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ETHYLENE REGULATION OF *Coffea arabica* FLOWERING INDUCED BY 1-METHYLCYCLOPROPENE

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Trabalho de Conclusão de Curso apresentado à Universidade Federal de Lavras, como parte das exigências do Curso de Agronomia, para a obtenção do título de Bacharel.

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REGULAÇÃO DE ETILENO EM *Coffea arabica* INDUZIDO PELO 1-METILCICLOPROPANO

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There is no greater example of dedication than our family. To my dearest family, whom I admire so much, I dedicate the results of the effort made along this journey.

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ABSTRACT

Plant flowering is orchestrated by hormones triggering a series of physiological events to induce anthesis. In coffee plants, it has been shown that ethylene plays an important role during flower opening among all hormones. Anthesis occurs after flower bud dormancy is broken, which involves the period of water deficit followed by rain, and increase of ethylene production. 1-methylcycloprone (1-MCP) is an inhibitor of endogenous ethylene production largely used in fruit storage to enlarge shelf life. However, in coffee plants, 1-MCP happens to modulate ethylene biosynthesis and signaling pathways and promote flowering. The objective of this work was to determine the effect 1-MCP has in *Coffea arabica* plants by analyzing phenological responses, ethylene and ACC production, ACO activity, and gene expression of the ethylene biosynthetic and signaling pathways. The experiment was a completely randomized design divided into two factorials of 2 x 2 x 3 and 2 x 2 x 2 (treatments x application x plant tissues) with four replicates. The first factorial was designed for ethylene, ACC, and ACO activity quantification, and the second one for ethylene biosynthetic and signaling pathways gene expression. The treatments used were water (control) and 1-MCP (50 mg a.i.), applications (before and after), and the plant tissues collected were leaf, flower bud, and root two hours later the spray. Each replicate had two coffee plants presenting mostly 3 mm (or more) length flower buds; two plants were used for phenotyping, and one of them to collect plant tissues. Flower bud, leaf, and root were used to analyze ethylene, ACC levels, and ACO, and for gene expression (RT-qPCR) of the ethylene biosynthetic pathway (*CaACS3* and *CaACO3*), perception (*CaETR1*, *CaETR2*, *CaETR4*, *CaEIN4* and *CaERS1*), and signaling (*CaCTR1*), only flower bud and leaf were analyzed. The results showed enhance of ethylene and ACC production levels, ACO activity, and levels of gene expression of the biosynthesis and signaling pathways after 1-MCP application in leaf and flower bud. The 1-MCP is efficient in promoting flowering in *C. arabica*. The water treatment did not promote anthesis in coffee plants. In conclusion, the 1-MCP binding induces more receptors production (*CaETR1*, *CaETR2*, *CaETR4*, *CaERS1*, and *CaEIN4*). Ethylene production is increased by negative *feedback* in the biosynthesis pathway (*CaACS3* and *CaACO3*). ACC and ACO activity are increased by the 1-MCP effect, which conforms the indirect correlation between 1-MCP receptors binding to induce ethylene production and flowering.

Keywords: coffee; anthesis; receptors; 1-MCP; water deficit; ACC.

RESUMO

O florescimento das plantas é orquestrado por hormônios que desencadeiam uma série de eventos fisiológicos para induzir a antese. No cafeeiro, foi demonstrado que o etileno desempenha um papel importante durante a abertura da flor entre todos os hormônios. A antese ocorre após a quebra da dormência das gemas florais, que envolve o período de déficit hídrico seguido de chuva e aumento da produção de etileno. A 1-metilciclopropeno (1-MCP) é um inibidor da produção de etileno endógeno amplamente utilizado no armazenamento de frutas para aumentar a vida útil. No entanto, no cafeeiro, o 1-MCP modula a biossíntese do etileno e as vias de sinalização e promove o florescimento. O objetivo deste trabalho foi determinar o efeito do 1-MCP em plantas de *Coffea arabica* por meio da análise de respostas fenológicas, produção de etileno e ACC, atividade de ACO e expressão gênica das vias de biossíntese e de sinalização do etileno. O experimento foi em delineamento inteiramente casualizado dividido em dois fatoriais de 2 x 2 x 3 e 2 x 2 x 2 (tratamentos x aplicação x tecidos vegetais) com quatro repetições. O primeiro fatorial foi desenhado para a quantificação da atividade do etileno, ACC e ACO, e o segundo para a expressão gênica das vias de sinalização e biossíntese do etileno. Os tratamentos utilizados foram água (controle) e 1-MCP (50 mg a.i.), as aplicações (antes e depois), e os tecidos vegetais coletados foram folha, gema floral e raiz duas horas após a pulverização. Cada repetição tinha duas plantas de café apresentando principalmente gemas de flores de 3 mm (ou mais) de comprimento; duas plantas foram utilizadas para fenotipagem e uma delas para coleta de tecidos vegetais. A gema floral, folha e raiz foram usados para analisar etileno, ACC e ACO, e para expressão gênica (RT-qPCR) da via de biossíntese do etileno (*CaACS3* e *CaACO3*), percepção (*CaETR1*, *CaETR2*, *CaETR4, CaEIN4* e *CaERS1*), e sinalização (*CaCTR1*), apenas a gema da flor e a folha foram analisados. Os resultados mostraram aumento nos níveis da produção de etileno e ACC, atividade de ACO e nos níveis da expressão gênica das vias de biossíntese e de sinalização após aplicação de 1-MCP em folhas e gemas florais. O 1-MCP é eficiente na promoção do florescimento em *C. arabica*. O tratamento com água não promoveu a antese do cafeeiro. Em conclusão, a ligação de 1-MCP induz mais produção de receptores (*CaETR1*, *CaETR2*, *CaETR4*, *CaERS1* e *CaEIN4*). A produção de etileno é aumentada por feedback negativo na via de biossíntese (*CaACS3* e *CaACO3*). A atividade de ACC e ACO é aumentada pelo efeito 1-MCP, que conforma a correlação indireta entre a ligação dos receptores 1-MCP para induzir a produção de etileno e o florescimento.

Palavras-chave: café; antese; receptores; 1-MCP; déficit hídrico; ACC.

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FIRST PART

1 INTRODUCTION

Coffee as one of the most important crops around the world has flowering as an important challenge to be understood physiologically. Ethylene the key hormone involved in coffee flowering is the main interest in this work since it is responsible for increasing flower bud sensibility to anthesis in coffee.

Nowadays, 1-MCP has been studied as an alternative tool that could help inducing flowering in coffee plants. The chemical molecules binds with the ethylene receptors in the signaling pathway and indirectly as a negative *feedback* acts in the biosynthesis pathway inducing ethylene production.

Understanding how 1-MCP work physiologically in coffee plants will help the development of application technologies in favor of concentrating flowering and managing plant health to improve coffee bean production, as well shorten the differences between productivity of the biennial years.

The objective of this work was to determine the effect 1-MCP has in *Coffea arabica* plants by analyzing phenological responses, ethylene and ACC production, ACO activity, and gene expression of the ethylene biosynthesis and signaling pathways.

2 LITERATURE REVIEW

2.1 Coffee economic aspects

Coffee species originates from Ethiopia, Africa, and when brought to Brazil by Francisco de Mello Palheta in 1727 the cultivation spread fast because of the optimum climatic conditions in the country. Coffee production started in Maranhão and later in Bahia, Rio de Janeiro, São Paulo, Paraná and Minas Gerais. The great acceptance of coffee in Brazil made it one of the economic base products up to nowadays (OLIVEIRA et al., 2012).

Since this discovery of coffee in Ethiopia, consumption has changed over the years as "waves". The first wave started after the First World War, where quality was not the main goal but the high production. The second wave came between the 60s and 90s, which was marked by the espresso coffee machines together with a concept of more quality and sophistication, followed by the act of consumption outside in cafeterias. The third wave, bring us to current days where the act of tasting superior quality of special coffees, and the differentiated service provided by coffee shops with blend coffee is highly demanded by the consumers (RICARDI, 2016; LAGES, 2017; SAMPAIO, 2019).

The most consumed coffee species around the world are Arabica (*Coffea arabica*) and Conilon (*Coffea canephora*). The differences between the two are about the more aromatic, slightly sweetened with flavor notes of chocolate and more acidity of Arabica, compared to the stronger, harsher, more bitter taste of Conilon, that is more soluble and widely used in manufacturing instant drinks (BUNN et al., 2015; RODRIGUES; DIAS et al., 2015; DURÁN et al., 2017).

C. arabica and *C. canephora* in Brazil represent an area of 2.16 million hectares in 2020, from this, a total of 277.3 thousand hectares were crop in formation and 1.88 million hectares in production, Minas Gerais has the largest planted area in the country. Although Brazil has a large cultivated area, coffee production is affected by the physiological biennial cycle of the plants, which consists of a year with great flowering followed by another year with less intense flowering, such influence reflects on the average productivity results each year. As 2020 was a positive biennial year especially for *Coffea arabica* the productivity reached 32.18 $bag.ha^{-1}$ representing an increase of 36% compared to 2019 (23.66 $bag.ha^{-1}$) (CONAB, 2020).

In 2021, the combination of a negative biennial year and adverse weather conditions such as drought, and frost in many regions increased the perspective for the average reduction in production due to lower blossoming, fruit setting, and plant development. The estimated productivity of *Coffea arabica* for 2021 is 21.6 $bag.ha^{-1}$, 33% less than 2020 (CONAB, 2021). The selling price per bag reached US$ 214.16 in September as a reflection of the damages caused by the weather conditions in 2021 (CEPEA, 2021). Besides the decrease in production, the exportation of green coffee beans has enhanced over the past five years (2017 to 2021) in Brazil ranging from 33,081 to 45,026 million bags (60 kg), and the estimation for 2022 is 56,3 million bags which maintain Brazil as the biggest green bean producer and exporter in the world (USDA, 2021).

2.2 Coffee biennial cycle

Rubiaceae family has 103 species described of the genus Coffea L., with most species being diploid and allogamous (DAVIS et al., 2006). *Coffea arabica* is an allotetraploid that comes from two diploid species *C. canephora* and *C. eugenioides* (2n=2x=22) (LASHERMES et al., 1999). The reproduction of *C. arabica* is mostly by self-fertilization that represents 90% of the flowers (FAZUOLI et al., 2000).

