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REGULATION OF COFFEE FLOWERING TIME: HORMONAL CROSSTALK AND TRANSCRIPTOMIC ANALYSIS OF REPRODUCTIVE DEVELOPMENT AND ANTHESIS

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Thesis submitted for the Degree of Doctor of Philosophy in Agronomy/Plant Physiology Postgraduate Program Federal University of Lavras

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MARLON ENRIQUE LOPEZ TORRES

REGULAÇÃO DO FLORESCIMENTO DE CAFÉ: *CROSSTALK* HORMONAL E ANÁLISE TRANSCRIPTOMICA NO DESENVOLVIMENTO REPRODUTIVO E ANTESE

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To my heavenly father for always guiding me in my Ph.D. project through the company of the holy ghost and my family for all the support, comprehension, and love

I dedicate

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GENERAL ABSTRACT

Flowering is one of the most critical steps during the plant´s life cycle; a synchronized interaction of endogenous and environmental signals triggers this process at the right time. In coffee (*Coffea arabica* L.), coffee flower development is asynchronous, resulting in an uneven fruit ripening. Understanding the mechanism by which coffee flowering is regulated may help adopt some agronomical practices to synchronize coffee production and provide better final product quality. This research aimed to investigate the influence of plant hormones and gene regulation in the control of coffee flowering. In this sense, two experiments were developed. In the first experiment, we evaluated the content of ethylene, Abscisic Acid (ABA), 1-aminocyclopropane-1-carboxylic acid oxidase (ACC), 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) activity, and Lysine Histidine Transporter 1 (LHT1) gene expression, as the ACC transporter, in a different key point of the coffee flower bud development in the Acauã (Late), Oeiras (Early) and *Semperflorens* (Continuous) coffee genotypes. Moreover, coffee plants supplied ACC exogenous application compared with 1-methilcyclopropene (1-MCP) and water treatments. ABA and ethylene content had an inverse profile in the rainy and dry period and, after rain before anthesis, the ACC content increased in all coffee tissues (Leaves, roots, and flower buds). A high relative LHT1 gene expression was observed at the same period for leaves and roots. At this point, increased ACC content was determined as a modulator of coffee anthesis. In an additional experiment, this activity was corroborated when coffee plants with 300 mM of exogenous ACC supply showed higher G6 flower bud stages. In the second experiment, a transcriptomic analysis (RNA-seq) from leaves, flower buds, and Shoot Apical Meristem (SAM) in the identical coffee genotypes of the first experiment was carried out in March and August. In addition, phenotypic (Flower bud development and flowering events) and biochemical analysis (Sugar content) were carried out. Starch content was higher for *Semperflorens* than in Oeiras and Acauâ, and sucrose content increased from June to August before anthesis in Acauã. Ethylene decreased in leaves from the rainy to dry period. *Semperflorens* presented more flowering events than Acauã, and Oeiras flowered more than Acauã. A total of 12748 Differentially Expressed Genes (DEGs) were found contrasting different tissues and sampling conditions associated with hormonal regulation, sugar metabolism, photosynthesis, circadian clock, temperature, endogenous and external stimulus, seed development, and flower development. 280 DEGs for sugar metabolism, 650 DEGs for ethylene biosynthesis and signaling, and 523 DEGs for floral organ identity and photoperiod were selected. Finally, 4 DEGs for sugar metabolism and 6 DEGs for photoperiod and flower development were identified to be congruent with the flowering pattern of coffee genotypes. 28 DEGs were identified for ethylene biosynthesis; however, no correlation was found with ethylene evolution in coffee tissues associated with flowering.

Keywords: Flowering, RNAseq, coffee tree, anthesis, ACC, ABA, ethylene

RESUMO GERAL

A floração é uma das etapas mais importantes do ciclo de vida das plantas, em que uma interação sincronizada de sinais endógenos e ambientais desencadeia esse processo no momento certo. No café (*Coffea arabica* L.), o desenvolvimento da flor é assíncrono, resultando em um amadurecimento desigual dos frutos. Compreender o mecanismo de regulação da floração do café pode contribuir para adotação de algumas práticas agronômicas para sincronizar a produção de café e consequentemente, diminuir os custos de produção e melhorar a qualidade final. O objetivo desta pesquisa foi investigar a influência dos hormônios vegetais e da regulação gênica no controle da floração do café. Nesse sentido, foram desenvolvidos dois experimentos. No primeiro experimento, foram avaliados o conteúdo de etileno, ácido abscísico (ABA), ácido carboxílico-1-aminociclopropano (ACC), a atividade do ácido 1-aminociclopropano-1-carboxílico oxidase (ACO), e a expressão do gene transportador de lisina histidina 1 (LHT1), como transportador de ACC, avaliadando-se em diferentes pontos-chave do desenvolvimento dos botões florais das cultivares Acauã (cultivar tardia), Oeiras (cultivar precoce) e *Semperflorens* (Cultivar Contínuo). Além disso, a aplicação de ACC exógeno em comparação com tratamentos de 1-metilciclopropeno (1-MCP) e de água foi testada em plantas de café. Os teores de ABA e etileno foram inversos no período chuvoso e seco e após a chuva, antes da antese, o teor de ACC aumentou em todos os tecidos do cafeeiro (folhas, raízes e botões florais). Uma alta expressão relativa do LHT1foi observada no mesmo período para folhas e raízes. Neste mesmo momento, o aumento do conteúdo de ACC foi determinado como um modulador da antese do café. Adicionalmente, essa atividade foi corroborada quando cafeeiros com 300 mM de ACC exógeno apresentaram maior número de botões florais no estado G6. No segundo experimento, foram realizadas análises transcriptômicas (RNA-seq) de folhas, botões florais e meristema apical (SAM) das mesmas cultivares utilizadas no primeiro experimento nos meses de março e agosto. Além disso, foram feitas análises fenotípicas (desenvolvimento do botão floral e eventos de floração) e bioquímicas (teor de açúcar). O teor de amido foi maior para *Semperflorens* do que para Oeiras e Acauã, o teor de sacarose aumentou de junho a agosto antes da antese em Acauã. O etileno diminuiu nas folhas do período chuvoso para o seco. A cultivar *Semperflorens* apresentou mais eventos de florescimento do que Acauã e Oeiras e, Oeiras, mais do que Acauã. Um total de 12748 Genes Diferencialmente Expressos (DEGs) foram encontrados contrastando diferentes tecidos e condições de amostragem, associados à regulação hormonal, metabolismo do açúcar, fotossíntese, relógio circadiano, temperatura, estímulo endógeno e externo, desenvolvimento de sementes e desenvolvimento de flores. Foram selecionados 280 DEGs para metabolismo do açúcar, 650 DEGs para biossíntese e sinalização de etileno e 523 DEGs para identidade de órgão floral e fotoperíodo. Finalmente, 4 DEGs para o metabolismo do açúcar e 6 DEGs para o fotoperíodo e desenvolvimento da flor foram identificados como sendo congruentes com o padrão de floração das cultivares de café. Ainda que, 28 DEGs foram identificados para a biossíntese de etileno.

Palavras-chave: Florescimento, RNAseq, cafeeiro, antese, ACC, ABA, etileno

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SUMMARY

CHAPTER 1

INTRODUCTION

Coffee is one of the most important agricultural products in the international market. It is cultivated in different latitudes around the world, but its distribution depends on many factors, such as climate, location, soil types, shading, and management practices (Carr 2001). Among approximately 103 Coffea species, *Coffea arabica* L. and *C. canephora* Pierre ex Froehner are the most important in the world coffee trade representing 60% and 40% of the world's coffee production, respectively (ICO 2020).

Required for reproductive success, coffee flowering is one the most important developmental process of the plant´s life cycle, synchronizing reproduction with appropriate environmental conditions, maximizing the number of progenies produced and thereby, contributing to its performance in natural environments for yield and quality. The maturation degree of coffee fruit is related to the flowering events that, in the coffee plant, may occur many times during the same production cycle. This sequential flowering event results from the asynchronous bud development, and is an intrinsic characteristic of the species, resulting in the uneven maturation of the fruits, which may give rise to losses in the coffee cup quality, and therefore less economical incomes.

Coffee flowering time is regulated by exogenous factors such as photoperiod, temperature, rain, shading system, nutritional plant status, as well as by endogenous ones, such as the age of the plant, water stress, plant hormones, and signaling genes activating floral induction and floral development until anthesis. An important aspect to be analyzed in coffee trees is the regulation of the internal factors of the plant such as the hormonal balance because information on how these substances act in the development of buds is still incipient. Lately, researchers have sought to increase knowledge about the events that occur in the development of floral buds at the anatomical and molecular level, and how those exogenous and internal factors are associated with coffee flowering development. In this research, was hypothesized that hormonal regulation, environmental and molecular factors are influencing the coffee flowering time. As result, three different papers aiming to elucidate knowledge related to those

complex relationships between molecular and physiological aspects regulating coffee flowering are presented.

In the first manuscript, chapter 2, we present the most recent information about endogenous and environmental factors influencing coffee flowering. Some models are shown to provide the possible molecular pathway involved in the flowering time and developmental process in *C. arabica*. Moreover, a proposed model of coffee floral induction under Brazilian environmental conditions showing the phenological cycle of early and late coffee flowering process and a proposed ABC model for floral organ identity genes is given. In the second manuscript, chapter 3, we present the hormonal crosstalk between Ethylene and ABA coffee in anthesis. 1-Aminocyclopropene-Acid (ACC) as an ethylene precursor increased in the re-watering period acting as the modulator in the anthesis process. Coffee plants with exogenous application of ACC showed a higher number of flower buds at the G6 stage compared with 1-MCP and water treatments. As regards Chapter 4, presents the manuscript related to the transcriptomic analysis (RNA-seq) and field data of ethylene evolution, sugars content, flower bud development, and flowering events of three coffee genotypes with contrasted flowering time. The objective was to identify some molecular and physiological factors regulating the flowering time in each coffee cultivar to explain the flowering pattern of Early, Late, and Continuous.

Results of this research show the involvement of ABA and ethylene pattern production in the flower bud development in coffee and present ACC as a modulator in the coffee anthesis process. On the other hand, the transcriptomic analysis displays the possible genetic complex regulation among sugar metabolism, hormonal regulation, identity floral, and photoperiod in coffee flowering.

CHAPTER 2

ARTICLE 1- AN OVERVIEW OF THE ENDOGENOUS AND ENVIRONMENTAL FACTORS RELATED TO THE COFFEE FLOWERING PROCESS

(DRAFT VERSION)

Article prepared and submitted to the *Beverage plant Research* Journal

An overview of the endogenous and environmental factors related to the coffee flowering process

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Abstract

The transition of vegetative meristems to a reproductive state followed by floral development and flowering is a complex process regulated by many regulatory pathways that, integrate (epi) genetic and environmental factors to guarantee reproductive success in angiosperms. In woody and perennial species, such as coffee trees, the extended life cycle reflects adaptive strategies, i.e., biannual cycle, sequential flowering, and bud latency during the winter, that ensure reproductive success in a variable environment. The dynamics of coffee flowering integrate multiple stimuli to induce morpho-physiological changes, especially during the reproductive phase. Using coffee as a model of crops, we discussed diverse stimuli which are directly and indirectly associated with molecular pathways related to the reproductive meristem transition and floral development until anthesis. Here, we propose to redefine some concepts regarding coffee floral development, such as i) A summary of genes possibly involved in the flowering pathways; ii) The phenological cycle considering early and late genotypes with differential flowering timing and fruit maturation; iii) A new classification of buds by position in the node (B1 - B4) to avoid misunderstandings with the uneven developmental stages; iv) comparative ABC model to *Arabidopsis* and tomato to allow evolutionary discussions of whorls development in *Coffea* sp. From this, we expect to collaborate directing future studies towards coffee breeding and production, for example, improving floral bud activation, controlling the flowering time, concentrating anthesis to produce more homogeneous ripening fruits, reducing abortion or early flower openings, and increasing fruitification. Moreover, we think that the explored relationship between physiological and molecular approaches during the coffee flowering may be extended to understand other tropical perennial species, especially those with asynchronous flowering behavior, and to understand evolutive aspects in *Rubiaceae.*

Key words: Floral development, *Coffea* sp., Flowering regulatory pathways; phenological cycle regulation; Crop breeding and production.

1. Introduction

Originated in the African continent, more precisely in Ethiopia, the *Coffea* genus includes 103 species belonging to the *Rubiaceae* family [1]. Among these species, *Coffea arabica* L. and *C. canephora* Pierre ex Froehner are commercially important, representing 60% and 40% of the world's coffee production 2018/19, respectively [2]. The coffee chain aggregates one of the most popular commodities worldwide, being estimated that world exports reached about 10.5 billion kg of fruits and the trades move around U$ 26,25 billion [2]. However, recent works suggest that coffee production will be very affected by warming in future predicted scenarios, especially in Brazil, the world's biggest producer [3, 4]. From the biological point of view, the coffee genus presents peculiar characteristics, such as a biannual cycle and uneven flowering and ripening processes, that were very few explored molecularly and could be useful to understand the flowering evolution in crops. Although recent advances regarding the environmental and endogenous factors affecting coffee production were reported [5], these aspects were less explored during the flowering process and a general overview to clarify and direct future studies is essential for coffee crop improvements.

Coffee plants present a biannual cycle, with the vegetative and reproductive phases occurring simultaneously after the first year of flowering [6], and the reproductive development can be divided as follows: development of branches and floral buds (FB) induction, FB development, anthesis, and fruit development [6, 7]. This organization was based on field observations and generalized to different coffee genotypes that present phenotypic differences. However, information at the molecular level of the coffee phenological cycle is scarce, and such information could help to unravel the biocycle and intraspecific differences [8]. For instance, there are genotypes considered to be early and others as late towards the flowering pattern [9]. In addition, this process is reported as being strictly related to a drought period and reduced temperatures (winter season) in the main Brazilian coffee-producing regions, with anthesis taking place as the rainy season, starts [10]. However, the interplay between reproductive development and its connection with environmental cues finds no molecular evidence supporting such an influence or discriminating its relationship with the metabolic and energetic status that could trigger developmental processes.

Flowering is one of the most important developmental processes of the plant life cycle, and inappropriate environmental conditions can negatively affect it, decreasing the number of progenies produced, and thereby, interfering in the plant performance in natural environments for yield and quality [11]. Coffee flowering is an asynchronous process, with this sequential flowering resulting from the asynchronous development of buds, an intrinsic characteristic of

the species. It often leads to uneven fruit maturation, which can greatly affect the final product and coffee cup quality [12, 13].

In coffee-producing areas around the equator, a higher number of flowering events are observed due to the absence of marked periods of water deficit whereas, in areas far from there, dry periods are longer and allow coffee trees to display one main flowering event [14, 10]. Water stress and rehydration are the most influential factors for coffee flowering [15-17]. Although water influences coffee anthesis, other external and internal factors are also involved, such as modifications in temperature and humidity [18], photoperiod [19, 20], plant nutrition status [21], production system (Shade or full sun) [22, 7], altitude [23], water stress deficit [17, 24-26] and phytohormones [27-30].

To facilitate comprehension of these processes we summarized the main changes occurring along the year, with the genes possibly related, and propose a new classification of buds based on their position in the node, which demonstrates the reported uneven floral buds' development. Even though many studies have been developed to better understand the effect of environmental factors affecting coffee flowering, less attention has been directed to the molecular aspects connecting all those processes. Based on transcription profiles, our knowledge until now is that different transcriptional factors seem to be involved in coffee flower development [13], and *in silico* analysis and expression profile showed that the *CaFLC* or *FRIGIDA* homologs might be involved in the coffee flowering regulation [31, 32]. However, due to the difficulties in coffee mutant plants, the function of these genes was not studied yet. Surely, many genes, such as *FT, LFY, SOC, CO,* and others are involved in the coffee flowering process (Fig. 1), as described in other species. Nevertheless, more studies are necessary to link all of these elements. Here, we reviewed the recent advances in the coffee flowering process and connected them to the historical knowledge about this crop to provide a better understanding of the coffee phenological cycle and perspectives to its control in different and challenging environmental scenarios.

2. Photoperiod, Light stimulus, and Shade

Coffee cultivation is carried out under two production systems, under full sun or under agroforestry systems, which depend on the geographic location and the type of agricultural exploitation desired in terms of costs and sustainability [33]. The photosynthesis rates are different in both conditions, the reduced light received under the shade system can directly affect reproductive organs, and flowering is drastically reduced [34]. The solar radiation intercepted by coffee plants during their growth has a direct effect on the development of

tissues, storage of photo assimilates, photosynthesis rate, flowering, and, above all, the distribution of the energy needed for the flowering time and fruit set [35]. In a plantation under full sun conditions, anthesis events are more intense, which has a positive impact on ripening uniformity, although trees present a biennial production pattern [22, 7]. On the other hand, shaded coffee trees tend to flower and produce fruits with the same intensity every year [33], but reductions of up to 20% have been reported in coffee plantations under shade production systems when compared to full sun conditions [36].

The decrease in flowering intensity is due to the lower carbon assimilation rates present under shade conditions, a greater growth stimulus of vegetative organs than the reproductive ones, and a lower number of nodes per branch and buds per node [37]. However, and curiously, larger bean size and a better cup coffee quality has been observed in this shade condition [38]. Physiologically, shading causes changes in the microclimate, especially in temperature and, due to the genetic plasticity of coffee trees, it can affect their anatomy [39] and physiology [40], and influence the process of floral induction, differentiation, and anthesis. The impacts depend on the type, density, duration, and shading time, as well as the prevailing climatic conditions.

The strength of flowering is a function of the monthly light intensity around flowering [34, 41]. Light quality and intensity, photoperiod, or day-length affect the plant growth, directly influencing flower development and many other traits [42]. In coffee, studies related to the photoperiod influence on flowering have found different results. In young and old coffee trees, floral initiation is stimulated by short days and delayed by long ones [27] and, together with phenological observations, coffee has been classified as a short-day plant [20]. However, other researchers have concluded that in equatorial regions, where day-length variation is small, coffee plants are not influenced by day-length and do not have a specific flowering season, as observed in Colombia, Costa Rica, and Central America [43, 44]. Thus, the inductive stimulus of the coffee reproductive cycle is still not clear and may be affected by the interplay of different environmental factors, in which the molecular aspects are poorly explored (Fig. 1).

As regards Brazil, the main coffee-producing regions of the country are characterized by marked seasons. After the March equinox, day length reduces to less than 13 light hours and this reduction acts as a signal for FB development, the transition from vegetative axillary buds to reproductive buds [20]. The perception of this stimulus constitutes the stage of floral evocation (Fig. 2A). Then, morphophysiological changes trigger floral development, enabling the phenotypic distinction between vegetative buds and FBs creating a range of different stages characterized from G1 till G4[6, 7, 16]. During FB development, consecutive transformations over two months (Fig. 2A) promote their growth until the G4 stage (> 4mm) (Fig 2B), the FB

developmental stage responsive to the water stimulus that triggers anthesis (Fig 2B, and Fig. 2C). This coincides with the winter season in the main coffee-producing areas of Brazil (Fig. 2A), where days are shorter and are accompanied by a lack of rain events, with G4 floral buds entering in a latent state. Then, after an increase in the leaf water potential due to rain or irrigation, anthesis is triggered [6, 7, 12].

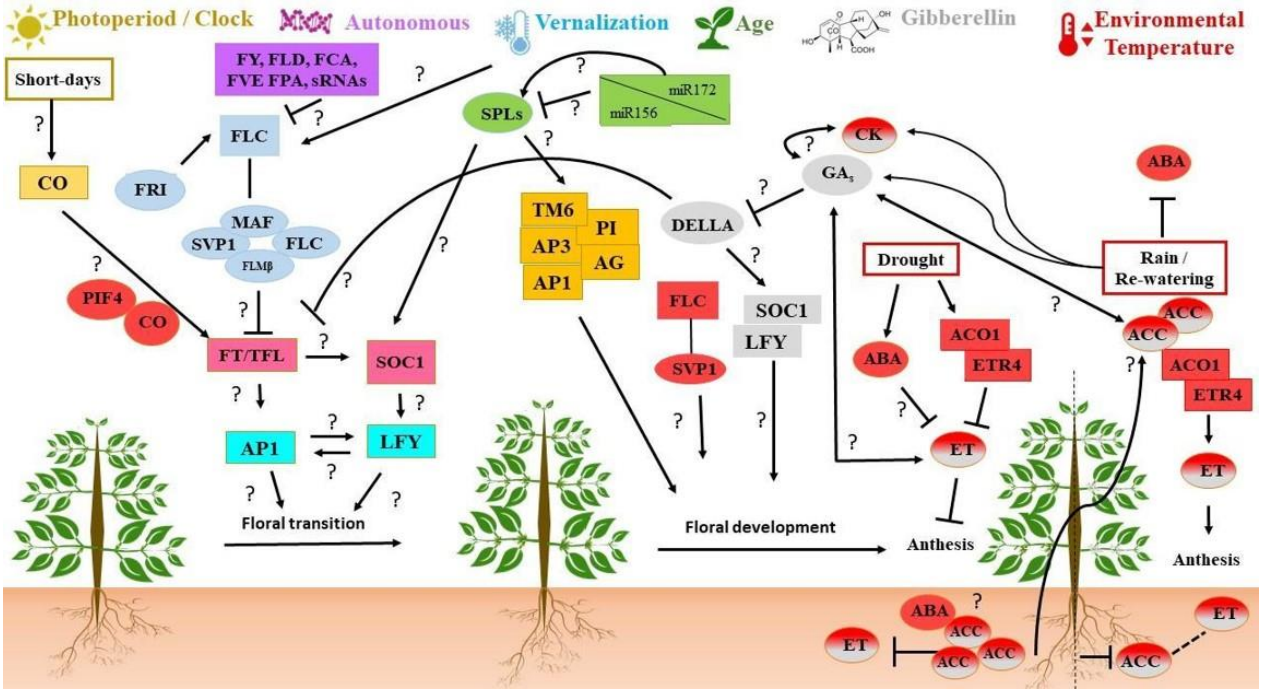

Fig 1. Summary of molecular pathways possibly involved in the flowering time and developmental processes in *Coffea arabica*: photoperiod/clock (yellow), autonomous (purple), vernalization (sky blue), age (green), gibberellin (gray), and environmental temperature (red). Pink boxes represent the main floral integrators genes. Light blue boxes represent the inflorescence meristem identity genes. Orange boxes represent the homeotic genes. Squared boxes represent genes having a pivotal role in the specific pathway. Circles represent several proteins or complexes. Solid lines indicate induction, dashed lines indicate no significant change in the relative amount and, the T end to the arrows indicate decreases. Interrogations indicate connections proposed in this review based on previous studies found in the literature for other species, but not confirmed for coffee. Relationships involving environmental changes associated with water (drought, rain, irrigation, re-watering) are connected in the environmental temperature pathway. The red and gray gradient in the circles indicates a possible interaction between the pathways. ABA: abscisic acid, ACC: 1-aminocyclopropane-1-carboxylic acid, ACO: 1-aminocyclopropane-1-carboxylic acid oxidase *AG: AGAMOUS, AP1: APETALA 1, AP3: APETALA 3*, CK: CYTOKININ, CO: CONSTANS, ET: ETHYLENE, ETR4: ETHYLENE RECEPTOR 4, *FCA: FLOWERING LOCUS CA, FLC: FLOWERING LOCUS C, FLD: FLOWERING LOCUS D, FLM: FLOWERING LOCUS M, FPA: FLOWERING LOCUS PA, FRI: FRIGIDA, FVE: FLOWERING LOCUS VE, FY: FLOWERING LOCUS Y*, GA: GIBBERELLIN, LFY: LEAFY, *MAF: MADS AFFECTING FLOWERING PI: PISTILLATA*, PIF4: Phytochrome Interacting Factor, *SOC1: SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*, SPL: SQUAMOSA PROMOTER-BINDING PROTEIN, sRNAs: SMALL RNAs, SVP: SHORT VEGETATIVE PHASE, TFL: TERMINAL FLOWER, TM6: *TOMATO MADS BOX GENE 6.*

The day length is detected by several components of the circadian clock that transform the inductive photoperiodic signal into chemical and molecular signaling directly associated with gene expression regulation related to flowering [45, 46]. Many studies show that *FLOWERING LOCUS T* (*FT*) promotes the conversion of the Shoot Apical Meristem (SAM) into inflorescence meristems, where floral meristems are originated [47-49]. The *FLOWERING LOCUS T* (*FT*) is expressed in the leaves, and its expression is under the control of the *CONSTANS* (*CO*), whose protein accumulates during the day. FT is translocated to the SAM through the phloem, forming a floral activation complex (FAC) from its interaction with a zip protein FLOWER LOCUS D (FD) and the 14-3-3 protein [50]. This protein complex is responsible for acting as a transcription factor to activate flowering identity genes, such as the *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA1* (*AP1*), and *FRUIT FULL* (*FUL*), which are directly associated with flower organ formation [51, 52].

Morphology changes related to the reproductive development in the SAM and Axillary meristem (AM) are preceded by changes in the transcriptional level and the protein abundance of around 300 genes [53]. Flower Interactive Database is responsible for the meristem architecture, which is characterized by the balance between reproductive and vegetative organ identity [45, 54, 55]. TERMINAL FLOWER 1 (TFL1) plays an antagonistic role with FT in the SAM, where it also interacts with the FD and 14-3-3 to form a floral repressor complex (FRC), responsible for keeping the SAM and AM under vegetative state [56]. The balance between these two proteins, FT and TFL1, known as flowering inducer and flowering repressor respectively, is directly associated with the inflorescence and branch architecture in annual and perennial species [56-58].

This balance is linked with the LEAFY (LFY) and AP1 regulation as shown that AP1 and LFY control *TFL1* expression, while *TFL1* is suppressed by AP1 and promoted by LFY [59]. This behavior might provide the basis for a better understanding of the determination of vegetative buds into reproductive ones in coffee (Fig. 1), as well as into the maintenance of vegetative branch growing since the asynchronous flowering and the development of new vegetative branches in coffee seems to be associated with a balance between vegetative and reproductive identity. LFY and AP1 play a central role in the determination of the SAM from its vegetative to its reproductive form [59] and are directly associated with the transcription regulation of key genes involved in floral identities, such as *TFL1* and *FD* [50, 60]. Although the closest homolog of AP1 was already described in coffee plants, named *CaAP1*, and its expression pattern agrees with its described function in other species [13] the FT/TFL1, FD, LFY, CO, and other homologs related to the circadian clock have not been yet described in coffee.

Studies involving these active hubs during the floral transition are necessary to a comprehensive overview regarding the control of flowering in coffee (Fig. 1) and helping to correlate to the flowering control in other tropical perennial species

3. Temperature: warming and vernalization

Several studies have shown the effect of temperature on coffee development and the overall impact on the flowering time and yield [18, 61-63], including a negative warming scenario, predicted for the next decades [64]. In coffee plants, the climate impacts are even more complex to evaluate because an "optimum" temperature also depends on the phenological and developmental stage of the plant [65]. The adequate mean annual temperature for *C. arabica* cultivars is usually at the range of 18–23 °C [20] whereas for *C. canephora* is from 22 to 26ºC [66, 67], showed that coffee floral initiation occurs when there is a day and night temperature relationship of 23°C/18°C and 18°C/13°C respectively, and flowering decreased when this relation was 33°C/28°C in a photoperiod <12h. In addition, inflorescence development time is also affected under higher temperatures, with 8-10 weeks at 23°C/18°C, 12-24 weeks at 18°C/13°C, and 4-6 weeks at 28°C/23°C, and with malformed inflorescences occurring at 33°C/28°C of day and night temperatures, respectively [18]. High temperatures associated with intense water deficit levels during the beginning of the anthesis period causes pollen tube dehydration and floral atrophy, leading to flower abortion due to their prematurely opening, with the petals remaining small and stiff, forming a star shape floral structure [6].

