required for floral induction, especially in dense plantation areas and thus, a management mediated through pruning would be essential, which, by promoting the regrowth of plant structures will help the plant to avoid photosynthetic downregulation, taking advantage of C fertilization effect provided by high atmospheric CO_2 levels.

2.4. Effect of supra-optimal temperatures on plant metabolism

It's known that high air temperatures may cause significant disturbances in metabolism and plant growth, since chemical reactions are accelerated, the chemical bonds are weakened, the lipid matrix membrane becomes more fluid and proteins denaturation and aggregation might occur, as well as the overproduction of reactive oxygen species (ROS) and inhibition of the transcription and translation processes (DaMatta and Ramalho, 2006). At photosynthesis level, the supra-optimal temperatures changes the use of captured solar energy, the gas diffusion through mesophyll (Lambers et al., 2008) and disturb the Calvin cycle activity (Pastenes and Horton, 1996), in particular by reducing the RuBiscO activity (Brandner-Crafts and Salvucci, 2000). The increase in temperature also leads to increased evaporative demand, which may cause stomatal closure and reduction of CO_2 supply to the chloroplast, reducing photosynthesis (Prasad et al., 2005).

Highly reactive molecules production at the chloroplast level is usually increased under environmental conditions that impose a reduction of the use of energy through photochemistry, while the light capture is little if any affected. This imbalance will then promote an over-production of excited molecules of chlorophyll (*e.g.*, $^3Chl^*$) and oxygen ($^1O_2$, $O_2^{\bullet-}$, $OH^{\bullet}$, H_2O_2) at the antennae and photosystems

(I and II) levels. If an uncontrolled ROS production occurs, oxidative stress conditions will be promoted causing extensive damages, namely through lipid peroxidation, protein degradation and inactivation, and DNA structure disruption, (Sairam et al., 2004, Logan, 2005; Smirnoff, 2005). The main defense mechanisms of plants against ROS is the presence of antioxidant enzymes (*e.g*., superoxide dismutase, ascorbate peroxidase, reductase and catalase, glutathione) (Logan, 2005; Smirnoff, 2005), making it an important mechanism at high temperatures (Allakhverdiev et al., 2008) and metabolites secondary plant itself (glutathione, ascorbic acid and carotenoids). To cope with environmental stressful conditions (*e.g*., cold and excess radiation), several coffee genotypes were observed to trigger antioxidant mechanisms (Ramalho et al., 1998; 2003; Fortunato et al., 2010; Batista-Santos et al., 2011; Ramalho et al., 2014). These, can contribute to prevent membrane lipid peroxidation (Campos et al., 2003). Furthermore, long term changes in the fatty acids and lipid class composition at membrane level are also often reported, helping to maintain and adequate membrane fluidity, namely through reduction of the fatty acid saturation level or by increasing the weight of phospholipids classes under low temperature exposure (Partelli et al., 2011; Scotti-Campos et al., 2014).However, such changes in plant metabolism have yet been no studied in coffee genotypes subjected to the interaction of high temperatures and increased [CO_2].

Thylakoid membranes are particularly sensitive to high temperatures, so impairments photochemical step of photosynthesis is among the first indicators of sensitivity to heat stress. This occurs, namely, because of electron transport uncoupling with the ATP synthesis and damage to PSII and to chloroplast ultrastructure (Larcher, 1995; Mano, 2002). In addition, there is loss of C assimilated in C3 plants due to the proportional higher increase of respiration and photorespiration than to photosynthesis (Long, 1991). Therefore, even moderately high temperatures may affect crop productivity (DaMatta and Ramalho, 2006). Concerning the photorespiration, it has been argued that the increased temperature increases more the O_2 solubility than that of CO_2, favoring RuBisCO oxygenase over carboxilase function, decreasing photosynthetic rates (Brandner-Crafts and Salvucci, 2000). However, under increased air [CO_2] photorespiration is reduced, being likely that under this conditions photosynthesis increase more at

higher that lower temperature, therefore, compensating (at least partially), the negative effects of high temperatures on the crop yield (Long, 1991; Polley, 2002).

The effects of supra-optimal temperatures have received specially attention regarding coffee crop, probably because such environmental factor is one of the most limiting for culture (DaMatta and Ramalho, 2006). Classical works (between 1950's and 1970's decades from the last century) pointed to a strong thermal sensitivity of coffee photosynthesis at temperatures above 20-25 °C (Wormer, 1965; Nunes et al., 1968; Kumar and Tieszen, 1976, 1980), estimating negligible photosynthesis close to 30 °C. However, such strong thermal sensitivity of photosynthesis is not usually observed under field conditions, what is consistent with frequently observed temperatures in suitable coffee regions (DaMatta and Ramalho, 2006). Indeed, there are reports of 40% reduction in photosynthesis when raising the temperature from 24 to 33 °C, however, this effect was restricted to the first six days as at 12 days of experiment, with the plants showed similar values to those obtained at 24 °C after at 12 days, although with increase of 50% in respiration rates (Frischknecht et al., 1982). Other studies have reported satisfactory values of both photosynthesis and stomatal conductance for leaf temperature close to 34 °C (Carelli et al., 1999; Carelli et al., 2006), that the temperature required to obtain the maximum photosynthesis rate reached 35 °C in *C. arabica* and *C. canephora* plants (DaMatta and Ramalho, 2006). Still, at high temperatures during flowering time may result in bud abortion or the development of infertile flowers, particularly when associated with prolonged dried periods (Camargo, 1985), what could have significant impact on yield.

2.5. Combined effect of CO_2 and supra-optimal temperature in energetic metabolism

As previously outlined, coffee responds positively to increased [CO_2] (Ramalho et al., 2013a), probably with a strong temperature dependence similar to other crops (Polley, 2002; Prasad et al., 2005). Thus, the largest positive effects of increased [CO_2] may occur at high temperatures (Morison and Lawlor, 1999; Long, 2001; Polley, 2002; DaMatta et al., 2010). For instance, it has been observed photosynthesis increases of *ca.* 60% in rice when the temperature increased from 32 to 38 ºC and 95% in soybeans with a rise from 28 to 40 ºC (Vu et al., 1997),

both grown at twice-ambient [CO_2]. These increases were independent of stomatal effect and changes may be related to the RuBisCO activation (Brandner-Crafts and Salvucci, 2000).

Another aspect crucial for plants acclimation to environmental stresses is related to strengthening of antioxidative system. The increase in [CO_2] may allow increased investments in such defense components, due to the greater assimilates availability, mitigating the effects of high temperatures (Morison and Lawlor, 1999; Martins et al., 2016). However, by increasing the energy use through photochemistry, the probability of occurrence of oxidative conditions will decrease (Martins et al., 2016). Nevertheless, depending on stress duration and intensity, as well on genotype, metabolic disorders and lipid peroxidation of cell membranes and chloroplast components may occur. Thus, it seems important that coffee acclimation response includes dynamic changes of membrane lipid matrix, which will enable to keep its function. As also referred, this can be achieved through modification of the fatty acids unsaturation level and the relative weight of the principal fatty acids and membrane lipid classes (Ramalho et al., 1998; Campos et al*.,* 2003; Partelli et al., 2011; Scotti-Campos et al., 2014). Such acclimation seems also include a dynamic mineral nutrients (Ramalho et al*.*, 2013b; Martins et al*.*, 2014a).

2.6. Whole-canopy gas exchanges

It is well documented that temperature and [CO_2] changes deeply effects of leaf gas exchanges, namely photosynthesis and stomatal. In general, so far, these impacts have been studied at leaf-scale, using one of many available traded equipments (Perez-Peña and Tarara, 2004). However, it can be very helpful to scale-up the photosynthesis measurements from single leaf to the whole-canopy level, as the latter integrates the output of leaves with different ages and degree of light exposure, as well as the contribution of other photosynthetic organs like fruit, shoots, and trunks (Long et al., 1996).

Perez Peña (2004) determined the whole-canopy gas exchange in vines plants under field conditions and found that photosynthetic rates values for whole plants were much lower compared to single leaf values. These differences are due to large differences in the amount of light received by leaves (inside and outside

the canopy, in the lower and upper part of the canopy), in the vapor pressure deficit inside the canopy and, in leaf age (Lambers et al., 2008). Thus, the use of specific techniques to measure the CO_2 flow from whole plants can increase the estimate accuracy of the real C-assimilation in the plants (Ferraz et al., 2016).

Plants with open architecture canopy, which enables inside canopy leaves to receive considerable irradiation, can show a closer relationship between whole-canopy and single leaf photosynthesis rates, as is the case for papaya plants (Ferraz et al., 2016). By opposition, coffee trees have a close canopy, with strong difference in light distribution inside and outside the canopy, therefore with quite relevant differences in leaf gas exchanges from different canopy positions (Araújo et al., 2008).

There are three types of systems for measuring gas exchanges at whole-canopy level, classified as closed, semi-closed and open system (Coombs et al., 1985; Bugbee, 1992; Mitchell, 1992). Since photosynthesis is [CO_2] dependent, closed chamber system is subjected to artifact because this method measures photosynthetic rates as a function of how long [CO_2] changes, which can deviates from the background concentration (Baldocchi and Amthor, 2001), consequently, it does not allow the measurements to be taken for a long time. Semi-closed systems can overcome such limitations, delivering regulated amounts of CO_2 to compensate for its withdraw as leaf photosynthesizes, or build up as it respires (Baldocchi and Amthor, 2001). In the open system, whole-canopy gas exchange is measured by entry *vs*. exit gas concentration differentials. This system allows to quickly determine small changes in gas exchange with precision (Baldocchi and Amthor, 2001; Ferraz et al., 2016). However, the measurement accuracy of the gas exchanges is directly related to the air flow entering the chamber, which must be constantly monitored (Baldocchi and Amthor, 2001).

A simple system with a single chamber to measure gas exchanges in coffee plants has been built by Marur and Faria (2007). However, a multi-chamber system extends the range of utility, allowing to simultaneously measure several levels of each environmental constraint, as drought (Perez-Peña and Tarara, 2004; Poni et al., 2014, Glenn, 2009; Baker et al., 2014) and the effect of the light distribution on canopy architecture (Glenn et al., 2003; Petrie et al., 2009).

3. CHAPTER 1

STOMATAL AND PHOTOCHEMICAL LIMITATIONS OF PHOTOSYNTHESIS IN *Coffea* SP. PLANTS SUBMITTED TO ELEVATED TEMPERATURES

INTRODUCTION

Global climate changes are a concern, especially as regards agricultural activities which strongly rely on environmental conditions. Estimates of significant air temperature increase suggests that heat will became one of the most detrimental stress to many crops. Estimates for global temperature rise might reach extreme increases between 3.7 and 4.8 °C by 2100, accompanied by an increase in atmospheric [CO_2], that will exceed 700 µL L^{-1} along the 2nd half of the present century (Collins et al., 2013; IPCC 2014). Since the optimal annual mean temperature for coffee plant development is in the range of 18-23 ºC for *C. arabica* and 22-26 °C for *C. canephora* (DaMatta and Ramalho, 2006), it has been argued that coffee, particularly *C. arabica*, will be highly sensitive to climate changes so that global warming could be a threat for coffee supply in the near future (Bunn et al., 2015; Magrach and Ghazoul, 2015). However, recent findings showed a surprisingly ability to coffee plant to endure quite high temperature, well above what was usually reported (for old genotypes), and that the increase of [CO_2] can

have a strong mitigating effect against high temperature in coffee species at the metabolic, mineral and defense mechanisms levels (Martins et al., 2014a; 2016), due to a strengthening of plant vigor (Ramalho et al., 2013a; Ghini et al., 2015). Still, it is considered that coffee yields are already been affect nowadays by climate changes, due to observed rising temperatures, often associated to lowered water availability (Bunn et al., 2015; Craparo et al., 2015), turning supra-optimal temperatures as a decisive environmental variable for the future of this crop.

High air temperatures may cause significant disturbances in plant metabolism and growth, since chemical reactions are accelerated and chemical bonds are weakened (Mano, 2000). Additionally, the lipid matrix membrane becomes more fluid and proteins denaturation and aggregation may occur in heat-stressed plants, as well as the overproduction of reactive oxygen species (ROS) and inhibition of the transcription and translation processes (Falcone et al., 2004; Dias et al.,2010). At photosynthesis level, supra-optimal temperatures change the use of captured solar energy, the gas diffusion through mesophyll (Lambers et al., 2008) and disturb the Calvin cycle activity (Pastenes and Horton, 1996), namely by reducing ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity, which is a key enzyme of the photosynthetic pathway (Brandner-Crafts and Salvucci, 2000). Thylakoid membranes are also sensitive to high temperatures, constituting one of the first indicators of heat sensitivity, due to electron transport uncoupling with the ATP synthesis and damage to both PSII and chloroplast ultra-structure(Larcher, 1995; Mano, 2002). In addition, there is higher C assimilated losses in C3 plants since both respiration and photorespiration rates increase more than photosynthesis does (Long, 1991), so that even moderately high temperatures may affect crop productivity (DaMatta and Ramalho, 2006).

High temperatures may also lead to increased evaporative demand, which may cause stomatal closure and reduction of CO_2 supply to the chloroplast, decreasing net photosynthesis (Prasad et al., 2005). Additionally, transpiration rates decline, thereby reducing latent heat loss and increasing leaf temperatures (Ainsworth and Rogers, 2007; DaMatta et al., 2010). The limitation of energy use through photochemistry can also promote an over energy status on the photosynthetic apparatus, with the increase of reactive oxygen species (ROS) production. These highly reactive molecules may cause in turn injuries in lipid membrane, contributing to increase electrolyte leakage through lipid membranes

(Wahid et al., 2007; Dias et al., 2010). Therefore, the triggering of antioxidant mechanisms, among them photoprotective pigments (*e.g*., zeaxanthin, lutein, carotenes), is of utmost importance to plant acclimation to environmental stresses and prevent lipid peroxidation, as it also the case of the coffee plant, namely when exposed cold and high irradiance (Ramalho et al., 1998; Batista-Santos et al., 2011; Martins et al., 2014b; Scotti-Campos et al., 2014).

Anatomic traits modifications are also triggered to help plants to cope with heat stress. Both increased and decreased specific leaf area have been reported in plants grown at elevated temperature (McDonald and Paulsen 1997; Xu and Zhou, 2006; Garrunna-Hernández et al., 2014; Wang et al., 2016), reflecting distinct mechanisms adopted by different groups of plants when grown at high temperatures.

Taking into account that temperature is one of the most limiting environmental conditions to the coffee crop (DaMatta and Ramalho, 2006), this work aims at study the impact and responses of this plant to seasonal changing temperatures, addressing two key issues: 1) Supra-optimal temperatures reduces photosynthesis through stomatal limitations in coffee genotypes? 2) Could summer supra-optimal temperatures cause sensitive impact at photochemical level, further contributing to reduce photosynthesis? To reach these goals, genotypes from the two main producing *Coffea* species extensively cropped in Brazil were used and several parameters were monitored, among them gas exchanges, chlorophyll *a* fluorescence, C-isotope composition, stomatal, and specific leaf area determinations, photosynthetic pigments and membrane permeability

MATERIAL E METHODS

Plant material and climatic variables

Plants of 1 to 2 year old from *C. arabica* cv. Catuaí Amarelo 65 (Catuaí 65) and *C. Canephora* cv. Emcapa 8111 Clone 02 (Clone 02) were grown in 100 L pots inside a greenhouse with natural fluctuations of light, temperature and relative humidity conditions. Substrate was composed of soil (dystrophic Yellow Latosol) and sand (1:1, v/v) and both fertilization and liming were carried out according to

soil analysis. Yet, 5 L of bovine manure were added each pot. Six plants from each species were assessed during two experimental periods, related to the existence of adequate temperature (September of 2014) and high temperatures (March of 2015) conditions. Plants were watered as required so that the soil moisture was maintained close to field capacity. Soil temperature and water tension were recorded using a soil sensor (model RT-1 Decagon Devices, Pullman WA, USA and Watermark 200SS, Irrometer Co., Riverside, CA, USA). Photosynthetically active radiation (PAR) was monitored using a quantum light sensor (model LightScout, Spectrum Technologies, Plainfield, Illinois, USA) and recorded (data logger Watch Dog Model 200 Spectrum Technologies, Plainfield, Illinois, USA).

Gas exchanges measurements

The net photosynthetic (A), stomatal conductance (g_s) and transpiration (Tr) rates were measured using a portable open-system infrared gas analyser (LI-6400, Li-COR, Lincoln, Nebraska, USA). The measurements were performed in the morning between 08:00 and 09:00 a.m. on full sunny days on the 2nd pair of recently mature leaves from branches from the upper 3rd part of the plant (fully sun exposed). Leaf instantaneous water-use efficiency (iWUE) was calculated as the A-to-Tr ratio, representing the units of assimilated CO_2 per unit of water lost through transpiration.

The photosynthetic capacity, A_{max} (representing the light- and CO_2-saturated rate of photosynthesis under optimal temperature), was measured through O_2 evolution in a Clark type O_2 electrode (LD2/2, Hansatech, UK) using leaf discs(10 cm^{2}each)from the 2rd pair of recently mature leaves. A_{max} was obtained at 35 ºC under saturating [CO_2] conditions (*ca*. 1%, supplied by 500 µL 1 M $NaHCO_3$) by exposing the leaf samples to increasing irradiances up to 1720µmol m^{-2} s^{-1} using a Björkman lamp (Hansatech) and neutral filters.

Chlorophyll a fluorescence measurement

Chlorophyll (Chl) *a* fluorescence was evaluated on the same non-detached leaves used for the gas exchange measurements, and on the same day, using a Pocket PEA fluorometer (Hansatech, King's Lynn, UK). For this analysis,

part of the sampled leaf was dark-adapted for *ca*. 30-40 min. using leaf clips (Hansatech) to turn the reaction centres into an "open" (oxidised Q_A) state (Bolhár-Nordenkampf et al., 1989). Thereafter, the leaf samples were exposed to saturating irradiance (3,500µmol m^{-2} s^{-1}) to obtain the Chl *a* PSII fast fluorescence kinetic transients. Measurements of maximal photochemical efficiency of PSII (F_v/F_m) and performance index (PI), which represents the energy cascade processes from the first absorption events until the reduction of plastoquinona (Strasser et al., 2004), were obtained. PI was calculated according to the equation:

$$PI = \frac{1-(F_0/F_m)}{M_0/V_j} \times \frac{F_m-F_0}{F_0} \times \frac{1-V_j}{V_j}$$

where: F_0 means fluorescence intensity at 50 µs, F_m represents maximal fluorescence intensity, V_J is relative variable fluorescence at 2 ms calculated as $V_J = (F_J - F_0)/(F_m - F_0)$, at which F_J is fluorescence intensity at the J step (at 2 ms), M_0 represents initial slope of fluorescence kinetics, which can be derived from the equation: $M_0 = 4^* (F_{300\,\mu s} - F_0)/(F_m - F_0)$.

C-isotope composition

Stable carbon ($\delta^{13}C$) isotope analysis was performed on oven-dried section samples of recently formed plagiotropic branches from the upper 3rd part of the plant (fully sun exposed), where the leaves used for gas exchanges measurements were inserted. Such samples were ground, weighted in tin capsules and folded. The carbon ($^{13}C/^{12}C$) isotope ratio of the samples were determined using an Isoprime (Micromass, UK) isotope ratio mass spectrometer coupled to an elemental analyzer (EuroVector, Italy) for online sample preparation by Dumas-combustion, according to Rodrigues et al. (2011). The isotopic C-ratio was calculated using the following standard δ notation:

$$\delta C = \left(\left(\frac{Rsample}{Rreference} \right) - 1 \right) * 1000(‰),$$

where $R = {}^{13}C/{}^{12}C$ for carbon. The isotope ratios were calibrated against the international standards IAEA CH6 and IAEA CH7. $\delta^{13}C$ results were referenced

against PeeDee Belemnite (PDB). Precision (the standard deviation of the set of standards analyzed in each batch) was 0.06‰.

Stomatal determinations

Leaf imprints from the abaxial leaf surface were taken and observed under a light microscope using two samples (10 cm^2 each) per replicate and two fields of view within each sampled area. Stomatal density SD was determined as previously described (Ramalho et al., 2013a).

Photosynthetic pigments

Photosynthetic pigments were analyzed as described by Torres Netto et al. (2005). Briefly, six leaf discs (28.26 mm^2each) was cut in fine strips and placed in a test tube containing 5 mL dimethyl sulfoxide (DMSO). The test tubes were then incubated at 70 ºC for 30 min. After cooling the extract in the dark, a 3 mL aliquot was analyzed spectrophotometrically at 480, 649 and 665 nm (Beckman DU640, Variant Inc., Walnut Creek, CA). The chlorophylls (Chls) *a* and *b* (Chl *b*), as well as total carotenoid (Car) contents were calculated according to the formulae of Wellburn (1994).

Membrane permeability

Ten freshly cut leaf discs (28.26 mm^2 each) were rinsed 3 times (1 min) with demineralized water and subsequently floated on 10 mL of demineralized water at 20ºC, following Dias et al. (2010). The electrolyte leakage was measured until leakage stabilization occurred, using a conductimeter (HANNA Instruments, HI 8819, UK). Total conductivity was obtained after exposing the flasks to 90 ºC, for 2 h in an oven followed by cooling. Membrane leakage was calculated as a percentage of total conductivity.