C. arabica is a small tree with the potential to reach 9 to 12 meters in height and grow at 1,300 to 2,000 m of altitude above sea level. The maturity of the tree takes three years to be achieved from seed to fruit production. The fruit is a cherry with a seed inside commonly known as a bean. The

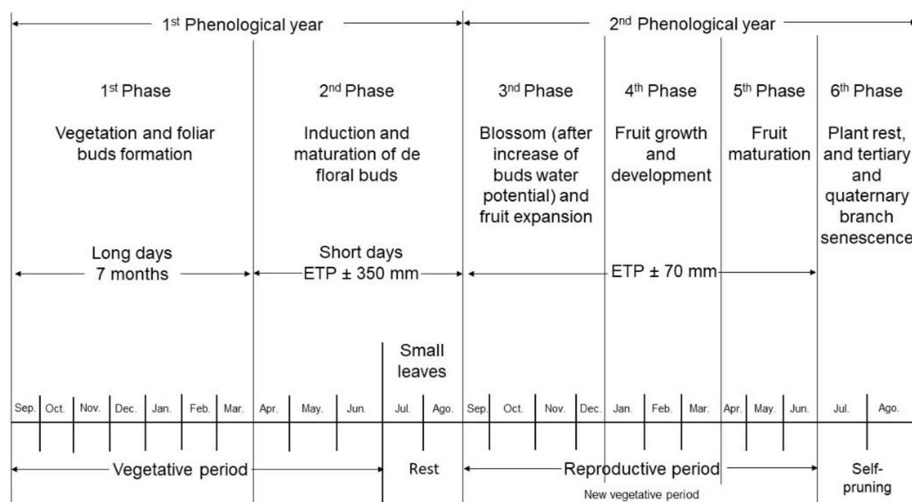
coffee plant structure has open branches system with a main orthotropic stem and plagiotropic branches. Each branch will generate a series of buds that can become flowers or orthotropic suckers. The flowers are white, with a five-lobed corolla, a calyx, five stamens, and a pistil. The ovary consists of ovules that after self-fertilization become beans (WINTGENS, 2009).

In Brazil, the climatic conditions divide the coffee cycle into six major phases (Figure 1): 1st phase - vegetation and foliar bud formation, 2nd phase - induction and maturation of floral bud, 3rd phase - blossom, 4th phase - fruit growth and development, 5th phase - fruit maturation and 6th phase - rest and senescence of the tertiary, and quaternary branches (CAMARGO; CAMARGO, 2001).

The first phase consists of the growth and development of nodes with axillary buds that will form the lateral branches responsible for fruit production in the next year. This phase takes place during September throw March of the first phenological year, also, physiologically prepares the plant for the following year. The second phase is characterized by the induction of axillary vegetative buds (1st phase) to floral buds, and their development until buttoning, this phase takes place during the period of short days (April to August) and closes the first phenological year of the coffee plant. At the end of phase two (July-August), the plants enter a period of dormancy (rest). The second physiological year begins with blossoming (September to December), after rain or irrigation, followed by fruit growth and development (January to March), and fruit maturation (April and June). In phase six (July to August) the branches enter a period of rest, senescence, and fall (CAMARGO; CAMARGO, 2001).

These two phenological cycles in Coffee are called biennial effect, which creates alternation between large and small bean production over time. This event is more pronounced in *C. arabica* than *C. canephora,* the alternation is a natural physiological response from coffee plants that need to vegetate more in one year to recover their energy and produce well in the following year (RENA; MAESTRI, 1985). In years of high productivity, photosynthesis is directs most to fruit formation and filling, and plant energy reserves will decrease, making a recovery period necessary in the next year when the plant spends more energy developing new plagiotropic branches, which consequently will lower fruit production (MENDONÇA et al., 2011; PEREIRA et al., 2011).

Figure 1. Scheme of the two phenological years of *C. arabica* plants cultivated in tropical climatic conditions of Brazil.



Source: Camargo and Camargo, (2001).

The physiological changes in the coffee bienniality can be influenced by a few factors, and the weather is an important one because it affects the growth and development of the plants (CAMARGO; CAMARGO, 2001). For instance, drought influences the development and productivity therefore will affect bienniality, since the plant needs time to recover from extreme periods of water deficit (COELHO; SILVA, 2005). The next factor that can affect bienniality is management growth; the nutritional supply can reduce the impact of the biennial production cycle (VALADARES et al., 2013).

The coffee plant's most required nutrient within the first eighteen months in order of need is nitrogen, potassium, calcium, magnesium, phosphorus, sulfur, iron, manganese, copper, boron, and zinc. As the crop gets older, the nutritional requirements will increase, making an adequate fertilizer supply important, in accordance to the crop growth and production (SANZONOWICZ et al., 2003).

This information shows the high correlation between nutrient management and bienniality, as in the year of high production the plants are depleted due to high metabolism, and to reduce the oscillation in fruit production, the nutrient replacement must be done to replenish soil fertility and make nutrients available for the coffee plants. Thus, when the plant resumption metabolism finds a well-fertilized soil it will make recovery faster than under conditions of low soil fertility availability (MATIELLO et al., 2002; MATIELLO et al., 2008; MENDONÇA et al., 2011).

Another factor related to bienniality is rust severity in coffee plants, high production demand higher expenses, which makes plants weaker due to low energy allocation in plant defense

mechanisms. This condition will increase the severity of the disease causing a fall of the damaged leaves, harm of flower buds formation, and intensify the biennial effect for next year's production (ZAMBOLIM, 2016; ZAMBOLIM et al., 2005).

Plant density has been used for greater stability in coffee production, as can reduce biennial cycles by inducing lower production per plant and lesser stress to the plants. The recovery time needed will be less in the next season, because the minor production in smaller spacing can condition plant to reduce the biennial effect due to less depletion per plant (MATIELLO, 2002; PREZOTTI; ROCHA, 2004; PEREIRA et al., 2011; DAMATTA et al., 2007).

Further studies are trying to use irrigation management to decrease the biennial effect in coffee plants; the volume of supplied water determines productivity and stability throughout the seasons. In irrigated crops, the biennial effects appear in the third or fourth year of production, however, depending on the increased production this effect can be anticipated by taking place in the first years (MATIELLO et al., 2002).

2.3 Coffee flowering

The plant reproductive stage has flowering as a crucial moment for species survival, and economically in agricultural crops. The success of fecundation guarantees the yield and quality of the finished product sold in the market (LIMA et al., 2021).

Flowering is associated with internal plant hormonal regulation and external sensitivity to the environment, based on these two factors plants are carefully regulated when the optimal moment to flowering is in order to succeed. Photoperiodism and vernalization are the two most important environmental mechanisms for floral evocation, however, quality of light, temperature, and abiotic stress can influence as well (AUSÍN et al., 2005).

In coffee plants, photoperiodism is controversial; some authors believe coffee needs short days with critical photoperiod of 13 to 14 hours (FRANCO, 1940, PIRINGER; BORTHWICK, 1955). However, some researchers have been point out that coffee is a neutral day plant, since in coffee regions throughout the world; the length of the day does not exceed the critical photoperiod. It is hard to imagine coffee as a short-day plant in an environment where the photoperiod is practically constant the whole year (RENA; MAESTRI, 1987, MAJEROWICZ; SÖNDAHL, 2005).

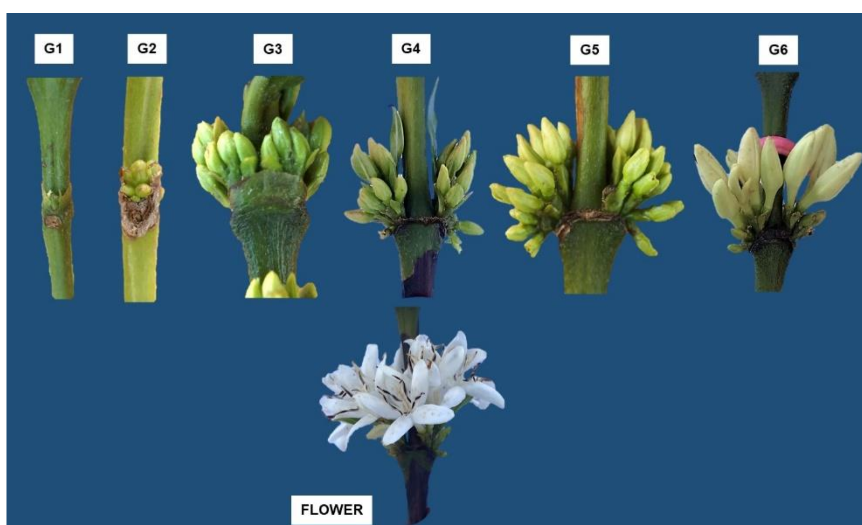
The asynchronism during flowering is another characteristic of coffee plants, flower buds will develop along the branches during vegetative and reproductive phases, resulting in buds with different stages of development (OLIVEIRA et al., 2014; MAJEROWICZ; SÖNDAHL, 2005). This

asynchronism associated with environmental factors can lead to multiple blooms if low rain showers happen during the dry season (SILVA et al., 2009; GUERRA et al., 2005). The consequence of the asynchronism is uneven fruit maturation, where branches will have buds, open flowers, green and ripe fruits at the same time (CRISOSTO et al., 1992; SOARES et al., 2005). This uneven flowering creates difficulties during harvest, disease, and pest control, besides reflects in reducing grain quality and increasing harvest costs (DAMATTA, 2007).

In addition, the bud flower after complete differentiation (4 to 6 mm) enters dormancy until environmental conditions are favorable for blooming. In Brazil, the period when coffee flower buds get dormant is during the dry season, they will return the competence of flower, and progress to anthesis after the rainy season starts (ALVIM, 1960; BARROS et al., 1978; CRISOSTO et al., 1992; MAGALHÃES; ANGELOCCI, 1976; RONCHI; MIRANDA, 2020).

The flower bud development is divided into seven phases to its size (Figure 2). Where, G1 - refers to nodes with undifferentiated buds; G2 - nodes with swollen buds; G3 - buds up to 3 mm in length; G4 - buds with 3.1 to 6 mm in length; G5 - 6.1 to 10 mm bud length (light green coloration); G6 - buds larger than 10 mm and white color; and FL – flowers. The flowers open early in the morning, which wilts on the second day and falls on the third (MORAIS et al., 2008).

Figure 2. Coffee flower bud development stages. G1 - undifferentiated buds, G2 - swollen buds, G3 - <3 mm buds, G4 - 3.1 to 6 mm buds, G5 - 6.1 to 10 mm bud, G6 - > 10 mm buds and Flower.



Source: author

Water stress participates as an important factor considered mandatory to concentrate flowering and reduce bud asynchronism. After dormancy is broken, the rain or irrigation stimulates growth

resumption of the flower buds to anthesis within 8 to 12 days (CANNELL, 1985, MES, 1957, ALVIM, 1960, PIRINGER; BORTHWICK, 1955, ALVIM, 1958, BROWNING, 1975, VAN DER VEEN, 1968).

Even though drought is mandatory for coffee plants, an accentuated and prolonged water deficit can negatively affect the phenological cycle and productivity of the plants, especially when associated with high temperatures (ALVIM, 1960; CAMARGO, 2001; DAMATTA, 2006). Coffee plants can have flowering stimulation when leaves' water potential are around -0.8 MPa, or -0.3 to -0.5 MPa when impose for a longer period, like two weeks (CRISOSTO et al., 1992). Likewise, the leaf water potential of -2.0MPa for 70 days can also induce anthesis and uniform flowering of about 80%, which is ideal for fruit harvest (GUERRA et al., 2005).