Associated with the circadian clock genes, the temperature stimulus is directly linked to flowering control, where day length together with TFL and FT transcription and protein accumulation induction controls flowering time in the photoperiod pathway via CO [50, 68]. Warmer temperatures also activate FT but have CO-independent regulation via PHYTOCHROME INTERACTION FACTOR 4 (PIF4), which directly binds and activates FT promoter expression [69]. Although the relationship between high temperatures, PIF4, and flowering is being well studied in *Arabidopsis* [70], in coffee plants this interaction has not yet been analyzed (Fig 1).

In contrast, a greater effort was devoted to the study of the *FLOWERING LOCUS C (FLC)* [31, 13], a gene described as being inductive under long cold days (vernalization) in *Arabidopsis* [71]. FLC is a central player that regulates flowering time in *Arabidopsis* and has a conserved role among species, acting as a key regulator of flowering target genes [72, 73]. This is a MADS-box transcription factor and acts in complex with the SHORT VEGETATIVE PHASE (SVP) [74-76] to suppress the transcription of a set of key genes in the formation of the floral

meristem, including *(FT), SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15 (SPL15), (SOC1)* and *SEPALLATAs (SEP)* [77, 75]. *CaFLC* is significantly expressed in vegetative tissues and this profile is similar for the paralogue's genes *CaSVP-1*, *CaSVP-2*, *CaSVP-3*[13], which might be associated with the function of these genes on vegetative and reproductive development [73, 75, 76]. Considering that *FLC* action is dependent on the formation of a complex with the SVP proteins [74, 76], it makes sense that the expression of *SVP* homologs also occurs during coffee flower bud development. However, *CaSVP-1* expression is higher during the early stages of bud development, a similar pattern to *AP1* responsible for floral meristem differentiation [13, 78] suggesting a functionalization for flowering induction.

In *Arabidopsis*, it was demonstrated that *FLC* is regulated by the vernalization pathway [79], so the prolonged exposure to cold reduces the *FLC* transcription promoting expression of the floral activator genes [80]. Therefore, exposure to winter cold determines the changes at the genetic level to synchronize the *Arabidopsis* reproductive period with warmer temperatures and shorter days during spring [81]. This suggests that the same could be occurring in coffee with CaFLC, however, *CaFLC* presents a variable expression pattern including vegetative and reproductive tissues, especially along with floral development [13] and upregulated in leaves under drought [31], which is the opposite to *Arabidopsis* [80]. At this point, the FLC's behavior is contrary to that expected for its function as a flower transition repressor. Although in citrus (*Poncirus trifoliata*) *FLC* is also positively regulated [82], in our understanding, there are two intriguing questions about *CaFLC*: i) if the decision for floral induction in coffee took place months before (Fig. 2A), why *FLC* transcript levels are expressed during all floral development? ii) why *FLC* is upregulated in leaves under drought which coincides with the cold temperatures of Brazilian winter? As discussed in this review, considering the complexity of coffee phenology (Fig. 1), most likely the divergent behavior of *CaFLC* contrasting to *Arabidopsis* and other cultivated species [76, 83] may reflect an adjustment to delay the coffee anthesis during the dry and cold period in Brazil [6, 12]. Nonetheless, the relationship of *CaFLC* and cold was not established yet in coffee, but it is a promising field to study because modulation of *FLC* transcript levels is interpreted as a key to regulating the progress of floral development programs in different species [80].

Drought has been reported for many years as an essential factor for the coffee flowering process, along with day shortening and temperature reduction (May to August), directly affecting the development of flower buds [6]. However, the absence of water deficit would compromise the final stages of flowering, and interestingly, constantly irrigated coffee trees also flourish [15], which makes the need for the dry period questionable. Our understanding on

this point is that the water deficit is possibly important to help the concentration of flowering, which would involve the action of hormones, such as ethylene and abscisic acid (ABA) (Discussed in item 5 of this review). Furthermore, during the drought period, in association with increasing temperature as the winter ends, there is a tendency for plants to decrease metabolism to delay energy (starch) expenditure under unfavorable conditions [8,84]. It seems plausible to interpret the dry period before anthesis as an adverse environmental condition, especially for the development of flower buds at more advanced stages (G4, Fig 2 C), than a direct positive effect on genes inducing flowering pathways.

Supporting this view, probably the increased expression level of *CaFLC* during drought conditions [31] does not represent a response to seasonal changes in temperature, but it is probably linked to other internal processes that are still unknown and unrelated to flower induction. Recent data support the idea of vernalization-independent regulation of *CaFLC*, in a pathway coordinated by *FRIGIDA* (*CaFLR-like*) [32]. FRIGIDA (FRI) has been shown to control flowering time in *Arabidopsis* (*AtFRI*) by cold-independent activation of *FLC* [85]. The interaction between *AtFLC* and *AtFRI* was related to ovule development in flowers of not vernalized plants [86], and the repression of *FLC* over *SOC1* activates *SEP3*, favoring the development of the late stages of the flower development [87]. *CaFRL-like* transcripts were detected in G4, G5, G6 flower buds and open flowers of coffee plants [32], showing an expression pattern very similar to *CaFLC* [32]. A sophisticated interaction between *CaFRL-like* and *CaFLC* may be taking place in coffee trees, which would justify the *CaFLC* constitutive expression.

Alternatively, it is also possible that the *CaFLC* expression in more advanced stages of flower bud development, and coincidentally during the dry period, is somehow associated with flower bud latency [13], a characteristic mechanism of the floral development of coffee [12]. Latency in coffee flower buds at the G4 stage may be related to the low availability of sugars to meet the energy demand of the more advanced developmental stages of the FBs stages. Following this principle, the dry period during the winter would be important to allow the advancement of buds in early stages (G1, G2, and G3, Fig 2C) until the G4 stage, and *CaFLC* could corroborate the synchronization of FB stages by delaying G4 bud progression.

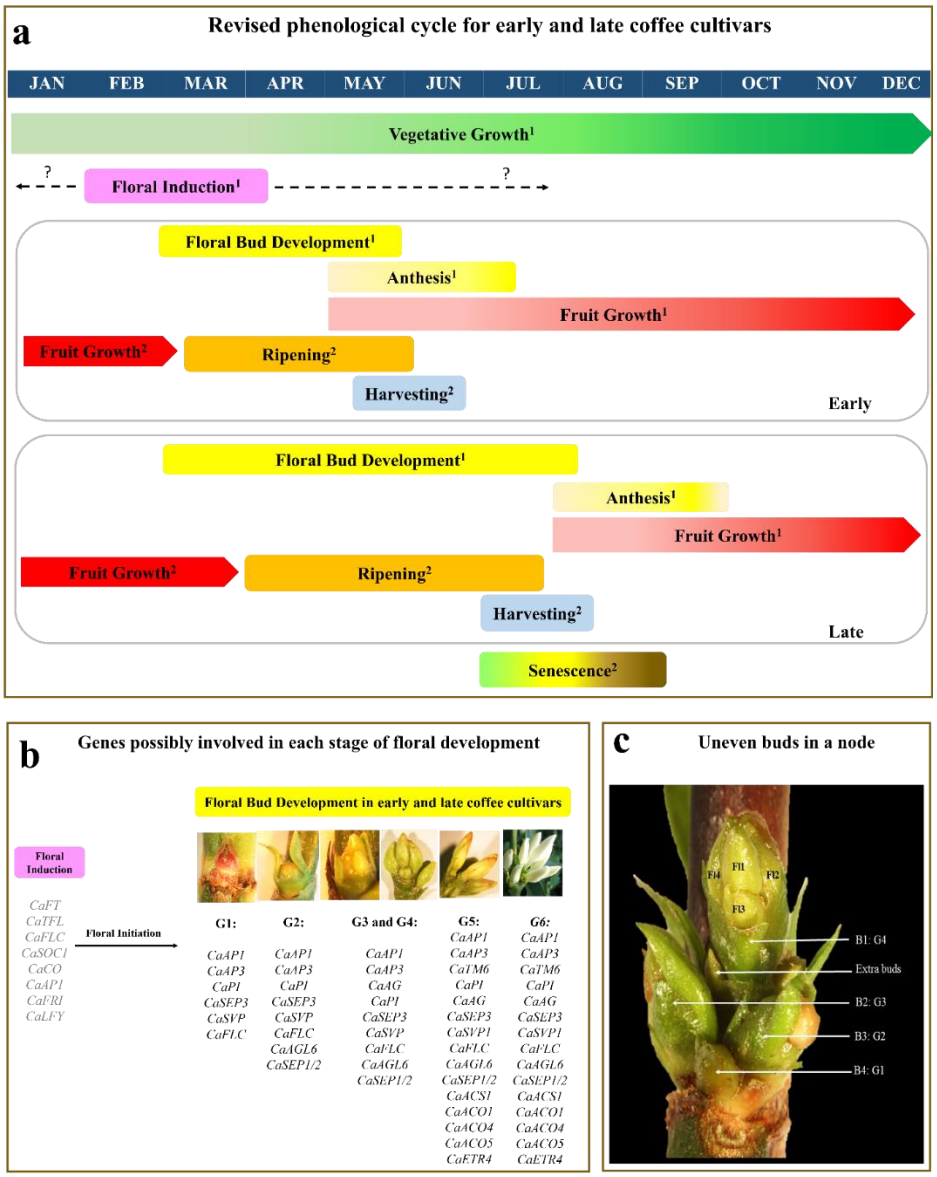

Fig. 2. Proposed model for the induction and floral *Coffea arabica* development under Brazilian environmental conditions. **a)** Revised model for vegetative and reproductive development in *Coffea arabica* cultivars regarding flowering time: early and late cultivars. The intensity of vegetative growth (leaf bud formation) for the two cultivars is shown by the gradient in the color of the green box. Floral induction (pink box) described in the literature between February and April is proposed to start in January, extending until July for early and late cultivars. The period of flower buds development (yellow box) is contrasting between cultivars, lasting 3 months in the early cultivar and 5 months in the late cultivar. Shortening in flower buds' development stages in the early cultivar promotes anticipation of anthesis (the yellow box with gradient: light yellow indicates less intense flowering and dark yellow indicates greater or main flowering). While the late cultivar starts the lesser intensity flowering, the early cultivar advances in fruit growth (red box). Fruit development stages (pinhead, expansion, graining) for both cultivars end at the beginning of the second phenological year, followed by fruit ripening and harvesting. Self-pruning is shown as the progress from senescence to death of tertiary and quaternary branches (box with green to black color gradient). A dashed arrow suggests change at the beginning and end of the respective phase. Interrogation indicates an unproven relationship. Superscript numbers indicate the first (1) or second (2) year in the coffee tree's phenological cycle. **b)** Genes are possibly involved in each stage of floral development in *Coffea arabica*. The floral induction process involves the activation of genes by environmental and endogenous pathways related to the differentiation of the Stem Apical Meristem (SAM) reproductive meristem. Then, flower initiation is initiated by combining

flower organ identity genes related to the specification of whorls in flower buds. In the G5 and G6 stages, genes of the ethylene pathway (*CaACS1*, *CaACO1*, *CaACO4*, *CaACO5*) are related to opening flower progress. Genes in gray represent the connection suggested in this review and genes in black are proposed based on the quantitative and qualitative gene expressions found in the literature [31, 13, 32]. **c)** Photo describing the uneven development of flower buds in a knot located on the branch of a coffee plant. Four flower buds (B1, B2, B3, B4) are shown in ascending order of emergence with the respective identification of flower bud development stages (G4, G3, G2, G1, according to [7]. In floral bud B1, the four flowers (Fl) originating from each floral bud are shown. In general, each node can produce 16 flowers, but in some cases, extra flower buds can be developed, as identified in this photo.

Until now, no role has been defined for *CaFLC* and the expression profiles of *CaFLC* suggest that it is far from being a regulator of coffee flowering. Many pathways remain unexplored and these connections open new avenues for discussion between points of conservation and divergences in regulatory mechanisms for the genetic pathways of flowering angiosperms, especially in perennial tropical woody species. Our understanding of how *FLC* regulates the multigene network of coffee floral induction is still fragmented. New insights are needed to provide a more consistent view of the integration of regulatory signals in this flourishing pathway.

4. Water stress

Coffee plantations are exposed to different climatic conditions around the world [14], with this wide range of environments affecting their growth, development, and production [88]. Water is one of the most limiting factors that coffee plants face during development [89] and it can directly influence coffee developmental processes, such as the time and the number of flowering events [14]. Differences in rainfall distribution led to a higher number of flowering events in coffee plantations close to the equator, whereas in areas relatively distant from this region, dry periods, which display a fundamental role in coffee flowering competence [90], are longer and allow coffee plants to display one main flowering event [14]. Different studies have focused on identifying the adequate levels of leaf water potential required for flower buds to restart their growth after the dry period in a uniform and synchronized manner, without affecting other physiological functions, such as photosynthesis, cell expansion, and leaf growth [15, 24, 28, 91, 92].

Although some studies mention that coffee flower buds enter in a dormancy period, which is broken by rain or irrigation, once they reach the G4 stage [7], no studies are showing the physiological basis of this type of dormancy, thus, the correct term that should be used is “Dormant or latent state”. It is possible that due to the decreased water availability during the dry period, the metabolism of reproductive tissues is reduced and only activated after coffee

trees are re-watered. In this sense, water transport through the xylem would activate physiological mechanisms for coffee anthesis. This supports the importance of water in coffee production sustainability [93]. The rate of opened flowers increased with the reduction of leaf water potential submitting coffee trees, with 60 % of their flower buds at the G4 stage, to predawn leaf water potential (Ψ_{pd}) levels from -0.04 to -2.82 MPa [17]. Besides, though the percentage of gas exchange in leaves was drastically reduced, the yield was not affected, and the proportion of fruits at the cherry stage increased with the reduction of the leaf water potential. On the other hand [91], showed that moderate levels of water deficit, Ψ_{pd} ranging from -0,34 to -0,82 MPa, had little or no effect on flowering synchronization and gas exchange rates. The same results were found by [94], with coffee trees displaying Ψ_{pd} levels from -0,8 to -1,9 MPa. In contrast, water deficit was not able to promote flowering concentration but enabled the saving of water and energy, as well as an increase in yield [95].

Interesting results have been obtained using leaf water potential and irrigation frequency, Ψ_{pd} from -1.2 to -1.6 MPa were sufficient to promote flowering synchronization, positively affecting uniformity and production rates in coffee, whereas a higher number of flowering events plants continuously irrigated suggest the importance of a period of water stress for a greater synchronization of coffee flowering [96].

The effect of the water stress and irrigation in coffee anthesis were demonstrated by Miranda et al., 2020, using the arabica cultivars "Catuai" and "Bourbon" grown under different irrigation treatments. The authors concluded that the suspension of irrigation promoted flowering synchronization, and consequently, a better ripening uniformity of the fruits for both cultivars. Similar results were found by [97], high soil moisture depletion followed with increased irrigation levels resulted in an increased number of flowers and the subsequent number of berries per bunch at given assessment. Irrigation caused significant effects on biometric parameters on coffee plant development, especially on the root system, stem, and subsequently in the number of branches and flower buds. This is reflected in higher yield levels (more than 50 %) in the treatments with irrigation [98]. Similarly, the application of irrigation in coffee trees can promote vegetative growth, thus mitigating the fluctuation of biennial production and then improving the sustainability of coffee production [93].

When comparing flowering intensity between irrigated and non-irrigated plants, both treatments differed only in anthesis time, which was earlier in the irrigated treatments [99, 100]. Water stress did not affect the timing of floral initiation, which occurred only when the photoperiod was lower than 12 h [16]. For the cultivars “Catuai Vermelho" and “Mundo Novo”, water stress (Ψ_{pd} = -2.5 MPa) significantly reduced the number of inflorescences, compared

with well-watered plants (Ψ_{pd} = -0.5 MPa), indicating the need for irrigation during floral initiation. Leaf water potential is associated with other environmental parameters, such as Evapotranspiration (ETp). In a parametrized agrometeorological model, it was determined that ETp values of 335 mm, Growing Degree Days (GDD) of 1579, and 7 mm of rainfall, are adequate parameters to indicate the period of the maximum flowering in coffee trees [101]. Floral initiation under Ψ_{pd} = -2.5 MPa was reduced significantly in the number of inflorescences, compared with well-watered (Ψ_{pd} from -0,5 to -1,5 MPa), and these reductions were associated with leaf shedding in stressed plants [16].

This suggests that regular irrigation can help in the period of coffee floral initiation for flowering synchronization. A similar finding was observed by [15], with frequent irrigation precluding flowering and subsequently controlled water deficit and re-irrigation to stimulate flowering. Flowering is usually observed in twelve to fifteen days after rain or irrigation of water-stressed plants [15, 24, 26, 97]. However, as far as we know, there is no molecular evidence demonstrating that water is directly activating genes related to the final stages of floral development and anthesis. Thus, we could also speculate that it is not water that activates coffee flowering, but its absence that reduces plant metabolism and consequently floral growth. Supporting this, early flowering is observed when water availability is not restricted or when unforeseen rains occur [24]. Anyway, the role of water activating flowering is rarely explored at the molecular level and the transcriptional targets were not described yet.

5. Hormonal regulation

The development of coffee flower buds in the drought period that precedes anthesis is associated with one or more significant rainfall events [34]. During this drought period, coffee flower buds stop growing, after reaching a size of 4 mm. At this point, flower buds are physiologically fully formed and the “dormant or latent state” is associated with high levels of Abscisic Acid (ABA) [34, 102] and rainfall causes FB growth resumption, which is associated with increased levels of gibberellic acid (GA) [27, 28].

Exogenous GA does not increase the number of flower buds coffee [103] but it anticipates anthesis, regardless of rain or irrigation, promoting fruit ripening synchronization [27]. In flower buds where the “dormant or latent state” was not completely broken by water stress, GA3 application stimulated anthesis, possibly by compensating a lack of endogenous GA [28], improving flowering synchronization in the cultivars "Typica" and "Yellow Catuai"[30]. After rain or irrigation, GA levels increase rapidly in the buds and gradually decrease with the expansion of the floral organ, whereas its levels in the xylem remain stable [103]. Similarly, after

rehydration, cytokinin levels increase in both and xylem, suggesting a synergistic effect with GA in the coffee flower buds development and anthesis [104]. Therefore, GAs can regulate coffee flower bud development in more advanced stages. Despite that, the genetic factors related to the gibberellin pathway in coffee are still not clear and the molecular mechanisms evidencing a probable regulation in this pathway to control flowering time are scarce.

Gibberellin-mediated flowering control is a hormonal regulation pathway with the effective participation of ethylene. In Arabidopsis, an increased level of ethylene induces the GAs degradation by the 26S proteasome, promoting the accumulation of DELLA protein and thus delaying flowering [105]. DELLA proteins have been shown to act as negative flowering regulators and their increased levels are associated with floral opening delay [106-108]. The DELLA-GA interaction is inversely proportional and in the absence of GA, these proteins repress the LFY and SOC1 transcription involved in the signaling to activate floral identity genes [105, 109].

Another hormone that seems to influence coffee floral development is ethylene [90] that presents divergent effects among species and is dependent on its concentration [105, 110]. To modulate the different processes in the plant's life cycle including responses during water stress [111], ethylene binds to its receptors and triggers a signaling cascade that ends with the activation of different transcription factors [112]. Previous studies suggest that coffee ethylene receptors form a multigene family in coffee [113]. These genes are regulated by the synthetic plant growth regulator 1-Methycyclopropene (1-MCP), a potent antagonist of the ethylene action [114]. Independently but dependent on the plant water condition and tissue-specific, the adjustment in the homolog's gene expression is crucial for endogenous control of ethylene levels in coffee [90]. 1-MCP regulates the gene expression in the ethylene biosynthesis and signaling pathways for leaves and flower buds in coffee, promoting synchronized anthesis during drought in coffee plants under natural conditions [90].

Moreover, the 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) enzymes integrate the ethylene biosynthesis pathway [115] had in Coffea arabica four genes identified: CaACS1, CaACO1, CaACO4, CaACO5[113]. The 1-MCP mechanism for coffee anthesis induction is still unclear, but a loss of regulation through feedback has been suggested to act on the ethylene biosynthesis pathway. This hypothesis was suggested because 1-MCP increased the expression of CaACS1-like, CaACO1-like, CaACO4-like, CaACO5-like, and reduced the gene receptor expression CaETR4-like [90]. The ethylene perception pathway is negatively regulated [116] so the reduction in the gene receptor expression increases the sensitivity to ethylene [117, 118]. In this sense,

adjustments in the gene receptor expression promoted by 1-MCP may have a significant contribution to anthesis through the adjustment of ethylene sensitivity in coffee flowering [90].

Considering that a dry period followed by rehydration is considered an important stimulus of coffee anthesis [12], it has been suggested that the floral opening is triggered by a signal produced in the roots during the dry season and activated after plant rehydration [15]. During the water deficit period, biosynthesis and flow of some plant hormones are altered, triggering important responses in plants under stress conditions [119]. The expression of CaACO1-like in leaves and roots is repressed by the water status in the soil (Ψ leaf from -2.3 and -4.0 MPa) but does not alter the gene profile expression of CaACO4-like and CaACS1-like [90]. The ACO enzyme may seem to play an important role in the coffee ethylene biosynthesis regulation, considering that the negative regulation of ACO homologs possibly reduces the ACC conversion rate, favoring the accumulation of this precursor [90]. The ethylene production in roots did not fluctuate significantly during the dry to wet seasons. However, ethylene levels in flower buds were higher during the dry period, before anthesis, and rehydration after precipitation promoted significant changes in the levels of it in leaves and branches [90]. Dehydration cycles followed by rehydration have already been linked to the differential ethylene gene expression, resulting in increased production of this hormone and the promotion of anthesis [110]. Therefore, under natural conditions, the coffee flower opening time regulation is associated with changes in the leaf water potential and gene expression in the ethylene biosynthesis and signaling pathways [90].

The model describing anthesis in coffee considering changes in the plant water potential associated with the effects of ethylene and 1-MCP suggests an interaction with ABA [90]. ABA has an antagonist effect on ethylene [120, 121], and due to the soil dry status, it was proposed that increased levels of ABA during the dry period may inhibit ethylene production in roots and shoots. In general, the ABA-ethylene antagonist relationship seems to be beneficial for coffee flowering, favoring the ACC accumulation and concentrated ethylene production in flower buds after rehydration [90].

Studies involving the effects of 1-MCP on coffee receptors and the ACC as a signaling molecule in an independent pathway on the metabolic network of coffee flowering are ongoing. Together, these data will be helpful to elucidate the hormonal crosstalk and connect flowering mechanisms in coffee plants and other perennial woody species. Additional investigations using a molecular approach are necessary to highlight the role of GAs as a protagonist in an independent pathway in the floral transition and its connection with flowering genes regulating.

6. Floral organs development in coffee plants

The transition of meristems to a reproductive state in coffee is regulated by a complex regulatory network, in which transcription factors, especially Flowering Locus T (FT), interact with environmental and endogenous cues to determine the coffee flowering time (Figs. 1 and 2). After this transition, the floral meristems arise and the floral organogenesis is initiated being coordinated by other transcription factors, such as APETALA1 and LEAFY [122, 123]. The core regulatory mechanisms are at some level conserved in angiosperms, but it has also diversified throughout gene duplications and functionalization changing phenotypic characteristics and expanding competitive strategies among species [124]. For example, the coffee flower is anatomically complete with five distinct whorls that develop centripetally, first sepals, followed by petals, stamens, and carpels.

This pattern of development is similar to model plants as Arabidopsis thaliana and tomato, but phenotypical differences are also observed as the biannual developmental cycle, epipetalous stamens, and secretory structures named colleters [8]. Several studies were dedicated to understanding floral diversification[125-127], however, this was poorly explored in the Coffea genus (*Rubiaceae*), which would clarify many biological aspects, such as i) floral development in perennial crops; ii) evolutionary changes related to new organs formation; iii) how to control the uneven bud development and flowering; iv) how to provide male sterility for breeding; v) how to decrease early openings of flowers and the high level of abortion after anthesis.

Key genes related to the development of the floral organs were described through mutant analysis, especially in A. thaliana, which gave rise to the classical ABC model that explains flower architecture [126, 128-130]. In this model, homeotic genes are grouped as functional factors that act in combinatorial protein-protein interactions to form tetrameric complexes that specify the identity of the four floral whorls. In detail, the A-function are encoded by APETALA1 and 2 (AP1 and 2), the B-function by APETALA3 (AP3) and PISTILLATA (PI), and the C-function by AGAMOUS (AG) and their single or combined action in each whorl results in the specification of floral organs: A in the first whorl specify sepals, whereas A+B in the second whorl determines the petals, B+C in the third whorl leads to the formation of the stamens and, finally, C in the fourth and most internal whorl specify the carpels. Subsequently, it was demonstrated that the SEPALLATAs (SEP1, 2, 3, and 4) act as connectors joining the ABC proteins throughout the K-box domain [131], adding a new factor named E-function and expanding the model to ABCE [132].

These cited genes are master regulators of transcriptional cascades that coordinate whorls development [133,134] and, except for AP2, all of them belong to the MADS-box family of

transcription factors, which is characterized by a MADS domain of 59 amino acids highly conserved [135]. This gene family displays a variable number of members in different species [136], which revealed an important evolutionary aspect of diversification, specially studied for ABC-function homologs [132, 137]. Based on that, the diversification of the MADS-box family, represented by changes in the gene sequence, expression profile, and functions, could explain the morphological variability of flowers, which has been associated with the relatively quick adaptation of Angiosperms to contrasting environments [138]. Despite its importance and the availability of coffee genomes [139, 140], few studies explored the MADS-box genes existing in Coffea sp. and their expression during development [31, 13, 32].

From a transcriptome database [141] followed by clone resequencing, 23 MADS-box full-length unigenes were identified in *C. arabica* by [13] and classified into the described Arabidopsis subfamilies [142]. Interestingly, all MADS proteins found in coffee belong to type II (or MICK proteins) and the lack of type I members is suggested to be due to a low expression level or expression in very specific tissues and developmental stages [143]. The expression profile from 18 of these coffee MADS-box genes showed that the majority of these genes are expressed in both vegetative and reproductive tissues, with six genes being expressed exclusively in vegetative tissues [13]. In the same work, the closest relative homologs for the ABCE-function genes (CaAP1, CaAP3, CaPI, CaAG, CaSEP1/2, and CaSEP3) were also determined, along with their Spatio-temporal expression profiles. For instance, CaAP1 (A-function) and CaAP3 (B-function) have similar expression patterns, with higher expression levels, which were restricted to sepals and only later in petals, in the first stages of floral development. In contrast, CaPI (B-function), CaAG (C-function), and CaSEP3 (E-function) are highly expressed at late stages of floral development, with the expression of the two first genes being restricted to stamens and carpels.

