UNIVERSIDADE FEDERAL DE LAVRAS

BÁRBARA CASTANHEIRA FERRARA BARBOSA

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF TWO *Coffea arabica* CULTIVARS UNDER DIFFERENT HEAT STRESS CONDITIONS

LAVRAS – MG

2016

BÁRBARA CASTANHEIRA FERRARA BARBOSA

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF TWO *Coffea arabica* CULTIVARS UNDER DIFFERENT HEAT STRESS CONDITIONS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para a obtenção do título de Doutor.

Prof. Antônio Chalfun Junior, PhD

Orientador

LAVRAS – MG

2016

BÁRBARA CASTANHEIRA FERRARA BARBOSA

CARACTERIZAÇÃO FISIOLÓGICA E MOLECULAR DE DUAS CULTIVARES DE *Coffea arabica* SOB DIFERENTES CONDIÇÕES DE ESTRESSE TÉMICO

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF TWO *Coffea arabica* CULTIVARS UNDER DIFFERENT HEAT STRESS CONDITIONS

> Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para a obtenção do título de Doutor.

APROVADA em 7 de abril de 2016.

Patricia Klein, PhD	Texas A&M University
Leonardo Lombardini, PhD	Texas A&M University
Prof. Dr. Luciano Vilela Paiva	UFLA
Cecilio Frois Caldeira Jr, PhD.	Instituto Tecnológico Vale

Prof. Antônio Chalfun Junior, PhD
Orientador

LAVRAS – MG

2016

Dedicado a minha mãe
Eunice Leite Castanheira,
razão do meu viver.

AGRADECIMENTOS

À Universidade Federal de Lavras e ao programa de Agronomia/Fisiologia Vegetal pela oportunidade de realização do doutorado.

À Texas A & M University pela oportunidade de realizar a pesquisa em suas instalações

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento Pessoal de Nível Superior pela concessão da bolsa de estudos.

Ao Professor Antônio Chalfun Junior pela a supervisão, orientação e confiança.

Ao Dr. Leonardo Lombardini por toda a ajuda e por estar sempre disponível.

À Dr. Patricia Klein pela ajuda com a análise de dados e por me permitir utilizar as instalações de seu laboratório para parte de meus experimentos.

À todos os membros do grupo do Laboratório de Fisiologia Molecular de Plantas pela amizade e companheirismo. Especialmente, ao Dr. Cecílio Frois, Andressa Spuri Azarias, Bruno Garcia e Carlos Cardon por toda a ajuda enquanto eu estava no exterior.

À todos os membros do grupo da Dr Klein na Texas A & M University por toda ajuda e receptividade..

Aos meus queridos amigos Samuel Chaves Silva e Juliana Pace Salimena, por sempre estarem presentes, mesmo quando estávamos a milhares de quilômetros de distância.

Aos meus amigos de College Station Vinícius Venânio e Paula Cipriano por todos os bons momentos juntos.

Ao meu querido namorado Aaron Harmon por ser sempre tão carinhoso e compreensivo.

À minha família pelo amor e apoio dado ao longo da minha vida.

Obrigada.

GENERAL ABSTRACT

Coffee is one of the most valuable primary products in world trade. Its cultivation, processing, transport, and marketing employs over 120 million people and significantly affects the gross national product (GNP) of 40 countries. Worldwide, coffee is grown on over 10,000,000 hectares of land. Smallholder farmers cultivate over 70% of production, making coffee crucial to the economy of many developing countries. Currently, Brazil is the world's leading producer of coffee, a position the country has held for the last 150 years, producing roughly 40% of the world's supply of Arabica beans across more than 2,000 farms in 16 states. According to the Intergovernmental Panel on Climate Change (2014), there is a high probability of temperature increases of 1 to 3°C in the tropics over the next 20 years. The losses to Arabica coffee production, as a result of increasing temperatures, are estimated to be as much as 10% of total production in 20 years. Therefore, in the face of imminent climate changes, the development of cultivars that are tolerant to adverse environmental conditions is essential. To that end, the identification of the key genes involved in plant responses to abiotic stress is critical for improving the crop by either traditional breeding or genetic transformation. Hence, we aimed to understand the impact of heat stress at the physiological and molecular levels on the growth and development of coffee. Moreover, we aimed to identify the genes that are differentially transcribed in response to high temperatures, which may lead to the future development of cultivars with improved quality and higher harvest security.

RESUMO GERAL

O café é um dos produtos primários mais valiosos no comércio mundial. Seu cultivo, processamento, transporte e comercialização emprega mais de 120 milhões de pessoas e afeta significativamente o produto interno bruto (PIB) de 40 países. Em todo o mundo, o café é cultivado em mais de 10 milhões de hectares. Os pequenos agricultores cultivam mais de 70% da produção, tornando o café crucial para a economia de muitos países em desenvolvimento. Atualmente, o brasil é o maior produtor mundial de café, uma posição que o país tem ocupado nos últimos 150 anos, produzindo cerca de 40% da oferta mundial de grãos arábica em mais de 2.000 fazendas em 16 estados. De acordo com o painel intergovernamental sobre mudanças climáticas (IPCC), há uma alta probabilidade de aumentos de temperatura de 1 a 3 ° c nos trópicos ao longo dos próximos 20 anos. As perdas na produção de café arábica, como resultado do aumento das temperaturas, são estimadas em até 10% da produção total em 20 anos. Portanto, em face das mudanças climáticas iminentes, o desenvolvimento de cultivares tolerantes a condições ambientais adversas é essencial. Para esse fim, a identificação dos principais genes envolvidos nos processos fisiológicos de interesse é fundamental para melhorar a cultura por seja por melhoramento tradicional ou transformação genética. Por isso, buscamos compreender o impacto do estresse térmico em níveis fisiológicos e moleculares no crescimento e desenvolvimento do café. Além disso, buscou-se identificar os genes que são diferencialmente transcritos em resposta a altas temperaturas, o que pode levar ao desenvolvimento futuro de cultivares com melhor qualidade e maior produção.

Palavras-chave: RNAseq. Café. Estresse abiótico.

SUMÁRIO

CHAPTER 1 – GENERAL INTRODUCTION

1 INTRODUCTION

Coffee is one of the most valuable primary products in world trade. Its cultivation, processing, transport, and marketing employs over 120 million people and significantly affects the gross national product (GNP) of 40 countries (LINGLE, 2008). Worldwide, coffee is grown on over 10,000,000 hectares of land. Smallholder farmers cultivate over 70% of production, making coffee crucial to the economy of many developing countries. Currently, Brazil is the world's leading producer of coffee (MISHRA; SLATER, 2012), a position the country has held for the last 150 years, producing roughly 40% of the world's supply of Arabica beans across more than 2,000 farms in 16 states.

Despite its economic importance, coffee's production and market are subjected to regular oscillations in both prices and volume of production. The biannual cycle of the crop and the adverse effects of weather conditions mainly explain these variations. Among them, drought and high temperatures are the factors that most affect plant development and production (ASSAD et al., 2004; Matta, 2004; MATTA; RAMALHO, 2006). According to the Intergovernmental Panel on Climate Change (2014), there is a high probability of temperature increases of 1 to 3°C in the tropics over the next 20 years. The losses to Arabica coffee production, as a result of increasing temperatures, are estimated to be as much as 10% of total production in 20 years (CAMARGO, 2010; DAVIS et al., 2012; ESTRADA; GAY; CONDE, 2012; LADERACH; JARVIS, 2008; RAMIREZ-VILLEGAS et al., 2012).

Therefore, in the face of imminent climate changes, the development of cultivars that are tolerant to adverse environmental conditions is essential. To that end, the identification of the key genes involved in plant responses to abiotic stress is critical for improving the crop by either traditional breeding or genetic

transformation. Previous studies found that the genetic basis of plant response to abiotic stress, such as extreme temperatures, is polygenic, meaning that more than one gene influence it. The transcription level of several genes is affected, resulting in physiological and biochemical responses, including altered patterns of photosynthesis (ZONIA; MUNNIK, 2006), respiration (DAVIDSON; SCHIESTL, 2001), and hormone production (LARKINDALE; KNIGHT, 2002; TEALE; PAPONOV; PALME, 2006).

Hence, we aimed to understand the impact of heat stress at the physiological and molecular levels on the growth and development of coffee. Moreover, we aimed to identify the genes that are differentially transcribed in response to high temperatures, which may lead to the future development of cultivars with improved quality and higher harvest security.

2 BACKGROUND

2.1 Coffee

Coffee is a perennial dicotyledonous tree belonging to the family Rubiaceae, which is the fourth largest family of angiosperms (STEVENS, 2012). The Rubiaceae family has about 500 genera and over 6,000 species, including the genus *Coffea*, the most important member in economic terms (INTERNATIONAL COFFEE ORGANIZATION, 2015b). Although the genus *Coffea* contains more than 124 species (DAVIS et al., 2012), only two have economic significance: *Coffea arabica* and *Coffea canephora*. *Coffea arabica* is native to the African continent and was originally found in southwestern Ethiopia, Sudan, and northern Kenya. The areas ideal for cultivation are usually above 600 meters in altitude in temperate zones (DAVIS et al., 2012). Despite its demanding production system, *C. arabica* represents 70% of total coffee production and has a better cup quality with low caffeine content (BARRETO et al., 2012).

As the second most highly traded commodity in the world (second only to oil), coffee exports account for a significant proportion of taxes and gross domestic product in many countries. The global coffee trade in 2014 reached 143 million 60 kg bags and accounted for exports worth an estimated $15.4 billion (ICO, 2015a). Brazil is the main producer and exporter of coffee, followed by Vietnam, Indonesia, Colombia, Ethiopia, Honduras, India, Mexico, Peru, Guatemala, Ivory Coast, Nicaragua, and El Salvador. In 2014, Brazil held the leading position in production and exportation and was the second largest consumer of coffee. The country harvested 45.4 million 60 kg bags of processed coffee. From January to December 2014, coffee accounted for 6.9% of all Brazilian exports, generating $6.66 billion in revenues and ranking fifth in national agribusiness exports.

In Brazil, climatic variability is the main factor causing oscillations in coffee grain yield. When it comes to the physiology of Arabica coffee, according to Camargo (2010), the optimal mean annual air temperature range is between 18 to 23°C. Above that temperature, there is an acceleration of fruit development and ripening, which can lead to quality loss. Depressed growth and abnormalities, such as yellowing of leaves, can result from continuous exposure to daily temperatures as high as 30°C. Additionally, flower abortion may occur when the air temperature during blooming is high, especially if the high temperature is associated with drought (MATTA; RAMALHO, 2006).

2.2 Climate change and the coffee crop

Global warming is undeniable, and it is mainly due to human interference in the climate system (INTERGOVERNMENTAL PANEL ON CLIMATE CHANGE, 2014). Increases in the emissions of greenhouse gases like carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) are causing wide changes in atmospheric events with critical impacts on vegetation (NUNEZ; SOLMAN; CABRE, 2009).

According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC, 2014), it is likely that semi-arid and sub-humid regions of Asia, Africa, and Latin America will warm, and freshwater availability is projected to decrease during this century. In Latin America, the air temperature is expected to increase 1.1 to 6.4°C. In Brazil, regions that are currently the largest producers of coffee may not be suitable for cultivation even with irrigation. It has been suggested that global warming could lead to industry losses of up to $2.9 billion in 2020 (IPCC, 2014).Increases in air temperature and shifts in weather patterns associated with climate change can result in drought conditions that affect plant growth, metabolism, and ultimately production.

In Brazil, coffee is usually cultivated in the open (i.e., not under the shade of large trees, as it is typical in Centro-American countries) and in this system leaves are constantly exposed to high irradiance levels. This means that more energy than can be used by photosynthesis is absorbed, which may cause an energy overcharge and overheating of the leaves. As a result, vegetative growth is reduced and lower tree vigor is observed. Floral abnormalities and low fruit quality can also occur. In addition, there is typically an increase in the incidence of pests and diseases in plants (MISHRA; SLATER, 2012). Ultimately, environmental changes induce low cup quality due to changes in the biochemical composition of grains by reducing sugars, proteins, and caffeine (MAZZAFERA, 2007). For example, caffeine content of grains was reduced when coffee plants were subjected to irrigation and high temperatures. Also, activities of proteases and high polyphenol oxidase were observed in conditions of high temperature and low precipitation (SILVA et al., 2005).

2.3 Coffee genomics

C. arabica is the only allotetraploid species (2n=4x=44 chromosomes) of the *Coffea* genus. A natural hybrid between the diploids *C. eugenioides* and *C. canephora*, it is mostly autogamous, while *C. canephora* and other known coffee species are diploids (2n=2x=22 chromosomes) and allogamous, multiplying mostly by cross-fertilization (FAZUOLI, 1999).

The process in which two genomes are adapted to coexist within the same nucleus is complex. To investigate the effects of genomic fusion and duplication, different studies have evaluated the divergence in the transcriptomes between parental plants and natural allopolyploids. In the additive model, it would be expected that the expression on allopolyploid was equivalent to the average expression of the parental species. However, gene expression analysis revealed a non-additive trend in allopolyploids such as *Gossypium hirsutum*

(CHAUDHARY et al., 2009) and *Triticum aestivum* (PUMPHREY et al., 2009). In coffee, recent microarray studies demonstrated a preferred expression of *C. canephora* homologs in plants subjected to high temperatures and *C. eugenioides* at low temperatures (BARDIL et al., 2011; PRIVAT et al., 2011).

Moreover, Arabica coffee is characterized by its low genetic variability (ANTHONY et al., 2001; LASHERMES et al., 1999), which is mainly a consequence of its autogamous reproductive biology (PRAKASH et al., 2002). The small area where coffee originated from and the small number of genotypes used during the dispersion process of the crop also contribute to the low variability (ANTHONY et al., 2002; VIDAL et al., 2010).

2.4 Abiotic stresses

Abiotic stresses are the primary cause of loss in agricultural productivity, as they alter plant optimal conditions causing functional disturbances in the cell (JASPERS; KANGASJARVI, 2010). Coffee production is subject to regular oscillations, which is explained by the biennial growth cycle and also by the adverse effects of weather conditions. Heat and drought represent the biggest climate challenges to be overcome to ensure the survival of crops and sustainable food production (ASSAD et al., 2004; JALEEL et al., 2009; MATTA, 2004; MATTA; RAMALHO, 2006).

Plants respond to drought stress and high and low temperatures by changing their development program and physiology (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000). One of a plant's first responses to stress is to express a vast number of genes whose products are involved in different adaptive modifications (THOMASHOW, 1999). The speed of signal perception and the consequent transcriptional changes that lead to a possible adaptation and stress tolerance vary among species and genotypes within the same species (SREENIVASULU; SOPORY; KISHOR, 2007).

To understand the nature and function of genes involved in stress tolerance, intense research has been performed to identify and characterize stress-induced genes. According to Shinozaki and Yamaguchi-Shinozaki (2007), gene products induced by stress can be classified into two groups (Figure 1).

Figure 1 - Functions of drought stress-inducible genes in stress tolerance and response
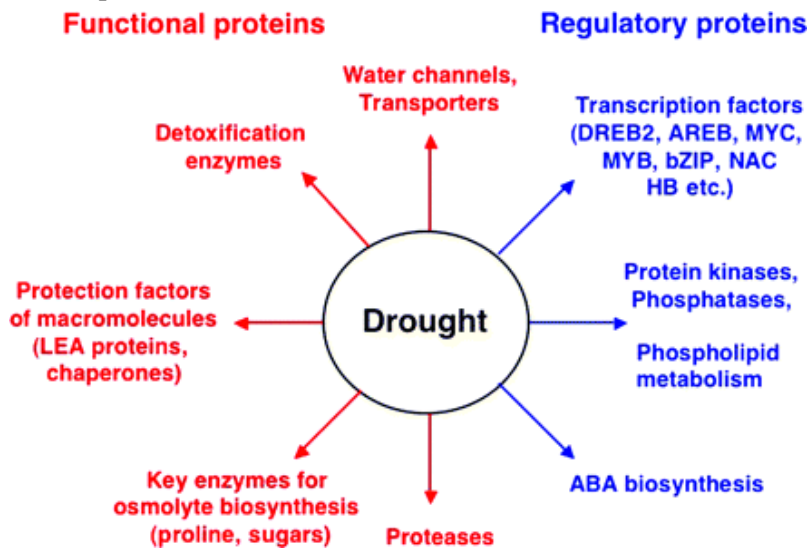
Source: Extracted from Shinozaki and Yamaguchi-Shinozaki (2007).

The first group includes genes that encode proteins involved in the synthesis and transport of osmoprotectants (proline, glycine betaine, polyamines, trehalose, mannitol, and galactinol) and the detoxification of reactive oxygen species (ROS) (glutathione S-transferases, hydrolases, superoxide dismutases, and ascorbate peroxidases). This group also includes genes coding for aquaporins, heat shock, and late embryogenesis abundant (LEA) proteins, and proteases.

The second group contains the transcription factors AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger, and WRKY. They are involved in

gene expression and signal transduction and play a fundamental role in the plants response to stress. This group also includes protein kinases, phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2006).