Statistical analysis

A completely randomized design with six replicates in a factorial scheme of 2 × 2 with two seasons and two genotypes was used. The various measured and calculated parameters were analyzed through a two-way ANOVA ($P \leq 0.05$),

to evaluate the differences between the seasons or genotypes, followed by a Tukey test for mean comparisons among the seasons for each genotype or between genotypes for each season. A 95% confidence level was used for all tests. Pearson's correlation coefficients among several variables were also estimated.

RESULTS

Climatic variables

Air temperature (T) and relative humidity (RH) were recorded using a data logger (model 200 Spectrum Technologies, Plainfield, Illinois, USA). The highest average T were obtained during summer with minimum and maximum values of 24.6 and 38.2 ºC (average of 30 consecutive days), respectively, while during the spring the minimum and maximum T averages were 19.5 and 32.4 ºC, respectively. Average daily temperature was 25.7 and 31.35 ºC during spring and summer, respectively (Figure 1A).The lowest RH values were observed near midday (*i.e*., close to 38%) in both seasons (Figure 1 B). The highest air vapor pressure deficit (VPD) values, calculated according to Jones (1992), were observed during summer (4.5 kPa) near midday (Figure 1 C), whereas during spring maximum value reached 2.7 kPa. Average daily VPD values were 1.5 and 2.4 kPa during spring and summer, respectively (Figure 1A).

The highest PAR values obtained were 1488 and 1314 µmol m^{-2} s^{-1}during summer and spring, respectively, whereas the average PAR values were 943 and 1084 µmol m^{-2} s^{-1}, on the same order (Figure 1D).

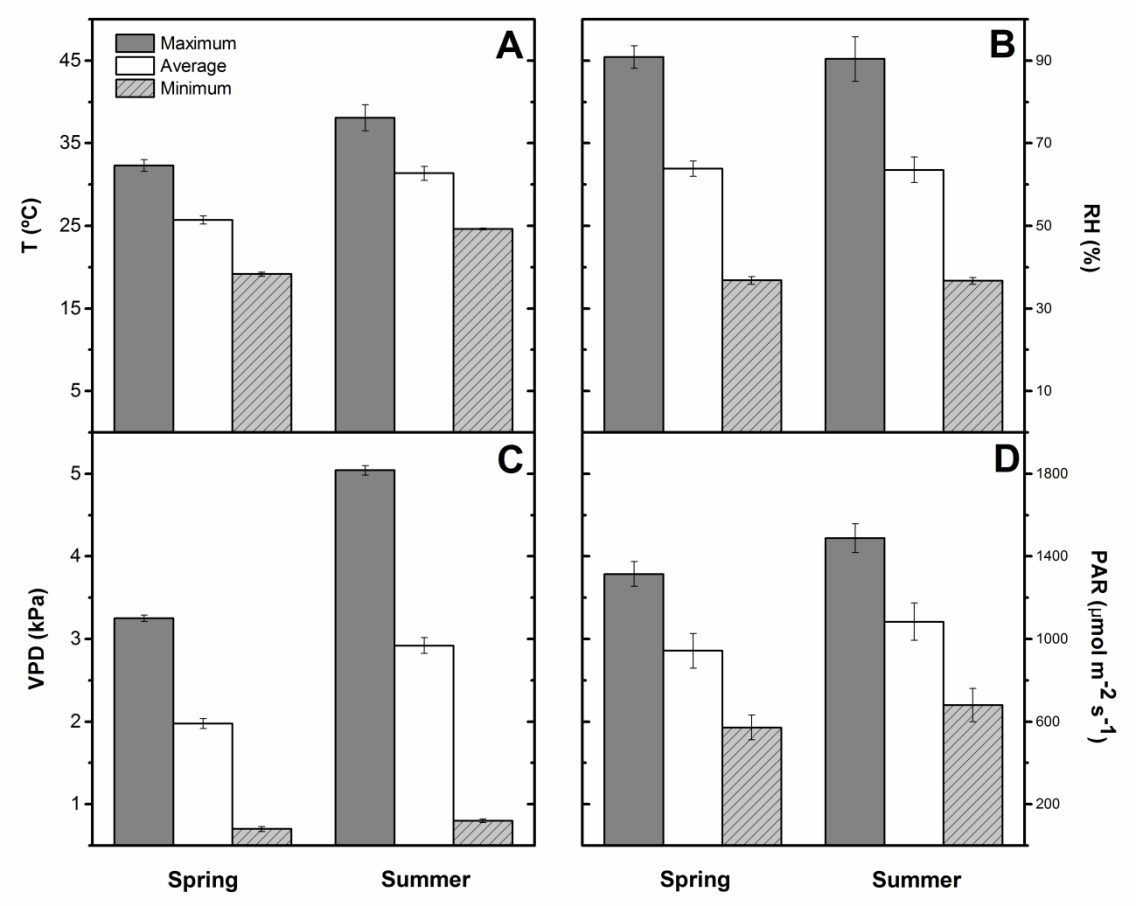

Figure 1 - Seasonal trends of air temperature –T (A), relative humidity – RH (B), air vapor pressure deficit – VPD (C) and photosynthetically active radiation- PAR (D). Each value represents the mean ± SE (n = 30), during spring (September 2014) and during summer (March 2015).

Photosynthetic rates and stomatal conductance

Catuaí 65 had higher A values in spring when compared to Clone 02, reaching C-assimilation values of nearly 7 μmol$CO_2$$m^{-2}$ s^{-1}(Figure 2A). Catuaí 65 showed decreases of *ca.* 28%in C-assimilation from spring to summer, whereas Clone 02 showed similar values in both seasons. gs values followed a similar pattern to that of A (Figure 2B). Catuaí 65 presented the highest g_s value in spring, and a marked reduction of *ca.* 65% in summer, whereas Clone 02 showed similar values in both seasons. As regards the photosynthetic apparatus potential (A_{max}), no significant differences were observed between genotypes in both seasons or between seasons for each genotype, with values ranging from 18.3 to 20.5 μmol O_2 m^{-2} s^{-1} (Figure 2C).

iWUE and C-isotope composition

No significant differences were found for iWUE values between genotypes in spring, whereas Catuaí 65 showed greater iWUE values than Clone 02 in summer (Table 1). Catuaí had higher iWUE in summer compared their values in spring, while Clone 02 showed no difference between seasons. Catuaí 65 had more negative $\delta^{13}C$ values than Clone 02 during spring, whereas the opposite situation was observed in summer (Table1). Regarding the season, Catuaí 65 had more negative $\delta^{13}C$ values during spring, while Clone 02 showed similar values in both seasons similarly to iWUE behavior (Table 1).

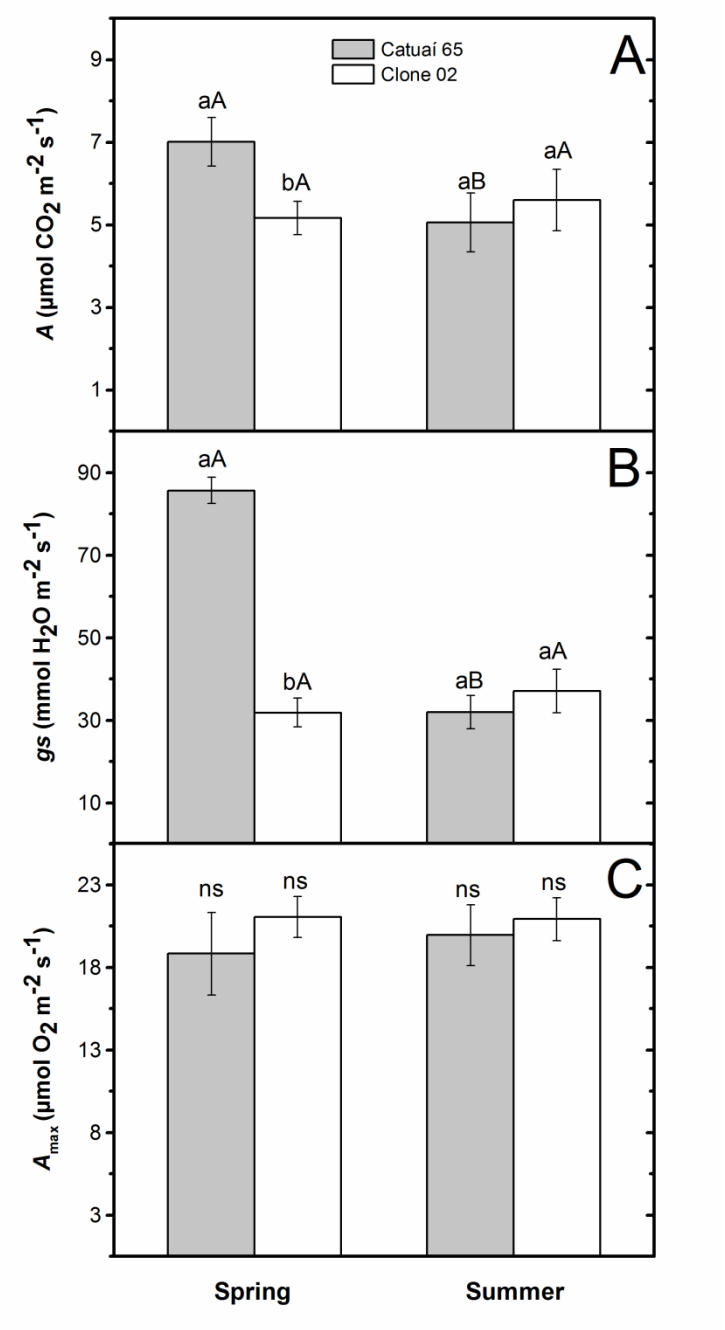

Figure 2 - Variation in the leaf gas exchange parameters, net photosynthesis rate (A), stomatal conductance (g_s) and photosynthetic capacity (A_{max}) in *Coffea canephora* cv. Conilon (Clone 02) and *Coffea arabica* (Catuaí 65) plants, during spring (September 2014) and during summer (March 2015).Each value represents the mean ± SE (n = 6); different letters indicate significant differences between seasons for the same genotype (A, B) or between genotypes for the same season (a, b), for a Tukey test at 5% probability. ns, non-significant.

Table 1 -Variation in the instantaneous water-use efficiency – iWUE (μmol CO_2/ mmol H_2O) and carbon isotope composition - $\delta^{13}C$ (‰) in *Coffea canephora* cv. Conilon (Clone 02) and *Coffea arabica* (Catuaí 65) plants, during spring (September 2014) and during summer (March 2015).

	Spring (September 2014)		Summer (March 2015)	
	Catuaí 65	Clone 02	Catuaí 65	Clone 02
iWUE	3.63 ± 0.1 aB	4.05 ± 0.7 aA	5.80 ± 0.7 aA	3.96 ± 0.5 bA
$\delta^{13}C$	-26.33 ± 0.3 bB	-25.73 ± 0.2 aA	-25.29 ± 0.3 aA	-26.12 ± 0.1 bA

Each value represents the mean ± SE (n = 6); different letters indicate significant differences between seasons for the same genotype (A, B) or between genotypes for the same season (a, b), for a Tukey test at 5% probability

Photosynthetic pigments, chlorophyll a fluorescence parameters and membrane permeability

Catuaí 65 showed higher Chl *a* content values than Clone 02, significantly only in spring (Table 2). Both genotypes did not show significant differences between seasons for Chl *a*, although with a tendency to lower values in summer (Table 2). Regarding the Chl *b*, although following similar trends than Chl *a*, no significant differences between genotypes for the same season and between seasons within the same genotype. Consequently, Total Chl values (Chl *a* + *b*) followed the same pattern of both Chl *a* and *b*, with higher values for Catuaí 65 than Clone 02 (significantly in spring), and with a tendency to higher values in spring than in summer within each genotype, but without any significant impact on the Chl (*a*/*b*). As regards carotenoid content, although no significant differences were observed between seasons and genotypes, Total Car values followed a consistent similar pattern than Total Chl but without significant changes. The Total Chl/Total Car ratio values were higher in Catuaí 65 than Clone 02 during spring, without significant differences.

Table 2 - Changes in the content of chlorophylls (Chl *a*; Chl b and Total Chl), total carotenoids (Total Car), ratios Chl (*a*/*b)* and Total Chl/Total Car ratios, maximum quantum yield of PSII (F_v/F_m), and photosynthetic index (PI) in *Coffea canephora* cv. Conilon (Clone 02) and *Coffea arabica* (Catuaí 65) plants, during spring (September 2014) and during summer (March 2015) seasons.

	Spring (September 2014)		Summer (March 2015)	
	Catuaí 65	Clone 02	Catuaí 65	Clone 02
	Pigments			
Chl *a* (µmol g^{-1})	1.97 ± 0.27 aA	1.27 ± 0.17 bA	1.47 ± 0.12 aA	1.11 ± 0.15 aA
Chl *b*(µmol g^{-1})	0.57 ± 0.09 aA	0.40 ± 0.06 aA	0.48 ± 0.04 aA	0.33 ± 0.05 aA
Total Chl (µmol g^{-1})	2.54 ± 0.36 aA	1.67 ± 0.22 bA	1.95 ± 0.16 aA	1.45 ± 0.19 aA
Total Car (µmol g^{-1})	0.26 ± 0.03 aA	0.20 ± 0.02 aA	0.22 ± 0.01 aA	0.18 ± 0.08 aA
Chl(*a*/*b*)	3.61 ± 0.30 aA	3.30 ± 0.24 aA	3.06 ± 0.12 aA	3.36 ± 0.08 aA
Total Chl/TotalCar	9.80 ± 0.47 aA	8.30 ± 045 bA	8.94 ± 0.35 aA	7.75 ± 0.39 aA
	Chlorophyll *a* fluorescence			
F_v/F_m	0.78 ± 0.01 aA	079 ± 0.01 aA	0.77 ± 0.01 bA	0.80 ± 0.01 aA
PI	5.01 ± 0.34 aA	4.28 ± 0.98 aA	4.50 ± 0.43 aA	4.22 ± 0.78 aA

Each value represents the mean ± SE (n = 6); different letters indicate significant differences between seasons for the same genotype (A, B) or between genotypes for the same season (a, b), for a Tukey test at 5% probability

.

Concerning the studied fluorescence parameters, Clone 02 had higher F_v/F_m values than Catuaí 65 during summer, whereas the genotypes showed similar F_v/F_m values during spring and no significant differences between seasons within each genotype (Table 2). PI values did not show any significant variation, ranging from 4.22 to 5.01.

Membrane selectivity through ion leakage evaluation showed to be quite stable between seasons for both genotypes, without relevant differences between genotypes in each season (Figure 3).

Stomatal traits

As regards stomata density (SD), Clone 02 showed higher values than Catuaí 65 regardless the season (Figure 4). Furthermore, opposite patterns were observed from spring to summer, since Catuaí65 presented a 22% reduction, in contrast to the 20% increase in Clone 02(Figure 4).

Pearson's Correlation

Positive correlations were observed between A and g_s and Total Car and Total Chl/Total Car total ratio. Positive correlations were also observed between Total Chl and Total Car, and Total Chl/Total Car ratio. However, no correlation among the other variables was observed (Table 3).

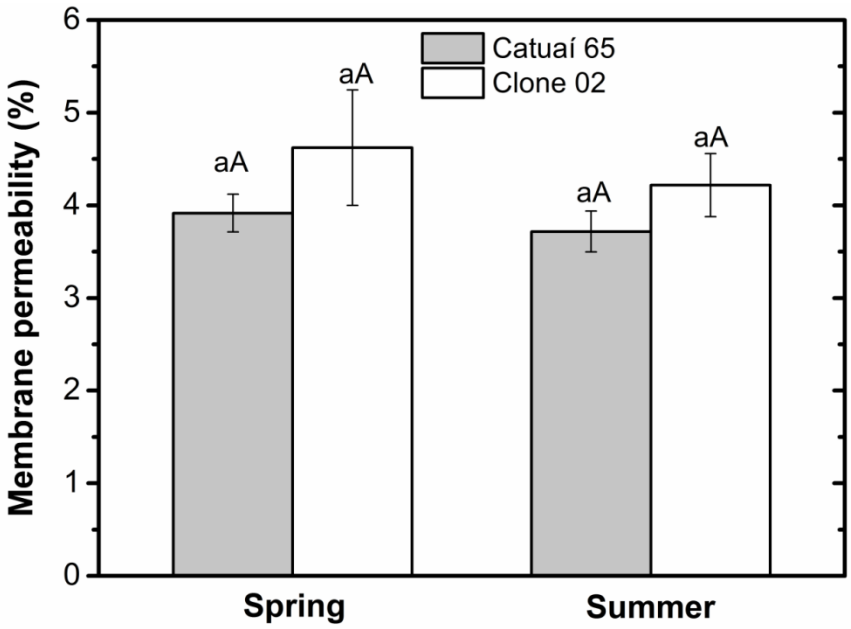

Figure 3 - Evaluation of membrane permeability in *Coffea canephora* cv. Conilon (Clone 02) and *Coffea arabica* (Catuaí 65) plants, during spring (September 2014) and during summer (March 2015).Each value represents the mean ± SE (n = 6); different letters indicate significant differences between seasons for the same genotype (A, B) or between genotypes for the same season (a, b), for a Tukey test at 5% probability.

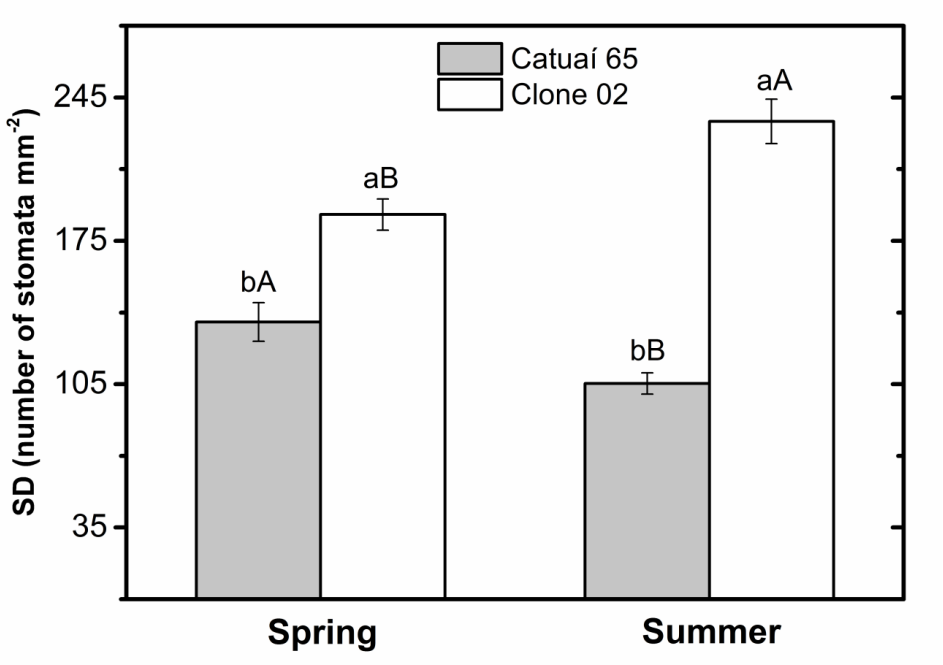

Figure 4- Variation in stomatal density (SD) in *Coffea canephora* cv. Conilon (Clone 02) and *Coffea arabica* (Catuaí 65) plants, during spring (September 2014) and during summer (March 2015). Each value represents the mean ± SE (n = 6); different letters indicate significant differences between seasons for the same genotype (A, B) or between genotypes for the same season (a, b), for a Tukey test at 5% probability.

Table 3 – Pearson's correlation between photosynthetic rates (A), stomatal conductance (g_s), maximum quantum yield (F_v/F_m), total chlorophylls (Total Chl) and carotenoids (Total Car), as well as the ratios (Chl (*a*/*b*) and Total Chl/Total Car), and stomatal density (SD) in *C.canephora* cv. Conilon (Clone 02) and *C.arabica* (Catuaí 65) plants, during spring (September 2014) and during summer (March 2015).

	A	g_s	F_v/F_m	PI	Total Chl	Total Car	Chl (*a*/*b*)	Total Chl/Total Car	SD
A	-	0.81**	-0.09	0.02	0.31	0.27	0.06	0.28	0.29
g_s	-	-	-0.02	0.11	0.37	0.36	0.16	0.29	0.34
F_v/F_m	-	-	-	0.39	-0.3	-0.3	-0.1	-0.04	0.05
PI	-	-	-	-	0.06	0.06	0.19	0.08	0.00
Total Chl	-	-	-	-	-	0.96**	-0.1	0.83**	-0.19
Total Carot	-	-	-	-	-	-	-0.08	0.70**	-0.12
Chl(*a*/*b*)	-	-	-	-	-	-	-	-0.17	0.08
Total Chl/Total Car	-	-	-	-	-	-	-	-	-0.34
SD	-	-	-	-	-	-	-	-	-

**Significant at 1% probability.

DISCUSSION

Several studies involving elevated temperature have mainly been performed in controlled environments, since it is possible to independently control each climatic variable. However, under natural fluctuations, the rising temperature during summer can results in decreased relative humidity and, in turn, increased VPD values, which may reduce stomatal conductance. Consequently, impairments can occur at the photochemical level caused by the excessive light energy, resulting in stomatal and photochemistry limitations to photosynthesis. This work was based on natural fluctuations of climatic variables. In this context, it was observed that temperature increased markedly during summer compared to spring, while both RH e PAR changed considerably less (Figure 1). Consequently, increased VPD values were mainly linked to increased temperature during summer. Also, irradiance level was high enough to saturate the photosynthetic apparatus at both leaf and whole-plant scale (DaMatta, 2004; Rodrigues et al., 2016b, which constitutes part of this thesis). Additionally, average water tension values were -15.4 ± 0.1 and-8.7± 0.1 kPa, during spring and summer, respectively, while mean soil temperatures were 25.1 ± 0.1 ºC and 27.7± 0.2 ºC, on the same order, which are within the range considered as not limiting to gas exchanges (Ziska, 1998; Thompson et al., 2007). Taking these into account the effects observed on leaf gas exchange will be driven mostly by supra-optimal temperature.