The described expression patterns of ABC-function genes in Coffea sp. are following the conserved function of these genes, in which the B and C-function genes guarantee the internal reproductive primordia cells [132, 144] and compete with A-function genes that, by its turn, reserve the external layer of cells to promote infertile organs formation of the perianth [126]. However, there are notable differences from the ABC model described in other species, suggesting functionalization of homeotic genes and/or participation of other genes to discriminate the perianth and reproductive organs in coffee flowers. Based on that, we reviewed the published expression profiles of coffee MADS-box genes to suggest their roles during reproductive development (Fig. 1) and propose an ABC model in Coffea sp. comparing it to Arabidopsis and a relatively closer species to coffee, tomato (*Solanum Lycopersicum*) (Fig. 3).

RNA and proteins are mobile signals that can act in different places than where they were produced [145], which could be a criticism of our proposal, however, it was already described that the expression of ABC genes follows the localization of their correspondent protein function [146].

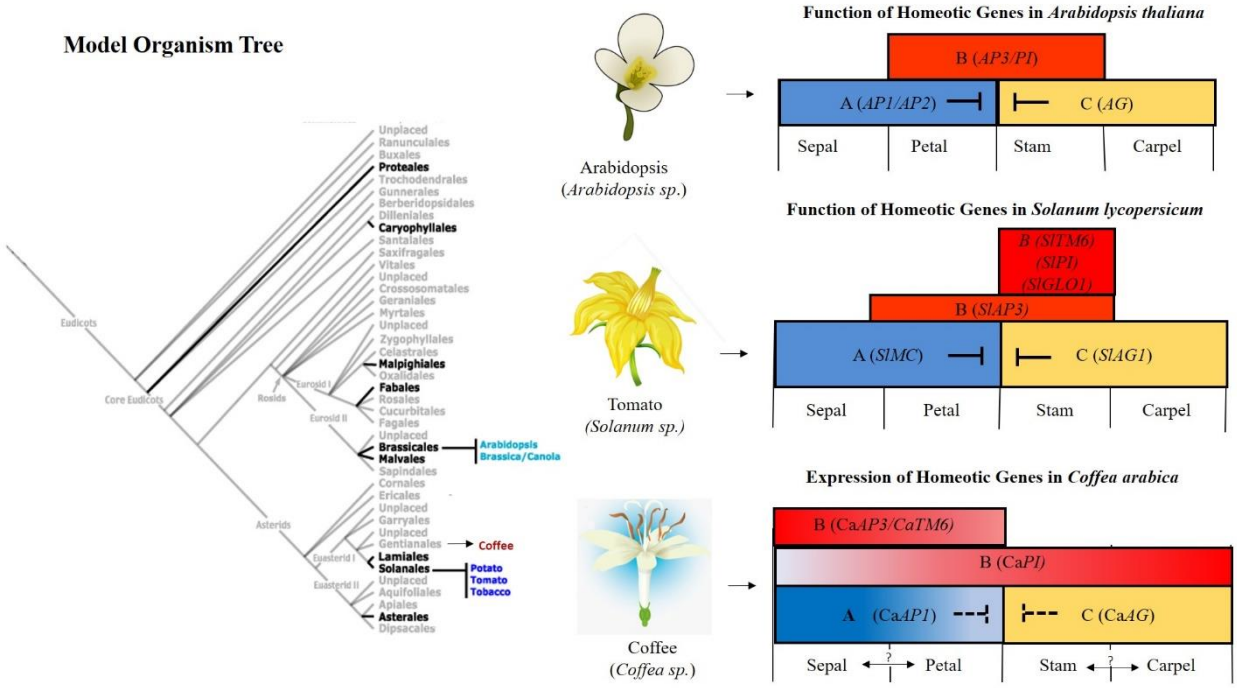

Fig. 3 Proposed ABC model for floral organ identity in *Coffea arabica*. On the left, phylogenetic classification of eudicots orders highlighting the model organisms (adapted from [147], which shows the relationship among three species compared: *Arabidopsis thaliana*, *Coffea arabica*, and *Solanum Lycopersicum*. A schematic representation of the respective flowers of the compared species is shown in the center. On the right, variations of the ABC model explaining floral organs differentiation are represented. The ABC model of *Arabidopsis thaliana* guides the investigation of orthologous homeotic genes in flowering species, in which the variation in the ABC model may occur due to cumulative differences along evolutionary time in sequences, expression patterns, and/or combination of these genes for the specification of the four floral whorls. The ABC model for *Arabidopsis thaliana* and *Solanum Lycopersicum* was built considering the functional analysis in mutant plants for specification floral organs [148-151]. For *Coffea arabica*, the ABC model is proposed based on the quantitative and qualitative gene expression (de Oliveira et al., 2014). The gradient color indicates the highest, lowest, or absent (white) expression of class A, B, and C genes in the initial, intermediate and final developmental phases of each floral whorl.

The floral development program is widely studied in model plants as *A.thaliana* and tomato, a model for fleshy fruit plants[148, 150-153]. Class A and C homeotic genes are highly conserved among these organisms, also sharing an antagonism in their functions to specify whorls (Fig. 3). *MACROCALYX (MC)* of tomato is the closest homolog of *AP1*[151] showing a similar function, with their mutants completely converting the development of the sepals into leaf-like structures and petals not being specified[151, 153]. Regarding the. Whereas it has been observed for C-function redundant expression patterns and functions among their

representatives *SHATTERPROOF1 (SHP1)* and *AG* in *Arabidopsis*[154, 155], which is similarly reported for the respective tomato closest homologs *SlAGL1* and *SlAG1*[154, 156]. Comparing to coffee plants, the *CaAP1* (A-function) is expressed in the perianth, far from *CaAG* (C-function), whose expression is restricted to the primordia of stamens and carpels[13]. This is consistent with the conserved expression of C-function genes and its ancestral function related to the internal fertile organs development in other species, including gymnosperms[144, 157-159]. This spatial separation in the expression of these genes is in agreement with their antagonistic action and competition for layers between A- and C-function genes[109], indicating that the A and C-factors in *Coffea sp.* keep functional similarities to *Arabidopsis* and tomato homologs (Fig. 3).

Nevertheless, an interesting characteristic of *CaAP1* expression is its expression only at late stages of petals development (gradient color in Fig. 3), after the arisen and separation of stamen primordia cells, suggesting a late A-function to provide fertile epipetalous stamens at the final of the flower developmental process[13]. Thus, because *CaAP1* is expressed in both primordia of sepals and petals, another homeotic gene seems to provide identity for coffee petals. *FRUITFULL* (*FUL*) is a close homolog of *AP1*, classified in the same SQUA subclade and known to display a redundant function since it is involved in the floral development activation and perianth formation[142, 160]. The putative ortholog of *FUL* was described in *C. arabica* (*CaFUL*) and its expression is higher in flowers than in fruits[13], suggesting that it could be involved in petal identity. Other candidates genes for coffee petals specification are *CaAGL6* and *CaSEPs* that make part of the conserved superclade *SEP*/*AGL6*/*AP1* and may perform similar functions to *AP1*[161-163]. *CaAGL6* and *CaSEP3* are exclusively expressed in flowers and fruits, whereas *CaSEP1/2* is expressed also in leaves and SAM[13], thus, it is possible that some functionalization has occurred in *Coffea sp*. Overall, these data suggest functional diversification of the *SEP*/*AGL6*/*AP1* subfamily during coffee evolution and, accordingly, the A-function is very diverse and arguably in angiosperms[164]. Functional studies of these genes is a promising field since it could be used to change the flowering time or to enhance floral activation of latent meristems providing more fruits, as well as, to explain the abnormal formation of petals and the premature opening, the “starlet flowers” phenomenon[165], that appears to be induced by warming conditions[6, 166, 167].

B-functions genes, represented by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis*, are related to the formation of petals and stamens and, accordingly, are reported that AP3 and PI interact with each other forming functional heterodimers in the second and third whorls[168]. Surprisingly, in coffee plants, *CaAP3* was expressed in sepals but not in stamens, with expression levels increasing progressively along with floral development. On the

other hand, *CaPI* expression was found in petals and stamens[13], in agreement with its conserved function and expression in *Arabidopsis* and tomato (Fig. 3), but also colleters. These results do not explain how stamens and carpels are differentiated in coffee plants since these organs show similar expression patterns of *CaPI* and *CaAG* (Fig. 3; and [13]. Duplication events are reported for the *DEF/AP3* subclade, which originated the divergent paralogous lineage *TOMATO MADS BOX GENE6* (*TM6*) [169]; related to functional diversification out of Brassicaceae[168, 170]. For example, *euAP3* and *PI* of Arabidopsis and its respective orthologs in tomato, *SlGLO1*[171] and *SlPI* [152] control the specification of petals and stamens in both species[172].

However, the silencing of these two genes in tomatoes does not affect the identity of the petals[148] and the complete differentiation of stamens requires the action of *TM6*, a sub-functionalization that is not present in *Arabidopsis*[152]. Similarly, this duplication event and the closest *CaTM6* homolog was reported in *C. arabica* with its expression being found exclusively in reproductive tissues and at higher levels at the final stages of floral development[13]. However, *CaTM6* expression was detected in the perianth, a similar pattern of *CaAP1*[13], suggesting a more complex regulation for coffee whorls differentiation and epipetalous stamens (Fig. 3).

Based on that, compensatory changes in B-function genes may have occurred in the *Rubiaceae* family with the participation of other players, non-described MADS-box genes, and/or miRNAs, as suggested for *Solanaceae*[148], which could result in functional evolution and novel structures found in coffee flowers.

7. Future perspectives

Considering the ever-growing demand for higher quality coffees and the threat of climate change, efforts should be urgently taken to better understand the coffee flowering process at the molecular level, since it can directly affect coffee yield and cup quality. As shown in this review, advances in the hormonal regulation of coffee flowering, particularly on ACC molecules and ethylene´s involvement during anthesis, reveal a new avenue for better comprehending the flowering process, as well as, manipulating it through the use of growth regulators. With the availability of coffee genomes and RNAseq global transcriptomics; which allows comparisons between different tissues, developmental stages, and growth conditions a better picture of the molecular regulation of coffee phenological cycle and floral development also starts to take place. Future studies should be dedicated to identifying all coffee MADS-box

genes, as well as, other possible players involved in floral development and whorls specification, along with their functional analysis using model species, easier to be transformed and manipulated. These approaches will certainly improve the knowledge towards the flowering process in such an important species that is *Coffea* sp. Through existing results from coffee reproductive development and possible path related to this behavior shown here, it will contribute to the next research regarding the understanding of coffee flowering behavior, also, other perennials with asynchronous flowering. The combination of environmental and endogenous cues related during this review associated with the coffee flowering control will clarify to researchers and farmers about exploring new techniques to better manipulate the coffee flowering induction and anthesis, and improve the final product quality.

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CHAPTER 3

ARTICLE 2- CROSSTALK BETWEEN ETHYLENE AND ABA DURING CHANGES IN SOIL WATER CONTENT REVEALS A NEW ROLE FOR ACC IN COFFEE ANTHESIS REGULATION

(DRAFT VERSION)

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Crosstalk between Ethylene and ABA during changes in soil water content reveals a new role for ACC in coffee anthesis regulation

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Abstract

Coffee flowering is regulated by endogenous and environmental stimulus and occurs once plants are rehydrated after a period of water deficit period. In this study, the effect of exogenous application of 1-aminocyclopropane-1-carboxylic acid (ACC) as a modulator in coffee anthesis was evaluated. Also, the evolution of Abscisic Acid (ABA), ethylene, ACC content, ACC oxidase (ACO) activity, and expression analysis of the *LHT1* (Lysine Histidine Transporter 1) transporter, in roots, leaves and, flower buds from coffee genotypes (*Coffea arabica* L. cv Oeiras, Acauã, and *Semperflorens*) cultivated under field conditions were evaluated. These analyses were carried out at three different times: at the end of the rainy period (May 18th), dry period (August 18th), and re-watering period (August 28th). Leaf and bud ABA content increased during the dry period in each coffee genotype, decreasing in Oeiras and *Semperflorens* and increasing in both tissues for Acauã in the re-watering period. Ethylene levels decreased from the rainy to re-watering period, similar to the ACO activity reduction observed in the same period. Remarkably, ACC increased in all tissues from the three coffee genotypes in the re-watering period just before anthesis. In a second experiment, covering plants under field conditions confirmed the importance of water in the coffee anthesis process, as the same pattern of the hormone evolution was observed when compared to the first experiment. A third field experiment of exogenous application of ACC confirmed the modulation of this non-protein amino acid in coffee anthesis since treatments with ACC showed a higher number of flower buds in the G6 stage. Finally, *LHT1* expression was higher in roots and flower buds in the re-watering period and no differences were found in leaves for field experiments I and II. An opposite ABA-ACO-ethylene interaction in coffee flower buds was observed, favoring the ACC level to be increased, and an intercellular ACC transport among leaves, buds, and roots is involved as a modulator in coffee anthesis.

Keywords: Flowering, *LHT1* Transporter, ACO, Acauã, Oerias, *Semperflorens*, ABA, Ethylene

1. INTRODUCTION

Worldwide, the production of most crops depends on flowering, and particularly in some species, such as coffee (*Coffea arabica* L.), this process can directly influence the quality of the final product (cup quality). Coffee asynchronous flower bud development leads to uneven flowering (De Oliveira *et al*., 2014) and, depending on the geographical location of the plantation, it can result in various flowering events. Flowering is an important step of the coffee reproductive phase, being influenced by different endogenous and environmental signals. In this process, one of the most important elements is water as a signaling transporter, as it has been shown that coffee anthesis occurs after rain events preceded by a period of moderate water stress (Drinnan & Menzel, 1994). Other factors, such as photoperiod, temperature, shade conditions, plant nutritional status, and phytohormones can also affect floral transition and plant development (De Camargo, 1985; Ramírez *et al*., 2010).

Information on the involvement of plant hormones in coffee flowering regulation is scarce. Previous studies have shown that Abscisic Acid (ABA) increases during the dry period and is associated with coffee flower bud dormant or "latent state", having plant rehydration decreasing ABA and increasing Gibberellin (GA) levels (Cueto & Dathe, 1986). After water withdrawing stress, dormant and non-dormant G4 flower buds (ranging from 3.2 to 6 mm in length) (Morais *et al*., 2008) could be distinguished based on their ethylene evolution (Schuch *et al*., 1992). In other species, changes in ethylene levels can either delay or promote flowering, as observed in Rice (*Oryza sativa* L.) (Wang *et al*., 2013), Arabidopsis (*Arabidopsis thaliana*) (Achard *et al*., 2007), Pineapple (A*nanas comusus* L.) (Trusov & Botella, 2006), and Roses (Meng *et al*., 2014).

The relationship between ABA and ethylene in the regulation of different biological processes is well known. Similar to ethylene, ABA accumulation accelerates the senescence of cut flowers and flowering in potted plants (Liu *et al*., 2014). Exogenous applications of ABA in Roses (*Rosa hybrida* L.) increased the ethylene sensitivity as well as the expression of some ethylene receptors (Müller *et al*., 2000). In hibiscus (*Hibiscus rosa-sinencis* L.), ABA negatively regulates the expression of the ethylene biosynthesis genes during flower development (Trivellini *et al*., 2011). On the other hand, the exogenous application of ABA positively regulates ethylene biosynthesis, increasing the ACC content in the abscission zone of the Lupine flower (*Lupinus luteus* L.) (Wilmowicz *et al*., 2016).

Recently, it was proposed that ethylene can play an important role in coffee flowering, since rehydrating droughted plants can increase ethylene levels and ethylene sensitivity, regulating coffee anthesis (Lima *et al*., 2020). Ethylene is a volatile compound that can be easily

transported by diffusion throughout cells and intercellular spaces (Alonso & Ecker, 2001). Ethylene is produced from the amino acid methionine, which is first converted into S-adenosylmethionine (S-AdoMet) through the S-AdoMet synthetase enzyme, and subsequently, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) converts S-AdoMet into 1-Aminocyclopropane-1-Carboxylic Acid (ACC), that is considered the limiting step of the ethylene biosynthesis pathway. Finally, ACC oxidase (ACO) generates ethylene by oxidizing ACC (Yang & Hoffman, 1984).

The role of ACC, as a signaling molecule independent of ethylene biosynthesis, has been reviewed, showing that ACC is part of many biological processes in plants such as in cell wall metabolism, vegetative development, stomatal development, and pollen tube attraction (De Poel & Van Der Straeten, 2014; Polko & Kieber, 2019; Vanderstraeten & Van Der Straeten, 2017; Mou *et al*., 2020). In addition, it was also found that ACC has a distinct function than ethylene in the non-seed plant *Marchantia polymorpha* L. (Katayose *et al*., 2021; Li *et al*., 2020), and in the induction of sexual reproduction of the Marine red alga (*Pyropia yezoensis)* (Rodophyta) (Uji *et al*., 2020).

These different biological functions attributed to ACC seem to have originated an evolutionarily conserved signal that predates its efficient conversion to ethylene in higher plants (Li *et al*., 2020). ACC transport throughout the plant occurs by two amino acid transporters, LHT1 and LHT2 (Choi *et al*., 2019; Shin *et al*., 2015), having the *LHT1* higher activity described in root tissues and flower development (Mou *et al*., 2020). In light of this knowledge, research on the role of ACC associated with the development and growth of plants should be expanded, including the reconsideration of physiological processes primarily attributed to ethylene.

The main objective of this research was to evaluate the modulation activity of ACC in the coffee flowering process quantifying the evolution of ABA, ACO, ACC, and ethylene in the most critical development period for coffee flower buds, including flower bud development, bud dormancy, and anthesis; and the *LHT1* gene expression evaluation as an ACC intracellular transporter. It is possible that ACC, in addition, to be the ethylene precursor, may be involved in the regulation of anthesis and fertilization of the coffee flower. To corroborate this hypothesis, field experiments were carried out complemented with laboratory analysis.

2. MATERIALS AND METHODS

To elucidate the modulation of ACC in coffee anthesis, three field experiments were designed. In each experiment, we sought to answer three complementary hypotheses related to hormonal crosstalk, the role of water, and the response of coffee plants in the exogenous application of ACC in the coffee anthesis process (Supporting information Table S1).

2.1 Field experiment I

To evaluate the evolution of phytohormones (ABA, Ethylene), intermediaries in the ethylene biosynthesis pathway (ACC and ACO), and relative expression of *LHT1* gene in coffee flowering development, three coffee genotypes (*Coffea arabica* L.) were selected: Oeiras, Acauã, and *Semperflorens*, classified as early, late and continuous, respectively, regarding their flowering and fruit ripening pattern. The experiment was conducted in a five-year-old coffee plantation at the Department of Agriculture of The Federal University of Lavras (UFLA) following a randomized block design with three biologicals repetitions, each one comprising ten plants. The experiment was carried out from May to August 2020, with samplings happening at the end of the rainy period (May 18th, late fall), end of dry period (-August 18th, winter), and re-watering period (August 28th, late winter, 10 days after the first rain event). For each period, sampling was comprising of roots (15 to 25 cm deep into the soil), leaves (young and fully expanded at the third or fourth node from plagiotropic branches), and flower buds (G2 buds with a broad and flat apex, G3 buds up to 3 mm in length, and G4 buds ranging from 3.2 to 6 mm in length) (Morais *et al*., 2008), taking place from 8:00 till 10:00 am. Samples were immediately frozen in liquid nitrogen and stored at -80 °C to be evaluated for ACC and ABA content, ACO activity, and *LHT1* expression analysis. For the ethylene analysis, leaves, buds, and root samples were collected in glass tubes. Plant water status was assessed by measuring predawn leaf water potential (from 03:30 till 05:30) using a Scholander-type pressure chamber (Fig. 1A). Both quantifications were made according to Lima *et al*. (2020).

2.2 Field Experiment II

Aiming to evaluate the effect of rain and hormonal balance in coffee flowering corroborating the results of the field experiment I, a second experiment was conducted in a three-year-old coffee (*Coffea arabica* cv. Paraiso 2) plantation. The experiment consisted of the evaluation of two treatments, plants under normal field conditions (Uncovered) and plants under rainfall exclusion (Covered), with three biologicals repetitions, each one composed of 10 plants in each treatment. Rainfall exclusion was achieved by the installation of translucent nylon

(polypropylene) fixed on twelve 3.2 m high eucalyptus wood logs, deep 0.60 m in the soil with three wood logs 3 m distant from each other. Every eucalyptus wood log was arranged on each posterior side and in the middle of the coffee line. A translucent nylon piece of 0,2 micrometers was fixed on top of wood logs using a wooden piece of 5x7 cm with a small hobnail, covering a total of 81 m^2 of rain exclusion area (Fig. S1). Leaf water potential measurement (Fig. 1B) and tissue sampling for the evaluation of Ethylene, ACC and ABA content, ACO activity, and *LHT1* expression analysis were performed similarly to field experiment I.

2.3 Field experiment III

To better understand the effect of ACC modulating coffee anthesis, exogenous treatments of ACC were evaluated. The experiment was carried out in the coffee germplasm collection of the experimental area of the Department of Agriculture (UFLA), on adult, four-year-old coffee cultivar (*Coffea arabica cv. Semperflorens*) trees. The experiment was conducted in a randomized design with four treatments (One plant per treatment) and ten replicates per treatment (Ten branches per plant) with 5-6 nodes per branch. Four treatments were applied, ACC (300 mMol), 1-Methylcyclopropene (1-MCP, 50 mg a.i. L^{-1}), ACC+1-MCP, and a control (water). It was added to all treatments a surfactant (Tween 20®, at 0,08%) to improve the solutions adherence and penetration in the coffee tissues; 200 mL of total solution per treatment was used during applications. ACC concentration applied was determined according to the ACC content found in coffee tissues in the re-watering period of field experiment I (Fig. 2(1C)). 1-MCP concentration applied was determined according to Lima *et al*. (2020). Each treatment was sprayed carefully in each branch, totally wetting leaves and flower buds in the upper third of the plant. Three days after applications, a rain event of 35 mm occurred, having all treatments influenced by the same quantity of water in the field. Flower bud differentiation was evaluated fifteen days after treatment application (Previous to anthesis) counting the number of flower buds in G2, G3, G4, and G6 stages (Morais *et al*., 2008) from all nodes (5-6) in each branch (240-250 flower buds per treatment).

2.4 Physiological and biochemical analysis

2.4.1. ACC quantification

Leaf, flower bud, and root ACC concentrations were determined by the Bulens method (Bulens *et al*., 2011), with minor modifications. Coffee tissues were grinded in liquid nitrogen using a mortar and pestle. 200 mg were transferred to 2 mL tubes for extraction with 1 mL of sulfosalicylic acid 5 % (p/v). After homogenization, tubes were maintained at 4 ºC for 30 min,

being gently mixed with 5 min intervals. After centrifugation at 3,090 g and 4 ºC for 30 min, 300 µL of the supernatant was transferred, in duplicates for each sample, to 10 mL vacutainer glass followed by 100 µL of 10 mM $HgCl_2$. The tubes were subsequently sealed with a serum cap, and 300 µL of cold NaOH-NaOCl solution (NaOH 0.434 M and NaOCl 0.173 M) was added using a syringe. The mixture was then homogenized for 5 s and incubated for 4 min on ice. After mixing the samples for 5 s, 5 mL of the headspace was withdrawn and ethylene levels were measured using the F-900 Portable Ethylene Analyzer (Felix Instrument USA) operating under the GC emulation mode. The ethylene ppm values were transformed to nmol using the gases equation, and the ACC content was expressed as nmol/g DW.

2.4.2. ACO enzymatic activity quantification

Leaf, flower bud, and root ACO activity were determined by the Bulens method (Bulens *et al*., (2011), with some modifications. After tissue grinding in liquid nitrogen, 250 mg were transferred to 2 mL tubes with 50 mg of polyvinylpolypyrrolidone. ACO extraction was performed with 1 mL of extraction buffer (MOPS 400 mM pH 7.2; 30 mM of ascorbic acid, and glycerol 10% (v/v)). Subsequently, samples were homogenized and centrifuged at 22,000 g for 30 minutes. Then, 800 µL of supernatant was collected and used for the subsequent analysis. 400 µL of the enzymatic extract was added to 3.6 mL of a reaction buffer containing: 2.8 mL of MOPS buffer (MOPS 64.3 mM pH 7.2; glycerol 12.86 % (v/v); sodium bicarbonate 25.8 mM; and iron sulfate 26 µM), 0.4 ml of ascorbic acid 45 mM, 0.1 mL of ACC 36 mM, and 0.3 mL of dithiothreitol (DDT) 12 mM. After sample homogenization, the reaction for ethylene release was carried out for 20 min at 30 °C. After homogenization by 5 s, 5 mL of the headspace air was withdrawn and used for the ethylene measurements using the F-900 Portable Ethylene Analyzer (Felix Instruments, USA) operating under the GC emulation mode. The ethylene ppm values were transformed to nmol using the gases equation, and the protein content was determined by the Bradford method (Bradford, 1976) in duplicate, using bovine serum albumin (BSA) as standard. One unit of ACO activity was defined as 1 nmol of ACC converted to 1 nmol of ethylene per min at 30 °C (Dong *et al*., 1992).

2.4.3. Ethylene measurement

For all sampling periods, leaves, flower buds, and root tissues were immediately incubated in 10 mL vacutainer glass tubes, containing a moist tissue placed on the bottom of each vial, sealed with serum caps, and incubated for 24 hours. For each biological sample, ethylene was quantified from the headspace gas using the F-900 Portable Ethylene Analyzer

(Felix Instruments, USA) operating under the GC emulation mode in triplicate. Plant material was incubated in two separate vials, and the headspace gas was withdrawn from the vials with a 10 mL plastic syringe. Samples, made of 2.5 mL of gas from each vial, were extracted using the same syringe and subsequently injected into the Ethylene Analyzer. After ethylene measurement, plant material was weighed and ethylene production rate was expressed as ppm/g FW. $h.^{-1}$ (Fresh weight per hour)

2.4.4. ABA extraction and concentration measurement

ABA content analysis in leaves, flower buds, and roots was carried out according to Liu *et al*. (2014). Samples were grinded in liquid nitrogen, then 500 μL of an extraction solution (methanol 90 % (v/v) and sodium diethyldithiocarbamate trihydrate 200 mg/L) was added to 200 mg of plant material in a siliconized borosilicate tube. Samples were incubated overnight under dark conditions at 4 °C and centrifuged at 8,000 g for 10 min at 4 °C. The supernatant was transferred to a pre-cold 1.5 mL Eppendorf tube and evaporated in a vacuum centrifuge at room temperature. The residue was dissolved in a methanolic Tris buffer solution containing methanol 10 % (v/v), Tris-HCl 50 mM pH 8.0, MgCl2 1 mM, and NaCl 150 mM. The ABA concentration in all tissues was measured with a Phytodetek ABA enzyme immunoassay test kit (Agdia; Elkhart, IN, USA, Catalog number: PDK 09347/0096), in duplicate according to the manufacturer's instructions.