2.4.1 Heat Stress

High temperatures greatly impact many physiological and metabolic processes in all living organisms (WAHID et al., 2007) (Figure 2). Heat stress is harmful to plant growth and productivity since it can disturb cellular homeostasis by causing the denaturation of many proteins. There are also indirect damages such as enzyme inactivation in chloroplasts and mitochondria, protein synthesis inhibition, protein degradation, and loss of membrane integrity (HOWARTH, 2005). Thermal stress also affects the organization of microtubules (SMERTENKO et al., 1997) and induces the production of secondary metabolites and phenolic compounds such as flavonoids and phenylpropanoids (BARTWAL et al., 2013). This damage eventually leads to a reduction in ion flux, production of toxic compounds and reactive oxygen species (HOWARTH, 2005), and consequent retardation in growth and development (HALL, 2001).

Figure 2 - Heat induced changes in plants
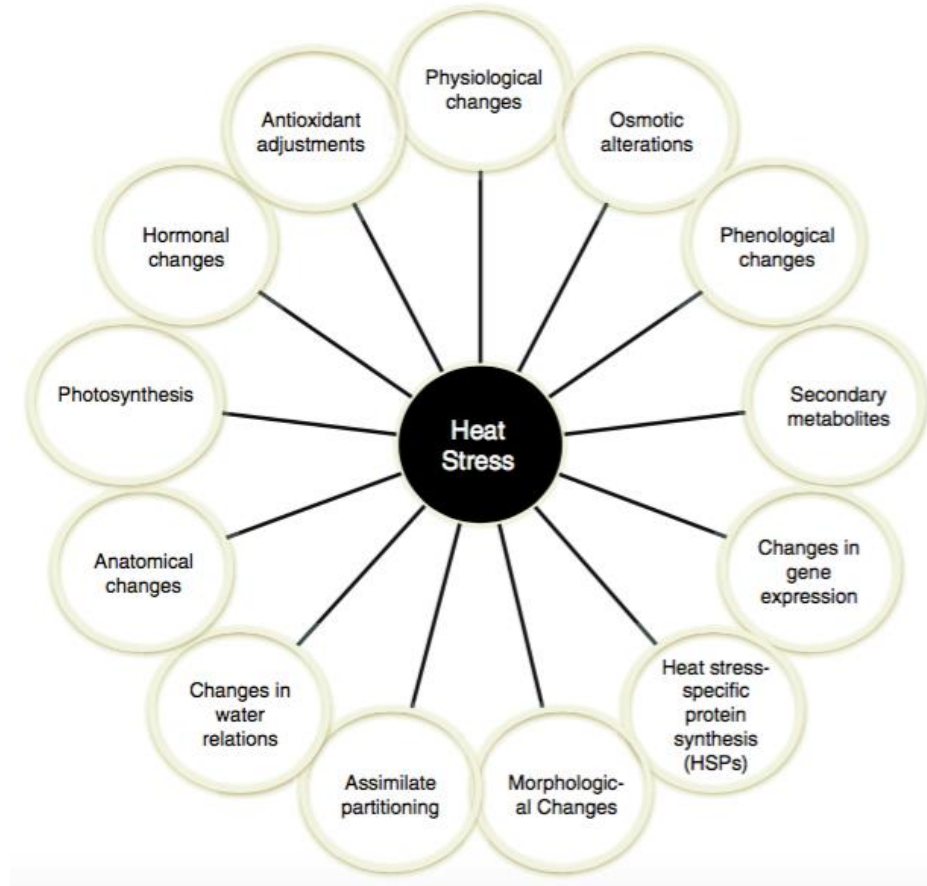
Source: Extracted from Tuteja (2012).

Additionally, an increase in temperature causes a reduction in photosynthetic rates because it favors photorespiration (PENUELAS; LLUSIA, 2002) and causes damages to the photosynthetic apparatus (ZHANG et al., 2014). The photosynthetic activity of chloroplasts is among the cellular functions most affected by heat (HU et al., 2015). Heat stress response in plants is a highly conserved process caused by exposure to high temperatures. Immediately after exposure to heat, there is a change in gene expression and

transcript accumulation, which leads to a higher synthesis of proteins related to stress tolerance (IBA, 2002).

Heat shock proteins (HSPs) are widely known as a major adaptive strategy to heat tolerance. HSPs have a molecular weight between 10 and 200kDa and are involved in signal transduction during stress. These proteins are chaperones that control the proper folding and conformation of structural and functional proteins, ensuring the maintenance of homeostasis during heat stress (PARK; SEO, 2015).

HSPs can be found in different cellular compartments such as organelles (nucleus, mitochondria, chloroplasts, and endoplasmic reticulum) and cytoplasm. The HSPs described so far can be grouped into five major families whose names are designated by the approximate molecular weights: the small HSPs (sHSP), the chaperonins (GroEL and HSP60), the HSP70 (DnaK), HSP90, and HSP100 (Clp) (SABEHAT; WEISS; LURIE, 1998; WANG et al., 2004).

Heat stress transcription factors (HSFs) are regulatory proteins mostly found in the cytoplasm that control the transcription of HSP encoding genes (BANIWAL et al., 2004). Among the most highly conserved transcriptional regulatory elements in nature, HSFs are activated in the presence of HSPs. They act by binding to heat shock elements in the promoters of target genes (HSPs), activating gene expression and therefore, controlling the heat stress response (HAHN et al., 2004).

The role of HSFs in heat stress has been reported in several studies. The HSFA2 gene for instance, was up regulated in tomato early development (GIORNO et al., 2010) and also improved heat tolerance in wild *Arabidopsis thaliana* (HU; HU; HAN, 2009). Additionally, in Arabidopsis, HSFs may be induced by cold, heat, water, and salt stresses (SWINDELL; HUEBNER; WEBER, 2007). Moreover, there have been reports on the role of HSFs in the regulation of other HSFs. In *A. thaliana*, under heat and high light stress,

HSFA1d and HSFA1e were identified as key regulators of HSFA2 (NISHIZAWA-YOKOI et al., 2011). Hahn et al. (2011) found a versatile regulatory mechanism in tomato, where HSP70 and HSP90 together regulate different HSFs. Some reports also showed a role for HSFs under other abiotic stresses (BANTI et al., 2010; CHAUHAN et al., 2011). In general, the heat stress response in plants was proven to be genetically complex, since the overexpression of a single HSF or HSP gene had limited impact on the increase of thermotolerance (VINOCUR; ALTMAN, 2005).

In addition to HSPs, other molecules such as LEA proteins play a fundamental role during periods of stress (WAHID et al., 2007). During stress, an increase in the influx of Ca^{+2} and cytoskeletal reorganization can occur, resulting in the regulation of mitogen-activated protein kinases (MAPK) and calcium dependent protein kinase (CDPK). Moreover, the production of ROS in organelles as a consequence of heat stress activates nuclear signaling cascades, and leads to the production of antioxidants and osmolytes that are crucial for proper water balance and osmotic adjustment (BOHNERT; JENSEN, 1996).

Besides being a concern for the cultivation of agronomically important crops in hot regions, global warming can increase the median temperature of the world, aggravating the problem of heat stress. Additionally, warm temperatures are also associated with drought conditions, which lead to water stress. So, understanding both heat and drought tolerance mechanisms and how they interact is critical to mitigate the effects of abiotic stresses on productivity (HUANG; XU, 2008).

2.4.2 Water stress

The importance of water for living beings is the result of its physical and chemical properties (SHAO et al., 2009). Water is a proton donor, which is absorbed and assimilated by plants during photosynthesis. Water is also a

solvent for many substances such as inorganic salts, sugars, and organic anions. When in the liquid form, water allows diffusion and mass flux of solutes and is essential for the transport and distribution of nutrients and metabolites (SANCHEZ-ROMERA et al., 2014).

To withstand environmental stresses such as drought, plants have evolved mechanisms of tolerance (CHAVES et al., 2002; SHVALEVA et al., 2006). Nogueira, Albuquerque e Silva (2005) stated that in *A. thaliana* at least 130 genes are involved in the mechanisms of drought tolerance. These mechanisms include morphological, structural and physiological changes, such as growth inhibition, control of transpiration, and stomatal closure.

In plants, drought conditions trigger the production of abscisic acid (ABA) that causes stomatal closure and induces the expression of genes related to stress. While some stress related genes may be induced by treatments with exogenous ABA, some do not respond to this stimulus, suggesting the existence of two different signaling pathways: ABA-dependent and ABA-independent.

According to Shinozaki and Yamaguchi-Shinozaki (2007), there are at least six signal transduction pathways that trigger the modulation of genes induced by drought, high salinity, and cold: three dependent and three independent of ABA concentrations (Figure 3). In ABA-dependent pathways, an ABA-responsive element (ABRE) is the main element that responds to ABA signaling. Other possible transcription factors in this pathway include ABRE-binding proteins (AREB) and ABRE-binding factors (ABFs). Likewise, MYB2, MYC2, and NAC are transcription factors that can be induced by ABA and affect gene expression. However, these genes are also responsive to jasmonic acid (JA) signaling and may be related to biotic stress responses.

Figure 3 - Transcriptional regulatory networks of abiotic stress signals and gene expression.
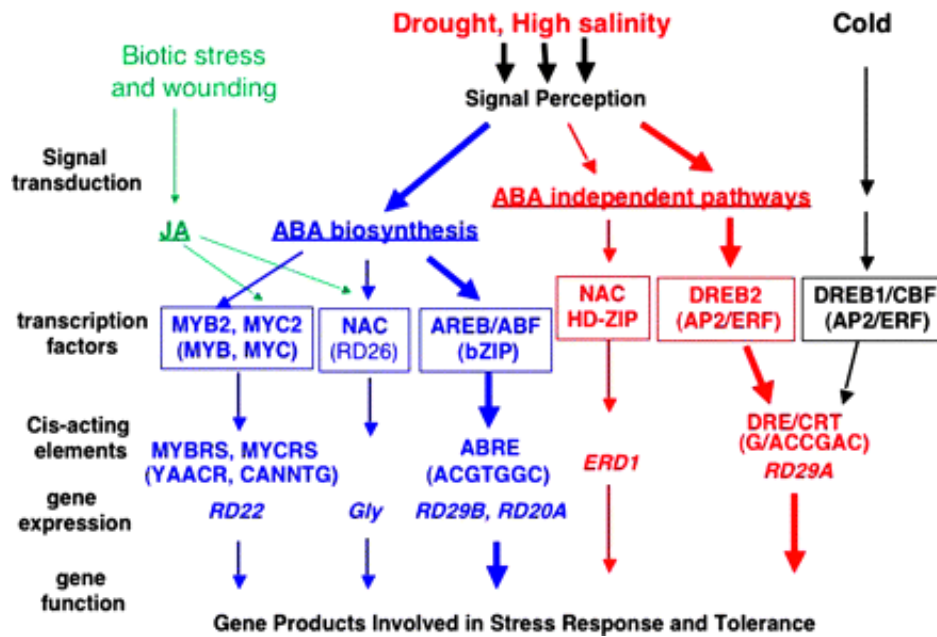
Source: Extracted from Shinozaki and Yamaguchi-Shinozaki (2007).

Furthermore, in ABA-independent pathways, dehydration-responsive elements (DRE) are the main elements involved in the regulation of genes induced by drought, salinity, and cold. DRE-binding protein (DREB1) and C-repeat-binding factors (CBFs) are involved in gene expression changes in response to cold, and DREB2s respond to dehydration and high salinity. The third ABA-independent pathway is controlled by drought and salinity, but not by cold and involves the transcription factors NAC and HD-ZIP (homeodomain Leucine zipper).

The understanding of all the factors involved in drought tolerance and plant responses to stress provides the information used as a basis for obtaining tolerant cultivars. The identification of genes involved in the response to abiotic stresses allows the isolation and introduction of new traits by genetic

transformation or conventional breeding (ZHANG, 2015). Therefore, next generation sequencing and especially RNA sequencing has emerged as an important tool for the discovery of stress related genes.

2.5 RNAseq

The transcriptome can be defined as the complete set of transcripts in a cell and their concentrations, in a specific stage of development or physiological condition. Thus, the transcriptome includes both coding RNA (mRNA) and non-coding RNA (rRNA, tRNA, structural RNA, regulatory RNA, and other types of RNA) (WANG et al., 2011). Several studies were conducted to understand the mechanisms involved in the transcription process in cells (SESHASAYEE et al., 2006), since the change in the levels of expression is directly related to changes in physiology and metabolism (VAN VLIET, 2010).

Until recently, DNA microarray was the most commonly used technique for determining a wide pattern of gene expression (HINTON et al., 2004). However, several methodological limitations were identified. Specific arrangements for each treatment, background saturation, and variable quality and density of the spots are all factors that hamper the comparative analysis of experiments and generally led to the need to develop complex normalizing methods (HINTON et al., 2004). Therefore, transcriptome sequencing emerged as an efficient alternative to solve these problems, and enables the analysis and interpretation of sequencing data without the need of a previous reference genome.

The RNAseq methodology has high sensitivity and can be used for characterizing the transcriptome of an organism (PINTO et al., 2011). This methodology is used to discover new transcripts, identify mutations, deletions, insertions, and alternative splicing sites. One of its major advantages is the almost total lack of noise and the ability to detect a large number of mRNA

copies per cell (XU; GAO; WANG, 2012). Several studies show that RNAseq data is very reliable and truly represents the complex integrated networks present in a plant cell (BLEEKER et al., 2011; XU; GAO; WANG, 2012).

Significant progress was made with this technology to better comprehend gene expression in plant species, such as those related to the development of the embryo (GAO et al., 2013; XU; GAO; WANG, 2012) and the responses to biotic stress (KAWAHARA et al., 2012). Therefore, RNAseq is a promising technique when it comes to understanding gene expression patterns, and it can also help in the generation of plants with higher resistance to abiotic stresses by breeding efforts.

REFERENCES

ANTHONY, F. et al. Genetic diversity of wild coffee (Coffea arabica L.) using molecular markers. **Euphytica,** Wageningen, v. 118, n. 1, p. 53-65, Mar. 2001.

ANTHONY, F. et al. The origin of cultivated Coffea arabica L. varieties revealed by AFLP and SSR markers. **Theoretical and Applied Genetics,** Berlin, v. 104, n. 5, p. 894-900, Apr. 2002.

ASSAD, E. D. et al. Impacto das mudanças climáticas no zoneamento agroclimático no café no Brasil. **Pesquisa Agropecuária Brasileira,** Brasília, v. 39, n. 11, p. 1057-1064, nov. 2004.

BANIWAL, S. K. et al. Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. **Journal of Biosciences,** Bangalore, v. 29, n. 4, p. 471-487, Dec. 2004.

BANTI, V. et al. The heat-inducible transcription factor HsfA2 enhances anoxia tolerance in arabidopsis. **Plant Physiology,** Bethesda, v. 152, n. 3, p. 1471-1483, Mar. 2010.

BARDIL, A. et al. Genomic expression dominance in the natural allopolyploid coffea arabica is massively affected by growth temperature. **New Phytologist,** London, v. 192, n. 3, p. 760-774, Nov. 2011.

BARRETO, H. G. et al. In silico and quantitative analyses of the putative FLC-like homologue in coffee (Coffea arabica L.). **Plant Molecular Biology Reporter,** Athens, v. 30, n. 1, p. 29-35, Feb. 2012.

BARTWAL, A. et al. Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. **Journal of Plant Growth Regulation,** New York, v. 32, n. 1, p. 216-232, Mar. 2013.

BLEEKER, P. M. et al. RNA-seq discovery, functional characterization, and comparison of sesquiterpene synthases from Solanum lycopersicum and Solanum habrochaites trichomes. **Plant Molecular Biology,** Dordrecht, v. 77, n. 4/5, p. 323-336, Nov. 2011.

BOHNERT, H. J.; JENSEN, R. G. Strategies for engineering water-stress tolerance in plants. **Trends in Biotechnology,** Amsterdam, v. 14, n. 3, p. 89-97, Mar. 1996.

28

CAMARGO, M. B. The impact of climatic variability and climate change on Arabica coffee crop in Brazil. **Bragantia,** Campinas, v. 69, n. 1, p. 239-247, 2010.

CHAUDHARY, B. et al. Reciprocal silencing, transcriptional bias and functional divergence of homeologs in polyploid cotton (Gossypium). **Genetics,** New York, v. 182, n. 2, p. 503-517, June 2009.

CHAUHAN, H. et al. Heat shock factors in rice (Oryza sativa L.): genome-wide expression analysis during reproductive development and abiotic stress. **Molecular Genetics and Genomics,** Berlin, v. 286, n. 2, p. 171-187, Aug. 2011.

CHAVES, M. M. et al. How plants cope with water stress in the field. Photosynthesis and growth. **Annals of Botany,** London, v. 89, n. 7, p. 907-916, June 2002.

DAVIDSON, J. F.; SCHIESTL, R. H. Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in Saccharomyces cerevisiae. **Molecular and Cellular Biology,** Washington, v. 21, n. 24, p. 8483-8489, Dec. 2001.

DAVIS, A. P. et al. Growing coffee: psilanthus (Rubiaceae) subsumed on the basis of molecular and morphological data; implications for the size, morphology, distribution and evolutionary history of Coffea. **Botanical Journal of the Linnean Society,** London, v. 167, n. 4, p. 357-377, Dec. 2011.

DAVIS, A. P. et al. The impact of climate change on indigenous arabica coffee (Coffea arabica): predicting future trends and identifying priorities. **Plos One,** San Francisco, v. 7, n. 11, p. e4981, Nov. 2012.

ESTRADA, F.; GAY, C.; CONDE, C. A methodology for the risk assessment of climate variability and change under uncertainty. A case study: coffee production in Veracruz, Mexico. **Climatic Change,** Dordrecht, v. 113, n. 2, p. 455-479, July 2012.

FAZUOLI, L. C. Cultivares do IAC. **O Agronômico,** Belo Horizonte, v. 51, n. 1, p. 24-27, 1999.

