ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

Department of Biological, Geological, and Environmental Sciences - BiGeA

Masters Degree in

Scienze e Gestione della Natura

Curriculum

Global Change Ecology & Sustainable Development Goals

Biochemical and molecular responses to supra-optimal temperatures and the potential mitigating effect of long-term elevated [CO_2] in *Coffea arabica* L.

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Session III

Academic year 2020-2021

ABSTRACT

Coffee is one of the most traded commodities of the world, grown in more than 80 tropical countries, but the ongoing climate change is expected to threaten coffee crop and productivity. This study aims at identifying coffee plant antioxidant and protective mechanisms under supra-optimal temperatures and the possible mitigating role of elevated atmospheric [CO_2] on heat stress. Potted plants of *Coffea arabica* cv. Geisha3, Marsellesa and Hybrid (Geisha3xMarsellesa) were grown at 25/20 °C (day/night), under 400 or 700 µL CO_2 L^{-1}, without water, nutrient, or root development restrictions, and then gradually subjected to temperature increase up to 42/30 °C, followed by a recovery period. The results showed a relevant heat resilience up to 37/28 °C regardless of genotype and [CO_2], suggesting that *Coffea spp*. can cope with temperatures well above what was previously believed. Heat tolerance was supported by an increase of photoprotective pigments (lutein, neoxanthin, and carotenes), antioxidant enzyme activities, such as ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT), and the upregulated expression of genes encoding for protective molecules (*HSP70*, *ELIP*, *Chape20*, *Chape60*). The further temperature rise up to 42/30 °C provoked an overall increase in transcript levels, GR and CAT activities, and de-epoxidation state (DEPS), with a concomitant decrease in several carotenoids, especially in Marsellesa, indicating a possible impairment of the photosystem components. Under enhanced [CO_2] such a drop in photoprotective pigments was not recorded. In addition, the lower upregulation of gene expression and lower activity of antioxidant enzymes (mainly CAT) found under high [CO_2] may suggest a possible reduction of energy overcharge in the photosynthetic apparatus. Although complementary studies are needed, coffee plants under elevated atmospheric [CO_2] could sustain better photosynthetic performances even under the predicted temperature increase.

TABLE OF CONTENTS

1. INTRODUCTION

1.1. COFFEE CROP AND PRODUCTION

Coffee is one of the largest tropical commodities in the world, being the economic basis of many countries, generating an income of *ca*. U.S. $ 173.000 million for the entire chain of value (DaMatta et al., 2018). It is one of the most popular beverages, consumed by about one-third of the world's population (DaMatta et al., 2019). It is estimated that involves about 100 million people from cultivation to the final product for consumption (Pendergast, 2010). Around 25 million farmers produce coffee, where most of the production is based on families and smallholders in low- and middle-income countries (Martins et al., 2017). The world annual production has been above 8 million tons of green coffee beans in the last years, reaching 10 million tons in 2018 (FAOSTAT, 2019; Fig. 1).

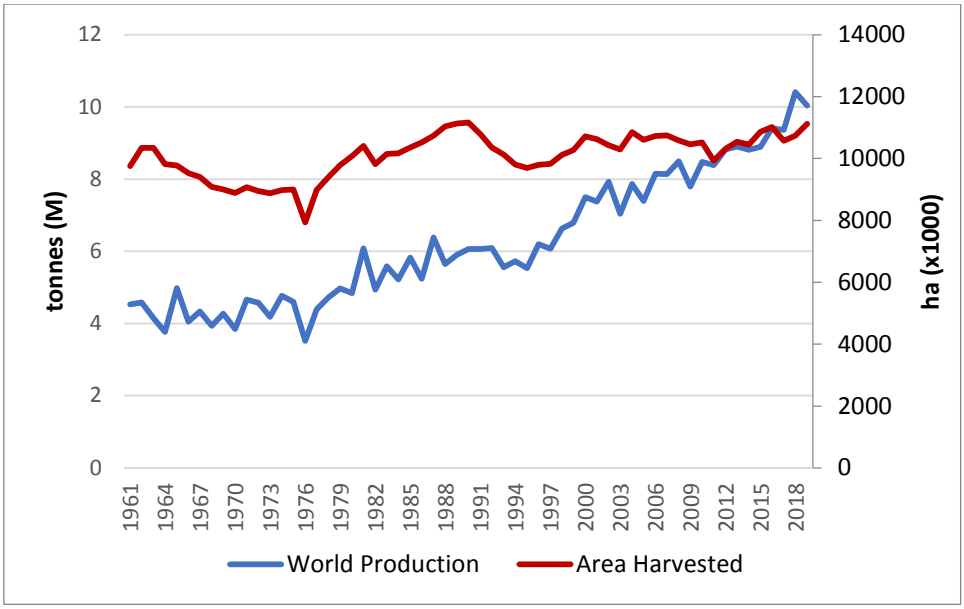


Figure 1: World Coffee (green) production and harvested area (FAOSTAT, 2019)

Coffee is produced in the tropical region of Africa (e.g., Uganda and Ethiopia), Asia (e.g., India, Indonesia, Vietnam), and America (e.g., Brazil, Colombia, Honduras), where Brazil is the world greater coffee producer, with more than 3 million tons (FAOSTAT, 2019; Fig. 2).

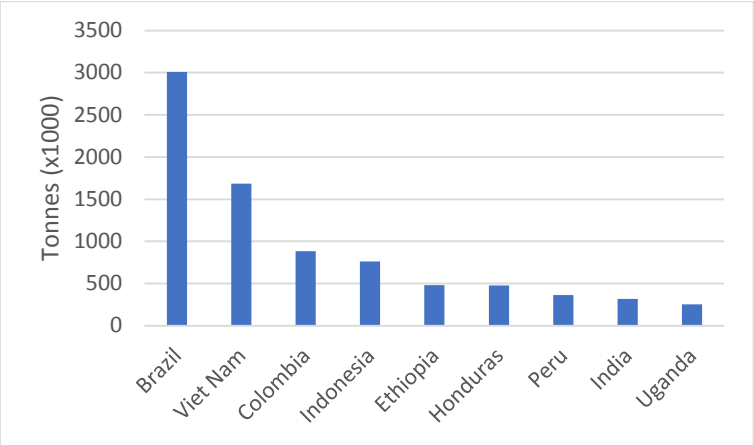


Figure 2: Top 2019 coffee producers (FAOSTAT, 2019)

Coffee market and demand grew significantly in the last 30 years: the global production (in volume) increased by more than 60% since the 1990s, especially in Asia and America, mainly due to the rise of coffee consumption (ICO, 2020). However, since 2016, a downward trend of the prices started to occur, caused primarily by the oversupply (ICO, 2020).

The world coffee consumption has risen in the last decade, reaching 1.19 kg/capita/year. Coffee is mainly an export product, since only 30% of the production is consumed in the producing countries (ICO, 2020). The main import countries in 2019 were USA, Germany, Italy, and France (Fig. 3), with a consumption of 4.46, 6.04, 5.54 and 5.23 kg/inhabitant/year respectively (FAOSTAT, 2018). The largest part of coffee is exported as green beans in the higher income regions (mainly EU and North America), which are responsible for the processing of coffee and the further export of roasted and soluble coffee (ICO, 2020).

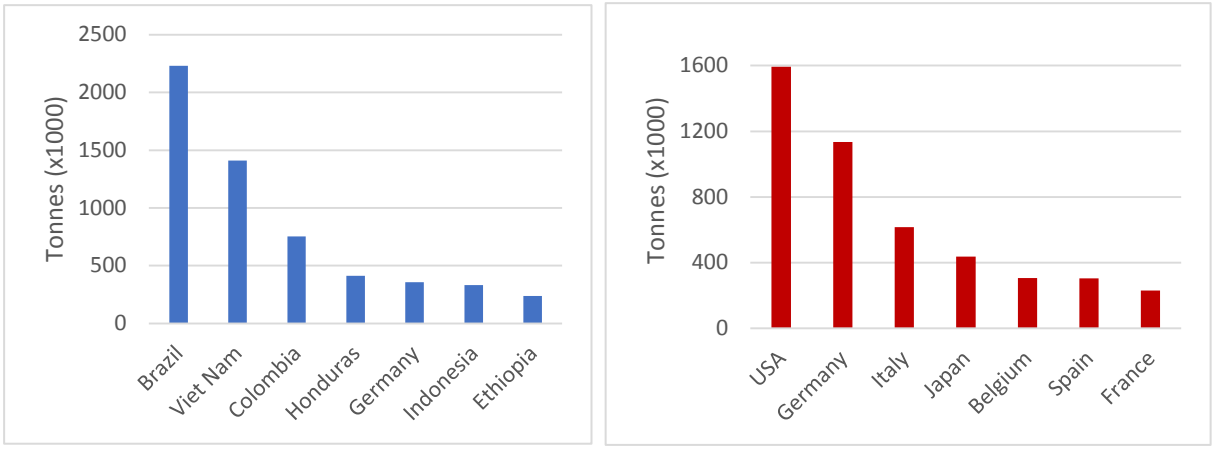


Figure 3: 2019 Export (left) and Import (right) quantity of coffee, green (FAOSTAT, 2019)

The coffee plant is an evergreen perennial C3 woody species belonging to the botanical genus *Coffea* of the Rubiaceae family. The coffee plant evolved in the understory of African tropical

forest, in south Abyssinia, and it is considered a shade-adapted species (Batista et al., 2012). It is a plant with a shrubby appearance, tall around 3 m to a maximum of 10 m, but generally maintained lower under cultivation. Branches can be distinguished between orthotropic branches, if they grow vertically, and plagiotropic/lateral branches, that grow horizontally. The inflorescence grows in the lateral branches, usually in plants of 3-4 years, turning into an oval drupe fruit. A coffee plantation usually has an average lifespan of *ca*. 30 years, but it can be productive more than 50 years (Bunn et al., 2015). It is grown in approximately 80 tropical countries, from South and Central America to Africa and Southwest Asia regions, extending from a latitude of 20-25° N in Hawaii down to 24° S in Brazil (DaMatta et al., 2018), mainly due to ecological constrains related to temperature and humidity (DaMatta & Ramalho, 2006).

Among all the 125 species of the *Coffea* genus, only two species are responsible for almost all of the world's coffee bean production: the allotetraploid *C. arabica* L. (arabica coffee; 2n = 4x = 44) and the diploid *C. canephora* Pierre ex A. Froehner (robusta coffee, 2n = 2x = 22). Around 60-70% of the global production is from arabica type of coffee, and the remaining 30-40% is from the robusta type of coffee. (Marques et al., 2021)

The most important cultivars of *C. arabica* are "Catuaí" and "Mundo Novo", while for *C. canephora* there are two "branches" related to "Robusta" and "Conilon" (also called Kouillou), the latter particularly found in Brazil (DaMatta & Ramalho, 2006). Additionally, breeding programs developed interspecific hybrids between the two coffee species to combine the organoleptic quality of *C. arabica* and the higher stress tolerance of *C. canephora*, generating important cultivars such as "Icatu".

C. arabica naturally grows in the highland forests of Ethiopia, at altitudes of 1600-2800 m above sea level (a.s.l.), where the average temperature is around 20°C (DaMatta et al., 2018). Currently it is cultivated mainly in Brazil, the largest coffee producer, but also in most of the coffee producing countries.

C. canephora, instead, is native to the lowland forests of the Congo River basin up to Lake Victoria in Uganda, with an altitude that varies from sea level up to 1200 m (DaMatta et al., 2018), thus growing in a lower altitude area than *C. arabica*.

The optimal annual mean temperature range is usually 18-21 °C for arabica cultivars and 22-26 °C for robusta coffee, with the latter being considered more adaptable to higher temperature than arabica coffee, since it can sustain temperatures up to 30 °C (DaMatta & Ramalho, 2006), although this can vary between species and genotypes.

Variation in the optimal range of temperature can affect the physiology and thus the growth of the plant. With mean temperatures above 23 °C fruit ripening of arabica cultivars is accelerated, leading to loss of quality, while higher temperature and dry seasons can cause flower abortion (Martins et al., 2014). However, the present arabica cultivars can grow in marginal regions, as in the northeast of Brazil, where the mean annual temperature can reach 25 °C. Temperatures below 17-18 °C can strongly decrease the crop growth, especially in robusta coffee, which is usually more susceptible to low temperatures (DaMatta & Ramalho, 2006).

The optimum annual rainfall range is 1200-1800 mm for arabica coffee, while robusta can adapt also to intensive rainfall exceeding 2000 mm. The demand largely depends on the characteristics of the soil, the cultivation practices, and the air humidity. Usually, a dry season of 3-4 months could occur, restraining vegetative growth, but being important to stimulate flowering for both species. An extreme condition of abundant rainfall and lack of a dry period can provoke lower yields (DaMatta & Ramalho, 2006).

Since the shaded plantations have usually lower yield due to the lower carbon assimilation, modern cultivars have been selected to have higher yields under full sunlight, and in some areas, shading is an abandoned practice. On the other hand, it is important to highlight that agroforestry systems (AFS) can have many beneficial effects, including the increase in biodiversity and cup quality (the quality of the beverage obtained after roasting), protection against environmental stresses, such as wind and high radiation. Consequently, shading could be an important practice in the present marginal areas, as well as an alternative to mitigate for future expected altered environmental conditions in a context of climate changes (DaMatta & Ramalho, 2006).

Arabica coffee, compared to robusta coffee, is generally less productive and less tolerant to biotic stresses, but the cup quality obtained from their beans is considered higher (DaMatta & Ramalho, 2006). For this reason, arabica prices are often twice the ones of robusta, which is also less expensive to produce. Nevertheless, a high-quality robusta coffee also exists, and it is used mainly in espressos or in blends with arabica coffee (ICO, 2020). In fact, the quality of robusta coffee has increased during the last years, as a result of the selection of new cultivars and better agronomic practices (Santos et al., 2015).

1.2. THE IMPACT OF CLIMATE CHANGES

Global change is one of the most alarming and critical phenomena of this century, which is altering ecosystems at local and global scale. It includes not only climate change but also air pollution and the diffusion of invasive plant and animal species. Climate changes are largely driven by the steady rise of greenhouse gases in the atmosphere, namely carbon dioxide, which increased almost by 50% since the pre-industrial times, from 280 µL L^{-1} in 1750 to the current levels that exceed 400 µL L^{-1} (DaMatta et al., 2019). Over the past 800,000 years, natural oscillations of atmospheric [CO_2] occurred following the ice ages, varying between 180 µL CO_2 L^{-1} during glacial periods and 280 µL CO_2 L^{-1} during interglacial periods (Martins et al., 2014), thus the ongoing increase is far beyond natural and excessively rapid. Anthropogenic activities strongly contributed to the [CO_2] increase, mostly associated with the use of fossil fuels since the eighteenth century, together with land use changes and deforestation. Agricultural activities are also particularly responsible for the rise of greenhouse gases, contributing for 1/3 of the CO_2 emissions, together with N_2O and CH_4 production (Dubberstein et al., 2018). Presently, atmospheric [CO_2] continues to increase at a rate close to 2 µL CO_2 L^{-1} per year, and it is estimated to reach values between *ca*. 450 and 600 µL L^{-1} by 2050 and between 730 and 1020 µL L^{-1} by 2100, depending on the greenhouse gas emission scenarios and measures that might be applied to counteract such rise (Santos et al., 2015).

Over the same period, the increase in atmospheric [CO_2] is estimated to be accompanied by the increase in the mean global temperatures, since greenhouse gas emissions are a major cause of global warming. Therefore, temperatures are expected to rise concomitantly with the [CO_2] increase, between 0.3-1.7 °C (best scenario) and 2.6-4.8 °C (worst scenario, without additional efforts to limit the emissions), relative to 1986-2005 (DaMatta et al., 2019).

In addition, the increase in global mean temperature can alter the water vapor retention capacity by the air, thus modifying the air humidity and the oceanic circulation. Global warming has a severe impact on the whole ecosystem, causing alteration of the precipitation patterns, melting of glacial ice, and rising of the sea level (Dubberstein et al., 2018). In fact, long-term climate changes are associated with climate anomalies and changes both in the frequency and the severity of extreme events, such as heat strokes and cold waves, longer and extreme droughts, floods and unpredictable rainfalls.

1.2.1. Plants and environmental stresses

As sessile organisms, plants cannot move to more suitable areas and avoid stressful environmental conditions. Facing these rapid climate changes, plants are exposed to new abiotic stresses and with greater intensity when compared to the natural environment in which they evolved (DaMatta et al., 2018). These alterations pose serious challenges on the agricultural sustainability, one of the human activities most vulnerable to global change, since the environmental alterations could decrease the crop quantity and quality usually achieved under the optimal growing conditions for a specific crop. These constraints may compromise world food security, causing food shortage especially in the most vulnerable countries, considering also that the increasing world population will require increased food production (Dubberstein et al., 2018). Climate changes are already becoming a threat in many tropical and subtropical countries, where the decrease in water availability and the increase of extreme events and new pressures are threatening farmers' livelihoods (IPCC, 2007).

Coffee crop and yield, especially in *C. arabica*, is strongly influenced by climatic variability. With the increase of global mean temperatures, the coffee industry might have to face serious challenges in the future (Davis et al., 2012), with negative consequences for the entire supply chain, especially the smallholders living in countries where coffee production is the main income. Considering also that coffee crop is a perennial crop, which requires 3-4 years to produce fruit and 5-8 years to reach the maximum reproductive potential (Davis et al., 2012), it is even more important to predict the future consequences of climate changes. Coffee production has already been affected in the past by severe droughts and high air temperatures, such as in the years 1962 and 1964 (Santos et al., 2015). Arabica cultivation in Ethiopia is predicted to be affected by climate changes, which will cause a decrease in the yield, especially in those areas that are already marginal nowadays, and, therefore, require additional improvement regarding management practices such as irrigation (Davis et al., 2012). According to the Global Agro-Ecological Zones (GAEZ) datasets, in the next 30 years 75% of available land suitable for arabica and 63% for robusta cultivation will be lost (ICO Report, 2020), leading to migration of coffee cultivation in higher latitudinal and altitudinal areas or even the abandonment of the production (Bunn et al., 2015). In Kenya, for instance, the present optimal areas for coffee cultivation that are located between 1400 and 1600 m.a.s.l. are projected to migrate to 1600-1800 m.a.s.l. by 2050 (Ziska et al., 2018). In Brazil and other countries, the suitable areas for coffee growth are expected to decrease as temperature rise

since yields would decrease too much. Future losses are expected to happen also in Mexico and other Central America's countries (Santos et al., 2015).

1.2.2. Drought and heat stress

If the optimal environmental conditions for the plant, including temperature, irradiation, water availability and mineral nutrition are changed, plant physiology would be impaired, causing a reduction or significant loss of yield, depending on the stress severity and on genotypes/species sensitivity.

Heat and drought stresses are one of the major environmental constraints to plant growth and productivity (Dubberstein et al., 2020).

Agricultural drought can be defined as an "extended period of deficient precipitation resulting in extensive damage to crops and yield losses" (Semedo et al., 2018). Drought events are one of the major threats to agricultural production. They are projected to increase in frequency and intensity in many agricultural areas, due to alteration of rainfall patterns, leading to physiological and metabolic impairments in plants (Avila et al., 2020). As referred, depending on the duration and the intensity of the stress, as well as on species tolerance, water deficit can cause a reduction in plant growth, nutrient uptake, photosynthesis and ultimately crop yields. Drought events may increase the leaf-to-air vapor pressure deficit (VPD) and limit the gas exchanges of plants (Avila et al., 2020). In fact, one of the first and major responses to drought stress is the stomatal closure, allowing the plant to avoid further water losses through transpiration. However, stomatal closure decreases the availability of CO_2 to carboxylation action of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), consequently reducing the photosynthetic performance, the production of sugars, and, therefore, the energy availability that sustains plant metabolism and growth. Coffee leaf stomata are known to be sensitive to both soil water availability decrease and air evaporative demand rise, due to an inherent low stomatal conductance; if the leaf water potential declines, the stomatal conductance (g_s) decreases as well (DaMatta et al., 2018). For instance, DaMatta et al. (2018) reported that as the leaf-to-air vapor pressure deficit (VPD) increases from 1.0 to 3.0 kPa, leaf stomatal conductance decreases by 60%.

Heat stress can be defined as the rise in temperature over the plant optimum for a period that is sufficient to damage plant development irreversibly; the produced damage is a function of the intensity and duration of the stress (Wahid et al., 2007). Supra-optimal temperatures can

affect several physiological processes and impact plants growth and productivity. Among heat impacts, membrane fluidity can be altered, since membrane lipid layer become more fluid, influencing cellular homeostasis and the capacity of pigments to capture the light (Dubberstein et al., 2020). At moderately high temperatures, cell damages, ion leakage and protein denaturation and aggregation can occur.

Photosynthesis is one of the most affected physiological processes under high temperatures. The activity of photosystems, especially PSII, which is more thermolabile, was shown to be reduced under high temperatures, due to alterations in thylakoid membranes (Wahid et al., 2007). In fact, heat stress can lead to dissociation of various photosystem components, such as the D1 protein and the oxygen evolving complex (OEC), impairing the thylakoid electron transport activity. Moreover, the biosynthesis of chlorophylls can be reduced, as a result of lipid peroxidation (Mathur et al., 2014). Furthermore, the metabolism of enzymes is impaired with supra-optimal temperature, and the affinity of RuBisCO for CO_2 declines, thus decreasing the carboxylation to oxygenation rate of the enzyme. In fact, heat stress stimulates photorespiration more than photosynthesis (DaMatta et al., 2019).

Under field conditions heat and drought stress usually occur in combination, that is, high temperature is usually associated with reduced water availability. For this reason, plant responses to drought and heat stress are difficult to disentangle. Like drought, heat stress increases the air evaporative demand, altering the stomatal conductance of leaves and modifying the diffusion of gases (DaMatta et al., 2019). Since water vapor saturation level increase exponentially with temperature, higher leaf temperatures have a strong impact on transpiration rates. Consequently, CO_2 flux and assimilation rate will decrease, impacting ultimately photosynthesis and the Calvin cycle (DaMatta et al., 2019).

In coffee plants, extreme temperatures can ultimately reduce tree growth and impair flowering and fruit set processes. Abnormal flower development can occur, if plants are frequently exposed to temperatures higher than 35 °C, or even flowering inhibition if temperatures are even higher, leading ultimately to a decrease in bean production and, consequently, yield reduction. Additionally, high temperatures can also affect coffee quality, since they accelerate the fruit maturation and reduce the accumulation of sucrose in the bean. Thus, the chemical composition, important for the beverage quality, can be altered, since the concentration of relevant compounds that contribute to taste, aroma, and flavor, is modified (DaMatta et al., 2018).

Plants have evolved several avoidance or acclimation mechanisms in order to survive under heat stress conditions. Short-term initial responses can include changes in leaf orientation, cooling trough increased transpiration rates, or progressive changes in the lipid composition of membranes (Wahid et al., 2007).

The maintenance of adequate lipid fluidity is essential for the correct functioning of membranes and cell homeostasis. Under heat stress plants can change the structure of the membrane, such as the lipid and protein components and the fatty acid saturation, with higher fatty acid saturation levels promoting a greater membranes insensitivity to lipoperoxidation (Santos et al., 2015).

Another level of response to heat stress is usually observed at the modification of expression of genes encoding for protective and stress-related proteins, such as the heat shock proteins (HSPs) and other molecular chaperones. The expression of *HSPs* genes has been observed in all organisms, from bacteria to humans (Wahid et al., 2007). *HSPs* overexpression can improve photosynthesis and membrane stability, increasing the plant tolerance under heat stress. These proteins can be subdivided into five classes according to their molecular weight: Hsp100, Hsp90, Hsp70, Hsp60 and sHSPs (small heat shock proteins) (Wang et al., 2004). Among these families, HSP70 proteins have a fundamental role in heat stress response. They can be found in many cell compartments, such as mitochondria and chloroplasts. They prevent proteins denaturation and protect their stability by controlling proteins folding during their transfer to the final localization (Wahid et al., 2007).