2.5 Molecular analysis

2.5.1. *In silico* Analysis

Genes encoding for Lysine Histidine Transporter (LHT1) in *Arabidopsis thaliana* were retrieved from The Arabidopsis Information Resource (https://www.arabidopsis.org/index.jsp) database. The protein sequences from these genes were used as input to perform similarity searches against the genomes of plant species from different orders such as *Rubiaceae, Solanales, Rosales, Gentianales, Malpighiales, Vitales, Poales,* and *Amborellale* by the Protein Basic Local Alignment Tool (BLASTp), at National Center for Biotechnology Information (NCBI, http://www.ncbi. nlm.nih.gov/). The sequences with significant similarity (e-value <10−5) were selected and the predicted proteins in which the inputted sequence identity was below 70% were removed. Protein sequences were aligned using the Clustal W program (Thompson *et al*., 1994) with the standard patterns. The phylogenetic tree was drawn using the MEGA software version 6.0 (Tamura et al., 2013), with a neighbor-joining comparison model (Saitou et al., 1987) and bootstrap values from 5,000 replicate to assess the robustness of the

tree. The *LHT1*primer (Forward 5`TTCGTCGGTTGCTCATCTCA and reverse: 5`TTGCCTTCTTCAGCCGTT) design was performed using the sequence obtained in the *in silico* analysis and the Primer Express v2.0 program (Applied Biosystems) (Supporting information Table S2).

2.5.2. RNA extraction, cDNA synthesis, and RT-qPCR assay

Total RNA from leaves, floral bud, and roots were extracted according to de Oliveira *et al*. (2015), with minor modifications. RNA samples (7,5 μg) were treated with DNase I using the Turbo DNA-free Kit (Ambion) to eliminate DNA contamination. RNA integrity was analyzed in 1% agarose gel, and RNA content, as well as quality, were accessed by spectroscopy (OD260/280 and OD260/230 > 1.8) (NanoVue GE Healthcare, Munich, Germany). One μg of the total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's protocol.

Real-time quantitative PCR (RT-qPCR) was performed using 15 ng of cDNA, with Rotor-Gene SYBR® Green PCR Kit (Qiagen), using a Rotor Gene-Q(R) thermocycler (Venlo, Netherlands). Reactions were carried out in 15 μL total reaction volume: 7.5 μL of SYBR-green (QuantiFast SYBR Green PCR Kit - Qiagen), 0.3 μL of forward and reverse gene-specific primers, 1.5 μL of cDNA at 10 ng/μL, and 5.7 of RNase-DNase-free water. Three biological repetitions were used, and reactions were run in duplicate as technical replicates. Amplification was performed with the following reaction conditions: enzyme activation with 5 min at 95 ˚C, then 40 cycles of 95 ˚C for 5 s, followed by 10 s at 60 ˚C and completed by a melting curve analysis to assess the specificity of the reaction by raising the temperature from 60 to 95 ˚C, with 1 ˚C increase in temperature every 5 s. Relative fold differences were calculated based on the ΔΔCT method (Pfaffl, 2001), using *MDH* and *RPL39* as reference genes (De Carvalho *et al*., 2013; Fernandes-Brum *et al*., 2017) (Supporting information Table S3).

2.6 Statistical analysis

For ACC, ethylene, ACO activity, and ABA, data analysis was performed using the InfoStat software (Di Rienzo *et al.*, 2020). The statistical difference was determined by one-way ANOVA, followed by the Tukey test. Results were expressed as the mean ±Standard Deviation (SD). The values marked with different letters are significantly different at $P<0.05$. For the gene expression, statistical analyses were performed by the R software (R Core Team, 2019). The expression rate and the confidence intervals were calculated according to the method

proposed by Steibel *et al*. (2009), which considers the linear mixed model given by the following equation: yijklm = μ + TGijk + Il + eijklm where, yijklm is the Cq (Quantification cycle) obtained from the thermocycler software for the kth gene (reference or target) from the mth well, corresponding to the lth plant subject to the *i*th treatment (Wet, Dry, and Rainy) at the *jth* tissues (Leaf, Bud, and Root); TGijk is the effect of the combination of the *ith* treatment (May 18th, August 18th, and August 28th) at the *jth* tissues (Leaf, Bud, and Root). Graphics were performed with SigmaPlot v. 14 (Systat Software Inc.).

3. RESULTS

3.1 Leaf water potential

In field experiment I, leaf water potential was measured from -0.35 to-0.45 MPa in the rainy period (May 18th), decreasing dramatically at the end of the dry period (August 18th), reaching values from -2.5 to -2.7 MPa. After 22 mm of rain in the re-watering period (August 28th), leaf water potential values increased to -1.5 MPa (Fig. 1A). In field experiment II, leaf water potential was measured only in the re-watering period, showing that values were higher in covered than in uncovered plants (Fig. 1B).

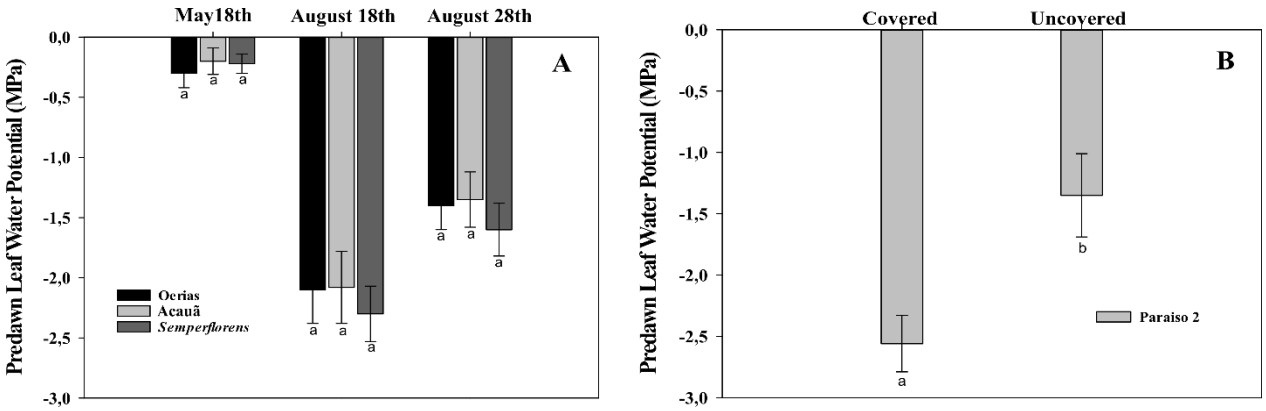

Fig. 1. Predawn leaf water potential for coffee genotypes. **A**) Coffee plants from field experiment I in the rainy period (May 18th), dry period (August 20th), and re-watering period (August 28th) for Oeiras, Acauã, and *Semperflorens* genotypes **B**) Coffee plants from field experiment II in the re-watering period (August 28th) for Paraiso 2 coffee cultivar. Data are the mean ± 95 % of Standard Deviation (SD) of the mean (n = 8). Different letters within the same period indicate statistical ($P < 0.05$) differences within the same period, respectively.

3.2 Field Experiment I

3.2.1 ACC content

In general, higher ACC levels were observed in the cultivar Oeiras when compared to the other genotypes (1.5 times more than Acauã and *Semperflorens*). Within each cultivar, there were different patterns of ACC production across the periods. For Oeiras, ACC showed an increase along the sampling periods for leaves, flower buds, and roots (Fig. 2 (1A)), whereas for Acauã, there was a decrease in the levels in the dry period for leaves and flower buds and an increase in the root, with increased levels being observed in all tissues after the rain event (Fig. 2 (1B)). As for the cultivar *Semperflorens*, a pattern of ACC increase in the leaves was observed during the dry period, slightly decreasing in flower buds, and remaining stable in roots. Similar to the previous genotypes, ACC levels increased after the rain event for leaves, flower buds, and roots (Fig.2 (1C)).

Although all genotypes presented different patterns of ACC production in their different tissues between the rainy and dry periods, the first rain after the dry period provided increases of ACC observed in all tissues from the three coffee genotypes (Fig.2 (1)). Oeiras ACC levels increased 93%, 277%, and 270% for leaves, flower buds, and roots, respectively, once plants were partially rehydrated. For Acauã plants, ACC also increased greatly in root and leaf (119 % and 713 %, respectively), as well in *Semperflorens*, rain promoted ACC increases of 72 % (leaves) and 388 % (roots) related to the dry period. When comparing the production of ACC among the three genotypes, it could be observed that there is a difference between Acauã and Oeiras where, although they showed similar ACC levels until the end of the dry period, ACC levels after the rain ranged from 800 to 1200 nmol ACC/gr DW (Acauã) and 1500 to 2300 nmol ACC/gr DW (Oeiras) (Fig. 2A and 2B). In *Semperflorens* plants, the pattern of ACC production was similar to the one observed for Acauã plants (Fig. 2 (1C)).

3.2.2 Ethylene production

Ethylene production showed different patterns among tissues and genotypes. Oeiras ethylene production decreased along the sampling periods for leaves and flower buds, with the opposite being observed for roots (Fig. 2 (2A). Acauã ethylene production decreased in leaves and increased for roots along the sampling periods, whereas for flower buds an increase was observed during the dry period, decreasing after the rain in the re-watering period (Fig. 2 (2B)). In *Semperflorens*, ethylene production showed a similar pattern for the three tissues, decreasing during the dry period and increasing once plants were partially rehydrated after the rain event (Fig. 2 (2C)). In general, ethylene production was similar for the three genotypes, showing a

decreasing pattern in leaves along the sampling periods and an increasing pattern in roots of Oeiras and Acauã plants (Fig. 2 (2)).

3.2.3 ACO activity

In Acauã and Oeiras the ACO activity had a similar pattern (Fig. 2 (3A and 3B)), decreasing from rainy period (May 18th) to re-watering period (August 28th) for leaves, and increasing in roots. Meanwhile, flower buds decreased ACO in the dry period (August 18th). In *Semperflorens* plants, ACO activity decreased from the rainy period (May 18th) to the dry period (August 18th) for all tissues and increased in the re-watering period (August 28th) for leaves and buds but continued decreasing for roots (Fig. 2 (3C)).

3.2.4 ABA content

ABA content was different among the analyzed sampling periods. In general, it was low in the rainy period (May 18th), increasing in the dry period (August 18th) and re-watering period (August 28th) observing different behaviors dependent on each cultivar. The ABA content was higher in *Semperflorens* than Oeiras and Acauã, especially for leaves. For Oeiras, the ABA level was similar for leaves, buds, and roots in the rainy period (May 18th), and showed higher levels for all tissues in the dry period but being even higher in leaves. Furthermore, in the re-watering period (August 28th) the ABA level decreased for all tissues, although was higher in leaves compared to other tissues, having levels similar to the dry period (Fig. 2 (4A)). In Acauã leaves ABA content increased from the end of the rainy period to the re-watering period (May 18th to August 28th), whereas, buds increased ABA from rainy to dry period (May 18th to August 18th) and decreased in the re-watering period (August 28th); ABA in roots was high in the rainy period (May 18th), and stable during the dry and re-watering periods (August 18th and August 28th) (Fig. 2 (4B)). In *Semperflorens* ABA content had a similar pattern to Oeiras, having low levels in the rainy period (May 18th), increasing in the dry period (August 18th), and decreasing in the re-watering period (August 28th) (Fig. 2 (4C)).

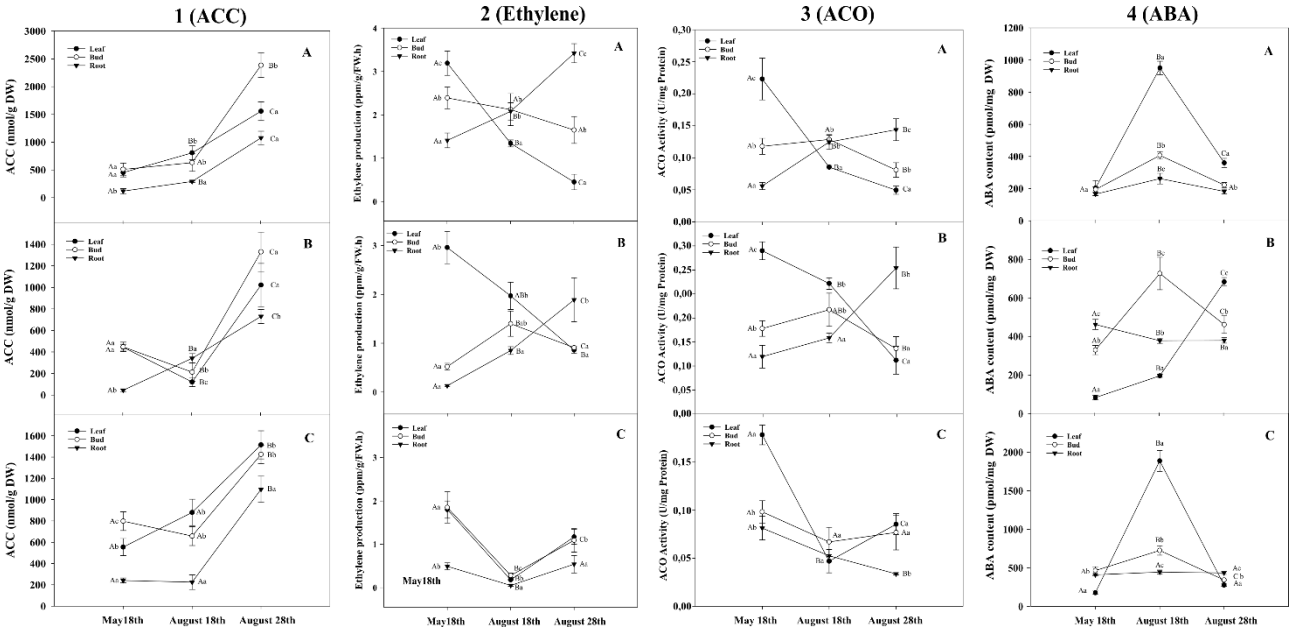

Fig. 2. Plant hormones evolution for coffee genotypes. ACC content (**1**), Ethylene production (**2**), ACO activity (**3**) and ABA content (**4**) in leaves, flower buds, and roots from (**A**) Oeiras, (**B**) Acauã, and (**C**) *Semperflorens* plants at the rainy period (May 18th), at the dry period (August 18th), and after rain in the re-watering period (August 28th). Data are means ± 95 % Standard Deviation (SD) of the mean (n = 6). Different upper-case and lower-case letters indicate the statistical difference of each tissue among the different periods and different tissues within the same period, respectively.

3.3 Lysine-Histidine Transporter 1 (*LHT1*) gene expression

In field experiment, I, the relative gene expression of the *LHT1* transporter was compared for the same tissues (Leaves, buds, and roots) among sampling periods. In general, differences were observed in the relative gene expression for root and bud tissues in the re-watering period (August 28th) when compared to rainy and dry periods (May 18th and August 18th). For leaves tissues, no differences were observed between sampling periods (May 18th, August 18^{th,} and August 28th) (Fig. 3A). In field experiment II, the comparison of the *LHT1* relative gene expression was made among leaves, buds, and roots for covered and uncovered plants in the re-watering period (August 28th). Results showed that *LHT1* expression was higher in uncovered plants for the root and flower bud tissues, whereas, no differences in the relative gene expression were observed in leaves (Fig. 3B), similarly to what was observed in leaves for field experiment I.

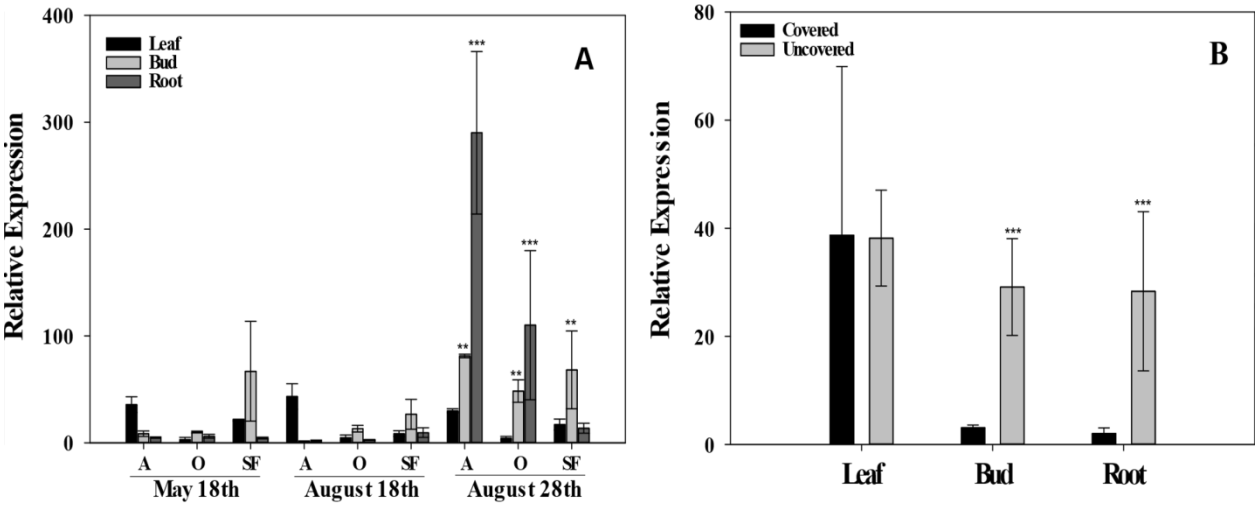

Fig. 3. Relative expression of the *LHT1*. (**A**) Field experiment I for leaves, buds, and roots in rainy period (May 18th), dry period (August 18th), and re-watering period (August 28th) for Acauã (A), Oeiras (O), and *Semperflorens* (SF). (**B**) *LHT1* relative expression in leaves, buds, and roots for field experiment II in re-watering period (August 28th) for Covered and Uncovered plants of coffee cultivar Paraiso 2. Data are means ± 95 % Standard Deviation (SD) of the mean (n=6). ** = significative difference of tissues between periods and *** = high significant difference of tissues between periods.

3.4 Field experiment II

The objective of field experiment II was to evaluate the effect of water on the evolution of ABA, ACC, ACO, and ethylene in the re-watering period (August 28th) using two treatments (Covered and uncovered plants). The content of ACC was higher in uncovered plants than in covered ones (Fig. 4A). However, the ethylene production did not vary between treatments, as well as the ACO activity, except for buds, that had higher production and activity in both parameters for uncovered plants (Fig. 4B and Fig. 4C). On the other hand, the ABA content decreased in the uncovered plants (Fig. 4D), possibly as an effect of the rehydration of the plant and the increase in the leaf water potential (Fig. 1B).

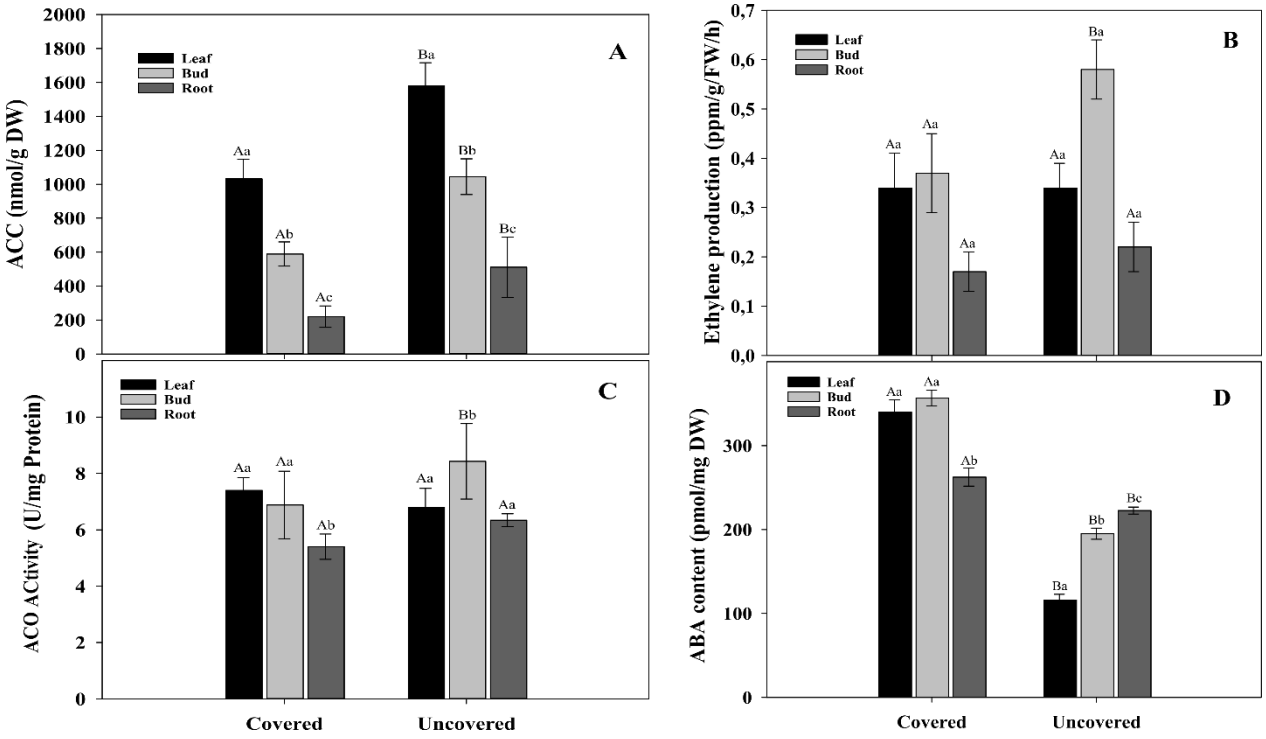

Fig. 4. Phytohormones contents. (**A**) ACC, (**B**) Ethylene, (**C**) ACO, and (**D**) ABA quantification in leaves, flower buds, and roots in the field experiment II (covered and uncovered plants of Paraiso 2 coffee cultivar). Data are means ± 95 % Standard Deviation (SD) of the mean (n = 6). Different upper-case and lower-case letters indicate statistical differences of each tissue among the different treatments and the different tissues within the same treatment, respectively.

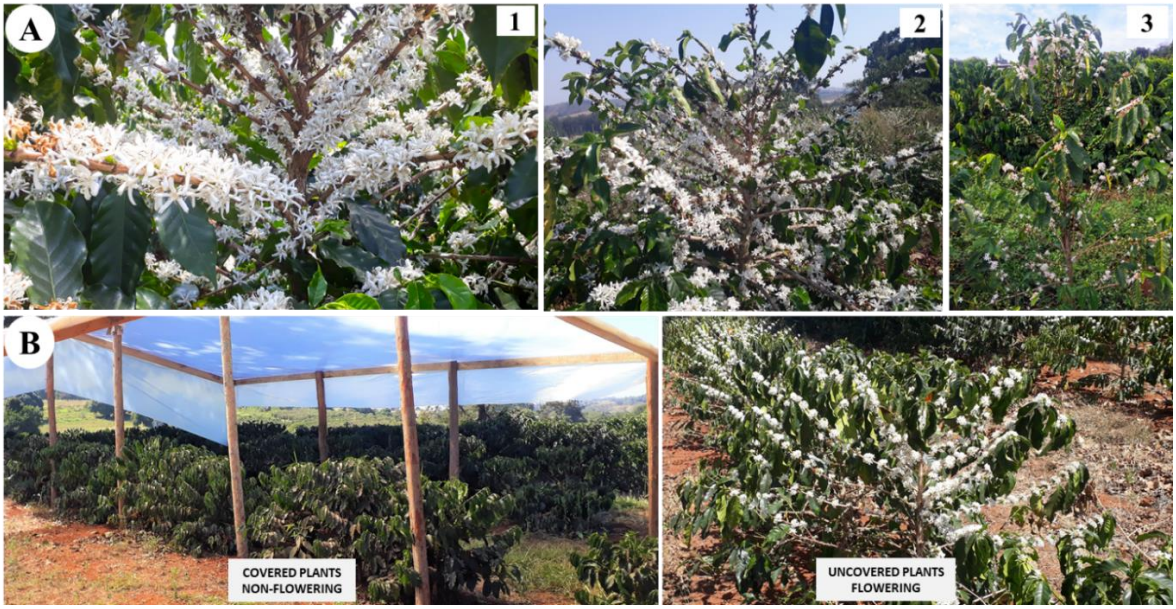

Fig. 5. Coffee anthesis in (**A**) Oeiras (1), Acauã (2), and *Semperflorens* (3) in field experiment I. (**B**) Non - Flowering and flowering plants of Paraiso 2 coffee cultivar using treatments of Covered and Uncovered environment at the field experiment II.

3.5 Field Experiment III

Coffee plants that had exogenous application of ACC showed an increase in the number of flower buds (at the G6 stage). The number of flower buds at the G6 stage was higher in the treatment with ACC (385%), followed by the treatment with 1-MCP + ACC (242%), and 1-MCP (185%) compared with control treatment (Figure 6A). These results are congruent with the statistical differences found among treatments for flower buds atG6 stage (Figure 6B). Because all coffee plants had after three days of treatments imposition, a 35 mm rain, the number of flowers bud at G6 stage in control treatment represent the effect of rain in coffee anthesis and the differences between control and the other treatments ((ACC), (1-MCP+ACC) and (1-MCP)) represent the effect those treatments on coffee anthesis.

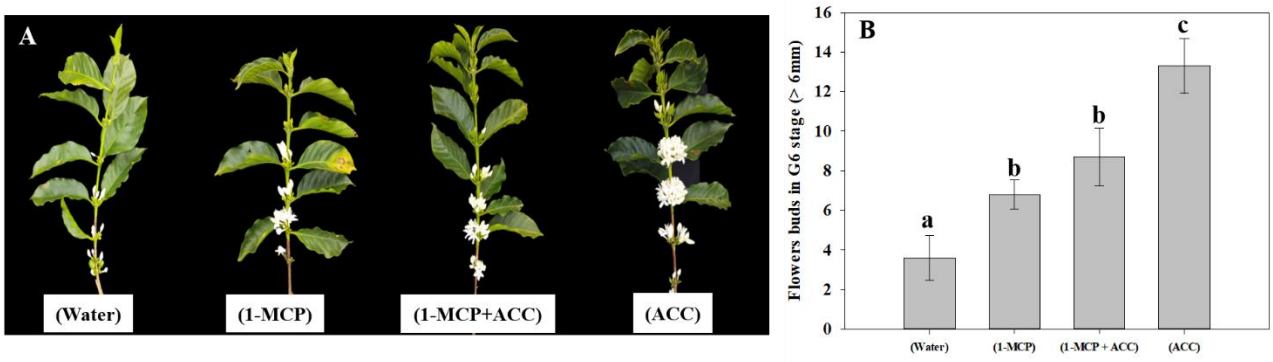

Fig. 6. Effect of ACC treatment in coffee anthesis (**A**) Branches with the opened flower as effect different treatments (**B**) Statistical differences among treatments with the application of exogenous ACC and other treatments in flower buds at G6 stage

4. DISCUSSION

4.1. Water stress and flowering

The transition from the vegetative to reproductive phase is a very important event for plants because flowering time is crucial to ensure the success of next-generation and species perpetuity (Romera-Branchat *et al*., 2014; Song *et al*., 2015). This phase is marked by different endogenous processes that respond to environmental stimuli. Diverse stress factors can induce, accelerate, inhibit or delay flowering in many species of plant including water deficit stress (Takeno, 2016). For field experiment, I, coffee plants of Oeiras, Acauã, and *Semperflorens* genotypes were under water deficit stress and leaf water potential reached -2.5 MPa in the dry period (August 18th) and then the average value of -1.5 MPa after rain in the re-watering period (August 28th) (Fig.1A) with anthesis occurring 10 days after 22 mm of rain (Fig. 5A). For field experiment II, covered and uncovered plants showed differences in leaf water potential (Fig.

1B), with only uncovered plants, had anthesis (Fig. 5B). Those results are corroborating that water is an important trigger of the coffee anthesis process.