GAO, Y. et al. Transcriptomic analysis of rice (Oryza sativa) endosperm using the RNA-Seq technique. **Plant Molecular Biology,** Dordrecht, v. 81, n. 4/5, p. 363-378, Mar. 2013.

GIORNO, F. et al. Developmental and heat stress-regulated expression of HsfA2 and small heat shock proteins in tomato anthers. **Journal of Experimental Botany,** Oxford, v. 61, n. 2, p. 453-462, Jan. 2010.

HAHN, A. et al. Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. **The Plant Cell,** Rockville, v. 23, n. 2, p. 741-755, Feb. 2011.

HAHN, J. S. et al. Genome-wide analysis of the biology of stress responses through heat shock transcription factor. **Molecular and Cellular Biology,** Washington, v. 24, n. 12, p. 5249-5256, June 2004.

HALL, A. E. **Crop responses to environment**. Boca Raton: CRC Press, 2001. 244 p.

HINTON, J. C. D. et al. Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection. **Current Opinion in Microbiology,** Oxford, v. 7, n. 3, p. 277-282, June 2004.

HOWARTH, C. J. Genetic improvements of tolerance to high temperature. In: ASHRAF, M.; HARRIS, P. J. C. (Ed.). **Abiotic stresses:** plant resistance through breeding and molecular approaches. New York: Howarth Press, 2005. p. 277-300.

HU, W.; HU, G.; HAN, B. Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice. **Plant Science,** Limerick, v. 176, n. 4, p. 583-590, Apr. 2009.

HU, X. et al. Protein sHSP26 improves chloroplast performance under heat stress by interacting with specific chloroplast proteins in maize (Zea mays). **Journal of Proteomics,** Amsterdam, v. 115, p. 81-92, Feb. 2015.

HUANG, B.; XU, C. Identification and characterization of proteins associated with plant tolerance to heat stress. **Journal of Integrative Plant Biology,** Amsterdam, v. 50, n. 10, p. 1230-1237, Oct. 2008.

IBA, K. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. **Annual Review of Plant Biology,** Palo Alto, v. 53, p. 225-245, 2002.

30

INTERNATIONAL COFFEE ORGANIZATION - ICO. **Indicator prices:** annual and monthly averages. London: ICO, 2015a. Disponível em: <http://www.ico.org/new_historical.asp? section=Statistics>. Acesso em: 15 mar 2015.

_____. **Trade statistics**. London: ICO, 2015b. Disponível em: <http://www.ico.org/trade_ statistics.asp>. Acesso em: 15 mar 2015.

INTERGOVERNMENTAL PANEL ON CLIMATE CHANGE - IPCC. **Fifth assessment report:** climate change. Geneva: IPCC, 2014.

JALEEL, C. A. et al. Drought stress in plants: a review on morphological characteristics and pigments composition. **International Journal of Agriculture and Biology,** Oxford, v. 11, n. 1, p. 100-105, Jan. 2009.

JASPERS, P.; KANGASJARVI, J. Reactive oxygen species in abiotic stress signaling. **Physiologia Plantarum,** Copenhagen, v. 138, n. 4, p. 405-413, Apr. 2010.

KAWAHARA, Y. et al. Simultaneous RNA-Seq analysis of a mixed transcriptome of rice and blast fungus interaction. **Plos One,** San Francisco, v. 7, n. 11, p. e9423, Nov. 2012.

LADERACH, P.; JARVIS, A. J. R. The impact of climate change in coffe-growing regions. In: (Ed.). **Taller de adaptacion al cambio climatico en las comunidades cafetaleras de la Sierra Madre de Chiapas**. Chiapas: Tuxtla Gutierrez, 2008. p. 17-18.

LARKINDALE, J.; KNIGHT, M. R. Protection against heat stress-induced oxidative damage in arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. **Plant Physiology,** Bethesda, v. 128, n. 2, p. 682-695, Feb. 2002.

LASHERMES, P. et al. Molecular characterisation and origin of the Coffea arabica L. genome. **Molecular and General Genetics,** New York, v. 261, n. 2, p. 259-266, Mar. 1999.

LINGLE, T. Preface. In: WINTGENS, J. N. (Ed.). **Coffee:** growing, processing, sustainable production. Weinheim: WILEY-VCH Verlag, 2008. p. 11-13.

MATTA, F. M. Exploring drought tolerance in coffee: a physiological approach with some insights for plant breeding. **Brazilian Journal of Plant Physiology, Campinas,** v. 16, n. 1, p. 1-6, jan. 2004.

MATTA, F. M. da; RAMALHO, J. D. C. Impacts of drought and temperature stress on coffee physiology and production: a review. **Brazilian Journal of Plant Physiology,** Campinas, v. 18, n. 1, p. 55-81, jan./mar. 2006.

MAZZAFERA, P. Estresse hídrico e conseqüências na composição das sementes de café e qualidade da bebida. In: SALVA, T. J. et al. (Ed.). **Cafés de qualidade:** aspectos tecnológicos, científicos e comerciais. Campinas: Instituto Agronômico de Campinas, 2007. p. 73-90.

MISHRA, M. K.; SLATER, A. Recent advances in the genetic transformation of coffee. **Biotechnology Research International**, Washington, v. 2012, p. 1-17, 2012.

NISHIZAWA-YOKOI, A. et al. HsfA1d and HsfA1e involved in the transcriptional regulation of HsfA2 function as key regulators for the hsf signaling network in response to environmental stress. **Plant and Cell Physiology,** Kyoto, v. 52, n. 5, p. 933-945, May 2011.

NOGUEIRA, R. J. M. C.; ALBUQUERQUE, M. B.; SILVA, E. C. Aspectos ecofisiológicos da tolerância à seca em plantas da caatinga. In: NOGUEIRA, R. J. M. C. E. A. (Ed.). **Estresses ambientais:** danos e benefícios em plantas. Recife: Imprensa Universitária, 2005. p. 329-361.

NUNEZ, M. N.; SOLMAN, S. A.; CABRE, M. F. Regional climate change experiments over southern South America. II: climate change scenarios in the late twenty-first century. **Climate Dynamics,** Berlin, v. 32, n. 7-8, p. 1081-1095, June 2009.

PARK, C.-J.; SEO, Y.-S. Heat shock proteins: a review of the molecular chaperones for plant immunity. **Plant Pathology,** Oxford, v. 31, n. 4, p. 323-333, Dec. 2015.

PENUELAS, J.; LLUSIA, J. Linking photorespiration, monoterpenes and thermotolerance in Quercus. **New Phytologist,** London, v. 155, n. 2, p. 227-237, Aug. 2002.

PINTO, A. C. et al. Application of RNA-seq to reveal the transcript profile in bacteria. **Genetics and Molecular Research,** Saint Paul, v. 10, n. 3, p. 1707-1718, 2011.

PRAKASH, N. S. et al. AFLP analysis of introgression in coffee cultivars (Coffea arabica L.) derived from a natural interspecific hybrid. **Euphytica,** Wageningen, v. 124, n. 3, p. 265-271, Apr. 2002.

PRIVAT, I. et al. The 'PUCE CAFE' project: the first 15K coffee microarray, a new tool for discovering candidate genes correlated to agronomic and quality traits. **BMC Genomics,** London, v. 12, p. 5, Jan. 2011.

PUMPHREY, M. et al. Nonadditive expression of homoeologous genes is established upon polyploidization in hexaploid wheat. **Genetics,** New York, v. 181, n. 3, p. 1147-1157, Mar. 2009.

RAMIREZ-VILLEGAS, J. et al. A way forward on adaptation to climate change in Colombian agriculture: perspectives towards 2050. **Climatic Change,** Dordrecht, v. 115, n. 3/4, p. 611-628, Dec. 2012.

SABEHAT, A.; WEISS, D.; LURIE, S. Heat-shock proteins and cross-tolerance in plants. **Physiologia Plantarum,** Copenhagen, v. 103, n. 3, p. 437-441, July 1998.

SANCHEZ-ROMERA, B. et al. Enhancement of root hydraulic conductivity by methyl jasmonate and the role of calcium and abscisic acid in this process. **Plant Cell and Environment,** Oxford, v. 37, n. 4, p. 995-1008, Apr. 2014.

SESHASAYEE, A. S. N. et al. Transcriptional regulatory networks in bacteria: from input signals to output responses. **Current Opinion in Microbiology,** Oxford, v. 9, n. 5, p. 511-519, Oct. 2006.

SHAO, H.-B. et al. Understanding water deficit stress-induced changes in the basic metabolism of higher plants: biotechnologically and sustainably improving agriculture and the ecoenvironment in arid regions of the globe. **Critical Reviews in Biotechnology,** Boca Raton, v. 29, n. 2, p. 131-151, June 2009.

SHINOZAKI, K.; YAMAGUCHI-SHINOZAKI, K. Gene networks involved in drought stress response and tolerance. **Journal of Experimental Botany,** Oxford, v. 58, n. 2, p. 221-227, Jan. 2007.

SHINOZAKI, K.; YAMAGUCHI-SHINOZAKI, K. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. **Current Opinion in Plant Biology,** Amsterdam, v. 3, n. 3, p. 217-223, June 2000.

HVALEVA, A. L. et al. Metabolic responses to water deficit in two Eucalyptus globulus clones with contrasting drought sensitivity. **Tree Physiology,** Oxford, v. 26, n. 2, p. 239-248, Feb. 2006.

SILVA, E. A. da et al. The influence of water management and environmental conditions on the chemicalcomposition and beverage quality of coffee beans. **Brazilian Journal of Plant Physiology,** Campinas, v. 17, n. 2, p. 229-238, abr./jun. 2005.

SMERTENKO, A. et al. Heat stress affects the organization of microtubules and cell division in Nicotiana tabacum cells. **Plant Cell and Environment,** Oxford, v. 20, n. 12, p. 1534-1542, Dec. 1997.

SREENIVASULU, N.; SOPORY, S. K.; KISHOR, P. B. K. Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. **Gene,** Amsterdam, v. 388, n. 1/2, p. 1-13, Feb. 2007.

STEVENS, P. F. **Angiosperm phylogeny website**. [S.l.: s.n.], 2012. Disponível em: <http:// www.mobot.org/MOBOT/research/APweb/>. Acesso em: 15 mar. 2015.

SWINDELL, W. R.; HUEBNER, M.; WEBER, A. P. Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. **BMC Genomics,** London, v. 8, p. 125, May 2007.

TEALE, W. D.; PAPONOV, I. A.; PALME, K. Auxin in action: signalling, transport and the control of plant growth and development. **Nature Reviews Molecular Cell Biology,** London, v. 7, n. 11, p. 847-859, Nov. 2006.

THOMASHOW, M. F. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. **Annual Review of Plant Physiology and Plant Molecular Biology,** Palo Alto, v. 50, p. 571-599, June 1999.

TUTEJA, N. **Improving crop resistance to abiotic stress**. New York: Weinheim, 2012. 1534 p.

VAN VLIET, A. Next generation sequencing of microbial transcriptome: challenge and opportunities. **FEMS Microbiology Letters,** Amsterdam, v. 302, n. 1, p. 1-7, Jan. 2010.

VIDAL, R. O. et al. A High-throughput data mining of single nucleotide polymorphisms in coffea species expressed sequence tags suggests differential homeologous gene expression in the allotetraploid coffea arabica. **Plant Physiology,** Bethesda, v. 154, n. 3, p. 1053-1066, Nov. 2010.

VINOCUR, B.; ALTMAN, A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. **Current Opinion in Biotechnology,** London, v. 16, n. 2, p. 123-132, Apr. 2005.

WAHID, A. et al. Heat tolerance in plants: an overview. **Environmental and Experimental Botany,** Elmsford, v. 61, n. 3, p. 199-223, Dec. 2007.

WANG, D. et al. Genome-wide temporal-spatial gene expression profiling of drought responsiveness in rice. **BMC Genomics,** London, v. 12, p. 149, Mar. 2011.

WANG, W. X. et al. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. **Trends in Plant Science,** Oxford, v. 9, n. 5, p. 244-252, May 2004.

XU, H.; GAO, Y.; WANG, J. Transcriptomic analysis of rice (Oryza sativa) developing embryos using the RNA-Seq technique. **Plos One,** San Francisco, v. 7, n. 2, p. e30646, Feb. 2012.

YAMAGUCHI-SHINOZAKI, K.; SHINOZAKI, K. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. **Annual Review of Plant Biology,** Palo Alto, v. 57, p. 781-803, 2006.

ZHANG, B. MicroRNA: a new target for improving plant tolerance to abiotic stress. **Journal of Experimental Botany,** Oxford, v. 66, n. 7, p. 1749-1761, Apr. 2015.

ZHANG, J. et al. Photosynthesis and ultrastructure of photosynthetic apparatus in tomato leaves under elevated temperature. **Photosynthetica,** Prague, v. 52, n. 3, p. 430-436, Sept. 2014.

ZONIA, L.; MUNNIK, T. Cracking the green paradigm: Functional coding of phosphoinositide signals in plant stress responses. **Subcellular Biochemistry,** London, v. 39, p. 207-237, 2006.

CHAPTER 2 PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF COFFEE PLANTS UNDER HEAT STRESS

ABSTRACT

Coffee is a perennial dicotyledonous tree belonging to the family Rubiaceae, which is the fourth largest family of angiosperms. Coffee is one of the most valuable primary products in world trade, being the second most highly traded commodity in the world (second only to oil). Coffee exports account for a significant proportion of taxes and gross domestic product in many countries. Despite its economic importance, coffee's production and market are subjected to regular oscillations in both prices and volume of production. The biannual cycle of the crop and the adverse effects of weather conditions mainly explain these variations. In Brazil, climatic variability is the main factor causing oscillations in coffee grain yield. The losses to Arabica coffee production, as a result of increasing temperatures, are estimated to be as much as 10% of total production in 20 years. Hence, we aimed to understand the impact of heat stress at the physiological and molecular levels on the growth and development of coffee. Moreover, we aimed at identifying the genes that are differentially transcribed in response to high temperatures. Gas exchange measurements and enzymatic assays were used to assess the physiological state of two coffee cultivars in response to two different environments and to heat stress treatments. At the molecular level, RNA sequencing made possible the identification of genes that are differentially expressed in response to temperatures. Several genes involved in metabolic pathways of stress tolerance have been identified, including cell signaling-related genes, transcription factors and cellular detoxification enzymes.

Keywords: RNAseq. Coffee. Abiotic stress.

RESUMO

O cafeeiro é uma dicotiledônea perene pertencente à família Rubiaceae, que é o quarta maior família de angiospermas. O café é um dos produtos primários mais valiosos no comércio mundial, sendo a segunda commodity mais comercializada no mundo (perdendo apenas para o petróleo), as exportações de café são responsáveis por uma proporção significativa dos impostos e produto interno bruto em muitos países. Apesar da sua importância econômica, a produção e o mercado de café estão sujeitos a oscilações regulares nos preços e volume de produção. O ciclo bianual da cultura e os efeitos adversos de condições climáticas são os principais fatores que explicam estas variações. No Brasil, a variabilidade climática é a principal causa de oscilações no rendimento de grãos de café. As perdas na produção de café arábica, como resultado do aumento das temperaturas, são estimados em até 10% da produção total em 20 anos. Por isso, buscamos compreender o impacto do estresse térmico em níveis fisiológicos e moleculares no crescimento e desenvolvimento do café. Além disso, objetivamos identificar os genes que são diferencialmente transcritos em resposta a altas temperaturas. Medições de trocas gasosas e ensaios enzimáticos foram utilizados a fim de avaliar o estado fisiológico de duas cultivares de café em resposta a dois ambientes diferentes e a tratamentos térmicos de stress. A nível molecular, o sequenciamento de RNA possibilitou a identificação de genes que são expressos diferencialmente em resposta a temperaturas. Vários genes envolvidos em vias metabólicas de tolerância ao stress foram identificados, incluindo genes relacionados a sinalização celular, fatores de transcrição, proteínas protetoras funcionais da células e enzimas de desintoxicação celulares.

Palavras-chave: RNAseq. Café. Estresse abiótico.

1 INTRODUCTION

Coffee is a perennial dicotyledonous tree belonging to the family Rubiaceae, which is the fourth largest family of angiosperms (STEVENS, 2012). The Rubiaceae family has about 500 genera and over 6,000 species, including the genus *Coffea*, the most important member in economic terms (INTERNATIONAL COFFEE ORGANIZATION, 2015b). Although the genus *Coffea* contains more than 124 species (DAVIS et al., 2012), only two have economic significance: *Coffea arabica* and *Coffea canephora*. *Coffea arabica* is native to the African continent and was originally found in southwestern Ethiopia, Sudan, and northern Kenya. The areas ideal for cultivation are usually above 600 meters in altitude in temperate zones (DAVIS et al., 2012). Despite its demanding production system, *C. arabica* represents 70% of total coffee production and has a better cup quality with low caffeine content (BARRETO et al., 2012).

Coffee is one of the most valuable primary products in world trade, being the second most highly traded commodity in the world (second only to oil). Coffee exports account for a significant proportion of taxes and gross domestic product in many countries. The global coffee trade in 2014 reached 143 million 60kg bags and accounted for exports worth an estimated $15.4 billion (ICO, 2015a). Moreover, its cultivation, processing, transport, and marketing employs over 120 million people and significantly affects the gross national product (GNP) of 40 countries (LINGLE, 2008). Worldwide, coffee is grown on over 10,000,000 hectares of land. Smallholder farmers cultivate over 70% of production, making coffee crucial to the economy of many developing countries. Currently, Brazil is the world's leading producer of coffee (MISHRA; SLATER, 2012), a position the country has held for the last 150 years, producing roughly 40% of the world's supply of Arabica beans across more than 2,000 farms in 16 states.