1.3. OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE

Living organisms must cope with oxidative stress from about 2.7 billion years ago, when oxygen appeared and accumulated in the atmosphere due to oxygenic photosynthesis. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ($^{*}O_2^{-}$), hydroxyl radical ($^{*}OH$) and singlet oxygen ($^{1}O_2$), are highly reactive species containing oxygen produced as natural by-products in the organisms during various metabolic pathways such as photosynthesis, photorespiration, and respiration. Its production should be kept under control, but the over production (and the occurrence of oxidative stress as a secondary stress) is a common consequence of the onset of environmental stress conditions, what can promote severe negative impacts in cell metabolism and structures of plants.

1.3.1 Thylakoid reactions and Z-scheme

Photosynthetic organisms use sunlight energy to synthesize C-skeletons that are then used for energy and other compound synthesis in the entire cell metabolism. The most active photosynthetic tissue in the plants is the leaf mesophyll. Mesophyll cells contain a large number of chloroplasts, where all the photosynthetic components are, such as pigments (chlorophylls and carotenoids, which are fundamental molecules specialized in absorbing light at different wavelengths), thylakoid electron carriers (e.g., plastoquinone and cytochromes), enzymes from the Calvin Cycle (e.g., RuBisCO).

Light energy is captured by the pigments from the antennae of two fundamental units called photosystems (PSI) I and II located within the thylakoid membranes. The two photosystems are interconnected by an electron transport chain (ETC), in which energy flows as an electron transport chain, along a series of molecules acting as donors and acceptors (Fig. 4). The general process of photosynthesis is a redox reaction in which electrons are removed from a chemical species, oxidizing it, and are given to another molecule, reducing it. At the end, most of the electrons reduce $NADP^{+}$ to NADPH. Additionally, part of the electron transport chain is also coupled with the transfer of protons (H^{+} ions) from the chloroplast stroma to the inner part of the thylakoids, thus creating an electrical (+) and chemical (H) gradient across the membrane, which will be used to produce energy as ATP through the action of the ATPase complex (Taiz et al., 2013).

When chlorophyll (Chl) absorbs a photon, the pigment switch from its basal state to a higher energy level or excited state (Chl^*). Excited chlorophylls have 4 different alternative ways of dissipating the energy and return to the basal state:

- Excited chlorophyll can emit a photon and return to its ground state, emitting fluorescence at a higher wavelength. Chlorophyll pigments emit fluorescence in the red-light region of the spectrum.
- Excited chlorophyll can go back to its basal state by directly converting the excitation energy in the form of heat, without the emission of photons.
- Chlorophylls can transfer the energy to another pigment.

Pigments of the LHC (light harvesting complex or antenna) of each PS transmit physically the light energy until they reach the reaction center (P_{680} and P_{700}), where two special pairs of chlorophyll *a* are able to transfer the electrons. In the reaction center of PSII, the excited chlorophylls quickly lose electrons to pheophytin (Pheo), and the oxidized P_{680} is reduced again by Yz (a tyrosine residue within protein D1), which received the electrons from water oxidation. Pheophytin, in turn, transfers the electrons to plastoquinone Qa and Qb. The electrons are passed to the Cytochrome b_6f complex, which then transfers them to plastocyanin (PC), and then to PSII. A series of Fe-S proteins transfers the electrons to the soluble ferredoxin (Fd), and finally, the flavoprotein ferredoxin-NADP reductase (FNR) reduces $NADP^+$ to NADPH, which, together with ATP, will be used in the Calvin Cycle to reduce CO_2 and produce C3 molecules that are thereafter used to sugar synthesis (CH_2O) (Taiz et al., 2013).

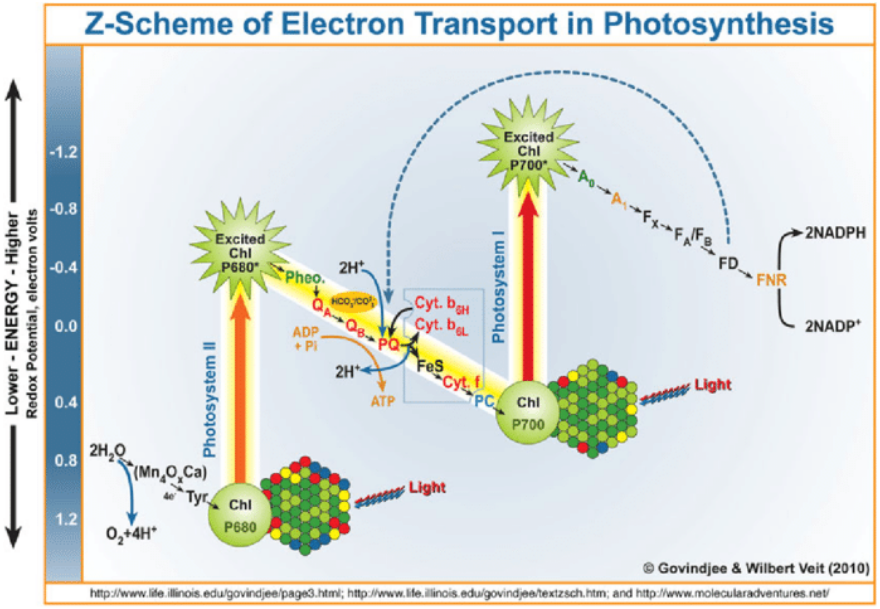


Figure 4: Z-Scheme of Electron Transport Chain (from Govindjee & Wilbert Veit, 2010)

1.3.2. ROS production and oxidative stress

In the chloroplast, molecular oxygen can accept electrons, thereby generating superoxide radical ($^*O_2^-$), the precursor of most other ROS:

$O_2 + e^- \rightarrow {}^*O_2^-$

$^*O_2^-$ is mostly produced during the non-cyclic electron transport in the ETC of chloroplasts and especially in PSI, being an alternative occurrence of the electron transfer from ferredoxin to $NADP^+$.

A superoxide radical, which does not have a long half-life, can receive an electron to another superoxide radical, forming hydrogen peroxide (H_2O_2) after protonation (Gill & Tuteja, 2010):

$2\ H^+ + 2\ {}^*O_2^- \rightarrow H_2O_2 + O_2$

Hydrogen peroxide, differently from other ROS, has a relatively long half-life. H_2O_2 can cause enzyme inactivation due to the oxidation of thiol groups (Gill & Tuteja, 2010). In fact, H_2O_2 has been recorded to cause photosynthesis inhibition, due to the oxidation of enzymes involved in the Calvin Cycle (Dumanović et al., 2021). However, at lower concentrations, H_2O_2 can also act as an important signaling molecule that activate plant responses upon the submission of plants to several stresses, such as gene expression.

Hydrogen peroxide in turn may be partially reduced to hydroxyl radical (${}^*OH$), one of the most highly reactive oxygen species, that may cause extensive impairments, among others, associated with lipid peroxidation and DNA damage:

$H_2O_2 + e^- \rightarrow HO^- + {}^*OH$

The production of ROS in plants is something unavoidable, hence plants have evolved several mechanisms to keep the balance between ROS production and scavenging. However, environmental stresses can lead to an excess of ROS levels in the cells which become out of control by plants. Most of the environmental stresses can cause an increase in ROS production. Drought, heat stress, salinity, chilling, nutrient deficiency, metal toxicity, UV radiation, but also biotic stresses such as pathogen attacks can indirectly provoke oxidative stress (DaMatta & Ramalho, 2006). If ROS increase dramatically, this may result in significant damage for the plant. ROS can damage all cellular structures, such as proteins (enzyme inactivation), nucleic acids (DNA breaks, mutations) and fatty acids in membranes, leading ultimately to cell apoptosis (Gill & Tuteja, 2010; Dumanović et al., 2021). Poly-unsaturated fatty acids (PUFAs) are one of the major targets of ROS, since they contain multiple double bonds with methylene bridges. Lipid peroxidation (LPO) can alter membrane fluidity and cause the production of lipid-derived radicals, generating an autocatalytic process and aggravating the oxidative stress (Gill & Tuteja, 2010).

The major source of ROS production in plant cells are chloroplasts (during photosynthesis), mitochondria (during respiration) and peroxisomes (associated with photorespiration). In chloroplast, oxygen is produced from H_2O, the first electron donor in photosynthesis. Oxygen

can accept electrons by triplet chlorophyll, photosystem PSI and PSII and by the electron transport chain (Gill & Tuteja, 2010).

The energy transfer from the chlorophylls of the light harvesting complex to the reaction center is not always 100% efficient. In some cases, the energy is not transferred to the next pigment but to oxygen, generating reactive species. Molecular oxygen, at its triplet state (in which the electrons have the same quantum number), is not very reactive and not toxic to aerobic organisms. To acquire higher reactivity O_2 requires an input of energy. The overexcitation of photosystems and the increase of reduced acceptors, such as plastoquinone, results in a surplus of excited chlorophyll $^1Chl^*$, which in turn can lead to the formation of chlorophyll in triplet state $^3Chl^*$. If oxygen reacts with $^3Chl^*$, it switched from triplet to singlet state $^1O_2$, a reactive species that can damage photosystems and their related proteins (Hassan et al., 2021), such as the D1 protein and causing pigment bleaching (Dumanović et al., 2021). This can happen because the energy normally directed to $NADP^+$ is in excess and it is not photochemically used, as occurs during stress conditions such as heat, drought, and salt stress (DaMatta & Ramalho, 2006). For instance, when stomatal closure occurs, the availability of CO_2 to the carboxylation sites is quite limited and C-assimilation is reduced due to a slowdown of the Calvin Cycle (DaMatta & Ramalho, 2006). In such circumstances, if it is not dissipated, the fraction of excitation energy that is not used in photochemistry will increase, what may result in serious damages, causing photoinhibition (Hassan et al., 2021).

Among the two photosystems, PSII is the usually more prone to be damaged, in comparison with PSI, which can be better protected by mechanisms such as the cycling electron transport/flow (CEF). Moreover, it is reported that an increase in the intracellular levels of H_2O_2 and $^1O_2$ can inhibit PSII repair damaged by photoinhibition, such as the synthesis of the D1 protein (Murata et al., 2007, Fig. 5).

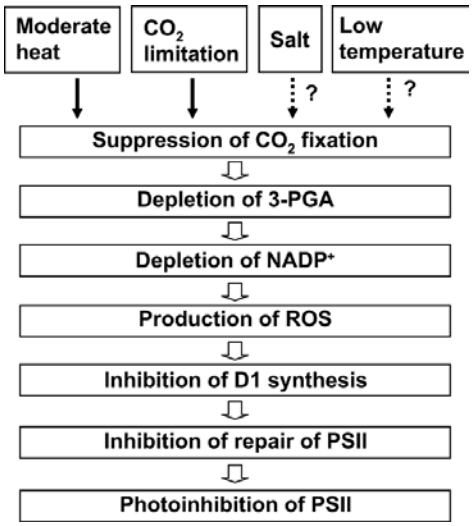


Figure 5: Hypothetical scheme of photoinhibition of PSII during various stresses (from Murata et al., 2007)

ROS production, and especially H_2O_2, can also take place in particular organelles, the peroxisomes, where H_2O_2 is generated by the oxidation of glycolate during photorespiration. Photorespiration increases under heat and drought stress, since stomata closure decreases the CO_2/O_2 ratio in mesophyll cells, leading to an increase in the oxygenation over the carboxylation of RuBisCO.

The extent to which ROS accumulate in plant cells is determined by the antioxidative defense mechanisms, which include the activity of enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and those involved in the ascorbate-glutathione cycle (also known as Halliwell-Asada pathway), but also non-enzymatic antioxidants, such as ascorbate, glutathione, α-tocopherol, xanthophylls, and flavonoids (Martins et al., 2016). In fact, the reinforcement of this antioxidative components was previously associated with increased plant stress tolerance to various abiotic and biotic stresses.

1.3.3. Enzymatic antioxidants

Among the enzymes involved in the antioxidant response, superoxide dismutase (SOD, E.C. 1.15.1.1) is an intracellular metalloenzyme which catalyzes the dismutation of superoxide into H_2O_2 and O_2, decreasing the risk of $^{*}OH$ formation (Dumanović et al., 2021), with the following reaction:

$$2\ H^{+} + 2\ {}^{*}O_2^{-} \rightarrow 2\ H_2O_2 + O_2$$

where one $^{*}O_2^{-}$ is reduced to H_2O_2 and the other is oxidized to O_2.

Plants possess several SOD isoforms, encoded by more than one gene and located in different cellular compartments. Three SOD isozymes are well known, which differ in the metal cofactor attached to the active site: Cu/Zn-SOD (in cytosol, peroxisome, and chloroplasts), Mn-SOD (in mitochondria) and Fe-SOD (in chloroplasts). It is well demonstrated that the enhanced stress tolerance can be related to the overproduction of SOD, which can be considered one of the first lines of defense against ROS accumulation, that is after ROS production (Gill & Tuteja, 2010).

In turn, catalases, enzymes containing heme groups, are able to eliminate H_2O_2 by decomposing it into water and oxygen:

$H_2O_2 \rightarrow 2\ H_2O + 1/2\ O_2$

Catalase (CAT, E.C. 1.11.1.6) is important to eliminate H_2O_2 produced in peroxisomes during photorespiration. There are several CAT isoforms that are differentially regulated and expressed. CAT efficiency in response to metal, drought, salt stress and high light conditions is demonstrated (Gill & Tuteja, 2010).

Another important antioxidant enzyme that also scavenges H_2O_2 is ascorbate peroxidase (APX, E.C. 1.1.11.1), mainly found in plants and algae. APX has higher affinity for H_2O_2 than CAT, and uses ascorbic acid (AA) as a reducing equivalent to reduce H_2O_2 to form water and dehydroascorbate (DHA):

$H_2O_2 + AA \rightarrow 2\ H_2O + DHA$

There are several isoforms of APX, with the names according to their cellular location, namely cytosol (APX-$_{Cyt}$), chloroplast (APX-$_{Chl}$), stromal (sAPX) and thylakoid (tAPX) forms. Correlation between APX overexpression and stress tolerance has been reported in several plants and for different environmental stresses; enhanced activity was found under drought stress in some bean cultivars (Zlatev et al., 2006).

Ascorbate reductase participates to the ascorbate-glutathione cycle (ASA-GSH). This cycle is a series of reaction that detoxifies H_2O_2 (Fig. 6). It involves several enzymes and molecules such as ascorbate, glutathione, and NADPH. The main goal of the pathways is to eliminate H_2O_2 using enzymes, ascorbate and glutathione as controlling agents.

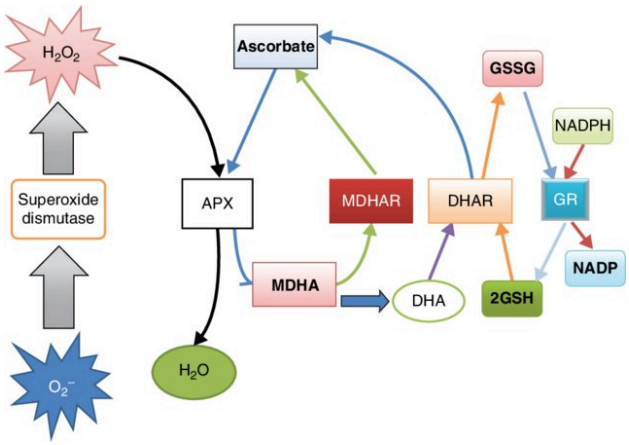


Figure 6: Schematic representation of ASA-GSH cycle (Banerjee & Roychoudhury, 2017)

Hydrogen peroxide is produced by superoxide dismutase to detoxify the superoxide radical. APX catalyzes the reduction of H_2O_2 producing water and using ascorbate as electron donor, which is oxidized into monodehydroascorbate (MDHA). Ascorbate can be regenerated from MDHA by monodehydroascorbate reductase (MDHAR); usually MDHA can also produce dehydroascorbate (DHA). Dehydroascorbate reductase (DHAR) catalyzes the reduction of DHA into ascorbate, using reduced glutathione GSH which is oxidized into GSSG. Glutathione reductase subsequently regenerate GSH from GSSG, using NADPH as a reducing equivalent (Banerjee & Roychoudhury, 2017).

Glutathione reductase (GR, E.C. 1.6.4.2) is a flavoprotein oxidoreductase found in many organisms, located especially in chloroplasts. It participates to the ascorbate-glutathione cycle (ASA-GSH), catalyzing the reduction of glutathione to the reduced form GSH, an important antioxidant molecule. In turn, GSH can be oxidized again by ROS and generate oxidized glutathione GSSG. Using NAD(P)H as reducing agent, the enzyme can regenerate GSH from GSSG:

$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$

The GSH-to-GSSG ratio can be used as a parameter to evaluate the state of the stress: the higher the better.

1.3.4. Non-enzymatic antioxidants

The main antioxidant molecules that play an important role in scavenging ROS are ascorbate and glutathione, as well as tocopherols, carotenoids, flavonoids and polyamines.

The reduced form of glutathione (GSH) is a ubiquitous antioxidant, present in both animals, plants, and microorganisms. It is localized in all cell organelles such as chloroplasts, peroxisomes, and mitochondria (Banerjee & Roychoudhury, 2017). Since it is very stable and soluble in water, GSH is considered the most important antioxidant, reducing the level of H_2O_2. Glutathione plays several roles in plant growth and metabolism, involved in signal transduction and expression of stress responsive genes, but also enzymatic regulation and cell differentiation (Gill & Tuteja, 2010). It is able to directly scavenge H_2O_2, $^{*}O_2^-$ and $^{*}OH$, and it plays a key role in the ASH-GSH cycle by contributing to regenerate ascorbate (Dumanović et al., 2021).

Ascorbic acid (ascorbate) is the most abundant and non-enzymatic antioxidant in plants, found in the cytosol and apoplast; high concentrations can be found in all plant tissues, especially in leaves and fruits. Ascorbate is a powerful antioxidant involved in the reduction of H_2O_2 together with APX, or it can directly scavenge $^{*}O_2^-$ and $^{*}OH$ radicals. Apart from its involvement in the ASA-GSH cycle, ascorbic acid also acts as a cofactor of violaxanthin de-epoxidase during the dissipation of the energy in excess (Gill & Tuteja, 2010).

α-tocopherol (vitamin E) is a lipophilic compound present in the thylakoid membranes. It is able to scavenge lipid radicals and ROS such as $^1O_2$ by energy transfer (Gill & Tuteja, 2010), playing an important role in the protection of PSII. The expression of tocopherol biosynthetic genes is reported to increase during oxidative stress.

1.3.5. Carotenoids

Carotenoids, also known as tetraterpenoids, are unsaturated pigments with conjugated double bonds, which give to them the ability to absorb energy (Bartley & Scolnik, 1995). They can be subdivided into two subclasses: hydrocarbon carotenes (such as α- and β-carotene), which are found both in the core complex and the LHC, and xanthophylls, which contain oxygen and are present only in the LHC subunits (Johnson et al., 1993). They are able to absorb wavelengths from 400 to 550 nm, in the blue-green light, and for this they are colored yellow, orange or red.

Carotenoids play an important role in regulating photosynthetic aspects. Together with chlorophyll, they participate in the capture of light energy at different wavelengths than chlorophylls, thus expanding the absorption spectrum (Bartley & Scolnik, 1995). As accessory pigments, they transfer the excitation energy from the light harvesting complex

(LHC) to the reaction centers. Apart from this, carotenoids such as β-carotene, lutein, and zeaxanthin, provide photoprotection of chloroplast structures by de-exciting $^{1}Chl^{*}$ states and though non-photochemical quenching (NPQ) of $^{3}Chl^{*}$, thus having an extremely important antioxidant function by dissipating the non-utilized excess of energy and preventing ROS formation (Johnson et al., 1993). They reduce the energy in excess by capturing the energy that triplet chlorophyll could transfer to oxygen, or by directly reacting with singlet oxygen (Dumanović et al., 2021).

When light increases (or the use of light energy is reduced) and electron transport becomes saturated, the pH decrease in the thylakoid lumen induces the conversion of the di-epoxide violaxanthin into the monoepoxide antheraxanthin and subsequently into the epoxide-free zeaxanthin, a reaction catalyzed by violaxanthin de-epoxidase (VDE) which requires O_2 and NADPH as cofactors (Fig. 7). Simultaneously, ascorbate is oxidized into dehydroascorbate (DHA) and reduced again into ascorbate through the ascorbate-glutathione cycle, which is highly interconnected with the xanthophyll cycle (Lambers et al., 2008).

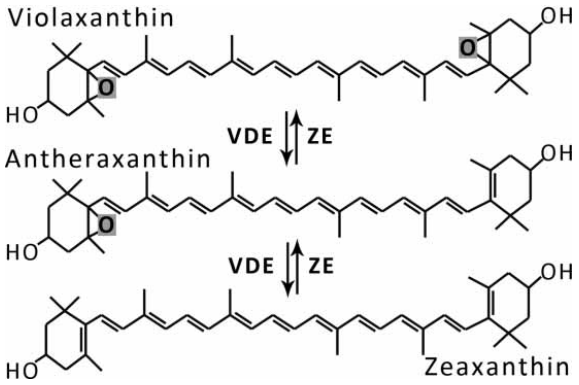


Figure 7: Xanthophyll cycle (VDE: Violaxanthin de-epoxidase; ZE: zeaxanthin epoxidase) (Latowski et al., 2011)

The xanthophyll cycle plays an important role in protecting plants from photoinhibition. Zeaxanthin, a hydrophobic pigment found at the periphery of the LHC (Wahid et al., 2007), is able to dissipate the energy directly by the de-excitation of chlorophylls (both singlet and triplet states), which prevent $^{1}O_2$ formation, and indirectly by inducing a conformational change in the PSII antenna, which facilitate thermal dissipation (Ramalho et al., 2000). Furthermore, zeaxanthin protects the thylakoid membranes from lipid peroxidation by decreasing the membrane fluidity (Wahid et al., 2007).

1.4. THE POTENTIAL ROLE OF ELEVATED [CO_2] IN THE MITIGATION OF HEAT STRESS IMPACTS

Photosynthetic C-assimilation is essential for the plant to produce biomass and grow. Plants can sense changes in atmospheric CO_2 concentration (C_a) and modify their physiological and biochemical processes, such as stomatal conductance, photosynthesis, and respiration (Ramalho et al., 2013). Plant responses to changes in [CO_2] are strongly species-dependent, but also in interaction with other environmental conditions, as well as nitrogen availability (Luo et al., 1999).

The current atmospheric CO_2 is still below the saturation level for photosynthesis in C3 crops. Consequently, plants can be positively affected by the rise in air [CO_2], directly through an increase in the net C-assimilation, whereas it will favor the competitive carboxylation (photosynthesis) over oxygenation (photorespiration), both performed by RuBisCO, what will further increase net photosynthesis while reduces H_2O_2 production (Martins et al., 2014; DaMatta et al., 2019).

Coffee plants usually display a moderately low photosynthetic rate (A) with respect to the other C3 plants, ranging from 4 to 11 μmol m^{-2} s^{-1}. This is mainly due to low stomatal conductance (g_s), since the maximum photosynthetic capacity (Amax), under saturating CO_2 and optimal light and temperature conditions, can reach as high as *ca*. 30 μmol m^{-2} s^{-1} (DaMatta et al., 2019). In such context, the enhancement of CO_2 concentration in coffee plants might increase photosynthetic rates more than what could occur to other plants (DaMatta et al., 2019)

In several plants different from coffee, it has been reported that elevated [CO_2] usually reduces stomatal closure, leading to a higher leaf temperature due to a reduction in transpiration flow (Fig. 8). Meta-analysis of experiments conducted with free-air CO_2 enhancement (FACE), where plants are grown under open-air conditions, reported that with a [CO_2] increase from *ca*. 366 to 567 μL L^{-1} air g_s decreased by 22% and light-saturated photosynthesis (A) increased by 31% in various C3 species (Ainsworth & Rogers, 2007). On the contrary, g_s in coffee leaves seemed to remain unchanged under high [CO_2], leading to

higher photosynthetic rates (A). Furthermore, leaf temperature is not expected to occur since the evaporative cooling is maintained through transpiration (DaMatta et al., 2018).