This effect can be observed in many tropical and subtropical trees, such as citrus, the most classical example. It was observed that experiments with lemon (*Citrus limon* L. Burm. f.) under a temperature range of 18-30 °C, flowering was controlled by drought stress (Chaikiattiyos *et al*., 1994), and similar results were reported for "Tahiti" lime (*Citrus latifolia* Tanaka) as well (Southwick & Davenport, 1986). In Litchi (*Litchi chinensis* Sonn) the autumnal water stress period increased significantly flowering intensity and yield (Stern *et al*., 1993), such as in Star fruit (*Averrhoa carambola* L.) (Wu *et al*., 2017), Longan (*Dimocarpus longan* Lour.) (Zhou *et al*., 2014), and Coffee (*Coffea arabica* L.) (Crisosto *et al*., 1992).

In the agroecological conditions of Brazil, coffee anthesis occurs after a short rainy event preceded by a water deficit period during the winter, which is necessary to break the bud "latent state", associated with endogenous and external factors (Ronchi & Miranda, 2020).
One of the most important factors that contribute to flowering under water deficit stress conditions are phytohormones (Izawa 2021). The main plant hormone studied in response to water stress is ABA, because acts as an important signaling mediator for plants adaptive response to a variety of environmental stresses regulating many physiological processes, including bud dormancy, seed germination, stomatal development, and transcriptional and post-transcriptional regulation of stress-responsive gene expression (Ali *et al*., 2020).

For the three coffee genotypes, ABA levels increased from rainy to dry period (May 18th to August 18th) in leaves and buds, which can be explained by the higher activity for stomatal closure in leaves to cope with the water restriction at this dry period. For Oeiras and *Semperflorens*, ABA content decreased after a re-watering period (August 28th), whereas in Acauã cultivar ABA content increased, as was observed by Martins *et al*. (2019). This contrast in ABA content can be associated with the intrinsic and phenotypic characteristics of flowering and ripening fruit patterns of each coffee cultivar, where Acauã is considered to be late, Oeiras is early, and *Semperflorens* is continuous. When the ABA level increases, it negatively modulates ethylene biosynthesis and ABA has shown a positive effect on floral transition under water stress, being able to participate in early flowering known as Drought Escape (Verslues & Juenger, 2011). Despite the ABA variation level between Acauã and the other genotypes, in this experiment for all coffee genotypes, ethylene production decreased from rainy to re-watering periods (May 18th to August 28th) in leaves, possibly due to the interaction of ABA in response to the water stress deficit.

4.2. ABA- Ethylene regulation

ABA and ethylene are well-known phytohormones involved in the regulation of physiological processes in plants (Müller, 2021). They participate mainly in plant growth regulation, biotic and abiotic stress responses, bud and seed dormancy, leaf and flower senescence, fruit ripening, germination, and flowering (Binder, 2020; Chen *et al*., 2020). In our study, in the field experiment I, basically ABA content was higher in leaves and flower buds than in root in the dry period for all genotypes (August 18th) (Fig.2(4)), probably because ABA is very active in leaves and buds due to water deficit to keep stomatal conductance in leaves (McAdam & Brodribb, 2018). Conversely, ethylene production decreased (Fig. 2 (2)) coinciding with a reduction of ACO activity, which serves as a catalyst in the oxidative process for the conversion of ACC into ethylene. This antagonism in response to water stress has been observed in other species directly related to the closing of the stomata to avoid dehydration (Daszkowska-Golec & Szarejko, 2013).

Concerning flowering, it is known that ethylene could act negatively by inhibiting flowering in *Arabidopsis* (Achard *et al*., 2007) or delaying in rice (Wang *et al*., 2013), demonstrating that ethylene signaling is delayed in both species. However, a positive effect on promoting flowering has been observed in pineapple (*Ananas comusus* L. Merr) (Trusov & Botella, 2006) and lilies (*Triteleia laxa* Benth) (Han *et al*., 1988). Recently, it was proposed that ethylene is involved directly in coffee anthesis by changes in the biosynthesis pathway and regulatory genes expression (Lima *et al*., 2020). In other words, the direct effect of ABA in flowering is not well understood, despite there being some reports about positive and negative influences in flowering (Izawa, 2021). Exogenous application of ABA negatively regulates flowering in *A. thaliana*, represented by *AtABI5* overexpression delaying floral transition by upregulating *FLOWERING LOCUS C (FLC)* expression (Wang *et al*., 2013).

In the same sense, ABA represses flowering by modulating SOC1 at the apex, when CO is needed by FT in the ABA-dependent floral induction (Riboni *et al*., 2020). The most known positive effect of ABA on flowering is the drought escape, which is a plant mechanism to avoid drought damage and reflecting in early flowering, to produce seeds before being affected by severe drought stress conditions (Franks, 2011; Gupta *et al*., 2020). Early flowering is characterized by an ABA increased level in response to water stress (Shavrukov *et al*., 2017; Sherrard & Maherali, 2006).

At the rainy period (May 18th), coffee plants showed no water deficit stress (-0.25 MPa) and the ethylene levels were higher than ABA, contrasting with the dry period (August 18th), where ethylene level decreased and ABA production increased in coffee plants under water

deficit stress (-2.5 MPa) in leaves and flower bud. These results show a clear and opposite behavior in phytohormones content related to water stress conditions in coffee plants (Fig. 2). This behavior suggests a direct ABA regulation on ethylene production, possibly by the downregulation of ACO activity. Negative ABA regulation in the ACO activity was observed in Hibiscus (*Hibiscus rosa-Sinensis* L.) when activation of the ethylene biosynthesis pathway was reduced by exogenous ABA treatment (Trivellini *et al*., 2011). Similar results were observed in Lepidio (*Lepidium sativum* L.) when ABA inhibited seed germination decreasing ethylene content by the *Lepidium ACO2* gene expression regulation (Linkies *et al*., 2009). Moreover, in Sugar beet, the radicle emergence is regulated by ABA-ethylene antagonism that affects ACC and ACO gene expression (Hermann *et al*., 2007) and, in Tomato (*Solanum Lycopersicum* L.) was observed upregulation in ripening (Zhang *et al*., 2009).

In our research, anthesis occurred after a re-watering period (August 28th) by rain, the ethylene content continued to decrease, coinciding with a decrease in the ACO activity. This behavior was observed for leaves and buds, whereas in the roots, it was the opposite. The ABA content decreased in the leaves and flower buds for Oeiras and *Semperflorens* genotypes, possibly because the water content for their rehydration was sufficient enough to reduce the water deficit stress in the plant, whereas in the Acauã cultivar, the ABA content increased in the leaves due to persistent water deficit stress.

In field experiment II, data from ACC, ABA, ethylene, and ACO were taken only after a re-watering period (August 28th) to support the previously hypothesized behavior in the coffee plant after rain. The results showed an increased level of ACC and decreased level of ethylene and ACO activity whereas ABA decreased after rain in response to rehydration of coffee plants (Fig. 4). These results corroborate with the results obtained in field experiment I, where an increased level of ACC was observed over an antagonistic relation between ABA and ethylene, participating in coffee anthesis after a water stress period. Also, PCA and correlation analysis reinforce the antagonism in the evolution of ABA and ethylene compared in the three sampled periods (Fig. S2).

4.3. ACC beyond ethylene precursor, acting as a possible flowering signaling

Since ACC was discovered (Adams & Yang, 1979), it has always been described as an ethylene precursor, and external applications of ACC are used for many experiments with ethylene (Elías *et al*., 2018). However, new pieces of evidence and recent discoveries showed that ACC can act as a signaling molecule in plants, independently of ethylene. One of the most remarkable results of our experiment is the increase of ACC in all tissues and coffee genotypes

after rain in the re-watering period (August 28th) (Fig. 2 (1)). In our initial hypothesis, we believed that ACC accumulated in the root during the dry period and transported to the plant aerial part to become ethylene after the rain would be involved in the coffee anthesis (Lima *et al*., 2020).

However, this study showed that the increase in ACC found in all tissues does not have a positive correlation with the ethylene production in the coffee plant aerial part (buds and leaves), which coincides with a low activity of the ACO enzyme in the field experiment I and corroborated with the results in the field experiment II. It is possible that ACC, in addition to being a precursor molecule of ethylene, is involved in the fecundation and anthesis process in coffee. Results of the field experiment III allow us to confirm this hypothesis because the treatment imposition with exogenous ACC showed a higher number of flower buds at the G6 stage as compared with control treatment (Water) (Fig. 6A and 6B). This high level of ACC observed in all tissues after rain in the re-watering period (August 28th) (Fig. 2(1)) could be derived from the amino acid methionine (Precursor of ACC) accumulated during the dry period as a plant response to alleviate the water stress period as observed in previous studies in coffee (Marcheafave *et al*., 2019), wheat (Le Roux *et al*., 2020), and Bitter gourd (Akram *et al*., 2020).

In *Arabidopsis*, Mou *et al*. (2020) found that ACC in ovules stimulates transient Ca^{2+} elevation and Ca^{2+} influx, and this signaling in ovular sporophytic tissue is involved in pollen tube attraction. ACC may participate as signaling in pollen tube attraction in the coffee flower fecundation process. This process is very important in the coffee crop since the anthesis only occurs between 10-12 days after the rain, at which time flower fecundation has already occurred in a 90% because the coffee plant is self-pollinated (Veddeler *et al*., 2006).

ACC has been shown to regulate some physiological mechanisms in plants such as stomatal development by guard cell differentiation (Yin *et al*., 2019), cell wall metabolism (Hofmann, 2008; Tsang *et al*., 2011; Xu *et al*., 2008), and vegetative development (Tsuchisaka *et al*., 2009; Vanderstraeten *et al*., 2019). In addition, it has been found that ACC has a distinct function than ethylene in the non-vascular plant *Marchantia polymorpha* (Katayose *et al*., 2021; Li *et al*., 2020) and sexual reproduction induction in the Marine red alga *Pyropia yezoensis* (Uji *et al*., 2020). In our study, the *LHT1*expression, an intracellular amino acid transporter that also transports ACC (Shin *et al*., 2015), was evaluated. Although its best-known function is amino acid transporters of ACC, it might be involved in many biological processes related to ethylene (Yang *et al*., 2020).

In field experiment I, *LHT1* expression was higher in all coffee genotypes after rain in the re-watering period (August 28th), especially for roots and buds (Fig. 3A), as well as in the

field experiment II, where in the uncovered coffee plants the *LHT1* expression was higher for buds and roots than in covered plants, whereas in leaves it did not show the difference (Fig. 3B). Notably, there was an increase in ACC in the coffee plant tissues (Fig. 2(1)), which coincides with a higher *LHT1* expression after the rain (Fig 4A), showing that LHT1 transporter might be associated with ACC intracellular transportation. The LHT amino acid transporters family are linked with flower development because *LHT2* and *LHT4* were expressed in the tapetum, suggesting their role in delivering amino acids to pollen grains in *Arabidopsis* (Foster *et al*., 2008; Lee *et al*., 2004). Moreover, *LHT5* and *LHT6* expressions were detected along the transmitting tract of the pistil and the pollen tube, pointing to a function in amino acid uptake for successful fertilization (Foster *et al*., 2008). The study represents the first report of *LHT1* transporter gene expression associated with ACC in coffee, suggesting intracellular transport and most likely, participating in the coffee anthesis. In light of these results, we propose that ACC participates in the anthesis process in the coffee plant, as represented in Fig. 7.

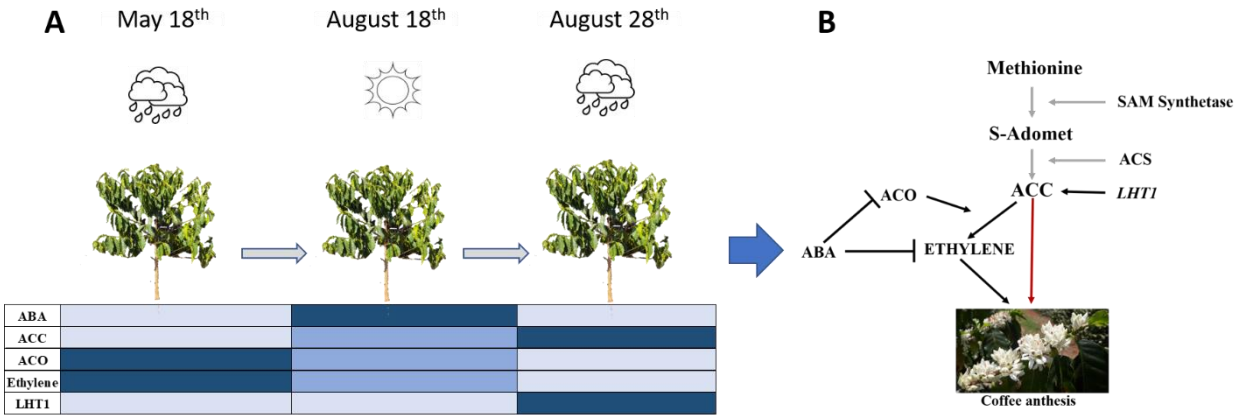

Fig. 7. Proposed model of ABA-ACO-Ethylene regulation favoring ACC increased level influencing coffee anthesis in the ethylene biosynthesis pathway. **A**) Evolution of phytohormones (ABA, ACC, ACO, Ethylene) and *LHT1* transporter measured in this study by the sampling times (May 18th, August 18th, and August 28th). The color intensity in the chart means the level of plant hormone in the coffee plant as a whole (Light blue = low, blue = medium, dark blue = high). **B**) Relationship among phytohormones according to our proposal, the black arrow means established plant hormones relation in this study, gray ones mean part of ethylene biosynthesis process described in the literature, the red arrow means a proposal of this study of ACC acting in coffee anthesis.

5. CONCLUSIONS

The results of this study showed that ACC acts as a modulator in the coffee flowering process, displaying more concentration in the anthesis period. We suggest that ACC has its activity modulated independently of the ethylene biosynthesis pathway. Furthermore, ABA is

involved in the process, especially, because coffee anthesis is preceded by a water stress deficit period in which this plant hormone plays an important role in stomatal closure. On the other hand, this is the first report of *LHT1* as an ACC transporter in coffee associated with anthesis. These results suggest that the increased ABA level during the dry period regulates the ACO activity decreasing the ethylene content and increasing the ACC level throughout an intracellular transport in roots, flower buds, and leaves, this may be directly associated with both fecundation and anthesis process in coffee in the re-watering period. Re-watering is an inductive stimulus for coffee anthesis after a water deficit period. Our results corroborate that making the water an important trigger for physiological processes of coffee anthesis is proven by covered plants not presenting anthesis while uncovered coffee plants bloomed.

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7. AUTHOR CONTRIBUTIONS

MEL, ISS, AAL, and ACJ designed the study, MEL, ISS, JME, and RM performed field experiments, MEL, RM, ISS, AJM and AJM performed laboratory analysis. CC curate and analyzed the data, MEL drafted the manuscript before all authors contributed to its improvement and agreed on its final content.

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ARTICLE 2 - SUPPLEMENTARY INFORMATION

Fig. S1 Design of rainfall exclusion treatment in field experiment II. Rainfall exclusion was achieved through the installation of translucent nylon (polypropylene) fixed on twelve 3.2 m high eucalyptus wood logs, buried 0.60 m deep in the soil with three wood logs 3 m distant from each other. Every eucalyptus wood log was arranged on each posterior side and in the middle of the coffee line. A translucent nylon piece of 0,2 micrometers was fixed on top of wood logs using a wooden piece of 5x7 cm with a small hobnail, covering a total of 81 m^2 of rain exclusion area.

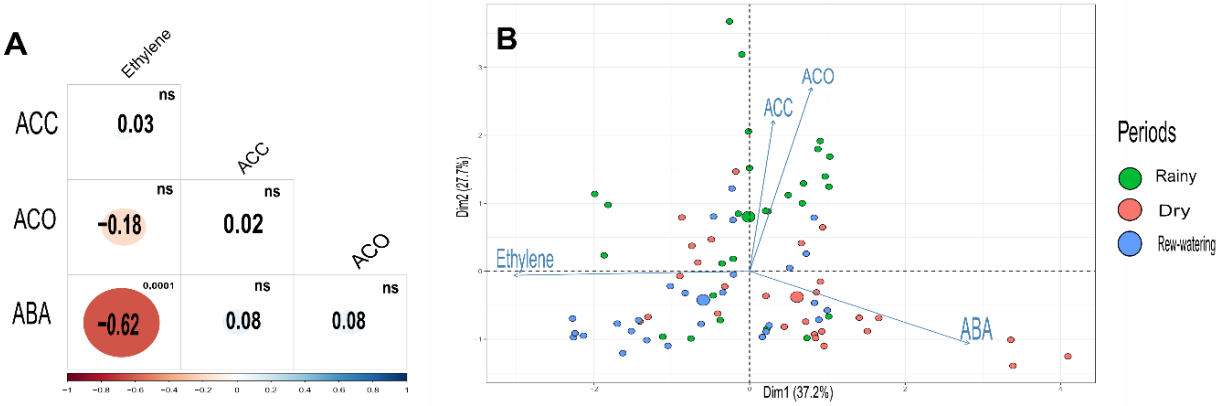

Fig. S2 Spearman Correlation (A) and PCA analysis (B) for ACC, ACO, ethylene, and ABA regulation in coffee anthesis. A principal component (PCA) and correlation analysis was carried out to understand the variation and relationship between the variables ABA, ACO, Ethylene, and ACC. The results showed that the biplot PCA represents 64,9% of the total variation. The main component 1 (37,2%) is the ethylene located in the positive part of the axis and by the ABA which is located in the negative part of the X-axis, which indicates that both have a negative correlation. The principal component 2 (27,7%) is

represented by the variables ACO and ACC, which are located on the positive part of the Y-axis and are closely related to each other (Fig. 3B). The results of the antagonistic relationship between ABA and ethylene are corroborated by the Spearman correlation analysis where its coefficient is r = -0.62, showing a high negative correlation for the two variables.

Table. S1 Experimental design of the different experiments conducted and their specific hypotheses.

Experiment	Treatments / Condition	Measurements	Hypothesis
Field I	Three coffee genotypes, Oeiras (Early), Acauã (Late), and *Semperflorens* (continuous) for coffee flowering pattern under field conditions in Rainy, Dry and Re-watering periods	-Predawn Leaf Water Potential - ACC, ABA, Ethylene content, ACO activity, and LHT1 gene expression	Hormonal crosstalk is involved in coffee anthesis
Field II	Rainfall exclusion derived in Covered and Uncovered coffee plants in the Re-watering period	-Predawn Leaf Water Potential - ACC, ABA, Ethylene content, ACO activity, and *LHT1* gene expression	Water is an important triggering transport signaling for coffee anthesis.
Field III	In coffee plants of *Semperflorens* were applied ACC, 1-MCP, (ACC+1-MCP), and Water treatments	-Flower bud in G6 stage	Exogenous application of ACC modulates coffee anthesis

Table S2 Sequence of primers used for RT-qPCR, where: TM = the melting temperature and E = efficiency of the primer annealing.

Gene	**Sequence of primers (5' – 3')**	**Concentration (µM)**	**Volume (µL)**	**TM (ºC)**	**E (%)**	**Reference**
Lysine histidine transporter (LHT1)	**F: TTCGTCGGTTGCTCATCTCA** **R: TTGCCTTCTCTTCAGCCGTT**	**1,5**	**2,25** **2,25**	**54** **52**	**100**	**This study**
Malate dehydrogenase (MDH)	**F: CCTGATGTCAACCACGCAACT** **R: GTGGTTATGAACTCTCCATTCAACC**	**2**	**3** **3**	**59** **60**	**87**	De Carvalho et al (2013)
Large ribosomal subunit 39 (RPL39)	**F: GCGAAGAAGCAGAGGCAGAA** **R: TTGGCATTGTAGCGGATGGT**	**2**	**3** **3**	**59** **60**	**87**	**Fernandes-Brum et al (2017)**

Table. S3 Quantitative real-time PCR parameters according to the Minimum Information for Publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin *et al*. (2009).

Experimental design / Sample	
Experimental group	Four- and five-years-old coffee (*Coffea arabica*) plants (Field experiment I, II) respectively.
Sample	Leaves, Roots, and flower buds for both experiments
Sampling procedure	Immediately frozen in liquid nitrogen (roots were first washed and then dried using paper towels)
Storage conditions / Time of storage	Freezer -80 °C / 3 weeks
RNA extraction	
Processing procedure	Grinding (mortar and pestle) in liquid nitrogen
Method	Organic extraction, according to De Oliveira et al., 2015.
RNA: DNA-free	TURBO DNA-*free*™ Kit (Ambion, Thermo Fisher Scientific)- Catalog number: AM1907 - Design of Intron-spanning primers whenever possible
Nucleic acid quantification	Spectroscopy (NanoVue GE Healthcare)
RNA integrity	Agarose gel (1 %)
Reverse transcription	
Kit	High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) - Catalog number: 4368814
Reaction conditions	25 °C 10’ / 37 °C 120’ / 85 °C 5’
Reverse transcriptase	MultiScrib™ MuLV
Amount of RNA / Reaction volume	1 µg / 20 µL
Priming strategy	Random primers
Storage conditions of cDNA	Freezer -20 °C
RT-qPCR	
Target information	Table S2

Reaction conditions	As stated in the Materials and Methods section
In silico	Primers were blasted using the BLAST tool at https://www.ncbi.nlm.nih.gov/
Empirical	Primer concentration of 1μM (final concentration on the reaction) Annealing temperature: 60 °C
PCR efficiency	5-fold dilution series of a mixed sample over at least five dilution points and verified to be higher than 80% (E = 10−1/slope).
Linear dynamic range	Samples are situated within the range of the efficiency curves for each primer
No template control (NTC)	Cq and dissociation curve verification
Data analysis	
Specialist software	Qiagen Rotor Gene-Q Series software (version 1.7)
Normalization	Two reference genes / $\Delta\Delta C_T$ method (Pfaffl, 2001)

CHAPTER 4

ARTICLE 3 - CONTRASTING FLOWERING-TIME GENOTYPES AND THE *SEMPERFLORENS* MUTANT REVEALED MOLECULAR PATHWAYS REGULATING THE FLORAL DEVELOPMENT IN *COFFEA ARABICA* L.

(DRAFT VERSION)

Article prepared and submitted to the *Planta* Journal

Contrasting flowering-time genotypes and the *Semperflorens* mutant revealed molecular pathways regulating the floral development in *Coffea arabica* L.

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Abstract

Coffee flowering is an important physiological process initiated by the transition of the vegetative to reproductive phase that results in the production of coffee beans, an important commodity. Although many aspects of flowering activation and development are described in model species, the related molecular pathways are very diversified in crops and poorly understood in coffee. To study such a process, three coffee genotypes were selected according to their contrasting flowering-time pattern: Acauã (Late), Oeiras (Early), and the *Semperflorens* mutant (Continuous). Coffee genotypes were compared firstly by phenotypic analysis, which proved their different nature of flowering-time and also determined specific developmental points. Then, genotypes were compared throughout RNA-seq analysis of Shoot Apical Meristem (SAM), Flower buds (G2 stage), and leaves (March and August). A total of 12478 DGEs were found among 18 contrasts performed, having these genes were related to sugar metabolism, hormonal regulation, photoperiod, endogenous and external stimulus, temperature, photosynthesis, flower development, seed development, and the circadian clock. From those, 280 genes associated with sugar metabolism, 650 genes associated with ethylene biosynthesis and signaling pathway, and 523 genes associated with flower development and photoperiodic responses in flowering time were selected. Thirteen candidate genes associated with endogenous and external stimulus in the flowering time process were selected (protein HEADING DATE 3A-like, protein MOTHER of FT and FTL 1 homolog 1-like, truncated transcription factor CAULIFLOWER A-LIKE, protein EARLY FLOWERING 5-like VIN-like protein 2, two components regulator-like APRR5, and MAD-box protein SOC1-like, Protein FLC Expressor, MADS-box protein SVP-like, FRIGIDA-like protein, Floral homeotic protein PMADS 2, Floral homeotic protein APETALA 2-like, SPL3-like). Those genes could explain

the regulation of flower development and anthesis events of coffee genotypes. On the other hand, in the physiological analysis, starch content was higher in *Semperflorens*, and two genes related to the enzymatic starch biosynthesis with high expression in March were identified (glucose-1-phosphate adenylyltransferase large subunit 1-like and glucose-6-phosphate/phosphate translocator 2%2C chloroplastic-like). Moreover, two genes related to sucrose transport in leaves (sugar transport protein 13-like and hexose carrier protein HEX6-like) correlated with the increase in sucrose content observed in Acauã from June to August. 28 DEGs related to the ethylene biosynthesis pathway were found, however, no correlation with ethylene measurement in leaves related to flowering time was observed. Genes associated with the endogenous stimulus, photoperiod, and sugar metabolism could be regulating coffee flowering time.

Keywords: RNA-seq, Coffee tree, Anthesis, Sugar metabolism, Plant hormones, Early, Late

1. INTRODUCTION

The flowering process is regulated by multiple external and endogenous signals to timing the transition from vegetative to reproductive phase (Coneva et al. 2012). In addition to well-known major pathways involved with flowering in plants (photoperiod, gibberellins, vernalization, age, and autonomous), other vias have been identified recently, including sugar metabolism, stress, thermosensory and plant hormones (Izawa 2021). The flowering process in angiosperms is complex, especially for woody species of the annual cycle under constant climatic variation. Coffee (*Coffea arabica* L.) flowering is a complex process regulated by external and endogenous stimulus due to flower bud formation requires a series of molecular and physiological changes in the differentiation pattern of apical or axillary buds (Thomas and Vince-Prue 1997; Crisosto et al. 1992; de Oliveira et al. 2014).

Based on its flowering pattern, coffee is classified as a gregarious species and represents one of the most important phases in its reproductive cycle associated with yield and coffee cup quality (Cannell 1985; Lima et al. 2020). Because coffee flowering is directly linked to production, studies have been carried out trying to synchronize the uneven fruit ripening caused by asynchronous flowering events (Miranda et al. 2020). Other factors, such as photoperiod, temperature, shade conditions, plant nutritional status, and phytohormones can also affect floral transition and plant development (de Camargo 1985; Ramírez et al. 2015).

Beyond flowering control well known by GA, other plant hormones have been described as influencing flowering time in plants (Izawa 2021). Recently, it was observed that the dry period following rehydration in coffee trees is associated with anthesis by increasing ethylene level in the shoot and, by increasing ethylene sensitivity through receptors such as *CaETR4-like* (Lima et al. 2020). Moreover, Abscisic Acid (ABA) increases during the dry period and is associated with coffee flower bud dormant or "latent state", having plant rehydration decreasing ABA and increasing Gibberellin (GA) levels (Cueto & Dathe 1986). Analysis of the ethylene receptor gene, *ETR2*, revealed that ethylene signals delay floral transition in rice and cause various developmental effects in panicle and seed-setting rate (Wuriyanghan et al. 2009; Wang et al. 2013). Conversely, the ethylene biosynthesis pathway induction positively regulated natural flowering in pineapple (Trusov and Botella 2006) and *Guzmania Lingulate* L. (Dukovski et al. 2006). Endogenous regulation in other plant hormones levels is also associated with flowering, such as brassinosteroids that promote a floral transition in *A. thaliana* (Li and He 2020), and abscisic acid that affects a floral transition under water deficit conditions in rice (Du et al. 2018). However, growth regulators can promote flowering, such as exogenous cytokinins (D'Aloia et al. 2011), gibberellin (Schuch et al. 1990), and 1-methyl cyclopropane (ethylene inhibitor) (Lima et al. 2020). Another important factor described as a flowering regulator is carbohydrates.

