ANDRÉALMEIDA LIMA

ETHYLENE REGULATION UNDER DIFFERENT WATERING CONDITIONS AND ITS POSSIBLE INVOLVEMENT IN COFFEE (*Coffea arabica* L.) FLOWERING

LAVRAS - MG

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Thesis submitted for the degree of Doctor of Philosophy as a Dual PhD with Agronomia/Fisiologia Vegetal Postgraduate Program, Universidade Federal de Lavras, Brazil, and Science of Tropical Environments Postgraduate program, Lancaster University, United Kingdom.

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To God for always guiding me through the PhD and to my family for all the support and love.

I dedicate

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"There is no such a thing as knowing more or knowing less, there are different kinds of knowledge"

Paulo Freire

GENERALABSTRACT

Coffee is one of the world's favourite beverage and the second most traded commodity after oil However, coffee quality can be greatly affected by fruit ripening stage at harvest time, which is often asynchronous due to the sequential flowering in this species. Coffee sequential flowering is a result of asynchronies in bud development and also environmental factors, since anthesis is triggered by precipitation after a period of water deficit. This series of events is commonly associated with an increase in ethylene production levels, suggesting that ethylene may be involved in the control of coffee anthesis promotion and its regulation might help to reduce the number of flowering events and thus promoting blossoming concentration. Thus, it was hypothesised that a drought-rewatering-induced ethylene burst might be the basis of the drought-rewatering-induced flowering in coffee trees. In order to test this hypothesis, this study aimed to access the effects of exogenous 1-MCP application on flowering of coffee trees under field conditions and, under greenhouse conditions, we aimed to study the ethylene regulation, through quantification of ethylene levels and expression levels of genes of the ethylene biosynthesis and signaling pathways, in coffee plants under well-watered and water-deficit conditions, as well as, after plant rehydration and 1-MCP treatment.1-MCP exogenous application, at the concentrations of 50 and 100 mg of a.i. per litre and with spray volume of one litre per plant, was able to promote coffee anthesis in trees of the cultivar ‘Acaiá Cerrado' under field conditions. Ethylene production rates in coffee seedlings from two cultivars, ‘Bourbon Amarelo' and ‘Obatã Vermelho’, growth under greenhouse conditions, were similar under well-watered and water-deficit conditions. Plant rehydration and 1-MCP treatment did not change ethylene release rates from both cultivars when compared to plants under well-watered and water-deficit conditions. However, the analysis of the ethylene regulation, through gene expression analysis, shortly after plant rehydration and 1-MCP treatment, suggest that increased levels may occur, helping to explain anthesis promotion upon 1-MCP application, and supporting the hypothesis that a drought-rewatering-induced ethylene burst might be the basis of the drought-rewatering-induced flowering in coffee trees.

Keywords: Flowering. Rrewatering. 1-MCP. Ethylene. Coffee tress.

RESUMO GERAL

O café é umas bebidas favoritas do mundo e a segunda commodity mais comercializada no mundo após o petróleo. Entretanto, a qualidade do café pode ser grandemente afetada pelo estádio de maturação dos frutos na época de colheita, se mostrando frequentemente bastante desuniforme devido ao florescimento sequencial nessa espécie. O florescimento sequencia do cafeeiroé resultante do desenvolvimento desuniforme de suas gemas e também devido a fatores ambientais, tendo em vista que a antese é desencadeada por precipitação após um período de restrição hídrica. Esta série de eventos é comumente associada com um aumento nos níveis de etileno, sugerindo que o etileno possa estar envolvido no controle da promoção da antese do cafeeiro e a regulação de seus níveis poderia auxiliar na redução do número de floradas a assim promover a concentração das floradas. Assim, foi proposto nesse trabalho, que um grande aumento na produção de etileno em resposta à reidratação após um período de seca promove o florescimento do cafeeiro. Para testar esta hipótese, analisou-se os efeitos da aplicação exógena de 1-MCP no florescimento do cafeeiro em condições de campo e em condições de casa de vegetação, objetivando-se estudar a regulação do etileno, por meio da quantificação de níveis e por meio de análises da expressão de genes das rotas de biossíntese e sinalização do etileno, em plantas mudas de cafeeiro bem-irrigadas e sob déficit-hídrico, assim como após a reidratação e aplicação do 1-MCP. A aplicação exógena de 1-MCP, nas concentrações de 50 e 100 mg de i. a. por litro e com um volume de aplicação de um litro por planta, foi capaz de induzir a antes do cafeeiro em plantas da cultivar ‘Acaiá Cerrado’ em condições de campo. As taxas de produção de etileno em mudas de cafeeiro de duas cultivares, ‘Bourbon Amarelo' e ‘Obatã Vermelho’, mantidas em casas de vegetação, foi similar em plantas bem-irrigadas e sob déficit-hídrico. A reidratação das plantas e o tratamento com 1-MCP não promoveu mudanças nas taxas de produção de etileno em ambas as cultivares e quando comparado com os níveis de etileno produzido em plantas bem-irrigadas e sob déficit-hídrico. Entretanto, a análise da regulação do etileno, por meio das análises da expressão gênica, logo após a reidratação das plantas e aplicação do 1-MCP, sugere que elevados níveis de etileno possam ocorrer, auxiliando na compreensão da promoção da antese pela aplicação de 1-MCP, e dando suporte a hipótese de que um grande aumento na produção de etileno em resposta à reidratação após um período de seca possa promover o florescimento do cafeeiro.