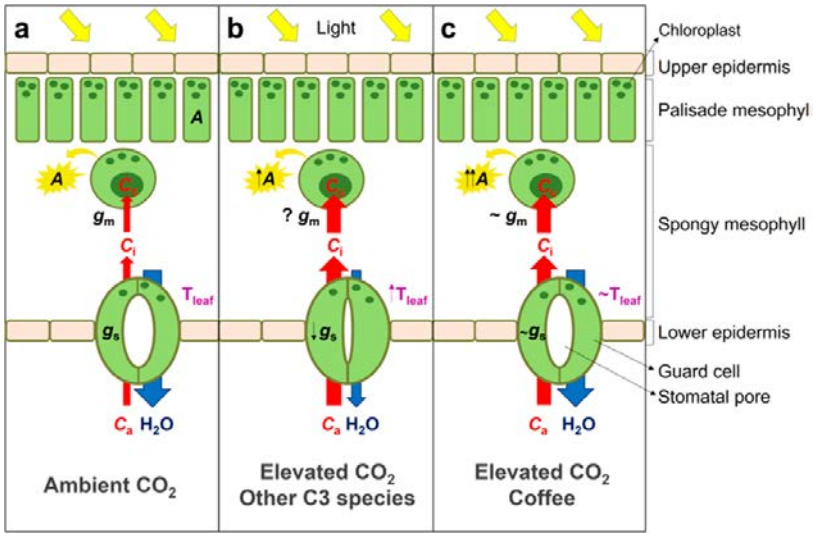


Figure 8: Schematic diagram of gas diffusion in different conditions (from DaMatta et al., 2018)

It has been recorded that the stimulation of CO_2 enrichment might be partially offset by a downregulation of photosynthesis, due to a lower sink strength that prevents plants from using the overproduced carbohydrates and to a variation in the allocation of assimilates to the photosynthetic components. On the other hand, high [CO_2] could decrease leaf nutrient concentrations, in a "dilution effect" that usually do not have negative implications to photosynthesis, as it was found in coffee (Ramalho et al., 2013; Martins et al., 2014). Coffee plants, however, seems be able to avoid the photosynthesis downregulation due to high capacity to continuously produce vegetative and reproductive structure, thus consuming the sugars resulting from the increased C-assimilation under elevated [CO_2]. In fact, the "mineral dilution" is believed to reflect qualitative physiological changes rather than nutrient deprivation, since coffee plants at higher [CO_2] displayed higher metabolic activity (Ramalho et al., 2013).

Several studies have reported the beneficial effect of CO_2 in mitigating the heat stress impact in some species, usually associated with reinforced protective mechanisms, as is also the case of coffee (Martins et al., 2016; Rodrigues et al., 2016). Enhanced [CO_2] could alleviate the negative impact of supra-optimal temperatures and counteract the increase in photorespiration, which is an energy cost for the plant (Martins et al., 2014). Furthermore, under elevated [CO_2] the optimal temperature level for leaf photosynthesis could increase (Wahid et al., 2007).

Classical studies on coffee plants suggested that above 20-25 °C photosynthetic performance is highly affected (Nunes et al, 1968). However, at least in some cases, other stress factors besides heat stress were present, such as high VPD. In recent experiments it was observed that coffee can tolerate heat stress and maintain the photosynthetic performance at temperatures up to 37/30 °C (day/night), especially under enhanced air [CO_2] (Martins et al., 2016). However, under temperatures of 42/34 °C plant performance was greatly affected, although in a lesser extent at elevated [CO_2] rather than ambient [CO_2]. Among the protective mechanisms, the antioxidant response seems to play a crucial role in providing heat stress tolerance in coffee plants until 37 °C, and the fall of these mechanisms at 42 °C might have been also associated with the greater physiological impacts observed at that temperature (Rodrigues et al., 2016; Semedo et al., 2018).

1.5. RESEARCH QUESTIONS AND THESIS OUTLINE

The aim of this study was to evaluate coffee plant capabilities to acclimate to supra-optimal temperatures in a context of climate changes, with a focus on the potential antioxidant mechanisms, and how these responses are affected by high CO_2 concentration. In particular, the work used biochemical and molecular approaches to study the effects of supra-optimal temperature and elevated [CO_2] on:

- Photosynthetic pigments concentration, in particular carotenoids
- Cellular activity of antioxidant enzymes
- Expression of genes related to antioxidant and other protective mechanisms

With the present investigation we attempt to understand how coffee plants respond to supra-optimal temperatures at ambient CO_2, if there are differences among ambient-CO_2 and elevated-CO_2 under control temperature, and how elevated CO_2 can mitigate the negative effects imposed by heat stress.

The research was made in collaboration with the Instituto Superior de Agronomía (ISA), University of Lisbon, thanks to a fellowship from ERASMUS+ program. The work received funding support from the European Union's Horizon 2020 research and innovation program (grant agreement No 727934, project BreedCAFS), and from national funds from Fundação para a Ciência e a Tecnologia, I.P. (FCT), Portugal, through the project PTDC/ASP-AGR/31257/2017, and the research units UIDB/00239/2020 (CEF), and UIDP/04035/2020 (GeoBioTec).

Coffee plant materials (cultivars Geisha, Marsellesa and their corresponding hybrid) was provided by Dr Hervé Etienne (Cirad-UMR DIADE, France) in the framework of the European Union's Horizon 2020 (H2020) research and innovation BREEDCAFS (Breeding Coffee for Agroforestry Systems, http://www.breedcafs.eu) project (2017-2021).

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL AND GROWTH CONDITIONS

Plants of three cultivated genotypes of *Coffea arabica* L., i.e., cv Marsellesa, cv. Geisha3 and their corresponding hybrid (Geisha3×Marsellesa) were used, using a similar experimental design as in Rodrigues et al. (2016), with minor modifications. Potted plants (20 L pots) were grown from the seedling stage for *ca.* two years in walk-in growth chambers (EHHF 10000; ARALAB, Portugal) under controlled environmental conditions of temperature (25/20 °C, day/night), irradiance (*ca*. 700-800 μmol m^{-2} s^{-1}), air humidity RH (75%), and photoperiod (12.30 h), and either ambient (400 μL CO_2 L^{-1} air, aCO_2), or elevated (700 μL CO_2 L^{-1} air, e CO_2) [CO_2] (Fig. 9).

Figure 9: Coffee plants in the walk-in growth chamber

For the entire experiment, the plants were kept well-watered plants, with predawn water potential higher than -0.3 MPa, with mineral nutrition provided as in Ramalho et al. (2013), and without space restrictions for root growth, as judged by visual examination at the end of the experiment after removing the plants from pots.

2.2. TEMPERATURE STRESS IMPLEMENTATION

Heat stress was progressively induced in order to allow plant acclimation, from 25/20 °C up to 42/30 °C at a rate of 0.5 °C day^{-1} (diurnal temperature), with 5-7 days of stabilization at 31/25 °C, 37/28 °C and 42/30 °C to allow for programmed data collection and evaluation. Subsequently, the temperature was readjusted to control (25/20 °C) and the plants were monitored over a recovery period of 4 (Rec4) and 14 (Rec14) days. The control conditions refer to plants grown at 25/20 °C and 400 μL CO_2 L^{-1}.

All determinations were performed on two newly matured leaves from the upper third part of 6-8 plants per treatment, at 25/20, 31/25, 37/28, 42/30 °C and at Rec4 and/or Rec14. The leaf material was flash frozen in liquid nitrogen and stored at -80 °C until analyses.

2.3. PHOTOSYNTHETIC PIGMENTS CHARACTERIZATION

2.3.1. Pigment extraction

Photosynthetic pigments were analyzed according to the methods described in Martins et al. (2016). In plant chloroplasts, pigments are associated to the membrane proteins that are parts of the photosystems PSII and PSI. To bring chlorophylls and carotenoids into solution, an extraction with a non-polar solvent, such as acetone, is needed. Chloroplast extracts contain a mixture of chlorophyll *a*, chlorophyll *b*, carotenoids, and other molecules.

Chlorophylls (Chl) and carotenoids (Car) were quickly extracted from 3 frozen leaf discs (0.5 cm^2 each) using an ice-cold mortar and pestle in cold 90% acetone. The samples were collected into 2-mL Eppendorf tubes, with a final volume of 1.5 mL, and kept for 30 minutes at 4 °C. The homogenate was then transferred for centrifugation (12000 *g*, 10 min, 4 °C). Subsequently, the supernatant was filtered using a Millipore nylon filter (0.45 μL).

2.3.2. HPLC determination of carotenoids

Chromatography is the science of separating chemical compounds from a sample in order to identify and subsequently quantify them. There are several techniques of chromatography, such as Gas Chromatography (GC), Liquid Chromatography (LC), High Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography (UPLC) etc.

HPLC is a column chromatography technique in which the compounds in the sample are separated into individual analytes as they pass through a column (Fig. 10). In this case analytes are separated according to their polarity.

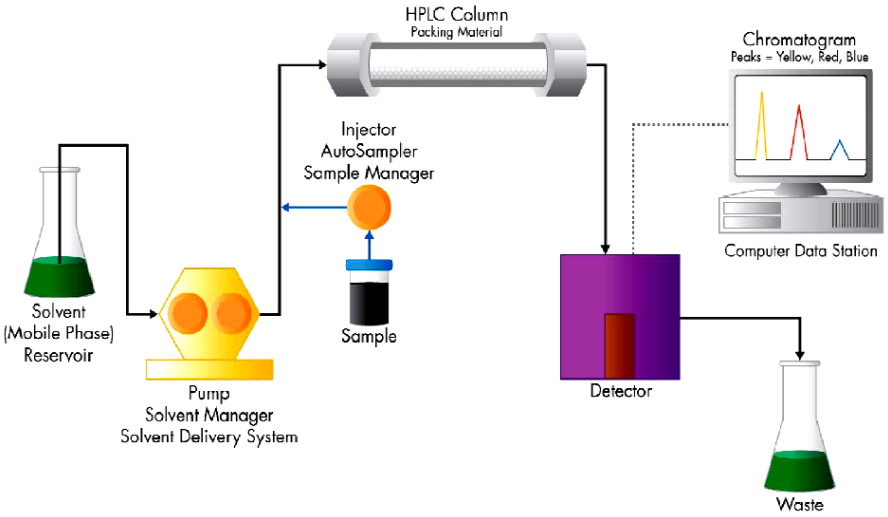


Figure 10: schematic diagram of High-Performance Liquid Chromatography (HPLC) system (from Water corporation, 2012)

As the sample passes through the column, a competition between the stationary phase and the mobile phase for different analytes occurs. This competition is set by having the mobile phase and the stationary phase with different polarities: the former is usually polar (e.g., H_2O), the latter is non-polar (e.g., C18). The more polar analytes will be more attracted to the mobile phase, therefore they will move faster and will elute first, while the more non-polar analytes will be more attracted to the stationary phase and will be more retained and come out last. Regarding the mobile phase used in this experiment, the polarity decreased in the following order: water, acetonitrile, triethylamine (solvent A), ethyl acetate (solvent B).

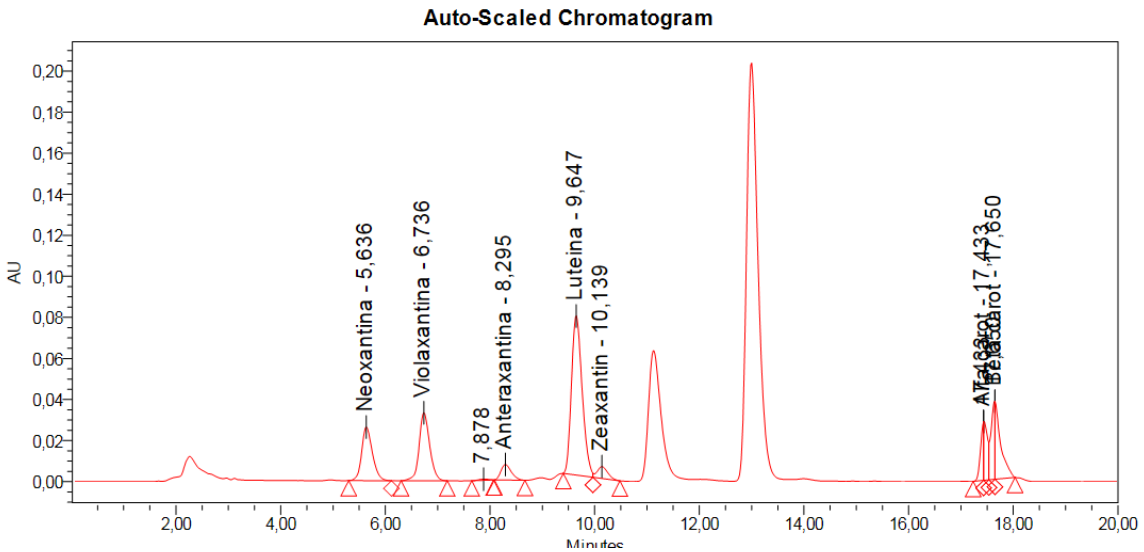


Figure 11: Example of a HPLC chromatograms. The x axis represents the retention time, and AU on the y axis represents the absorbance units at 440 nm.

Note that polar analytes, more attracted to the mobile phase, will be the first to elute (Fig. 11). α- and β-carotene, which are hydrocarbon carotenoids, differs substantially from xanthophylls, and will elute only after the increase of a more non-polar solution in the column (i.e., ethyl acetate).

For separation, identification and quantification of carotenoids, a reverse-phase HPLC was carried out according to the method optimized for coffee by Ramalho et al. (1997), with minor adjustments, using an end-capped (C18) 5-µm Spherisorb ODS-2 column (250 × 4.6 mm). The elution of the 20 µL injection volume for each sample was performed at 20 °C (20 °C column temperature, 4 °C sample temperature) over 30 min, with a flow rate of 1 mL min^{-1}. Carotenoids were separated with a polar to non-polar gradient, using a non-linear gradient of 25-100% ethyl acetate in acetonitrile/H_2O (9:1 [v/v], containing 0.1% triethylamine). The sequence of the procedure was: 0-10.50 min, linear gradient 75-25% ethyl acetate; 10.50-20 min, linear gradient 59-41% ethyl acetate; 20-21 min, linear gradient 0-100%; 21-30 min, isocratic with 100% ethyl acetate, in order to separate α and β-carotene.

Pigments were detected with a photodiode-array detector. The carotenoids were identified by comparing their retention time and absorption spectra with standards prepared under the same analytical conditions. Peak areas of each of the carotenoid standards were used to obtain the standard curve to quantify the carotenoids (See Annex). The identification of the various compounds in the sample is based on their retention time. It is known that for a given mobile phase and at a given flow rate with a given column, a pure standard of an analyte (e.g., lutein)

elutes at a certain time. Compound quantification is based on the measure of the area under the peak, which is related to how much analyte was on the sample. The integrated areas of carotenoids were calculated from the chromatograms, and results in mg L^{-1} were obtained using the standard curves. Giving the fixed proportion of 1 cm^2 (diameter of leaf discs) for 1 mL of solution, each mg m^{-2} of carotenoids were obtained by multiplying by 10 the results in mg L^{-1}. To obtain the value in mg g^{-1}, the value of the Specific Leaf Area (m^2 g^{-1} DW) of the leaf sampled, which varies according to the day and genotype and calculated from relative water content (RWC) determination for each temperature, was multiplied. The de-epoxidation state, involving xanthophyll cycle components was calculated as DEPS = (zeaxanthin (Z) + 0.5 antheraxanthin (A)) / (violaxanthin (V) + A + Z).

2.4. ACTIVITY OF ANTIOXIDATIVE ENZYMES

Enzyme activities were determined in chloroplast extracts, obtained using 1 g FW of leaf tissue from three plants per treatment (the enzyme activity assays were not performed for the 31/25 °C and Rec4 treatments). All the procedures were performed according to the method described in Ramalho et al. (2018) with some modifications.

A 100 mg sample of leaf tissue was weighed and placed in a 2-mL Eppendorf tube. For the enzymatic extraction I used 1 mL of buffer containing 200 mM Tris-HCl (pH = 8), 10 mM $MgCl_2$*$6H_2O$, 30 mM β-mercaptoethanol, 4 mM DTT, 2% Triton X-1, "Complete cocktail EDTA" (2 pills) and 10% Glycerol, adding 1% (1 mL) of polyvinylpolypyrrolidone (PVPP) to each sample in the homogenization phase. The samples were then centrifuged (13,000 *g*, 20 min, 4 °C). The supernatant was used to evaluate the enzymatic activities.

Enzyme activity was measured using a UV-Visible spectrophotometer, following the rate at which the reaction occurs, and thus the rate at which the absorbance changes over a period of time. During the assay, as the selected enzyme acts, the intensity of the light absorbed by the substrate will decrease, because its concentration will decline as the substrate will be converted into product.

The spectrophotometer will provide the absorbance values (OD) at different times (s). By dividing the absorbance change by time, using at least three time points, it is possible to know how much the absorbance of the sample changes as a function of time (OD s^{-1}). According to

the Beer-Lambert law, that correlates the light absorbance to the concentration, it is possible to calculate the enzyme activity (in μmol g^{-1} DW min^{-1}) using specific extinction coefficients ε for every reaction studied:

μmol g^{-1} DW min^{-1} = -1xOD s^{-1} x (1xε^{-1}) x (1000 μ^{-1} L^{-1} of sample extract) x (1xg^{-1} DW) x 60

2.4.1. Catalase activity

Catalase (CAT, EC 1.11.1.6) reacts with hydrogen peroxide and dissociates it into molecular oxygen and water:

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

The enzyme assay reaction mixture contained 40 mM H_2O_2 in 50 mM phosphate buffer (pH 7.8) and 10 μL of the enzyme extract in a total volume of 1 mL. In relation to the H_2O_2 consumption, catalase activity was estimated by reading the absorbance at 240nm, and calculated using an extinction coefficient of 3.94 mM^{-1} cm^{-1}.

2.4.2. Ascorbate peroxidase activity

Ascorbate peroxidase (APX, EC 1.11.1.11) reacts with ascorbate and H_2O_2 to produce dehydroascorbate:

$AA + H_2O_2 \rightarrow DHA + 2H_2O$

The enzyme reaction mixture contained 20 mM ascorbate and 0.1 mM H_2O_2 in 50 mM phosphate buffer (pH 7.8) and 10 μL of the enzyme extract in a total volume of 1 mL. Tubes were read at 290nm, and the enzyme activity was determined through the H_2O_2-dependent oxidation of ascorbate, using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for calculations (Ramalho et al., 2018).

2.4.3. Glutathione reductase activity

Glutathione reductase (GR, EC 1.6.4.2) catalyzes the reduction of the oxidized form of glutathione (GSSG) to the reduced form (GSH), using NADPH as a cofactor:

$GSSG + NADPH \rightarrow GSH + NADP^+$

The enzyme reaction mixture contained 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), 3 mM $MgCl_2$ in 50 mM phosphate buffer (pH 7.8), and 10 μL of enzyme extract in a total volume of 1 mL. GR activity was evaluated through the NADPH oxidation rate reading

the decrease in absorbance at 340nm, using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for calculations.

2.4.4. Superoxide dismutase activity

Superoxide dismutase (SOD; EC 1.15.1.1) catalyzes the conversion of the superoxide anion radical to hydrogen peroxide, as the following reaction:

$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

SOD assays are more technically complex than the other enzyme assays, due to the short life of the substrate and the difficulty to detect O_2^- (Ewing & Janero, 1995). For this, indirect SOD assays are often used, monitoring the change of an indicator substance that reacts with O_2^-, in this case adrenaline. The enzyme reaction mixture contained 20 mM adrenaline in 50 mM phosphate buffer (pH 7.8) and sodium carbonate buffer (pH 10.4) with EDTA 0.125 mM, and 50 μL of the enzyme extract in a final volume of 1 mL. The reaction starts with the addiction of 50 μL of adrenaline and tubes were read at 480nm.

2.6. EXPRESSION STUDIES OF SELECTED GENES

Genes related to the antioxidative system and other protective proteins were selected for the expression studies, based on Martins et al. (2016) and Ramalho et al. (2018).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) is a technique that allows the study of gene expression by quantifying the number of mRNA transcripts present in a sample. The technique is based on the amplification of a targeted cDNA (complementary DNA, produced with a reverse transcription) using specific primers. It is based on three fundamental stages repeated several times: denaturation and separation of the double chain, annealing of primers with the cDNA, extension, and polymerization by DNA polymerase. During the process, a certain quantity of fluorescence related to the amount of RNA transcript is emitted. The fluorescent signal is given by the presence of the SYBR Green detector in the reaction mixture, a fluorescent dye that binds to cDNA. As the PCR runs, the fluorescence will start to increase; samples with higher amounts of transcripts will produce a faster amplification curve, while samples with lower transcripts will delay the exponential phase (Fig. 12).

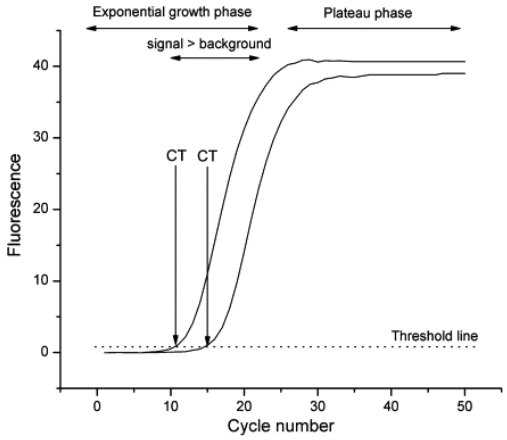


Figure 12: Real-Time PCR amplification curves (from Kubista et al., 2006)

Total RNA from the three genotypes (Geisha3, Marsellesa, Hybrid) x the two [CO_2] levels (aCO_2 and eCO_2) x 4 temperatures (25/20, 37/30, 42/34 °C and Rec14), was isolated from 100 mg of plant material and quantified as described in Marques et al. (2020), using the kit RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol, and digested with DNase I, an enzyme that catalyze the DNA hydrolysis, using an on-column Qiagen DNase set to remove putative genomic DNA. Total RNA concentration was checked with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA) to verify the RNA quality. This control step allows the identification of possible contamination, such as proteins, which absorbs at different wavelength compared to RNA.

To synthesize the first-strand cDNAs 1 µg of DNA-free total RNA was used, using oligo-(dT)18 primers and the SuperScript II first-strand synthesis system (Invitrogen, USA). Primer sequences are given in Table 1. The primers were dissolved in a volume of sterile distilled water that is 10x the amount of nmoles and diluted to give a concentration of 10 µM.

The qRT-PCR reactions were performed in 96-well plates using qPCR SYBR® Green Supermix (BioRad, USA) in an iQ™ 5 Real-Time Detection System (BioRad), using the following quantities in the plates:

- 1 µL samples
- 5 µL SYBR Green
- 1 µL FW (forward primers) and 1 µL RV (reverse primers)
- 2 µL water

The PCR program was as follows: hot start activation of the Taq DNA polymerase at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s; plate read.

To determine the specificity of the primer pairs, a melting/dissociation curve analysis was performed following the RT-qPCR experiment. To verify the specificity of each amplification, and the absence of primer dimers that could interact with SYBR Green, dissociation curves were obtained for each amplicon at the end of the PCR run, by continuous fluorescence measurement from 55° to 95°C, with sequential steps of 0.5°C for 15 s.

Three technical replicates were used for each sample. The mean Ct (threshold cycle) was used for data analyses, which is the cycle at which the amplification curve intercepts the threshold line. The full sample set was always included in each technical replicate to exclude any artifacts consequential of between-run variations. No signals were detected in non-template controls run in parallel for each primer set. Two negative controls were included for each primer pair, in which cDNA was replaced by water or total RNA.

Table 1: Selected genes used for real-time qPCR studies, related to protective mechanisms and/or oxidative stress control, primer sequences and amplicon size (bp).