Environmental conditions such as drought undergo changes in a series of processes at physiological, biochemical, and molecular levels, such as photosynthesis, nutrient absorption, and changes in gene expression (FAROOQ et al., 2009). The hormones play a crucial role in the adaptation of plants to environmental stresses (PELEG; BLUMWALD, 2011), and it has been shown that abscisic acid and ethylene are the two main hormones that link their responses to stress (ALBACETE et al., 2014; DOLFERUS, 2014), and flowering.

2.4 Ethylene biosynthesis and signaling path

A plant growth hormone is a substance produced within the plant at low concentration levels, also can be transported to another parts of the plant where it promotes a response; however, similar, if not identical, compounds can be synthesized by other organisms or by man (OPIK; ROLFE, 2005).

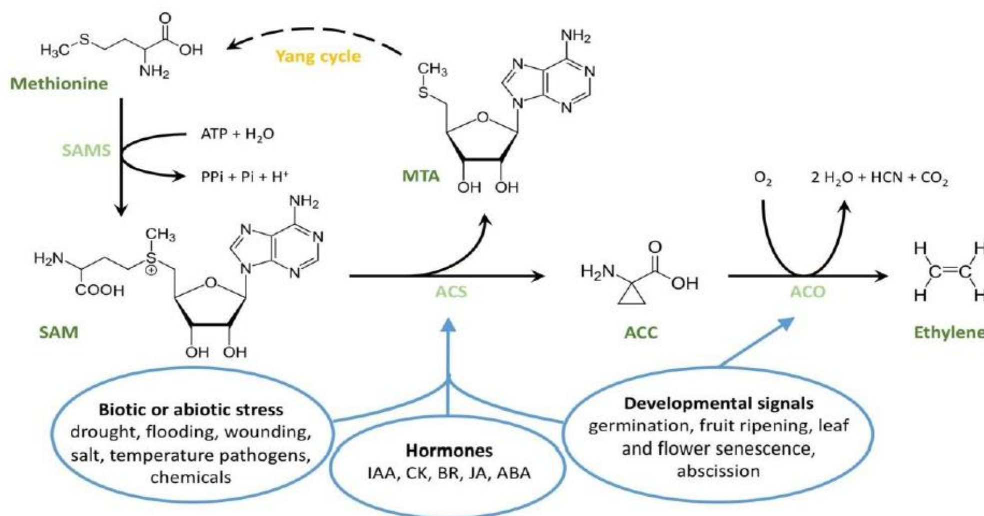
The gaseous plant hormone ethylene is an important regulator of plant growth, development, and responses to abiotic/biotic stresses. Although all plants produce ethylene, the overall ethylene level is generally low. During various developmental stages and stress events, ethylene production can induce plant senescence, fruit ripening, or favors root cutback and infections spread; all of which can affect local or neighboring cells (YANG; HOFFMAN, 1984; KENDE, 1993; WANG et al., 2002).

The identification of methionine (Figure 3), SAM (S-adenosylmethionine), and ACC (1-aminocyclopropane-1-carboxylic acid) as pathway precursors/intermediates were major advances in defining the ethylene biosynthetic pathway in higher plants (LIEBERMAN et al., 1966, ADAMS; YANG 1977; 1979). Ethylene is synthesized from SAM, an activated form of methionine and a common precursor to many biosynthetic pathways. SAM is converted to ACC by the enzyme ACS

(ACC synthase), and ACC is then oxidized by the enzyme ACO (ACC oxidase) to form ethylene (YANG; HOFFMAN, 1984, KENDE, 1993).

Two main reactions that are specific to the ethylene biosynthesis pathway are the conversion of SAM to ACC and then ACC to ethylene, catalyzed by ACS and ACO, respectively (KENDE, 1993). ACS activity is unstable and presents at lower levels in tissues that do not produce large amounts of ethylene, although its activity is high under conditions that promote the formation of ethylene. In contrast, ACO is constitutively present in most vegetative tissues. As a result, ACS is thought to be primarily compromising and generally rate-limiting enzyme of ethylene biosynthesis (YANG; HOFFMAN, 1984, SATO; THEOLOGIS, 1989, ZAREMBINSKI; THEOLOGIS, 1994, WANG et al., 2002).

Figure 3. Ethylene biosynthetic pathway and Yang cycle - (SAM) S-adenosyl-L-methionine, (SANS) SAM synthase, (ACC)-1-aminocyclopropane-1-carboxylic acid, (MTA) 5'-methylthioadenosine, (ACS) ACC synthase and (ACO) ACC oxidase.



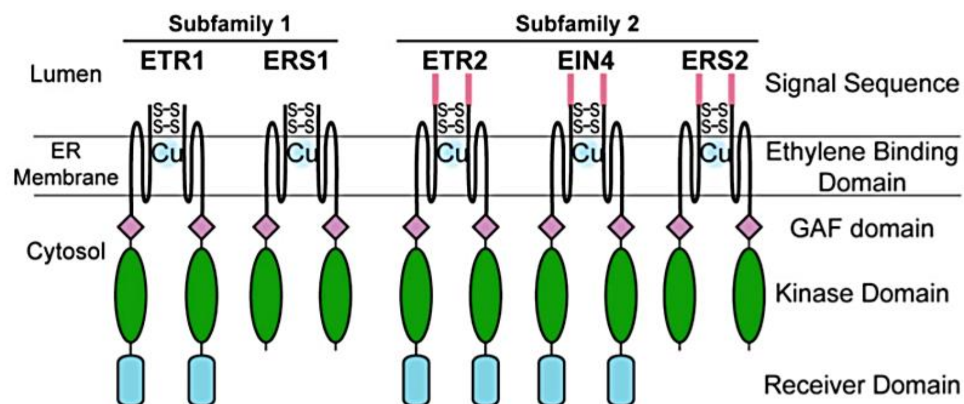
Source: Vanderstraeten and Straeten (2017)

Hormones act as internal signals within the plant. In the same way as environmental signals, they must be noticed and initiate a series of responses. The steps between initial perception and final response are known as signal transduction - another set of chemical changes, this time taking place within the cell, that alter the biochemistry and/or gene expression patterns of that cell. These changes can then act as signals initiating even more responses (OPIK; ROLFE, 2005).

Plant cells must have receptors on molecules that can detect varying amounts of ethylene. The effectiveness of ethylene at nanomolar concentrations indicates that some receptors have a high binding affinity to it (Figure 4). Its encoded proteins are divided into subfamily 1 consisting of ETR1 and ERS1 receptors and subfamily 2 consisting of ETR2, EIN4, and ERS2 receptors. All of them have a similar unique N-terminal domain, a histidine kinase-like domain, and show histidine kinase activity *in vitro*. ETR1, ETR2, and EIN4 have degenerate histidine kinase domains and show serine/threonine kinase activity *in vitro* (WEN, 2015, MOUSSATCHE et al., 2004).

Ethylene receptors can be found in all plant tissues, which means any part of the plant is responsive to ethylene, and since ethylene is lipophilic can diffuse in an aqueous and lipid environment, the endomembrane location does not prevent perception. Although, the response and sensitivity are dependable on the plant development stage and organ perception capacity of each species, that will be regulated according to receptors expression (BARRY; GIOVANNONI, 2007, CIARDI; KLEE, 2001, JONES et al., 2001, LACEY; BINDER, 2014)

Figure 4. Ethylene receptor structure and subfamilies in Arabidopsis.

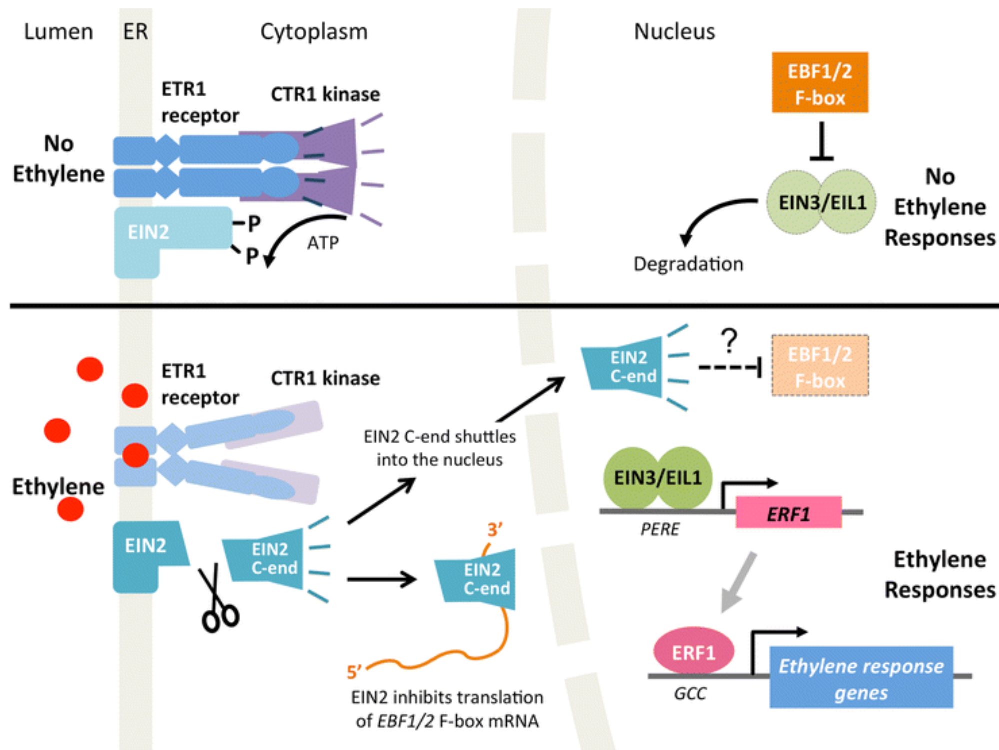


Source: Lacey and Binder (2014)

When ethylene is present, the signaling pathway works negatively because each receptor is a negative regulator in the presence of ethylene. However, when ethylene is absent the receptors will remain active and interact with the N-terminal region of the protein kinase Constitutive Triple Response 1 (CTR1) (Figure 5). CTR1 works as a negative regulator in the signaling pathway phosphorylating the C-terminal domain of the Ethylene Insensitive protein 2 (EIN2) downstream of the receptors. Thus, the via 26S proteosome will degrade the transcription factor Ethylene Insensitive 3 (EIN3) or EIN3-like 1 (EIL1) inside of the nucleus. The proteins F-box will bind with EIN3 1/2

(EBF1/2), repressing the ethylene responses in the tissues (HUANG et al., 2003, KIEBER et al., 1993, AN et al., 2010, POTUSCHAK et al., 2003, JU et al., 2012, WEN et al., 2012, ALONSO et al., 1999, HUA; MEYEROWITZ, 1998; KIEBER et al., 1993; TIEMAN et al., 1999).