Stomatal limitations to leaf gas exchanges

Photosynthesis was affected by declines in g_s during summer in Catuaí 65 plants, and not to an impact at mesophyll level, since A_{max}, F_v/F_m, and membrane permeability values were not affected. Elevated temperature increases the air evaporative demand, often resulting in stomatal closure and, thereafter, reducing photosynthesis due a lower CO_2 influx to the leaves (DaMatta et al., 2010). Such stomatal closure can be linked to an inability of plant vascular system to quickly restore the water status, due to a low leaf hydraulic conductivity, even in plants that are well supplied with soil water, as it was found in coffee plants compared to

others tropical plants (Martins et al., 2014c; Nardini, 2014). Therefore, it is likely that such summer higher air temperature imposed a high air vapor pressure deficit on the leaves. This was followed by a strong g_s reduction, which in the long term lead to a higher water use efficiency reflected in the $\delta^{13}C$ (and iWUE) increase only in Catuaí 65 plants. In fact, less negative $\delta^{13}C$ value in leaf tissue is associated with higher WUE and linked to prolonged stomatal closure periods, what restrict both CO_2 availability at carboxylation sites and H_2O consumption (Drake et al., 1997), but suggesting that g_s was proportionally more reduced than A. That was not the case of Clone 02 plants, which were quite insensitive regarding g_s and A, what is accordance with the absence of significant changes of iWUE and $\delta^{13}C$ from spring to summer.

Stomata controls leaf gas exchanges by a fine balance between the CO_2 required for photosynthesis and the level of water availability, so that stomatal size and density determine the maximum leaf diffusive conductance to CO_2. In fact, higher SD of small stomata will increase g_s for the same total pore area, due to shorter diffusion path length (Franks and Beerling, 2009). Interestingly, summer conditions promoted changes in stomatal traits in a species-dependent manner, as recently noted for *C. arabica* and *C. canephora* plants under high temperature (Chapter 2). In our work, SD reduction in Catuaí 65 in summer is in line to the observed g_s reduction. On the other hand, Clone 02 showed an increased SD in summer (Fig. 4), as also observed earlier for other *C. canephora* genotype (Chapter 2). However, since g_s was maintained unchanged (Fig. 2B), that suggested that stomatal opening control overcomes the effects of SD on total g_s. In fact, plants growing under moderate stress can show increased SD while maintaining g_s to that observed under control (Xu and Zhou, 2008).

Are there limitations linked to photochemistry pathway?

Increased temperature accelerates the kinetic energy and movement of molecules across membranes, so that chemical reactions are accelerated, the chemical bonds are weakened, the lipid matrix membrane becomes more fluid and proteins denaturation and aggregation might occur, as well as the overproduction of ROS (Wahid et al., 2007).Additionally, elevated temperatures have been

associated to decreases in both g_s and CO_2 uptake, resulting in non-efficiently light use (Lambers et al., 2008).

As stated above, in our study net C-assimilation seemed to be affected by stomata control only in Catuaí 65 (Clone 02 did not show impact on g_s and A). Furthermore, non-stomatal impacts on F_v/F_m, PI (Table 2), and membrane permeability (Fig. 3) were absent, and, most of all, the A_{max} values remained completely unchanged between seasons (Fig. 2). This allow us to discard the possibility of non-stomatal/mesophyll impacts at specific sites of the photosynthetic apparatus, considering the imposed temperature conditions and is in accordance with the resilience of the photosynthetic apparatus in coffee plants up to 37ºC (Chapter 2; Martins et al., 2016). Notably, such A reduction in Catuaí 65 (*ca.* 28%) during summer was similar to decline in canopy photosynthesis in the same season (Chapter 3).

The cell membrane thermo-stability was previously observed in coffee genotypes, and were related to the reinforcement of protective mechanisms (Martins et al., 2016), whereas PSII was found to be quite tolerant to heat stress, being affected only after harsh exposure to 42 ºC along the entire diurnal period. These impacts were higher in plants grown under normal air [CO_2], but showed a much lower extent when growing at elevated [CO_2] (Chapter 2).

Supra-optimal temperature can cause photosynthetic pigments degradation, what can be associated to the production of active oxygen species (Todorov et al., 2003; Guo et al., 2006), with photobleaching of pigments and declines in the Chl (a/b) ratio when PSI is particularly affected, namely under chilling (Batista-Santos et al., 2011) and high irradiance (Nunes et al., 1993) stresses in coffee plants. That was not the case since only non-significant changes were found on the photosynthetic pigments from spring to summer (Table 2). This suggests the absence of impact at PSI level, what agrees with previous assessment of its heat (but not cold) tolerance to temperatures as high as 37 ºC (Batista-Santos et al., 2011; Rodrigues et al., 2016c), and confirms the maintenance of membrane systems and the processes link to then, as reflected in stable F_v/F_m which is related to PSII activity in the thylakoid electron transport.

In conclusion, these results showed an absence of mesophyll limitation to the photosynthetic pathway in both genotypes, when compared spring to summer values. Furthermore, stomatal control was the main limiting process to A in Catuaí

65, whereas no significant impact was observed in Clone 02 as corroborated by the obtained Pearson’s correlation.

4. CHAPTER 2

LONG-TERM ELEVATED AIR [CO_2] STRENGTHENS PHOTOSYNTHETIC FUNCTIONING AND MITIGATES THE IMPACT OF SUPRA-OPTIMAL TEMPERATURES IN TROPICAL *Coffea arabica* AND *C. Canephora* SPECIES[1]

INTRODUCTION

The actual atmospheric [CO_2] is below the optimal concentration for photosynthesis in C3 crops. Therefore, net photosynthesis rates (A) are usually increased in response to [CO_2] enhancement (Zhu et al., 2012), often above 50%, with trees showing the largest rise (Ainsworth and Rogers, 2007). Such stimulation by CO_2 results from a higher RuBisCO carboxylation rate, which is both related to higher substrate (CO_2) availability and to the competitive inhibition of CO_2 over O_2 at the carboxylation sites of RuBisCO, which reduces the photorespiration rate. This ultimately leads to decreases in CO_2 loss and energy costs associated with

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the photorespiration pathway (Long et al., 2004; Ainsworth and Rogers, 2007; Kirschbaum, 2011).

The beneficial effects of elevated [CO_2] on crop photosynthesis, growth and production can be strongly attenuated by other effects of climate changes, such as global warming and lower water availability. Supra-optimal temperatures can alter gas diffusion in the mesophyll (Lambers et al., 2008) and disrupt the activity of the Calvin cycle (Pastenes and Horton, 1996), namely by reducing RuBisCO activity (Crafts-Brandner and Salvucci, 2000). Furthermore, elevated temperatures stimulate respiration and photorespiration more than photosynthesis, due to decreases in the affinity of RuBisCO for CO_2 and in the solubility of CO_2, both relative to O_2, which reduces the relative rate of carboxylation to oxygenation and C assimilation (Crafts-Brandner and Salvucci, 2000; Ainsworth and Rogers, 2007). Higher temperatures also affect water relations and evaporative demand, and alter the fluidity and stability of membrane systems, hormone balance and, ultimately, primary and secondary metabolism (Wise et al., 2004; Wahid et al., 2007). By increasing the evaporative demand in the air, higher temperatures are often implicated in stomatal closure, which further decreases photosynthesis due to decreases in CO_2 flux to leaves (DaMatta et al., 2010). Collectively, these responses can have pronounced impacts on plant growth and crop yields (Prasad et al., 2003). Nonetheless, the reduction in photorespiration associated with high air [CO_2] in C3 plants is expected to stimulate A to a greater extent at high temperatures than at moderate ones, thereby offsetting (at least partially) the impact of supra-optimal temperatures on A and yield (DaMatta et al., 2010; Kirschbaum, 2011).

Under field conditions, environmental constraints (*e.g.*, water availability and extreme temperatures) are often superimposed. However, relatively few studies have devoted attention to how leaf physiology responds to a combination of high [CO_2] and warming. These two factors interact in ways that can either exacerbate or cancel their independent effects (Way et al., 2015), but some reports have highlighted the mitigation effects of enhanced CO_2 on the impact of temperature rise, by increasing tree growth and forest productivity (Boisvenue and Running, 2006).

The optimal annual mean temperatures for plant development are between 18 and 23 °C for C. arabica and between 22 and 26 °C for *C. canephora*

(DaMatta and Ramalho, 2006). It has been argued that coffee, particularly C. arabica, is highly sensitive to climate changes and that global warming will threaten the coffee supply in the near future (Davis et al., 2012; Bunn et al., 2015). Indeed, there is a general belief that the coffee production has already been affected by climate changes in several coffee-growing countries, especially by adverse events associated with severe drought spells in combination with high temperatures (Bunn et al., 2015; Van Der Vossen et al., 2015) or related to time series of temperature increase (Craparo et al., 2015). These problems are expected to be exacerbated given that the actual plantations, which have an average lifespan of *ca.* 30 years, will be in fact increasingly exposed to such new environmental conditions (Bunn et al., 2015). Furthermore, modeling studies have even foreseen dramatic effects on the coffee crop, including significant changes in agroclimatic zoning and the loss of suitable areas in the largest coffee-producing countries, Brazil and Vietnam (Assad et al., 2004; Zullo et al., 2011; Bunn et al., 2015); productivity reductions (Gay et al., 2006; Bunn et al., 2015; Craparo et al., 2015; Ovalle-Rivera et al., 2015); the extinction of wild populations of *C. arabica* (Davis et al., 2012); and increased agricultural, social and economic vulnerabilities (Baca et al., 2014). Nevertheless, these studies have been mostly based on rising temperature scenarios without considering (1) the recognized ability of the coffee plant to metabolically adjust to harsh environments (DaMatta and Ramalho, 2006; Ramalho et al., 2014) or (2) the possible mitigating effects of elevated [CO_2] on the adverse impacts of increased temperatures, as the interactive effects of increased air CO_2 with other environmental variables (as supra-optimal temperature) remain to be fully elucidated in coffee. Despite the agronomic importance of coffee and the relevance of potential impacts of the elevated [CO_2] per se, only very recently the direct effects of enhanced [CO_2] on the coffee physiology have been addressed (Ramalho et al., 2013a; Martins et al., 2014a; Ghini et al., 2015). These efforts have revealed significant improvements in photosynthetic performance without any apparent photosynthetic downregulation.

Here, we advance our previous studies by assessing the concomitant effects of elevated [CO_2] and supra-optimal temperatures on coffee physiology, providing the first insights into the physiological and biochemical mechanisms underlying the responses of the key photosynthetic pathway to predicted environmental conditions. To reach these goals, it were used three coffee

genotypes (from the two main producing Coffea species) extensively cropped in Brazil. Our results highlight the key role of elevated [CO_2] to mitigate the harmful effects of supra-optimal temperatures on the physiology of the coffee crop, providing valuable information to advance our comprehension of the crop performance in climate change scenarios.

MATERIAL AND METHODS

Plant material and experimental design

Coffea arabica L. (cv. IPR108 and Icatu) and *Coffea canephora* Pierre ex A. Froehner cv. Conilon Clone 153 (CL153) plants, *ca*. 1.5 years of age, were transferred into walk-in growth chambers (EHHF 10000, ARALAB, Portugal) and grown for 1 year in 28 L pots under controlled temperature (25/20 ºC, day/night), irradiance (*ca*. 700-800 µmol m^{-2} s^{-1}), RH (75%), photoperiod (12 h), and either 380 µL CO_2 L^{-1} air (380-plants) or 700 µL CO_2 L^{-1} air (700-plants). Thereafter, temperature was increased from 25/20 ºC up to 42/34 ºC at a rate of 0.5 ºC day^{-1}, with 7 days of stabilization at 31/25, 37/30 and 42/34 ºC to allow for programmed evaluations. Determinations were performed on newly matured leaves from the upper part of the plant. For biochemical analyses, leaf material was collected after *ca*. 2 h of illumination from 6 to 8 plants of each genotype, flash frozen in liquid nitrogen and stored at -80°C until analysis. Whenever possible, all analyses were performed on the same leaves. The plants were maintained without restrictions in water, nutrients, or root development.

Stomatal determinations

Leaf imprints from the abaxial leaf surface were taken and observed under a light microscope using five samples per replicate and three fields of view within each sampled area. Stomatal density (SD), stomatal index (SI) and stomatal size (SS) were determined exactly as previously described (Ramalho et al., 2013a).

Leaf biomass C-isotope composition

Stable carbon ($\delta^{13}C$) isotope analysis was performed on oven-dried leaf samples, which were weighted in tin capsules and folded. The carbon ($^{13}C/^{12}C$) isotope ratio of the samples were determined using an Isoprime (Micromass, UK) isotope ratio mass spectrometer coupled to an elemental analyzer (EuroVector, Italy) for online sample preparation by Dumas-combustion, according to Rodrigues et al. (2011). The isotopic C-ratio was calculated using the following standard δ notation:

$$\delta C = \left(\left(\frac{\text{Rsample}}{\text{Rreference}}\right) - 1\right) * 1000(‰),$$

where R = $^{13}C/^{12}C$ for carbon. The isotope ratios were calibrated against the international standards IAEA CH6 and IAEA CH7. $\delta^{13}C$ results were referenced against PeeDee Belemnite (PDB). Precision (the standard deviation of the set of standards analyzed in each batch) was 0.06‰.

Leaf gas exchanges

The leaf rates of net photosynthesis (A), stomatal conductance to water vapor (g_s) and transpiration (Tr) were obtained under photosynthetic steady-state conditions after *ca*. 2 h of illumination (in middle of the morning). A portable open-system infrared gas analyzer (Li-Cor 6400, LiCor, Lincoln, USA) was used, with external [CO_2] supply of 380 and 700 µL L^{-1}, and irradiance of *ca*. 700-800 µmol m^{-2} s^{-1}. Leaf instantaneous water-use efficiency (iWUE) was calculated as the A-to-Tr ratio, representing the units of assimilated CO_2 per unit of water lost through transpiration.

The photosynthetic capacity, A_{max} (representing the light- and CO_2-saturated rate of photosynthesis under optimal temperature), was measured in leaf discs (1.86 cm^2) via O_2 evolution using a Clark type O_2 electrode (LD2/2, Hansatech, UK). A_{max} was obtained at 25 °C, *ca*. 7% CO_2 (supplied by 400 µL of 2 M $KHCO_3$) and increasing irradiances 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 used for gas exchange measurements, using a PAM-2000 system (H. Walz, Effeltrich, Germany), following the formulae discussed elsewhere (Kramer et al., 2004; Krause and Jahns, 2004; Schreiber, 2004). Measurements of minimal fluorescence from excited antennae Chl *a* molecules before excitation energy migrates to the reaction centers (F_o), maximal fluorescence corresponding to the complete reduction of primary photosystem (PS) II acceptors (F_m), and maximal photochemical efficiency of PSII (F_v/F_m) were performed on overnight dark-adapted leaves. F_o was determined using a weak light (< 0.5 µmol m^{-2} s^{-1}); F_m was obtained using a 0.8 s saturating pulse of *ca.* 7500 µmol m^{-2} s^{-1} of actinic light. Another set of parameters was evaluated under photosynthetic steady-state conditions, under 700-800 µmol m^{-2} s^{-1} of actinic light and superimposed saturating flashes. This included q_P, q_L, q_N, $Y_{(II)}$ (=ϕ_e), $Y_{(NPQ)}$, $Y_{(NO)}$ and F_v'/F_m' (Kramer et al., 2004; Klughammer and Schreiber, 2008), F_s/F_m' (Stirbet and Govindjee, 2011) and the PSII photoinhibition indexes (Werner *et al*., 2002). F_o', needed for the quenchings determination was obtained in the dark immediately after actinic light was switched off and before the first fast phase of fluorescence relaxation kinetics. F_v'/F_m' represents the actual PSII efficiency of energy conversion under light exposure. q_P and q_L denote the proportion of energy trapped by PSII open centers and driven to photochemical events based on the concept of separated (q_P) or interconnected (q_L) PSII antennae. q_N is the non-photochemical quenching and represents the sustained, photoprotective thermal energy dissipation. Estimates of the quantum yields of photosynthetic non-cyclic electron transport ($Y_{(II)}$), of non-regulated energy (heat and fluorescence) dissipation of PSII ($Y_{(NO)}$), and of regulated energy dissipation of PSII ($Y_{(NPQ)}$), where [$Y_{II} + Y_{(NPQ)} + Y_{(NO)} = 1$], were also performed (Kramer et al., 2004; Huang et al., 2011). Finally, it were evaluated the predictor of the rate constant of PSII inactivation, F_s/F_m' (Stirbet and Govindjee, 2011), and the PSII photoinhibition indexes (Werner *et al*., 2002). The latter included the A) chronic photoinhibition (PI_{Chr}), representing the percent reduction in F_v/F_m at each temperature relative to the maximal F_v/F_m obtained during the entire experiment, B) dynamic photoinhibition (PI_{Dyn}), representing the decline in F_v/F_m that is fully reversible

overnight, that is measured as the percent reduction in midday F_v'/F_m' relative to F_v/F_m at each temperature, relative to the maximal F_v/F_m from the entire experiment, and C) total photoinhibition ($PI_{Total} = PI_{Chr} + PI_{Dyn}$).

Thylakoid electron transport rates

Sub chloroplast fractions were obtained from a leaf pool (*ca*. 5 g_{FW}) from 5-6 plants as described in 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 measured with an O_2 electrode (LW2, Hansatech) at 25 °C, under a PPFD of *ca*. 3000 µmol m^{-2} s^{-1} supplied by a Björkman lamp (Hansatech), and using 1 mL of the reaction mixture containing *ca*. 100 mg Chl.

Photosynthetic and respiratory enzyme activities

Four freshly cut leaf discs (0.5 cm^2 each) were used to measure the activity of several enzymes involved in C-metabolism. Optimized methods for coffee (Ramalho et al., 2013a) were used for all enzyme assays, which were based on NADH oxidation at 340 nm, at 25 °C, with 1 mL final volume in the cuvette. For the enzymes involved in the photosynthetic pathway, the total activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO: EC 4.1.1.39) was assayed following the method of Gerard and Driscoll (1996), and the activity of ribulose 5-phosphate kinase (RuB5PK: EC 2.7.1.19) was determined according to the method of Souza et al. (2005). The activities of pyruvate kinase (PK: EC 2.7.1.40) and NADH-dependent malate dehydrogenase (MDH: EC 1.1.1.37), which are enzymes involved in the respiratory pathway, were assessed following the methods of Diaz et al. (1996) and López-Millán et al. (2000), respectively.

Statistical analysis

The various measured and calculated parameters were analyzed using a two-way ANOVA to evaluate the differences between the two atmospheric CO_2 conditions, between the several temperatures, and their interaction, followed by a

Tukey's test for mean comparisons. Each ANOVA was performed independently for each of the studied genotypes. A 95% confidence level was adopted for all tests.

RESULTS

Stomatal traits

Under control conditions (25/20 ºC), plants from the three genotypes grown at elevated [CO_2] tended to present lower SD (*ca*. 5-14%) and greater SS (*ca*. 3-7%) values, although statistical significance was observed only in Icatu (Fig. 1). With high [CO_2], SI values increased modestly (5.5%) in CL153, but a 14% reduction was found in Icatu and IPR108.

In response to a gradual increase in temperature to 42/34 ºC (*ca*. 8 weeks), newly expanded leaves showed species-dependent and [CO_2]-independent changes in SD and SS, with no significant differences between the two [CO_2] growth conditions. On the other hand, elevated temperature induced similar trends in CL153 plants regardless of the [CO_2] condition, but significant increases in SD (27%) and SI (27%) and reduction in SS (13%) were found only under elevated [CO_2], when compared to their respective values at 25/20 ºC. In contrast, IPR108 showed lower SD (*ca*. 30%) and SI (between 24-27%) values, together with higher SS (6-9%) values, in both [CO_2] conditions. Under normal [CO_2], Icatu closely followed IPR108, with significant reductions in SD (26%) and SI (19%), whereas with elevated [CO_2], only SS was significantly altered (a decrease of 7.3%).