Gene symbol	Gene description	Primer sequence (5'–3')	Amplicon size (bp)
HSP70	Stromal 70 kDa heat shock-related protein, chloroplastic	F: GGGAAGCAATTGACACCAAG	150
		R: AGCCACCAGATACTGCATCC	
ELIP	Chloroplast early light-induced protein	F: GCCATGATAGGGTTTGTTGC	101
		R: GTCCCAATGAACCATTGCAG	
Chape 20	Chloroplast 20 kDa chaperonin	F: GTTAAAGCTGCCGCTGTTG	150
		R: CTCACCTCCTTGAGGTTTCG	
Chape 60	Mitochondria chaperonin CPN60	F: GGATAGTGAAGCCCTTGC	80
		R: CCCAGGAGCTTTTATTGCAC	
CAT	Catalase isozyme 1	F: CTACTTCCCCTCGCGGTAT	150
		R: CTGTCTGGTGCAAATGAACG	
CuSOD1	Superoxide dismutase [Cu-Zn]	F: CCCTTGGAGACACAACGAAT	141
		R: GGCAGTACCATCTTGACCA	
CuSOD2	Superoxide dismutase [Cu-Zn]	F: GGGGCTCTATCCAATTCCTC	150
		R: GGTTAAAATGAGGCCCAGTG	
APX Cyt	Cytosol ascorbate peroxidase	F: TCTGGATTTGAGGGACCTTG	108
		R: GTCAGATGGAAGCCGGATAA	
APX Chl	Chloroplast ascorbate peroxidase	F: CACCTGCTGCTCATTTACG	100

		R: GACCTTCCCAATGTGTGTG	
APXt+s	Stromatic ascorbate peroxidase (sAPX) mRNA	F: AGGGCAGAATATGAAGGATTGG	112
		R: CCAAGCAAGGATGTCAAAATAGCC	
VDE2	Violaxanthin de-epoxidase	F: GGGTTCAAAATGCACAAGACTG	86
		R: CCCTCTTTTACCTCAGGCATTG	

2.7 STATISTICAL ANALYSIS

Data were analyzed using two-way ANOVAs ($P < 0.05$) to evaluate the differences between the two atmospheric [CO_2] and among the different temperature treatments, as well as their interaction, followed by Tukey's tests for mean comparisons. Each ANOVA was performed independently for each of the studied genotypes. The relative expression ratio of each target gene was computed based on its real-time PCR efficiency and the crossing point (CP) difference of a target sample *vs.* a control (25/20 °C, 400 µL CO_2 L^{-1} air) within each genotype. A 95% confidence level was adopted for all tests.

3. RESULTS

3.1. PHOTOSYNTHETIC PIGMENTS

Several carotenoid concentrations were obtained through HPLC determination. Figure 13 shows a representative chromatogram of an extract of *C. arabica* leaves. Mean values in mg g^{-1} DW and standard errors (n = 4-8) of each carotenoid (neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, α-carotene, β-carotene) for all treatments and genotypes are shown in the figures below. In addition, some indexes were also calculated: the sum of α- and β-carotene, the α- to- β-carotene ratio, the sum of violaxanthin, antheraxanthin and zeaxanthin (VAZ), total carotenoids, the VAZ pool to total carotenoids ratio, and the conversion state of violaxanthin into zeaxanthin (i.e., de-epoxidation state, DEPS).

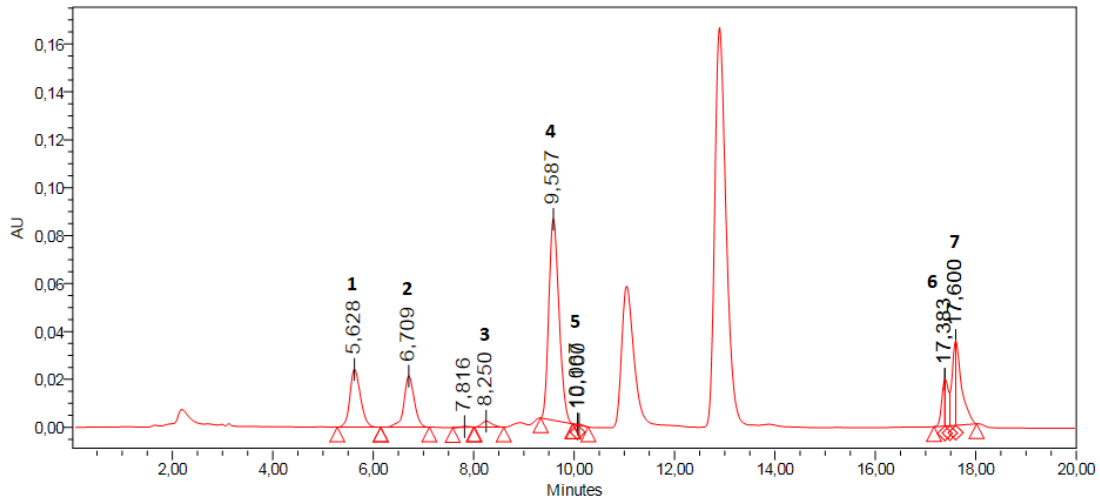


Figure 13: HPLC chromatogram of a *C. arabica* spp. leaf extract with detection at 440 nm. Identification of compounds: neoxanthin (1), violaxanthin (2) antheraxanthin (3), lutein (4), zeaxanthin (5), α-carotene (6), β-carotene (7). α- and β-carotene areas were calculated by multiplying each outer half peak x2.

3.1.1. Effect of CO_2 treatments at control temperature

At control temperature (25/20 °C), enhanced [CO_2] promoted a tendency toward higher contents of several carotenoids in Marsellesa plants (Fig. 15a, 15b). In fact, high [CO_2] increased by *ca*. 45% neoxanthin, violaxanthin, antheraxanthin and β-carotene contents; a great increase in lutein (53%), zeaxanthin (493%), VAZ (64%), DEPS (74%), and total carotenoids (54%) was observed too. On the contrary, for the other genotypes, no differences were observed between the two CO_2 treatments; for some carotenoids the values for plants grown under 700 µL CO_2 L^{-1} air ("700-plants") were even lower, such as the case of zeaxanthin (almost 40% less) in Geisha (Fig. 14a). In Hybrid, no large differences were observed for most of the carotenoids, except for β-carotene, which was lower under high [CO_2], and antheraxanthin, α-carotene, and consequently the α/β-carotene ratio, which were higher in the 700-plants (Fig. 16a, 16b).

3.1.2. Effects of increasing temperature at ambient CO_2 concentration

When temperature was increased from 25/20 °C to 31/25 °C, the general tendency of the plants at ambient [CO_2] is towards an increase in the content of several carotenoids, as indicated by the increase in total carotenoids in all genotypes. This is the case of neoxanthin, violaxanthin (although significant only in Geisha, with a 50% and 77% increase respectively) (Fig. 14a), but also of lutein (38% and 24% in Geisha and Hybrid, respectively), α-carotene (105% in Geisha) and β-carotene, and thus α+β carotene contents and the α/β-carotene ratios.

Regarding zeaxanthin, the response was genotype dependent: in Geisha, zeaxanthin content and DEPS decreased almost by 70%, whereas in Hybrid and Marsellesa no significant change was observed.

As temperature was raised further from 31/25 °C to 37/28 °C, pigment composition was differentially affected in the different genotypes. Total carotenoids content slightly increased in Marsellesa (Fig. 15b), while it decreased in Geisha, although remaining higher than control conditions (Fig. 14b). In fact, Marsellesa plants showed an overall increase of carotenoids compared to the values observed at 31/25 °C, especially for antheraxanthin (45%), zeaxanthin (230%), and DEPS (66%). In Geisha, on the contrary, a decrease in several carotenoids was observed, especially in violaxanthin (30% decrease) and β-carotene (with a consequent decrease in α+β carotene content and increase in α/β-carotene ratio). However, a strong increase in zeaxanthin content (206%) and DEPS (184%) was observed, while neoxanthin and antheraxanthin content did not change significantly. In Hybrid, no significant changes were observed (Fig. 16a, 16b).

When temperature reached the maximum value (42/30 °C), the overall content of most carotenoids dropped, reaching or even being lower than control conditions. This is the case of neoxanthin and violaxanthin (*ca*. 30% and 60% decrease respectively, compared to the values observed at 37/30 °C, with lower values than control conditions), antheraxanthin (only in Geisha) and thus the VAZ pool, although zeaxanthin contents increased relative to control, which led to a substantial increase in DEPS (314% in Marsellesa and 98% in Hybrid). Furthermore, α- and β-carotenes decreased too, especially in Marsellesa (62% and 52% respectively), thus reducing total α+β content (56% in Marsellesa). In addition, α/β–carotene ratio also decreased (63% in Geisha).

Surprisingly, the single pigment contents did not decrease when plants were checked after 4 (Rec4) and 14 days (Rec14) of recovery at control temperature, and sometimes they even increased from 42/30 °C, such as neoxanthin in Marsellesa (53% increase at Rec4). However, the overall carotenoids concentration returned to control conditions at the end of the experiment, with some exceptions. In Hybrid, for instance, antheraxanthin content was higher than control conditions even after 14 days of recovery (Fig. 16a), while in Geisha a 60% decrease relative to controls was observed (Fig. 14a). Violaxanthin content slightly increased in Geisha and Marsellesa from 42/30 °C to Rec14, but the final content was lower than the one at the beginning (40% decrease in Marsellesa, Fig. 15a). Zeaxanthin content at Rec4 and

Rec14 increased by 347% and 123% in Hybrid and by 860% and 720% in Marsellesa, compared to control conditions, whereas in Geisha the content reached almost zero at Rec14. In turn, DEPS, increased by 196% and 82% at Rec4 and Rec14 in Hybrid, and by 246% and 175% in Marsellesa, whereas a 73% decrease was observed in Geisha. In such conditions, the VAZ pool slightly increased at Rec4 in Marsellesa and Hybrid, but returned to control conditions at Rec14, whereas in Geisha it remained lower than that of 25/20 °C (Fig. 14a). Regarding lutein, its content increased from 42/30 °C, especially in Marsellesa (133%), reaching the highest values at Rec4 in all genotypes and decreasing at Rec14; in Geisha and Hybrid lutein content at Rec14 was higher than control conditions. On the contrary, after the recovery period, an overall decrease in carotenes content was observed: In Geisha and Hybrid, α-carotene at Rec14 was *ca*. 64% and 53% less than control condition, respectively, whereas in Marsellesa an increase of 54% was recorded from 42/30 °C to Rec4, although at Rec14 it decreased again. The variation of β-carotene was strongly genotype dependent: in Hybrid it slightly decreased at Rec4, in Geisha it remained stable, while in Marsellesa it increased remarkably and returned to control values at Rec14.

3.1.3. Interaction between high temperature and CO_2 enhancement

The differences between the two CO_2 treatments at control temperature were highly genotype dependent; however, when plants were exposed to supra-optimal temperatures, some general trends can be seen. Overall, when temperature was raised from 25/20 °C to 31/25 °C, while in the plants grown under 400 μL CO_2 L^{-1} air ("400-plants") the total carotenoids content increased, in the 700-plants it remained stable or even decreased slightly. In Geisha, enhanced [CO_2] decreased neoxanthin content by 42%, compared to ambient [CO_2]. Violaxanthin content increased only in Hybrid (26%, Fig. 16a). In Geisha-700, violaxanthin, which remained stable, was 43% less than the 400-plant counterparts (Fig. 14a), although an opposite trend was observed in Marsellesa (Fig. 15a). On the other hand, antheraxanthin content was not altered in Geisha-700, but high [CO_2] increased its content by 37% with respect to ambient [CO_2] (Fig. 14a); in contrast, in Marsellesa no differences between the two CO_2 treatments were observed, although antheraxanthin decreased from 25/20 °C to 31/25 °C (35%) in Marsellesa-700 (Fig. 15a). Zeaxanthin content decreased by 66% in Hybrid-700, differently from the 400-plant counterparts in which it slightly increased; consequently, DEPS was 64% less in the 700-plants compared to the 400-plants (Fig. 16a). However, an opposite trend was observed in Geisha, where, contrarily to the remarkable decrease observed in the 400-plants, plants under high [CO_2] did not alter zeaxanthin and DEPS from 25/20 °C to

31/25 °C; hence, they were more than double than in the 400-plants (Fig. 15a). In Marsellesa, however, even if zeaxanthin content almost halved under high [CO_2], no significant differences were observed in DEPS between the two CO_2 treatments at 31/25 °C (Fig. 16a). Lutein, which increased in the 400-plants of Geisha and Hybrid, remained almost unchanged in the 700-plant counterparts; consequently, enhanced [CO_2] decreased lutein content by 40% in Geisha (Fig. 14b) and 22% in Hybrid (Fig. 16b), whereas in Marsellesa-700 a slight increase was observed compared to the 400-plant counterparts (Fig. 15b). Regarding α-carotene, substantial differences can be observed in Geisha, since this carotene remained stable in the 700-plant: at 31/25 °C the pigment content in the 400- and 700-plants was 0.265 and 0.113 mg g^{-1}, respectively (Fig. 14b). The same trend can be observed in Marsellesa plants, where high [CO_2] decreased α-carotene content by 60% compared to ambient [CO_2] (Fig. 15b). In Geisha, β-carotene content, together with α+β content and a α/β-carotene ratio, differed significantly between the two CO_2 treatments: while a substantial increase was observed in the 400-plants, they remained almost unchanged in the 700-plant counterparts, being remarkably lower than the ones at ambient [CO_2] (Fig. 14b). An opposite trend occurred in Marsellesa, where β-carotene and α+β content remained higher in the 700-plants compared to the 400-plants, differently from α/β-carotene ratio which slightly decrease under high [CO_2] (Fig. 15b).

Nevertheless, an overall increase in pigment concentration was observed in the 700-plants when temperature increased to 37/28 °C, with a different response depending on the genotypes. For example, neoxanthin content slightly increased only in Geisha (Fig. 14a), while for the other genotypes it remained unchanged, maintaining the same difference from the 400-plant counterparts as the previous temperature (37% difference in Hybrid). Violaxanthin slightly decreased both in Marsellesa and Hybrid, but if in the former it remained higher than the 400-plants, in the latter was slightly lower. Antheraxanthin, in turn, increased in all genotypes, reaching the same values as the 400-plant counterparts. Zeaxanthin content slightly differed between the two CO_2 treatments in Marsellesa (Fig. 15a) and Hybrid (Fig. 16a). However, no substantial differences were observed in VAZ pool and DEPS between the 400- and 700-plants, although DEPS increased in Hybrid-700 (83%, relative to 31/28 °C), in contrast to the 400-plants which remained stable (Fig. 16a). α-carotene almost doubled in Geisha-700, reaching the same values as the 400-plant counterparts, differently from the high difference observed at 31/25 °C (Fig. 14b). On the other hand, in Marsellesa-700 α-carotene content, although it increased, remained lower at high [CO_2] (Fig. 15b).

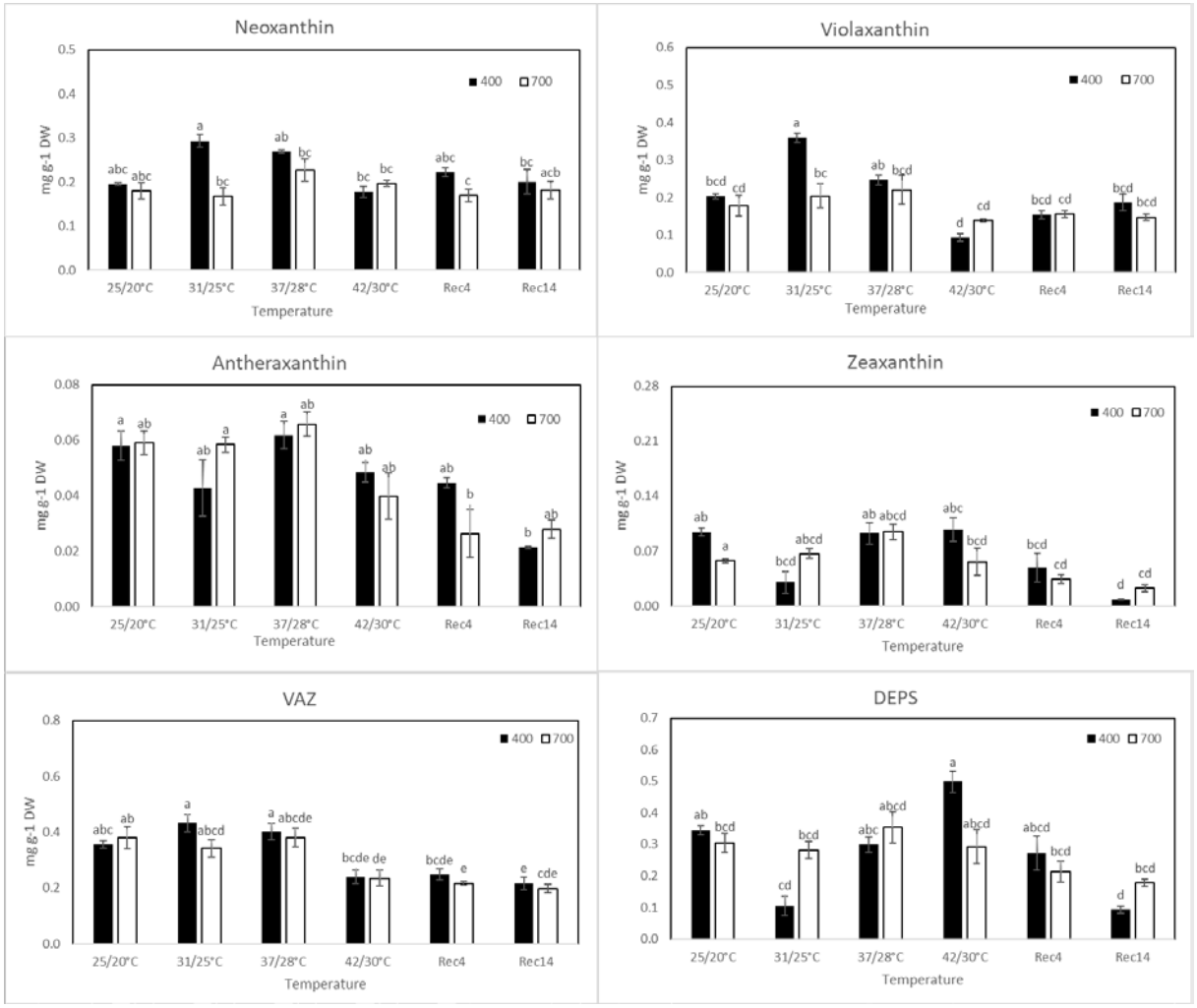
Similarly, an increase in β-carotene, together with α+β content and α/β-carotene ratio, was recorded in Geisha-700, thus reducing the difference between the two CO_2 treatments observed at 31/25 °C (Fig. 14b). However, α/β-carotene ratio slightly decreased in Hybrid-700, being lower than the 400-plant counterparts (Fig. 16b).

As temperature reached the maximum values of 42/30 °C, the variation in total carotenoids was not significant in the 700-plants, except in Geisha where a slight decrease was observed (Fig. 14b). Geisha-700 and Hybrid-700 reached similar values of the 400-plant counterparts, whereas in Marsellesa a higher carotenoid content was observed at high [CO_2] (Fig. 15b). However, some differences in the single pigment concentrations can be observed between the two CO_2 treatments. In Marsellesa and Hybrid, for example, enhanced [CO_2] allowed a greater content of neoxanthin compared to ambient [CO_2]. A similar trend occurred for violaxanthin, which decreased in Geisha-700 and Marsellesa-700, but not as much as the 400-plant counterparts. On the contrary, in Hybrid-700 a 32% decrease of antheraxanthin was observed, differently from the 400-plants where it increased (Fig. 16a). Zeaxanthin content increased by five times in Marsellesa-700, in contrast to what occurred in Geisha and Hybrid, where a decrease and a significant difference between the 400- and 700-plants was observed, especially in Hybrid. On the contrary, DEPS was markedly lower at high [CO_2], as it slightly decreased from 37/28 °C to 42/30 °C (except in Marsellesa in which it doubled), in contrast to the sharp increase in the 400-plants. In Marsellesa enhanced [CO_2] caused a 60% increase of VAZ pool compared to ambient [CO_2], whereas in the other genotypes it reached similar values than the 400-plant counterparts. Lutein content increased or remained stable in the 700-plants, in contrast to the decrease in the 400-plants, although only in Marsellesa a significant difference between the CO_2 treatment was observed (Fig. 15b). In Geisha-700, a decrease in β-carotene, and especially α-carotene, was observed at 42/30 °C, reaching similar values of the 400-plant counterparts, although α/β-carotene ratio was slightly higher in the 700-plants (Fig. 14b). Large difference between the two CO_2 treatments can be observed in the content of β-carotene and α+β of Marsellesa and α-carotene and α/β-carotene ratio in Hybrid, which were substantially higher in the 700-plants.

An opposite trend seemed to occur during the recovery periods: if under ambient [CO_2] a general rise in the concentration was recorded (at least at Rec4), under enhanced [CO_2] the overall carotenoids content decreased, especially in Hybrid (Fig. 16b). This is the case of carotenes, in which the values at Rec14 were lower than control conditions (except for

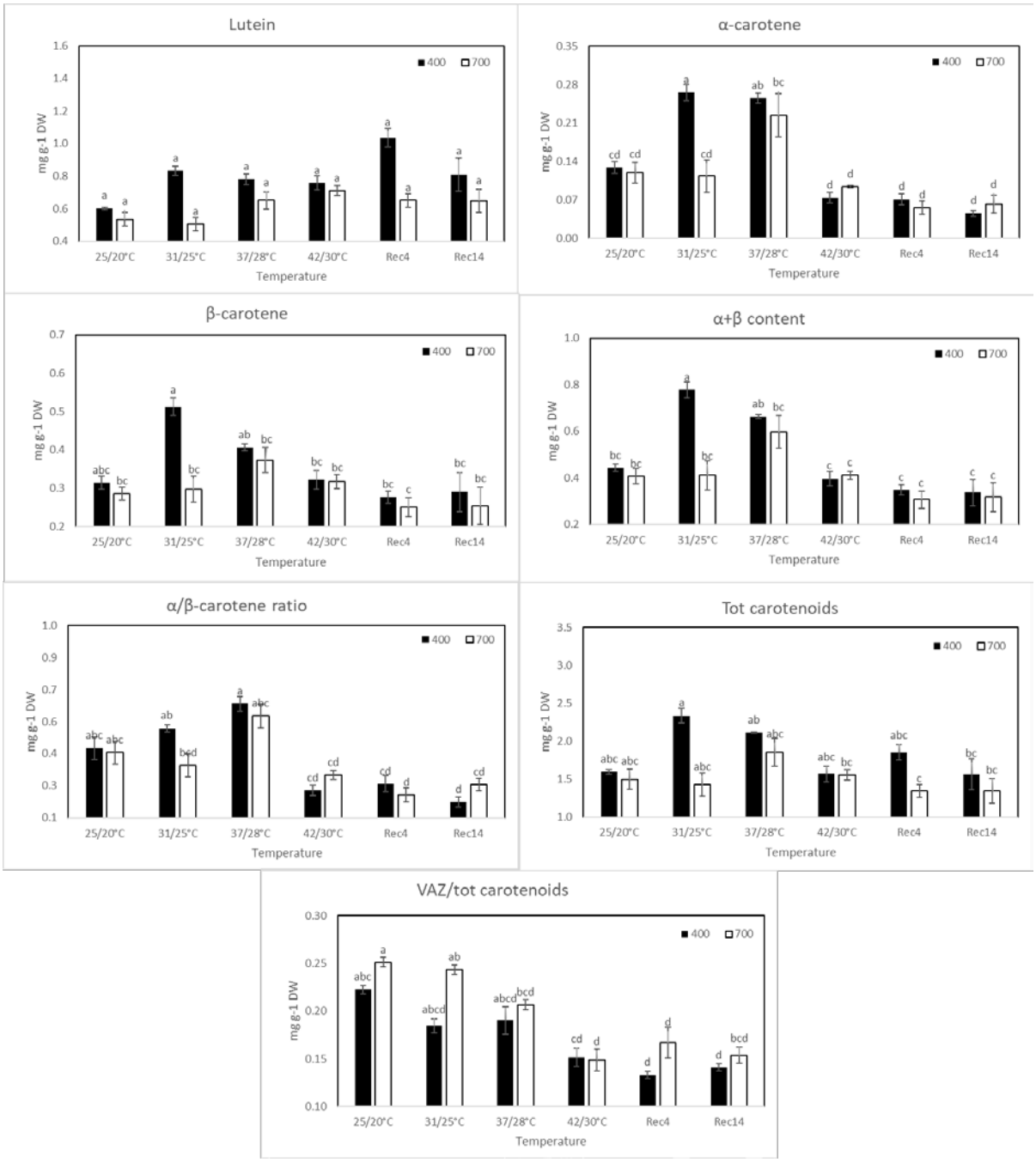
β-carotene in Hybrid-700), although the content did not differ remarkably from the 400-plant counterparts. Lutein content at Rec14 reached similar concentrations than the ones observed at 25/20 °C, being moderately lower in the 700-plants of Geisha and Hybrid, and higher in Marsellesa-700, compared to the concentrations under ambient [CO_2]. At Rec4 neoxanthin content decreased in Hybrid-700, even if at Rec14 a slight increase was observed, restoring the control concentrations (Fig. 16a). At Rec14, violaxanthin content was lower than control conditions in Marsellesa-700 (55% decrease) and Hybrid-700 (31% decrease). On the other hand, in Marsellesa-700 and Hybrid-700, zeaxanthin content was higher at Rec14 than at 25/20 °C (doubled in Marsellesa and 60% more in Hybrid), since it continued to increase even after the heat imposition ended, as occurred in the 400-plant counterparts. Overall, at Rec14 VAZ pool was slightly lower in 700-plants than in 400-plants, except for Marsellesa; an opposite trend occurred for DEPS, especially in Geisha, in which almost double values are found in 700-plants compared with the 400-plant counterparts. In Marsellesa and Hybrid, however, DEPS remained higher than control conditions, similarly to what occurred in the 400-plants.