Despite its economic importance, coffee's production and market are subjected to regular oscillations in both prices and volume of production. The biannual cycle of the crop and the adverse effects of weather conditions mainly explain these variations. In Brazil, climatic variability is the main factor causing oscillations in coffee grain yield (ASSAD ET AL., 2004; MATTA, 2004; MATTA; RAMALHO, 2006). When it comes to the physiology of Arabica coffee, according to Camargo (2010), the optimal mean annual air temperature for growth is in the range of 18 to 23°C. Above that temperature, there is an acceleration of fruit development and ripening, which can lead to quality loss. Depressed growth and abnormalities, such as yellowing of leaves, can result from continuous exposure to daily temperatures as high as 30°C. Additionally, flower abortion may occur when the air temperature during blooming is high, especially if the high temperature is associated with drought (MATTA; RAMALHO, 2006).

According to the Intergovernmental Panel on Climate Change (2014), there is a high probability of temperature increases of 1 to 3°C in the tropics over the next 20 years. The losses to Arabica coffee production, as a result of increasing temperatures, are estimated to be as much as 10% of total production in 20 years (CAMARGO, 2010; DAVIS et al., 2012; ESTRADA; GAY; CONDE, 2012; LADERACH; JARVIS, 2008; RAMIREZ-VILLEGAS et al., 2012).

Therefore, heat stress is one major factor to be overcome in order to maintain crop productivity. The occurrence and intensity of heat waves is expected to increase with global warming, making the development of crop plants with improved heat tolerance a priority. To that end, it is necessary to have a better understanding of (i) the various plant responses to high-temperatures, (ii) the heat stress tolerance mechanisms, and (iii) the key genes involved in the physiological responses to heat stress.

Hence, we aimed to understand the impact of heat stress at the physiological and molecular levels on the growth and development of coffee. Moreover, we aimed at identifying the genes that are differentially transcribed in response to high temperatures, which, in the future, can lead to the development of cultivars with improved quality and thus higher harvest security.

2 MATERIALS AND METHODS

2.1 Plant Material and Gas exchange

Experiment 1: Field experiment

The study compared two *C. arabica* varieties (Catuaí IAC 144 and Acauã) that are hypothesized to differ in heat tolerance. Physiological parameters, such as carbon assimilation rate, stomatal conductance and transpiration rate were measured for each group of plants grown at two different locations (Pirapora and Varginha) at two distinct developmental stages (flowering in September-October 2014 and at harvest time in March-April 2015). The experimental field in Varginha (21°3356.8"S, 45°24'07.5"W) is located at 976.2m above the sea level, while the Pirapora (17°3420.7"S, 44°5817.2"W) field stands at a much lower altitude at 535.8 meters above the sea level. It is also important to note that a central pivot irrigated the in Pirapora, whereas the plants from Varginha depended only on the rain regimen for water supply. Additionally, the coffee plants in both experimental fields were about 7 years old at the time of the first experiments.

The experimental design was of randomized blocks (3 blocks $\times$ 3 plants $\times$ 2 cultivars $\times$ 2 altitudes $\times$ 2 stages, n = 72 plants total). Gas exchange parameters were measured with a portable infrared CO_2 analyzer (IRGA LCA-4 ADC Hoddesdon, UK), mostly on clear typical mornings, between 9 and 12, on three completely expanded leaves (third pairs of leaves of the plagiotropic branch, from superior third) per plant.

Experiment 2: Growth chamber experiment

Two hundred seeds of two *C. arabica* cultivars (Acauã and Catuaí IAC 144) were sown in 1-gallon pots filled with commercial greenhouse mix (Professional Growing Mix, Sun-Gro Horticulture). Thirty days after sowing,

the sprouts were individually transferred to 2 litter pots and shaded with a 50% shade factor cloth. The seedlings grew in the greenhouses of the Department of Horticultural Sciences at Texas A&M University, College Station, TX, USA. Once the seedlings reached the three pair leaf stage, they were transferred to two growth chambers at the Texas A&M AgriLife Research and Extension Center at Overton, TX. After fifteen days of acclimation (at 23/19 °C day/night temperatures and 12 hour days), seedlings were subjected to the optimal and higher-than-optimal temperature regime treatments (23/19 °C and 30/26 °C day/night, respectively, 12 hour days) for 4 weeks. The experimental design was a randomized complete block design with a split plot restriction. Each growth chamber was a main plot. In each chamber, 5 blocks with 1 plant of each cultivar in each block was laid out lengthwise across the chamber. Thus, n=2 for the main plots and n=5 for the sub-plots so that a total of 20 plants was studied. Physiological parameters (net carbon assimilation rate, stomatal conductance, transpiration rate, and chlorophyll fluorescence) were monitored at weekly intervals (to control plant physiological conditions) with a portable infrared CO_2 analyzer (LI-6400XT, LI-COR®). The analyses were performed in the morning between 9 and 12, with CO_2 concentration fixed in 400μM mol^{-1}, inside the building where the growth chambers were located. Each completely expanded leaf used for the measurements was marked and used for all subsequent assessments. However, for data analysis purposes only the last measurement (after 4 weeks of temperature treatments) was taken into account.

In both experiments, the photon flux density inside the measuring chamber was adjusted to 1000 μmol $m^{-2}s^{-1}$. Means were compared by student t tests and significance was assessed by the Tukey's test (P <0.05).

2.2 Antioxidant metabolism

Only plant materials collected from the field experiment were subjected to antioxidant metabolism analysis. Leaf samples were collected for each tree analyzed (9 trees x 2 cultivars x 2 locations x 2 phonological stages = total of 72).

The enzymes Superoxide Dismutase (SOD), Catalase (CAT) and Ascorbate peroxidase (APX) were extracted according to Biemelt, Keetman e Albrecht (1998): 0.2 g of leaf fresh weight were macerated in liquid nitrogen and homogenized in 1.5ml of extraction buffer containing: 1.47ml of 0.1M potassium phosphate buffer (pH 7.0), 15μl of 0.1M ethylenediaminetetraacetic acid (EDTA, pH 7.0), 6μl of 0.5M dithiothreitol (DTT), 12μl of 0.1M phenylmethylsulfonyl fluoride (PMSF), 0.001M ascorbic acid and 22 mg polyvinylpolypyrrolidone (PVPP). The extract was centrifuged at 12,000g for 10 min at 4°C and the supernatant was collected and stored at −20°C during the analysis period.

For the statistical analysis, means were compared by student t tests and significance was assessed by the Tukey test ($P < 0.05$).

2.2.1 SOD activity

SOD (EC 1.15.1.1) activity was assessed based on a method previously published by Nishikim, Appaji e Yagi (1972) with modifications. SOD activity was assessed according to its ability to inhibit the photoreduction of nitro-blue tetrazolium (NBT) (GIANNOPOLITIS; RIES, 1977). A 100μl volume of enzyme extract was added to 1.9ml of incubation medium composed of 50mM potassium phosphate buffer (pH 7.8), 14mM methionine, 0.1μM EDTA, 75μM NBT and 2μM riboflavin. The samples were then illuminated with a 20W fluorescent lamp for 10 min, and readings were performed at 560nm. One unit of SOD corresponds to the amount of enzyme that is able to inhibit the

photoreduction of NBT by 50% under the assay conditions. Analysis for estimation of protein concentrations was carried out using bovine serum albumin as a standard (BRADFORD, 1976).

2.2.2 CAT activity

CAT was evaluated according to Havir and Mchale (1987): aliquots (10μl) of enzyme extract were added to 170μl of incubation medium containing 90μl of potassium phosphate 200mM (pH 7.0), 71μl of water and 9μl of hydrogen peroxide 250mM, incubated at 28°C. Enzyme activity was determined by the decrease in absorbance at 240nm every 15s for 3 min, monitored by the consumption of hydrogen peroxide. The molar extinction coefficient used was $36mM^{-1}cm^{-1}$.

2.2.3 APX activity

APX activity was determined by monitoring of the rate of oxidation of ascorbate at 290nm every 15s for 3 min. Aliquots (10μl) of enzyme extract were added to 170μl of incubation buffer, consisting of 90μl of potassium phosphate 200mM (pH 7.0), 9μl ascorbic acid 10mM, 62μl of water and 9μl of hydrogen peroxide 2mM (NAKANO; ASADA, 1981).The molar extinction coefficient was $2.8mM^{-1}cm^{-1}$.

2.3 RNA extraction and sequencing

Samples consisting of three young, completely expanded leaves from each plant were ground with liquid nitrogen using a mortar and pestle. Approximately 100 mg of pulverized tissue was used for each RNA extraction, which was performed according to the recommendations of the Concert[TM] Kit Plant RNA Reagent (Invitrogen®) manual. The samples were subsequently treated with the Turbo DNA-free Kit (Ambion®) to remove residual DNA contamination. RNA integrity was assessed by 1.0% agarose gel electrophoresis.

The samples were quantified at 260nm with a spectrophotometer. Further, RNA Integrity Number (RIN) values were determined using a Bioanalyzer 2100 (Aligent Technologies, Santa Clara, CA) to make sure all samples had a RIN greater than 7.0. Samples that exhibited high levels of integrity and purity were used for TruSeq library preparations. Libraries were constructed for each sample using a cDNA Synthesis kit (Illumina Inc., San Digo, CA, USA) following the manufacturer's instructions. Two lanes of paired-end (2×150 bp) sequencing of the cDNA libraries was performed on the Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA). Library preparation and sequencing was performed by AgriLife Genomics and Bioinformatics Services (Texas A&M University). Sequence cluster identification, quality prefiltering, base calling and uncertainty assessment were done in real time using Illumina's HCS 2.2.58 and RTA 1.18.64 software with default parameter settings.

2.3.1 Alignment to the reference genome

The sequencing reads were mapped to the annotated genes of *C. canephora* (available at http://coffee-genome.org) using the CLC Genomics Workbench (GWB) RNA-Seq analysis tool (http://www.clcbio.com).

2.3.2 Empiric analysis of differential gene expression (EDGE)

CLC GWB reports gene expression as "Reads Per Kilobase of Transcript per Million Mapped Reads" (RPKM). The RPKM values are then used by the edgeR package in the CLC GWB (v.8.0) in order to find differentially expressed genes. The resulting list of genes was then filtered for a p-value adjusted by multiple-hypothesis testing of less than 0.05 and a log-fold change of ≥ 2 or ≤ -2.

2.3.3 Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper

All the differentially expressed genes were submitted to a blast search using the BlastKOALA (available at http://www.kegg.jp/blastkoala/) with default parameters for a K number assignment. As the *C. canephora* genome is still not deposited in this database, the *Solanum lycopersicum* genome was used as a reference to find coffee orthologs.

All genes that were assigned a K number identifier were then submitted to the KEGG mapper tool, in order to find the biological pathways the differentially expressed genes are associated with.

2.3.4 Gene Ontology

The Gene Onthology (GO) Analysis Toolkit (available at http://bioinfo.cau.edu.cn/agriGO/analysis.php) was used in order to evaluate gene ontology. A single enrichment analysis was performed for all the differentially expressed genes previously identified.

3 RESULTS

As this research was divided into two major experiments, the results will also be divided accordingly.

3.1 Field experiment

The data and samples for the field experiment were collected during two different stages: harvesting in April 2014 and flowering in October 2014.

3.1.1 Physiological data

With regards to the photosynthetic rates, for Catuaí cultivar (Figure 4), the average measurements at harvesting time (April 2014) were: 9.6 μmol m^{-2} s^{-1} in Pirapora and 4.0 μmol m^{-2} s^{-1} in Varginha. Likewise, at flowering time (October 2014), the averages photosynthetic rates for Pirapora and Varginha were respectively: 2.7 and 3.8 μmol m^{-2} s^{-1}.

Moreover, for the transpiration rates in April, plants of the Catuaí cultivar presented for the Pirapora and Varginha locations respectively 3.0 and 1.2 mmol m^{-2} sec^{-1} during the day, and 0.08 and 0.08 mmol m^{-2} sec^{-1} at night. On the other hand, in October, Pirapora and Varginha rates were respectively: 0.6 and 1.0 mmol m^{-2} sec^{-1} during the day and 0.12 and 0.04 mmol m^{-2} sec^{-1} at night.

The day stomatal conductance rates of Catuaí plants were of 143.1 mmol m^{-2} sec^{-1} in Pirapora and 43.1 mmol m^{-2} sec^{-1} in Varginha (both on April 2014). Whereas, the night rates for Piorapora were 5.7 mmol m^{-2} sec^{-1} and for Varginha 7.5 mmol m^{-2} sec^{-1}. Additonally, in October the day rates were of 12.2 and 29.3 mmol m^{-2} sec^{-1} for Pirapora and Varginha respectively and the night rates were 11.7 and 4.9 mmol m^{-2} sec^{-1}.

Furthermore, the day leaf temperatures in April were of 31.7°C (Pirapora) and 32.1°C (Varginha), while the night ones were of 28.4°C (Pirapora) and 22.4°C (Varginha). In comparison, in October the day and night

temperatures for Pirapora and Varginha were respectively: 35.1 and 32.2°C (day) and 20.1 and 17.8°C (night).

Lastly, the dark respiration rates for Pirapora and Varginha were, respectively: 0.53 and 0.48 µmol m^{-2} s^{-1} (April), and 0.93 and 0.51 µmol m^{-2} s^{-1}.

When it comes to the Acauã cultivar, the average photosynthetic rates (Figure 5) at harvesting time (April 2014) were: 8.5 µmol m^{-2} s^{-1} in Pirapora and 4.7 µmol m^{-2} s^{-1} in Varginha. Likewise, at flowering time (October 2014), the averages photosynthetic rates for Pirapora and Varginha were respectively: 2.3 and 4.0 µmol m^{-2} s^{-1}.

Moreover, for the transpiration rates in April, plants of the Catuaí cultivar presented for the Pirapora and Varginha locations respectively 2.6 and 1.9 mmol m^{-2} sec^{-1} during the day, and 0.12 and 0.08 mmol m^{-2} sec^{-1} at night. On the other hand, in October, Pirapora and Varginha rates were respectively: 0.5 and 1.0 mmol m^{-2} sec^{-1} during the day and 0.21 and 0.06 mmol m^{-2} sec^{-1} at night.

The day stomatal conductance rates of Catuaí plants were of 117.6 mmol m^{-2} sec^{-1} in Pirapora and 53.6 mmol m^{-2} sec^{-1} in Varginha (both on April 2014). Whereas, the night rates for Piorapora were 9.9 mmol m^{-2} sec^{-1} and for Varginha 7.1 mmol m^{-2} sec^{-1}. Additonally, in October the day rates were of 10.5 and 37.1 mmol m^{-2} sec^{-1} for Pirapora and Varginha respectively and the night rates were 15.9 and 7.8 mmol m^{-2} sec^{-1}.

Furthermore, the day leaf temperatures in April were of 31.8°C (Pirapora) and 34.1°C (Varginha), while the night ones were of 28.3°C (Pirapora) and 22.0°C (Varginha). In comparison, in October the day and night temperatures for Pirapora and Varginha were respectively: 35.2 and 30.0°C (day) and 19.7. and 19.5°C (night).

Lastly, the dark respiration rates for Pirapora and Varginha were, respectively: 0.89 and 0.66 µmol m^{-2} s^{-1} (April), and 0.92 and 0.53 µmol m^{-2} s^{-1}.

Figure 4 - Gas exchange measurements for the Catuaí cultivar. Different letters represent statistically different means (p=0.05).
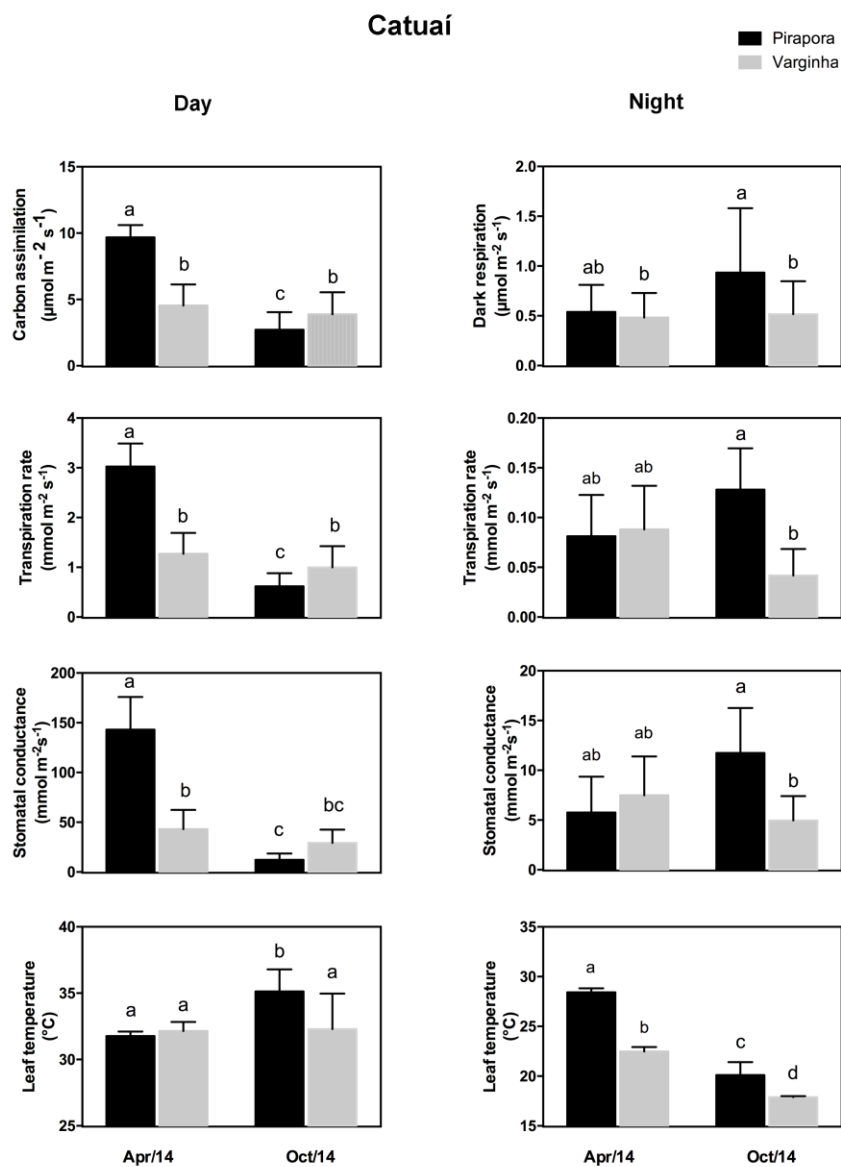
Source: Do autor (2016).