Figure 5. Model of the core ethylene signaling pathway. Top: in the absence of the ethylene signal, the ethylene receptors (represented by the isoform ETR1) activate the CTR1 protein kinase, which represses EIN2 function. In the nucleus, the master transcription factors EIN3/EIL1 are degraded. Bottom: when ethylene is detected, the ethylene receptors no longer activate CTR1, resulting in the proteolytic release of the EIN2 C-END, which inhibits protein translation of the F-box proteins EBF1/2. EIN3/EIL1 are consequently stabilized and regulate an extensive transcriptional cascade involving the ERF1 transcription factor. Other elements that regulate the pathway can be found in MERCHANTE et al (2013).



Source: Chang (2016)

According to Opik and Rolfe (2005) plant cells will respond in different ways (or not) to a given hormone, which raises the concept of “competence to respond” - that is, whether a given type of cell will respond to a given concentration of a plant hormone or not. This process have the potential to be regulated by the environment, it can be seen that plant growth hormones act as a means of integrating environmental signals and distributing them around the plant.

2.5 1-methylcyclopropene

1-MCP (1-methylcyclopropene) is a cyclic olefin, which binds to ethylene receptors and prevents ethylene from inducing a conformational change in the plant. The compound is non-toxic, odorless, stable at room temperature, and has been used to protect cut flowers, potted plants and other vegetables against senescence ethylene effect (SISLER; SEREK, 1997; 2003).

The 1-MCP is the first non-toxic patented product that inhibits the action of ethylene, which was developed for commercial use by two American companies FloraLife Inc. and AgroFresh Inc. (Rohmand Haas) under the names EthylBloc® and SmartFresh ™, respectively. EthylBloc® was especially developed for ornamental crops, while Smart-Fresh ™ for edible products. Both compounds were produced as powders where 1-MCP was complexed with γ-cyclodextrin, when mixed with water or buffer solution, it releases the gas (SEREK; SISLER, 2005).

The efficiency of 1-MCP comes from the 10 times greater affinity to the ethylene receptors than the ethylene itself. Only lower concentrations of the 1-MCP is needed for a fasten diffusion in plant tissues, also, the time where it stays bonded to the receptors is greater than the ethylene, about 7 to 12 days (BLANKENSHIP; DOLE, 2003; KEBENEI et al., 2003).

The effect of 1-MCP treatment is limited in duration due to a breakdown of the receptor protein and the synthesis by the cell of new receptors molecules, which is induced by the absence of the ethylene in the signaling pathway (SISLER et al., 1996). According to Lima et al. (2021) and Santos (2016), chemical treatment with 1-MCP might overcome the need for rehydration of water restricted *C. arabica* plants to promote flowering, justified by the loss of regulation by negetive *feedback* in biosynthesis pathway and perception. This information suggests that 1-MCP could be working as a stress factor blocking the receptors and indirectly inducing ethylene production.

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SECOND PART – ARTICLE

1 INTRODUCTION

Coffee is the world's most valuable agricultural commodity trade with Brazil as the biggest producer and exporter. The advantage for high production comes from Brazil's unique semi-tropical climate, which provides the optimum condition for coffee plants to grow in substantial rainfall, and temperature (TOPIK, 2019, VOLSI et. al., 2019). The estimation of *Coffea arabica* production for 2022 is 56,3 million bags, which will maintain Brazil as the biggest producer and exporter in the world (USDA, 2021).

The importance of coffee around the world has increased the researches about its phenology and physiology characteristics. Today there are 130 species of Coffee cataloged, however, only two species are economically produced *C. arabica* and *C. canephora* (Robusta) (DAVIS; RAKOTONASOLO, 2021, DAVIS et al., 2019). *C. arabica* has a superior taste characterized as aromatic, slightly sweetened with flavor notes of chocolate, this superiority makes it more cultivated than *C. canephora* (BUNN et al., 2015).

Coffee is classified as a perennial crop with bienniality growth that affects production because of high and low years of productivity, related to a natural physiological response from the need to vegetate more in one year to recover energy and produce better in the following year (RENA; MAESTRI, 1985, CARVALHO et al., 2020).

Flowering in coffee plants as directly related to the bienniality has been the subject of studies because of its asynchronism in flower bud development, what consequently lowers bean production and quality. The flowering event can happen multiple times when there is not a marked period of drought, which is mandatory to concentrate coffee bloom (DA MATTA et al., 2007).

The period of water deficit necessary for a coffee plant to flower has been related to ethylene production. Changes in ethylene sensitivity has been contributing for coffee flower buds acquiring the competence to flower in response to drought and rehydration (LIMA et al., 2021, LOPEZ et al., 2021). In citrus, drought seems to stimulate the accumulation of ACC (the precursor of ethylene) in the roots, while rehydration stimulates the transportation of ACC to the shoot, causing ethylene production (G'OMEZ-CADENAS et al., 1996) which is a similar mechanism that happens in coffee plants.

1-MCP (1-methylcyclopropene) a cyclic olefin has been used in the market to avoid flower abscission in roses and lilies (WEI et al., 2020, CORDEIRO et al., 2020) by binding with the receptors of the ethylene-signaling pathway (SISLER; SEREK, 1997, 2003). However, in coffee plants, 1-MCP

seems to have the inverse effect and induces flowering as observed by Lima et al. (2021). It could be hypothesized that when 1-MCP binds with the receptors makes it unavailable for ethylene binding, coffee plants would sense the lack of ethylene binding in the signaling pathway, and indirectly by feedback stimulating more biosynthesis of ethylene, which would enhance flower bud sensitivity and promote flowering. Identifying which receptors 1-MCP binds and associate with ethylene and ACC production, and ACO activity could help to understand how this process works after 1-MCP application in the coffee plants.

Economically, inducing flowering in coffee plants with 1-MCP could be an alternative for irrigated systems, since water restriction could be better managed. Also, could help reduce diseases incidence by promoting flowering before the rainy season arrives. The coffee flower when opened during the rainy season provides a microclimate around the branches elevating humidity, which could favor diseases incidence (ZAMBOLIN, 2016). Using 1-MCP to help with the uniformity of flowering could be promising to coffee crops especially during negative years of production.

The improvement that 1-MCP could have in coffee flowering makes necessary a deep understanding of how ethylene production is influenced phenologically and physiologically by this chemical compound. The objective of this work was to determine the effect 1-MCP has in *Coffea arabica* plants by analyzing phenological responses, ethylene and ACC production, ACO activity, and gene expression of the ethylene biosynthesis and signaling pathways.

2 MATERIAL AND METHODS

2.1 Field experiment

The experiment was carried out in a coffee plantation (1 hectare) located at the Federal University of Lavras, Lavras, Minas Gerais, Brazil in 2021. The cultivar used was “MGS Paraíso 2”, characterized as a hybridization of “Catuaí Amarelo IAC 30” with the hybrid “Timor UFV 445-46”, low height, resistance to rust (*Hemileia vastatrix*), intermediate maturation, yellow fruit, and high drink quality. The plants had an average of 1.2 m in height. The soil texture in the area was 71.4% clay, 6.5% silt, and 22.1% sand, and fertility is described in Table 1.

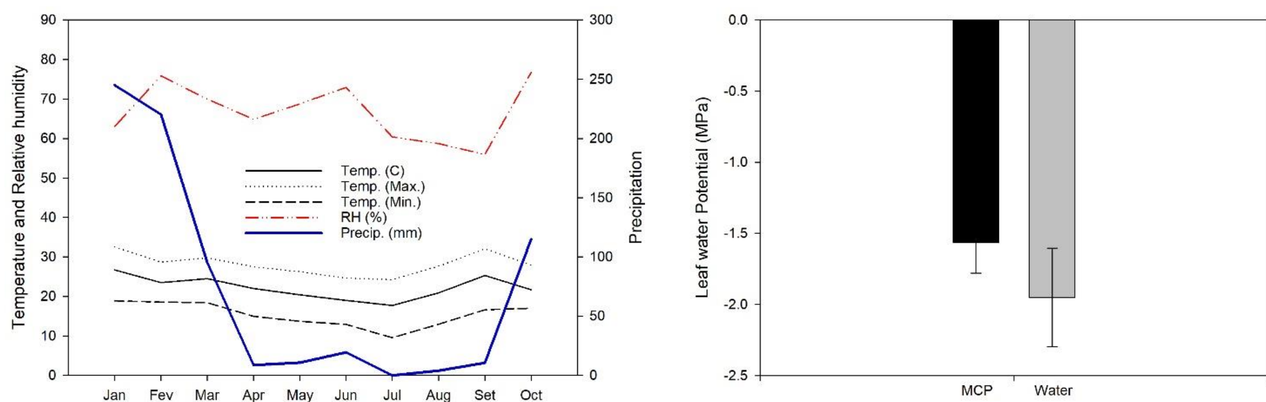
Table 1. Analyses of the soil fertility in a coffee plantation at the Federal University of Lavras, Brazil, 2021.

Soil depth (cm)	pH (water)	Al	Ca	Mg	K	P(1)	O.M. (2)
		cmloc/dm^3			mg/dm^3		%
0-20	5.1	0.2	3.3	0.5	32.5	8.4	2.5
20-40	5.1	0.7	2.1	0.4	37.6	28.9	2.8

(1) Extractor P-mehlich1. (2) Loss of weight per combustion method. O.M. - organic matter.

The temperature, relative humidity, and precipitation data were collected from the university weather station (January to October 2021) to track the period of the dry and rainy seasons. Predawn leaf water potential was measured by the end of the dry season with Scholander-type pressure chamber equipment to analyze the level of plant stress, the measures (MPa) were taken between 4:00 to 5:00 am before sunrise, two leaves of each treatment were used, collected from the middle part of the coffee plant (Figure 1).

Figure 1. Means of temperature (°C), maximum and minimum temperature (°C), relative humidity (%), precipitation (mm) and Predawn Leaf water potential (MPa) of a Coffee plantation at the Federal University of Lavras, Brazil, 2021.



The experiment was a randomized design factorial of 2 x 2 x 3 with four replicates, the treatments were the application of 1-MCP and Water (control), two periods of plant tissue collection (before and two hours after spraying, August 2021), and tissue collection (leaf, flower bud, and root). The analyses were Ethylene, ACC, and ACO activity quantifications. In gene expression, the factorial was changed to 2 x 2 x 2 with four replicates where the tissue collection was from leaf and flower bud.

The 1-MCP named Harvista TM from AgroFresh Inc. had 17.15 $g.L^{-1}$ of the active ingredient. A dilution of 50 mg a. i. L^{-1} was prepared according to Lima et al. (2021) and 200 $mL.plant^{-1}$ was sprayed for 20 s using a back sprayer pump in each plant at 8:00 am.

2.2 Phenotypic characterization

Two coffee plants with a predominance of 3 mm (or bigger) flower buds per replicate of each treatment were randomly chosen in the area. Four branches were identified around the middle third part of the plant (two on each opposite side) for a phenotipic count of the flower buds' development before and after application for 12 days. After the first rainfall (on September), the control treatment was counted again for 12 days to track the development of the flower buds under the influence of the rain. Each branch had three nodes identified for counting. Classification by size was adopted to count the flower buds used by Moraes et al. (2008), where G3 - <3 mm flower bud, G4 - 3.1 to 6 mm flower bud, G5 - 6.1 to 10 mm flower bud, G6 - > 10 mm flower bud and flower (anthesis). The results were presented as the average of the number of flower buds and flowers per node in each evaluation day.