Figure 14a*:* Changes in leaf carotenoids content (mg g^{-1} DW), obtained through HPLC determinations in *C. arabica* cv. Geisha3 plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20°C, day/night) and supra-optimal temperatures of 31/25 °C, 37/28 °C, and 42/30 °C. and after 4 (Rec4) and 14 days of recovery (Rec14).



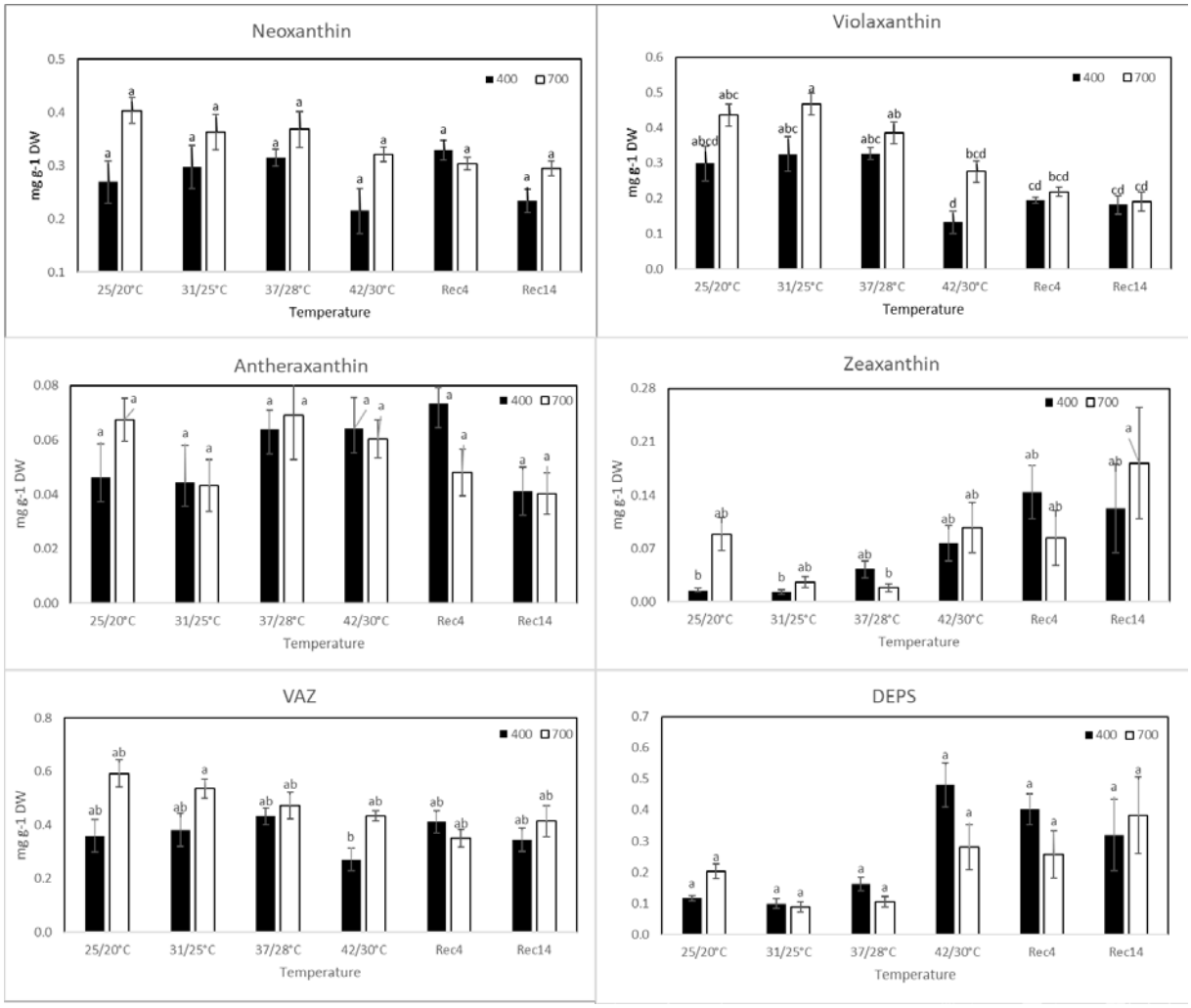
For each carotenoid concentration, the mean values ± SE (n= 4-8) followed by different letters express significant differences ($p>0.05$) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

Figure 14b: Changes in leaf carotenoids content (mg g^{-1} DW), obtained through HPLC determinations in *C. arabica* cv. Geisha3 plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 31/25 °C, 37/28 °C, and 42/30 °C, and after 4 (Rec4) and 14 days of recovery (Rec14).



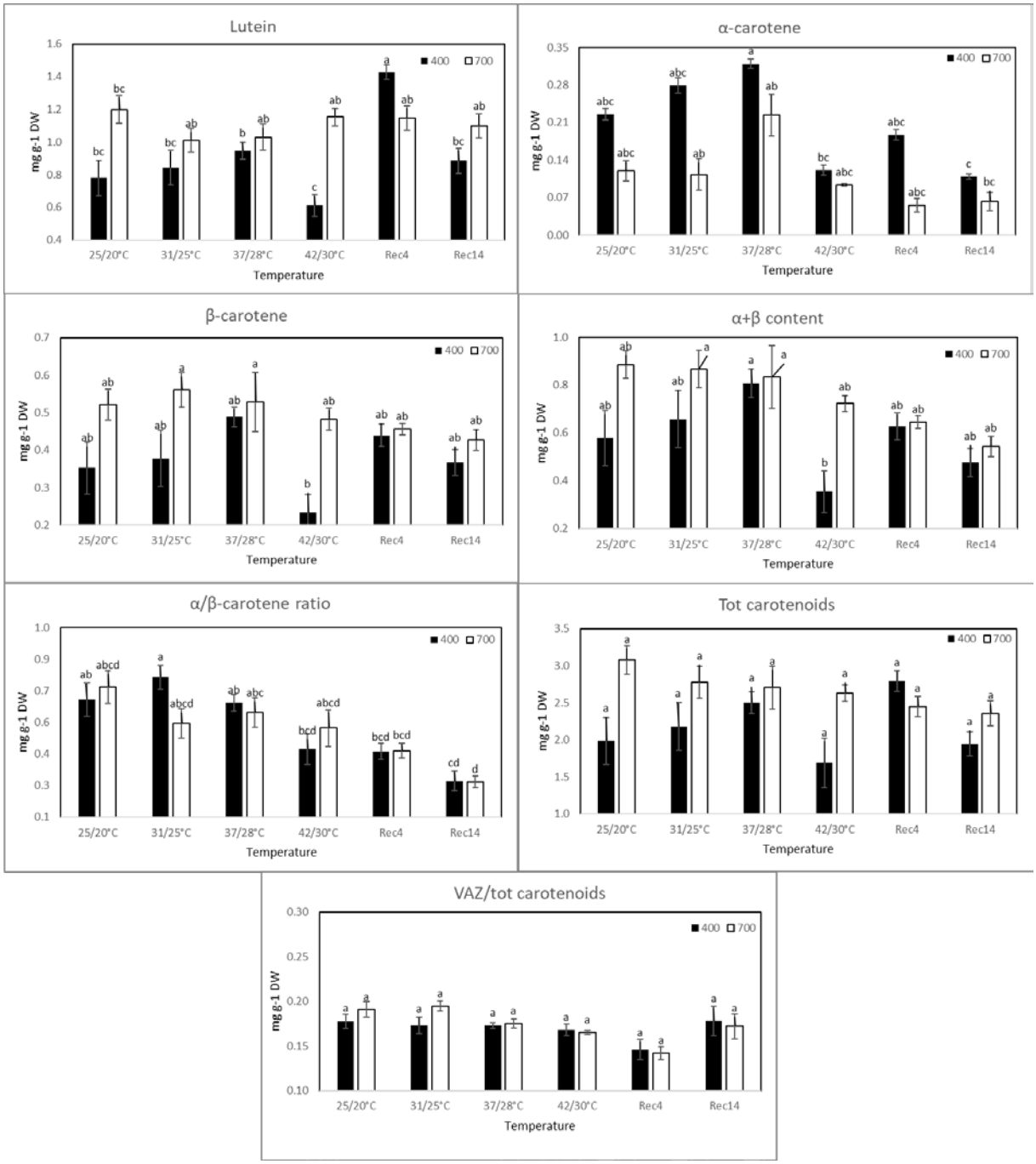
For each carotenoid concentration, the mean values ± SE (n= 4-8) followed by different letters express significant differences ($p>0.05$) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

Figure 15a: Changes in leaf carotenoids content (mg g^{-1} DW), obtained through HPLC determinations in *C. arabica* cv. Marsellesa plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 31/25 °C, 37/28 °C, and 42/30 °C. and after 4 (Rec4) and 14 days of recovery (Rec14).



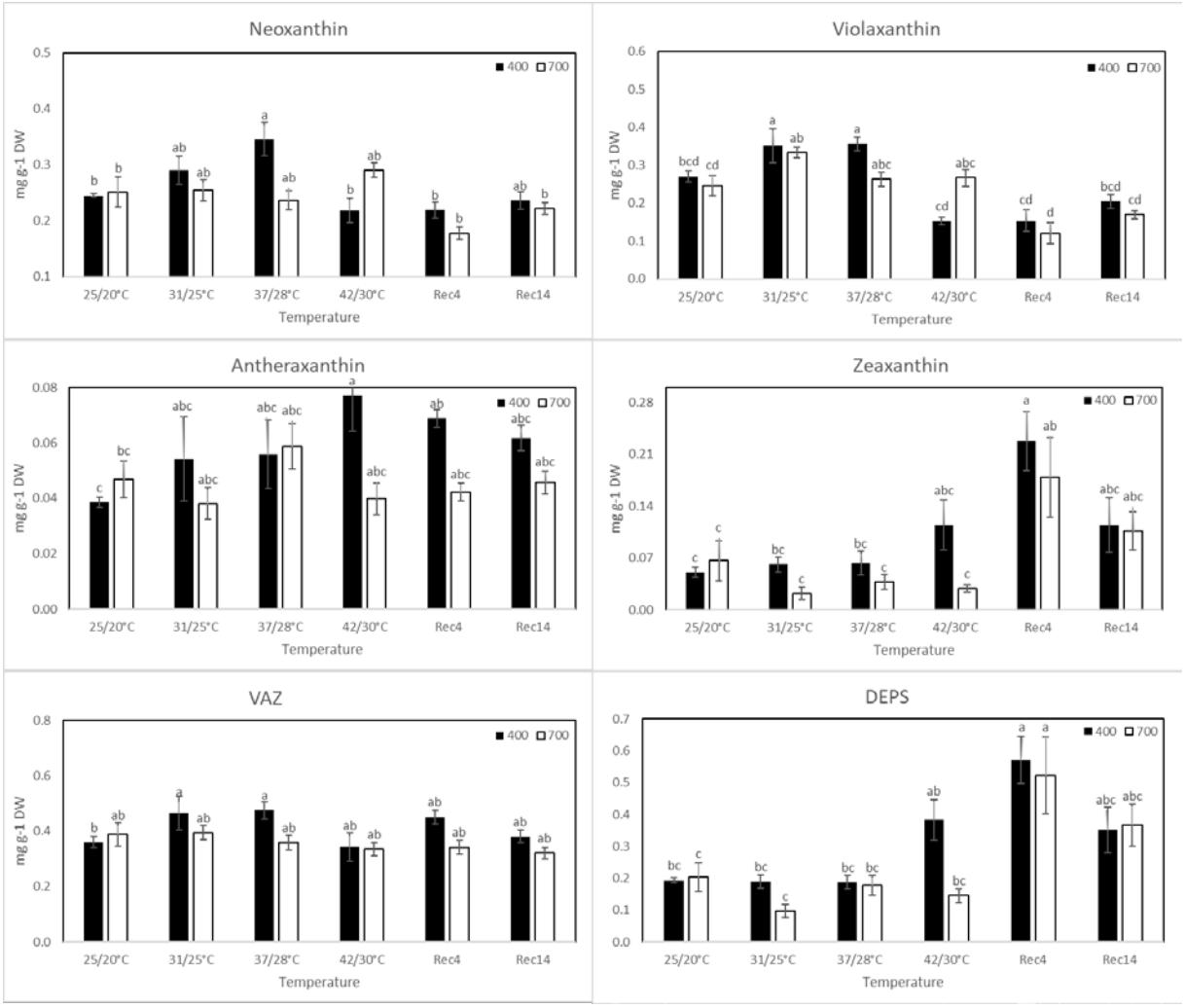
For each carotenoid concentration, the mean values ± SE (n= 4-8) followed by different letters express significant differences (p>0.05) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

Figure 15b*:* Changes in leaf carotenoids content (mg g^{-1} DW), obtained through HPLC determinations in *C. arabica* cv. Marsellesa plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 31/25 °C, 37/28 °C, and 42/30 °C, and after 4 (Rec4) and 14 days of recovery (Rec14).



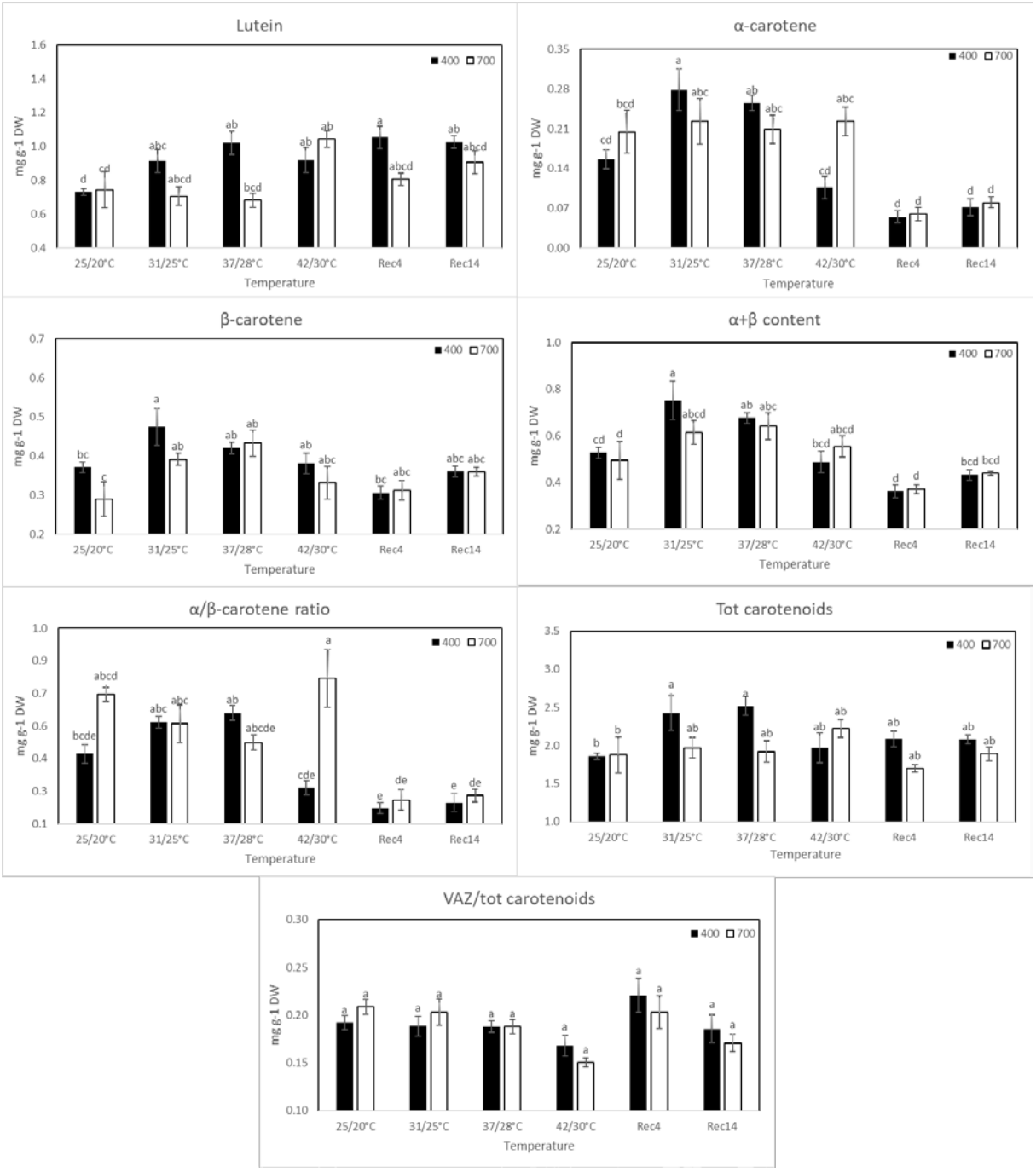
For each carotenoid concentration, the mean values ± SE (n= 4-8) followed by different letters express significant differences ($p>0.05$) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

Figure 16a*:* Changes in leaf carotenoids content (mg g^{-1} DW) obtained through HPLC determinations in *C. arabica* cv. Geisha3xMarsellesa (Hybrid) plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 31/25 °C, 37/28 °C, and 42/30 °C, and after 4 (Rec4) and 14 days of recovery (Rec14).



For each carotenoid concentration, the mean values ± SE (n= 4-8) followed by different letters express significant differences ($p>0.05$) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

Figure 16b: Changes in leaf carotenoids content (mg g^{-1} DW), obtained through HPLC determinations in *C. arabica* cv. Geisha3xMarsellesa (Hybrid) plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 31/25 °C, 37/28 °C, and 42/30 °C, and after 4 (Rec4) and 14 days of recovery (Rec14).



For each carotenoid concentration, the mean values ± SE (n= 4-8) followed by different letters express significant differences (p>0.05) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

3.2. ANTIOXIDANT ENZYMES ACTIVITY

The maximal cellular activity of four enzymes contributing to remove ROS, ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and catalase (CAT) was assessed in the two different CO_2 conditions and four different temperatures: 25/20 °C (control), 37/28 °C, 42/30 °C and Rec14 (after 14 days of recovery). Some differences can be observed across genotypes, but overall, the activity of the enzymes, apart from SOD, were enhanced under supra-optimal temperatures.

3.2.1 Ascorbate peroxidase (APX)

Under control temperature, the potential activity of APX under enhanced [CO_2], compared to ambient [CO_2], was strongly genotype dependent (Fig. 17). In Geisha, APX activity doubled under high [CO_2]; in Hybrid-700 it was slightly lower than in the 400-plant counterparts, while no significant differences were observed in Marsellesa.

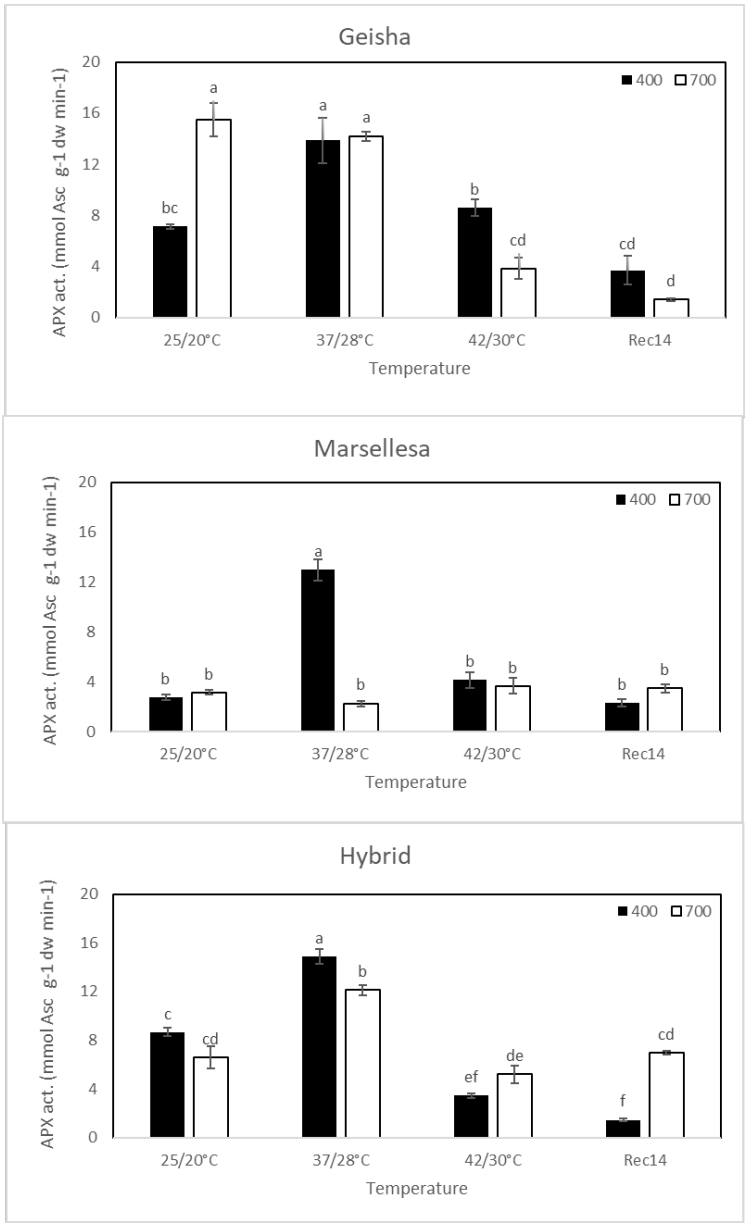
At 37/28 °C, the enzyme activity at ambient [CO_2] increased remarkably in all genotypes, reaching its maximal activity: 94% increase in Geisha, 71% in Hybrid and 367% in Marsellesa. Under elevated [CO_2], the increase in APX activity was observed only in Hybrid plants (83%), even though it remained lower than the 400-plants. In Geisha-700 and Marsellesa-700 APX activity remained stable, although in Geisha it was already uncommonly high at control condition (which could be caused by an error during the experiment).

At 42/30 °C the enzyme activity was severely impacted in all genotypes, irrespective of [CO_2] treatments, apart from Marsellesa-700 in which the activity remained relatively stable under all the experiment, while in the 400-plants counterparts a significant decrease (almost 70%) was observed, although the activity returned to control conditions. In Geisha-400 and Geisha-700 the enzyme activity decreased by *ca*. 40% and 70%, respectively from 37/28°C, being lower under elevated [CO_2]. On the contrary, in Hybrid plants an opposite trend was observed, since the activity decreased by *ca*. 80% in the 400-plants and 60% in the 700-plants, being higher under elevated [CO_2].