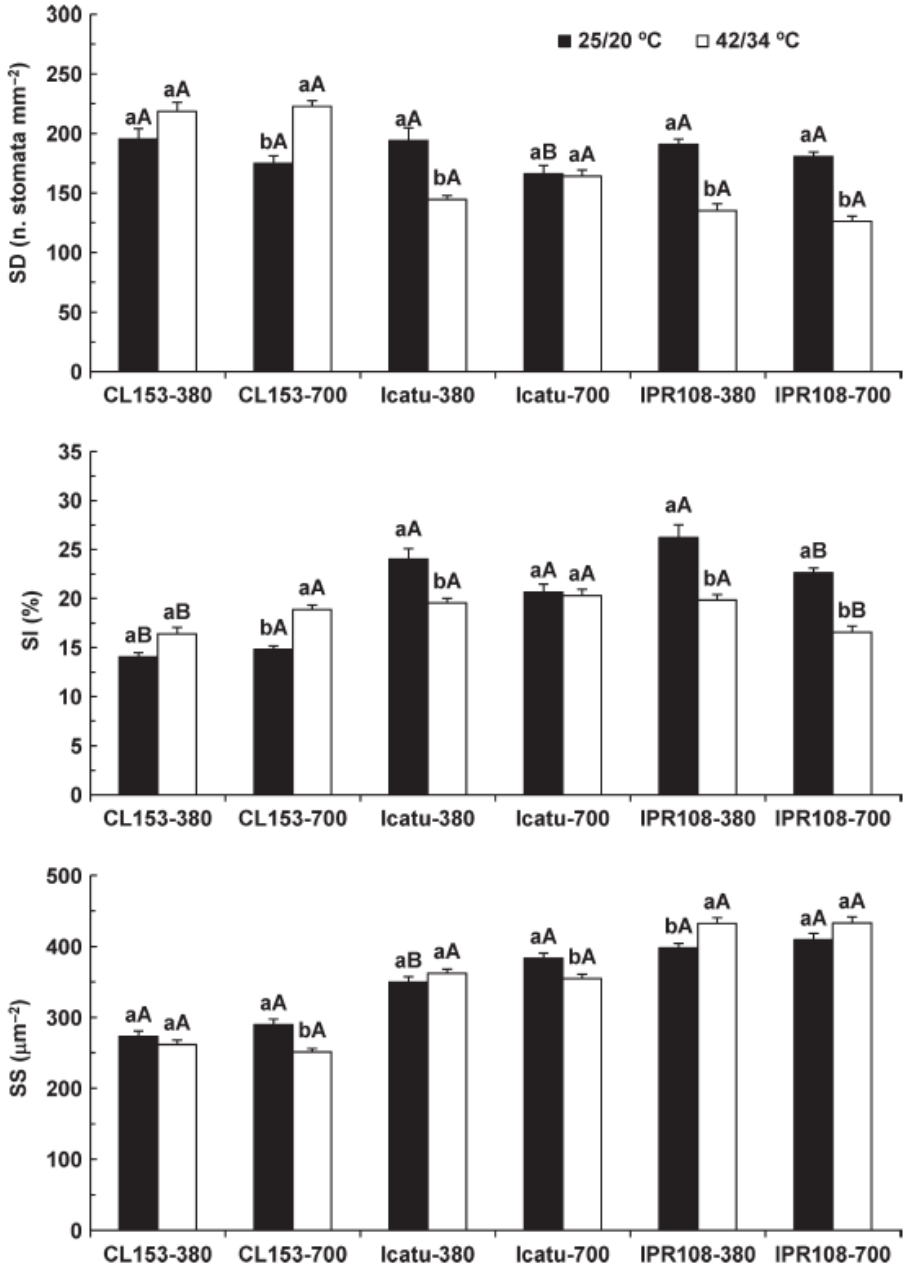

Figure. 1 – Variation in stomatal density (SD), size (SS) and index (SI) in the leaves of *C. canephora* cv. Conilon (CL153) and *C. arabica* (Icatu and IPR108) plants grown under 380 or 700 µL CO_2 L^{-1}, which were grown at 25/20 ºC (control temperature – black bar) or 42/34 ºC (maximum temperature – white bar). For each parameter, the mean values ± SE (n = 30) followed by different letters express significant differences between temperatures for the same CO_2 treatment, within each genotype (a, b), or between CO_2 treatments for each temperature, separately for each genotype (A, B).

Leaf gas exchanges and $\delta^{13}C$ composition

Under 25/20ºC no significant differences were observed in A and g_s between the two [CO_2] in the three genotypes, when measured with an external CO_2 supply of 380 and 700 µL L^{-1} (data not shown). Also at 25/20 ºC, the A values obtained at 700 µL L^{-1} in the 700-plants of CL153, Icatu and IPR108 (representing future estimated environmental conditions) were 48%, 61% and 74% greater, respectively, than those obtained with 380 µL L^{-1} in the 380-plants (representing present CO_2 conditions) (Figure 2).

Along the gradual temperature increase, significantly higher A values were always observed at enhanced [CO_2], irrespective of temperature and genotype. Up to 37/30 ºC, A values did not significantly decrease. In fact, A values were higher at 31/25 ºC (for all the genotypes grown at 700 µL L^{-1}) and/or 37/30 ºC (*e.g.*, CL153-380 plants). However, at 42/34 ºC, A was strongly reduced in all genotypes, especially in 380-plants, what increased the difference between the plants grown under the two [CO_2] conditions. In fact, the A values of 700-plants were 205% (CL153), 223% (Icatu), and 495% (IPR108) greater than those observed for 380-plants when measured at their respective [CO_2] conditions (Figure 2). Notably, at such extreme temperature of 42/34 ºC, 700-plants maintained relevant photosynthetic functioning, especially those from CL153 and IPR108 genotypes that showed A values close to those observed in 380-plants at control temperature.

Under enhanced temperatures, increases in g_s were observed mostly at 37/30 ºC (except in IPR108 380-plants) and 42/34 ºC (except in Icatu 380-plants). At 37/30 ºC maximal g_s increases were observed in 380-plants of CL153 (92%) and Icatu (56%), whereas at 42/34 ºC maximal values were found in 700-plants of CL153 (62%), Icatu (31%) and IPR108 (89%), together with IPR108 380-plants (92%). These temperature-related changes in g_s (and transpiration, data not shown) provoked strong decreases in iWUE, which were irrespective of genotype and [CO_2], although the 700-plants maintained higher iWUE values throughout the experiment, significantly only up to 31/25 ºC (Figure 2).

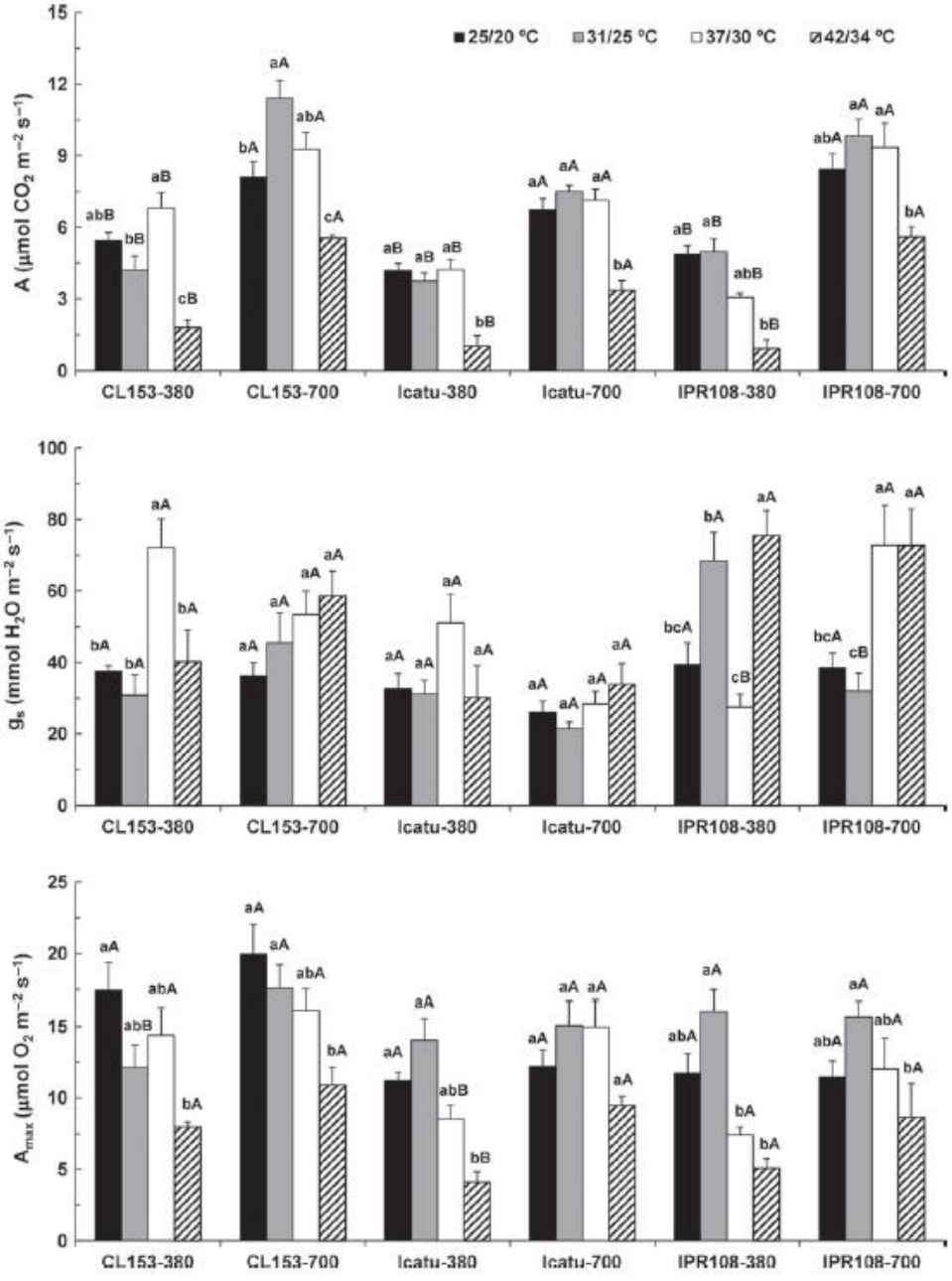

Figure 2 - Variation in the leaf gas exchange parameters, net photosynthesis rate (A), stomatal conductance to water vapor (gs) and photosynthetic capacity (Amax) in Coffea canephora cv. Conilon (CL153) and Coffea arabica (Icatu and IPR108) plants, which were grown under 380 or 700 μL CO_2 l^{-1} and submitted to control (25/20 °C, day/night) or supra-optimal temperatures of 31/25, 37/30 and 42/34 °C. For each parameter, the mean values ± SE (n = 5–8) followed by different letters express significant differences between temperature treatments for the same CO_2 level, separately for each genotype (a, b), or between CO_2 levels for each temperature treatment, separately for each genotype (A, B).

In most cases, A_{max} did not statistically differ between [CO_2] conditions. Nevertheless, a tendency for higher A_{max} values was observed in the 700-plants of CL153, Icatu (throughout the entire experiment) and IPR108 (in the two highest temperatures) (Figure 2). Concerning the effect of temperature, A_{max} did not significantly decrease in CL153 and Icatu plants up to 37/30 ºC. In other words, photosynthetic potential was clearly maintained at supra-optimal temperatures. In IPR108 significant heat sensitivity was observed at this temperature compared to 31/25 ºC only in 380-plants (Figure 2). At 42/34 ºC, all genotypes had reductions in A_{max}, but higher absolute values and lower impacts were observed in 700-plants (reductions between 22 and 45%) than in 380-plants (reductions between 54 and 63%). Consequently, the differences in A_{max} between 380- and 700-plants increased at high temperature. Under control temperature these differences were at most 14% (CL153), but at 42/34 ºC, A_{max} values in 700-plants were 37% (CL153), 130% (Icatu) and 70% (IPR108) greater than those in 380-plants, although significantly only for Icatu. Moreover, the saturating irradiance at which A_{max} was obtained was consistently higher in plants grown under elevated CO_2 (data not shown), suggesting that these plants might endure higher irradiance levels.

Under control conditions, $\delta^{13}C$ in 380-plants ranged between *ca*. -24.9 and 27.2‰, but the plants grown under high [CO_2] had much more negative $\delta^{13}C$ values, between *ca*. -35.5 and 38.2‰ (Table 1). These latter values are an "artifact" that can be largely attributable to the $\delta^{13}C$ differences in air CO_2 sources, since the 700-plants consumed a higher proportion of CO_2 supplied by a pure CO_2 pressurized bottle that was less enriched in $^{13}CO_2$ (-37.6‰ $\delta^{13}C$), whereas the 380-plants received mostly common air ($\delta^{13}C$ = -8‰) supplemented with the same CO_2 bottle (Table 1). Therefore, direct comparisons between the two [CO_2] conditions are meaningless. In any case, changes in $\delta^{13}C$ due to variations in thermal regimes within each [CO_2] condition can provide useful information. $\delta^{13}C$ values significantly and consistently decreased with increasing temperature, showing the lowest levels at 42/34 ºC, independent of genotype and growth [CO_2].

Table 1 - Variation in the instantaneous water-use efficiency (iWUE) and carbon isotope composition (d13C) in *Coffea canephora* cv. Conilon (CL153) and *Coffea arabica* (Icatu and IPR108) plants, which were grown under 380 or 700 µL CO_2 l^{-1} and submitted to control (25/20 °C, day/night) or supra-optimal temperatures of 31/25, 37/30 and 42/34 °C. For each parameter, the mean values ± SE (n = 5–8) followed by different letters express significant differences between temperature treatments for the same CO_2 level, separately for each genotype (a, b, c, d), or between CO_2 levels for each temperature treatment, separately for each genotype (A, B)

			Temperature (day/night)			
Parameter	Genotype	[CO_2] (µL l^{-1})	25/20 ºC	31/25 ºC	37/30 ºC	42/37 ºC
iWUE (mmol CO_2/mol H_2O)	CL 153	380	16.23 ± 2.59 aA	6.60 ± 1.62 bA	3.88 ± 0.18 bA	1.67 ± 0.32 bA
		700	14.02 ± 1.90 aA	11.32 ± 1.37 aA	4.93 ± 0.53 bA	2.50 ± 0.49 bA
	Icatu	380	17.40 ± 3.73 aA	4.48 ± 0.29 bB	5.02 ± 0.81 bA	1.11 ± 0.21 bA
		700	24.14 ± 4.5 aA	15.66 ± 1.11 bA	6.44 ± 0.33 cA	2.53 ± 0.54 cA
	IPR 108	380	15.46 ± 1.70 aA	3.31 ± 0.60 bB	3.63 ± 0.31 bA	1.10 ± 0.14 bA
		700	18.30 ± 1.41 aA	12.32 ± 1.27 bA	4.93 ± 0.36 cA	2.17 ± 0.30 cA
$\delta^{13}C$ (‰)	CL 153	380	-26.1 ± 0.0 aA	-25.9 ± 0.3 aA	-27.7 ± 0.0 bA	-28.8 ± 0.0 cA
		700	-36.9 ± 0.2 bB	-36.5 ± 0.0 aB	-38.9 ± 0.1 cB	-39.5 ± 0.1 dB
	Icatu	380	-24.9 ± 0.1 aA	-26.6 ± 0.1bA	-27.6 ± 0.0 cA	-28.1 ± 0.1 dA
		700	-35.5 ± 0.1 aB	-36.2 ± 0.1bB	-37.6 ± ,0.0 cB	37.5 ± 0.0 cB
	IPR 108	380	-27.2 ± 0.1 aA	-27.1 ± 0.1 aA	-28.5 ± 0.0 bA	-29.0 ± 0.1 cA
		700	-38.2 ± 0.1 aB	-39.1 ± 0.0 bB	-39.3 ± 0.1 bB	-40.0 ± 0.0 cB

Chlorophyll a fluorescence analysis

The majority of Chl fluorescence parameters did not change significantly until 37/30 ºC, but were usually affected at 42/34 ºC. Therefore, our attention will be particularly focused on impacts at the two highest temperatures (Table 2).

Thylakoid electron transport rates

At 25/20 ºC, the thylakoid electron transport rates at PSI and PSII levels increased under high [CO_2] in all genotypes (Table 4). The activity of PSII including the OEC (PSII+OEC) increased *ca*. 30% in CL153 and 10% in the *C. arabica* genotypes. Similarly, increases of 25% (CL153), 15% (Icatu) and 11% (IPR108) were also found for PSII without OEC (PSII-OEC), whereas PSI activity was promoted by 20% (CL153), 9% (Icatu) and 6% (IPR108). PSI activity was 2.5- to 3.5-fold greater than that of PSII. Up to 37/30 ºC, the electron transport activity of both photosystems was maintained or marginally stimulated, and some differences between [CO_2] treatments were still present in all genotypes.

Upon exposure to 42/34 ºC, the potential activity of both photosystems was significantly decreased, but high activity levels were maintained, as compared to control. *C. arabica* plants grown under 700 µL CO_2 L^{-1} maintained some advantage over 380-plants, showing activities up to *ca*. 30% higher for PSII-OEC and PSI in IPR108. Unexpectedly, CL153 700-plants showed the strongest reduction, to values below those observed for 380-plants.

Photosynthetic and respiratory enzymes activity

Under control temperature, the potential activities of the enzymes related to the photosynthetic pathway were significantly increased by elevated [CO_2] conditions, between 36% (IPR108) and 46% (Icatu) for RuBisCO and between 42% (Icatu) and 63% (IPR108) for Ru5PK (Table 5).

Table 2 - Variation in the leaf chlorophyll a fluorescence parameters in *Coffea canephora* cv. Conilon (CL153) and *Coffea arabica* (Icatú and IPR108) plants, which were grown under 380 or 700 µL CO_2 l^{-1} and submitted to control (25/20 °C, day/night) or supra-optimal temperatures of 31/25, 37/30 and 42/34 °C. The parameters included the minimal fluorescence, F_o; maximal photochemical efficiency of PSII, F_v/F_m; the estimate of the quantum yield of noncyclic electron transport, Y(II); the quantum yield of regulated energy dissipation of PSII, Y(NPQ); the quantum yield of nonregulated energy (heat and fluorescence) dissipation of PSII, Y(NO); the photoprotective sustained thermal dissipation, q_N; photochemical quenching based on the concept of interconnected PSII antennae, q_L; the actual PSII photochemical efficiency of energy conversion, F_v'/F_m'; and the predictor of the rate constant of PSII inactivation, F_s/F_m'. For each parameter, the mean values ± SE (n = 5–8) followed by different letters express significant differences between temperature treatments for the same CO_2 level, separately for each genotype (a, b, c), or between CO_2 levels for each temperature treatment, separately for each genotype (A, B).

			Temperature (Day/night)			
Parameter	Genotype	µL L^{-1}	25/20ºC	31/25ºC	37/30ºC	42/34ºC
F_o	Clone 153	380	0.20 ± 0.01 bB	0.25 ± 0.01 bA	0.37 ± 0.01 bA	0.61 ± 0.08 aA
		700	0.37 ± 0.01 abA	0.25 ± 0.01 bA	0.38 ± 0.01 abA	0.41 ± 0.01 aB
	Icatu	380	0.23 ± 0.01 bA	0.27 ± 0.01 bA	0.38 ± 0.01 bA	0.78 ± 0.12 aA
		700	0.38 ± 0.04 aA	0.29 ± 0.01 aA	0.39 ± 0.02 aA	0.48 ± 0.03 aB
	IPR 108	380	0.23 ± 0.01 bB	0.24 ± 0.01 bA	0.36 ± 0.01 bA	0.73 ± 0.09 aA
		700	0.41 ± 0.01 abA	0.29 ± 0.00 bA	0.38 ± 0.02 abA	0.50 ± 0.05 aB
F_v/F_m	Clone 153	380	0.78 ± 0.01 aA	0.80 ± 0.01 aA	0.80 ± 0.01 aA	0.62 ± 0.03 bB
		700	0.75 ± 0.01 aA	0.79 ± 0.00 aA	0.80 ± 0.01 aA	0.76 ± 0.01 aA

Table 2. Cont.;

	Icatu	380	0.75 ± 0.01 aA	0.76 ± 0.00 aA	0.73 ± 0.02 aA	0.37 ± 0.1 bB
		700	0.74 ± 0.01 aA	0.78 ± 0.00 aA	0.77 ± 0.00 aA	0.70 ± 0.01 aA
	IPR 108	380	0.76 ± 0.00 aA	0.79 ± 0.00 aA	0.78 ± 0.00 aA	0.57 ± 0.05 bB
		700	0.75 ± 0.01 aA	0.77 ± 0.00 aA	0.79 ± 0.01 aA	0.71 ± 0.03 aA
$Y_{(II)}$	Clone 153	380	0.48 ± 0.02 aA	0.45 ± 0.02 aA	0.42 ± 0.02 aA	0.24 ± 0.03 bA
		700	0.42 ± 0.01 abA	0.51 ± 0.03 aA	0.41 ± 0.04 abA	0.35 ± 0.03 bA
	Icatu	380	0.43 ± 0.05 aA	0.37 ± 0.02 aA	0.32 ± 0.02 aA	0.05 ± 0.01 bA
		700	0.49 ± 0.04 aA	0.46 ± 0.04 aA	0.35 ± 0.02 abA	0.24 ± 0.07 bA
	IPR 108	380	0.34 ± 0.02 bA	0.50 ± 0.02 aA	0.41 ± 0.01 abA	0.14 ± 0.02 cA
		700	0.46 ± 0.06 aA	0.49 ± 0.02 aA	0.44 ± 0.01 aA	0.21 ± 0.03 bA
$Y_{(NPQ)}$	Clone 153	380	0.13 ± 0.01 cB	0.22 ± 0.02 bcA	0.27 ± 0.02 bA	0.43 ± 0.04 aA
		700	0.31 ± 0.01 aA	0.12 ± 0.01 bA	0.29 ± 0.04 aA	0.25 ± 0.04 aB
	Icatu	380	0.19 ± 0.02 aA	0.21 ± 0.02 aA	0.27 ± 0.02 aA	0.33 ± 0.06 aA
		700	0.21 ± 0.03 aA	0.17 ± 0.01 aA	0.27 ± 0.04 aA	0.33 ± 0.05 aA
	IPR 108	380	0.21 ± 0.02 bB	0.15 ± 0.01 bA	0.23 ± 0.01 bA	0.48 ± 0.02 aA
		700	0.31 ± 0.03 abA	0.16 ± 0.02 bcA	0.26 ± 0.02 bcA	0.39 ± 0.03 aA
$Y_{(NO)}$	Clone 153	380	0.39 ± 0.02 aA	0.33 ± 0.03 aA	0.31 ± 0.02 aA	0.33 ± 0.01 aA
		700	0.27 ± 0.02 cB	0.37 ± 0.02 abA	0.29 ± 0.01 bcA	0.39 ± 0.01 aA
	Icatu	380	0.37 ± 0.03 bA	0.42 ± 0.01 bA	0.42 ± 0.02 bA	0.62 ± 0.06 aA
		700	0.26 ± 0.03 bA	0.37 ± 0.03 abA	0.37 ± 0.04 abA	0.43 ± 0.05 aB