Figure 5 - Gas exchange measurements for the Acauã cultivar. Different letters represent statistically different means (p=0.05).
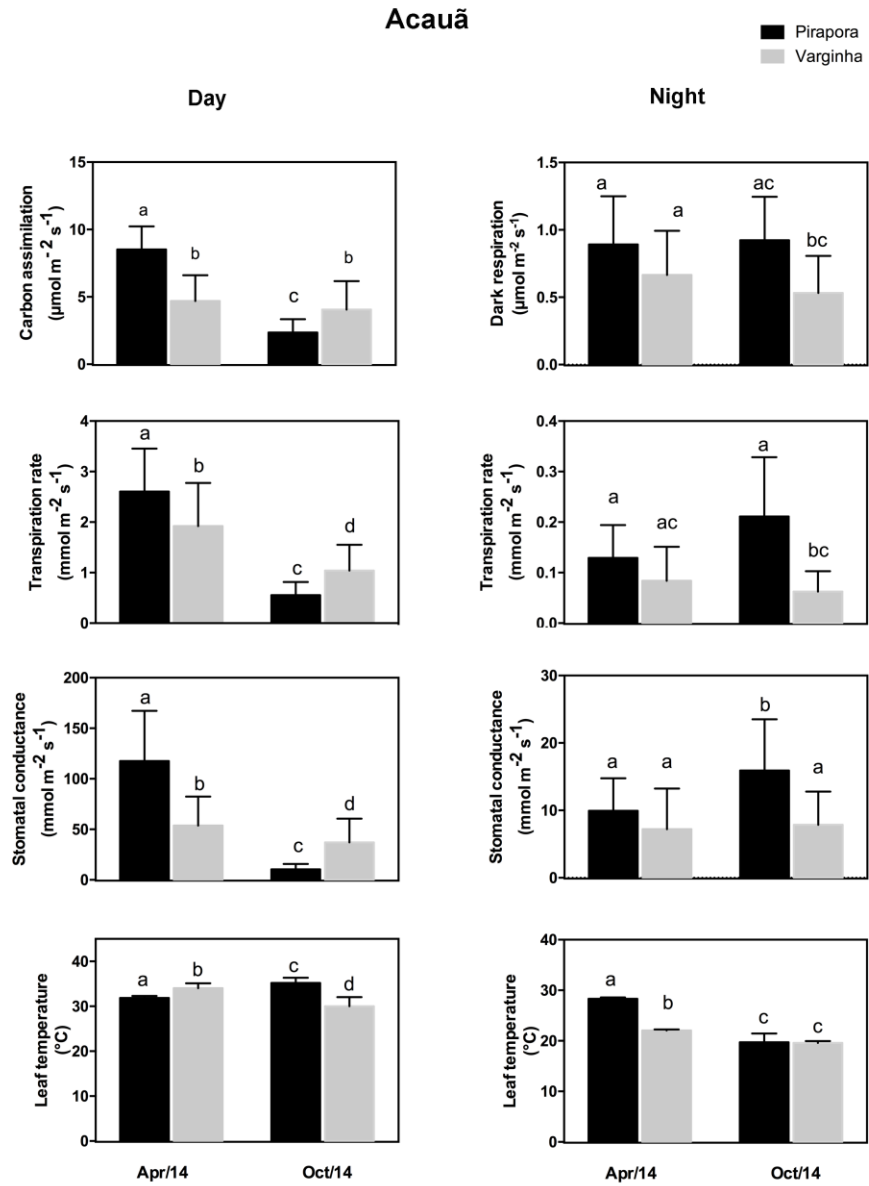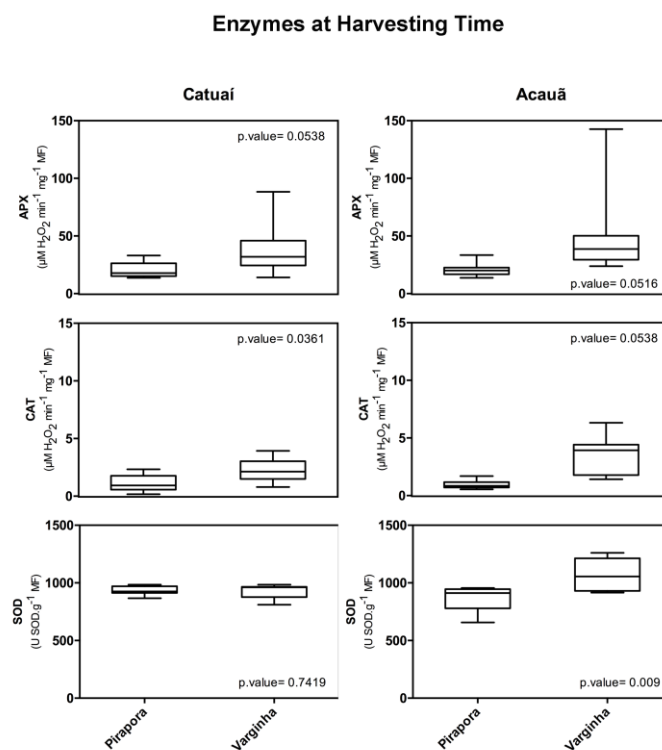

Source: Do autor (2016).

3.1.2 Enzyme Assay

The analysis of enzymatic activity was divided in two different assays, one for the samples collected at the harvesting stage (Figure 6) and one for the samples collected during flowering (Figure 7).

No statistical differences were observed when the locations were compared, except for the CAT activity in Catuaí at harvesting and the SOD activity in Acauã at both harvesting and flowering. However, even though there was not a statistical difference, higher antioxidant enzyme activities could be observed for Varginha samples.

Figure 6 - Antioxidant metabolism enzyme activity for the samples collected in April 2014

Source: Do autor (2016).

Figure 7 - Antioxidant metabolism enzyme activity for the samples collected in
October 2014

Enzymes at Flowering Time
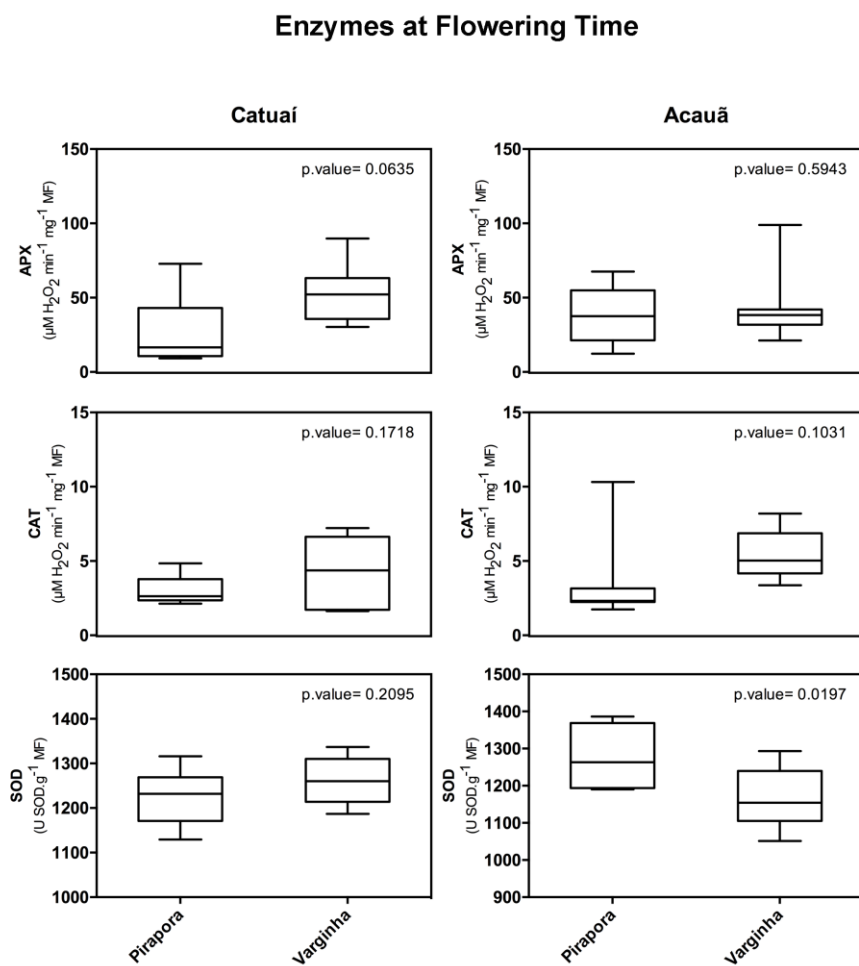
Source: Do autor (2016).

3.1.3 RNAseq

A total of 1,016,158,978 (105 bp long) paired-end reads were generated
by the 24 RNAseq libraries (Table 1). The total number of reads mapped to the
C. canephora reference genome by the CLC software was on average 85.94%
(considering only reads mapped in pairs). In Catuaí, 501,035,060 reads were

sequenced, of which 85.92% mapped to the reference genome, while in Acauã, 515,122,918 reads were sequenced and 85.96% mapped.

Table 1 -   Analysis of RNAseq data mapped to the *C. canephora* genome for Catuaí and Acauã cultivars from two different locations and two different phenological stages

Sample ID	Rep #	Number of reads mapped in pairs	Number of reads mapped in broken pairs	Number of reads not mapped	Total	% of reads mapped in pairs	% of reads mapped in broken pairs	% of reads not mapped	% Total
Catuaí Pirapora 1	1	33952580	3428973	1545999	38927552	87.22	8.81	3.97	100
	2	40556714	4396243	1825713	46778670	86.7	9.40	3.90	100
	3	34308158	3565978	1539286	39413422	87.05	9.05	3.91	100
Acauã Pirapora 1	1	41158248	5089748	2046706	48294702	85.22	10.54	4.24	100
	2	44635044	5134461	2023611	51793116	86.18	9.91	3.91	100
	3	36951598	4084528	1800562	42836688	86.26	9.54	4.2	100
Catuaí Varginha 1	1	40641720	4479669	2020419	47141808	86.21	9.50	4.29	100
	2	32302952	3562205	1683157	37548314	86.03	9.49	4.48	100
	3	32472982	3599405	1560039	37632426	86.29	9.56	4.15	100
Acauã Varginha 1	1	34696960	4527451	1801725	41026136	84.57	11.04	4.39	100
	2	31013234	3202335	1426493	35642062	87.01	8.98	4.00	100
	3	35631820	3469568	1584344	40685732	87.58	8.53	3.89	100
Catuaí Pirapora 2	1	36634322	3243389	1809421	41687132	87.88	7.78	4.34	100
	2	36684514	4002920	2035470	42722904	85.87	9.37	4.76	100
	3	35818862	4701953	2423993	42944808	83.41	10.95	5.64	100
Acauã Pirapora 2	1	36728754	4299515	1774233	42802502	85.81	10.05	4.15	100
	2	32208996	3893648	2159234	38261878	84.18	10.18	5.64	100
	3	38677652	4209123	2124789	45011564	85.93	9.35	4.72	100
Catuaí Varginha 2	1	35085364	4566858	1727834	41380056	84.79	11.04	4.18	100
	2	35668156	4709512	1875226	42252894	84.42	11.15	4.44	100
	3	36296750	4457896	1850428	42605074	85.19	10.46	4.34	100
Acauã Varginha 2	1	39885660	4268107	2142951	46296718	86.15	9.22	4.63	100
	2	36786376	3909591	2001209	42697176	86.16	9.16	4.69	100
	3	34415482	3469786	1889376	39774644	86.53	8.72	4.75	100
Mean	-	36383870.75	4094702.583	1861342.417	42339915.75	85.94	9.66	4.40	100
Total	-	873212898	98272862	44672218	1016157978	-	-	-	-

3.1.3.1 Sample correlations

All samples were plotted in a Pearson correlation matrix (supplementary figures 1 and 2) in order to guarantee equality among sample replicates.

As expected, all correlations were high between samples belonging to the same experimental group, meaning that they are representative as biological replicates.

3.1.3.2 Identification of differentially expressed genes (DEGs)

Since the most representative factor for our experiments is temperature, we grouped the two phonological stages in order to obtain a more detailed profile of genes differentially expressed in response to diverse environmental

field conditions. Therefore, we compared the two field locations of Pirapora and Varginha for both Catuaí and Acauã cultivars. Considering the Catuaí Cultivar, the comparison between samples from Pirapora and samples from Varginha resulted in the identification of 2146 DEGs (1383 up-regulated and 763 down-regulated). Meanwhile, for the Acauã cultivar 1,679 DEGs were identified (914 up-regulated and 765 down-regulated). Therefore the number of DEGs was greaterin Catuaí, and 707 genes were common for both cultivars (Figure 8).

Figure 8 - Venn diagram of differentially expressed genes between the two cultivars in response to the different environments, and their overlap
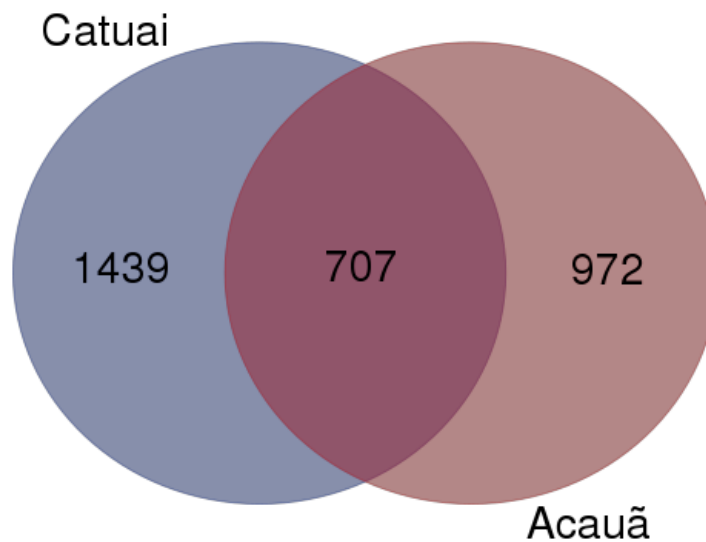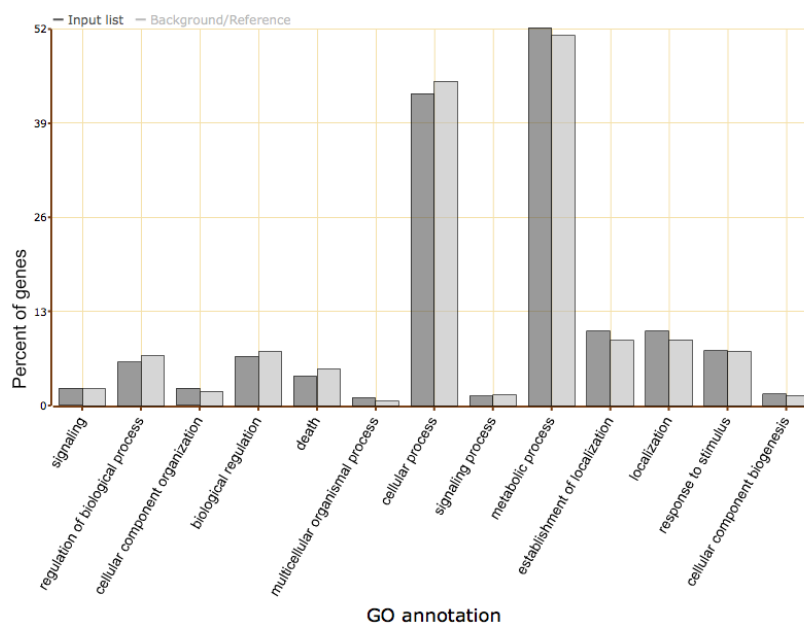

Source: Do autor (2016).

3.1.3.2.1 Gene Ontology (GO) annotation of DEGs

For the Catuaí cultivar, of the 2146 identified DEGs, 1278 were annotated by GO in 13 enriched subcategories (Figure 9). The main subcategories with a percentage of genes higher than the background were: *metabolic process* (667 genes), *establishment of localization* (131 genes) and *localization* (132 genes).