After 14 days of recovery at control temperature a decrease of APX activity of *ca*. 50% was reported in all plants at ambient [CO_2], thus being lower than control conditions, especially in

Marsellesa-400. In Marsellesa-700, no significant changes were observed. In Hybrid-700, an opposite trend was observed compared to the 400-plant counterparts: APX activity increased from 42/30 °C to Rec14 by *ca*. 30%, returning to control conditions and being almost 5 times higher than in the 400-plants.

Figure 17*:* Changes in maximal activity of ascorbate peroxidase (APX) in C. arabica (Geisha3, Marsellesa and the correspondent hybrid) plants, grown under 400 or 700 μL CO_2 L^{-1}, at control (25/20 °C, day/night), supra-optimal temperatures (37/28 °C, 42/30 °C) and after 14 days of recovery (Rec14).



Mean values with ± SE (n=3) followed by different letters express significant differences (p>0.05) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

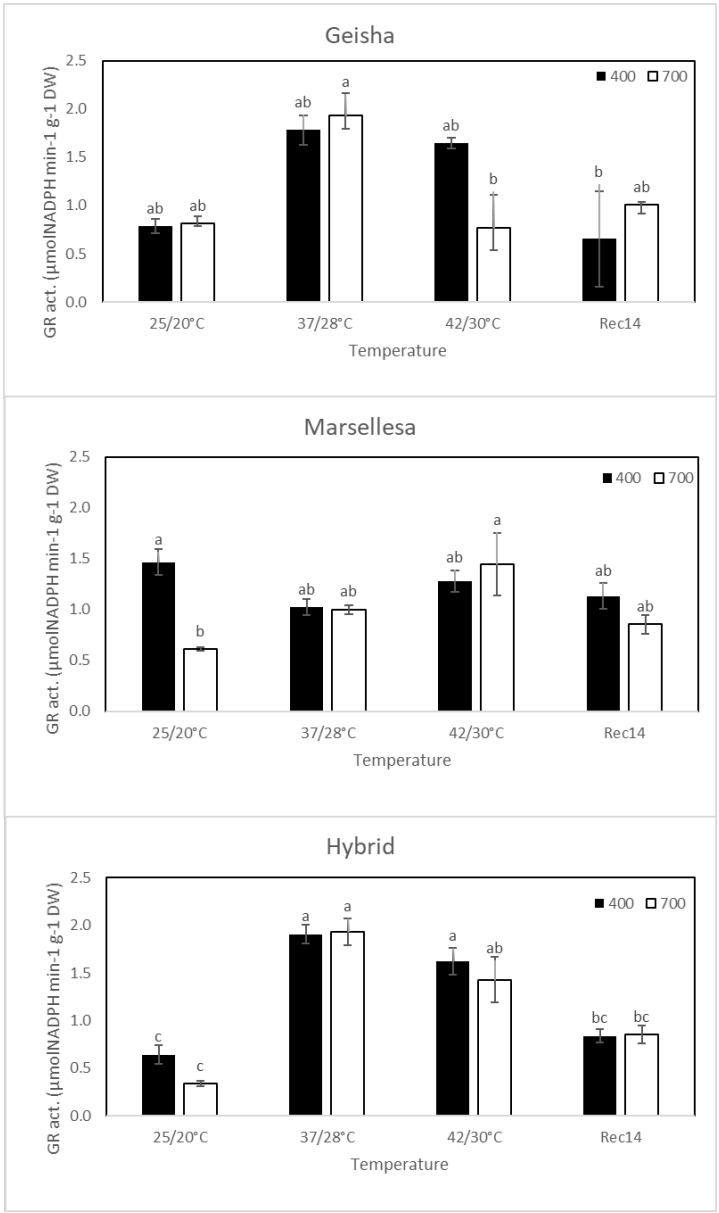
3.2.2. Glutathione reductase (GR)

Under control temperature, the potential activity of GR under enhanced [CO_2] was remarkably lower than in the 400-plant counterparts of Marsellesa, whereas it did not differ significantly in Geisha (Fig. 18). In Hybrid-400, GR activity was almost double compared to Hybrid-700, and in Marsellesa the difference was even higher, although the activity in Marsellesa-400 was unusually high. In fact, as heat stress was imposed, the enzyme activity in Marsellesa under ambient [CO_2] decreased by 30%. In Geisha and Hybrid plants, GR reached the maximal activity at 37/28 °C, with a 125% and 136% increase in Geisha-400 and Geisha-700, and a 195% and 352% increase in Hybrid-400 and Hybrid-700, respectively. No statistical differences were observed between the two CO_2 condition at this temperature.

At ambient [CO_2], when temperature reached its maximum value – 42/30 °C – a decrease in the activity of GR was observed in Geisha and Hybrid, although the activity remained higher than control condition. In Marsellesa the activity increased again, returning almost to control conditions. The same trend was observed under enhanced [CO_2], with a 70% and 26% decrease in Geisha and Hybrid, respectively, and a 45% increase in Marsellesa.

After 14 days of recovery, the enzyme activity decreased from 42/30 °C, irrespective of [CO_2] conditions. The greatest decrease was observed in Hybrid-400, with 50% decrease with respect to the value at 42/30 °C, although remaining slightly higher than control conditions. In Marsellesa-400 the activity remained high. GR activity decreased by 40% also in Hybrid-700 and Marsellesa-700, although it remained higher than control conditions, whereas in Geisha it slightly increased, although not significantly.

Figure 18: Changes in maximal activity of glutathione reductase (GR) in C. arabica (Geisha3, Marsellesa and the correspondent hybrid) plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night), supra-optimal temperatures (37/28 °C, 42/30 °C) and after 14 days of recovery (Rec14).



Mean values with ± SE (n=3) followed by different letters express significant differences (p>0.05) for the interaction between [CO_2] and/or temperature treatments, separately for each genotype.

3.2.3. Catalase (CAT)

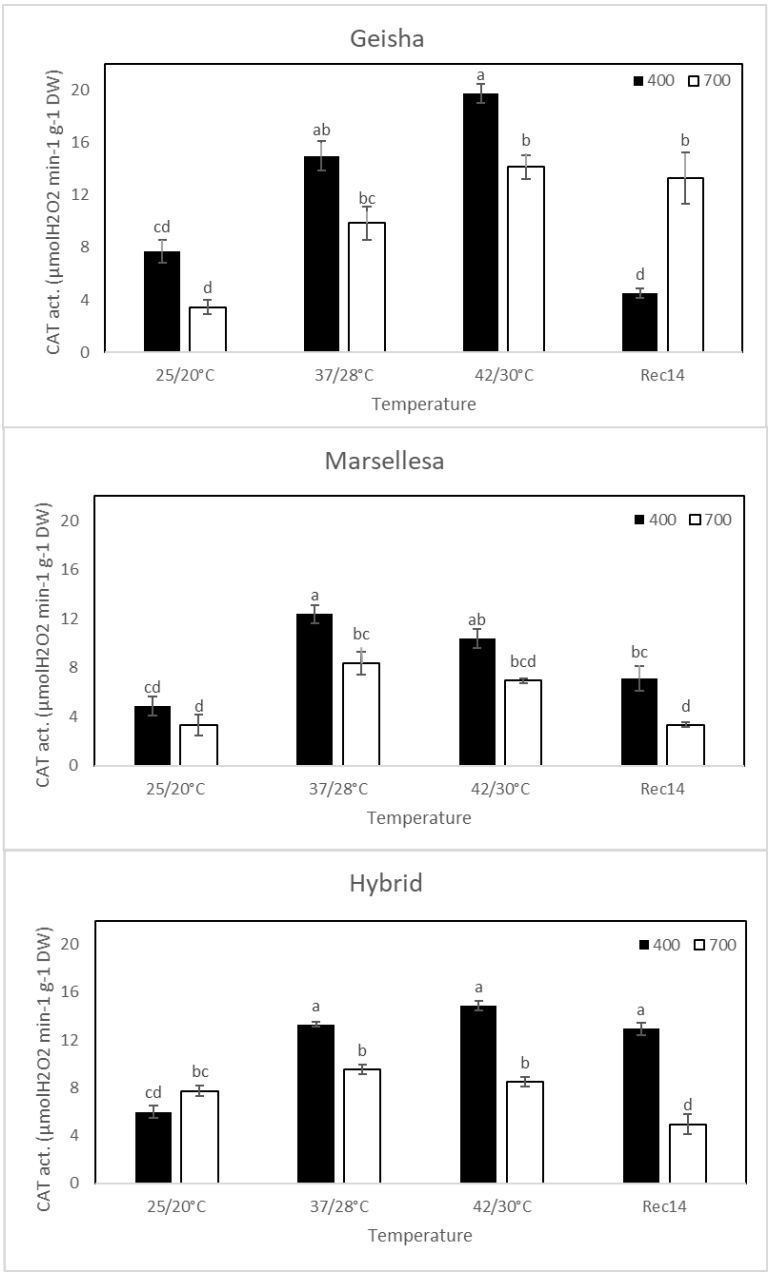
At 25/20 °C, small differences between the two [CO_2] conditions were recorded in all genotypes (Fig. 19). In Geisha and Marsellesa, the 400-plants exhibited higher CAT activity relative to the 700-plant counterparts, (7.71 against 3.46 µmol H_2O_2 g^{-1} DW min^{-1} in Geisha). Opposite trends were observed in Hybrid, where the enzyme activity was moderately higher in the 700-plants.

As temperature increased from 25/20 °C to 37/28 °C, the potential activity of CAT increased sharply in all genotypes, irrespective of [CO_2] conditions. In Marsellesa, maximal activity was reached at this temperature, increasing by 153% both in the 400- and 700-plants. In all genotypes, plants under enhanced [CO_2] showed lower activities than the 400-plant counterparts.

At 42/30 °C, the enzyme activity in plants grown at ambient [CO_2] slightly increased by 31% and 11% in Geisha and Hybrid, respectively, reaching its maximal activity. On the contrary, in Marsellesa-400 a 16% decrease was observed, although remaining higher than control conditions. The same trend was observed under enhanced [CO_2] in Geisha and Marsellesa, but not in Hybrid-700 in which CAT remained stable.

After the recovery period at control temperature, an overall decrease in CAT activity was observed, particularly in Geisha-400, where the activity decreased by 77% and reached lower values than control conditions, whereas in the other genotypes CAT activity only slightly decreased, and it remained higher than control conditions (double in Hybrid-400). While under enhanced [CO_2] the activity of CAT decreased in Marsellesa (52%) and Hybrid (41%) from 42/30 °C, it remained high and unchanged in Geisha-700, being three times higher than in the 400-plant counterparts. In Hybrid and Marsellesa, instead, the activity of the 400-plants was double that of the 700-plants, which returned to basal conditions (Marsellesa) or even lower (Hybrid).

Figure 19: Changes in maximal activity of catalase (CAT) in C. arabica (Geisha3, Marsellesa and the correspondent hybrid) plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night), supra-optimal temperatures (37/28 °C, 42/30 °C) and after 14 days of recovery (Rec14).



Mean values with ± SE (n=3) followed by different letters express significant differences ($p>0.05$) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

3.2.4. Superoxide dismutase (SOD)

During the whole experiment, SOD activity varied differently according to the different genotypes and [CO_2] treatments (Fig. 20). At control temperature, enhanced [CO_2] reduced the activity of SOD in Hybrid and Marsellesa, although significantly only in Marsellesa

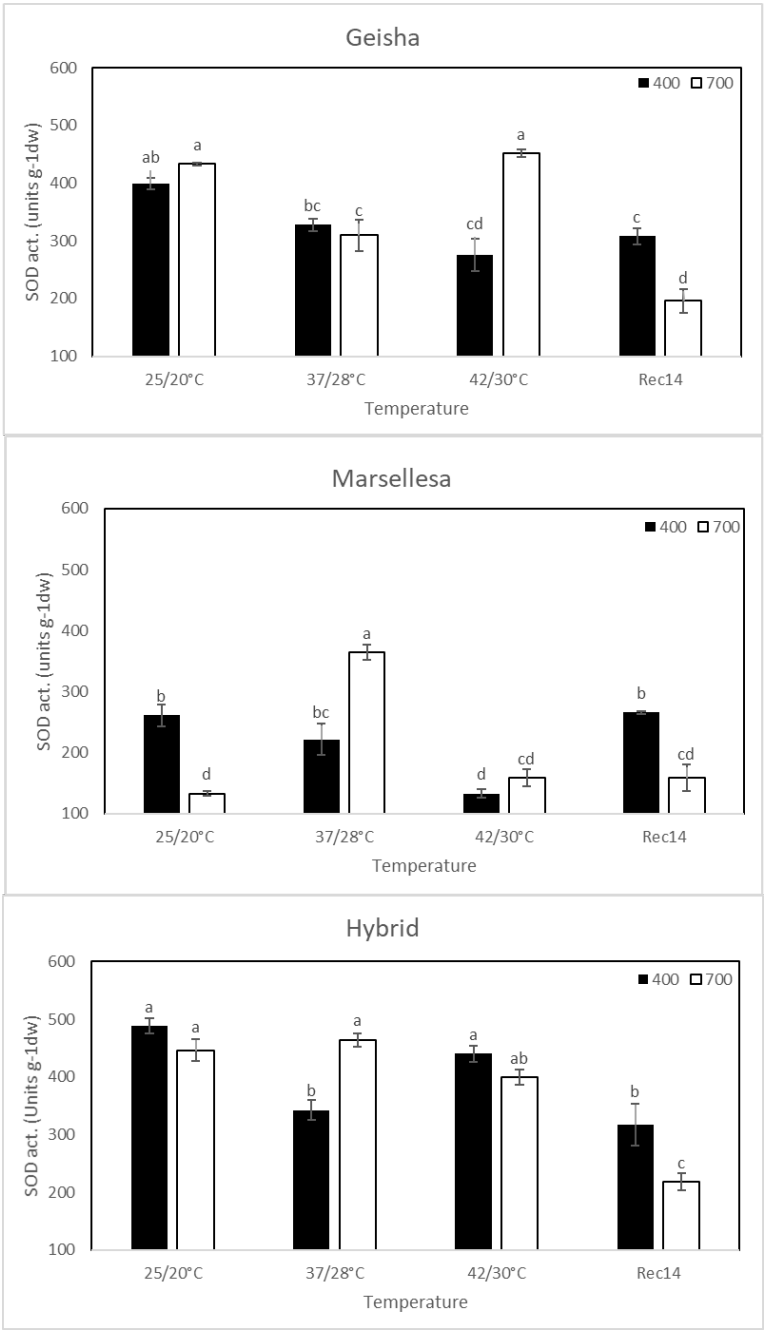
(almost double at ambient [CO_2]). On the contrary, in Geisha, SOD activity was slightly higher under elevated [CO_2].

As temperature was increased to 37/28 °C, plants under ambient [CO_2] showed an overall decrease in the activity of SOD, especially in Hybrid. On the contrary, different responses were observed under enhanced [CO_2]: in Geisha it decreased by *ca*. 30%, reaching the same values of the correspondent 400-plants; in Hybrid no significant changes were observed; in Marsellesa the activity sharply increased by 174%. Consequently, SOD activity was substantially higher in the 700-plants of Marsellesa and Hybrid.

When temperature reached the maximum value, the enzyme activity under control [CO_2] decreased in Geisha (15%) and Marsellesa (40%), but not in Hybrid, in which an increase of almost 30% was recorded; however, all genotyped showed lower SOD activity relative to controls. The same trend was observed in Marsellesa under high [CO_2], where the activity drastically decreased by 56% returning almost to control conditions. In Hybrid-700 SOD activity decreased too, in contrast to what occurred in the 400-plant counterparts, being remarkably lower than the activity observed at 25/20 °C. Large differences were observed especially in Geisha, where the enzyme activity increased by almost 50%, being remarkably higher than the 400-plant counterparts and reaching the control values.

After the recovery period, SOD activity continued to rise in Geisha-400 and Marsellesa-400, although significant only in Marsellesa, with double values with respect to the ones at 42/30 °C, thus restoring the basal condition. On the contrary, in Hybrid-400 a decrease in the activity of *ca*. 30% was recorded, reaching values lower than the control conditions. Under enhanced [CO_2] SOD activity decreased in Geisha (56%) and Hybrid (45%), reaching values remarkably lower than the ones at 25/20 °C, whereas in Marsellesa it remained stable. Nevertheless, in all genotypes SOD activity was lower in the 700-plants compared to the 400-plants.

Figure 20: Changes in maximal activity of superoxide dismutase (SOD) in C. arabica (Geisha3, Marsellesa and the correspondent hybrid) plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night), supra-optimal temperatures (37/28 °C, 42/30 °C) and after 14 days of recovery (Rec14).



Mean values with ± SE (n=3) followed by different letters express significant differences ($p>0.05$) for the interaction between [CO_2] and temperature treatments, separately for each genotype.

3.3. EXPRESSION OF SELECTED GENES

For my thesis work, gene transcripts related to the oxidative stress control and other protective mechanisms were studied. In particular, I studied the expression of genes encoding for antioxidant enzymes, such as catalase (*CAT*), superoxide dismutase (*CuSOD1* and *CuSOD2*), ascorbate peroxidases (APX_{-Cyt}, APX_{-Chl} and APX_{-t+s}), and violaxanthin de-epoxidase (*VDE2*), together with genes encoding for protective proteins related to heat stress, such as heat-shock proteins (*HSP70*), early light-induced proteins (*ELIP*), and chaperonins (*Chape20* and *Chape60*). As temperature increased in time, numerous changes in gene expression patterns were observed, in plants growth under both 400 and 700 µL CO_2 L^{-1}.

3.3.1. Effect of CO_2 treatments at control temperature

Under control temperature – 25/20 °C – the expression of all the studied genes was statistically similar in the 400-plants compared with their 700-plant counterparts for all genotypes (Fig. 21, 22, 23).

3.3.2. Effects of increasing temperature at ambient CO_2 concentration

At ambient [CO_2], as temperature increased from 25/20 to 37/28 °C, most of the genes were largely upregulated and reached the maximum values at 42/30 °C. Generally, after 14 days of recovery at control temperature (Rec14), most transcript levels decreased compared to the highest temperature, often restoring the basal expression.

Regarding genes encoding for protective molecules, such as *HSP70* and *ELIP*, the highest values were attained at 42/30 °C, with a great increase especially in Hybrid (twice the one at 37/28°C for *HSP70*, 60% increase in *ELIP*). After the recovery period the expression of the genes decreased, although they remained higher than control values, especially in Hybrid (Fig. 23). Chaperonin gene expression (*Chape20* and *Chape60*) was upregulated until 42/30 °C: the highest values can be observed in Hybrid (26-fold in *Chape20* and 11-fold in *Chape60*; Fig. 23).

High increase in the expression can be observed also in genes related to the antioxidative system components (SOD, APX, CAT). The expression of genes of the superoxide dismutase family, *CuSOD1* and *CuSOD2*, but also *CAT* gene expression, was upregulated especially in Geisha and Hybrid, reaching the highest values at 42/30 °C and subsequently decreasing at Rec14. In Geisha, *CAT* expression almost doubled from 37/28 °C to 42/30 °C and remained

high even after the stress imposition (Fig. 21), whereas in Marsellesa no significant differences were observed from 37/28 °C to Rec14 (Fig. 22). Significant values can be found especially for *CuSOD2*, where at 42/30 °C Geisha and Hybrid reached values of 28- and 55-fold, respectively. After this dramatic increase, the plants could not return to the basal conditions, although the values decreased substantially: a great decrease in *CuSOD1* expression was observed for Hybrid and Geisha; *CuSOD2* expression decreased by 90% in Geisha and 75% in Hybrid, although remaining unchanged in Marsellesa (14-fold from 37/28 °C to 42/30 °C). One of the strongest responses was observed in the expression of the *APX* gene family. High values were reached at 37/28 °C, especially for the cytosolic enzyme APX_{-Cyt} (48- in Geisha and Marsellesa) and continued to rise at 42/30 °C. APX_{-Cyt} upregulation in Marsellesa (104-fold at 42/30 °C) was the largest observed over the entire experiment for all the studied genes (Fig. 22). At Rec14, APX transcripts decreased (*ca*. 80% of APX_{-Chl} and APX_{-t+s} in Marsellesa) but remained much higher compared to control conditions.

Unlike the general rising trend of most gene expression, *VDE2* expression behaved differently depending on the genotypes. A downregulation of *VDE2* occurred in Marsellesa and Hybrid at 42/30 °C, both returning to basal levels at Rec14 (Fig. 22 and 23). In Geisha the downregulation occurred at 37/28 °C (44% decrease), but at 42/30 °C the basal expression was restored (Fig. 21).

3.3.3. Interaction between high temperature and CO_2 enhancement

As in the plants treated with ambient [CO_2], the general trend observed under elevated [CO_2] was an upregulation of most of the selected genes. Nevertheless, the expression patterns tended to be lower in the 700-plants than the 400-plant counterparts at every temperature treatment. A schematic representation of differences in genes upregulation at 42/30 °C between the two [CO_2] treatments is shown in Fig. 24. Gene expression of the protective molecule *Chape60* in Hybrid-700 was half of Hybrid-400 at 37/28 °C and continued to be higher at 42/30 °C and Rec14 (Fig. 23). The same trend of *Chape60* can be observed also in Geisha and Marsellesa (Fig. 21 and 22). Similarly, *Chape20* was three times lower at 42/30 °C in the 700-plants of Marsellesa and Hybrid (Fig. 22 and 23). If at 42/30 °C the *HSP70* expression reached 13-fold in Hybrid-400, the 700-plant counterparts increased the expression only by 2-fold at 37/28 °C and remained stable at 42/30 °C, although at Rec14 the difference between the two CO_2 treatments diminished; the same trend can be observed for *ELIP* gene expression (Fig. 23). In Geisha, differences in *HSP70* and *ELIP* gene expression

between the 400- and 700-plants were observed only up to 42/30 °C (Fig. 21). In Marsellesa, instead, *HSP70* was higher in the 400-plants under supra-optimal temperature, but if plants under ambient [CO_2] recovered the basal expression, the 700-plants did not change the expression from 42/30 °C to Rec14 (Fig. 22).

Regarding the genes encoding for antioxidant enzymes, strong differences between the two [CO_2] treatments were observed too. In all genotypes under high [CO_2], *CAT* expression slightly increased at 37/28 °C, but remained stable as temperature was increased to 42/30 °C, in contrast with the 400-plant counterparts where *CAT* continued to increase (65% reduction in Geisha-700, Fig. 21). Difference in *CAT* expression can be observed also after the recovery period: in Geisha, high [CO_2] provoked a reduction of 50% with respect to the 400-plant counterparts.