Table 2. Cont.;

	IPR 108	380	0.45 ± 0.04 aA	0.36 ± 0.02 aA	0.36 ± 0.02 aA	0.38 ± 0.02 aB
		700	0.23 ± 0.03 bB	0.35 ± 0.02 abA	0.3 ± 0.01 abA	0.40 ± 0.02 aA
q_N	Clone 153	380	0.41 ± 0.02 abB	0.5 ± 0.03 abA	0.55 ± 0.03 abA	0.69 ± 0.04 aA
		700	0.6 ± 0.02 aA	0.29 ± 0.03 bB	0.56 ± 0.03 aA	0.45 ± 0.05 aB
	Icatu	380	0.42 ± 0.04 aA	0.36 ± 0.03 aA	0.47 ± 0.03 aA	0.42 ± 0.16 aA
		700	0.46 ± 0.04 aA	0.39 ± 0.02 aA	0.52 ± 0.06 aA	0.55 ± 0.04 aA
	IPR 108	380	0.45 ± 0.06 aB	0.35 ± 0.04 bA	0.50 ± 0.02 aA	0.63 ± 0.03 aA
		700	0.63 ± 0.02 aA	0.36 ± 0.07 aA	0.56 ± 0.02 aA	0.60 ± 0.03 aA
q_L	Clone 153	380	0.60 ± 0.04 aA	0.44 ± 0.03 abA	0.37 ± 0.02 bA	0.41 ± 0.02 bA
		700	0.38 ± 0.04 aB	0.35 ± 0.07 aA	0.32 ± 0.03 aA	0.29 ± 0.03 aA
	Icatu	380	0.41 ± 0.06 aA	0.22 ± 0.03 aA	0.27 ± 0.02 aA	0.17 ± 0.06 aA
		700	0.37 ± 0.04 aA	0.39 ± 0.06 aA	0.32 ± 0.04 aA	0.27 ± 0.10 aA
	IPR 108	380	0.35 ± 0.06 abA	0.43 ± 0.05 aA	0.39 ± 0.01 abA	0.19 ± 0.03 bA
		700	0.45 ± 0.07 aA	0.4 ± 0.04 abA	0.45 ± 0.01 abA	0.22 ± 0.03 bA
F_v'/F_m'	Clone 153	380	0.61 ± 0.02 aA	0.66 ± 0.01 aA	0.66 ± 0.00 aA	0.44 ± 0.05 bB
		700	0.66 ± 0.02 aA	0.76 ± 0.01 aA	0.69 ± 0.01 aA	0.66 ± 0.02 aA
	Icatu	380	0.65 ± 0.04 aA	0.73 ± 0.01 aA	0.63 ± 0.02 abA	0.27 ± 0.08 bB
		700	0.73 ± 0.02 aA	0.70 ± 0.01 abA	0.63 ± 0.02 abA	0.56 ± 0.01 bA
	IPR 108	380	0.61 ± 0.04 aA	0.71 ± 0.02 aA	0.64 ± 0.01 aA	0.47 ± 0.02 bA
		700	0.67 ± 0.03 aA	0.70 ± 0.02 abA	0.64 ± 0.01 abA	0.55 ± 0.04 bA

Table 2. Cont.;

F_s/F_m'	Clone 153	380	0.52 ± 0.02 bA	0.55 ± 0.02 bA	0.58 ± 0.02 bA	0.76 ± 0.03 aA
		700	0.58 ± 0.01 abA	0.49 ± 0.03 bA	0.59 ± 0.04 abA	0.65 ± 0.03 aA
	Icatu	380	0.58 ± 0.05 bA	0.63 ± 0.02 bA	0.68 ± 0.02 bA	0.95 ± 0.01 aA
		700	0.51 ± 0.04 bA	0.54 ± 0.04 bA	0.65 ± 0.02 abA	0.76 ± 0.07 aA
	IPR 108	380	0.66 ± 0.02 bA	0.50 ± 0.02 cA	0.59 ± 0.01 bcA	0.86 ± 0.02 aA
		700	0.54 ± 0.06 bA	0.51 ± 0.02 bA	0.56 ± 0.01 bA	0.79 ± 0.03 aA

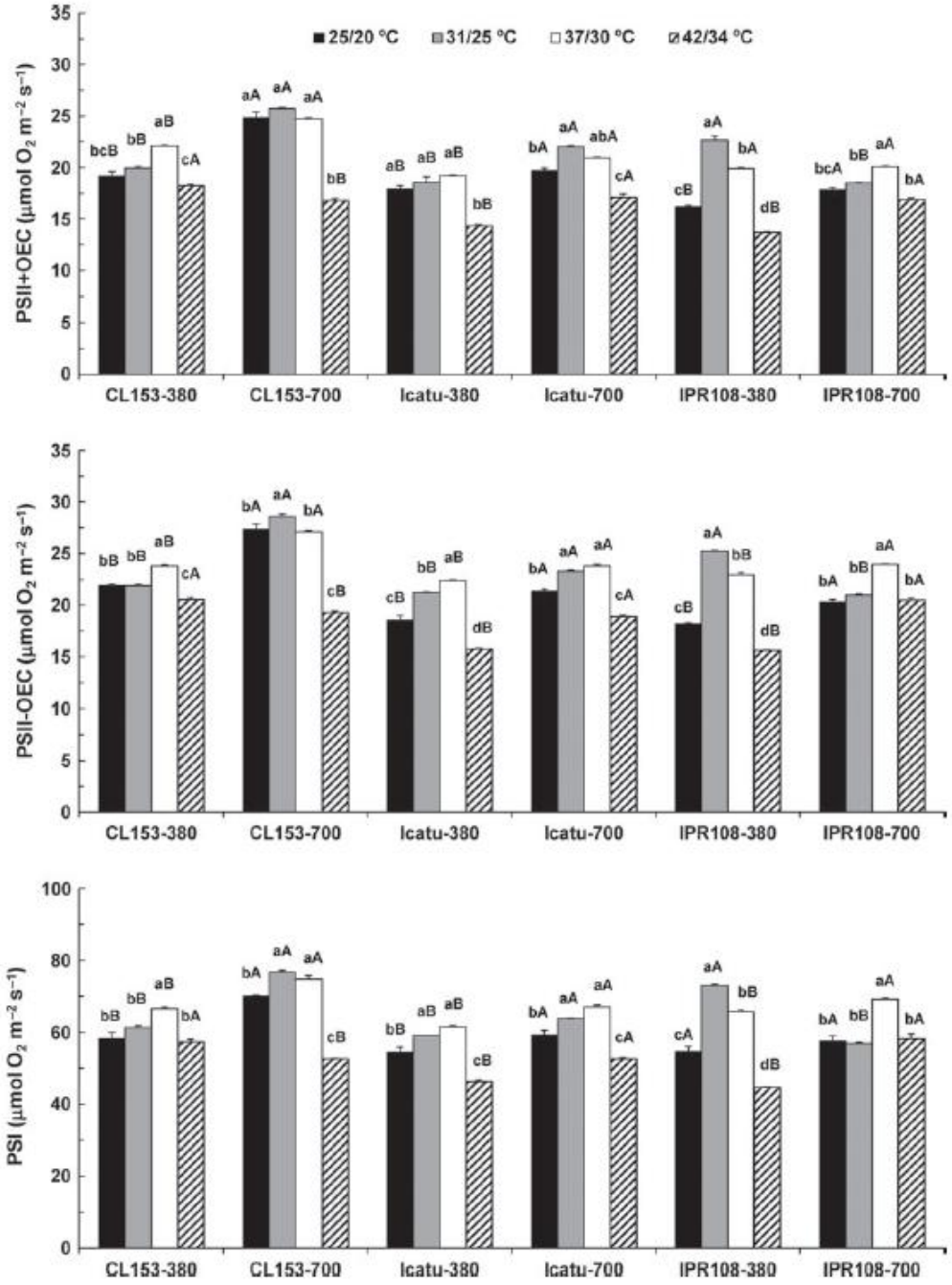

Figure 3 - Rates of thylakoid electron transport potential associated with PSII (with and without OEC) and PSI in *C. canephora* cv. Conilon (CL153) and *C. arabica* (Icatu and IPR108) plants, which were grown under 380 or 700 µL CO_2 L^{-1} and submitted to control (25/20 ºC, day/night) or supra-optimal temperatures of 31/25 ºC, 37/30 ºC and 42/34 ºC. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between temperatures for the same CO_2 treatment, separately for each genotype (a, b, c, d), or between CO_2 treatments for each temperature, separately for each genotype (A, B).

At higher temperatures the activities of these enzymes increased under both [CO_2] conditions. Maximal absolute activities were mostly found at 31/25 ºC, or, in a few cases (*e.g.*, for RuBisCO in 380-plants of CL153 and Icatu), at 37/30 ºC. The 700-plants maintained higher activity values up to 37/30 ºC. However, at 42/34 ºC, severe impacts on the activities of both enzymes were observed, with reductions between 75% (Icatu 700-plants) and 41% (CL153 380-plants) in RuBisCO, and between 75% (Icatu 700-plants) and 44% (IPR108 380-plants) in Ru5PK. Notably, greater impacts on both enzymes were usually observed in 700-plants than in 380-plants on the three genotypes. In fact, 700-plants maintained higher absolute values only in IPR108 for RuBisCO, and in CL153 and IPR108 for Ru5PK.

The activities of PK and MDH varied in a manner similar to that observed for photosynthetic enzymes. At 25/20 ºC, elevated [CO_2] resulted in significantly higher activities, between 20% (CL153) and 75% (Icatu) for MDH, and between 76% (CL153) and 86% (Icatu) for PK. These were the greatest differences found between [CO_2] conditions among all parameters.

At 31/25 ºC, MDH and PK activities were either enhanced or not affected, but at 37/30 ºC some reductions were already found when compared to the previous temperature. With a further increase to 42/34 ºC, strong reductions in activity were observed. At this extreme temperature, activities were reduced between 74% (IPR108 700-plants) and 54% (CL153 380-plants) for MDH, and between 52% (IPR108) and 29% (Icatu 380-plants) for PK. At this temperature, 700-plants usually showed the stronger activity impacts, despite that somewhat higher activities were maintained.

When comparing the two species grown under 380 µL L^{-1}, maximal activities for all enzymes were observed in CL153 plants at 37/30 ºC. For Icatu and IPR108 such maximal values were found at 31/25 ºC, with decreases at higher temperatures (except in Icatu for RuBisCO and PK). However, under 700 µL L^{-1} maximal activities in CL153 were consistently found at 31/25 ºC instead of 37/30 ºC. In contrast, IPR108 plants maintained RuBisCO activity and showed maximum MDH activity at 37/30 ºC. In Icatu maximal values were still found at 31/25 ºC.

Table 3 - Leaf maximal activities of some photosynthetic (ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO; ribulose 5-phosphate kinase, Ru5PK) and respiratory (NADH-dependent malate dehydrogenase, MDH; pyruvate kinase, PK) enzymes in *C. canephora* cv. Conilon (CL153) and *C. arabica* (Icatu and IPR108) plants, which were grown under 380 or 700 µL CO_2 L^{-1} and submitted to control (25/20 ºC, day/night) or supra-optimal temperatures of 31/25 ºC, 37/30 ºC and 42/34 ºC. For each parameter, the mean values ± SE (n = 4) followed by different letters express significant differences between temperatures for the same CO_2 treatment, separately for each genotype (a, b, c, d), or between CO_2 treatments for each temperature, separately for each genotype (A, B).

			Temperature (day/night)			
Parameter	Genotype	µl L^{-1}	25/20ºC	31/25ºC	37/30ºC	42/34ºC
RuBisCO (µmol m^{-2} s^{-1})	Clone 153	380	17.98 ± 0.23 bcB	20.33 ± 0.86 bB	27.89 ± 1.37 aB	10.56 ± 0.63 cA
		700	24.88 ± 1.29 bA	41.69 ± 1.75 aA	35.44 ± 4.52 aA	9.37 ± 1.36 cA
	Icatu	380	15.34 ± 1.22 bB	21.1 ± 0.66 aB	23.45 ± 0.89 aA	8.04 ± 0.71 cA
		700	22.41 ± 0.67 bA	29.53 ± 2.02 aA	25.31 ± 0.98 bA	5.69 ± 0.54 cA
	IPR 108	380	15.11 ± 0.57 bB	22.97 ± 0.87 aB	18.22 ± 0.59 bB	5.13 ± 0.41 cB
		700	20.68 ± 1.47 bA	29.1 ± 1.66 aA	27.95 ± 0.55 aA	10.7 ± 0.55 cA
RU5Pk (µmol m^{-2} s^{-1})	Clone 153	380	83.13 ± 4.7 cB	101.96 ± 1.54 bB	117.64 ± 3.25 aB	29.93 ± 1.75 dB
		700	126.28 ± 5.02 bA	187.62 ± 4.65 aA	135.48 ± 3.07 bA	42.67 ± 5.53 cA
	Icatu	380	74.63 ± 2.38 cB	107.23 ± 3.04 aB	88.71 ± 3.23 bB	29.96 ± 2.04 dA
		700	105.95 ± 3.16 cA	161.18 ± 1.81 aA	123.97 ± 3.5 bA	27.03 ± 0.9 dA
	IPR 108	380	66.22 ± 2.74 bB	104.36 ± 2.16 aB	74.92 ± 2.52 bB	36.99 ± 1.04 cB
		700	107.72 ± 4.68 bA	186.13 ± 5.37 aA	101.48 ± 2.01 bA	53.94 ± 2.76 cA

Table 3. Cont.;

MDH ($\mu mol\ m^{-2}\ s^{-1}$)	Clone 153	380	95.49 ± 2.74 bB	96.89 ± 3.64 bB	118.87 ± 3.18 aB	43.54 ± 2.05 cA
		700	128.43 ± 2.24 cA	170.94 ± 7.91 aA	145.73 ± 2.55 bA	51.09 ± 3.71 dA
	Icatu	380	74.43 ± 3.66 bB	102.99 ± 3.08 aB	93.54 ± 3.86 aB	31.54 ± 1.68 cB
		700	130.08 ± 2.33 aA	125.79 ± 5.42 aA	120.51 ± 1.02 aA	41.14 ± 1.14 bA
	IPR 108	380	82.3 ± 3.4 aB	96.21 ± 2.63 aB	47.31 ± 2.79 bB	36.47 ± 1.46 bA
		700	112.14 ± 3.45 bA	149.43 ± 11.65 aA	170.39 ± 10.42 aA	29.68 ± 3.93 cA
PK ($\mu mol\ m^{-2}\ s^{-1}$)	Clone 153	380	23.8 ± 1.28 bcB	25.85 ± 0.8 bB	35.7 ± 2.33 aB	16.25 ± 2.88 cB
		700	42 ± 2.26 bA	64.72 ± 2.05 aA	47.97 ± 2.06 bA	23.27 ± 2.53 cA
	Icatu	380	18.04 ± 0.78 bcB	24.13 ± 1.62 bB	31.95 ± 3.11 aA	12.79 ± 0.7 cA
		700	33.53 ± 1.89 aA	38.16 ± 2.22 aA	31.36 ± 1.87 aA	17.21 ± 0.95 bA
	IPR 108	380	20.14 ± 0.47 bB	31.87 ± 1.88 aA	13.94 ± 0.78 bcB	13.51 ± 1.75 cA
		700	36.54 ± 1.6 aA	33.43 ± 2.78 aA	24.75 ± 1.07 bA	17.47 ± 1.7 cA

DISCUSSION

Studies involving elevated CO_2 in greenhouse conditions or controlled environments have been considered difficult to interpret due to the potential limitations associated with light quality and quantity, root growth volume and nutrient levels (Drake et al., 1997; Kirschbaum, 2011). These limitations were not present in our study, as root growth remained unrestricted in large pots, an adequate mineral nutrition was assured and the PPFD was close to that of some shaded coffee production systems. Indeed, the PPFD was high enough to saturate the photosynthetic apparatus even in coffee plants grown in open fields (DaMatta, 2004). Also, by maintaining an invariant air relative humidity, increases in temperature are expected to cause no major changes in air evaporative demand. Therefore, we were able to properly interpret the physiological responses to increasing temperature without the confounding effects associated with increases in both temperature and vapor pressure deficit, as often occurs under field conditions.

Stomatal traits and $\delta^{13}C$ responses to elevated [CO_2] and supra-optimal temperature

Stomata controls leaf gas exchanges by a fine balance between the CO_2 required for photosynthesis and the level of water availability. Stomatal size and density determine the maximum leaf diffusive conductance to CO_2 (Woodward and Kelly, 1995; Franks and Beerling, 2009), and plants can adjust to increases in air [CO_2] by decreasing SD and SI, contributing to long-term decreases in g_s (Woodward and Kelly, 1995; Tricker et al., 2005; Ainsworth and Rogers 2007). In coffee species under adequate temperature (25/20 ºC), elevated [CO_2] had a marginal impact in SD (except in Icatu) and SI (Fig. 1), and, above all, g_s remained mostly unaffected (Table 1). Similar results were recently reported for coffee plants grown under elevated [CO_2] under controlled environmental conditions in growth chambers (Ramalho et al., 2013a) or under field conditions in FACE systems (Ghini et al., 2015). Such limited g_s responsiveness, if any, contrasts with reports of g_s decreases (stomatal acclimation) under elevated [CO_2] in most plants from all

functional groups. However, our findings do agree with the minor g_s responses found in other woody species (Ainsworth and Rogers, 2007; Moutinho-Pereira et al., 2009). Taking into account that g_s values in coffee are typically low and that stomatal limitations represent the main constraint to photosynthesis (Martins et al., 2014c), the absence of stomatal acclimation is expected to allow for greater potential photosynthetic gains associated with [CO_2] enhancement.

Interestingly, high temperature promoted changes in stomatal traits in a species-dependent manner. Higher SD and SI and lower SS values were observed in CL153 (Fig. 1) at 42/34 ºC (significant only in 700-plants). These changes may have contributed to sustain the tendency of higher g_s, given that higher densities of small stomata lead to increases in g_s for the same total pore area, due to shorter diffusion path length (Franks and Beerling, 2009). In sharp contrast, SD and SI were reduced and SS increased in IPR108. This would lower g_s, in contrast to our findings, suggesting that the control of stomatal opening overcomes the effects of SD and SS. This might be related to fact that plants had an unrestricted water supply coupled with a relatively high relative humidity. Stomatal trait modifications in Icatu 380-plants were similar to those in IPR108. Since g_s values at 42/34 ºC were similar to those at 25/20 ºC, the control of stomatal opening overrode changes in stomatal morphology.

As a consequence of the dichotomous responses of A and g_s to enhanced [CO_2], increases in water-use efficiency are usually observed (Leakey et al., 2009; DaMatta et al., 2010), as also found in the present work. iWUE increased in 700-plants compared to 380-plants under control temperature (Table 1), due to increases of A coupled with invariant g_s values. Also, changes in SD, SI and SS might have contributed to the observed iWUE values in CL153, but only to a limited extent in the *C. arabica* genotypes as found by Tricker et al. (2005) in poplar. However, due to predicted global warming, increases in water loss through transpiration flow should be expected. Such losses will be likely associated with increases in both g_s and in leaf-to-air deficit pressure vapor, which, together with A reductions, will reduce WUE. In fact, even under stable %RH in our experimental conditions, iWUE was reduced to less than 1/3 at 37/30 ºC and below 19% at 42/34 ºC, although the 700-plants maintained iWUE values that more than doubled those of 380-plants at the highest temperature. The observed changes in iWUE were further corroborated by $\delta^{13}C$ results. Less depletion of ^{13}C (less negative

$\delta^{13}C$) in leaf tissue is associated with higher WUE and linked to prolonged stomatal closure periods that restrict both CO_2 availability at carboxylation sites and H_2O consumption (Drake et al., 1997). Here, the temperature rise resulted in slight but consistent decreases in leaf $\delta^{13}C$, independent of growth [CO_2] and genotype (Table 1). This reflects a WUE decline and suggests that, on a long-term basis, C-assimilation was proportionally more reduced than g_s, confirming our instantaneous gas exchange determinations.

Impacts of elevated [CO_2] and temperature on photosynthesis

In coffee, A is largely controlled by diffusive rather than biochemical limitations and has large photorespiration rates (Martins et al., 2014c). This, together with the finding that A_{max} values were quite similar in both [CO_2] conditions, suggests that the remarkable increases in A (close or above 50% under control temperature) were preferentially supported by a reduction in photorespiration and diffusive resistances rather than by the observed reinforcement of photosynthetic components. These increases in A are in line with the potential stimulation of *ca*. 50% predicted for C3 trees (Drake et al., 1997; Ainsworth and Rodgers, 2007). It is also known that increases in A promoted by high [CO_2] would increase ATP demand (required for RuBP regeneration), and that the control of photosynthesis shifts from RuBisCO limited to RuBP regeneration limited (Ainsworth and Rogers, 2007). This may not have happened with coffee plants as both electron transport and Ru5PK activity were reinforced (Tables 4 and 5).