Moreover, taking into account the molecular function, 49 genes were classified as transcription factors, all up regulated, and 631 genes were classified in the catalytic activity.

Figure 9 - Functional classification of DEGs from the Pirapora vs. Varginha comparison for the Catuaí cultivar based on GO terms

Source: Do autor (2016).

Additionally, for Acauã, out of the 1679 identified DEGs, 999 were annotated by GO in 12 enriched subcategories (Figure 10). The main subcategories with a percentage of genes higher than the background were: *regulation of biological process* (76 genes), *biological regulation* (83 genes), *establishment of localization* (98 genes) and *localization* (99 genes). It is also important to note that 44 transcription factors we up regulated in Acauã plants.
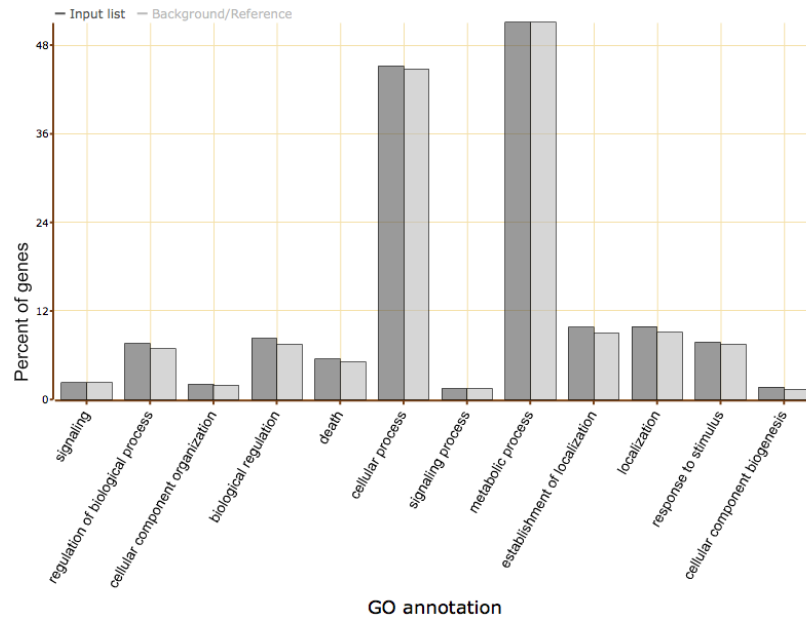
Figure 10 - Functional classification of DEGs from the Pirapora vs. Varginha
comparison for the Acauã cultivar based on GO terms

Source: Do autor (2016).

Moreover, some of the genes that could not be annotated by GO are
known to play a major role in heat stress response, like heat shock and LEA
proteins. Thus they are represented in Table 2, which includes the fold change
observed in response to diverse environmental conditions.

Table 2 - DEGs of interest that were not annotated by the GO tool

| Feature ID	EDGE test: Pirapora vs Varginha, tagwise dispersions - P-value	EDGE test: Pirapora vs Varginha, tagwise dispersions - Fold change	Annotation
Cc08_g10810_1	7.94097E-13	23.93	Heat shock factor protein HSF30
Cc10_g04530_1	0.000546884	12.06	Heat stress transcription factor B-4
Cc02_g02350_1	5.34568E-11	10.84	Heat shock protein 83
Cc08_g12550_1	4.12487E-10	9.19	Small heat shock protein, chloroplastic
Cc02_g07140_1	1.17729E-05	5.53	18.2 kDa class I heat shock protein
Cc00_g20010_1	0.011874308	5.18	Putative Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
Cc01_g12670_1	8.8687E-15	4.57	Heat shock cognate 70 kDa protein
Cc01_g08580_1	0.001146234	4.17	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
Cc04_g04290_1	6.34432E-05	3.69	26.5 kDa heat shock protein, mitochondrial
Cc02_g04250_1	9.22524E-09	3.39	Heat shock 22 kDa protein, mitochondrial
Cc04_g00900_1	8.2463E-08	2.97	17.4 kDa class III heat shock protein
Cc02_g23670_1	0.010078967	2.76	22.7 kDa class IV heat shock protein
Cc08_g15400_1	0.004911937	2.51	Putative Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
Cc03_g03680_1	0.000451414	2.47	Heat shock 70 kDa protein 8
Cc02_g07150_1	0.000315483	2.42	17.6 kDa class I heat shock protein 3
Cc01_g13580_1	0.009720589	2.40	17.5 kDa class I heat shock protein
Cc09_g01840_1	3.79415E-06	2.39	15.4 kDa class V heat shock protein
Cc01_g12750_1	0.00901962	2.36	17.6 kDa class I heat shock protein 3
Cc10_g04530_1	0.01147763	2.26	Heat stress transcription factor B-4
Cc01_g12720_1	1.00766E-12	2.21	Heat shock cognate 70 kDa protein
Cc01_g19020_1	4.24157E-05	2.19	Heat stress transcription factor A-6b
Cc08_g13040_1	1.80588E-07	2.17	Heat shock protein STI
Cc02_g07120_1	5.50763E-09	2.07	18.5 kDa class I heat shock protein

3.1.3.2.2 KEGG mapper and pathway annotation

The pathways identified by the KEGG mapper with the highest number of differentially expressed genes (both up and down regulated) were plant hormone signal transduction (Figure 11) and phenylpropanoid biosynthesis (Figure 12) with 16 and 41 genes, respectively.

In the plant hormone signal Transduction pathway three ABA related genes were up regulated and one down regulated. Additionally, genes from the ethylene, jasmonic and salicylic acids, and Bbrassinosteroids pathways were also up regulated, while the genes from the cytokine pathway were down regulated.

Regarding the phenylpropanoid biosynthesis pathway, key genes involved in the production of secondary metabolites were also differentially expressed, including the phenylalanine ammonia lyase (PAL) encoding gene. Moreover, many genes involved in the biosynthesis of flavonoids, anthocyanins, and plant steroids were, predominantly up regulated.

Figure 11 - DEGs identified in the plant hormone response pathway in response to the different environments. Blue boxes represent up regulated genes and red boxes down regulated genes
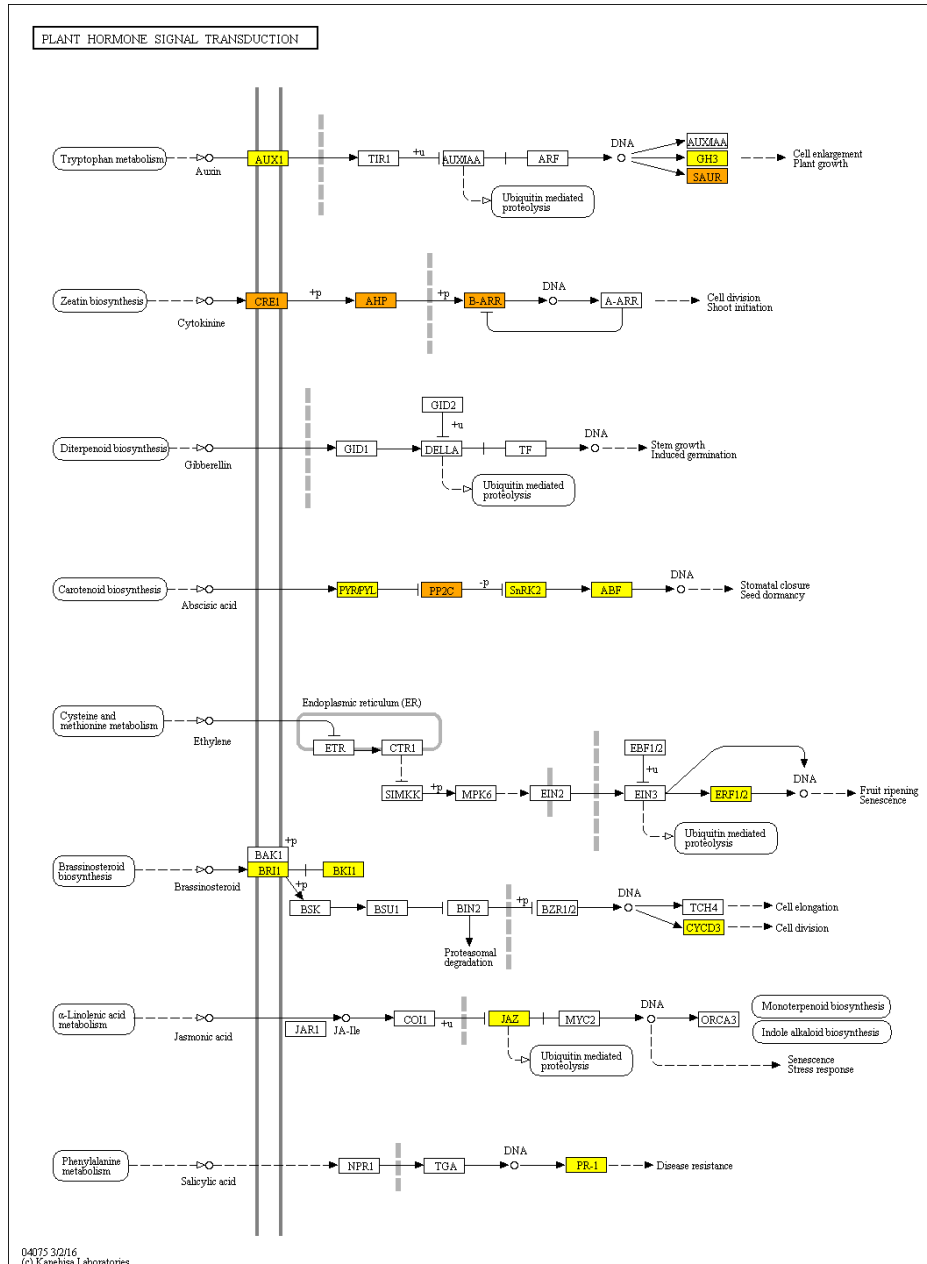
Source: Do autor (2016).

Figure 12 - DEGs identified in the phenylpropanoid biosynthesis pathway in response to the different environments. Yellow boxes represent up regulated genes and orange boxes down regulated
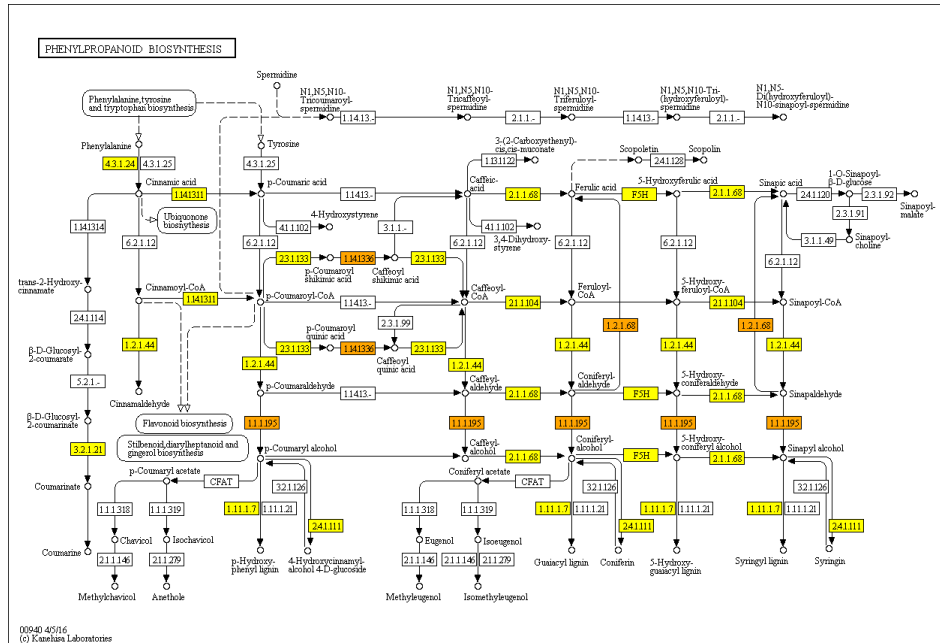
Source: Do autor (2016).

3.2 Growth Chamber Experiment

3.2.1 Physiological data

Gas exchange measurements were used to assess photosynthetic rate (A), transpiration rate (E) and stomatal conductance (gs), in Catuaí and Acauã plants, in response to two different temperature treatments: ambient (control) and high temperature (Figure 13). For the Catuaí cultivar we observed photosynthetic rates of approximately 5.7 μmol CO_2 m^{-2} s^{-1} and 4.3 μmol CO_2 m^{-2} s^{-1} were observed in normal and hot conditions, respectively. Moreover, the transpiration rate was 1.0 mmol H_2O m^{-2} sec^{-1} for the control condition and 0.7 mmol H_2O m^{-2} sec^{-1} for the hot condition, while the stomatal conductance was of

49.7μmol m^{-2} s^{-1} and 35.9 μmol m^{-2} s^{-1} for control and hot conditions, respectively.

Figure 13 - Gas exchange measurements for both Catuaí and Acauã cultivars in control and high temperature conditions.
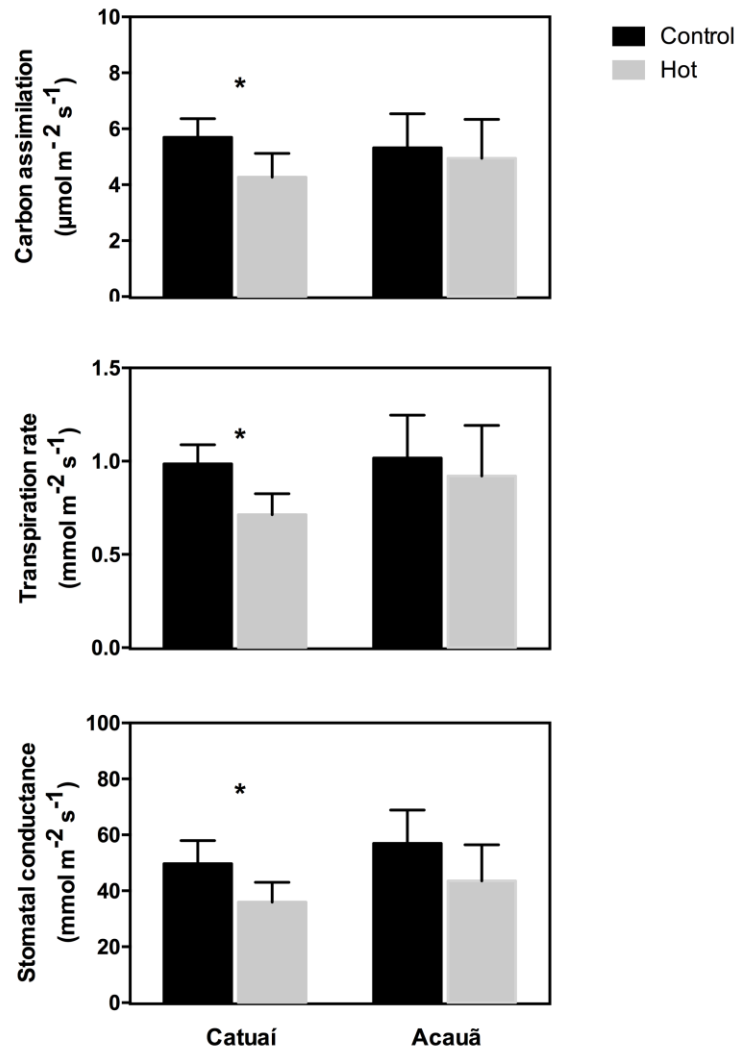
Source: Do autor (2016)

With regards to the Acauã cultivar (Figure 13), the observed rates for control and hot conditions were, respectively: 5.3 μmol CO_2 m^{-2} s^{-1} and 4.9 μmol CO_2 m^{-2} s^{-1} for photosynthesis, 0.9 mmol H_2O m^{-2} sec^{-1} and 0.8mmol H_2O m^{-2} sec^{-1} for transpiration, and 56.9 μmol m^{-2} s^{-1} and 43.5 μmol m^{-2} s^{-1} for stomatal conductance.

3.2.2 RNAseq

A total of ~713M (105 bp long) paired-end reads were generated by the 20 RNAseq libraries (Table 3). The total number of reads mapped to the *C. canephora* reference genome by the CLC software was on average 87.62% (considering only reads mapped in pairs). In Catuaí plants, ~342M reads were sequenced, of which 86.39% mapped to the reference genome, while in Acauã plants ~371M reads were sequenced and 88.85% of those mapped.