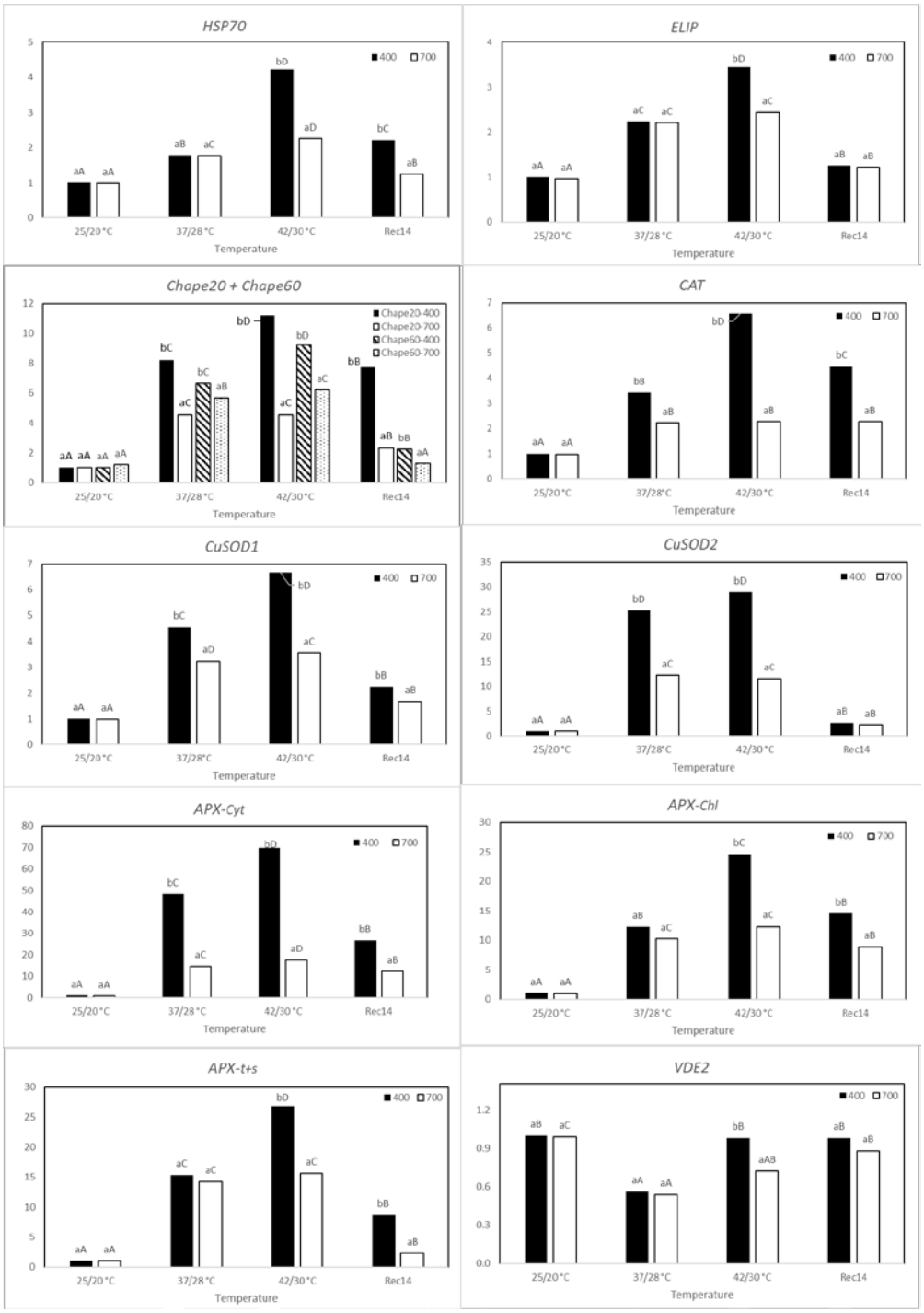
Unlike *CAT*, maximal *CuSOD1* gene expression was reached at 42/30 °C also under enhanced [CO_2]: large increases can be observed especially in Hybrid, where *CuSOD1* at 42/30 °C was more than three times higher than the value at 37/28 °C (Fig. 23). However, as for the other genes, also *CuSOD1* was always lower in the 700-plants compared to the 400-plant counterparts. In contrast to the great upregulation of *CuSOD2* observed in the 400-plants, gene expression did not change significantly in Marsellesa-700, although a slight increase at Rec14 was observed (Fig. 22). In fact, elevated [CO_2] only slightly increased *CuSOD2* gene expression under supra-optimal temperatures, differently from the substantial increase observed in the 400-plant counterparts; in Hybrid-700, *CuSOD2* expression returned to basal level at Rec14, while in Hybrid-400 it remained substantially higher (13-fold).

APX-$_{Cyt}$ was upregulated also in the 700-plants, although not remarkably as the 400-plant counterparts: at 37/28 °C, enhanced [CO_2] reduced the gene expression in Geisha (70%), Marsellesa (77%) and Hybrid (60%); this difference continued to occur also at 42/30 °C and Rec14. In Geisha and Hybrid, the expression of *APX-*$_{Chl}$ at 37/28 °C, together with *APX-*$_{t+s}$ in Geisha, was similar in the two [CO_2] levels; however, it varied markedly between the two [CO_2] treatments at 42/30 °C, as well as at Rec14 (Fig. 21).

Unlike the expression pattern of most of the other genes, *VDE2* expression in the 700-plants did not differ substantially from the 400-plant counterparts, especially in Geisha (Fig. 21). In Marsellesa, the downregulation of *VDE2* expression was slightly higher in the 700-plants than the 400-plants (Fig. 22). In Hybrid, the 700-plants had higher transcript levels of *VDE2* than

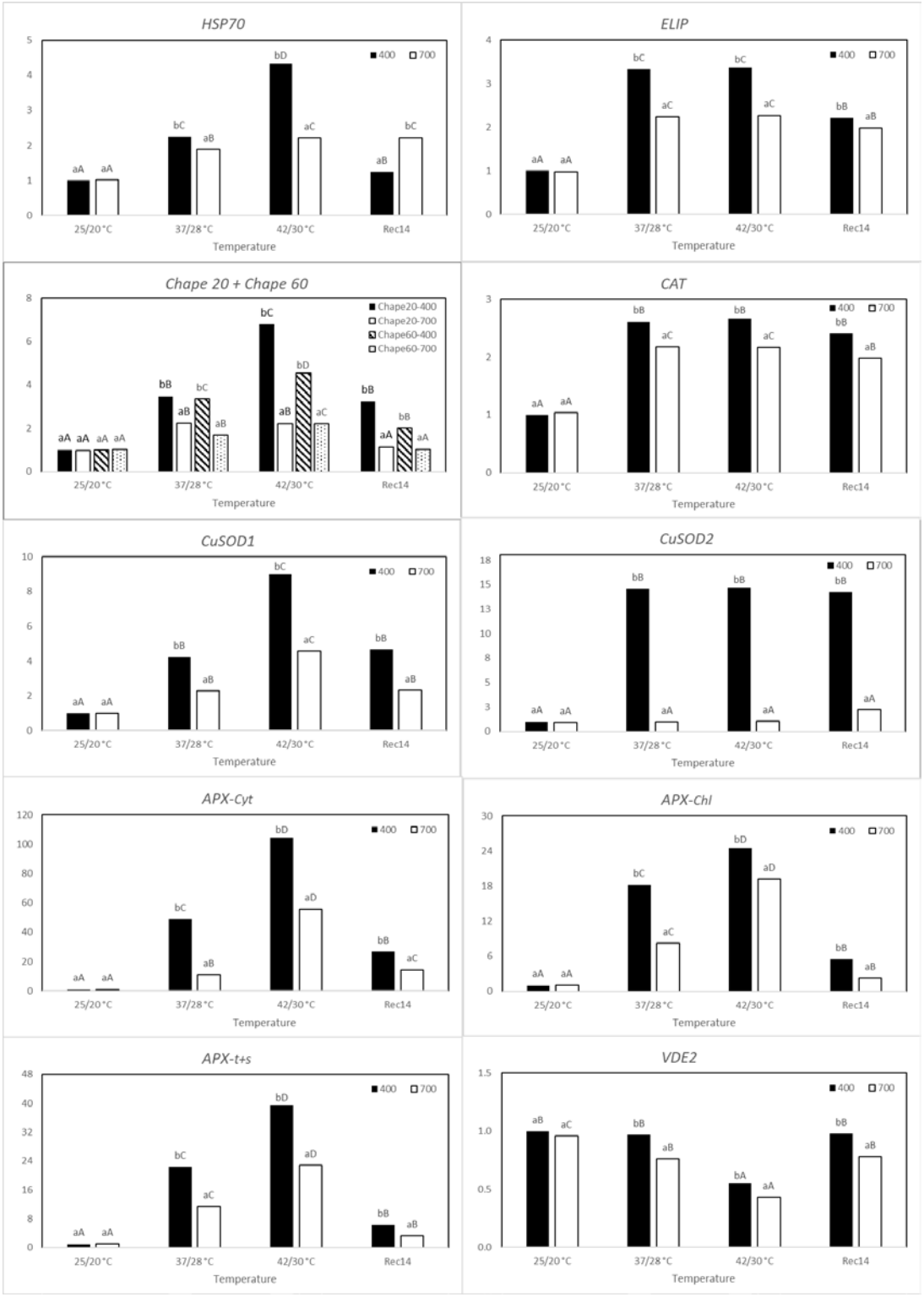
the 400-plants at 42/30 °C; however, at Rec14 the gene expression of the 700-plants continued to be downregulated, while the 400-plants returned to control conditions (Fig. 23).

Figure 21: Real-time PCR expression studies relative to the expression value observed under control conditions of temperature and CO_2 (25/20 °C,400 µL CO_2 L^{-1}) from leaves of *C. arabica* cv. Geisha3 plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 37/28 °C, 42/30 °C and after 14 days of recovery (Rec14).



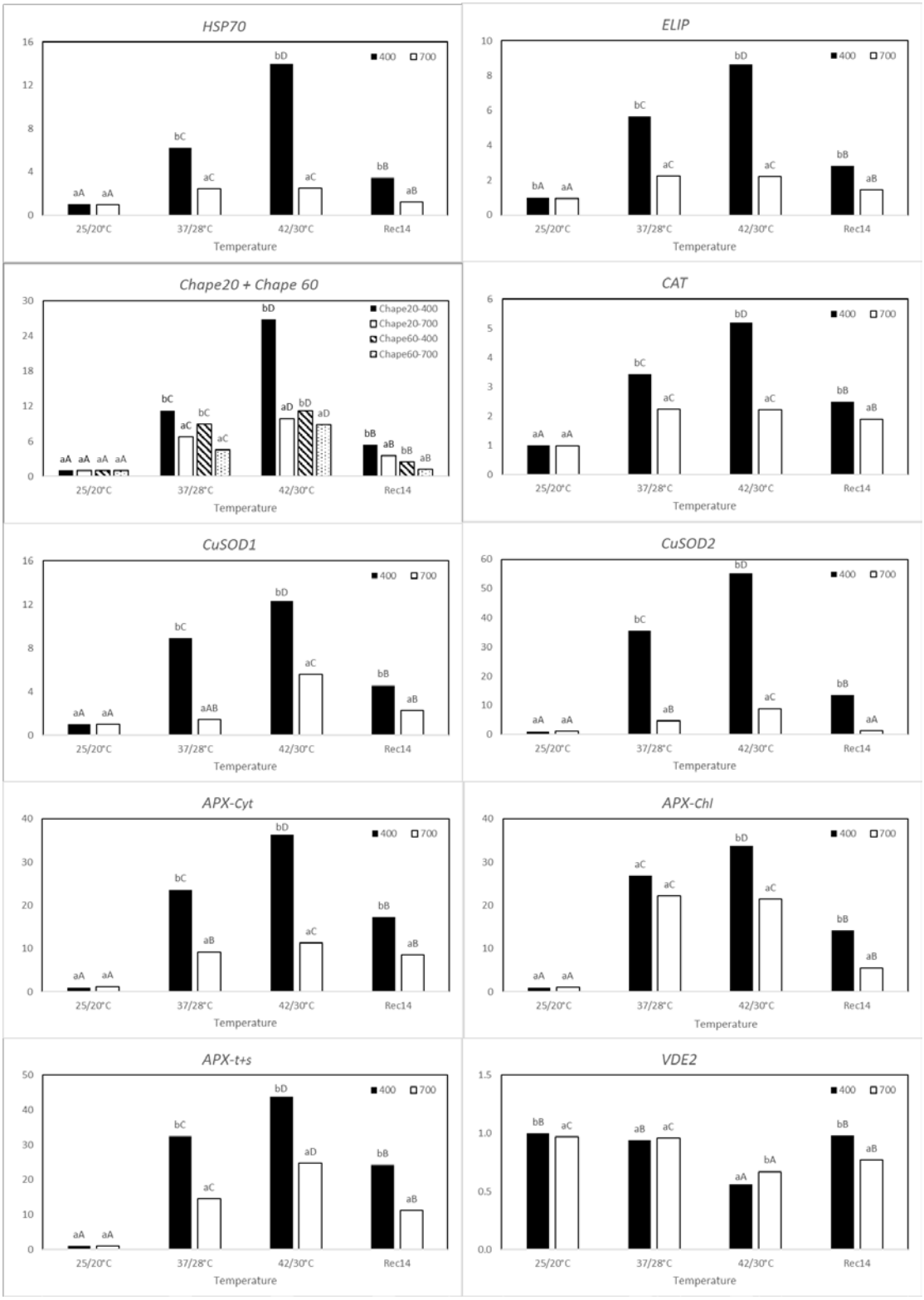
For each gene transcript, the mean values ± SE (n=3) followed by different letters express significant differences between [CO_2] levels for each temperature treatment (a, b), or between temperature treatments for the same [CO_2] level (A, B, C, D).

Figure 22: Real-time PCR expression studies relative to the expression value observed under control conditions of temperature and CO_2 (25/20 °C,400 µL CO_2 L^{-1}) from leaves of *C. arabica* cv. Marsellesa plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 37/28 °C, 42/30 °C and after 14 days of recovery (Rec14).



For each gene transcript, the mean values ± SE (n=3) followed by different letters express significant differences between [CO_2] levels for each temperature treatment (a, b), or between temperature treatments for the same [CO_2] level (A, B, C, D).

Figure 23: Real-time PCR expression studies relative to the expression value observed under control conditions of temperature and CO_2 (25/20 °C,400 µL CO_2 L^{-1}) from leaves of *C. arabica* cv. Geisha3xMarsellesa (Hybrid) plants, grown under 400 or 700 µL CO_2 L^{-1}, at control (25/20 °C, day/night) and supra-optimal temperatures of 37/28 °C, 42/30 °C and after 14 days of recovery (Rec14).



For each gene transcript, the mean values ± SE (n=3) followed by different letters express significant differences between [CO_2] levels for each temperature treatment (a, b), or between temperature treatments for the same [CO_2] level (A, B, C, D).

Figure 24: Heat map representing differential expression of genes observed at 42/30 °C from leaves of *C. arabica* cv. Geisha3, Marsellesa, Geisha3xMarsellesa (Hybrid), grown under 400 or 700 µL CO_2 L^{-1}. Colors represent fold changes in the expression relative to the control temperature and [CO_2] (25/20 °C and 400 µL CO_2 L^{-1}. From yellow to red: positive fold changes; from yellow to blue: negative fold changes.

Gene	Cultivar	42/30 °C	
		400 ppm	700 ppm
HSP70	Geisha		
	Marsellesa		
	Hybrid		
ELIP	Geisha		
	Marsellesa		
	Hybrid		
Chape20	Geisha		
	Marsellesa		
	Hybrid		
Chape60	Geisha		
	Marsellesa		
	Hybrid		
CAT	Geisha		
	Marsellesa		
	Hybrid		
CuSOD1	Geisha		
	Marsellesa		
	Hybrid		
CuSOD2	Geisha		
	Marsellesa		
	Hybrid		
APXcyt	Geisha		
	Marsellesa		
	Hybrid		
APXchl	Geisha		
	Marsellesa		
	Hybrid		
APXt+s	Geisha		
	Marsellesa		
	Hybrid		
VDE2	Geisha		
	Marsellesa		
	Hybrid		

4. DISCUSSION

The control of oxidative stress through the increase in photoprotective pigments, antioxidative enzymes and other protective compounds could play a fundamental role in stress acclimation of coffee plants, confirmed by the studies about coffee plant responses under several environmental stresses, including high irradiance (Ramalho et al., 2000; Ramalho et al., 1997), cold (Ramalho et al., 2003), drought (Ramalho et al., 2018), and heat stress (Martins et al., 2016). The crucial role of the oxidative stress control could be explained by the fact that coffee plants evolved in a shaded environment and low values of irradiance are able to saturate the photosynthetic apparatus (DaMatta et al., 2019). In addition, photosynthetic performance has been shown to be maintained even at 42/30 °C under high ambient CO_2 concentrations (Rodrigues et al., 2016). Starting from these assumptions, the present work was undertaken to test the ability of coffee plants to cope with day/night temperatures up to 37/28 °C at ambient [CO_2], and up to 42/30 °C under elevated [CO_2].

4.1. PHOTOPROTECTIVE PIGMENTS

Carotenoids play a crucial role under conditions that can increase oxidative stress, such as supra-optimal temperatures, since they are able to dissipate the excess of energy or directly scavenge reactive oxygen species (ROS) and chlorophyll triplets ($^3Chl^*$), thus protecting the photosynthetic components and the lipids of chloroplast membranes.

4.1.1. Xanthophyll cycle

Adequate levels of xanthophylls, and especially zeaxanthin, in the light harvesting complexes (LHC), are associated with higher capability of reducing excess excitation energy, thus controlling the formation of ROS. During the xanthophyll cycle, violaxanthin is reversibly converted to antheraxanthin and zeaxanthin by the enzyme violaxanthin de-epoxidase (VDE). Zeaxanthin and antheraxanthin are involved in the protection of PSII, directly by quenching $^1Chl^*$, thus preventing the formation of $^3Chl^*$, or indirectly by changing the conformation state of LHCII, which facilitates the energy dissipation (Ramalho et al., 2000; Johnson et al., 1993). Zeaxanthin content is expected to increase under heat stress, since it is associated with a higher need for thermal dissipation (non-photochemical quenching, NPQ) and prevention of lipid peroxidation, by removing the epoxy groups from the oxidized double bonds of thylakoid fatty acids (Ramalho et al., 2003, Martins et al., 2016). Several studies

correlated the ability of thermal dissipation to the de-epoxidation state (DEPS) of the violaxanthin cycle (Jahns & Holzwarth, 2012).

In this work, enhanced [CO_2] under control temperature promoted an increase in VAZ pool and DEPS in Marsellesa, although the unusually high values of zeaxanthin observed at 25/20 °C could represent an experimental error. On the contrary, in the other genotypes, high [CO_2] did not significantly alter the level of xanthophylls.

Previous studies reported that reduction in photosynthetic potential did not occur up to 37/30 °C, and maximal RuBisCO and Ru5PK activities were found at 31/25 °C in *C. arabica* (Rodrigues et al., 2016). Nevertheless, at 31/25 °C, an increase in VAZ pool was recorded in the 400-plants (except in Marsellesa), mostly due to an increase of violaxanthin in Geisha, and of antheraxanthin in Hybrid, although not accompanied by an increase in the de-epoxidation state, which drastically decreased in Geisha. This could suggest that coffee plants are not yet stressed at 31/25 °C, although this is a temperature already above the optimal values. In Hybrid, enhanced [CO_2] could promote a higher photosynthetic efficiency in plants exposed to higher temperatures, since an increase in violaxanthin, followed by a decrease in antheraxanthin, zeaxanthin and DEPS, was observed. In fact, a reduction in photoprotective pigments has been associated with a higher C-assimilation under high [CO_2], that ultimately led to a reduction of excess energy and a minor need to dissipate it (Martins et al., 2016).

As temperature was raised up to 37/28 °C, an increase in antheraxanthin, zeaxanthin and DEPS relative to 31/25 °C was observed in Geisha, regardless of [CO_2], which suggests that violaxanthin could have been converted to zeaxanthin to protect PSII components. Zeaxanthin (and DEPS) were also reported to increase in *Coffea sp*. under low-temperature stress (Ramalho et al., 2003). Unlike Geisha, in Marsellesa a relevant increase in zeaxanthin and DEPS did not occur until 42/30 °C irrespective of CO_2 treatments, conferring a higher thermotolerance than Geisha. At 42/30 °C, a temperature well above the optimal conditions, photosynthetic performance is largely impaired in coffee plants (Rodrigues et al., 2016). In an earlier study, Martins et al. (2016) reported an increase in zeaxanthin (and DEPS) in genotypes Icatu and IPR108, irrespective of [CO_2]. In line with these results, plants grown at ambient [CO_2] showed a moderate increase in zeaxanthin and DEPS, indicating a conversion of violaxanthin into zeaxanthin, since a concomitant decrease in violaxanthin was observed. The same trend, although less pronounced, was recorded in Marsellesa plants under high [CO_2], which could suggest lower need of thermal dissipation, but not in the other genotypes,

where a decrease in VAZ and DEPS was observed, reaching values lower than control conditions.

A prompt heat stress recovery could have occurred in Marsellesa and Hybrid, regardless of [CO_2] treatments, as an increase in zeaxanthin and DEPS (only in Hybrid) was observed, especially after 4 days of recovery (Rec4) remaining higher than in controls at the end of the experiment, giving an advantage for possible future heat exposures. Geisha, on the other hand, showed the worst recovery, with antheraxanthin and especially zeaxanthin contents much lower than the initial control value.

In contrast to the recorded increase in zeaxanthin and DEPS, violaxanthin de-epoxidase (*VDE2*) gene expression was even downregulated by heat stress, regardless of CO_2 treatments. This discrepancy between transcript levels and enzyme activity was also reported by Ramalho et al. (2018), hence highlighting the need to combine physiological and molecular studies, together with morphological assessments, to better understand the plant's response to stress.

4.1.2. Carotenes

α- and β-carotene are two accessory pigments found in the reaction centers and core antennae of photosystems PSI and PSII (Johnson et al., 1993; Ramalho et al., 2003). Besides the function of absorbing light (especially blue light) together with chlorophylls, thereby expanding the spectrum of light that the plant can absorb, carotenes, and especially β-carotene, are important photoprotective molecules. β-carotene protects lipid membranes and chlorophyll *a* from oxidation (Lichtenthaler, 1987) by forming triplet carotenes, which quench $^1O_2$ and $^3Chl^*$ and dissipate energy by heat (Ramalho et al., 2003). Furthermore, β-carotene protects the chlorophyll *a* of Cyt b6/f complex from photobleaching promoted by $^1O_2$ (Zhang et al., 1999). Lower levels of β-carotene in the reaction centers have been associated with higher vulnerability to photodamage (De Las Rivas et al, 1993).

Under control temperature, higher β-carotene contents were found under enhanced [CO_2] in Marsellesa, which also increased the α- plus β-carotene (α+β) content. Higher level of carotenes under elevated [CO_2] have been reported also in *C. canephora* cv. CL153 (Martins et al., 2016). By contrast, in Geisha and Hybrid carotene contents did not differ between the

two CO_2 treatments, except for a moderately lower β-carotene concentration in Hybrid 700-plants which ultimately exhibited a higher α/β-carotene ratio.

Under supra-optimal temperatures, plants under ambient [CO_2] showed an overall increase in carotene contents, especially in Geisha and Hybrid which reached maximal values of α- and β-carotene at 31/25 °C, associated with a higher α/β-carotene ratio. Consequently, the total carotene content was higher at ambient rather than elevated [CO_2], indicating a higher need of protecting the photosynthetic components. However, in these two genotypes, carotenes started to moderately decrease at 37/28 °C and even more at 42/30 °C, where noticeable low values of α+β content and α/β ratio were observed. Similarly, Ramalho et al. (2003) observed a decrease in carotenes, and especially α-carotene, in *Coffea sp*. under cold stress. The decrease in α/β ratio has been interpreted as a protective mechanism against the energy in excess (Ramalho et al., 2003). In contrast, plants grown under enhanced [CO_2] attained maximal values of β-carotene at 37/28 °C, which could suggest a higher thermotolerance than the 400-plants; remarkable differences between the two CO_2 treatments can be observed in α-carotene values (and α/β-carotene ratio) in Hybrid at 42/30 °C, where the 700-plants reached the maximum values, although a substantial decrease was observed after the recovery period. A different trend occurred in Marsellesa plants: at ambient [CO_2] carotene contents increased as temperature was raised, which could confer higher thermotolerance at least until 37/28 °C, since a substantial decrease was recorded at 42/30 °C, although plants recovered after the experiment. Under enhanced [CO_2], α-carotene decreased too, but β-carotene content was markedly higher in the 400-plant counterparts, which could be due to a replacement of α-carotene by β-carotene, previously found to occur under light stress conditions (Demmig-Adams & Adams, 1996).

4.1.3. Other protective pigments

Neoxanthin and lutein are found in the periphery of the LHC of photosystems; they play important functions as they maintain the correct assembly and stability of antenna proteins (Ramalho et al., 2002). They are commonly considered as accessory pigments that transfer the absorbed light to chlorophyll (Lichtenthaler, 1987). However, apart from the primary function of providing stability, it has been shown that lutein could improve the protection of photosystems against photooxidation (Pogson et al., 1998) by quenching $^1Chl^*$ though thermal dissipation and even the triplet state of chlorophyll (Jahns & Holzwarth, 2012).