Sugars, the final products of photosynthesis, are mobilized to sink organs to support plant growth and development tissues. Sugars do not only act as a source of energy, but they also act as signals molecules to regulate developmental phases in plants (Cho et al. 2018; Bolouri Moghaddam and Van den Ende 2013). The sucrose level, the major photosynthetic product, increased during the transition of the vegetative to reproductive phase and acted as a signal to control tissue growth and differentiation (Tognetti et al. 2013). It seems that in these transitions the required level of energy in the reproductive system is superior to the vegetative phase, because of the formation of new florals, seeds, and fruit organs. The activation of energy-consuming processes such as mitosis increased sucrose supply to the meristem seems like a signal for the sucrose role of flowering transition in plants (Bernier 1993). Another reason for sugar accumulation in flower formation is related to ecological functions since flowers should provide nectar to attract pollinators agents for pollen exchange (Fornoff et al. 2017).

There are many shreds of evidence on sugar participating as signaling on the flowering process in plants, however, the mechanism of how it participates and transmits signals remain largely unknown. Previous studies reported that sucrose has the main function in the leaf phloem for the generation of florigens such as Flowering Locus T (FT), meanwhile, trehalose-

6-phosphate acts in the shoot apical meristem to promote the flowering signal of those florigens (Cho et al. 2018). Sugar transporters are involved in the movement of sucrose from sink to source in long-distance, which are important in the phloem loading (Smeekens et al. 2010).

There are two types of sugar transporters: SUCROSE TRANSPORTERS (SUTs) and SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERs (SWEETs) (Cho et al. 2018). In *Arabidopsis thaliana,* the overexpression of the *SWEET10* sugar transporter causes early flowering, indicating that transcriptional activation of this transporter by FT and SOC1 occurs during the promotion of flowering in inductive photoperiod (Andrés 2020). Hyperaccumulation of sugars in mature leaves of maize (Zea mays L.) defective in SUT1 caused a reduced structure and delay in flowering (Slewinski et al. 2009).

In grapevines, drastic flower abortion is caused by a lack of sugar in flowers during female meiosis caused by environmental or physiological fluctuations (Lebon et al. 2008). Exogenous application of sucrose has shown floral induction followed by a high expression of *Flowering Locus T (FT)* homologs, in Chrysanthemum *(Chrysanthemum indicum)* cultivar "Floral Yuuka" confirming the signaling functions of sucrose in this species (Sun et al. 2017). Conversely, the application of 6% of glucose delays flowering in Arabidopsis associated with Long Day (LD) photoperiod (Zhou et al. 1998).

Although some connections about hormonal regulation and flowering have been proposed for a coffee tree, information about how sugar metabolism and hormones influence the process is scarce. Many kinds of research have focused on elucidating the role of sugars in the development of the grain and its effect on the quality of the final product (cup quality). *Coffea arabica*, being an angiosperm, must share the same mechanisms that activate and develop the flowering process that has been mostly studied in other species. Similar to sugar metabolism, the involvement of plant hormones and coffee flowering is not well elucidated. In this study, we use three coffee genotypes contrasting in flowering patterns, Oeiras (Early), Acauã (Late) (Carvalho 2008), and *Semperflorens,* a natural mutant that presents continuous flowering all year (Antunes 1960). We hypothesized that differences between those coffee genotypes might be associated with sugar metabolism, floral identity genes, and plant hormone (Ethylene) evolution during the coffee flowering process. To validate this hypothesis, transcriptomic analysis (RNA-seq), ethylene evolution, and sugar content was performed in leaves, flower buds, and Shoot Apical Meristem (SAM). This study will help to elucidate how those factors are influencing the coffee flowering process.

2. MATERIAL AND METHODS

2.1. Plant Material

Three *Coffea arabica* genotypes classified as early, late, and continuous about their flowering and fruit ripening pattern were selected: cv. Oeiras, Acauã, and *Semperflorens*, respectively. Cultivar Acauã comes from the cross between 'Mundo Novo IAC 388-17' and 'Sarchimor' (IAC 1668) carried out by IBC technicians in 1975/76, in Paraná, where it was registered with the number IBC – PR 82010. Oeiras, it was developed by the genealogical method from the hybrid CIFC HW 26/5, resulting from the cross between 'Catura Vermelho' (CIFC 19/1) and 'Híbrido de Timor' (CIFC 832/1), being, therefore, a cultivar belonging to the Catimor germplasm. *Semperflorens*, is a natural mutant identified by the IAC, which flowering almost continuously throughout the year. It presents with dense foliage, leaves a little smaller than those of the Típica cultivar, of a more intense green, shorter branches and, apparently, more resistant to drought, genetic analysis showed that this mutation is from “Bourbon” cultivar (Antunes 1960; Carvalho et al. 2008)

The experiment was conducted in a five-year-old coffee plantation at the Department of Agriculture of the Federal University of Lavras (UFLA) following a randomized block design with three biological replicates, each one comprising ten plants. For RNA-seq analysis, sampling of Flower Bud (FB) (G1, G2 stage for Oeiras and Acauã), and G1 till G3 for *Semperflorens*) (Morais et al., 2008), and Shoot Apical Meristem (SAM), which took place from 8:00 to 10:00 AM in March and three leaves (young and fully expanded at the third or fourth node from plagiotropic branches), were collected in March and August (Fig. 1). Samples were collected and immediately frozen in liquid nitrogen and stored at -80 °C for maceration and RNA extraction. For sugar content analysis, leaves were collected monthly from March to September, frozen in liquid nitrogen, and stored at -80°C. To evaluate the flowering pattern of every coffee genotype, FB development stages and anthesis events were recorded monthly from March to September.

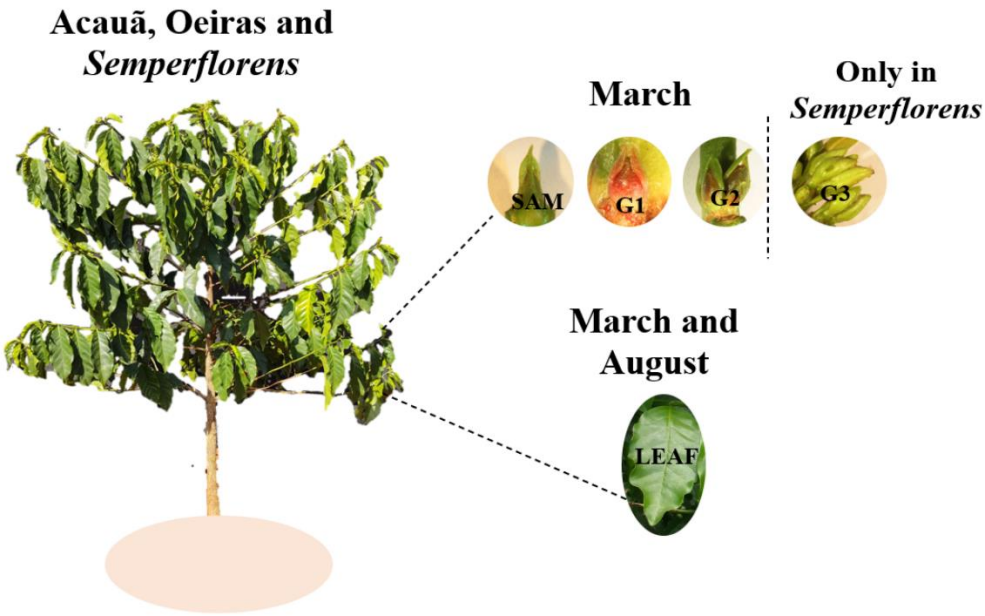

Fig. 1. Tissue sampling in Acauã, Oeiras, and *Semperflorens* coffee genotypes. For Acauã and Oeiras FB in G1 and G2 stages and SAM were collected in March. For *Semperflorens*, in addition to the same tissues collected for Acauã and Oeiras, FB in the G3 stage were collected. Leaves were collected in March and August for all coffee genotypes.

2.2. Analysis of flower bud development and anthesis

Coffee genotypes in this study are defined as Late (Acauã), early (Oeiras), and continuous (*Semperflorens*) regarding their flowering pattern (Carvalho et al. 2008). To evaluate those differences, two physiological analyses were carried out in the field: FB development and anthesis events. For the first analysis, in every coffee genotype was selected four plants, four branches per plant, and three nodes in every branch. In every node, FB in different stages was evaluated monthly from March to September, and the number of flower buds in each developmental stage was evaluated according to Morais et al. (2008). For the second analysis, in every anthesis event, the number of opened flowers were recorded. Three branches per plant and three nodes in every branch of each coffee genotype were selected. Evaluations were made according to the flowering events, which happened concerning FB development and the flowering pattern of each coffee genotype (generally after a rain event).

2.3. RNA extraction

Total RNA from leaves, FB, and SAM were extracted according to de Oliveira et al. (2015), with minor modifications. RNA samples (7,5 µg) were treated with DNase I using the Turbo DNA-free Kit (Ambion) to eliminate DNA contamination. RNA integrity was analyzed

in 1 % agarose gel, and RNA content, as well as quality, were accessed by spectroscopy ($OD^{260/280}$ and $OD^{260/230}$ > 1.8) (NanoVue GE Healthcare, Munich, Germany) and validated by using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). All samples presented standard values and RNA integrity numbers (RIN) higher than 6.0.

2.4. RNAseq analysis

A total of 2,765,320,286 non-strand-specific paired-end reads (2 x 150 bp) were sequenced from Leaves, SAM, and FB of *Coffea arabica* L. (3 genotypes, and, for leaves samples, two different months of 2020 (March and August) with 4 biological replicates each) using an MGI-tech DNBSEQ-G400 sequencer. The libraries were sequenced, and quality controlled done by BGI Genomics Co., Ltd. The method for filtering coding RNAs from other classes of RNAs was the oligo dT selection (also called polyA selection). In the subsequent steps, parameters such as overall sequence quality, sequence length distribution, duplication levels, overrepresented sequences, and adapter content were accessed using FastQC version 0.11.8 (Andrews, 2010). All RNAseq libraries were aligned to the *Coffea arabica* L. genome (available at https://www.ncbi.nlm.nih.gov/genome/?term=txid13443[orgn]) using the annotation Cara_1.0 with STAR version 2.7.8a (Dobin et al. 2013). The resulting alignment files in. bam format was sorted and inspected for highly duplicate fragments with Picard tools version 2.25.4 (Picard toolkit, 2019). Fragments uniquely mapped to exons were quantified with the HTSeq-count script (Anders et al. 2015). After quantification, fragment counts were summarized with inhouse R scripts, and edgeR (GLM mode) Bioconductor package (Robinson et al., 2010; Huber et al., 2015) was used to normalize fragment counts and infer genes Up and Down-regulated across relevant contrasts. Genes were considered Differentially Expressed (DE) if their false discovery rate - FDR (Benjamini and Hochberg, 1995) - were below 0.05 and had a minimum expression fold change of two. In other words, a given gene is called DE if the difference in expression between two conditions is at least two times the expression in the condition with the lower expression value and passed in a stringent statistical procedure called FDR.

2.5. Ethylene measurement

In every sampling time (From March till September), leaves, FB, and root tissues were collected and immediately incubated in 10 mL vacutainer glass tubes, containing a moist tissue placed on the bottom of each vial, sealed with serum caps, and incubated for 24 hours. For each biological sample ethylene was quantified from the headspace gas using the F-900 Portable

Ethylene Analyzer (Felix Instruments, USA) operating under the GC emulation mode in triplicate. Plant material was incubated in two separate vials, and the headspace gas was withdrawn from the vials with a 10 mL plastic syringe. Samples made of 2.5 mL of gas from each vial were extracted using the same syringe and subsequently injected into the F-900 (Lima et al. 2020). After ethylene measurement, plant material was weighed, and ethylene production rate was expressed as ppm g^{-1} FW h^{-1}.

2.6. Carbohydrate analysis

Carbohydrates were extracted from 0.2 g of completely expanded leaves as described by Campos et al. (2019). Macerated samples had added 5mL of potassium phosphate buffer solution 100 mM at pH 7.0, then the samples were heated at 40ºC for 30 minutes and centrifuged at 5.000 g for 10 minutes. The supernatant was collected for sucrose quantification by the anthrone reagent method described by Van Handel (1968), total soluble sugars (TSS) analysis using the protocol described by Yemm and Willis (1954), and reducing sugars (RS) by the dinitrosalicylic acid method (DNS) described by Miller (1959). The pellets resulting from the centrifugation were resuspended in 4mL of potassium acetate buffer solution 200 mM pH 4.8 and added with 1 mL of amyloglucosidase enzyme solution 1 mg/mL, incubating in a double boiler at 40 ºC for two hours. Thereafter, the extract was centrifuged at 5.000 g, for 20 minutes. The resulting solution was used for starch quantification by the anthrone method described by Yemm and Willis (1954).

2.7. Statistical Analysis

For Sugar and ethylene content data analyses were performed using the InfoStat software (Di Rienzo et al. 2020). The statistical difference was determined by one-way ANOVA, followed by the Tukey test. Results were expressed as the mean ±Standard Deviation (SD).

3. RESULTS

3.1. Flowering bud development and anthesis

The floral development of coffee genotypes was evaluated monthly from April to October (Fig. 2), which correspond to the main period of floral bud (FB) development until flowering (Camargo and Camargo 2010; reviewed by Lopez 2021 under submission), and the FB classified according to its size (Morais et al, 2008). Firstly, in April, Acauã and Oeiras showed most FB at the G2 stage, whereas *Semperflorens* present FB at many different

developmental stages (37% in G2, 32% in G3, 14% in G4, 2% in G5, 3% of flowers and 11% of fruits) revealing its typical continuous flowering (Fig. 2A). Then, in May, Acauã and Oeiras presented 96% and 81% of G2 buds respectively, but Oeiras also showed FB in more advanced stages as G3 (16%) and G4 (2%), which agrees with its reported early-flowering pattern. For *Semperflorens*, once again, the mixed pattern of developmental stages was found, 21% of FB in the G2 stage, 28% in G3, 15% in G4, 1% in G6, 31% in fruit, and 4% as a vegetative branch (Fig. 2A).

During the floral development in the next month, the same pattern happened with Acauã showing more FBs at late stages of development compared to Oeiras and *Semperflorens* presenting many stages at the same time including fruits, according to the flowering-time characteristic of each genotype (Fig. 2A). Noteworthy, the harvesting of the previous producing year (coffee plants are biennial) occurred in July concomitantly with analysis of FBs at the new branches of the year, which causes a loss of FBs and reset in the development of latent buds. The continuity of evaluations showed that despite this FBs loss, the same developmental patterns were maintained between genotypes. For example, in July, Acauã presented 97% of G2 buds and 3% in G3, whereas Oeiras 70% in G2 and 27% in G3 and *Semperflorens* 88% in fruit, 1% in G2, 9% in G3, and 1% in G4 stage (Fig. 2A). The same pattern occurred in August and September, with Oeiras showing FBs at more advanced stages of development. Finally, the last step of FBs development was evaluated by counting the major flowering events observed for coffee genotypes along the period (Fig. 2B). *Semperflorens* was the more precocious and with a higher number of flowering events presenting five antheses, whereas Oeiras presented four antheses with the first occurring on May 29th contrasting to Acauã with three events being the first on July 20th (Fig. 2B). Additionally, in October the flowering process for all coffee genotypes was over and the following process was the filling and development of grain (Fig. 2A). Thus, from these results, we could prove the contrasting floral development and flowering-time between genotypes, which suggests differences in the related regulatory molecular pathways and the next analysis explored this hypothesis.

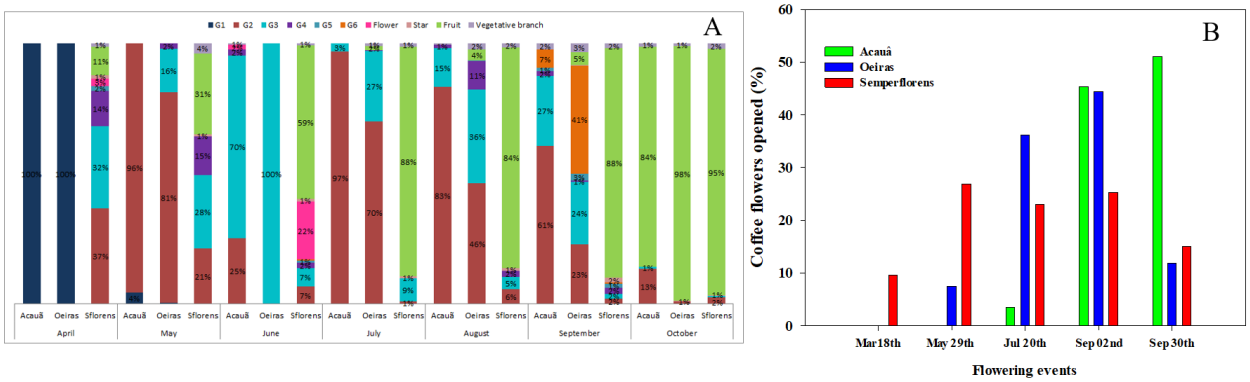

Fig. 2. Representation of coffee FB development at different stages (A) and Flowering events in a year (B) for Acauã, Oeiras, and *Semperflorens*

3.2. RNAseq analysis

From all sequenced reads, 2,068,658,034 (75% of the sequenced fragments) were uniquely mapped to exons in the genome. During the quality assessment, we identified that the majority of libraries had an excess of duplicated fragments. If quantified, a duplicate fragment can inflate the expression of a given gene and impair the Differential Expression (DE) analysis (Peng et al. 2015). For that reason, we scanned all libraries for duplicates with Picard tools to remove all duplicated fragments from the downstream analysis. In this way, a total of 876,719,229 high-quality, individual, and uniquely mapped fragments were quantified for DE analysis which represents a mean value of 18.3 million fragments uniquely mapped to exons in the *C. arabica* genome. The remaining fragments represent a subset of 32% of the total sequenced fragments (Figure 3).

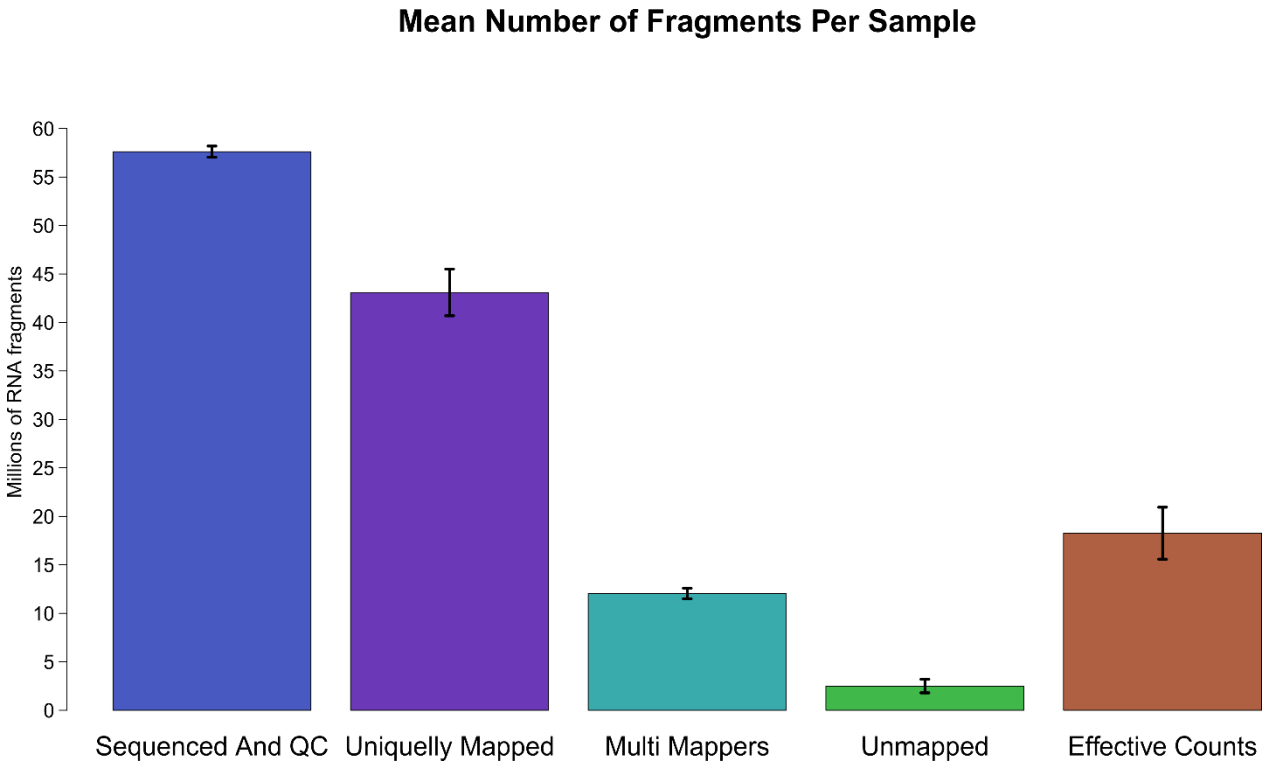

Figure 3. Bar plot depicting the mean values of the number of fragments per sample that were sequenced, uniquely mapped, multi mapped, or unmapped to the *Coffea arabica* genome. The effective counts bar represents the sub-set of RNA-seq fragments that were effectively used *per* sample in the differential expression analysis. Error bars represent the standard deviation, values are presented as millions of RNA fragments.

From the quantified fragments, we applied normalization procedures to infer the relationship between different samples. Firstly, we applied a Multidimensional Scaling (MDS) approach to access the Euclidean distance between the expressed genes in each sample (Fig. 4A), those values were computed using edgeR in the GLM model (Robinson et al. 2010). Doing so, we found that samples of the same organ in different varieties tend to cluster together such as leaves (Leaf) in the left of dim1 and Shot Apical Meristem (SAM) in the right of dim1, Oeiras (Oei) and Acauã (Aca) Flowering Buds (FB) clustered together in the bottom of Dim2 whereas, surprisingly, FB from the *Semperflorens* are closer to the SAM of all Varieties. Additionally, we calculated the correlation between the normalized expression values (in Counts Per Million – CPM) of all genes in all samples using the spearman method and plotted the result as a Clustering Heatmap (Figure 4B). Following the multidimensional scaling approach, we found that the expression of genes differs mainly as a function of the organ, however, a special case can be verified, as pointed out in the MDS plot, where FB of *Semperflorens* is more meristem-

like when compared to the other two genotypes. Three clear distinctive clusters could be identified in the clustering tree, one composed of leaves samples, the other from FB of Oeiras and Acauã, and a third one composed of all SAM and *Semperflorens* FB.

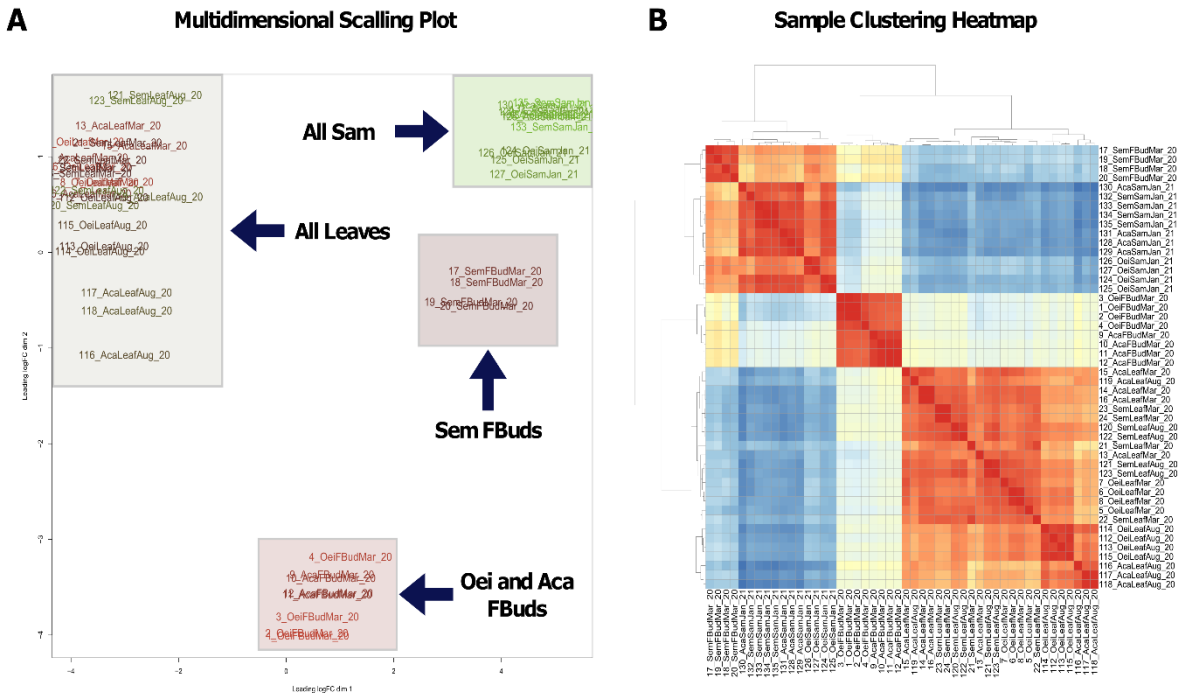

Figure 4. A Multidimensional scaling plot for all libraries after edgeR GLM normalization. **A)** Rectangles are an approximation of the dimensional space of a given organ. Horizontal and vertical axes are principal components explaining the variance of the data. The distance between each pair of samples is the Euclidean distance for the expressed genes. Distances between two samples are in log2-fold-change. The closer the distance the more similar the expression of the expressed genes in the samples. **B)** Heatmap representation of the Spearman correlation of all expressed genes across all samples. Hierarchical Clustering Tree was performed with the hclcust algorithm. Colors are coded as levels of correlation between two libraries.

3.3. Differential Expression

A total of 12478 genes, 15% of all annotated in the reference genome, was found to be Differentially Expressed (DE) in the analyzed conditions (Fig. 5). The most relevant contrasts are those comparing leaves from the same genotype in different months; leaves from different genotypes in March 2020; leaves from different genotypes in August 2020; FB from different genotypes; SAM from different genotypes and FB versus SAM in the same genotype. In this way, we analyzed 18 contrasts involving the 3 organs, 3 varieties, and 3 months. It is important to note that, if a Differentially Expressed (DE) gene is Up-regulated in this comparison, it means

that it is more expressed in the condition after the "_x_". In other words, the second element in the comparison is always the one being referred to. For example, a gene is reported to be Up-regulated in AcaSAM x OeiSAM which means that this gene is more expressed in the SAM of Oeiras plants rather than in the SAM of Acauã plants in the same condition, the same analogy can be made for the Down-regulated genes.