Palavras-chave: Florescimento. Re-irrigação. 1-MCP. Etileno. Cafeeiro.

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CHAPTER 1

Coffee flowering regulation

ABSTRACT

Coffee quality, among other factors, is directly associated to the fruit ripening stage at harvest time, which is often highly asynchronous due to the sequential flowering found in coffee trees, and usually leads to higher production costs and also a lower cup quality. Coffee sequential flowering is a result of asynchronies in bud development and also environmental factors, since anthesis is triggered by precipitation after a period of water deficit. Once flower buds complete their differentiation, usually in the beginning of the dry season, they enter in a dormant state, which is broken by rain or irrigation. Certain levels of water deficit during the dry season seem to be required for coffee flower buds to become sensitive to respond to the stimulus of regrowth upon plant rehydration. This series of events, water deficit followed by rehydration, is commonly associated to changes in root-to-shoot hormonal signaling, especially for the plant hormone ethylene. Rapid and transient increases in ethylene production have been shown to occur after plant rewatering following water deficit periods. Some studies have shown that these increased levels of ethylene are important for water recovery in flowers and flower opening, controlling these processes by regulating the expression of rehydration responsive genes. Thus, in this study, it is proposed that a drought-rewatering-induced ethylene burst is the basis of the drought-rewatering-induced flowering of coffee trees.

Keywords: Coffee. Flowering. Ethylene.

RESUMO

A qualidade do café, dentre outro fatores, está diretamente relacionada com o estádio de maturação dos frutos na época da colheita, o qual é altamente desuniforme devido ao florescimento sequencial encontrado no cafeeiro, e geralmente leva a maiores custos de produção e uma menor qualidade da bebida.O florescimento sequencial do café é resultante da não uniformidade no desenvolvimento das gemas e também a fatores ambientais, visto que a antese é desencadeada por precipitação após um período de déficit hídrico. Assim que as gemas florais completam a sua diferenciação, geralmente no inicio da estação da seca, elas entram em um estado de dormência, o qual é quebrado pela chuva ou irrigação. Certos níveis de déficit hídrico parecem ser necessários para as gemas florais se tornarem sensíveis a responder o estimulo para a retomada do crescimento após a reidratação da planta. Essa série de eventos, déficit hídrico seguido de reidratação, é comumente associada a mudanças na sinalização hormonal entre raiz e parte aérea, especialmente do fitormônio etileno. Aumentos rápidos e transientes nos níveis de etileno têm sido observados após a reidratação de plantas após períodos de deficit hídrico. Alguns estudos mostram que estes maiores níveis de etileno são importantes na recuperação hídrica de flores e para a abertura de flores, controlando esses processos pela regulação de genes responsivos à reidratação.Assim, neste estudo, é proposto que um grande aumento na produção de etileno em resposta à reidratação após um período de seca promove o florescimento do cafeeiro.

Palavras-chave: Café. Florescimento. Etileno.

1.1 INTRODUCTION

Coffee is one of the most important crops in the world being classified, after oil, as the second most valuable traded commodity worldwide. Brazil poses as an important country in this scenario as the world's largest coffee producer, being responsible for 32% of coffee world's production, and exporter, with 34% from total coffee exportations coming from Brazil, the largest consumer after the United States (International Coffee Organisation (ICO) 2014).