Under control temperature, higher level of neoxanthin and lutein were observed in Marsellesa plants grown under enhanced [CO_2], as reported by Martins et al. (2016) in *C. canephora*. In plants grown under ambient [CO_2], as temperature was raised an overall increase of the two xanthophylls was observed, reaching maximal values at 37/28 °C in Marsellesa and Hybrid and at 31/25°C in Geisha. However, drastic decreases were observed as temperature reached the maximum values, except for lutein which remained higher than control condition in Geisha and Hybrid, similarly with the values of lutein observed in Martins et al. (2016). Under supra-optimal temperatures, enhanced [CO_2] promoted higher content of neoxanthin and lutein in Marsellesa; remarkable differences between the two CO_2 treatments can be observed in lutein content at 42/30 °C, where Marsellesa 700-plants retained higher pigment values. Thus, in Marsellesa the concomitant decrease of lutein and β-carotene, which have complementary actions in protecting the photosynthetic apparatus (Martins et al., 2016), under increasing temperature regimes at ambient [CO_2] indicates that this genotype was highly affected by supra-optimal temperature, and that elevated [CO_2] strongly mitigated the heat stress impact. On the other hand, in the other genotypes, lutein and neoxanthin were generally lower in the 700-plants compared to the 400-plant counterparts.

4.2. ANTIOXIDANT ENZYMES

The enzymes selected in this study play an important role in the control of oxidative stress since they are able to react and reduce the concentration of several reactive oxygen species, such as H_2O_2, $^{*}O_2^{-}$ and $^{*}OH$. The control of ROS accumulation is accomplished through the ascorbate-glutathione cycle, with the participation of enzymes such as SOD, APX and GR, and extra-chloroplastic scavenging systems such as CAT. For example, Koussevitzky et al. (2008) reported the fundamental role of APX1 in the response of *Arabidopsis thaliana* to drought and heat stress, while GR was found to play a key role in the regeneration of GSH and the maintenance of the ascorbate pool (Ding et al., 2009). Therefore, the activity of APX, GR, SOD and CAT under supra-optimal temperatures is expected to increase. A summary of the different response of the three genotypes under the different temperatures and [CO_2] treatments is shown in Fig. 25.

At control temperature, the effect of elevated [CO_2] on the antioxidant enzymes was largely genotype dependent: in Geisha it provoked an upregulation of the activity of SOD and APX; in Marsellesa, except for APX, the activity decreased, while in Hybrid, higher [CO_2] resulted in a lower activity of the enzymes, except CAT. Lower values at elevated [CO_2] were

reported also by Martins et al. (2016) and were associated with a lower need of developing a robust antioxidant system due to higher photosynthetic and lower photorespiration rates. However, in contrast with the observed responses of enzyme activity under enhanced CO_2 levels, the corresponding gene expression at elevated [CO_2] of *CAT*, *CuSOD1* and *CuSOD2*, *APX-*$_{cyt}$, *APX-*$_{chl}$ and *APX-*$_{t+s}$ was not statistically different from the one at ambient [CO_2].

At 37/28 °C, coffee plants start to perceive the heat stress, yet they are not severely impacted as when they are exposed to 42/30 °C, as confirmed by previous studies (Rodrigues et al., 2016). Therefore, changes that occur at this temperature could be important to reveal which genotype has the strongest antioxidant system. Under supra-optimal temperatures, the activity of antioxidant enzymes is expected to increase to promote ROS scavenging, in agreement with the increase at 37/28 °C of GR (except the 400-plants of Marsellesa) and CAT activity in every genotype and both CO_2 treatments, APX in Hybrid (both CO_2 treatments) and in 400-plants of Geisha and Marsellesa. The increasing trend is in agreement with the results observed by Martins et al. (2016), where an increase in APX, GR and CAT in *C. canephora* cv. CL153 380-plants, GR and CAT in *C. arabica* cv. Icatu, and Cu,Zn-SOD and CAT in *C. arabica* cv. IPR108 700-plants was observed. These results are consistent with the upregulation of genes related to the antioxidative system components, especially in the 400-plants. CAT and APX activity, and the corresponding gene expression, was usually higher in the 400-plants, in line with the findings of Martins et al. (2016), where CAT and APX activity were higher in the 380-plants of CL153. Higher enzyme activity at ambient [CO_2] could suggest a greater production of ROS, thus a higher need to control them. The greatest difference between the two CO_2 treatments was recorded in APX activity in Marsellesa, in agreement with the great upregulation of *APX* (especially the cytosolic isozyme). In Marsellesa, while in the 400-plants SOD activity decreased, enhanced [CO_2] promoted an increase in the activity of this enzyme, differently from the higher upregulation of *CuSOD1* and *CuSOD2* in the 400-plants. A greater SOD activity could indicate a stronger control of superoxide radical and the increase in H_2O_2 production, which may act as an important signaling molecule and trigger further antioxidative responses. The most common response to oxidative stress under this temperature seemed to be that of CAT, denoting a strong extra-chloroplastic control of H_2O_2 which can diffuse across membranes (Fortunato et al., 2010).

With the additional temperature increase up to 42/30 °C, the further changes in enzyme activities varied across genotypes, but the prevalent reaction was a decrease in most of the

enzyme activities, in agreement with the deleterious effects on coffee plant observed by Rodrigues et al. (2016) at 42/34 °C. Nevertheless, in Geisha, CAT activity increased irrespective of CO_2 conditions, concomitant with enhanced gene expression (only in Geisha-400 and Hybrid-400), suggesting that the extra-chloroplastic control of H_2O_2 still remained the most relevant response, since APX and GR activity decreased, similar to what was reported by Martins et al. (2016) (except in IPR108), although *APX* was strongly upregulated in the 400-plants. In turn, in the 700-plants of Geisha an increase in SOD activity was observed, contrary to the decrease observed in the 400-plants, suggesting that SOD was not affected by supra-optimal temperature under enhanced [CO_2] or that the need of O_2^- scavenging was needed only at this temperature. However, except for SOD, the enzyme activities, together with their RNA transcripts, were usually lower in 700-plants than the 400-counterparts. An increase in photorespiration rate, thus a stronger need to scavenge H_2O_2, could explain the greater CAT activity observed at ambient [CO_2]. On the other hand, in Marsellesa, CAT activity decreased in both [CO_2] conditions, but in the 700-plants APX activity increased, in contrast with the sharp decrease in the 400-counterparts, suggesting a stronger ROS control under enhanced [CO_2]. Differently from the increase in *CAT*, *SOD* and *APX* gene expression, Hybrid plants exhibited a decrease in all enzyme activities, except CAT and SOD in the 400-plants, implying a lower thermotolerance and a possible photoinhibition status even in plants growth at elevated [CO_2]. In fact, a decrease in APX activity, but not in SOD, which produces H_2O_2, could lead to greater heat sensitivity and less efficient control of $^*O_2$ and $^*OH$. Overall, APX was the most affected enzyme at 42/34 °C, contrasting to the increase in *APX* gene expression, one of the most responsive genes to high temperatures. This could be explained by the fact that APX is a thermosensitive enzyme, hence the removal of H_2O_2 in the chloroplast could be impaired even under high [CO_2].

Along the recovery period (Rec14), in Geisha plants under ambient [CO_2], a remarkable decrease of the antioxidant enzymes (except SOD) was observed. In fact, the enzymes reached values lower than control conditions, suggesting that they were highly affected by the heat stress and that the plant could not return to the normal functioning of balancing ROS production and scavenging. However, this trend was in contrast with the observed results of gene expression, since both *CAT* and *APX* transcripts were remarkably higher than the basal level, especially *APX*-$_{Cyt}$. On the other hand, under enhanced [CO_2] CAT and GR activity remained considerably high, even higher than control conditions, in accordance with the high *CAT* transcript at Rec14, which could be helpful for possible future heat stress episodes. APX

and SOD, in turn, were very low compared to control condition even under high [CO_2], but this could be due to an error in the experiment at 25/20 °C, since the activity of the two enzymes in Geisha-700 is excessively high. In Marsellesa and Hybrid a decrease in enzyme activities was observed too, with the exception of APX in Hybrid-700 and SOD in Marsellesa-400 (in accordance with the maintenance of high transcript levels of *CuSOD2*), suggesting that plants could be permanently affected. Nevertheless, GR and SOD activity in Marsellesa, together with GR in Hybrid-700, were higher than control conditions, in line with gene expression results, likely giving a future advantage to the plants and conferring a possible stress memory with a consequent increase in the constitutive enzyme activity.

The antioxidant defense system in coffee plants was previously reported to increase under several stress conditions. Fortunato et al. (2010) found that Cu,Zn-SOD and APX activities were enhanced in some coffee genotypes subjected to a gradual cold treatment. An efficient ROS control was also observed in Ramalho et al. (2018), in which *C. arabica* and *C. canephora* cultivars were exposed simultaneously to drought and cold stress. Apart from coffee plants, chilling tolerance was reported by Kang & Saltveit (2002) in cucumber seedling radicles, where higher APX and CAT enzyme activity was observed after the stress imposition.

The antioxidant response under high [CO_2] has been studied also in plants different from coffee, showing variable results. For example, *A. thaliana* under heat and drought stress showed high levels of ascorbate and CAT activity under elevated [CO_2] (Zinta et al., 2014). In several species a reduction of antioxidant molecules was observed: during alfalfa (*Medicago sativa* L. cv. Aragòn) regrowth, CAT activity was reduced by high [CO_2] (Erice et al., 2007), while in soybean (*Glycine max* (L.) Merr.) elevated [CO_2] decreased the activity of SOD, CAT, APX, and GR (Pritchard et al., 2000). Therefore, the response to elevated CO_2 and its potential mitigating effect under stress conditions is strongly species-dependent and it appears to vary also between different cultivars, as reported in this study.

Not always transcript levels of genes coding for antioxidant enzymes were in line with the enzyme activity trend. The notion that gene expression does not always perfectly follow biochemical patterns has been previously mentioned (Martins et al., 2016; Ramalho, et al., 2018). This is not surprising due to post-transcriptional and post-translational mechanisms regulating protein synthesis and enzyme function. For example, *CuSOD1* and *CuSOD2* expression was upregulated in all three genotypes and under both CO_2 treatments, in contrast

to the observed decrease in SOD activity in several samples. Nevertheless, the upregulation of genes related to the antioxidative system, as observed during the experiment, could suggest a stronger ROS control under stress. Transgenic tobacco (*Nicotiana tabacum* L.) plants that over-express a gene encoding for Cu,Zn-SOD were reported to be more tolerant to oxidative stress, having higher SOD and APX activities, than the plants that did not express the SOD isoform (Gupta et al., 1993).

Figure 25: Schematic representation of APX, GR, CAT, and SOD activity changes in C. arabica cv. Geisha3, Marsellesa and Hybrid (Geisha3xMarsellesa), under 400 or 700 µL CO_2 L^{-1} and supra-optimal temperatures of 37/28°C, 42/30 °C, and after 14 days of recovery, relative to control temperature (25/20 °C).

TRAITS	RESPONSES						
	eCO2 vs aCO2 (25/20 °C)	37/28 °C		42/30 °C		Rec14	
		400 ppm	700 ppm	400 ppm	700 ppm	400 ppm	700 ppm
APX	+	+	=	=	-	-	-
	=	+	=	=	=	=	=
	-	+	+	-	-	-	=
GR	=	+	+	+	=	=	=
	-	-	+	=	+	-	+
	-	+	+	+	+	+	+
CAT	-	+	+	+	+	-	+
	-	+	+	+	+	+	=
	+	+	+	+	=	+	-
SOD	+	-	-	-	=	-	-
	-	-	+	-	+	=	=
	-	-	=	=	=	-	-

Geisha
Marsellesa
Hybrid

\+ Increase*
\- Decrease*
= Unchanged*

*Relative to 25/20 °C

4.3 OTHER PROTECTIVE MOLECULES

Besides the antioxidant enzymes and photoprotective pigments, other protective molecules were assessed through the gene expression studies. These molecules are reported to have crucial roles in the maintenance of cellular homeostasis under several environmental stresses, including heat stress. However, it should be mentioned that future biochemical studies regarding these molecules could strengthen the obtained results, by corroborating or contradicting the observed changes at the transcriptional level.

The 70-kD Heat Shock Proteins (HSP70s) are one of the most important protective molecules in plants subjected to heat stress. The over-expression of these molecular chaperones was related to higher protection against DNA degradation in transgenic plants under heat stress conditions (Grover et al., 2013). In addition, overexpression of *HSP70* genes is correlated with higher tolerance to heat stress (Wang et al., 2004). Present results show that in coffee plants grown under optimal temperature, enhanced [CO_2] did not increase *HSP70* gene

expression, similar to the findings of Martins et al. (2016), where even a downregulation occurred in the 700-plants. With rising temperature, *HSP70* gene upregulation was observed in all genotypes and CO_2 treatments, although higher in the 400-plants, and remained moderately higher than control conditions even after the recovery period. Previous studies showed how HSP70 synthesis is one of the first response to high temperatures (Martins et al., 2016). However, maximal values are found only at 42/30 °C, especially in the 400-plants of Marsellesa. The lower values recorded in the 700-plants suggest that high [CO_2] could enhance thermotolerance in coffee plants, since *HSP70* genes are usually upregulated under stress conditions, as previously observed by Marques et al. (2021). HSP70s, since they are involved in PSII repair, might protect plants from oxidative stress by stimulating antioxidant enzyme activities under supra-optimal temperature (Martins et al., 2016), as observed with the increase in CAT and GR activities. Panchuk et al. (2002), in fact, found a positive correlation between the expression of the gene encoding for APX and heat shock transcription factors (HSFs) in transgenic plants of Arabidopsis under heat stress.

Transcript levels of other protective molecules, i.e., chaperonins 20 and 60 (*Chape20* and *Chape60*) and early light-induced protein (*ELIP*) were also evaluated in this work. These chaperonins support the correct folding of new proteins, especially plastid proteins like RuBisCO, playing an important role in heat stress tolerance (Wang et al., 2004). As for all other the genes, under control temperature *Chape20* and *Chape60* were not significantly altered by enhanced [CO_2], as also reported by Martins et al. (2016) in which even a downregulation occurred in some 700-plants. Similar to *HSP70* expression, as temperature was increased the expression of *Chape20* and *Chape60* genes was upregulated in all genotypes and CO_2 treatments, with maximal transcript values at 42/30 °C and a subsequent decrease after the heat stress imposition (Rec14).

ELIPs are found in thylakoid membranes and protect plants under different environmental stresses. They can participate in the oxidative stress response by dissipating excess energy and preventing the formation of radicals (Liu et al., 2020). As reported by Martins et al. (2016), higher *ELIP* upregulation was expected to occur in the 700-plants. However, elevated [CO_2] provoked lower transcript levels than in the 400-plant counterparts during all temperature treatments; large differences between the CO_2 treatments were found especially in Marsellesa. The lower upregulation of these protective molecules found in the 700-plants

compared to the 400-plant counterparts could suggest a lower need of protecting photosynthetic components from photoinhibition.

5. CONCLUSIONS

Under an optimal temperature regime (25/20 °C), enhanced [CO_2] did not significantly alter the expression of genes coding for antioxidant enzymes and protective molecules, despite the large differences and the reinforcement of the photosynthetic functioning observed in previous studies (Ramalho et al. 2013).

As heat stress was imposed, upregulation of the selected genes was recorded in all genotypes and in both CO_2 treatments, although much more pronounced in plants grown at ambient [CO_2]. However, some post-transcriptional and post-translational mechanisms might have occurred since the observed enzyme activities did not perfectly follow the general upregulation of the corresponding genes.

Generally speaking, coffee plants were most affected at the most extreme temperature; among the selected genotypes, Marsellesa showed higher vulnerability to supra-optimal temperatures. However, at elevated [CO_2], Marsellesa and Hybrid maintained higher levels of some carotenoids relative to the ones at ambient [CO_2], conferring a possible higher thermotolerance in these genotypes. Furthermore, the significant increase in DEPS observed in all plants grown under ambient [CO_2], but not under elevated [CO_2], could represent a fundamental marker for photoinhibition and a PSII transition from a photosynthetic to photoprotective conformation. The reduced activity of APX and CAT recorded in Geisha plants under enhanced [CO_2], together with the lower upregulation of all selected genes, could represent a reduction in the oxidative pressure and photoinhibition status. In fact, under elevated [CO_2], a greater photosynthetic functioning, with a concomitant reduction in photorespiration rate, could reduce the probability of ROS accumulation and the need of antioxidant enzymes. Future research on net photosynthesis, photosynthetic enzymes (RuBisCO and Ru5PK) activity, PSII photoinhibition, and ultimately yield and coffee bean quality will confirm if enhanced [CO_2] is able to promote an increase in photosynthetic capacity of coffee plants under supra-optimal temperatures.

In conclusion, present results are in line with the previously observed tolerance of *C. arabica* plants to temperatures up to 37/28 °C, as confirmed by the overall increase both in photoprotective pigments, especially carotenes, which protect thylakoid membranes by dissipating energy, and antioxidant enzymes, which are important to reduce ROS accumulation. Oxidative stress is an indirect consequence related to both drought and heat stress, which usually occur concomitantly under field conditions, especially in tropical areas. Together with the rise in global mean temperatures, extreme events and drought episodes are predicted to increase, which could exacerbate the impacts of high temperatures on coffee plants performance. Under these circumstances, there is no doubt that oxidative stress control will play a crucial role in a climate change scenario. As a result, by reinforcing the antioxidant protection, coffee plants could be more tolerant to global warming well above what was traditionally stated.

It is, therefore, of outermost importance to improve knowledge regarding plant antioxidant defense under abiotic stresses, and to select the most tolerant genotypes able to cope with future climate changes.

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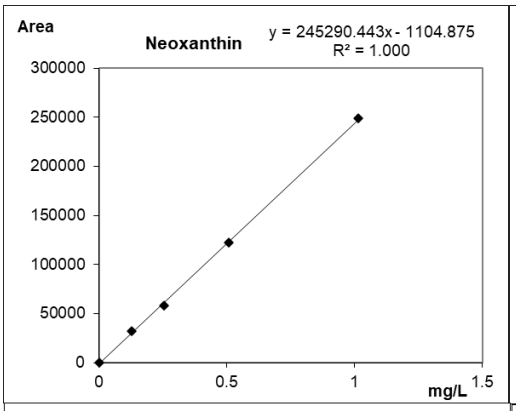
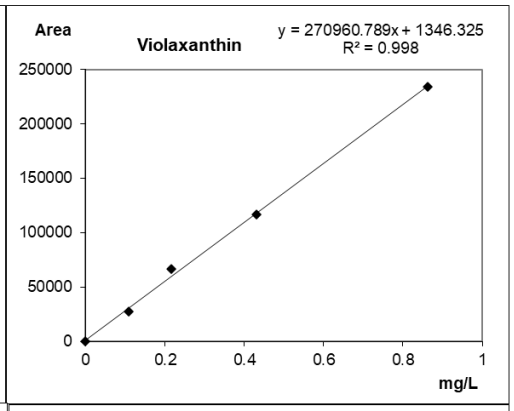
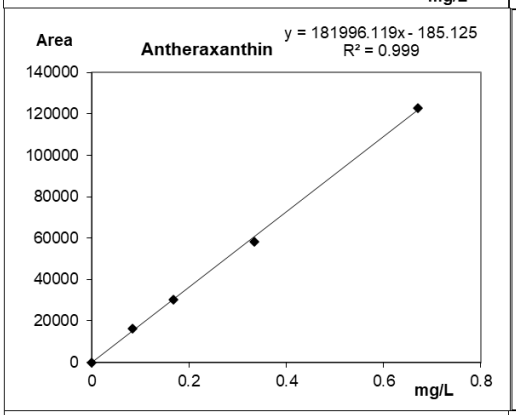
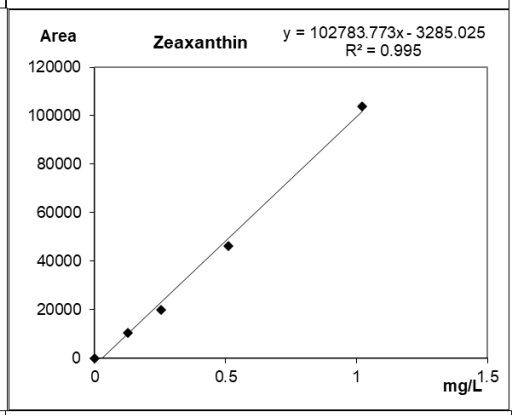
ANNEX

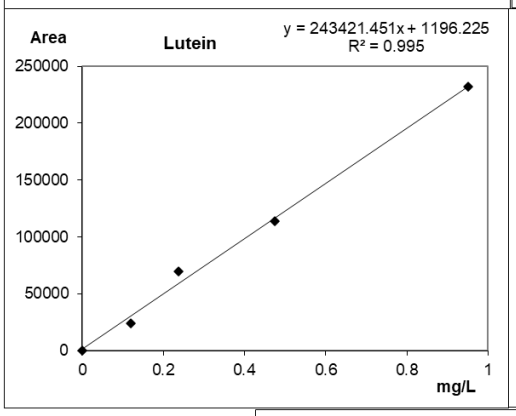
Calibration curves of pigments built using individual carotenoid standards for HPLC quantification, using the same column condition and gradient.

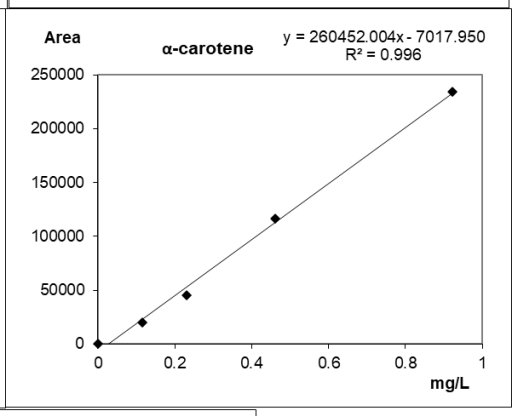

Area
Neoxanthin
y = 245290.443x - 1104.875
R² = 1.000
300000
250000
200000
150000
100000
50000
0
0
0.5
1
1.5
mg/L

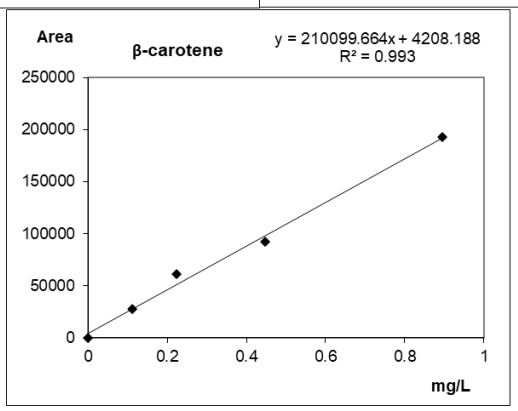

Area
Violaxanthin
y = 270960.789x + 1346.325
R² = 0.998
250000
200000
150000
100000
50000
0
0
0.2
0.4
0.6
0.8
1
mg/L


Area
Antheraxanthin
y = 181996.119x - 185.125
R² = 0.999
140000
120000
100000
80000
60000
40000
20000
0
0
0.2
0.4
0.6
0.8
mg/L


Area
Zeaxanthin
y = 102783.773x - 3285.025
R² = 0.995
120000
100000
80000
60000
40000
20000
0
0
0.5
1
1.5
mg/L


Area
Lutein
y = 243421.451x + 1196.225
R² = 0.995
250000
200000
150000
100000
50000
0
0
0.2
0.4
0.6
0.8
1
mg/L


Area
α-carotene
y = 260452.004x - 7017.950
R² = 0.996
250000
200000
150000
100000
50000
0
0
0.2
0.4
0.6
0.8
1
mg/L


Area
β-carotene
y = 210099.664x + 4208.188
R² = 0.993
250000
200000
150000
100000
50000
0
0
0.2
0.4
0.6
0.8
1
mg/L