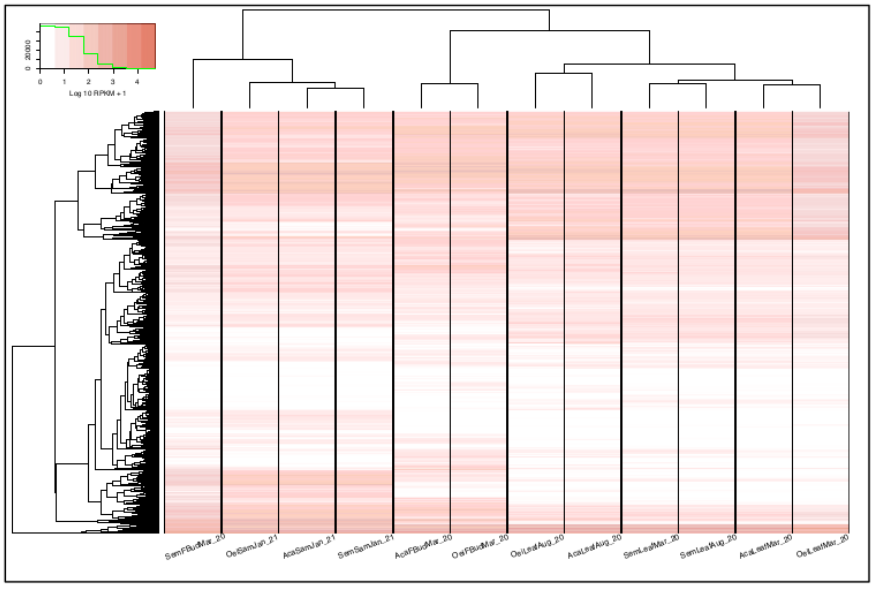

Figure 5. Heat map representation of all differentially expressed genes (lines) across all conditions (columns). The dendrogram on top of the heatmap reflects the relationship of different conditions whereas the dendrogram on the left reflects the relationship of different genes across conditions. Colors are coded as a function of logarithmic base 10 of expression values normalized in FKPM (Fragments per kilobase per million mapped reads).

3.4. Differential expressed Genes (DEGs) in Flower Buds (FB) and Shoot Apical Meristem (SAM)

The total of differential expressed genes (DEG) for FB, Shoot Apical Meristem (SAM), and (FB x SAM) were compared for all coffee genotypes. For the graph interpretation in each contrast, the number of genes in each circle represents the difference between the second genotype against the first. For example, in contrast to FB_ Acauã x Oeiras, there is 937 DEG in Oeiras against Acauã. The Venn diagram shows the number of the same DEG expressed concerning other contrasts. From 937 DEG, 194 are differentially expressed in the contrast FB_ Acauã x SF, 323 DEG are differentially expressed in all contrast, 296 DEG are differentially expressed in the contrast FB_OeirasxSF and only 124 DEG are exclusives and different in the contrast FB_ Acauã x Oeiras.

Results show that the number of DEG exclusives between Acauã and Oeiras are inferior to in the other contrast (FB_ Acauã x SF and FB_Oeiras x SF), meaning that Acauã and Oeiras are more similar in DEG in FB compared with *Semperflorens*. On the other hand, there are more DEG in FB_Oeiras x SF contrast than FB_ Acauã x SF showing a marked difference of Oeiras in relation to *Semperflorens* that the other coffee genotypes. Only 323 DEGs are shared in all contrast (Fig. 6A). For SAM there are 1226 DEG in the contrast SAM_ Acauã x Oeiras, 1097 DEG in the contrast SAM_Oeiras x SF, and 479 DEG in the contrast SAM_ Acauã x SF and only 56 DEG are shared by all contrast. There is more similarity between SAM of Acauã and Semperflorens because it presents less DEG in comparison with other contrast (Fig. 6B). In the comparison of FB x SAM contrast in the same coffee genotype, the genotype Oeiras presented more DEG in SAM against FB, but there is a high quantity of DEG shared between all contrast (2700). Notably, Oeiras and Acauã contrast have more DEG in common (3408) compared with *Semperflorens*, and fewer DEG exclusives are observed in *Semperflorens*, possibly because DEG in SAM and FB in this genotype is presented a similar expression as showed in (Fig. 6C).

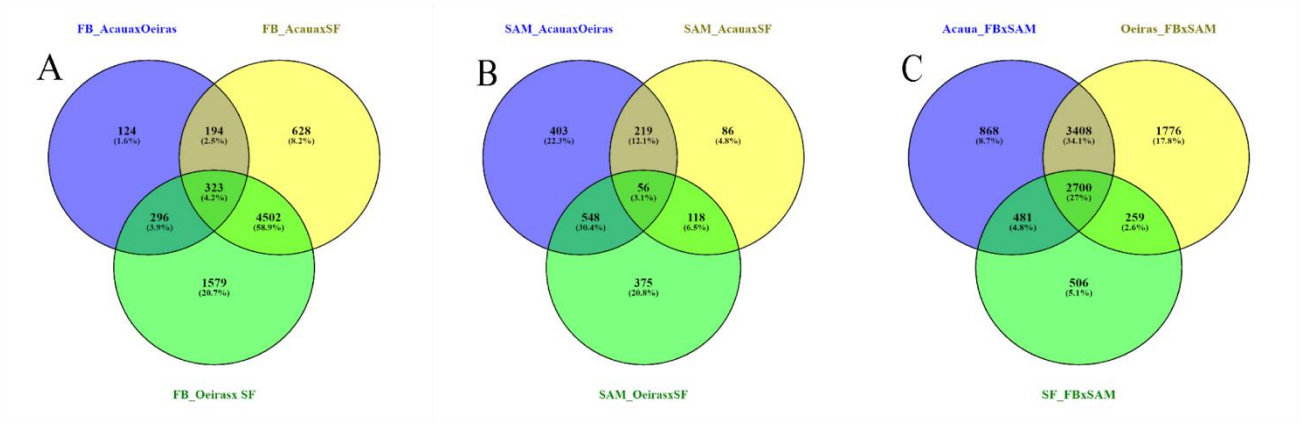

Figure 6. Venn diagram representing the total DEG in each contrast for A) FB, B) Shoot Apical Meristem (SAM), and C) FB vs SAM

3.5 Differential Expressed Genes (DEGs) in Leaves in March and August

For leaves, the total of DEG was compared in March and August, for all coffee genotypes (Oeiras, Acauã, and *Semperflorens*). A comparison was made using the contrast of all DEG between coffee genotypes in each and between months. Then, the contrasts were grouped to compare the number of DEG between them (Fig. 7). The number of exclusive DEG in March for LM_ Acauã x Oeiras contrast was inferior concerning the other contrast (LM_ Acauã x SF and LM_Oeiras x SF), showing that Acauã and Oeiras are very similar compared with other contrast involving *Semperflorens*. Meanwhile, the number of DEGs shared for all

the contrasts (50) is inferior compared with August (LA) and March x August (L_MxA) (Fig. 7A). In August, there was an increase in the number of exclusives and shared DEG for all contrasts compared with March (Fig. 7B). The number of exclusive DEGs (626) between March and August for *Semperflorens* was inferior compared with Oeiras and Acauã and the number of DEGs shared between Acauã and Oeiras is superior to the number of DEG shared of both genotypes with *Semperflorens* (Fig. 7C).

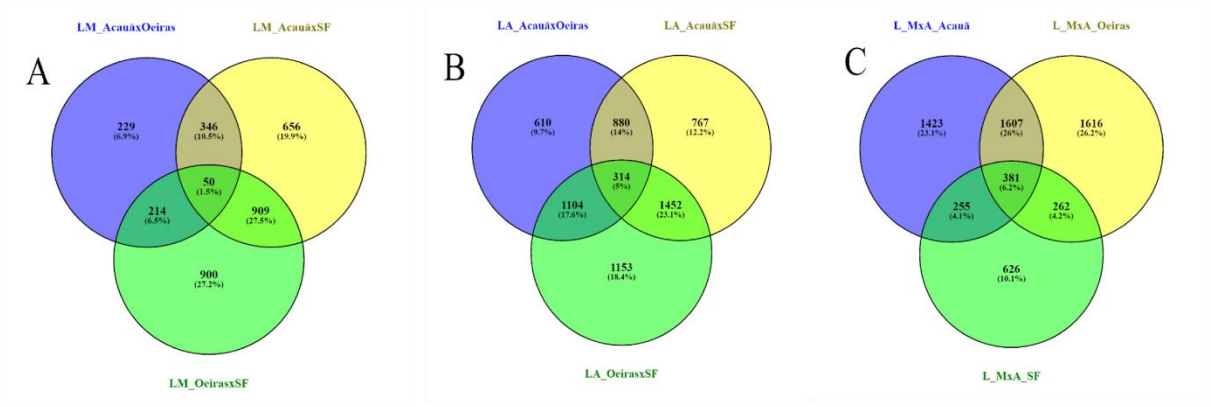

Figure 7. Venn diagram representing the total DEG for A) Leaves in March (LM), B) Leaves in August (LA), and C) Leaves compared between March and August for each genotype (L_MxA).

3.6. Identification of Gene Ontology (GO) in Flower Buds and Shoot Apical Meristem (SAM)

Aiming to describe the main metabolic function of sequenced genes found in this study, we summarize the number of the Gene Ontology description (GO) of UP and DOWN regulated genes for each function in FB and SAM. At this point, the objective was to compare metabolic differences associated with the flowering process between Early (Oeiras), Late (Acauã), and (*Semperflorens*) continuous coffee genotypes. In every contrast was possible to observe in commonly expressed genes for the following biological functions: Hormonal regulation, flower development, temperature stimulus, external stimulus, external stimulus, sugar metabolism, photosynthesis, and seed development. For Acauã and Oeiras (AcaxOei) contrast, 415, 115, and 125 UP-regulated genes for hormonal regulation, flower development, and endogenous stimulus, respectively were detected.

In addition, UP and DOWN-regulated genes related to temperature stimulus and external stimulus (Fig. 8 (A1)) could also be seen. The most important in this contrast is the UP-regulated genes in Oeiras associated with hormonal regulation and flower development because sampling tissue was in march, which means that differences in coffee flowering time of those genotypes could be associated with those metabolic processes, which are more active

in Oeiras. On the other hand, the expression pattern in the number of UP and DOWN-regulated genes observed in the contrast Acauã x *Semperflorens* (AcaxSF) and Oeiras x *Semperflorens* (Oeix SF) is very similar (Fig. 8 (A2 and A3)).

That means the *Semperflorens* genotype is metabolically different from Oeiras and Acauã because UP and DOWN-regulated genes for other metabolic processes such as seed development, sugar metabolism, and photosynthesis are expressed. For Shoot Apical Meristem (SAM), it was evaluated the same metabolic processes as FB. In the contrast Acauã x Oeiras (AcaxOei), we found only Up-regulated genes in Oeiras for all metabolic processes evaluated, highlighted the number of genes observed for photosynthesis, hormonal regulation, and flower development (Fig. 8 (B1)). In the contrast Acauã x *Semperflorens* (AcaxSF), only Down-regulated genes were found for temperature and external stimulus in *Semperflorens*, indicating that SAM for both genotypes is similar in DEG compared with other contrast (Fig 8 (B2)). In the contrast to Oeiras x *Semperflorens* (OeixSF), we observed having only DOWN-regulated genes for Oeiras except for sugar metabolism (Fig. 8 B3). FB and SAM were contrasted in the same coffee genotype, aiming to compare the number of DEG in both tissues. The number of UP and DOWN-regulated genes in the three contrasts is similar (Fig. 8 (C1, C2, and C3)). However, the major gene activity is in FB, because more DOWN-regulated genes in sugar metabolism, hormonal regulation, flower development, temperature, endogenous and external stimulus were observed.

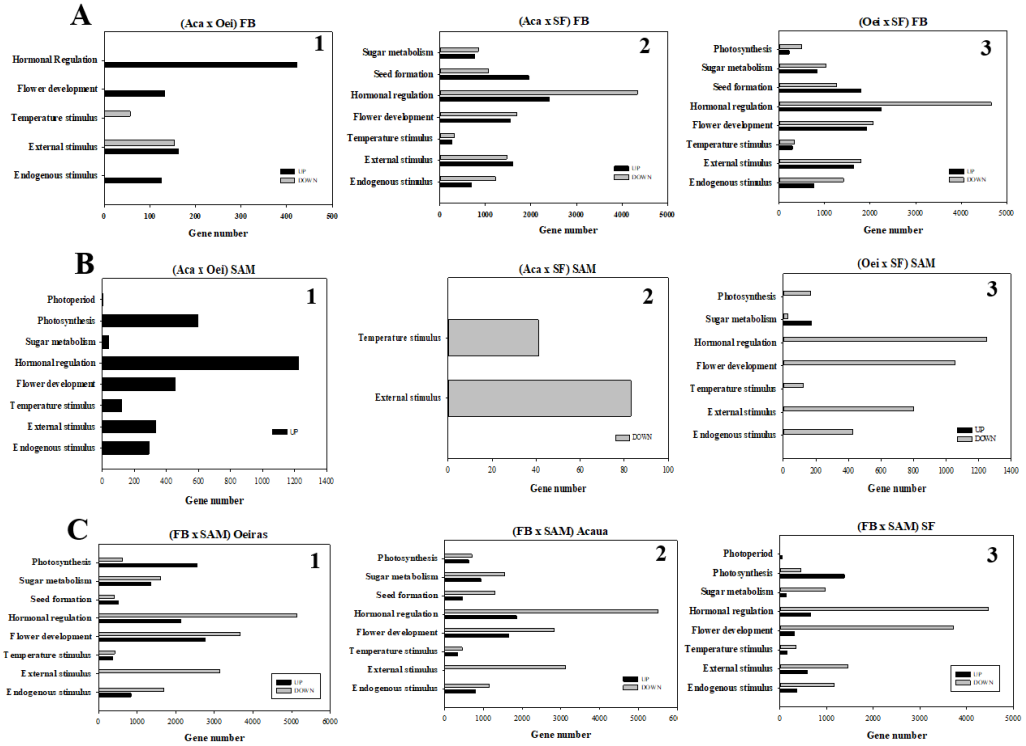

Fig. 8. Summary of the most important UP and DOWN-Regulated genes and Gene Ontology (GO) functions found in Acauã, Oeiras, and *Semperflorens* and their contrasts (A) Flower bud (FB), (B) Shoot Apical Meristem (SAM), and (C) Contrast of Flower Bud x Shoot Apical Meristem (FB x SAM).

3.7. Identification of Gene Ontology (GO) in Leaves

For leaves, the number of UP and DOWN-regulated genes were evaluated by contrasting coffee genotypes in March, August, and March x August. Both months, March and August, represent the beginning and final process of coffee flower development, respectively. Moreover, in March wheather is prevalent are summer conditions and rainy, whereas in August represents winter, low temperatures, and a water deficit period. In the contrast Acauã x Oeiras (AcaxOei) in March, we found UP-regulated genes for flower development and hormonal regulation in Oeiras, however, for hormonal regulation, there are more DOWN-regulated for Acauã indicating that possibly that phenotypical differences between Oeiras and Acauã in flowering time could be regulated by those metabolic processes (Fig. 9 (A1)), and highlighted the number of DOWN-regulated genes for external stimulus. There are only DOWN-regulated genes for Acauã in contrast to Acauã x *Semperflorens* (AcaxSF), highlighting the number of genes for hormonal regulation and external stimulus (Fig. 9 (A2)).

In the contrast, Oeiras x *Semperflorens* (OeixSF), was observed DOWN-regulated genes in Oeiras for all metabolic processes being higher the number for external stimulus, hormonal regulation, and vegetative to reproductive phase (Fig. 9 (2C)). In August, DOWN-regulated genes were observed for sugar metabolism, hormonal regulation, flower development, and external stimulus in Acauã, and in the contrast, Acauã x Oeiras (AcaxOei) (Fig. 9(B1)) being consequent in the metabolic status with the flowering stage of both genotypes because Oeiras has experimented some flowering events, whereas Acauã is next to its first flowering event. In the contrast Acauã x *Semperflorens* (AcaxSF), there are UP-regulated genes for the photosynthesis process in SF, whereas the majority of genes are DOWN-regulated for Acauã, especially for hormonal regulation, sugar metabolism, external stimulus, and flower development (Fig. 9(B2)).

In the contrast Oeiras x *Semperflorens* (OeixSF), a balance between UP and DOWN-regulated genes was observed for endogenous stimulus, external stimulus, temperature stimulus, and photosynthesis, however, a higher number of DOWN-regulated genes were observed for flower development and hormonal regulation, and vegetative to reproductive phase (Fig.9 (B3)). Some UP-regulated genes for the circadian clock were observed in this contrast for *Semperforens.* For the comparison of UP and DOWN-regulated genes between

March and August in leaves, there are some differences in coffee genotypes. For Acauã, the majority number of expressed genes was in March, especially for hormonal regulation and external stimulus (Fig. 9 (C1)). Oeiras showed a similar number of UP and DOWN-regulated genes in March and August, except for the vegetative to reproductive phase, photosynthesis, and endogenous stimulus (Fig. 9 (C2)). For *Semperflorens*, a higher number of UP-regulated genes were observed in August, especially for hormonal regulation and flower development (Fig.9 (C3)).

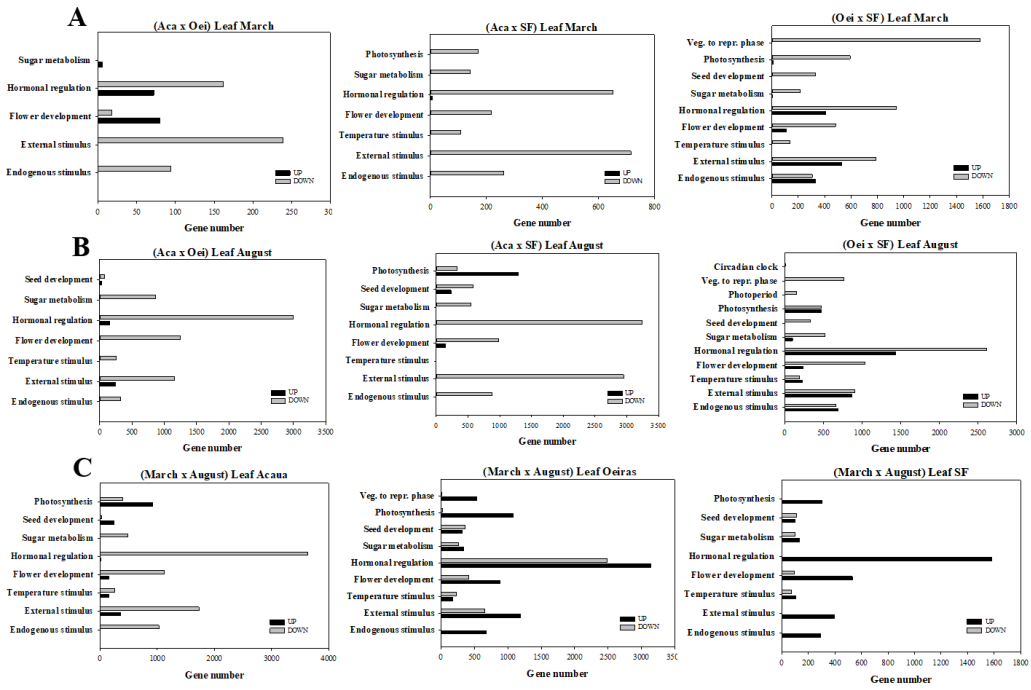

Figure 9. Summary of the most important UP and DOWN-Regulated genes and Gene Ontology (GO) functions found in Acauã, Oeiras, and *Semperflorens* and their contrast in (A) Leaves in March, (B) Leaves in August, and (C) Contrast of leaves between March and August (March x August).

3.8. Sugar Analysis

Leaves Carbohydrates were evaluated during the reproductive coffee tissues development for three coffee genotypes (Acauã, Oeiras, and *Semperflorens*). For the starch content, a gradual increase for all genotypes with a peak in august was observed, and the coffee genotype *Semperflorens* differed in starch concentration, showing a greater accumulation of carbohydrates, which may favor the development of buds and flowering at various times of the year (Fig. 10A). Sucrose content had a higher concentration in July and August, tending to an upper concentration in Oeiras and *Semperflorens* in April and May. Acauã showed an increase of sucrose from June to August, showing a differential behavior in the evolution of this carbohydrate between Oeiras and *Semperflorens* (Fig. 10B). For total sugars, there was a peak

in July for all coffee genotypes, a period that precedes the flowering time that occurred in August, although Oeiras genotype presented a higher concentration in May (Fig. 10C). For reducing sugars there was also an increase until September, with no significant difference between genotypes (Fig. 10D).

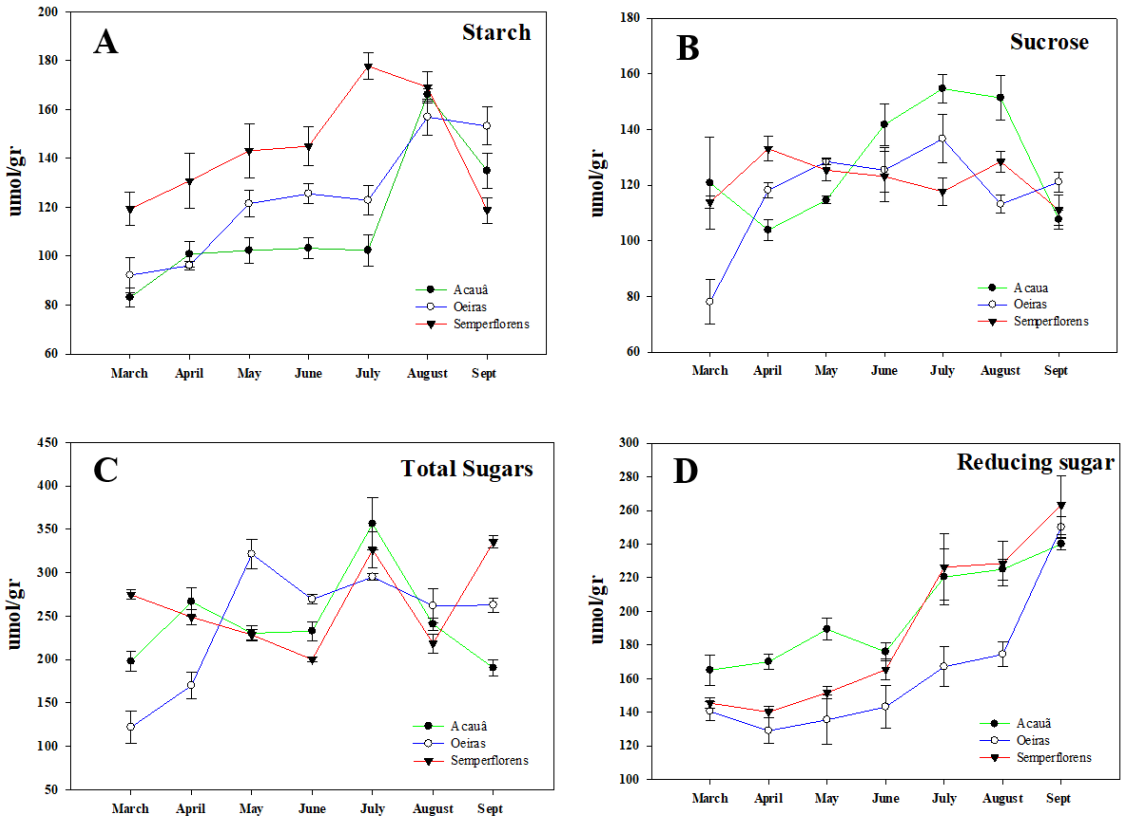

Figure 10. Carbohydrate content (A) Starch, (B) Sucrose, (C) Total sugar, and (D) Reducing sugar from leaves of Acauã, Oeiras, and *Semperflorens*.

3.9. Ethylene evolution

Ethylene evolution was measured in leaves and FB from March to September when this is the most critical period in the development of coffee FB including anthesis. Ethylene evolution in FB between Acauã and Oeiras was similar in all months, except for July where ethylene content showed a peak for Oeiras. Conversely, the ethylene evolution for *Semperflorens* was different compared with Oeiras and Acauã between March and May. Increased levels of ethylene were observed in *Semperflorens*, but it decreasing from June to September, a similar pattern evolution in Acauã and Oeiras (Fig. 11A). The ethylene evolution pattern in leaves was different than in FB. All coffee genotypes showed a decrease from April to September, however, Acauã presented less content than Oeiras and *Semperflorens*. Ethylene content between Oeiras and *Semperflorens* was similar in all sampling periods (Fig. 11B).

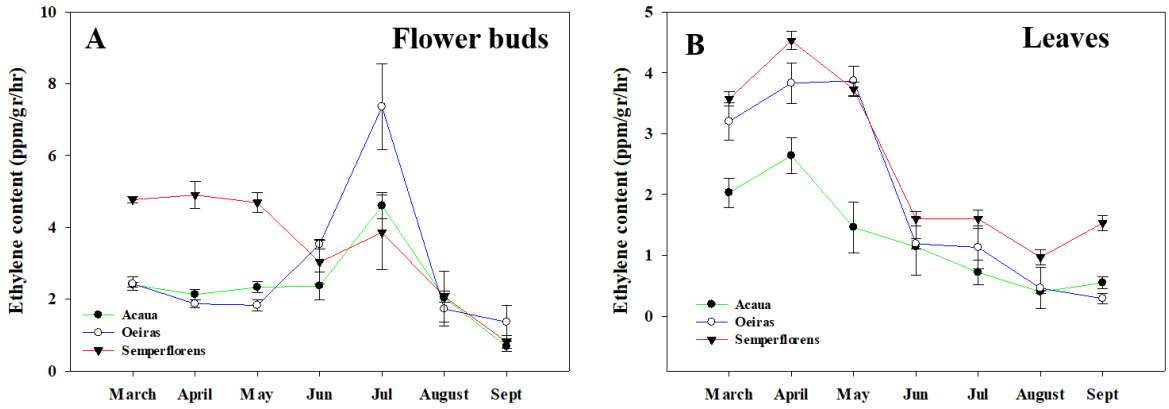

Figure 11. Ethylene evolution in (A)Flower buds and (B) Leaves for Acauã, Oeiras, and *Semperflorens*.

4. DISCUSSION

The coffee flowering process represents one of the most important steps in the cultivation production chain. It is influenced by endogenous and environmental stimuli, which regulate the flowering time in each production cycle. Furthermore, it is directly related to the yield, and the final product (Cup quality). In this sense, the selection of a coffee genotype to be planted as early or late is directly related to these factors in the harvest time. Coffee producers need to combine coffee genotypes with different flowering patterns in the field to synchronize the harvest time and avoid excessive costs and reduction of product quality.

Understanding the main factors determining the coffee flowering time is important in terms of genetic improvement, selecting those coffee genotypes adjusted to the producer requirements, and adaptability to the frequent climatic variations. In this study, three coffee genotypes contrasting in their flowering pattern, Oeiras (Early), Acauã (Late), and *Semperflorens* (Continuous) were selected. The core objective was to elucidate the main factors influencing this coffee flowering pattern. We were focused on hormonal regulation genes, especially ethylene, floral identity, control of flowering time, and sugar metabolism. We hypothesized that those differences in the flowering pattern of the coffee genotypes selected for this study are associated with all or some of those biological processes. To complement the RNA-seq analysis, we measured the sugar and ethylene content behavior from March to September which represents the most critical period in flower development and coffee anthesis. Moreover, we quantified the flower development evolution and flowering events in each coffee genotype.

4.1. Candidate genes involved sugar metabolism and flowering process

Sugars are the main product of photosynthesis and are mobilized to different plant organs for growth tissue differentiation. The role of sugars in flowering has been studied, determining that sucrose seems to function primarily in the leaf phloem to enhance the production florigens as Flowering Locus T (FT), whereas Trehalose-6-phosphate acts in the SAM to promote the signaling pathway downstream for the flowering process (Cho et al. 2018). The content of starch, sucrose, total sugars, and reducing sugars was evaluated for all coffee genotypes and the result showed that the sugar level increased from March to August, during the FB development period.