Originated in the African continent, more precisely in Ethiopia, coffee belongs to the Rubiaceae family and its genus, Coffea, is represented by more than 100 species (DAVIS et al. 2006), among which only Coffea arabica L. and C. canephora are commercially important, representing 60% and 40% of the world's coffee production (International Coffee Organization (ICO) 2014).

Coffee quality, among other factors, is directly associated to fruit ripening stage at harvest time, which is often highly asynchronous due to sequential flowering found in this species. This may lead to a lower cup quality and also to increased productions costs, since more than one harvesting operation will be necessary, and post-harvest fruit selection may also be required (DAMATTA et al., 2007)

Coffee sequential flowering is a result of asynchronies in bud development along the branches, at the vegetative and reproductive levels (DE OLIVEIRA et al., 2014; MAJEROVICZ & SONDAHL, 2005), and the influence of environmental factors, since a period of water deficit seems to be required for flower bud competence and coffee anthesis is triggered by rain after a period of water deficit (Alvin, 1960; Barros et al., 1999; Rena & Maestri, 1985). Once flower buds complete their differentiation, growth is stopped, and buds enter in a dormant or latent state. Coffee flower bud dormancy coincides with the beginning of the dry season under Brazilian tropical conditions, and

dormancy breaking is triggered by rain or irrigation after this period of water restriction (Alvin, 1960; Barros et al., 1978). Certain level of water deficit seems to be required for flower buds to acquire competence to progress to anthesis upon rewatering (CRISOSTO et al., 1992; MAGALHÃES; ANGELOCCI, 1976), and blossoming concentration may be achieved after more prolonged water deficit conditions or on irrigated crops (Guerra et al. 2005; Silva et al. 2009).

Apparently, coffee flower bud competence, dormancy breaking, and consequent growth resumption, are mediated by changes in root-to-shoot chemical signaling during and after water stress relief upon rewatering (CRISOSTO et al., 1992). Considering the requirement of a water deficit period followed by rainfall to break flower bud dormancy, a series of events well-known to induce an ethylene burst in woody species (GOMEZ-CADENAS, et al., 1996), and fact that flower bud competence and regrowth are probably controlled by a root-sourced signal (CRISOSTO et a., 1992), ethylene is a good candidate to participate in coffee flowering promotion.

Ethylene is a plant hormone that is involved in the regulation of several developmental processes, such as organ abscission, seed germination, growth transition from vegetative phase to reproductive phase, flowering, fruit ripening, senescence, and is also involved in biotic and abiotic stress responses (ABELES et al., 1992). Ethylene can inhibit (ACHARD et al., 2007) or promote (TRUSOV; BOTELA, 2006; WANG et al., 2007) flowering depending on the species, and it regulates different aspects of flower development, including pollen and ovule development (DE MARTINS; MARIANI, 1999; HOLDEN et al., 2003), flower opening (ÇELIKEL et al., 2012; REID et al., 1998), and flower senescence (SHAHRI; TAHIR, 2013).

A recent study has shown that a rapid and transient elevation in ethylene production, upon rewatering after a period of water stress, serves as a signal to

ensure water recovery in flowers and promotes flower opening by influencing the expression of a set of rehydration-responsive genes (MENG et al., 2014). Thus, in this study, we propose that a drought-rewatering-induced ethylene burst would be the basis of the drought-rewatering-induced flowering of coffee trees.

1.2 LITERATURE REVIEW

1.2.1 Coffee: botanical and economical aspects

Being originated in the African continent (Ethiopia), coffee belongs to the *Coffea* L. genus, from the *Rubiaceae* family, which has more 100 species identified so far (DAVIS et al., 2006). All species from this genus are diploid (2n=2x=22) and present cross-fertilization, being highly self-incompatible due to the presence of a gametophydic system of incompatibility controlled by one gene with multiple alleles (CHARRIER; BERTHAUD, 1985). The species *Coffea arabica* L. (*C. arabica*) represents an exception in this context and consists of an alotetraploid (2n=4x=44) species, originated from the natural cross between *Coffea eugeniodes* and *Coffea canephora* (LASHERMES et al., 1999), which reproduces mainly (around 90%) by self-fertilization (CONAGIN; MENDES, 1961).