Photosynthetic down-regulation, defined as a reduction in photosynthetic potential (Woodward, 2002), was absent in all coffee genotypes up to 37/30 ºC. This was reflected in the similar A values obtained in the plants from both growth [CO_2], when the photosynthetic rates were measured both at either 380 and 700 μL CO_2 L^{-1} (data not shown). High metabolic activity and sink strength to utilize photosynthates are decisive to sustain high C-acquisition in response to increasing [CO_2] (Stitt, 1991; Long et al., 2004; Ainsworth and Rogers, 2007). This is apparently the case in coffee plants under our experimental conditions, since plants displayed a high allocation of resources to continuous vegetative growth of leaves and branches (which can be reinforced by regular pruning) and to the

growth of reproductive structures, with fruit production occurring twice annually. Additionally, the down-regulation of photosynthesis is often associated with declines in leaf N (Stitt, 1991; Bader et al., 2010) and lower N-allocation to RuBisCO, RuBP regeneration and to proteins associated with electron transport (Ainsworth and Rogers, 2007; Bader et al., 2010; Zhu et al., 2012), none of which was observed in coffee plants at 25/20 ºC. In fact, somewhat higher investments in photochemistry and biochemistry components were observed under high [CO_2] conditions, related to photosystems (I and II) electron transport potential and to RuBisCO and Ru5PK activities. Reinforcement was also observed for enzymes related to the respiratory pathway (MDH and PK). This contrasts with findings that respiration rates usually decline or remain unchanged under elevated [CO_2] (Woodward, 2002; Crous et al., 2012), usually due to the inhibition of mitochondrial enzymes (Drake et al., 1997).

At 37/30 ºC, which was well above optimal temperature, higher enzyme activities were observed in all genotypes (except for PK in IPR108). These enhanced activities were particularly obvious in 700-plants, which reflect the greater investment made in response to C-fertilization (Long et al., 2004). This would likely allow a higher potential level of metabolic functioning (A_{max}) and, by strengthening the plant, alter its tolerance limits to environmental constraints. In fact, the tolerance of the photosynthetic pathway is of crucial importance to plant survival and the acclimation to environmental stresses. It is usually accepted that *C. canephora* displays greater heat tolerance than *C. arabica* (DaMatta and Ramalho, 2006). However, A (and A_{max}), as well as many photosynthesis-related parameters (see below), were not significantly altered up to 37/30 ºC in both species. These findings reflect a tolerance to temperatures well above those considered adequate for the coffee crop (DaMatta and Ramalho, 2006). Such heat-tolerance agrees with a few studies that reported the maintenance of relevant A and A_{max} values in coffee plants up to 34-35 ºC, provided that g_s is kept high (see DaMatta and Ramalho, 2006), as was the case in our study. Collectively, these data are consistent with the high coffee productivity observed in tropical environments where long diurnal periods with air temperatures above 35 ºC are relatively common (DaMatta and Ramalho, 2006). However, it should be noted that, under field conditions, temperature increases are usually associated with

rises in the atmospheric vapor pressure deficit, which may result in stomatal closure and compromise photosynthetic performance.

Despite the significant impacts on A at 42/34 ºC, 700-plants maintained A values that were 2-fold (CL153 and Icatu) and 5-fold (IPR108) greater than those found in 380-plants. This agrees with the assumption that a greater reduction in photorespiration will occur at high rather than low temperatures (DaMatta et al., 2010; Kirschbaumm, 2011), partially offsetting the negative effects of supra-optimal temperatures on A. Also, the preservation of A_{max} was clear in the 700-plants of *C. arabica* genotypes, as no significant differences were observed between the values at 42/34 ºC and at control temperature. Therefore, high [CO_2] remarkably mitigated the effect of heat on A and A_{max}.

Given that no stomatal closure occurred under higher temperatures and A_{max} (assessed under saturating CO_2, in the absence of diffusion-mediated limitations of photosynthesis) was much less compromised than A at the highest temperature, the reductions in A were likely related to photosynthetic dysfunctions at the mesophyll level, as discussed further below.

Photochemical and biochemical components functioning

High temperature inhibition of whole leaf photosynthesis is usually caused by the disruption of the functional integrity of the photosynthetic apparatus at the chloroplast level (Berry and Björkman, 1980). Thylakoid membranes are particularly sensitive to heat, and impacts on photochemistry constitute one of the first indicators of sensitivity, with damages occurring at PSII and in the chloroplast ultrastructure (Mano, 2002).

The activity of PSI tend to higher values up to 37/30 ºC (Table 4) and Chl values also increased (up to 20%; data not shown). Therefore, increases in the energy capturing processes (F_o) up to that temperature would be plausible and will not reflect impairments at the light capture level, as also confirmed by the maintenance of F_v/F_m in all plants and under both [CO_2] conditions. However, at the highest temperature treatment, PSI activity decreased and the Chl content was stable. This was concomitant to significant F_o increases and F_v/F_m declines in 380-plants, especially in *C. arabica* species, suggesting that a threshold for irreversible damage had been reached (Pastenes and Horton, 1999). This could be

attributable to photoinhibition on the PSII centers, which is possibly related to D1 protein loss. This was been suggested to occur in coffee under high irradiance (see Ramalho et al., 2000), and is in agreement with the highest total photoinhibition values (PI_{Total}) observed in 380-plants (Table 3). Though, it should be emphasized that PSII maintained relevant activity levels (Table 4) even with such a significant increase in F_o. Therefore, it should not be discarded that to such increase in F_o might have accounted a reduction in the fluidity of thylakoid membranes (Tovuu et al., 2013). This was in fact observed at high temperatures in all genotypes and both [CO_2] conditions, due to an increase in fatty acid saturation (data not shown).

The high heat tolerance observed up to 37/30 ºC for A and A_{max} would rely on (and was confirmed by) the global low impact on photochemical efficiency and functioning (which is expressed by, *e.g.*, F_v/F_m, F_v'/F_m', $Y_{(II)}$, and q_P) and on photosynthetic components (*e.g.*, PSI, PSII, RuBisCO and Ru5PK activities). These responses were largely unrelated to [CO_2], but the 700-plants usually maintained higher functional levels.

Nevertheless, exposure to temperature as high as 42/34 ºC provoked reductions in almost all of the parameters related to photosynthetic performance. This included decreases in RuBisCO and Ru5PK activities, which showed to be the most sensitive components of the photosynthetic apparatus. This loss of enzyme activity was probably associated with modifications of native protein conformation, and contrasted to the high heat tolerance reported for RuBisCO in some species (Berry and Björkman, 1980; Wise et al., 2004). These negative effects were also observed in the activities of the enzymes PK and MDH. However, despite these strong impacts at 42/34 ºC, high [CO_2] clearly preserved the PSII efficiency, the proportion of energy trapped by open PSII centers and driven to photochemical events, and the electron transport activities (Table 2 and 4). In fact, in the 700-plants only marginal effects were observed in several parameters (*e.g.*, F_v/F_m, F_v'/F_m', $Y_{(II)}$, q_P, and q_L) in *C. canephora*. In *C. arabica* 700-plants most parameters were also less affected than in 380-plants, namely the PSs activities that did not differ from those found in controls. Since significant activity levels were maintained by both PSs in 380-plants and OEC was not affected (even with the strong F_o rise), it can therefore be concluded that these are not key sensitive components and are not responsible for the severe reduction in

A at observed at 42/34 ºC in these coffee genotypes. These results are in contrast with reports suggesting the water splitting system, PSs and electron transport as particularly heat sensitive targets (Berry and Björkman, 1980; Wise et al., 2004).

Complementary to the photosynthetic energy use decreases, energy dissipation processes (q_N, $Y_{(NPQ)}$, and $Y_{(NO)}$) were enhanced, often only at the highest temperature and strongly in 380-plants. For instance, q_N and $Y_{(NPQ)}$ mechanisms were triggered in CL153 380-plants, accompanied by a reduction in $Y_{(NO)}$, but this was not enough to fully protect the photochemical efficiency of PSII. In the Icatu genotype, $Y_{(II)}$ was reduced to almost residual values, accompanied by major increases in $Y_{(NPQ)}$ and $Y_{(NO)}$. The latter represented 62% of the total quantum yield and suggested the greatest sensitivity to heat. $Y_{(NO)}$ is usually remarkably stable in spite of environmental stress (Busch et al., 2009), reflecting non-photochemical quenching (other than that caused by the down-regulation of light-harvesting) attributable to photoinactivation and non-regulated energy (heat and fluorescence) dissipation in PSII (Kramer et al., 2004; Busch et al., 2009; Huang et al., 2011). In this way, a high $Y_{(NO)}$ value is indicative of constraints in the use of incident radiation, which results from insufficiency of both photochemical energy conversion ($Y_{(II)}$) (which decreased in all 380-plants) and non-photochemical energy loss in PSII, related to the protective down-regulation of light-harvesting function ($Y_{(NPQ)}$) (Huang et al., 2011). Such heat sensitivity in Icatu 380-plants indicated by $Y_{(NO)}$ fully agrees with the highest values of the rate constant for PSII inactivation (F_s/F_m'), chronic (PI_{Chr}) and total (PI_{Total}) photoinhibition, and with the lowest photochemical efficiencies of PSII (F_v/F_m and F_v'/F_m'). In addition to the impact on photosynthetic enzymes, these findings reveal a global impairment of the photosynthetic apparatus, responsible by the low photosynthetic rates. A comparison with 700-plants of all genotypes, especially Icatu, further emphasizes the mitigation effect caused by increased growth [CO_2]. In fact, the parameters associated to photosynthetic impairments ($Y_{(NO)}$, F_s/F_m', PI_{Chr} and PI_{Total}) were reduced, which was consistent with a decreased impact on photochemical energy use, PSII functioning and enzyme activities, as well as on A.

Species-specific responses to elevated [CO_2] and high temperatures

Although *C. canephora* was globally less affected by the highest temperatures (including the 380-plants), other patterns in both species should be highlighted. When grown under 380 µL CO_2 L^{-1}, *C. canephora* plants showed maximal activities for all studied enzymes at 37/30 ºC, whereas in *C. arabica* genotypes such maximal values were usually displayed at 31/25 ºC, usually decreasing at higher temperature. This is in agreement with the higher optimum temperature for *C. canephora* compared to *C. arabica* (DaMatta and Ramalho, 2006). However, elevated [CO_2] allowed the *C. arabica* genotypes to sustain or (rarely) increase several parameters at 37/30 ºC compared to their values at 31/25 ºC. This was clear in IPR108, where similar values were observed for A, q_P and RuBisCO activity for the two temperatures, whereas q_L and the activities of PSs and MDH increased at 37/30 ºC. These findings suggest a positive drift in tolerance and greater mitigation due to C-fertilization, at least at moderately high temperatures.

Our findings provide important evidence regarding the individual impacts of elevated [CO_2] and temperature and how they interact on coffee species at the photosynthetic level. In this pioneer study it was demonstrated the following: 1) Both *C. canephora* and *C. arabica* genotypes were remarkably tolerant up to 37/30 ºC, above what might be expected. However, a threshold for irreversible damage was reached at extreme temperatures of 42/34 ºC, for plants grown under normal [CO_2]. Impacts on A resulted from multiple impairments, particularly in RuBisCO and Ru5PK, which were the most sensitive photosynthetic components. The photosystems were highly heat tolerant at both the physical (energy capture) and photochemical (electron transport) level. In general, Icatu was the most sensitive genotype, but different responses in *C. arabica* genotypes suggest intraspecific variability that can be exploited in breeding programs. In CL153 plants, several parameters remained unaffected even at 42/34 ºC, mostly in 700-plants. 2) Stomatal traits were irresponsive to [CO_2], except in Icatu (SD and SS at 25/20 ºC) and IPR108 (SI at 25/20 ºC and 42/34 ºC). On the other hand, significant changes in these traits were clearly driven by temperature, in a species dependent manner. SD and SI increased and SS tended to decrease in CL153, whereas opposite patterns were observed in *C. arabica*. Increased temperatures strongly reduced

iWUE, but plants under elevated [CO_2] presented higher water use efficiency at all temperatures. 3) Elevated [CO_2] strongly attenuated the impact of temperature on both species, particularly at 42/34ºC, modifying the response of coffee plants to supra-optimal temperatures. 4) High [CO_2] did not down-regulate photosynthesis. Instead, C-assimilation, photochemical and biochemical functioning were improved at all temperatures. These changes likely contributed to prevent an energy overcharge in the photosynthetic apparatus, ultimately reducing the need for energy dissipation and PSII photoinhibition.

Our data offer novel and timely results concerning the remarkable mitigating effects of elevated [CO_2] on the presumable harmful effects of supra-optimal temperatures in coffee plants. Although much remains to be studied, prediction of the future of coffee crop in climate change and global warming scenarios are seriously unreliable if the role of CO_2 is not considered to be a key player in coffee heat tolerance. In this regard, and fortunately, future perspectives on the sustainability of this crop should not be as catastrophic as previously predicted based mostly in rising temperatures scenarios.

5. CHAPTER 3

WHOLE-CANOPY GAS EXCHANGES IN *Coffea* SP. IS AFFECTED BY SUPRA-OPTIMAL TEMPERATURE AND LIGHT DISTRIBUTION WITHIN THE CANOPY: THE INSIGHTS FROM AN IMPROVED MULTI-CHAMBER[2]

INTRODUCTION

Sunlight energy is transformed into chemical energy by means of photosynthesis in higher plants. During the process, plants fix carbon dioxide and release oxygen with concurrent water loss. The balance of these processes is essential to the impacts of the environmental and genetic variables on crop productivity (Baker et al., 2014).

Gas exchange determinations have been widely reported on single leaves since there are several commercial instruments available (Perez-Peña and Tarara, 2004). However, it is quite difficult to scale-up the photosynthesis evaluation from single leaf to whole-canopy level, as the latter integrates the

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output of leaves with different ages and degree of light exposure, as well as from other photosynthetic organs like fruit, shoots, and trunks (Long et al., 1996). In this way, leaf-level photosynthesis measurements can only provide incomplete (and potentially misleading) information if extrapolated to quantify photosynthesis or infer differences in water demand, and crop productivity at the whole plant level. On the other hand, whole-canopy gas exchange measurements can overcome some of these limitations related to single leaf evaluations.

Plant gas exchanges at canopy scale have been measured using a number of approaches, including both open and closed system chambers with some requiring a fetch or homogeneous upwind land cover in some measurement systems. In an open system, accurate measurement of canopy gas exchange depends on the ability to measure entry *vs*. exit gas concentration differentials, as well as airflow rate through the chamber. Typically, at a constant airflow rate, these gas concentration differentials are higher during the day (*e.g*., photosynthetic CO_2 uptake) than at night (*e.g*., respiratory CO_2 loss), with a similar diurnal trend also for transpiration (Baker et al., 2014).

Canopy photosynthesis is a closely related determinant of productivity as it takes into account the genotypic variation in both light conversion and interception efficiencies (Petrie et al., 2009). Photon flux density at different levels in a canopy is often the major factor determining the rate of CO_2 assimilation of individual leaves (Petrie et al., 2009). Long et al. (2006) developed mathematical models to design optimum distributions of leaves to maximize efficiency, which have been used as a guide for selecting improved crop genotypes. Older varieties with more horizontal leaves been replaced by newer varieties that have been bred to have more vertical leaves in the top layer (Zhu et al., 2010; Evans, 2013).

C. canephora generally has a larger canopy and individual leaves and the leaves are more rugose and crenate than *C. arabica* ones. *C. canephora* is typically a multi-stem tree whereas *C. arabica* is managed mostly with a single primary stem (Souza et al., 2004). The optimal annual mean temperatures for plant development are in the range of 18-23 ºC for *C. arabica* and of 22-26 °C for *C. canephora* (DaMatta and Ramalho, 2006). It has been argued that coffee, particularly *C. arabica*, will be highly sensitive to climate changes and that global warming will threaten the coffee supply in the near future (Davis et al., 2012; Bunn et al., 2015). Climate changes, particularly as regards temperature, were

estimated to affect the coffee trees (Assad et al., 2004; Davis et al., 2012; Baca et al., 2014; Ovalle-Rivera et al., 2015; Gay et al., 2006; Craparo et al., 2015).

A wealth of studies of gas leaf-scale exchanges of coffee plants can be found, since early pioneer works (Nunes et al., 1968; Kumar and Tieszen, 1980) until present (Martins et al., 2014a; Ramalho et al., 2013a; Martins et al., 2016; Silva et al., 2017), but a total absence of whole-canopy gas exchanges (using the multi-chamber open system approach) was noted for this crop. For our studies we developed an open, multi-chamber system to continuously measure whole canopy gas exchange. With this system we could evaluate whole-canopy gas exchanges of genotypes from the two most important producing *Coffea* species: *Coffea arabica* cv. Catuaí Amarelo (heat sensitive) and *Coffea canephora* cv. Emcapa 8111 Clone 02 (heat tolerant)).We aim at study the impact of supra-optimal temperature and light distribution within the canopy on whole plant gas exchanges level, and it is hypothesized that whole-canopy gas exchanges would be reduced to a greater extent in *C. arabica* than *C. canephora* during summer, although the latter has a denser canopy architecture, which could reduce the light scatter through the canopy.

MATERIAL AND METHODS

Plant material and climatic variables

Plant material and substrate used are exactly as described in Chapter 1. In brief, plants of 1 to 2 year old from *C. arabica* cv. Catuaí Amarelo – Catuaí (heat sensitive) and *C. canephora* cv. Emcapa 8111 Clone 02- Clone 02 (heat tolerant) were grown in 100 L pots under greenhouse conditions, with natural fluctuations of light, temperature and relative air humidity. For the two experimental periods on spring (September 2014) and summer (February 2015) the plants presented a height of 0.66 ± 0.03 and 1.06 ± 0.06 for *C. arabica*, and 0.92 ± 0.03 and 1.23 ± 0.01 m for *C. canephora*, respectively.

Plants were watered as required so that the soil moisture was maintained close to field capacity. Air temperature and humidity were recorded using a data

logger (model 200 Spectrum Technologies, Plainfield, Illinois, USA), whereas soil temperature and water tension were recorded using a soil sensor (model RT-1 Decagon Devices, Pullman WA, USA and Watermark 200SS, Irrometer Co., Riverside, CA, USA). Relative air humidity inside the chamber was calculated based on vapor pressure measured by the gas analyzer cited below. Photosynthetic photon flux density (PPFD) was monitored using a quantum light sensor (model LightScout, Spectrum Technologies, Plainfield, Illinois, USA) and recorded (data logger Watch Dog Model 200 Spectrum Technologies, Plainfield, Illinois, USA). Plant fertilization followed specific literature based on soil analyses (Matiello et al., 2010).

Whole-canopy gas exchange measurements

Whole-canopy gas exchange measurements were performed using six plants per genotype during two seasons (spring: September 2014; summer February 2015) with the multi-chamber open system, previously described by Miller et al. (1996), and adapted by Glenn et al. (2003). Briefly, twelve chambers were built with a volume of *ca.*0.78 and *ca*.1.1 m^3, in spring and summer, respectively, using a transparent polyester film chamber (Mylar® - Dupont, Wilmington, DE, USA; PAR transmittance of 90%) (Corelli and Magnanini, 1993). One centrifugal blower provided air flow to four chambers with a constant flow rate of 20 Ls^{-1} (September 2014: Catuaí: 11.9 L s^{-1} m^{-2} leaf area, Clone 02: 6.34 L s^{-1} m^{-2} leaf area; February 2015: Catuaí: 5.91 L s^{-1} m^{-2} leaf area, Clone 02: 4.6 L s^{-1} m^{-2} leaf area). The volume air exchange rate was *ca.* 39 and 55 s during spring and summer, respectively, due to the different size chambers that were increased as the plants increased in size. The data were collected (CR1000 Campbell Scientific, Logan, Utah, USA) along the 10 consecutive days of the experiment in each season.

Flow rates were monitored daily with a flow meter (model AZ®9871 Anemometer Printer, Taichung City 406, Taiwan) measuring the outlet air stream. The water vapor pressure and CO_2 concentrations of the inlet and outlet air streams were monitored by a portable infrared gas analysis (IRGA) system (Ciras – DC Gas Analyzer PP Systems, Amesbury, Massachusetts, USA,). Apump sampled inlet and outlet air flow to the IRGA. Every hour, twelve readings were

recorded (six from each genotype). Solenoid valves were controlled by the data logger to sample a single chamber every 5 min. Gas concentration data were collected every 30 s, but the first two minutes of each 5 min sampling were discarded as the time required for purging the previous sample. The flux of CO_2 and H_2O were converted to photosynthesis (A_c) or respiration (Rd_c) (µmol CO_2 m^{-2} s^{-1}), and transpiration-E_c (mmol H_2O m^{-2} s^{-1}) of the whole canopy. Water-use efficiency (WUE_c) was calculated as A_c/E_c (µmol CO_2mmol H_2O^{-1}). The canopy conductance (g_c) was estimated using the formula $g_c = (E_c/VPD_{air})$ x 101.325 according to Perez-Peña (2004). Thermocouples measured the internal chamber temperature, which was simultaneously reported with gas exchange. The whole-canopy gas exchange system is showed in figure 1.

Figure 1 - View of the whole-canopy gas exchange system used during the experiment.