The genotype *Semperflorens* presented a higher level of starch as a sugar reserve for its metabolic processes compared with Acauã and Oeiras. However, before flowering time (August) all genotypes presented the same starch content. The sucrose content in Oeiras and *Semperflorens* is very similar in all months, but for Acauã, the sucrose content increased from June to August, coinciding with the flowering time of this. The role of sugar in the flowering time has been widely studied since sugar represents energy for the metabolic processes in plants. Sucrose promotes flowering in several species (Bernier et al. 1993). Transcriptome analysis using mature leaves in maize (*Zea mays* spp *mays*) sampled at the floral transition stage has shown that expression levels of several key genes involved in starch and sucrose metabolism are altered in the *id1* mutant (Coneva et al. 2012). Those findings suggested that the balance between transitory starch and sucrose is important for controlling flowering time. Increased level of endogenous sucrose also enhances flowering in tomatoes (*Solanum Lycopersicum*) (Micallef et al. 1995).

The flowering events were higher in *Semperflorens* than Acauã and Oeiras, whereas Oeiras presented more flowering events than Acauã (Fig. 2B). It seems that sucrose content in Acauã is needed close to the main flowering event in September and for that reason, we observed an increase from June to August, whereas for *Semperflorens* and Oeiras, sucrose content is not concentrated in a specific period because flowering events are more frequent. Total sugars increased in may for Oeiras but not for Acauã and *Semperflorens*, showing a peak in July, decreasing before anthesis in august. Reducing sugars showed an increased pattern but no differences were observed between the coffee genotypes. The RNA-seq analysis showed a high number of UP and DOWN-Regulated genes involved in sugar metabolism. Meanly those associated with enzymatic processes in cleaving and sugar production, and intracellular sugar transport.

In the Gene Ontology analysis for leaves, FB and SAM, we found some interesting contrasts showing that sugar is involved in those tissues possibly as signaling of flowering time. A higher number of DEGs were found in leaves than FB and SAM because the photosynthetic process is superior in leaves. Finally, in figures (12 A and B), the expression of two genes as candidates for starch biosynthesis in *Semperflorens* in March (glucose-6-phosphate/phosphate translocator 2%2C chloroplastic-like and glucose-1-phosphate adenylyltransferase large subunit 1-like) is shown. In figure 12 (C and D) is presented the expression of two candidate genes as sucrose transporter in August (Sugar transport protein 13-like and Hexose carrier protein HEX6-like). The expression of those genes is congruent with the high starch content observed in *Semperflorens* in March (Fig. 10A) and the increased level of sucrose for Acauã in August (Fig. 10B), respectively.

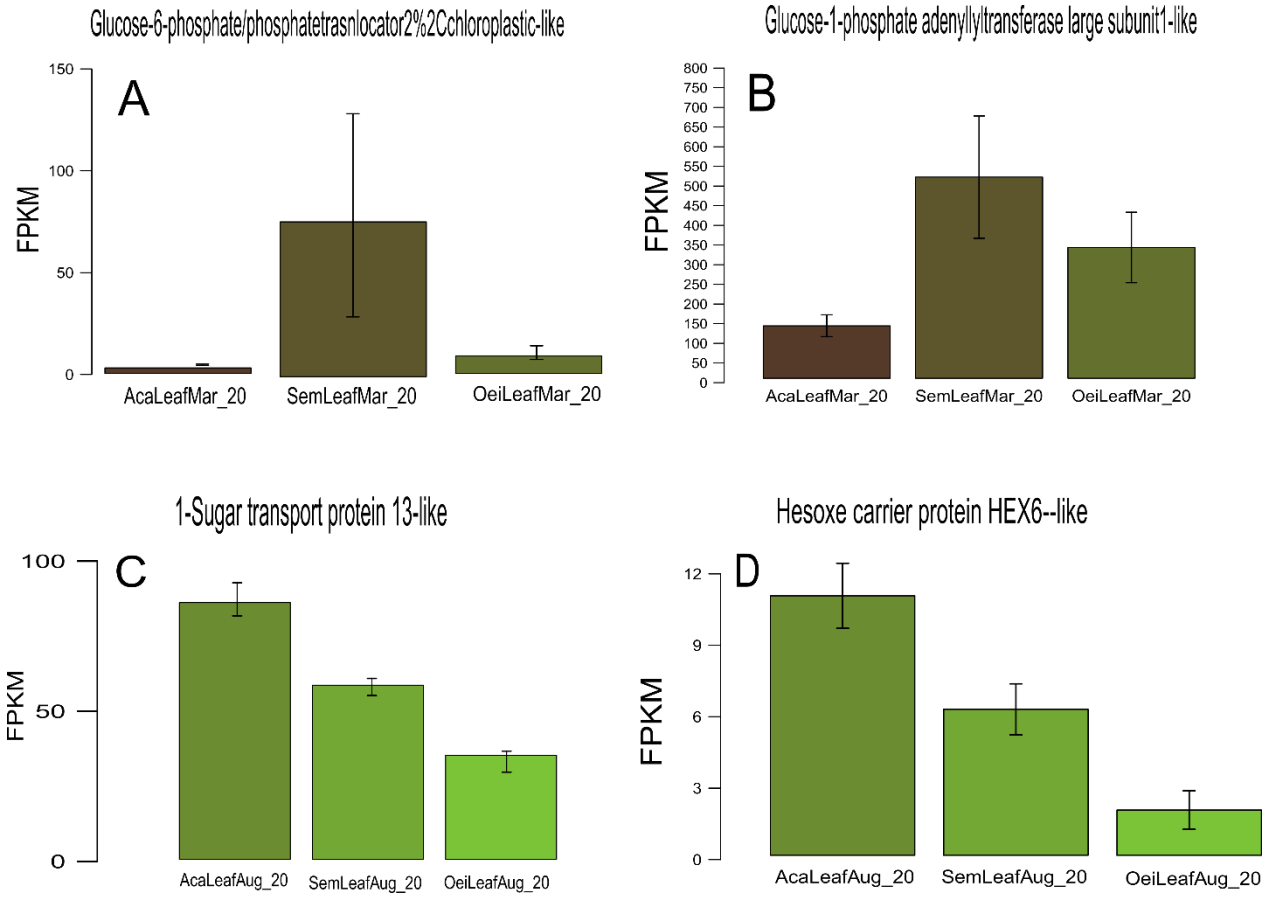

Figure 12. Candidate expressed genes for enzymatic starch biosynthesis for *Semperflorens* in March (A and B), and candidate genes for sugar transport in Acauã in August (C and D).

4.2 Hormonal regulation in coffee flowering

Despite, any effective models for the hormonal controls of flowering have not been established yet, many studies are showing the individual effect of plant hormones in flowering (Izawa 2021). In coffee, previous studies have shown that Abscisic Acid (ABA) increases during the dry period and is associated with coffee flower bud dormant or "latent state", having plant rehydration decreasing ABA and increasing Gibberellin (GA) levels (Cueto & Dathe 1986). After water withdrawing stress, dormant and non-dormant G4 flower buds (ranging from 3.2 to 6 mm in length) (Morais et al. 2008) could be distinguished based on their ethylene evolution (Schuch et al. 1992). In other species, changes in ethylene levels can either delay or promote flowering, as observed in Rice (*Oryza sativa* L.) (Wang et al. 2013), Arabidopsis (*Arabidopsis thaliana*) (Achard et al. 2007), Pineapple (A*nanas comusus* L.) (Trusov & Botella 2006), and Roses (Meng et al. 2014).

In our RNA-seq analysis, were observed a high number of UP and DOWN-regulated genes in the biosynthesis and signaling pathway of ethylene, and for this study, ethylene evolution was measured in leaves of Oeiras, Acauã, and *Semperflorens* coffee genotypes. Our initial hypothesis was that ethylene is involved not only in the anthesis process but in all the coffee flowering processes. Results of ethylene evolution in the field showed that there are some differences between coffee genotypes in the ethylene content by the flower development process. For FB, ethylene presented similar pattern production for all genotypes, except for *Semperflorens* which presented high ethylene production from March till May. For leaves, a reduction in ethylene content from April till August was observed, having a pattern production of Oeiras and *Semperflorens* similar, whereas Acauã presented a low level of ethylene production (Fig. 11). Possibly, low ethylene production in leaves is a characteristic of a late coffee genotype.

In the Gene Ontology analysis, the most remarkable for all evaluated contrasts in leaves, FB, and SAM is the amount of UP and DOWN-Regulated genes associated with hormonal regulation (Fig. 8 and 9). It is possible that not only ethylene is involved in the coffee flowering time regulation, but gibberellins, auxins, strigolactones, cytokinins, and abscisic acid as signaling in response to the environmental cues or endogenous stimulus. However, for this study we evaluated those expressed genes related to ethylene production, the majority of these genes are involved in biosynthesis pathway (aminocyclopropane-1-carboxylate-like), and receptors for signaling cascade (Ethylene-response transcription factor RAP2-12-like, ethylene-responsive transcription factor 1B-like, EIN3-binding F-box protein 1-like, ethylene-responsive transcription factor 4-like, and ethylene-responsive transcription factor 5-like). In the used

genome annotation, Cara_1.0, there are sixty-three 1-aminocyclopropane-1-carboxylate oxidase (ACO) and eleven 1-aminocyclopropane-1-carboxylate synthases (ACS).

Twenty-eight ACO genes were found to be differentially expressed in at least one contrast whereas two ACS were DE in at least one contrast. Recently, it was proposed that ethylene can play an important role in coffee flowering, because rehydrating droughted plants can increase ethylene levels and ethylene sensitivity, regulating coffee anthesis (Lima et al. 2020). Other plant hormones such as GA, JA, ABA, and cytokinins have been studied in flowering time. In Arabidopsis, under long-day conditions, GA in leaf and shoot apex tissues can promote flowering, whereas apex-specific depletion of GA results in non-flowering phenotypes under short-day conditions (Porri et al. 2012). On the other hand, a genetic link between GA and Jasmonic Acid (A) was established, because DELLA protein can enhance the activity of TOE1 and TOE2 indirectly via sequestration of JAZ. As a result, DELLA degradation by GAs frees multiple repression sites in the FT promoter, controlling flowering time in *A. thaliana* (Browse and Wallis 2019). Exogenous Cytokinin can promote flowering under short-day conditions. TSF (another florigen in *A. thaliana*), FD, and SOC1 are required for Cytokinin-mediated flowering (D'Aloia et al. 2011). Drought escape (DE) is a response to drought stress-inducing early flowering in several plant species. The fact that ABA biosynthesis is increased under drought stress suggests that ABA could be involved in the DE response (Verslues and Juenger 2011).

4.3. Candidate genes involved in coffee flowering time in response to endogenous and external stimulus

The timing of flowering is determined by endogenous genetic components as well as various environmental factors, such as day length, temperature, and stress. Plants respond to these stimuli by a signaling cascade, directing to the transition of the vegetative to reproductive phase. In this sense, there are many interconnected genes for specific functions. In our study it was found many genes associated with different functions in the flowering process, having those genes being associated to explain the differences in flowering time of the coffee genotypes. 250 UP and DOWN-Regulated genes related to floral identity and flower development were selected as candidates to explain those differences.

In the classification of Gene Ontology (GO) for flower development, we can note some differences between coffee genotypes contrasted. In FB, there are more UP-regulated genes for Oeiras than Acauã in March, when, at this point, they are phenotypically similar in flower development (Figure 2A), but the higher number of DEG in Oeiras than Acauã could explain

that genetically they are different in their flowering pattern. The pattern of UP and DOWN-regulated genes for flower development comparing Acauã and Oeiras against *Semperflorens* is similar. It seems that this difference is marked in the SAM because only UP-regulated genes of flower development were observed when comparing the SAM of Oeiras and Acauã (Fig. (10 B1)).

There are more UP and DOWN-Regulated genes in FB than in SAM (Fig. 8C). Aiming to know the identity of those genes involved in the flower development process in leaves, FB, and SAM, the 42 most expressed genes were selected. Moreover, thirteen genes as the most important candidates to explain the flowering time between coffee genotypes according to their expression pattern were selected (Fig. 13, 14, and 15). The protein Heading 3A-like is a homolog of FT in rice, promoting flowering in short days. Due to the high level of expression observed in *Semperflorens* FB compared with Acauã and Oeiras, we can speculate that this expression could explain why *Semperflorens* are different in flowering time (Tamaki et al. 2007; Taoka et al. 2011) (Fig. 13A). The truncated transcription factor CAULIFLOWER A-like is described as a probable transcription factor that promotes early floral meristem identity in synergy with APETALA1, FRUITFULL, and LEAFY. It is required subsequently for the transition of an inflorescence meristem into a floral meristem. It seems to be partially redundant to the function of APETALA1 (Li et al. 2000).

CAULIFLOWER GENE (CAL) primarily specifies the floral meristem identity in a redundant way to AP1; FUL has a function in several processes, such as the development of carpel structure and specification of inflorescence meristem (IM) identity (Ferrándiz et al. 2000)

This gene is highly expressed in FB of *Semperflorens*, possibly regulating early flowering for this genotype (Fig. 13B). The MADS-box protein SOC1-like is higher expressed in FB of Oeiras than in Acauã and *Semperflorens* (Fig. 13C), this could explain the difference in flowering time of Oeiras compared with Acauã because this is a transcription activator acting in flowering time control when associated with AGL24, mediates the effect of gibberellins on flowering under short-day conditions, and regulates the expression of LEAFY (LFY), which links floral induction and floral development (Liu et al. 2008). The K-domain of a blueberry-derived SOC1-like gene promotes flowering in tobacco (Song et al. 2013). Overexpression of *ZmSOC1* resulted in early flowering in Arabidopsis through increasing the expression of *AtLFY* and *AtAP1*. Overall, these results suggest that *ZmSOC1* is a flowering promoter in Arabidopsis (Zhao et al. 2014).

The gene VIN3-like protein 2 is highly expressed in FB of *Semperflorens* and represents other candidate genes involved in coffee regulation in this genotype (Fig. 13D). This gene may

be involved in both the vernalization and photoperiod pathways by regulating gene expression and promoting flowering in non-inductive photoperiods (Sung et al. 2007). The two-component response regulator-like APRR5 gene is more expressed in Acauã than Oeiras and *Semperflorens* and is a candidate gene controlling the late flowering pattern in Acauã concerning Oeiras (Fig. 13E). This gene is involved in the positive and negative feedback loops of the circadian clock. With RVE8, it forms a negative feedback loop of the circadian clock, a repressor of CCA1 and LHY, thereby controlling photoperiodic flowering response (Nakamichi et al. 2012; Rawat et al. 2011).

The MOTHER of FT protein and TFL1 homolog 1-like is most expressed in *Semperflorens* than in Acauã and Oeiras (Fig. 13F), being a candidate gene regulating flowering pattern in this genotype. This gene regulates identities of the determinate and indeterminate meristems, and ultimately affects flowering time and plant architecture (Yu et al. 2019). MOTHER of FT AND TFL1 functions as a floral inducer and that it may act redundantly in the determination of flowering time in Arabidopsis (Yoo et al. 2004).

On the other hand, six candidates' genes were identified acting in the autonomous pathway in the flowering time control (Fig. 14). Finally, the EARLY FLOWERING 5-like protein was selected, a gene that has been described as a flowering repressor in Arabidopsis (Noh et al. 2004). However, it is possible that due to the pattern expression in all tissues of coffee genotypes (Leaves, FB and SAM), this gene could be acting as a flowering promoter in *Semperflorens* (Fig. 15). At this point, we cannot explain how this could happen but is an interesting proposal for future studies.

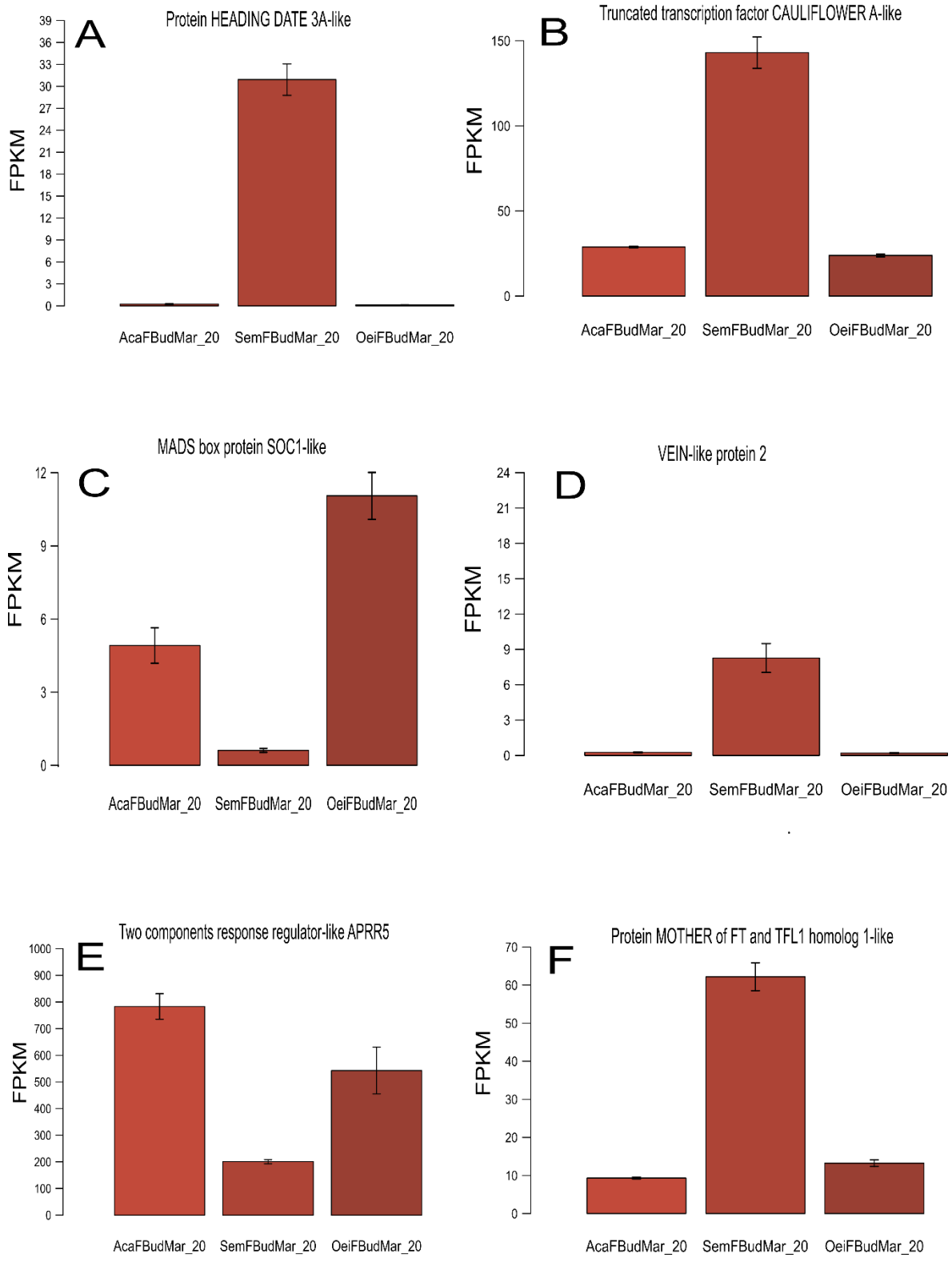

Figure 13. The candidate expressed genes influencing flowering time in coffee genotypes

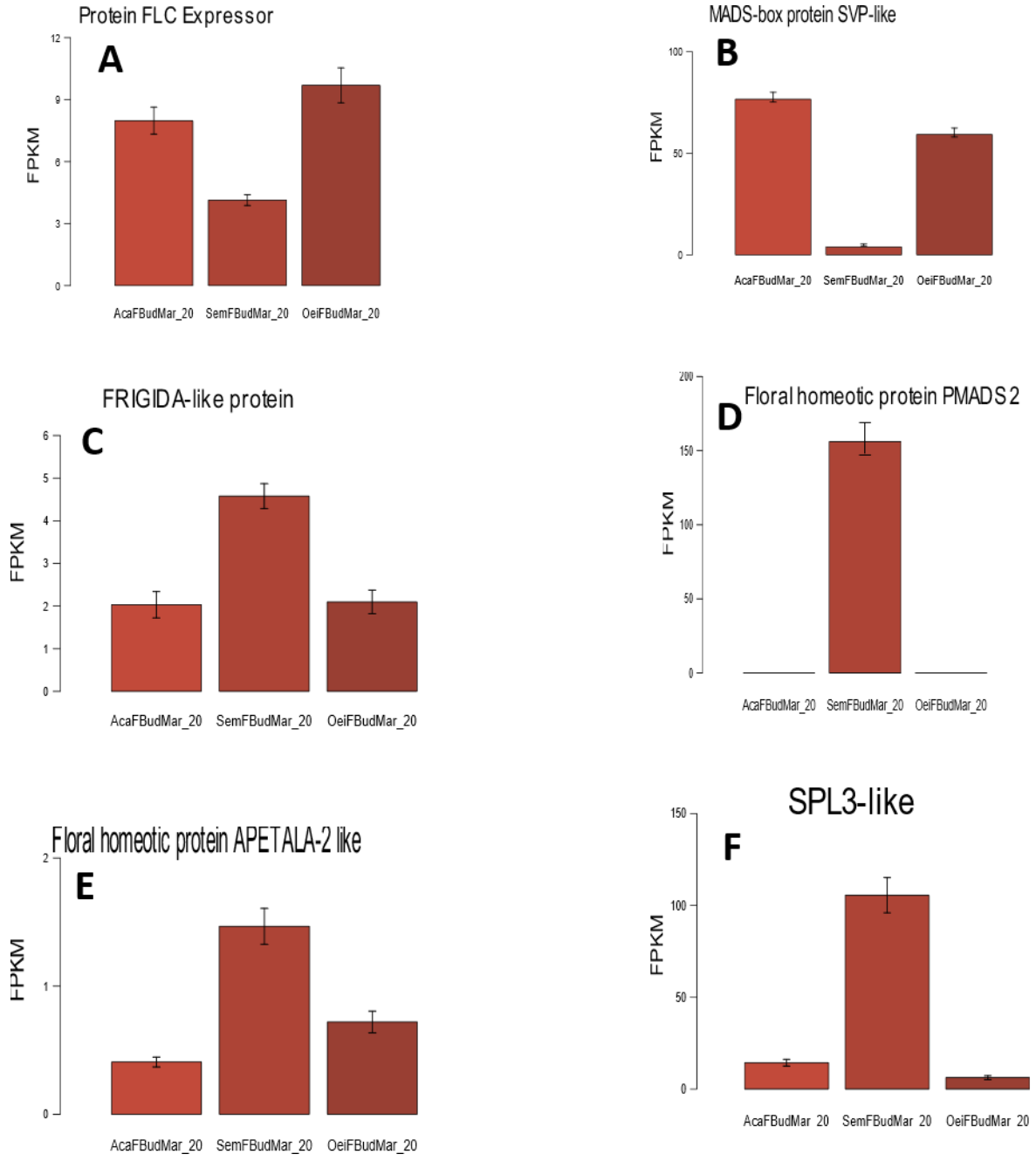

Fig. 14. Candidate expressed gene associated with the Autonomous Pathway in the flowering process control

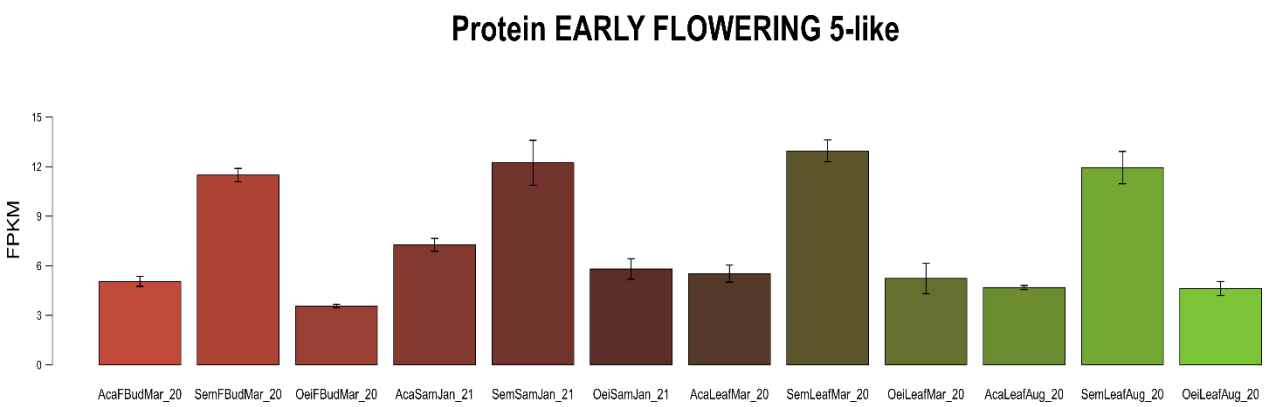

Figure 15. Candidate gene promoting early flowering in *Semperflorens*

5. CONCLUDED REMARKS

Differences in the coffee flowering process among Acauã, Oeiras, and *Semperflorens* seem to be associated with genes controlling sugar metabolism, photoperiod, hormonal regulation, and flower development that might be controlling flowering time in coffee. Candidate genes were selected to explain those differences because they were more expressed in some contrasts and are congruent with the phenotypical evaluations in the field (Sugar content and flower bud development). In this sense, four genes were associated with sugar metabolism to explain the high content of starch in *Semperflorens* (Possibly utilized as an energy reserve for metabolic processes in continuous flowering) and the increase of sucrose in Acauã from June to August before anthesis (Time for Late flowering). Moreover, thirteen candidate genes were associated with photoperiodic and flower development responses in the flowering pattern among coffee genotypes. Further analysis is needed to elucidate the metabolic pathway affected by those genes.

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CHAPTER 5

FINAL CONSIDERATIONS

In general, the coffee flowering process has not been widely studied yet. Especially, how the physiological and molecular factors interact for anthesis occurring in each production cycle. Studying how these factors interact, may help to understand the mechanisms that the coffee plants use to adapt to an environment in constant climatic variance. Being coffee a world trade commodity, its asynchronous flowering pattern has a direct impact on yield and quality, and finding the key flowering regulator may help to manipulate this mechanism and by genetic breeding, release new coffee genotypes with a synchronized flowering and production.

Our results showed that there is a hormonal regulation in the coffee flowering process. 1-Aminocyclopropene-Acid (ACC), the ethylene precursor molecule functions as a modulator in the coffee anthesis, however, this is the last step in the flower bud development process. ABA content is increased in the dry period and ethylene content decrease from rainy to dry period. Transcriptomic analysis showed that there is a strong hormonal regulation possibly interacting as a coordinated symphony for the coffee flowering process. At this point, we have more questions than answers, in who? and how? all those plant hormones are regulating different metabolic pathways in the plant performance as a whole. Additional studies will be needed to elucidate the answer to these questions.

Possibly, the best way to understand the regulation of flowering in coffee is by doing a deep study of the physiological and genetic plant responses to environmental stimuli such as water stress, temperature, relative humidity, nutrition, among others. Genes related to photoperiod, circadian clock, sugar metabolism, flower development, and floral organ identity are involved in the coffee flowering time. Even though, all of them may be acting such as a network, a deep study of individual mechanisms is needed. Future researches should focus on elucidating the participation of other plant hormones, and the isolated effect of genes regulating external and endogenous stimulus in coffee flowering time.