Coffee trees may reach up to four meters high and their branches can be divided in two groups, orthotropic branches, vertical growing, and plagiotropic branches, lateral growing (CASTRO et al., 1987). They show deep primary roots that branch in the upper layers of the soil. The leaves are opposite and show an elliptic format (ALVEZ; LIVRAMENTO, 2003). Arabica coffee inflorescences are formed in the leaf axilla from plagiotropic branches developed in the previous growing season (from September to March) (*C.canephora* plants may display inflorescences on branches formed in the current growing season). Each leaf axilla harbors 4 to 5 buds arranged in a linear series, resulting in up to 10 buds per node. These buds rarely develop into vegetative branches and usually form inflorescences or remain dormant. Each inflorescence gives rise to up to four flower buds (MAJEROVICZ & SONDAHL, 2005).

Coffee fruits are classified as drupes that, in a simplified way, can be characterized as fleshy fruits, indehiscent, with the pericarp clearly differentiated in exocarp, mesocarp and endocarp, which involves the bilocular ovary where two seeds can be found (RODRIGUES, 2001; SILVA, 2002). The exocarp (peel) is the most external tissue of the fruit and is formed by a single layer of compacted, polygonal, and parenchymatous cells, with a variable number of chloroplasts and stomata evenly distributed on its surface. Exocarp is green during most part of coffee fruit development and turns red or yellow, depending on the cultivar or progeny, during the ripening process (BORÉM, 2008). The mesocarp, also referred as mucilage, is a tissue with a gelatinous consistency composed by parenchymatous cells containing vascular tissues (xylem and phloem). In green fruit, the mesocarp consists of a hard tissue that, due to the action of pectinolytic enzymes, undergoes softening during ripening (CASTRO; MARRACCINI, 2006). The endocarp, also referred as parchment, is composed of a hard and lignified cover that involves the seeds. As the endocarp develops, its cells changes into sclerenchyma cells, conferring the fibrous texture observed in ripened fruits. Coffee seeds are formed by the silver skin or spermoderm, which is the outermost layer that wraps the seeds and whose function remains to be discovered, the endosperm, which constitute the storage tissue of coffee seeds, and embryo, formed by a hypocotyl and two cotyledons (BORÉM, 2008).

Among the cultivated species, only *C. arabica* and *C. canephora* show economical importance, being responsible for 60% and 40% of world's coffee production. According to the ICO, Brazil is the first and second largest producer and consumer of coffee in the world, respectively. More than 70% of the Brazilian coffee production is made of arabica coffee and the Minas Gerais state is the national leader in coffee production, with more than 50% of all the coffee produced in Brazil. According to Conab (Companhia Nacional de Abastecimento), the Brazilian agency responsible for crop forecasts, coffee

production in 2014/2015 came to 45.3 million bags, its lowest level in the last three years. This reduction in the coffee production last year was probably caused by the severe drought and high temperatures faced last year. In spite of that, coffee exports from Brazil reached a record high of 36.8 million bags in crop year 2014/2015 (April to March) and was worth more than US$6.5 billion.

1.2.2 Coffee phenology

The coffee phenological cycle presents a succession of vegetative and reproductive phases that occur in approximately two years, differently from the majority of plants where the inflorescences are emitted in the spring and the fruits are formed in the same phenological year (CAMARGO, 1985). Coffee trees are classified as biennial plants and, the phenological cycle for *C.arabica*, under the tropical climate conditions of Brazil, can be divided into six different phases (Figure 1.1): (1) vegetative growth and flower bud formation; (2) flower bud induction and maturation; (3) Anthesis; (4) fruit growing; (5) fruit ripening; (6) senescence of tertiary and quaternary branches (CAMARGO; CAMARGO, 2001).