2.3 Physiological and Biochemical analyses

2.3.1 Ethylene quantification

Ethylene quantification was made by collecting flower buds, leaves, and roots and storing them inside of 10 mL glass vial, each replicate was composed of two sub replicates of each tissue. Each tube was stored with ~10 flower buds, one leaf (middle part of the plant), and ~10 cm root (from 10 to 20 cm soil depth) and sealed with the vial cap for 24 hours. An F-900 Portable Ethylene analyzer (Felix Instrument, USA) was used to quantify ethylene, a syringe (10 mL) was used to remove the headspace gas inside of the vials, a total of 6 mL of gas was removed (3 mL from each sub replicate) and injected into the equipment operating under the GC (gas chromatography) emulation mode. After quantification, the glass vials containing the tissues were weighted to express ethylene evolution rate as ppm.g-1.FW. h^{-1} (fresh weight per hour) (LIMA et al., 2021).

2.3.2 ACC (1-aminocyclopropane-1-carboxylic acid) quantification

ACC quantification was performed according to the Bulens et al. (2011) method with a few modifications. Flower buds, leaves, and roots from each treatment were collected in the field (harvest in nitrogen), grinded in nitrogen with 50 mg of polyvinylpolypyrrolidone using mortar and pestle, and

stored at -86°C deep freezer. To extraction, 200 mg of each grinded treatment were transferred to a 2 mL microtube and 1 mL of sulfosalicylic acid 5% (p/v) was added and homogenized in a vortex and kept at 4 °C for 30 min. with gently inversions of the microtubes every 5 min. The microtubes were centrifuged at 15,200 g for 10 min, and 300 µL of the supernatant was transferred to a 10 mL glass vial along with 100 µL of 10 mM $HgCl^2$ and sealed with the vial cap. To start the reaction, 300 µL of cold NaOH 6M and NaOCl 1.2M solution (proportion of 2:1) was added in sequence with a syringe homogenized in a vortex for 5s and incubated for 4 min in ice. After 4 min., the vial was vortex for 5s and 6 mL headspace was withdrawn with a syringe and injected in the F-900 Portable Ethylene Analyzer equipment operating under GC emulation mode. The ethylene ppm values were transformed to nmol using the gases equation, and the ACC content was expressed as $nmol.g^{-1}$ DW (dry weight).

2.3.3 ACO (ACC oxidase) enzymatic activity quantification

ACO quantification was performed according to the Bulens et al. (2011) method with a few modifications. Flower buds, leaves, and roots from each treatment were collected in the field, grinded in nitrogen with 50 mg of polyvinylpolypyrrolidone using mortar and pestle, and stored at -86 °C deep freezer. To extraction, 250 mg each the grinded treatment was transferred to a 2 mL microtube, and 1mL of extraction buffer (MPOS 400 mM pH 7.2, Ascorbic acid 30 mM, and Glycerol 10% (v/v)) was added, homogenized in a vortex, and centrifuged at 22,000 g. for 30 min. The supernatant (800 µL) was collected and stored at -86 °C deep freezer. In a glass vial (10 mL) 3.6 mL of a reaction buffer was added 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 12 mM. For the quantification, 0.4 mL of the stored extract was added into the vial and a gentle homogenization for 5s before incubating the vial for 20 min at 30 °C. Another homogenization for 5s was made before removing 6 mL of the headspace air with a syringe and injected in the F-900 Portable Ethylene Analyzer equipment operating under GC emulation mode. The ethylene ppm data was transformed to nmol by the gases equation and the protein content from the remaining extraction was determined by the Bradford method (BRADFORD, 1976) in duplicates, bovine serum albumin was used as standard. One unit of ACO activity was defined as one nmol of ACC converted to 1nmol of ethylene per min. at 30 °C (DONG et al., 1992). The results were expressed in $U.mg^{-1}$ of protein.

2.4 Molecular analysis

2.4.1 *In silico* analysis

The genes receptors of the ethylene signaling pathway identified in *Coffea arabica* found in the literature and used were *CaETR1* (Ethylene receptor 1), *CaETR4* (Ethylene receptor), and *CaEIN4*(Ethylene insensitive 4). However, to cover all receptors that could be binding with 1-MCP a search for the missing genes of the subfamilies 1 and 2 of the ethylene signaling pathway was done by doing a gene encoding search in *Arabidopsis thaliana* and retrieved from The Arabidopsis Information Resource (https://www.arabidopsis.org/index.jsp) database. The genes encoding of *CaETR2e* (Ethylene receptor 2), *CaERS1* (Ethylene response sensor 1), and *CaERS2* (Ethylene response sensor 2) were found and the protein sequence of these genes were used as input to perform similarity searches with the genome of plant species including *Coffea arabica*. The gene protein search was made 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. No similarity was found for the gene protein *CaERS2* in *Coffea arabica* and was removed from the in silico analysis.

The *CaERS1* and *CaETR2* amino acid sequences were aligned by the Clustal W program (THOMPSON et al., 1994) and the phylogenetic tree was construsted for each one using the Neighbour-joining method using MEGA software (TAMURA et al., 2013) and bootstrap values from 5,000 randomization replicates to assess the robustness of the tree. The species used to align with *CaERS1* and *CaETR2* and in the phylogenetic trees are identified in figures 2 and 3.

Figure 2. Phylogenetic tree of the probable Ehylene Response Sensor 1 (*ERS1*) gene in *Coffea arabica* (Car) and homologous sequences from 17 species: *Ananas comosus* (Ac), *Arabidopsis thaliana* (Ath), *Brassica rapa* (Bra), *Cannabis sativa* (Cas), *Coffea canephora* (Cc), *Cucumis sativus* (Cus), *Glycine max* (Gma), *Hordeum vulgare* (Hvu), *Malus domestica* (Md), *Manihot esculenta* (Me), *Medicago truncatula* (Mtr), *Nicotiana tabacum* (Nta), *Oryza sativa* (Osa), *Setaria italica* (Sit), *Solanum lycopersicum* (Sol), *Solanum pennellii* (Vv), *Vitis vinifera* (Vv), *Zea mays* (Zma).

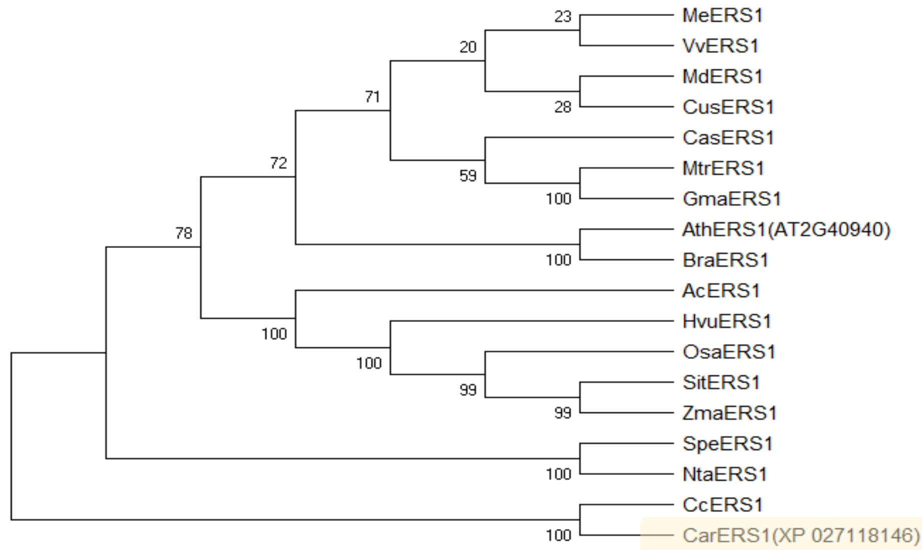
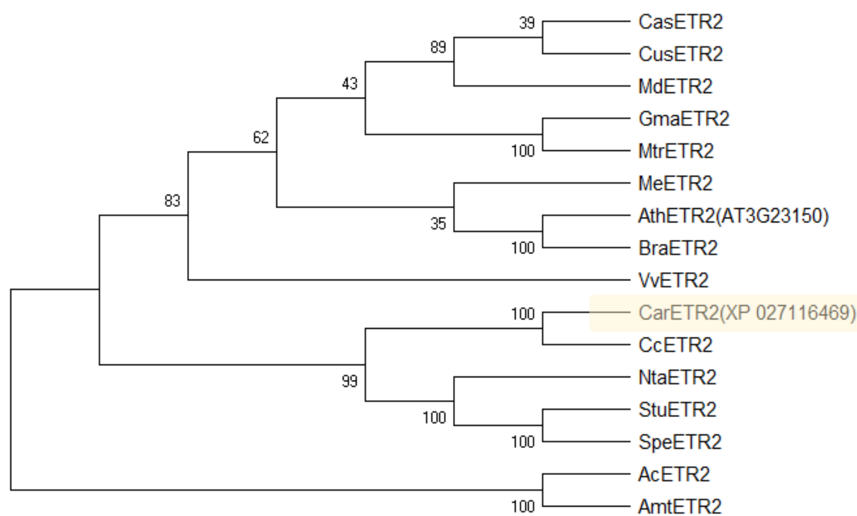


Figure 3. Phylogenetic tree of the probable Ehylene Receptor 2 (*ETR2*) gene in *Coffea arabica* (Car) and homologous sequences of 16 species: *Ananas comosus* (Ac), *Arabidopsis thaliana* (Ath), *Brassica rapa* (Bra), *Cannabis sativa* (Cas), *Coffea canephora* (Cc), *Cucumis sativus* (Cus), *Glycine max* (Gma), *Malus domestica* (Md), *Manihot esculenta* (Me), *Medicago truncatula* (Mtr), *Nicotiana tabacum* (Nta), *Oryza sativa* (Osa), *Setaria italica* (Sit),, *Solanum pennellii* (Vv), *Vitis vinifera* (Vv), *Zea mays* (Zma).



To make sure the ethylene signaling pathway was blocked by 1-MCP the gene expression of *CaCTR1* (Constitutive triple response) was analyzed. Also, to track ethylene production the *CaACO3* (1-aminocyclopropane-1-carboxylate oxidase 3) and *CaACS3* (1-aminocyclopropane-1-carboxylate synthase 3) genes were analyzed as well. Two reference genes were used *MDH* (Malate dehydrogenase) and *RLP39* (Ribosomal Protein L39).

The primers forward and reverse were performed using the *in silico* and literature sequences and designed using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) and IDT (OligoAnalyzer) (https://www.idtdna.com/pages/tools/oligoanalyzer) (Table 2).

Table 2. Gene primers sequence used for amplification of cDNA fragments via RT-qPCR. Conc.: Concentration, Vol.: Volume, TM: Temperature of Melting, E: Efficiency.