Table 3 - Analysis of RNAseq data mapped to the *C. canephora* genome

Sample ID	Rep #	Number of reads mapped in pairs	Number of reads mapped in broken pairs	Number of reads not mapped	Total	% of reads mapped in pairs	% of reads mapped in broken pairs	% of reads not mapped	% Total
Catuaí Control	1	23313602	1861159	658095	25832856	90.25	7.2	2.55	100
	2	21547854	1634033	627781	23809668	90.5	6.86	2.64	100
	3	30022864	2921213	849909	33793986	88.84	8.64	2.51	100
	4	16128570	2388949	549521	19067040	84.59	12.53	2.88	100
	5	34842466	4797473	1101483	40741422	85.52	11.78	2.7	100
Catuaí Warm	1	24947280	2239275	664843	27851398	89.57	8.04	2.39	100
	2	34750180	4508297	1054251	40312728	86.2	11.18	2.62	100
	3	32898934	2879864	893158	36671956	89.71	7.85	2.44	100
	4	34213810	5822703	1006691	41043204	83.36	14.19	2.45	100
	5	39836758	11670059	1349175	52855992	75.37	22.08	2.55	100
Acauã Control	1	19137964	2295811	748907	22182682	86.27	10.35	3.38	100
	2	31474876	3587107	1036155	36098138	87.19	9.94	2.87	100
	3	41876240	4522257	1139563	47538060	88.09	9.51	2.4	100
	4	22982400	1277373	624637	24884410	92.36	5.13	2.51	100
	5	33005586	2741479	920929	36667994	90.01	7.48	2.51	100
Acauã Warm	1	36240836	3156507	953853	40351196	89.81	7.82	2.36	100
	2	31047348	2754063	783197	34584608	89.77	7.96	2.26	100
	3	41024838	3767770	1131490	45924098	89.33	8.2	2.46	100
	4	36114960	3581000	991872	40687832	88.76	8.8	2.44	100
	5	36902038	4227877	1290275	42420190	86.99	9.97	3.04	100
Mean	-	31115470.2	3631713.45	918789.25	35665972.9	87.62	9.78	2.60	100
Total	-	622309404	72634269	18375785	713319458	-	-	-	-

3.2.2.1 Sample correlations

All samples were plotted using a Pearson correlation matrix (supplementary figures 3 and 4), in order to guarantee equality among sample replicates. As expected, all correlations were high between samples belonging to the same experimental group, except for one Acauã sample obtained from the optimal temperature treatment. Said sample was therefore excluded from further analysis.

3.2.2.2 Identification of differentially expressed genes (DEGs)

When analyzing the Catuaí cultivar, the comparison between control and plants subjected to high temperatures resulted in the identification of 548 DEGs (433 up regulated and 115 down regulated). In Acauã we identified 702 DEGs (550 up regulated and 152 down regulated). Therefore the number of DEGs was greater in Acauã, and 24 genes were common among cultivars (Figure 14).

Figure 14 - Venn diagram of differentially expressed genes in Catuaí and Acauã plants, in response to temperature treatments and their overlap.
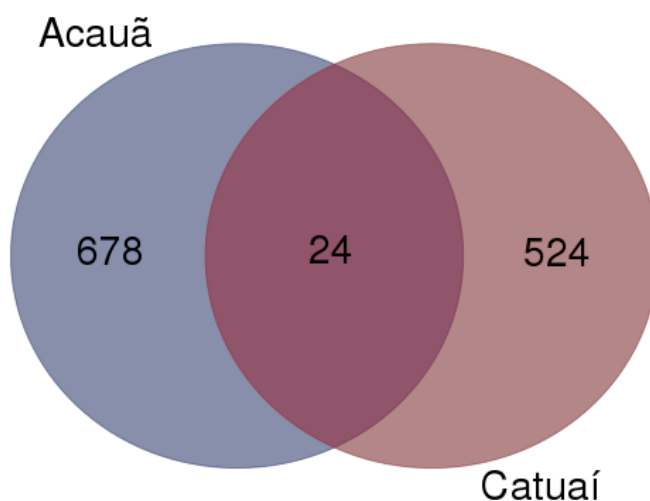
Source: Do autor (2016).

3.2.2.2.1 Gene Ontology (GO) annotation of DEGs

The gene ontology analysis for the Catuaí cultivar resulted in the annotation of 342 out of the 548 DEGs identified. The genes were divided in 11 enriched subcategories (Figure 15). The main subcategories with a percentage of genes higher than the background were: *cellular component organization* (12 genes), *regulation of biological process* (28 genes) and *biological regulation* (31 genes).

Among the genes annotated in the subcategory of *cellular component organization*, all 12 genes were up regulated, whereas for the *regulation of biological process* subcategory 21 were up regulated and 7 down regulated, in response to the temperature treatment. Moreover, for the subcategory *biological regulation* 8 genes were down regulated and 23 were up regulated (of which 11 were classified in the *transcription regulator activity* category).

Figure 15 - Functional classification of DEGs from the Catuaí cultivar in response to the heat treatment based on GO terms.
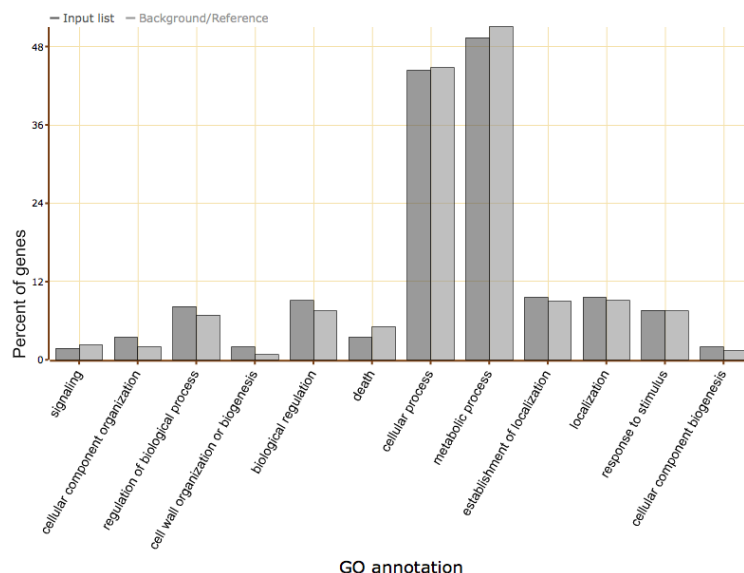
Source: Do autor (2016).

Furthermore, the gene ontology analysis for the Acauã cultivar resulted in the annotation of 397 out of the 702 DEGs identified. The genes were divided in 11 enriched subcategories (Figure 16). The main subcategories with a percentage of genes higher than the background were: *biological regulation* (31 genes), *death* (24 genes) and *response to stimulus* (32 genes).

Among the genes annotated in the subcategory of *biological regulation*, 24 genes were up regulated and 7 down regulated, whereas for the *death* subcategory 15 were up regulated and 9 down regulated, in response to the temperature treatment. Moreover, for the subcategory *response to stimulus* 8 genes were down regulated and 24 were up regulated.

Figure 16 - Functional classification of DEGs from the Acauã cultivar in response to the heat treatment based on GO terms.

Source: Do autor (2016).
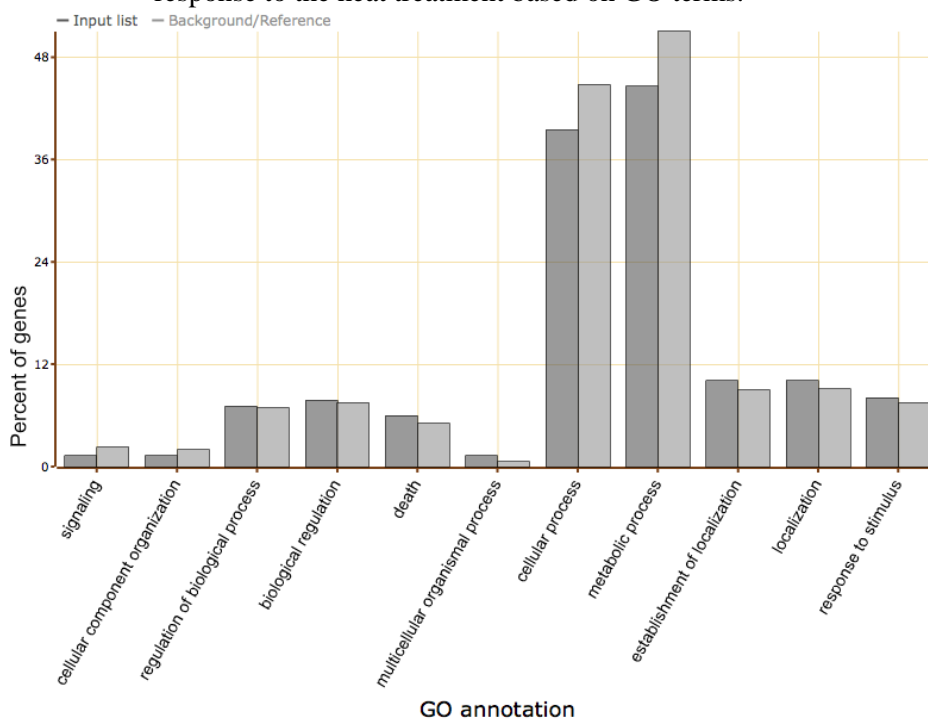
3.2.2.2.2 KEGG mapper and pathway annotation

When taking into account the response of both cultivars to high temperatures, the pathway identified by the KEGG mapper with the highest number of differentially expressed genes was the phenylpropanoid biosynthesis pathway (Figure 17) with 22 genes.

In the phenylpropanoid biosynthesis pathway, key genes involved in the production of secondary metabolites such as flavonoids, anthocyanin and plant steroids were up regulated, including the phenylalanine ammonia lyase (PAL) encoding gene.

Figure 17 - DEGs identified in the Phenylpropanoid Biosynthesis pathway in response to the heat treatment. Yellow boxes represent up regulated genes

4 DISCUSSION

Plants are able to respond and adapt to various environmental changes to which they are exposed during their life cycle (AHUJA et al., 2010). This fact is due to the regulation of gene expression at the transcriptional and post-transcriptional levels. This study allowed the identification, through RNAseq, of the differentially expressed genes in two coffee cultivars in response to different environmental conditions and temperature treatments, in addition to analyzing the plants physiological attributes in response to these treatments.

The gas exchange measurements taken in field conditons in April 2014 (Figures 4 and 5) indicate that the plants from both cultivars had higher photosynthesis, respiration, transpiration, and stomatal conductance rates when grown in the Pirapora location. Pirapora has higher than optimal temperatures for coffee, what has a direct impact in plant energy balance (JONES; ROTENBERG, 2001). However, it is important to note that the fields are irrigated by central pivots, therefore water availability is not a constraint. Varginha, on the contrary, has ideal temperatures and altitude, but the plants depend only on the rain regimen for water supply. The years of 2013 and 2014 (first semester) were marked by one of the longest and most intense droughts Brazil has ever experienced, and that resulted in a coffee production 30% lower than predicted. Thus, we believe that the lower rates observed in Varginha are also related to the drought conditions, but it is impossible to isolate the effects of abiotic factors in field experiments. According to Chaves and Oliveira (2004), the decrease in photosynthetic rates under field conditions in response to water deficit is primarily due to stomatal closure and the phytohormone abscisic acid (ABA) is known as the main factor that controls the opening and closing of stomata (LIZANA et al., 2006). Beebe et al. (2013) stated that stomatal closure may occur before the change on the leaf water status, suggesting the existence of an early communication between shoot and root system when the soil dries.

Phytohormones such as ABA and ethylene are believed to be the mediators of the root-shoot signaling and thus are crucial for the plant metabolism under adverse conditions such as low water availability and increased ozone concentration in the atmosphere(WILKINSON; DAVIES, 2010).

On the other hand, the measurements obtained for October (Figures 4 and 5) indicate higher photosynthesis, day transpiration and day stomatal conductance rates for both cultivars in Varginha. This could be explained by the way irrigated coffee plantations in Pirapora are managed: in order to obtain more uniform flowering, it is usual to suspend the irrigation for a period up to 100 days (GUERRA et al., 2005). Associated with the extremely high temperatures observed for the Pirapora region in October (average of 35.2°C), the drought conditions resulted in stomatal closure, and consequently, lower transpiration and photosynthesis rates. At night, however, as the temperatures dropped to around 19°C an increase in the stomatal conductance, transpiration and respiration rates could be observed. The activity of the antioxidant system enzymes was also higher for the October samples, corroborating the gas exchange results.

In the controlled conditions of the growth chambers, the hot temperature treatment resulted in statistically different gas exchange rates for Catuaí plants when compared to the control, but for the Acauã cultivar the differences were not statistically different, which could represent a higher tolerance of this cultivar to heat stress.

With regards to the enzymes of the antioxidant system it is important to note their role in plant stress responses, as the tolerance to several different environmental stresses have been correlated with the activity of ROS scavenging enzymes (MASSACCI et al., 1995). The SOD enzyme is key to the defense against oxidative stress in aerobic organisms, as it is responsible for the dismutation of the superoxide radical in O_2 and H_2O_2 (SHARMA et al., 2012)

Likewise, APX is a key component of the glutathione ascorbate cycle, playing an essential role in the control of intracellular levels of ROS, using two molecules of ascorbic acid to reduce H_2O_2 to water with a concomitant generation of two molecules of dehydroascorbate. APX is recognized as one of the antioxidant enzymes widely distributed in plants, with a high affinity for H_2O_2, which makes it efficient under stressful conditions (SHARMA et al., 2012). Moreover, CAT is considered one of the primary defenses against oxidative stress induced by senescence (ZIMMERMANN et al., 2006), catalyzing the dismutation of two H_2O_2 molecules to water and oxygen. Although catalase has a high specificity for H_2O_2, the affinity is much smaller than APX (SHARMA et al., 2012), which might explain its lower activity when compared to APX.

Chaitanya et al. (2002) found higher levels of SOD, CAT and APX in plants treated with high temperatures when compared to the control, and stated that there is a correlation between all the enzymes activities, as they increased in a coordinated way. As mentioned before, overall, in the field experiment the enzyme activity was higher for SOD, CAT and APX in the samples collected in October. Moreover, SOD concentrations for Acauã were statistically different at both harvesting and flowering stages, being higher in Varginha samples in April and higher in Pirapora samples in October.

Besides producing antioxidant enzymes, plants have other mechanisms to cope with heat stress, one being hormonal response. Hormones play an important role in plant adaptation to adverse environmental conditions and their stability, production and homeostasis are altered in response to heat stress (MAESTRI et al., 2002). ABA is implicated in the responses to osmotic stress as it mediates a dehydration-signaling pathway in plants by modulating the transcription of several genes (NEILL, 1992; XIONG et al., 2002). ABA mediates the plant response to dehydration. In field conditions heat and drought

stresses usually occur simultaneously, so ABA induction can be an important component of heat tolerance as well. Ethylene, a gaseous hormone is also involved in the tolerance to environmental stresses. The ethylene-responsive factor (ERF) is a family of transcription factors that regulates plant responses to biotic and abiotic stress. Despite being characterized as ethylene responsive, ERFs can also be regulated in an independent manner, by stress or by other phytohormones like jasmonic acid (JA) and ABA (DEY; VLOT, 2015).

In the KEGG annotation, the Plant Hormone Response was identified as containing genes that were differentially transcribed. The pattern observed for the genes related to ABA suggests higher levels of signal transduction, which is indicative of the stressed state of the plants. Moreover, the up regulation of ERF genes is additional evidence that the plants are responding to heat conditions.

Although usually underestimated, secondary metabolites also play a fundamental role in plant stress tolerance. Mostly synthesized from the intermediates of primary carbon metabolism via the phenylpropanoid pathway (WAHID et al., 2007), secondary metabolites such as flavonoids and phenylpropanoids are induced by high-temperature. PAL is the principal enzyme of the phenylpropanoid pathway and its increased activity in response to thermal stress is considered as the main response of cells to heat stress (BITA; GERATS, 2013).

Phenolic compounds such as lignin, anthocyanin, and flavonoids are the most important class of secondary metabolites in plants as they play a variety of roles in plant physiology, including tolerance to abiotic stresses (WAHID, 2006). Rivero et al. (2001) found that in tomato plants, thermal stress results in increased PAL; decreased peroxidase and polyphenol oxidase activity and accumulation of soluble phenolics.

Genes that are part of the phenylpropanoid pathway were found to be overexpressed in response to both environmental conditions and heat treatment.

The KEGG annotation identified the up regulation of key genes for the synthesis of flavonoids and anthocyanin, the most important one being the PAL gene, since this enzyme is part of the first step of the secondary metabolites synthesis pathway.

Lastly, the molecular mechanisms controlling the tolerance to abiotic stress are based on the regulation and activation of specific genes (WANG; VINOCUR; ALTMAN, 2003), and are classified into two major categories: (1) genes involved in signaling cascades, (2) genes that contribute directly to the protection of membranes and proteins, such as HSPs, chaperones, and LEA proteins (late embryogenesis abundant) (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2007).

The first group consists of genes involved in signal transduction, such as transcription factors that play a fundamental role in the plant response to stress, and also include kinases, protein phosphatases, enzymes of phospholipid metabolism and other signaling molecules (Seki et al., 2003). Many transcription factors are induced by stress, suggesting that different transcriptional regulatory mechanisms can regulate the signal transduction pathways of drought, cold and salinity stresses (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2007).

In this study, as evidenced by the GO category analysis, several genes related to different families of transcription factors were up regulated in response to different environmental conditions. Among these we identified some genes of the families, DREB, ERF, MYB, WRKY and bZIP, besides heat shock transcription factors and proteins (Table 2), which play an important role in plants by regulating the expression of target genes in response to stress (AHUJA et al., 2010). These families can be subdivided into ABA dependent (MYB and bZIP) and independent (AP2, DREB and ERF) pathways.

The DREB (Dehydration Responsive Element Binding proteins) transcription factors are included in the superfamily AP2/ERF, which is

characterized by the presence of the DNA binding domain AP2/ERF (MAGNANI; SJOLANDER; HAKE, 2004; RIECHMANN et al., 2000). The overexpression of some DREB transcription factors has been linked to the regulation of target genes in the tolerance to various abiotic stresses, especially membrane proteins, and chaperones (MATSUKURA et al., 2010; WANG et al., 2008).

The second group of genes that respond to high temperatures consists of those encoding protective proteins of cell damage caused by thermal stress. In this group the following genes were identified as being up regulated in our experiments: late embryogenesis abundant protein - LEA and HVA22. LEA protein function is thought to be related to the protein degradation pathway. They can prevent aggregation and protect citrate synthase from conditions such as heat and drought stress. The HVA22 proteins are part of a class of LEAs that are induced by ABA and that protect cells against apoptosis (GUO; HO, 2008; SHEN et al., 2001).