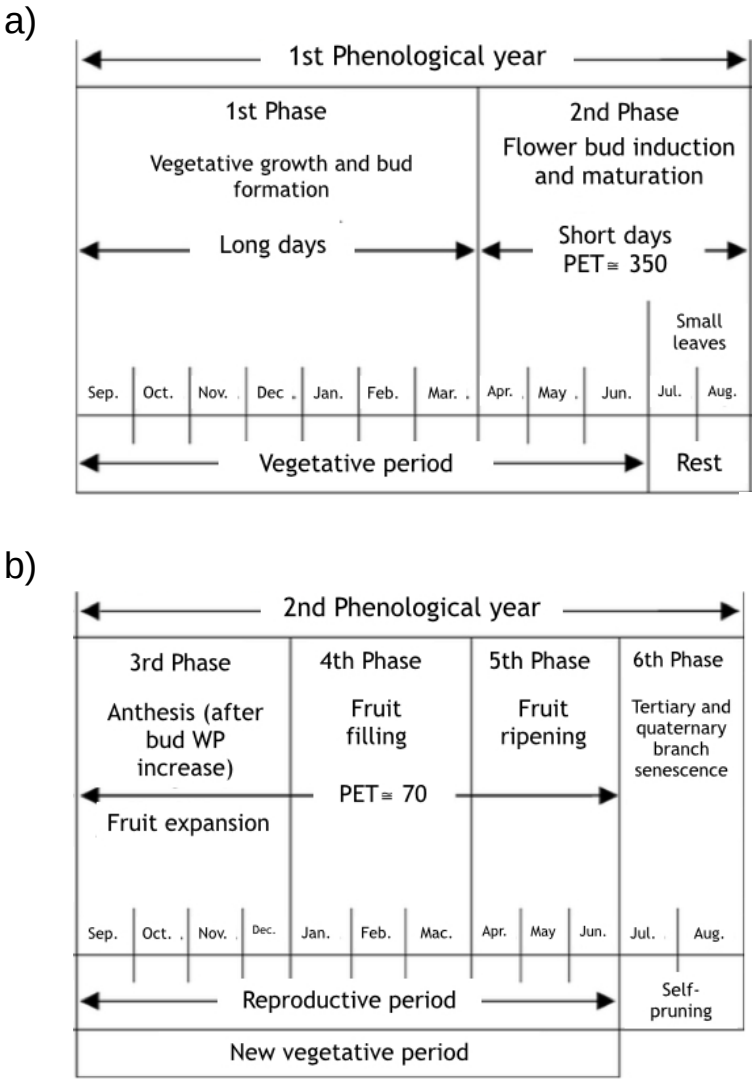

Figure 1.1 Representation of the six phenological phases between the first (a) and second (b) phenological years of Arabica coffee under Brazilian tropical climate conditions (adapted from CAMARGO; CAMARGO, 2001). PET stands for Potential Evapotranspiration.

The first phenological year is characterized by vegetative growth (Phase 1), which usually takes place from September to March, time characterized by

the occurrence of long days, and by the formation and maturation of flower buds (Phase 2) from April to August, when day length becomes shorter. After their complete development, flower buds enter in a dormant phase and become ready for anthesis, which is promoted by a substantial increase in their water potential in response to precipitation or irrigation. In the two last months of Phase 2, July and August, the dormant buds produce a pair of small leaves, separating the first from the second phenological year (CAMARGO; CAMARGO, 2001; MES, 1957; GOUVEA, 1984).

The second phenological year begins with anthesis (Phase 3), after an increase in the water potential of the mature flower buds, followed by the growing and expansion of coffee fruit between September and December. In water deficit conditions, coffee trees usually show three flowering events, with the first taking place around the end of August, the second at the end of September / beginning of October (usually responsible for more than 90% from all the production), and the third occurs around the middle of November. These sequential flowering events lead to fruits at different ripening stages at harvest time, which can greatly affect cup quality (REIS; CUNHA, 2010). Phase 4 corresponds period fruit growing and development, where fruit expansion reaches its maximum. From April to June, fruits undergo the process of ripening (Phase 5), where fruits slightly increase their size and a change in their colour can clearly be observed. Approximately 24 to 34 weeks after anthesis, fruit ripening is completed and thus the seeds are completely developed. During Phase 6, from July to August, there is the senescence of tertiary and quaternary branches (CAMARGO; CAMARGO, 2001).

1.2.3 Coffee flowering

Coffee flowering is a complex process that comprises a sequence of biochemical, physiological, and morphological events, which are affected by several factors such as temperature, light, soil, water availability, carbon/nitrogen ratio, and genotype (RENA; BARROS, 2004). It may be separated into four different phases with some overlap among them in some cases: (1) flower induction, (2) flower differentiation, (3) dormancy, (4) Anthesis (RENA & MAESTRI, 1985).