Leaf area, branch angle and light scatter

The total number of leaves on each plant was counted at the end of each

period of measurements in the two seasons. Leaf area was measured on a pool of 100 leaves per plant in both seasons using a portable leaf area meter (Li-Cor 360A, Lincoln, Nebraska, USA) and the average of area per leaf was used to estimate the total plant leaf area. The branch angle of six branches from the upper third of the canopy was measured in each plant using a protractor.

The light distribution through the canopy was measured using a ceptometer (Quantum Meter, Spectrum Technologies, Model LQS-QM, Plainfield, Illinois, USA) every 0.1m from the apex of the canopy until 0.8m, when the intercepted light by the leaves reached values close to 100%.

Statistical analysis

Whole plant chambers were arranged in a completely randomized design, with 6 replicates. Data were analyzed in a 2 x 2 factorial scheme with two seasons and two species. Branch angle data were analyzed in a completely randomized design by species. Whole-canopy gas exchange data were analyzed using two-way ANOVAs ($P < 0.05$) to evaluate the differences between seasons or species, followed by a Tukey test for mean comparison among seasons for the same species or between species within the same season. A 95% confidence level was adopted for all tests. Only the significant differences related to season, species or the season x species interaction are noted in the tables and figures. Differences in light scatter within the canopy are indicated with 95% confidence level errors bars. The A_c x E_c, A_c x PAR, g_c x VPD and Rd_c x A_c regression analyses used SAS software (version 8, Cary, North Carolina, USA).

RESULTS

The highest average temperatures outside the chamber were obtained during summer with minimum and maximum values of 23.6 and 37.1 ºC (average of 10 consecutive days), respectively. In spring time, minimum and maximum temperature averages were 18.2 and 31.3 ºC, respectively. That corresponded to average daily temperatures of 23.3 and 28.6 ºC outside the chamber in spring and

summer, respectively. Inside the chambers daily averages were 24.1 and 31.0 ºC for Catuaí and 24.0 and 29.9 ºC for Clone 02 during spring and summer, respectively. In this way, the daily average difference between the inside and the outside of the chamber was ca. 0.7-0.8 ºC in spring and 1.3-1.4 ºC in summer, therefore with a low potential impact.

The lowest relative humidity outside the chamber (close to 38%) was observed near midday in both seasons (Figure 2A, B). The highest air VPD values were observed during summer (4.04 kPa) near midday (Figure 2 C, D), whereas during spring maximum value reached 2.9 kPa. PPFD values were similar in spring and summer, reaching values of *ca.* 830 µmol m^{-2} s^{-1} near midday (Figure 2 C, D).

Catuaí showed the highest whole-canopy photosynthesis rates (A_c) during most of the day, in both seasons, when compared to Clone 02, with a much greater difference was observed between these genotypes in spring (Figure 3A).In this season, Catuaí maintained A_c values between 4 and 5 µmol m^{-2} $s^{-1}$for most of the diurnal period (8:30-15:30), whereas Clone 02 showed A_c values close to half (between 2 and 2.5 µmol m^{-2} s^{-1}). Although showing strong reductions in summer in comparison to spring, Catuaí still maintained higher A_c values than Clone 02, which in turn showed no-significant changes between seasons. The pattern of whole canopy transpiration, E_c, followed a close variation pattern to that of A_c (Figure 3B), with the highest values around 12:00-13:00. The highest average values were observed in Catuaí (2.24 mmol m^{-2} s^{-1}) during spring, which were reduced to 1.43 mmol m^{-2} s^{-1} in summer. These plants showed as well higher whole canopy conductance (gc) values than Clone 02 plants in both seasons (Figure 3C), although without significant differences in summer. The highest values of g_c were 171 and 102 mmol H_2O m^{-2} $s^{-1}$for Catuaí and Clone 02 plants, respectively. These values were observed in early morning in the spring and decreased over the course of the day. Both genotypes had similar values during summer (Figure 3C).

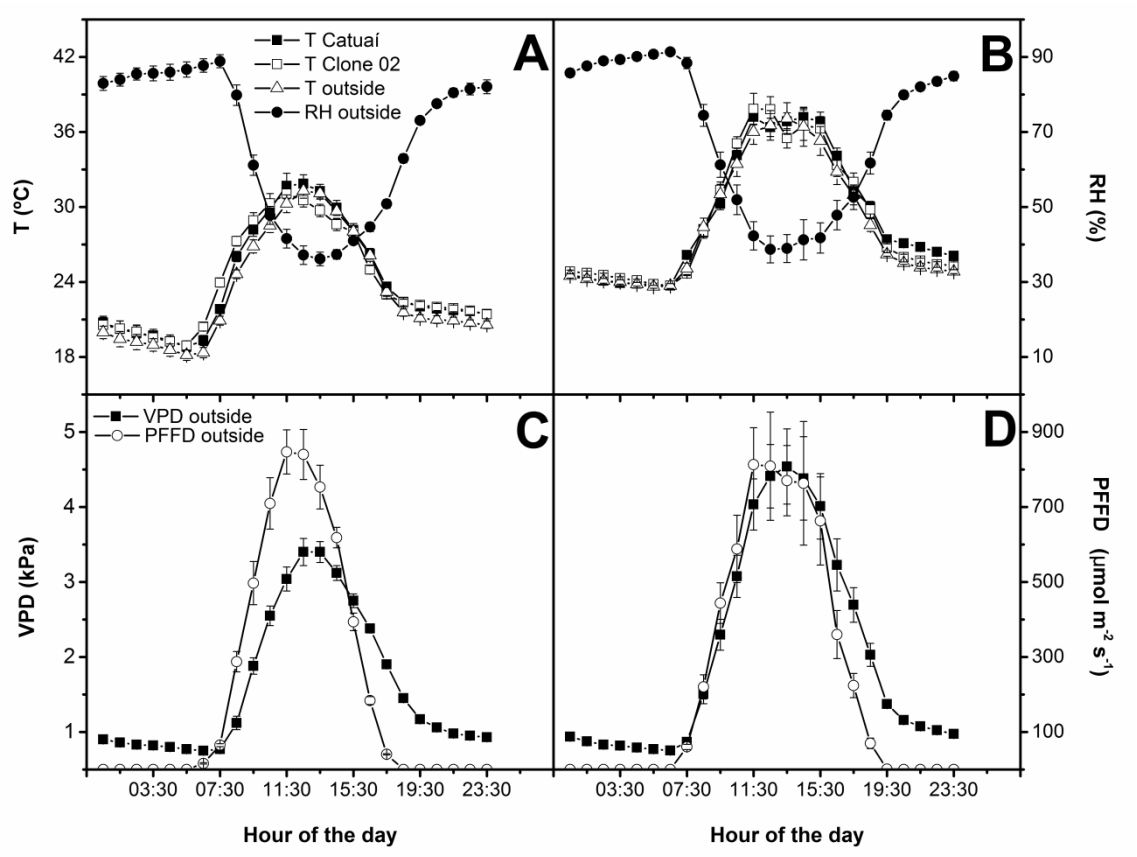

Figure 2 - Seasonal trends of air temperature (T) in and outside the chamber of *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02), relative humidity (RH), air vapor pressure deficit (VPD) and photosynthetically active radiation (PFFD). Each value represents the mean ± SE (n=10) of ten uninterrupted days, during spring (September) 2014 (A and C) and during summer (February) 2015 (B and D). The legend of symbols are similar between A and B, and between C and D.

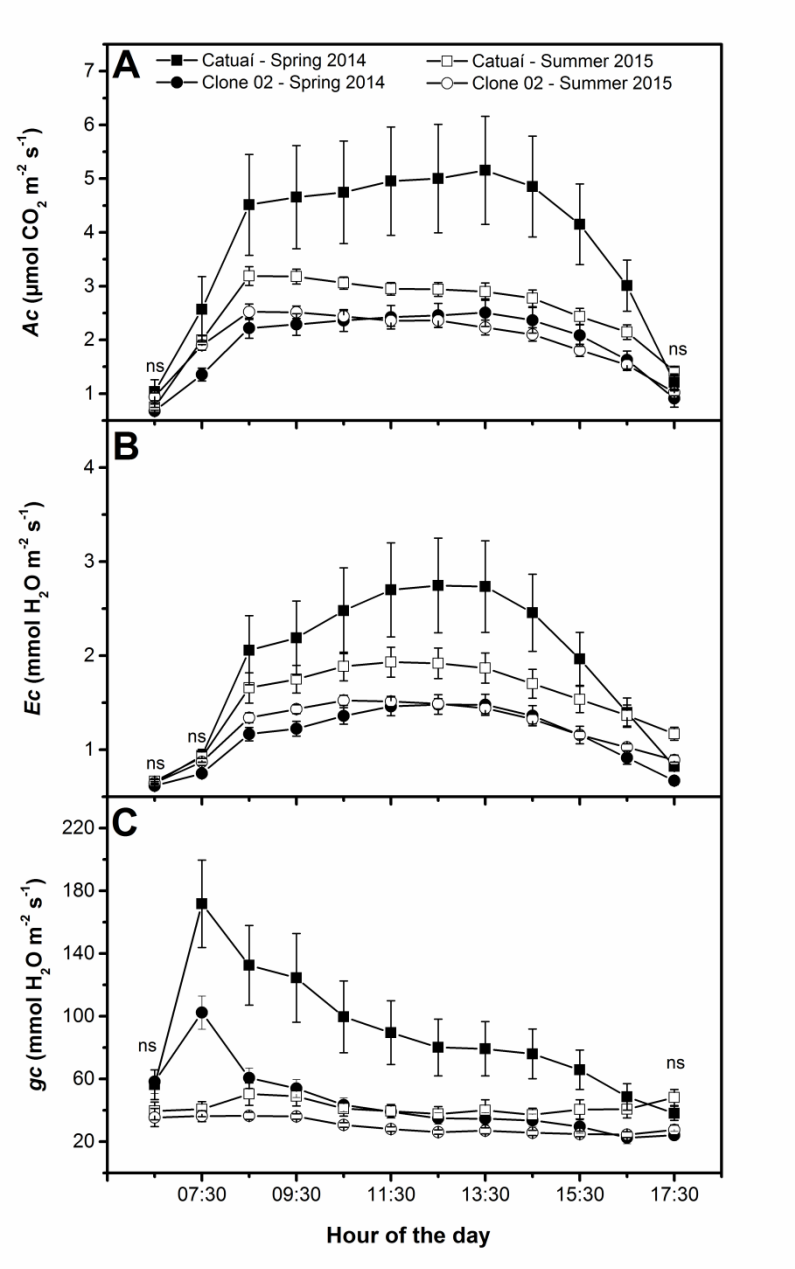

Figure 3 - Diurnal trends of CO_2 assimilation, A_c, (A), transpiration, E_c, (B) and canopy conductance, g_c, (C) of the whole-canopy, obtained during ten uninterrupted days during spring and summer, in *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02). Each value represents the mean ± S.E. (n=6). There was a significant interaction between species and seasons. ns-not significant. There is significant difference ($p<0.05$) between genotypes within the same season and/or between season for the same genotype when the lower limit of standard error bar is higher than the upper limit at the same hour of the day.

As a result of the differences of A_c values along the day, Catuaí showed significantly higher daily accumulated whole-canopy photosynthesis (A_{ca}- given by the integrated area below the respective A_c curves) than Clone 02 (Table 1). Reflecting the stability of A_c between seasons, Clone 02 did not show A_{ca} differences between spring and summer, contrary to Catuaí that showed a significant 28% reduction in summer.

Following the same integration method, daily accumulated whole-canopy transpiration (E_{ca}) and night respiration (Rd_c) had the same pattern as A_{ca} for both genotypes, showing significant reductions (E_{ca}– 29%; Rd_c– 38%) in Catuaí, and only marginal changes in Clone 02 (Table1). Still, Catuaí maintained higher values than Clone 02 of all daily accumulated whole-canopy parameters on both seasons.

Resulting from their different A_c and E_c values in spring, the whole-canopy water use efficiency (WUE_c= A_c/E_c) was higher in Catuaí than in Clone 02 (Figure 4). In summer both species had significantly lower WUE_c than in spring, without relevant species differences (Figure 4).

The photosynthetic light response of the two genotypes was significantly different in both spring and summer (Figure 5A and B). In spring the maximal photosynthetic rates obtained through the adjusted curves was 7.2 and 2.6 µmol CO_2 m^{-2} $s^{-1}$for Catuaí and Clone 02, respectively, whereas for summer those values decreased to 2.6 and 1.8 µmol CO_2 m^{-2} s^{-1}, respectively. The apparent quantum efficiency (ϕ), estimated from the initial (linear) part of the light response curve (up to a PPFD of 100 µmol m^{-2} s^{-1}),reached 0.035 and 0.019 µmol CO_2 µmol Q^{-1} during the spring, but decreased to 0.014 and 0.010 in summer for Catuaí and Clone 02, respectively.

Clone 02 intercepted *ca*. 70% of incident PPFD at the first 0.1 m, irrespective of season. On the other hand, Catuaí intercepted *ca.*40% (spring) and 52% (summer) of incident PPFD at the same canopy level (Figure 6). Catuaí maintained a lower PPFD interception than Clone 02 until 0.5m from the apex in spring and 0.3 m in summer. Such higher light interception at the more external leaf levels of the canopy in Clone 02 would be likely related to its greater leaf area per plant than Catuaí, in both seasons (*ca.*47 and 22% by spring and summer, respectively), and to its lower branch angle (Table 2). Good relationship between gc and VPD (Figure 7 A) as well as between Ac and Rdc (Figure 7B) were observed in both genotypes.

Table 1 - Daily accumulated photosynthesis (A_{ca}) and transpiration (E_{ca}) and night (dark) respiration (Rd_c) of whole-canopy for *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02) during spring (September) 2014 and summer (February) 2015 samplings.

Genotypes	Spring	Summer
	A_{ca} (g CO_2 m^{-2} day^{-1})	
Catuaí	6.21 ± 1.38 aA	4.46 ± 0.85 aB
Clone 02	2.70 ± 0.31 bA	2.73 ± 0.18 bA
	E_{ca} (L H_2O m^{-2} day^{-1})	
Catuaí	1.09 ± 0.23 aA	0.77 ± 0.09 aB
Clone 02	0.49 ± 0.05 bA	0.54 ± 0.03 bA
	Rd_c (µmol m^{-2} s^{-1})	
Catuaí	0.40 ± 0.04 aA	0.25 ± 0.01 aB
Clone 02	0.15 ± 0.03 bA	0.18 ± 0.01 bA

Each value represents the mean ± SE (n=6); different letters indicate significant differences between seasons for the same genotype (A, B) or between genotypes for the same season (a, b), for a Tukey test at 5% probability. The ANOVA showed significant differences between genotypes within the same season and between seasons for the same genotype for leaf area and significant differences significant differences for angle of insertion of plagiotropic branch.

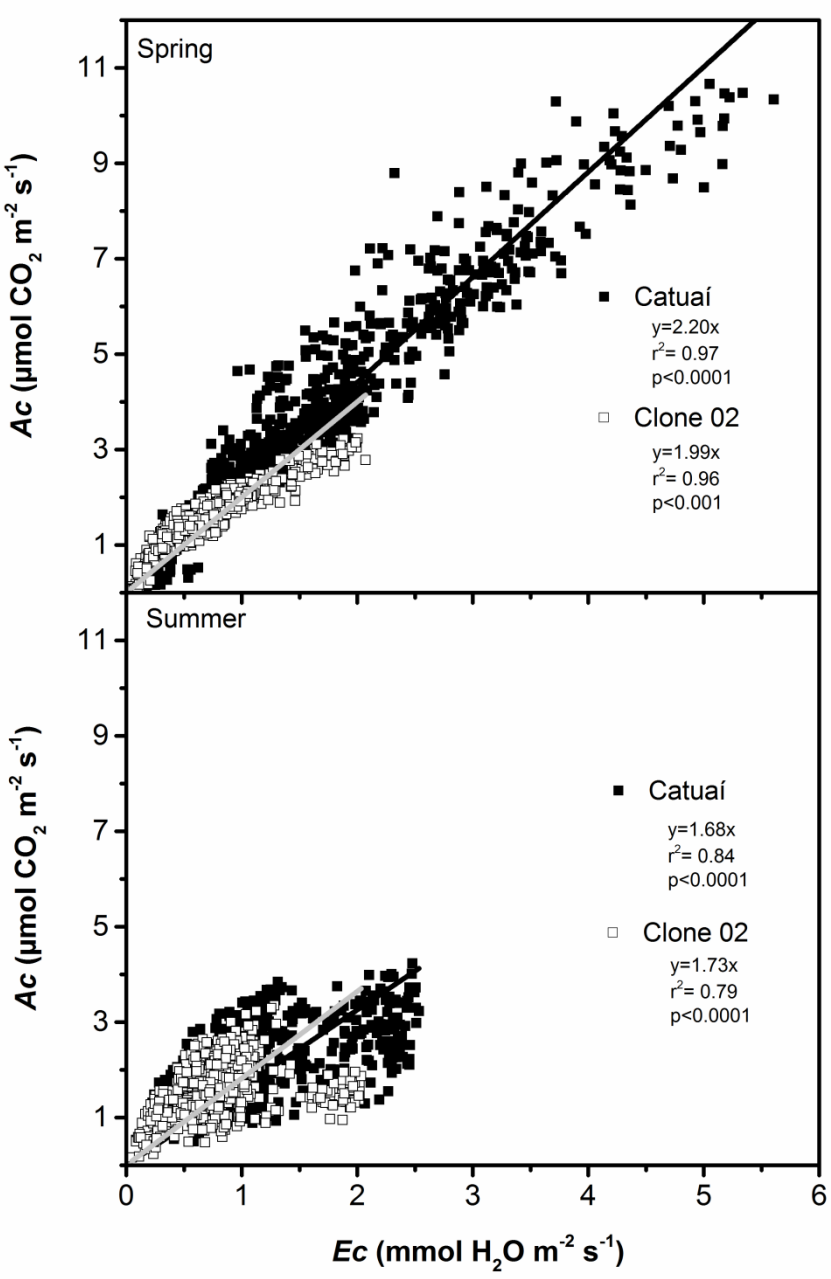

Figure 4 - Relationships between CO_2 assimilation-A_c and transpiration –Ec, rates in *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02), during spring (September) 2014 and summer (February) 2015 (n=720).

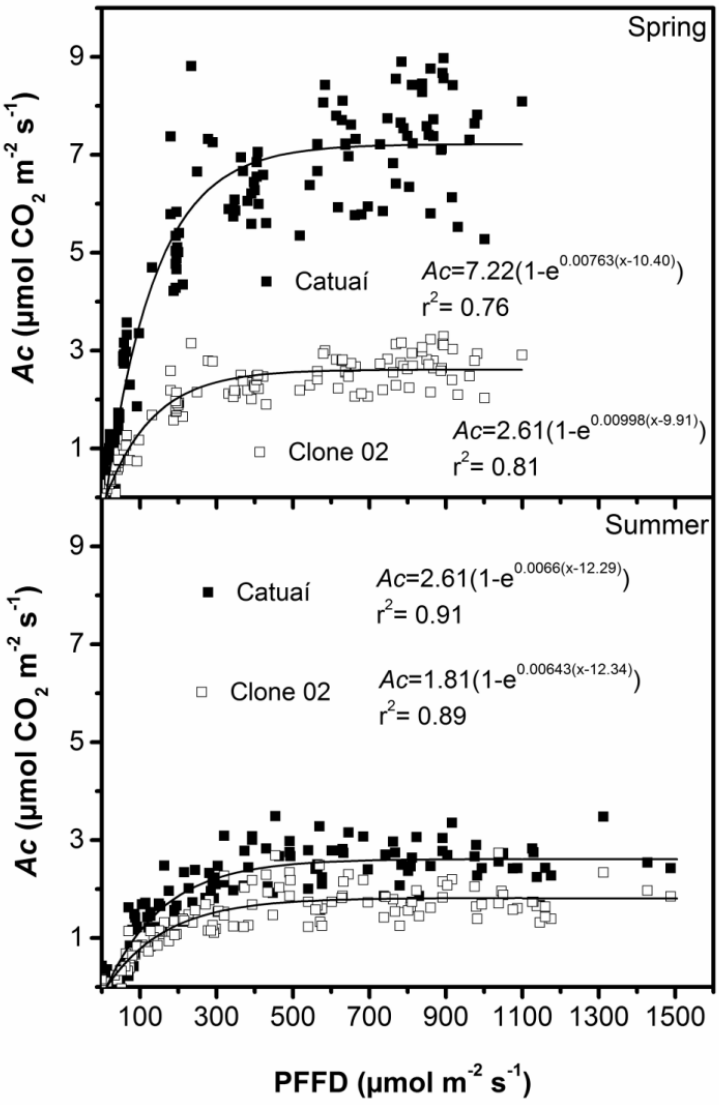

Figure 5 - Relationship between CO_2 assimilated by whole-canopy, A_c, and photosynthetic photon flux density, PPFD, in *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02), using mean hourly values in spring (September) 2014 and summer (February) 2015. (n= 120 for each curve). The CO_2 assimilated values were obtained using mean values outside chamber temperature and relative humidity of 26.1 ± 1.2 and 54.8 ± 5.8 during spring (September) 2014 and 32.2 ± 1.3 and 55.9 ± 5.5 during summer (February) 2015, respectively.