Gene	Sequence of primers (5'-3')	Conc. (µM)	Vol. (µL)	TM (°C)	E (%)	References
Ethylene receptor 1 (*CaETR1*)	F:GATGGGTGCTTGTGCAGTTT R: AGCATTAGAGCTGTCGCACAT	1.5	2.25	56 57	67	Santos (2020)
Ethylene receptor 2 (*CaETR2*)	F: TTGCCTTCATCGTCCTGTGT R:ACCAGAGCTGTGAGCGTTTTA	1.5	2.25	57 57	83	This study
Ethylene receptor 4 (*CaETR4*)	F: TTGGTCCATTCAGGAACTCG R: GCATCCTGTTTTGCTTGTTG	0.2	0.3	59 60	79	Lima et al. (2021)
Ethylene response sensor 1 (*CaERS1*)	F: TTGGAGACTCTCTCGCACTTT R:GGAAGAGGGTGCCAAGATTGA	1.5	2.25	56 57	100	This study
Ethylene insensitive 4 (*CaEIN4*)	F:TATTTGGGACTGGGGTTTGGG R: AGCAGCCCCTTTTCAATCCAT	2	3	57 57	93	Santos (2020)
Constitutive triple response (*CaCTR1*)	F:TAAGCCAACCGAAACCTCCTG R:TGGAAACCCTAAAAGCAGCCAT	2	3	57 57	93	Santos (2020)
1-aminocyclopropane-1-carboxylate synthase 3 (*CaACS3*)	F: GCTGCTTCTTCTCTTTTTGCCT R: CTGCCATCCCAGGAAGTACG	2	3	59 60	93	Santos (2020)
1-aminocyclopropane-1-carboxylate oxidase 3 (*CaACO3*)	F: ACGTGGAAGCCAATGTTACC R:GAGGGAGAAGAAAACATCCTAGC	1	1.5	56 55	96	Ságio et al (2014)
Malate dehydrogenase (*MDH*)	F: CCTGATGTCAACCACGCAACT R:GTGGTTATGAACTCTCCATTCAACC	2	3	59 60	100	De Carvalho et al. (2013)
Ribosomal Protein L39 (*RPL39*)	F: GCGAAGAAGCAGAGGCAGAA R: TTGGCATTGTAGCGGATGGT	2	3	59 60	100	Fernandes-Brum et al. (2017)

2.4.2 RNA extraction, cDAN synthesis and RT-qPCR assay

Leaves and flower buds of each treatment were collected and grinded in nitrogen with 50 mg of polyvinylpolypyrrolidone for total RNA extraction according to Oliveira et al. (2015) protocol. RNA samples (7.5 µg) were treated with DNase I from the Turbo DNA-free kit (Ambion) to eliminate DNA contamination. A 1% agarose gel was prepared to infer RNA integrity, and to analyze the content and quality of the RNA samples (OD 260/280 and OD 260/230 > 1.8)

Spectroscopy (Nano Vue GE Healthcare, Munich, Germany) was used. One µg of the total RNA was reverse transcribed into cDNA, the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, USA) was used 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), 1.5 µL of cDNA at 10 ng/µL, and the added forward and reverse gene-specific primers volume are described in (Table 2) when necessary RNase-DNase-free water was added to complete the final volume. For each biological replicate of the treatments, a technical triplicate reaction was to run. 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. The results were presented in relative expression.

2.5 Statistical analyses

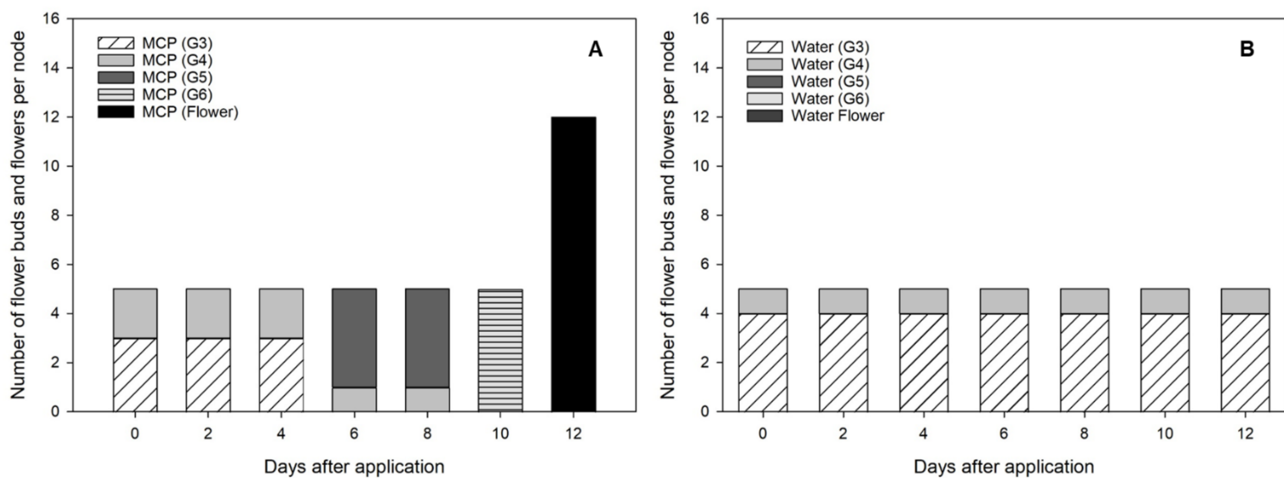
The phenotypic data were analyzed by means, no statistical analyses were made. The Ethylene, ACC, and ACO activity quantification results were submitted to analysis of variance by the F test, and comparison of means by Tukey test at 95% of probability. The SISVAR software was used as a computational aid for data analysis (FERREIRA, 2014). 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). Graphics were performed with SigmaPlot v. 14 (Systat Software Inc.).

3 RESULTS

Phenotypic characterization

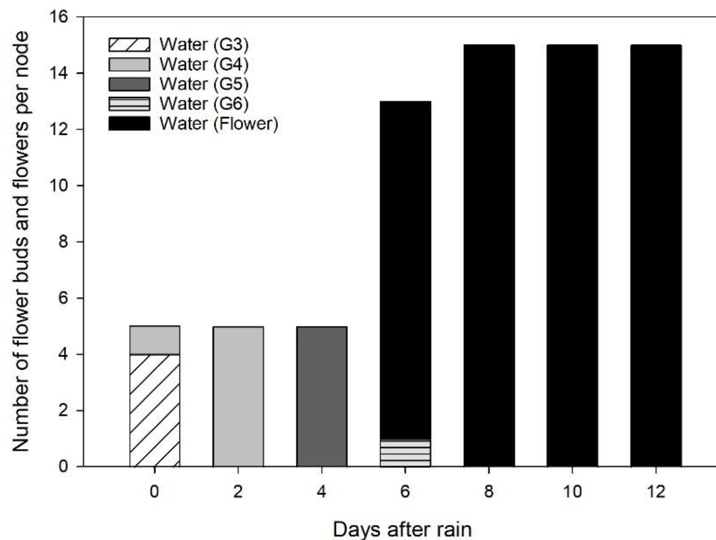
The results obtained from the flower bud count development after the 1-MCP and Water application had a direct relation to the 1-MCP effect (Figure 4). It took an average of six days for flower buds to break dormancy and increase size moving from G3 and G4 to anthesis (flower) in 12 days after the spray (A), while the control maintained flower buds dormant in G3 and G4 stages (B).

Figure 4. Number of flower buds (G3, G4, G5 and G6) and flowers per node after 1-MCP and water application in *C. Arabica* plants located at the Federal University of Lavras, 2021.



When the first rainfall arrived (Figure 5), the control plants that kept their flower buds dormant were counted again to observe how fast rain stimulate flower buds to anthesis compared to 1- MCP. The results presented showed, only two days after rain stimulation was already happening to move all flower bud to the G4 stage, and six days later flowers were almost finished blooming. An average of six days was necessary for the control treatment to blossom under the effect of the rain.

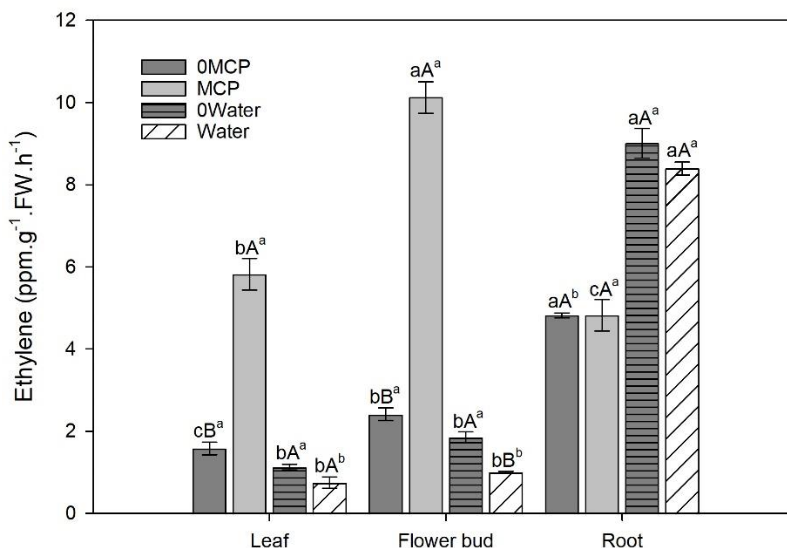
Figure 5. Number of flower buds (G3, G4, G5 and G6) and flowers per node after rainfall (Control - water) in *C. Arabica* plants located at the Federal University of Lavras, 2021.



Physiological and Biochemical analyses

Ethylene quantification results (Figure 6) were statistically different in the triple interaction between the studied factors. The 1 - MCP treatment before and after (two hours later) application was different in leaf and flower bud, not changing in roots. An increase of ethylene production was observed after the spray, in leaf increased 4 $ppm.g^{-1}.FW.h^{-1}$ compared to before application, while flower buds increased around 8 $ppm.g^{-1}.FW.h^{-1}$ compared to before. The water treatment results were not different in ethylene production, except in flower bud that had a decrease of ethylene production after application. The difference of ethylene production after application of 1-MCP compared to Water in leaf was 5 $ppm.g^{-1}.FW.h^{-1}$ and flower bud 9 $ppm.g^{-1}.FW.h^{-1}$.

Figure 6. Ethylene quantification in leaf, flower bud, and root before (0MCP and 0Water) and after (MCP and Water) 1-MCP and water application in *C. arabica* plants located at the Federal University of Lavras, 2021.