In general, flower induction, which ends with the formation of the flower primordia, which is recognizable only under microscope, occurs in response to floral inductive cues, where a vegetative bud becomes genetically determined to produce a flower or an inflorescence (ZIK & IRISH, 2003). Although some authors suggest that photoperiod, temperature, precipitation, and internal factors such as plant hormones and carbohydrates may be involved in coffee flower induction, the mechanism that triggers the perception of these stimuli and activation of reproductive development is still poorly understood (CRISOSTO et al., 1992; SOARES et al., 2005).

As mentioned above, each leaf axil has four to five buds, which are at different developmental stages from their inception (MAJEROVICZ; SONDAHL, 2005; DE OLIVEIRA et al., 2014). After induction, four floral meristems are formed inside each bud and their development is also asynchronous (DE OLIVEIRA et al., 2014). These asynchronies are considered to be as the main cause non-uniform flowering in coffee, which will then lead to fruits at different ripening stages at harvest time. In addition, it should be mentioned that there is an age gradient on coffee branches, where closer to the branch apex, younger the leaves and buds (Figure 1.2).

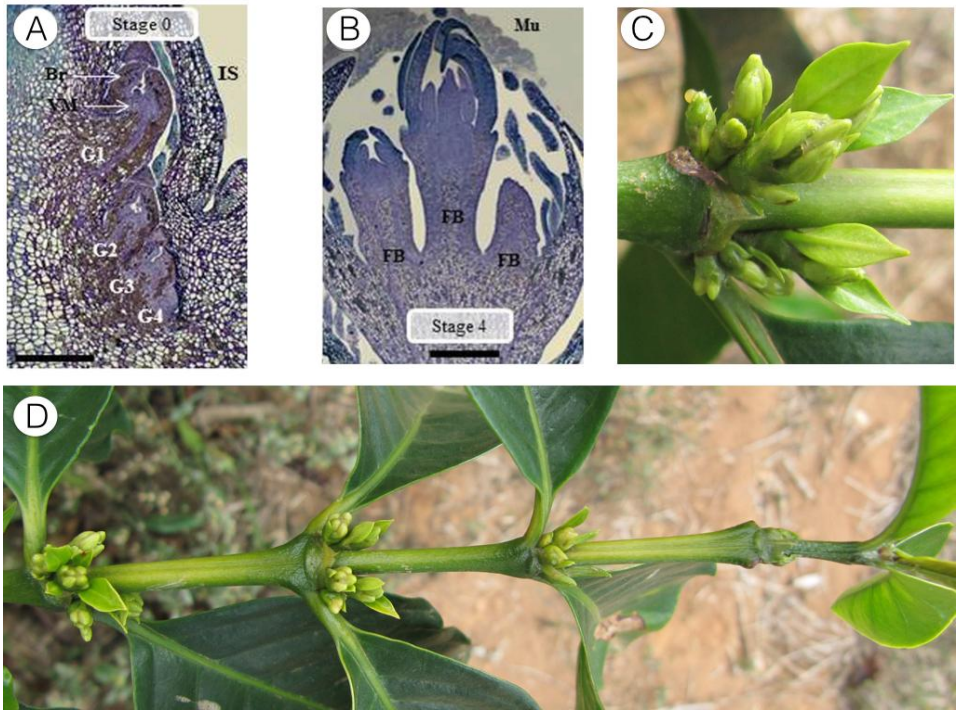

Figure 1.2 Representation of the different levels of bud developmental asynchronies found in coffee trees. Morphological analysis of four vegetative buds (G1-G4) (A) and an inflorescence with three flower buds represented (B) (DE OLIVEIRA et al., 2014). *Br* bracts, *VM* vegetative meristem, *IS* interpetiolar stipules, *FB* flower buds, *Mu* mucilage. Inflorescences at different developmental stages on the same node (C). Developmental gradient of flower buds along a plagiotropic branch (D).

After induction, flower differentiation takes place as a continuous process for around two months, until flower buds reach 4 to 6 mm. Morais et al. (2008) characterized the transition to the reproductive state dividing coffee flower growing and development into six different stages or sub-phases (Figure 1.3): (G1) non-differentiated buds; (G2) buds with a broad and flat apex; (G3) flower buds with up to 3 mm in length; (G4) flower buds ranging from 3.1 to 6 mm in length; (G5) flower buds ranging from 6.1 to 10 mm in length (light green colour); (G6) flower buds bigger than 10 mm in length (white colour).