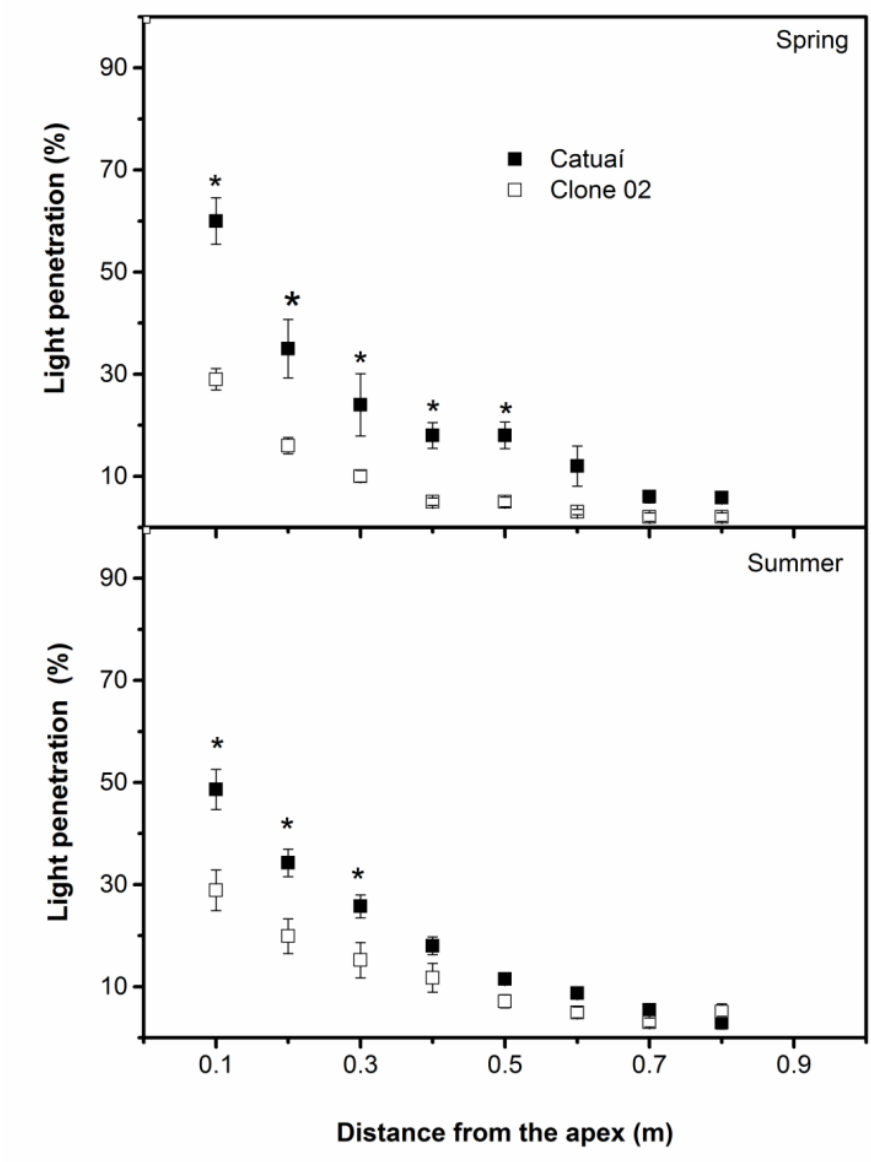

Figure 6 - Canopy light penetration in *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02), during spring and summer. Each value represents the mean ± S.E. (n=6).

Table 2 - Mean leaf area per plant and branch angle for *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02) during spring (September) 2014 and summer (February) 2015 samplings.

Genotypes	Spring	Summer
	Leaf area (m^2)	
Catuaí	1.67 ± 0.31 bB	3.38 ± 0.13 bA
Clone 02	3.15 ±0.26 aB	4.34 ± 0.24 aA
	Branch Angle(º)	
Catuaí	-	33.2 ± 0.4 a
Clone 02	-	31.2 ± 0.5 b

Each value represents the mean ± SE (n=6); different letters indicate significant differences between seasons for the same genotype (A, B) or between genotypes for the same season (a, b), for a Tukey test at 5% probability. The ANOVA showed significant differences between genotypes within the same season and between seasons for the same genotype for leaf area and significant differences significant differences for angle of insertion of plagiotropic branch.

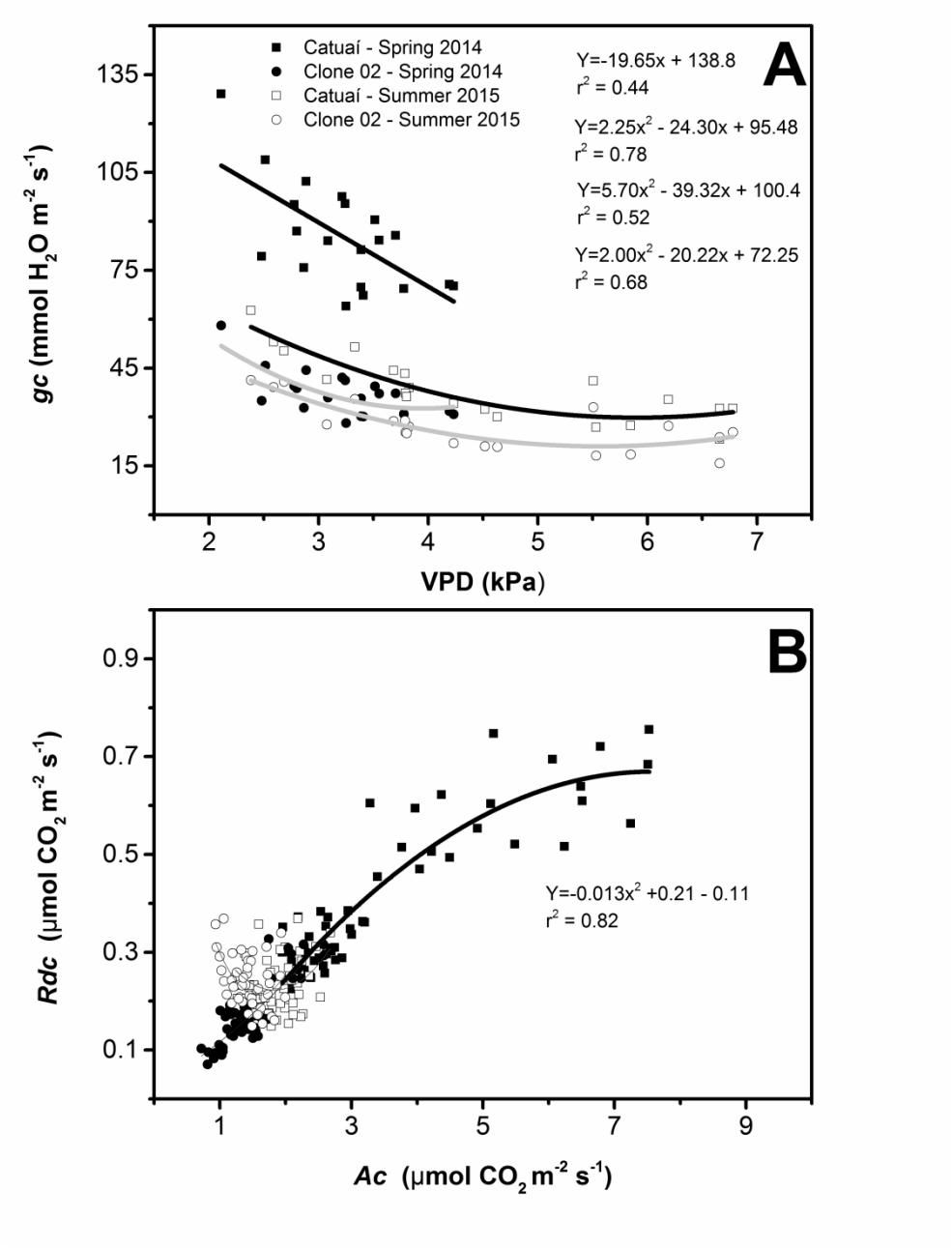

Figure 7 - Relationship between canopy conductance and midday vapor pressure deficit (A) and between mean daily photosynthesis and nightly respiration rates (B) for *C. arabica* cv. Catuaí Amarelo (Catuaí) and *C. canephora* cv. Emcapa 8111 Clone 02 (Clone 02)in spring and summer samplings. For each curve n= 20 (A) and 50 (B).

DISCUSSION

It was not within the scope of the study to determine the effect of the elevated chamber temperature on gas exchange, but the somewhat higher temperatures inside the chamber in comparison to the outside (0.7-0.8 ºC in spring and 1.3-1.4 ºC in summer) are believed to not affect the leaf gas exchanges *per se* (Perez-Peña and Tarara, 2004). Instead, the increase of daily mean temperature inside the chambers from spring to summer (6.9ºC for Catuaí and 5.9 ºC for Clone 02) would have governed the plant gas exchange responses, since it clearly configures an increase from adequate (spring) to supra-optimal (summer) temperatures.

Transpiration rates and air flow were sufficient during the day to maintain chamber air temperature close to that of ambient air via removal of latent energy from the chambers (Baker et al., 2014). Soil water tension during spring and summer (-15.4 ± 0.1 kPa and -9.8 ± 0.04 kPa, respectively) are within the range considered as not limiting to gas exchanges (Thompson et al., 2007). Soil temperatures were 25.1 ± 0.02 ºC and 28.1 ± 0.1ºC during spring and summer, respectively. This variation did not likely limit A_c, since soil temperatures within the range of 20-30ºC do not generally limit photosynthetic rates in tropical plants (Ziska, 1998).

Using the soil water balance technique, Pereira et al. (2011) measured transpiration rates of 1.65 L H_2O $plant^{-1}$ day^{-1} in 1.5 year old plants of *C. Arabica* with 1.45 leaf area, similar to the whole-canopy transpiration rates of 1.70 for Catuaí with 1.68 leaf area during spring indicating that our chamber system provided reliable information.

Although canopy photosynthesis would be saturated in the field at irradiances considerably higher than 600-700 µmol m^{-2} s^{-1} (DaMatta, 2004), or close to 700 µmol m^{-2} s^{-1} under environmental controlled conditions (Batista-Santos et. al., 2011), both species were light saturated in this case at 400 µmol m^{-2} s^{-1} (Figure 5). PPFD and relative humidity were very similar in the spring and summer (Figure 2). Therefore, the key seasonal difference between the spring and summer environmental conditions seemed to rely predominantly on air temperature variations, which, by increasing in summer, lead to a vapor pressure

deficit rise of *ca.* 25% (Figure 2).

In the spring, maximum air temperatures outside the chamber reached values between 30 and 33 ºC during some part of the day. These values are within the range of temperatures without negative impact on net photosynthesis, although would promote an increase in water loss through transpiration. However, in the summer, air temperature was maintained for several hours at 36-37 ºC, which seems to be the tolerance limit for coffee genotypes of *C. arabica* and *C. canephora* under ambient [CO_2], whereas transpiration was promoted (Chapter 1).

Although the optimum mean annual temperature range for *C. arabica* is 18-21ºC, selected cultivars have been satisfactory established under intensive management conditions in marginal regions with average temperatures as high as 24-25ºC, such as in the northeast of Brazil (DaMatta, 2004; DaMatta and Ramalho, 2006). In addition, *C. canephora* showed the highest growth rate when the mean growing season temperature increased from 21 to 27.5 ºC (Partelli et al., 2013). Others reported strong thermal sensitivity of coffee photosynthesis at temperatures above 25 °C (Nunes et al., 1968; Kumar and Tieszen, 1980). However, this strong thermal sensitivity of photosynthesis is not usually observed under field (DaMatta and Ramalho, 2006). Work conducted by Carelli et al. (1999) reported satisfactory photosynthesis and stomatal conductance values for leaf temperatures up to 34 ºC. Although the data were measured on single leaves, the results obtained were similar to the present study, where satisfactory photosynthetic rates were observed up to 33 °C (Figures 2 and 3). Furthermore, under environmental controlled conditions and high relative humidity (75%), *C. arabica* cv. Icatu and *C. canephora* cv. Clone 153 maintained photosynthetic rates without strong negative impacts up to 37 ºC (Chapter 1).

In fact, in Catuaí the net canopy photosynthesis rates for most part of the diurnal period in spring (4 to 5 µmol CO_2 m^{-2} s^{-1}) were in the range obtained for single leaf photosynthesis measurements, which reported values ranging from 4 up to 11 µmol CO_2 m^{-2} s^{-1} (DaMatta and Ramalho, 2006). However, the values observed in summer (mostly below 3 µmol CO_2 m^{-2} s^{-1}) could already denote some heat impact. On the other hand Clone 02 did not show changes on A_c (and E_c), likely related to its known higher heat tolerance (DaMatta and Ramalho, 2006).

Catuaí had higher A_c than Clone 02 plants in both seasons probably related to canopy architecture with a more open habit that resulted in a higher

PPFD penetration into the canopy (Figure 6), and in a higher C-gain at the whole plant scale (Glenn and Puterka, 2007; Zhu et al., 2010). From the light response curves, greater light distribution inside canopy of Catuaí trees resulted in higher maximum photosynthesis and higher apparent quantum efficiency (ϕ), the latter in the range of values found in Catuaí submitted the different N-nutrition and high irradiance (Nunes et al., 1993). Therefore, the higher A_c values in Catuaí were likely related primarily to the lower leaf density and, to a lesser extent, a greater branch angle measured in these plants (Figure 3A and Table 2).The higher A_c values further resulted in higher daily accumulated photosynthesis (Table 1). In contrast, the low A_c in Clone 02 (particularly in spring) can be associated with reduced light distribution within the canopy (Figure 6).

Higher g_c was observed in Catuaí during the spring, which resulted in increased E_c when compared to Clone 02. Single leaf studies documented similar stomatal conductance values ranging from 10 and 200 mmol H_2O m^{-2} s^{-1} (Barros et al., 1997). When midday air VPD exceeded *ca.*2 kPa, there was a decrease of g_c, and E_c (Figure 7), similar to the g_s reductions reported in coffee plants when air VPD reached *ca*. 2.2 kPa under field conditions (Barros et al., 1997),. During the summer the E_{ca} decrease was likely related to water availability and transport. Although plants were irrigated, increased air VPD imposed a greater environmental water demand, which was not accompanied by an equivalent replacement, since coffee plants have a low leaf hydraulic conductivity compared other tropical plants (Martins et al., 2014c; Nardini et al., 2014). Under conditions of high irradiance/temperature and high atmospheric VPD, the high transpiration rate can lower leaf water potential and produce a similar diurnal A_c and E_c hysteresis, presumably via partial stomatal closure, even in plants that are well supplied with soil water (Bunce, 1990). This would indicate that in some situations plants would be unable to meet the evaporative demand of the atmosphere (Bunce, 2006) and this response is probably linked to the decline of Catuaí plants gas exchanges in summer. Clone 02 g_c declined in the summer, however, similar A_c and E_c values were observed for both spring and summer (Figure 3) demonstrating a relative tolerance to supra-optimal temperatures in this species (DaMatta and Ramalho, 2006).The stomatal sensitivity to evaporative demand appears to be weaker in *C. canephora* than in *C. arabica* since, in the latter, stomatal conductance decreases in a curvilinear manner with increasing leaf-to-air

vapour pressure deficit (Gutierrez et al., 1994; Figure 7).

The relationship between A_c and E_c indicated that Catuaí had a greater instantaneous WUE_c than Clone 02 in the spring (Figure 4A), and there were no significant differences between species in summer (Figure 4B). WUE_c results from both seasons were within the range of 2.1 to 2.2 mol CO_2/mol H_2O found in the fourth and last completely expanded leaf of *C. arabica* (Carvalho et al., 2014), and similar to 2.1µmol CO_2/mmol H_2O observed in *C. canephora* grown under high water and nitrogen availability conditions (DaMatta et al., 2002).

Catuaí had greater whole-canopy gas exchange as well a greater sensitivity to high temperature than Clone 02, similar to reported by DaMatta and Ramalho (2006,) but contrary to reports of relevant thermal tolerance of genotypes from both species up to 37 ºC (Chapter 1). Data reported in the present study support the mechanism that A_c decreases in Catuaí were linked to decreases in g_c. Some key enzymes of the Calvin cycle, as well as the thylakoid electron transport were not likely affected in *C. arabica* and *C. canephora* when temperature was increased from 25/20 ºC (day/night) up to 37/30 ºC (Chapter 1).

Dark respiration was linearly related to the daily A_c rate (Figure 7) as also observed in other plants (Raghavendra et al., 1994; Hoefnagel et al., 1998). This indicated a biochemical link between the two processes and suggests that respiration can be limited by substrate availability (Atkin and Tjoelke, 2003; Atkin et al., 2005).

Although the multi-chamber system worked suitably, the need to clarify some points remains. First, the amount of incident light on the coffee trees was only one third of that which occurs naturally under field conditions, which may have contributed to reducing the gas exchange in Clone 02, and therefore contributing to a poor light penetration within the canopy. Second, there was a remarkable reduction of A_{ca} in Catuaí (*ca*. 28%) during summer. During this season, fruit filling occurs which will increase the photosynthetic demand and, consequently, the rate in producing plants, what was not the case in our young non-yielding plants. Thus, it is likely that the reduction of photosynthesis during summer in reproductive Catuaí could present a lower magnitude since photosynthesis can likely increase from the strengthening of the reproductive sinks (Vaast et al., 2005). Finally, it's well documented that *C. canephora* has a higher yield potential than *C. arabica* under field conditions in low altitude areas which

contradicts somewhat the measured gas exchanges. However, Clone 02 had lower respiration rates, which might result in higher photoassimilate availability to growth (data not showed) and yield. Thus, pruning *C. Canephora* to improve canopy light distribution could increase yield (Morais et al., 2012; Verdin-Filho et al., 2014).

In summary, our data showed whole-canopy gas exchange data from a multi-chamber system demonstrated that: 1) the canopy architecture of Clone 02 imposed a limitation to whole-canopy gas exchange linked to light distribution within the canopy and 2) Catuaí had greater heat sensitivity in the summer than Clone 02, and this response was linked to g_c decreases and the subsequent decrease of A_c and Rd_c. Thus, pruning systems need to be developed to mitigate the effects of lower light distribution within the canopy of Clone 02 and take advantage of the species heat tolerance. Conversely, Catuaí is a productive species with an architectural habit that promotes increased canopy light distribution but the species lacks heat tolerance.

6. CONCLUDING REMARKS

Under controlled conditions, coffee plant has the ability to adjust the photosynthetic machinery to increased atmospheric [CO_2] that are expected to occur in the near future during this century. Regarding temperature, coffee trees present a certain tolerance, occurring relevant physiological/biochemical damages only above 37/30 ºC (Chapter 2). *C. arabica* and *C. canephora* genotypes showed similar or even higher A values at 37/30 ºC (day/night), compared to their control (25/20ºC), when relative humidity was maintained at 75%. The photosystems were highly heat tolerant at both the physical (energy capture) and photochemical (electron transport) levels, when the electron transport (including or not OEC), chlorophyll *a* fluorescence parameters and A*max* values at 37/30 ºC were similar to or even higher than the control. However, under a fluctuating environment conditions, rising temperature may lead to increases in air VPD, decreasing both SD and g_s, during warming season, especially in *C. arabica*. however without damages to photochemical pathway as observed by A_{max}, Fv/Fm, PI and membrane permeability at leaf–scale (Chapter 1). Concerning whole-plant, similar pattern was observed with declines in g_c during warming season (Chapter 3). Although such decrease in gc resulted in lower A_c during summer in *C. arabica*, both species showed reduced iWUEc during summer, corroborating with marked reduction in iWUE at leaf-scale in coffee genotypes grown under controlled conditions at elevated temperature. However, their counterparts grown under elevated [CO_2] presented higher iWUE at leaf-scale at all temperatures (Chapter

2), as well as *C. arabica* under a fluctuating environment conditions during summer (Chapter 1). Elevated [CO_2] strongly alleviated the effects of high temperature on both species, particularly at 42/34ºC, and coffee genotypes did not down-regulate photosynthesis since leaves and shoot presented continuous growth. Otherwise, C-assimilation, photochemical and biochemical functioning were improved at all temperatures (Chapter 2). These changes likely contributed to prevent an energy overcharge in the photosynthetic apparatus, eventually reducing the need for energy dissipation and PSII photoinhibition. Impacts on photosynthesis at 42/34 ºC resulted from multiple impairments, particularly in RuBisCO and Ru5PK, which were the most sensitive photosynthetic components. In general, responses were genotype-depend, although in *C. canephora* several parameters remained unaffected even at 42/34 ºC, mostly at growth [CO_2] (Chapter 2), corroborating with A and gs values at leaf-scale (Chapter 1) and even with unaffected A_c, E_c and g_c values at whole-plant scale (Chapter 3), further reinforcing heat-tolerance of *C. canephora.* Although Catuaí had greater heat sensitivity, its canopy architecture promotes increased light distribution within the canopy, whereas Clone 02 showed higher heat-tolerance than Catuaí but limited canopy light distribution, so that pruning systems need to be developed to mitigate the effects of lower light distribution within their canopy.

In summary, when the relative humidity remained at 75%, coffee genotypes showed the ability to cope with greater temperature than that previously reported, showing satisfactory photosynthetic performance up to 37/30ºC. Additionally, enhanced CO_2 that is predicted to occur in this century can mitigate the effects of temperature on physiological traits even at 42/34 ºC. However, when the coffee genotypes experience increased VPD linked to elevated temperature, decreases in canopy and leaf photosynthetic rates occur due to reduced canopy and conductance stomatal, respectively, even under well-watered conditions.

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