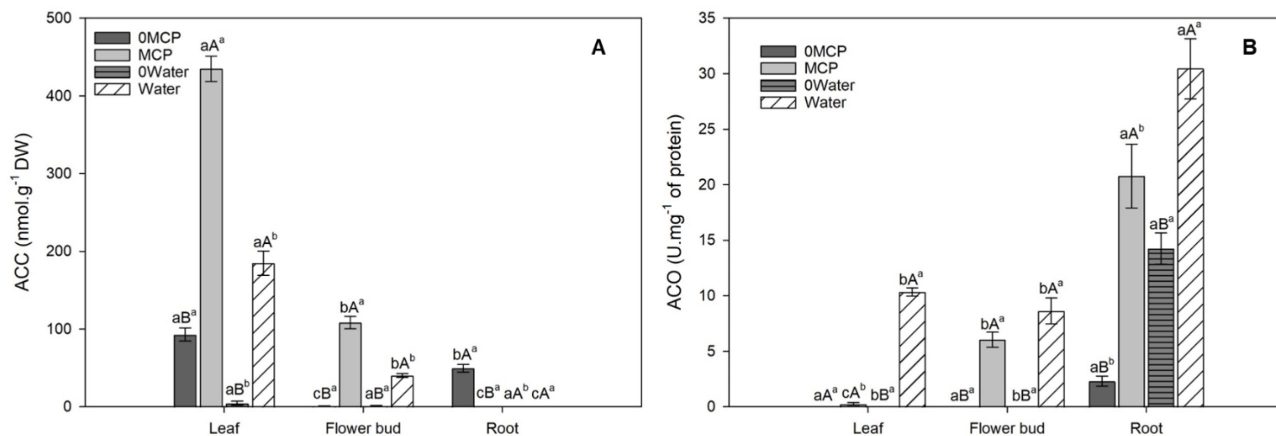


*Means followed by distinct lowercase letters differ from different tissues in the same application period and application treatment; uppercase letters differ in the same tissue, between application period in the same application treatment; and superscript letters differ in the same tissue, same application period, and different application treatment by Tukey test at $P<0.05$. Coefficient of variation: 11.16%.

The quantification of ACC (Figure 7.A) a precursor of ethylene production as well had results with triple significant interaction between the treatments. The application of 1-MCP increased by 341.69 $nmol.g^{-1}$ DW in leaf and 107.21 $nmols.g^{-1}$ DW in flower buds compared to no treatment. The root had 49.59 $nmol.g^{-1}$ DW before the application and none two hours later, which could be an indication of ACC translocation to the areal part of the plant.

The water application also showed an increase of ACC production like 1-MCP, however, in lower levels. In the leaf, ACC was higher at 180.26 $nmol.g^{-1}$ DW and flower buds 39.12 $nmol.g^{-1}$ DW compared to no treatment. The ACC in the root tissue was not detected before and after water application. The difference between 1-MCP and Water application (after) showed higher ACC levels in the 1-MCP treatment, where the leaf had 250.03 $nmol.g^{-1}$ DW and flower bud 68.15 $nmol.g^{-1}$ DW against the Water control.

Figure 7. ACC (A) and ACO activity (B) quantification in leaf, flower bud, and root before and after (two hours) 1-MCP and water application in *C. arabica* plants located at the Federal University of Lavras, 2021.



*Means followed by distinct lowercase letters differ from different tissues in the same application period and application treatment; uppercase letters differ in the same tissue, between application period in the same application treatment; and superscript letters differ in the same tissue, same application period, and different application treatment by Tukey test at P<0.05. Coefficient of variation: 19.84% (ACC) and 33.21%. (ACO).

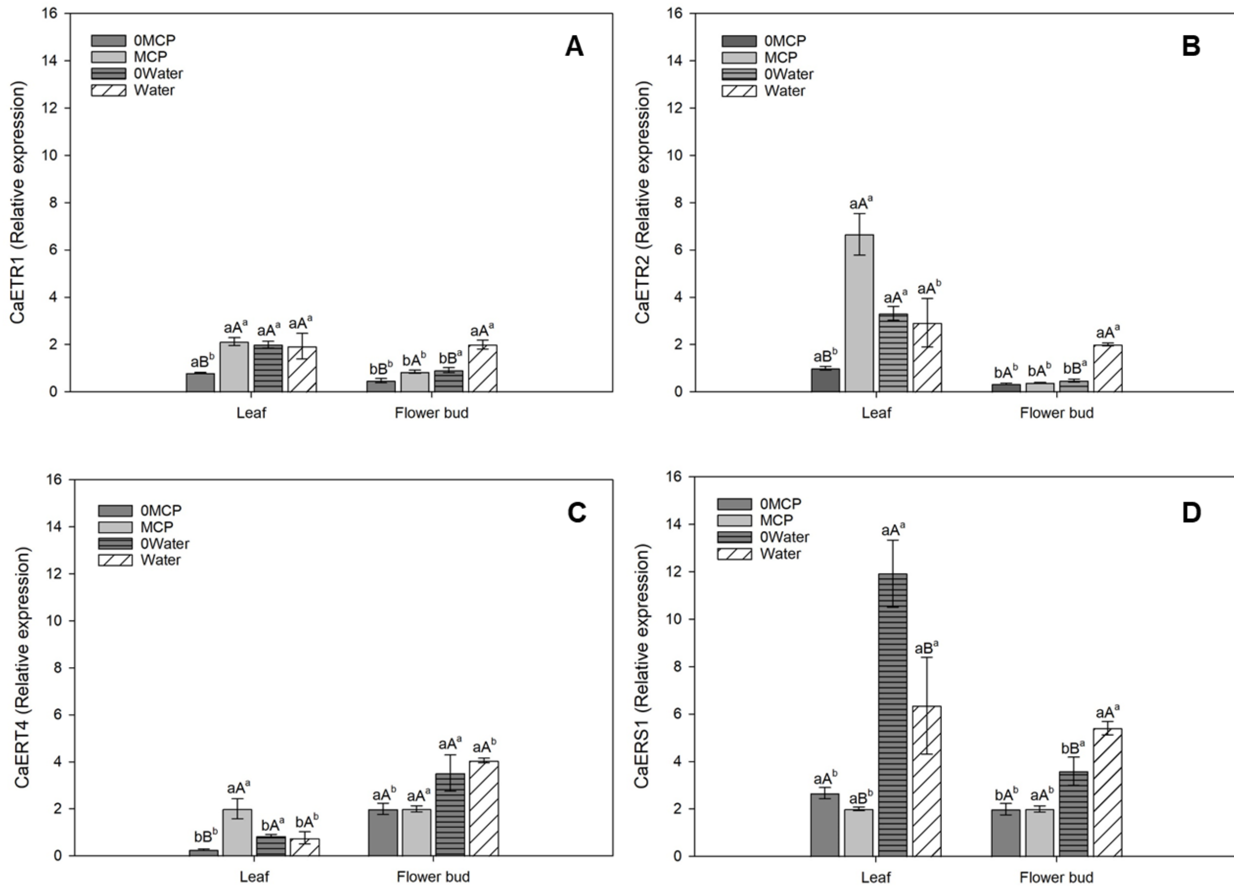
The ACO enzymatic activity (Figure 7.B) had triple interaction between the treatments and no activity was detected before the application of 1-MCP and water treatment for leaf and flower bud, however, ACO activity was observed in the root tissue before the spray. After 1-MCP and water were applied enzymatic activity increased especially in the water treatment plants for all tissues. While no difference was detected in the leaf after 1-MCP was applied, a significant increase was verified in flower bud and root, which represent six and nine times more activity compared to before application, respectively. The water treatment plants showed an increase of ACO activity after application in leaf, flower bud, and root, where there was 10, 9, and 2 times more activity, respectively.

Molecular analyses

The molecular analyses for the gene *CaETR1* (Figure 8.A) showed an increase of the gene expression after the 1 - MCP application in leaf and flower bud, the increase was higher in leaf with three times more expression compared to before application. The water treatment was not different in expression in leaf tissue; however, there was more expression after water spray in flower bud, this increase was four times more compared to no treatment.

CaETR2 (Figure 8.B) expression showed similar differences between the treatments, although the expression was higher in leaf tissue, where 1-MCP expression was six times more expressed after two hours of the spray. The flower bud did not differ between 1 -MCP applications. Water treatment only upregulated *CaETR2* leading to four times more expression compared to before the application.

Figure 8. *CaETR1* (A), *CaETR2* (B), *CaETR4* (C), and *CaERS1* (D) relative expression in leaf and flower bud before and after (two hours) 1-MCP and water application in *C. arabica* plants located at the Federal University of Lavras, 2021.



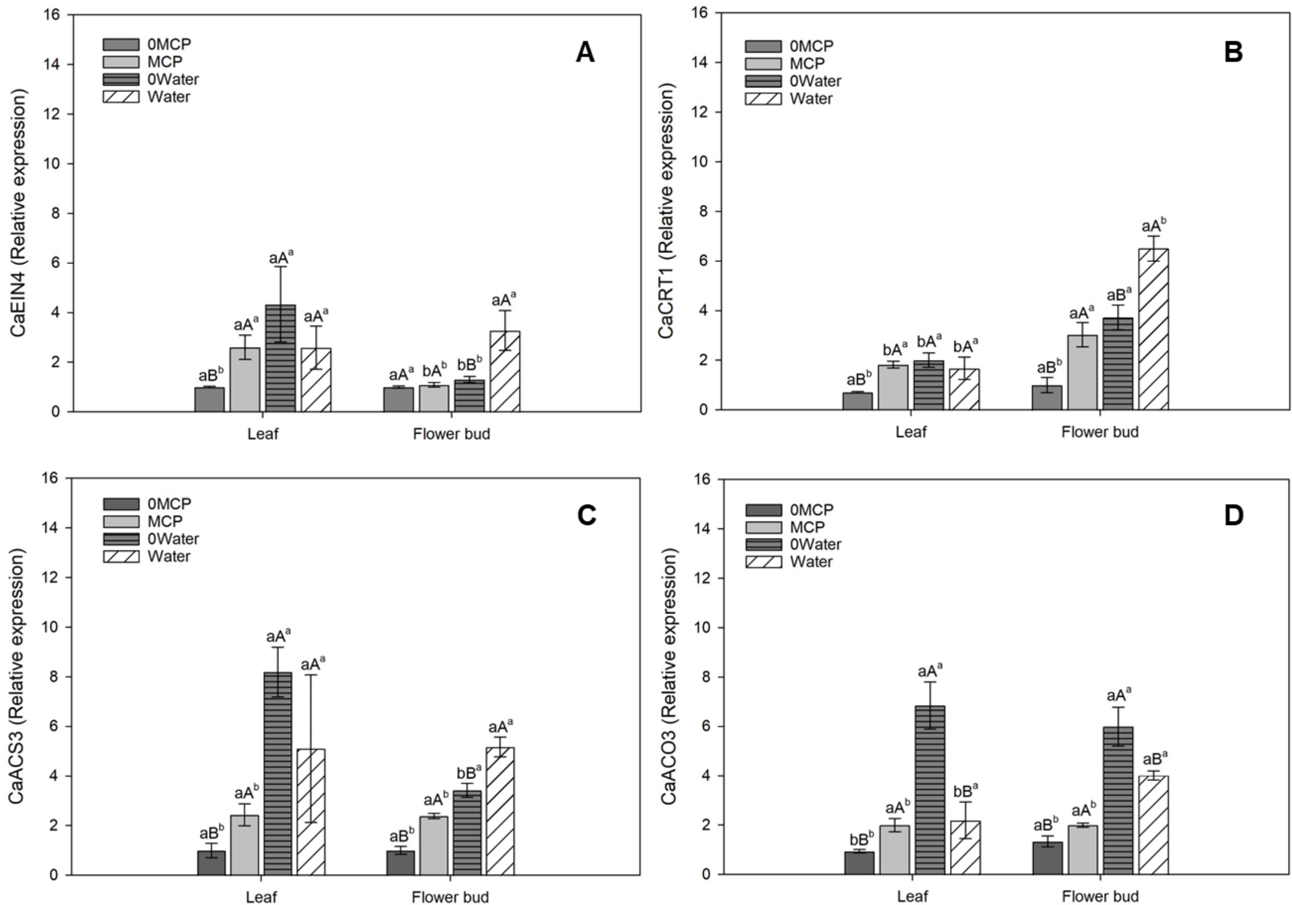
*Means followed by distinct lowercase letters differ from different tissues in the same application period and application treatment; uppercase letters differ in the same tissue, between application period in the same application treatment; and superscript letters differ in the same tissue, same application period, and different application treatment at P<0.001 of significance.