5 FINAL REMARKS

When a plant is subjected to heat stress, a number of physiological and molecular traits are changed, various genes are turned on or off, resulting in altered levels of metabolites and proteins, some of which may be responsible for conferring a certain degree of protection against abiotic stresses. A key to progress toward breeding better crops under stress is to understand the changes in molecular machinery that occur in response to stress. The use of various biotechnological approaches provides a way to reduce the losses caused by high temperature, but it requires background biological knowledge of the tolerance mechanism. With the advances in genome sequencing techniques, we have the genomic information that can be exploited to improve heat tolerance in crop species. Cultural practices such as planting time, planting methods, and soil and irrigation management have long been in use to minimize the effects of stress. However, in practice, to be successful in improving agricultural productivity in stress environments, both genetic improvement and adjustment in cultural practices must be employed simultaneously.

REFERENCES

AHUJA, I. et al. Plant molecular stress responses face climate change. **Trends in Plant Science,** Oxford, v. 15, n. 12, p. 664-674, Dec. 2010.

ASSAD, E. D. et al. Impacto das mudanças climáticas no zoneamento agroclimático no café no Brasil. **Pesquisa Agropecuária Brasileira,** Brasília, v. 39, n. 11, p. 1057-1064, 2004.

BARRETO, H. G. et al. In silico and quantitative analyses of the putative FLC-like homologue in coffee (Coffea arabica L.). **Plant Molecular Biology Reporter,** Athens, v. 30, n. 1, p. 29-35, Feb. 2012.

BEEBE, S. E. et al. Phenotyping common beans for adaptation to drought. **Frontiers in Physiology,** Lausanne, v. 4, p. 35, Mar. 2013.

BIEMELT, S.; KEETMAN, U.; ALBRECHT, G. Re-aeration following hypoxia or anoxia leads to activation of the antioxidative defense system in roots of wheat seedlings. **Plant Physiology,** Bethesda, v. 116, n. 2, p. 651-658, Feb. 1998.

BITA, C.; GERATS, T. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress tolerant crops. **Frontiers in Plant Science,** Lausanne, v. 4, p. 273, 2013, July 2013.

BRADFORD, M. M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. **Analytical Biochemistry,** New York, v. 72, n. 1/2, p. 248-254, May 1976.

CAMARGO, M. B. The impact of climatic variability and climate change on arabica coffee crop in Brazil. **Bragantia,** Campinas, v. 69, n. 1, p. 239-247, 2010.

CHAITANYA, K. V. et al. Variation in heat stress-induced antioxidant enzyme activities among three mulberry cultivars. **Plant Growth Regulation,** Dordrecht, v. 36, n. 2, p. 175-180, Feb. 2002.

CHAVES, M. M.; OLIVEIRA, M. M. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. **Journal of Experimental Botany,** Oxford, v. 55, n. 407, p. 2365-2384, Nov. 2004.

DAVIS, A. P. et al. Growing coffee: psilanthus (Rubiaceae) subsumed on the basis of molecular and morphological data; implications for the size,

morphology, distribution and evolutionary history of Coffea. **Botanical Journal of the Linnean Society,** London, v. 167, n. 4, p. 357-377, Dec. 2011.

DAVIS, A. P. et al. The impact of climate change on indigenous arabica coffee (Coffea arabica): predicting future trends and identifying priorities. **Plos One,** San Francisco, v. 7, n. 11, p. e4981, Nov. 2012.

DEY, S.; VLOT, A. C. Ethylene responsive factors (ERFs) in the orchestration of stress responses in monocotyledonous plants. **Frontiers in Plant Science,** Lausanne, v. 6, p. 640, Aug. 2015.

ESTRADA, F.; GAY, C.; CONDE, C. A methodology for the risk assessment of climate variability and change under uncertainty. A case study: coffee production in Veracruz, Mexico. **Climatic Change,** Dordrecht, v. 113, n. 2, p. 455-479, July 2012.

GIANNOPOLITIS, C. N.; RIES, S. K. Superoxide dismutases. 1. Occurrence in higher-plants. **Plant Physiology,** Lancaster, v. 59, n. 2, p. 309-314, Feb. 1977.

GUERRA, A. F. et al. **Irrigação do cafeeiro no cerrado: e**stratégia de manejo de água para uniformização de florada. Planaltina: Ministério da Agricultura, 2005. 4 p. (Comunicado Técnico, 122).

GUO, W.-J.; HO, T.-H. D. An abscisic acid-induced protein, HVA22, inhibits gibberellin-mediated programmed cell death in cereal aleurone cells. **Plant Physiology,** Bethesda, v. 147, n. 4, p. 1710-1722, Aug. 2008.

HAVIR, E. A.; MCHALE, N. A. Biochemical and developmental characterization of multiple forms of catalase in tobacco-leaves. **Plant Physiology,** Lancaster, v. 84, n. 2, p. 450-455, June 1987.

INTERGOVERNMENTAL PANEL ON CLIMATE CHANGE - IPCC. **Fifth assessment report:** climate change. Geneva: IPCC, 2014.

INTERNATIONAL COFFEE ORGANIZATION - ICO. **Indicator prices:** annual and monthly averages. London: ICO, 2015a. Disponível em: <http://www.ico.org/new_historical.asp? section=Statistics>. Acesso em: 15 mar 2015.

_____. **Trade statistics**. London: ICO, 2015b. Disponível em: <http://www.ico.org/trade_ statistics.asp>. Acesso em: 15 mar 2015.

JONES, H. G.; ROTENBERG, E. **Energy, radiation and temperature regulation in plants.** New York: John Wiley & Sons, 2001.

LADERACH, P.; JARVIS, A. J. R. The impact of climate change in coffe-growing regions. In: (Ed.). **Taller de adaptacion al cambio climatico en las comunidades cafetaleras de la Sierra Madre de Chiapas**. Chiapas: Tuxtla Gutierrez, 2008. p. 17-18.

LINGLE, T. Preface. In: WINTGENS, J. N. (Ed.). **Coffee:** growing, processing, sustainable production. Weinheim: WILEY-VCH Verlag, 2008. p. 11-13.

LIZANA, C. et al. Differential adaptation of two varieties of common bean to abiotic stress - I. Effects of drought on yield and photosynthesis. **Journal of Experimental Botany,** Oxford, v. 57, n. 3, p. 685-697, Feb. 2006.

MAESTRI, E. et al. Molecular genetics of heat tolerance and heat shock proteins in cereals. **Plant Molecular Biology,** Dordrecht, v. 48, n. 5, p. 667-681, Mar. 2002.

MAGNANI, E.; SJOLANDER, K.; HAKE, S. From endonucleases to transcription factors: Evolution of the AP2 DNA binding domain in plants. **The Plant Cell,** Rockville, v. 16, n. 9, p. 2265-2277, Sept. 2004.

MASSACCI, A. et al. The effect of growth at low temperature on photosynthetic characteristics and mechanisms of photoprotection of maize leaves. **Journal of Experimental Botany,** Oxford, v. 46, n. 1, p. 119-127, Jan. 1995.

MATTA, F. M. da. Exploring drought tolerance in coffee: a physiological approach with some insights for plant breeding. **Brazilian Journal of Plant Physiology,** Campinas, v. 16, n. 1, p. 1-6, jan. 2004.

MATTA, F. M. da; RAMALHO, J. D. C. Impacts of drought and temperature stress on coffee physiology and production: a review. **Brazilian Journal of Plant Physiology, Campinas,** v. 18, n. 1, p. 55-81, jan./mar. 2006.

MATSUKURA, S. et al. Comprehensive analysis of rice DREB2-type genes that encode transcription factors involved in the expression of abiotic stress-responsive genes. **Molecular Genetics and Genomics,** Berlin, v. 283, n. 2, p. 185-196, Feb. 2010.

78

MISHRA, M. K.; SLATER, A. Recent advances in the genetic transformation of coffee. **Biotechnology Research International**, Washington, v. 2012, p. 1-17, 2012.

NAKANO, Y.; ASADA, K. Hydrogen-peroxide is scavenged by ascorbate-specific peroxidase in spinach-chloroplasts. **Plant and Cell Physiology,** Kyoto, v. 22, n. 5, p. 867-880, 1981.

NEILL, S. J. Abscisic acid: physiology and biochemistry, eds W. J. Davies and H. G. Jones, xiv + 266 pp. Oxford: BIOS Scientific Publishers (1991). £39.95 or $79.00 (hardback). ISBN 1 872748 65 1. **The Journal of Agricultural Science,** Amsterdam, v. 118, n. 03, p. 404-404, 1992.

NISHIKIM, M.; APPAJI, N.; YAGI, K. Occurrence of superoxide anion in reaction of reduced phenazine methosulfate and molecular-oxygen. **Biochemical and Biophysical Research Communications,** New York, v. 46, n. 2, p. 849-854, Jan, 1972.

RAMIREZ-VILLEGAS, J. et al. A way forward on adaptation to climate change in Colombian agriculture: perspectives towards 2050. **Climatic Change,** Dordrecht, v. 115, n. 3/4, p. 611-628, Dec. 2012.

RIECHMANN, J. L. et al. Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. **Science,** London, v. 290, n. 5499, p. 2105-2110, Dec. 2000.

RIVERO, R. M. et al. Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. **Plant Science,** Limerick, v. 160, n. 2, p. 315-321, Jan. 2001.

SEKI, M. et al. Molecular responses to drought, salinity and frost: common and different paths for plant protection. **Current Opinion in Biotechnology,** London, v. 14, n. 2, p. 194-199, Apr. 2003.

SHARMA, P. et al. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. **Journal of Botany,** London, v. 2012, p. 1-26, 2012.

SHEN, Q. X. et al. The stress- and abscisic acid-induced barley gene HVA22: developmental regulation and homologues in diverse organisms. **Plant Molecular Biology,** Dordrecht, v. 45, n. 3, p. 327-340, Feb. 2001.

SHINOZAKI, K.; YAMAGUCHI-SHINOZAKI, K. Gene networks involved in drought stress response and tolerance. **Journal of Experimental Botany,** Oxford, v. 58, n. 2, p. 221-227, Jan. 2007.

STEVENS, P. F. **Angiosperm phylogeny website**. [S.l.: s.n.], 2012. Disponível em: <http:// www.mobot.org/MOBOT/research/APweb/>. Acesso em: 15 mar. 2015.

WAHID, A. et al. Heat tolerance in plants: an overview. **Environmental and Experimental Botany,** Elmsford, v. 61, n. 3, p. 199-223, Dec. 2007.

WAHID, A. Physiological implications of metabolite biosynthesis for net assimilation and heat-stress tolerance of sugarcane (Saccharum officinarum) sprouts. **Journal of Plant Research,** Tokyo, v. 120, n. 2, p. 219-228, Mar. 2006.

WANG, Q. et al. Overexpression of a rice OsDREB1F gene increases salt, drought, and low temperature tolerance in both Arabidopsis and rice. **Plant Molecular Biology,** Dordrecht, v. 67, n. 6, p. 589-602, Aug. 2008.

WANG, W. X.; VINOCUR, B.; ALTMAN, A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. **Planta,** Berlin, v. 218, n. 1, p. 1-14, Nov. 2003.

WILKINSON, S.; DAVIES, W. J. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. **Plant Cell and Environment,** Oxford, v. 33, n. 4, p. 510-525, Apr. 2010.

XIONG, L. M. et al. Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis. **Journal of Biological Chemistry,** Bethesda, v. 277, n. 10, p. 8588-8596, Mar. 2002.

ZIMMERMANN, P. et al. Senescence-specific regulation of catalases in Arabidopsis thaliana (L.) Heynh. **Plant Cell and Environment,** Oxford, v. 29, n. 6, p. 1049-1060, June 2006.

SUPLEMENTARY FILES

	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10	P11	P12
P01	1	0.97	0.99	0.94	0.98	0.98	0.95	0.96	0.93	0.95	0.96	0.97
P02	0.97	1	0.93	0.93	0.97	0.98	0.95	0.93	0.91	0.95	0.95	0.97
P03	0.99	0.93	1	0.97	0.99	0.99	0.97	0.95	0.93	0.96	0.97	0.98
P04	0.94	0.93	0.97	1	0.98	0.98	0.96	0.91	0.92	0.93	0.93	0.96
P05	0.98	0.97	0.99	0.98	1	0.99	0.97	0.93	0.93	0.96	0.95	0.93
P06	0.98	0.98	0.99	0.98	0.99	1	0.97	0.94	0.93	0.95	0.95	0.97
P07	0.95	0.95	0.97	0.96	0.97	0.97	1	0.95	0.97	0.97	0.96	0.98
P08	0.96	0.93	0.95	0.91	0.93	0.94	0.95	1	0.93	0.91	0.95	0.95
P09	0.93	0.91	0.93	0.92	0.93	0.93	0.97	0.93	1	0.95	0.95	0.95
P10	0.95	0.95	0.96	0.93	0.96	0.95	0.97	0.91	0.95	1	0.97	0.98
P11	0.96	0.95	0.97	0.93	0.95	0.95	0.96	0.95	0.95	0.97	1	0.99
P12	0.97	0.97	0.98	0.96	0.97	0.97	0.98	0.95	0.95	0.98	0.99	1

Table 1. Pearson's correlation matrix of all samples from the harvesting collection

	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24
P13	1	0.97	0.96	0.68	0.88	0.9	0.95	0.93	0.93	0.93	0.93	0.94
P14	0.97	1	0.98	0.7	0.97	0.91	0.89	0.94	0.91	0.92	0.92	0.95
P15	0.96	0.98	1	0.72	0.96	0.92	0.88	0.93	0.9	0.93	0.92	0.96
P16	0.68	0.7	0.72	1	0.72	0.86	0.66	0.96	0.74	0.77	0.72	0.75
P17	0.98	0.97	0.96	0.72	1	0.9	0.92	0.96	0.94	0.95	0.95	0.96
P18	0.88	0.91	0.92	0.86	0.9	1	0.86	0.89	0.9	0.92	0.86	0.93
P19	0.9	0.89	0.88	0.66	0.92	0.86	1	0.98	0.98	0.95	0.95	0.95
P20	0.95	0.94	0.93	0.96	0.96	0.89	0.98	1	0.98	0.97	0.97	0.97
P21	0.93	0.91	0.9	0.74	0.94	0.9	0.98	0.98	1	0.97	0.97	0.97
P22	0.93	0.92	0.93	0.77	0.95	0.92	0.95	0.97	0.97	1	0.98	0.99
P23	0.93	0.92	0.92	0.72	0.95	0.86	0.95	0.97	0.97	0.98	1	0.97
P24	0.94	0.95	0.96	0.75	0.96	0.93	0.95	0.97	0.96	0.99	0.97	1

Table 2. Pearson's correlation matrix of all samples from the flowering collection

	C1P1	C1P2	C1P3	C1P4	C1P5	C1P6	C1P7	C1P8	C1P9	C1P10
C1P1	1	0.98	0.99	0.98	0.99	0.99	0.92	0.96	0.97	0.95
C1P2	0.98	1	0.98	0.95	0.97	0.98	0.91	0.97	0.97	0.97
C1P3	0.99	0.98	1	0.98	0.99	0.98	0.92	0.97	0.98	0.96
C1P4	0.98	0.95	0.98	1	0.97	0.96	0.9	0.95	0.95	0.93
C1P5	0.99	0.97	0.99	0.97	1	0.98	0.92	0.96	0.97	0.96
C1P6	0.99	0.98	0.98	0.96	0.98	1	0.93	0.98	0.98	0.97
C1P7	0.92	0.91	0.92	0.9	0.92	0.93	1	0.91	0.92	0.9
C1P8	0.96	0.97	0.97	0.95	0.96	0.98	0.91	1	0.99	0.99
C1P9	0.97	0.97	0.98	0.95	0.97	0.98	0.92	0.99	1	0.99
C1P10	0.95	0.97	0.96	0.93	0.96	0.97	0.9	0.99	0.99	1

Table 3. Pearson's correlation matrix of all samples from the Catuaí Cultivar (growth chamber experiment)

	C2P1	C2P2	C2P3	C2P4	C2P5	C2P6	C2P7	C2P8	C2P9	C2P10
C2P1	1	0.52	0.54	0.56	0.55	0.53	0.55	0.51	0.54	0.53
C2P2	0.52	1	0.96	0.96	0.95	0.96	0.96	0.94	0.93	0.97
C2P3	0.54	0.96	1	0.99	0.99	0.97	0.98	0.94	0.97	0.98
C2P4	0.56	0.96	0.99	1	0.99	0.97	0.98	0.92	0.96	0.96
C2P5	0.55	0.95	0.99	0.99	1	0.97	0.98	0.92	0.98	0.97
C2P6	0.53	0.96	0.97	0.97	0.97	1	0.99	0.96	0.99	0.97
C2P7	0.55	0.96	0.98	0.98	0.98	0.99	1	0.96	0.98	0.97
C2P8	0.51	0.94	0.94	0.92	0.92	0.96	0.96	1	0.95	0.97
C2P9	0.54	0.93	0.97	0.96	0.98	0.99	0.98	0.95	1	0.97
C2P10	0.53	0.97	0.98	0.96	0.97	0.97	0.97	0.97	0.97	1

Table 4. Pearson's correlation matrix of all samples from the Acauã Cultivar (growth chamber experiment)