In *CaETR4* (Figure 8.C), only leaf treated with 1-MCP showed a difference in gene expression where after application there was seven times more expression compared to before, which maintain the tendency observed in the genes described previously. *CaERS1* (Figure 8.D) was more expressed before application (1-MCP) in the leaf tissue for both treatments and decreased after application as well. The expression kept the same in flower bud for 1-MCP (before and after) and increased after water application. *CaEIN4* (Figure 9.A) followed the same pattern of *CaETR1*, *CaETR2*, and *CaETR4* with 1-MCP application increasing the gene expression in leaf tissue but not in flower bud. The same

increase of expression after water application was observed in flower bud as in the described previous genes.

CaCTR1 expression (Figure 9.B) for 1-MCP treatment presented higher expression after the application in leaf and flower bud tissues, the expression was three times more expressed in both tissues. For the water treatment, only in flower bud tissue there was an increase of expression after application. *CaACS3* and *CaACO3* genes (Figure 9.C/D) increased twice the expression after 1-MCP application in both tissues, while coffee plants treated with water had a decrease of gene expression in both tissues, except in *CaACS3* where gene expression was higher after water spray in flower bud.

Figure 9. *CaEIN4* (A), *CaCRT1* (B), *CaACS3* (C), and *CaACO3* (B), relative expression in leaf and flower bud before and after (two hours) 1-MCP and water application in *C. arabica* plants located at the Federal University of Lavras, 2021.



*Means followed by distinct lowercase letters differ from different tissues in the same application period and application treatment; uppercase letters differ in the same tissue, between application period in the same application treatment; and superscript letters differ in the same tissue, same application period and different application treatment at P<0.001 of significance.

4 DISCUSSION

The application of 1 - MCP as a flowering inducer in *C. Arabica* plants by the end of the dry season (winter in Brazil) to verify its efficiency showed promising results statistically significant ($P<0.05$), with a direct correlation between phenotypic flower bud development, physiological stimulation and molecular expression.

Phenotypic characterization

The 1-MCP effect took six days to break flower bud dormancy and promote anthesis. The sensitivity for flower bud development happened slower in 1-MCP (Figure 4.A) than the control (water) after the rainfall (Figure 5), while the control without rain (Figure 4.B) flower bud remained dormant. This might have occurred because the 1-MCP causes an indirect stimulation by negative *feedback* in the ethylene biosynthesis since the cyclic olefin function is to block the receptors of the ethylene signaling pathway (SISLER; SEREK, 1997; 2003). This stimulation may happen in the lack of ethylene entering the signaling pathway sensed by the plant, which will stimulate ethylene production.

The motive that could justify the difference in speed for flower bud development under the effect of 1-MCP and rain, might be the indirect (1-MCP) and direct (rain) stimulation caused by each. In coffee, the rain is known as an important factor that stimulates anthesis after the dry season and has been suggested that interacts directly with ACC, the precursor for ethylene production, as is a soluble molecule (CRISOSTO et al., 1992, BRADFORD; YANG, 1980, GÓMEZ-CADENAS et al., 1996; MORRIS; LARCOMBE, 1995, TUDELA; PRIMO-MILLO, 1992).

Physiological and Biochemical analyses

Ethylene production confirmed the indirect stimulation promoted by 1-MCP two hours later in the application, while the water treatment decreased production (Figure 6). The ethylene increase was higher in flower bud than leaf and the presence of ACC (Figure 7A) in flower bud and leaf, also was directly increased with the application of 1-MCP. Although ethylene production decreased when water was applied in leaf and flower bud, the water stimulated ACC translocation to those tissues probably due to the affinity with water.

The low detection of ACC in the root could be due to ethylene production in the same tissue indicating the stress situation of the plant. The higher levels of ACO activity (Figure 7.B) in the root

than leaf and flower bud confirm the stress, together with the levels of ethylene in the root observed previously (Figure 6). The dry season causes high levels of stress in coffee plants and ethylene is responsible for root growth inhibition (WEN, 2015), which was verified in the area during the root collection for the experiment.

ACO activity was not detected before 1-MCP and water application in leaf and flower bud; however, two hours later there was a small (not significant) activity expression in the leaf of 1-MCP treatment and a high increase in the water treatment. In flower bud, the ACO activity showed an increase for both treatments after application indicating that ACO enzymes were present in all tissues before the application. ACO is always present in all tissues of the plant and the amount of enzymes does not limit the oxidation of ACC into ethylene because one enzyme can make many reactions, as long as they have ACC for oxidation (YANG; HOFFMAN, 1984; KENDE, 1993).

Molecular analyses

The genes responsible for the perception of the ethylene *CaETR1*, *CaETR2*, *CaETR4* (Figure 8), and *CaEIN4* (Figure 9.A) had an increase of expression two hours later after 1-MCP application compared to before in the leaf. This shows that 1-MCP besides blocking the receptors stimulated the production of more receptors, which configured the plant sensitive to the lack of ethylene entering the pathway. However, in the flower bud, the expression did not differ from before and after application for all receptors including *CaERS1*, the response for the lack of ethylene entering in flower bud could take more time to stimulate more receptors production. *CaERS1* expression in leaf was reduced after 1-MCP spray, probably by the blocking effect of the product.

When we look back to ethylene results (Figure 6), it is possible to see that leaf increased receptors expression but ethylene increase was not as high as the flower bud ethylene production. It seems that if receptors are replaced faster, the ethylene production increase will not be as higher as observed in flower buds.

Now looking in flower bud perception genes (CaETR1, *CaETR2*, *CaETR4*, *CaERS1*, and *CaEIN4*) expression did not differ (Figure 8/9) but ethylene production was 1.7 times higher than leaf ethylene production (Figure 6). The lack of change in gene expression after 1-MCP application could promote a higher stress response in flower bud to induce more ethylene production by nagetive *feedback* in the biosynthetic pathway since all gene receptors were binding with 1-MCP and blocking ethylene from entering the signaling pathway. These observations confirm the effect of the

1-MCP in indirectly promoting ethylene production in coffee plants and inducing flowering slower than the rain (Figure 4/5), and leaf recovers faster from the 1-MCP binding than flower buds.

Plant cells must have receptors on molecules that can detect varying amounts of ethylene. (WEN, 2015, MOUSSATCHE et al., 2004). The functional efficacy of 1-MCP results from its affinity for the ethylene receptors, which is about 10 times greater than that of ethylene. This justifies the greater connection with the receptors and fast diffusion in plant tissues and half-life ranging between 7 and 12 days while the duration of binding of the ethylene is effective for a maximum of 10 minutes (BLANKENSHIP; DOLE, 2003; KEBENEI et al., 2003).

The water application did not differ in expression before and after spray in leaf tissue for all receptors (Figure 8) genes, except for *CaERS1* (Figure 8.D) where there was a decrease in expression after the application. In flower bud there was an increase of gene expression in all receptors, except *CaERT4*, that could have happened due to the affinity of the water with ACC (Figure 7.A) that could be stimulating ethylene production, however, this stimulation were not enough to induce flowering (Figure 4.B) and to produce more ethylene (Figure 6). It could be hypothesized, that the plant increase receptor availability (expression) to bind with ethylene, as an environment perception of rain even dough is not there yet.

The amount of rain needed to stimulate flowering in generally is between 10 and 35 mm (PEREIRA et al., 2008), and as suggested by Lima et al. (2021) rain would enable ACC translocation via the xylem to the shoot, consequently increasing ethylene concentration in the flower buds and promoting flowering.

The *CaCRT1* gene showed an increase of expression after the 1-MCP application that can be justified by the binging happing in the receptors. A greater expression happened in flower bud than leaf tissue, probably due to less expression of the receptors. The water treatment *CaCRT1* expression did not differ in leaf before and after application however increased in flower bud, which indicates that there was less ethylene (Figure 6) to be binding with the receptors in the signaling pathway.

After seeing that 1-MCP interferes in the ethylene perception and signaling pathway by binding and stimulating more receptors expression due to the lack of available receptors, the biosynthetic pathway was analyzed as well to conform to the possibility of negative *feedback* be happening. The expression of the genes responsible for the enzymatic activity *CaACS3* and *CaACO3* (Figure 9 C/D) that converts and oxidize ACC in ethylene showed higher expression after 1-MCP application compared to before in the two tissues (leaf and flower bud) this increase can be directly related with the ethylene production quantified in this work (Figure 6). The

water treatment showed the inverse results with decreased expression in both genes, which can also be related with the ethylene quantification as in both tissue, a decrease was also observed.

Two main reactions are specific to the ethylene biosynthesis pathway, conversion of SAM to ACC and ACC to ethylene, catalyzed by ACS and ACO enzymes, respectively (KENDE, 1993). The ACS and ACO activity are stimulated under conditions that promote the formation of ethylene (YANG; HOFFMAN, 1984; SATO; THEOLOGIS, 1989; ZAREMBINSKI; THEOLOGIS, 1994; WANG et al., 2002).

5 CONCLUSION

1-MCP is efficient in promoting flowering after six days of application in *Coffea arabica* plants by binding with the ethylene perception receptors (*CaETR1*, *CaETR2*, *CaETR4*, *CaERS1*, and *CaEIN4*), and blocking the signaling (*CaCTR1*) pathway.

The 1-MCP binding induces more receptors production (*CaETR1*, *CaETR2*, *CaETR4*, *CaERS1*, and *CaEIN4*). Ethylene production is increased by negative *feedback* in the biosynthetic pathway (*CaACS3* and *CaACO3*). ACC and ACO activity is increased by the 1-MCP effect, confirming the indirect correlation between 1-MCP receptors binding to induce ethylene production and anthesis.

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