After G6 flowers open, start to wilt on the next day, and fall on the third day after opening. At the stage G4, all floral whorls have been formed and flower differentiation is completed. Once flower buds receive the stimulus for regrowth, through elongation, they disrupt a mucilage barrier, which is secreted by colleters, and flower opening is allowed to occur (MAJEROVICZ; SONDAHL, 2005; DE OLIVEIRA et al., 2014).

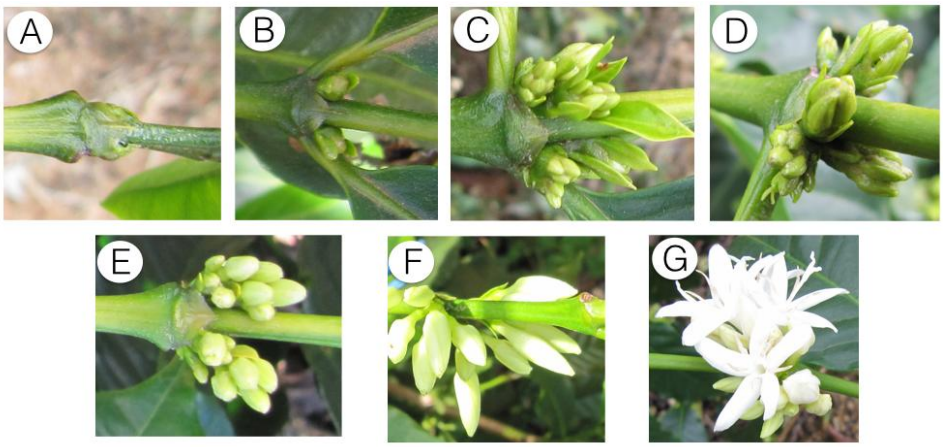

Figure 1.3 Coffee flower development. (A) Non-differentiated buds (G1 stage). (B) Buds with a broad and flat apex (G2 stage). (C) Flower buds with up to 3 mm in length (G3 stage). (D) Flower buds ranging from 3.1 to 6 mm in length (G4) stage. (E) Flower buds ranging from 6.1 to 10 mm in length (light green colour) (G5 stage). (F) Flower buds bigger than 10 mm in length (white colour) (G6 stage). (G) Anthesis.

Once flower buds reach the G4 stage, growth is stopped and flower buds enter in a dormant or quiescent state. Under Brazilian tropical conditions, dormancy of the coffee flower buds coincides with the start of the dry season and the decline of the temperatures, which begin around May and last until August/September. Under natural conditions, dormancy is broken after the first rains or irrigation following the dry period and anthesis occurs after 10-12 days (ALVIN, 1960; RENA & MAESTRI, 1985; BARROS et al., 1999). Thus, the

occurrence of sporadic and sometimes low-intensity rains during the latter phases of flower bud development is believed to be one the uncontrolled factors responsible for several blossom periods in coffee and consequently asynchronous fruit ripening (RENA et al., 2001; RENA AND BARROS, 2004).

Coffee shows a gregarious flowering process, in which all plants within a certain region flower simultaneously. In countries where coffee is grown in regions with a well-defined long dry season, e.g. Brazil, anthesis will occur with the return of rainfall, and coffee trees show intense blossoming events, usually two to four in the main coffee production areas of Brazil (BARROS et al., 1978; RENA AND BARROS, 2004). This is the main reason for which Brazil has one strong crop peak per year. This pattern of concentrated bloom season allows that harvest extends for a relatively short period of approximately three months every year, lead to a higher percentage of fruits at ideal ripening stage at harvest time, and thus allow the use of harvesting practices, such as the use of mechanical harvesting machines, which may lower production costs. On the other hand, in places where the dry period is not pronounced or extended, as observed on the equatorial region of the globe, where important coffee producing countries, such as Colombia, Vietnam, Indonesia and Ethiopia, are located, anthesis can be induced throughout the year. Although the main blossoming events take place from January to April, in these countries anthesis may be triggered every time there is a dry period followed by blossoming showers. This pattern of flowering directly affects coffee quality, since fruits at different ripening stages will be present at harvest time, increase costs, caused by the increased number of harvest events, and because of this fruits need to be hand-picked or post-harvest selected, and disease control is more difficult under these conditions (RENA & MAESTRI, 1985). In general, precipitations between5to 10 mm are considered to be sufficient to trigger anthesis (CARVALHO, 2008).

During the dry period, coffee flower buds are thought to become sensitive to respond to the stimulus for regrowth (ALVIN, 1960; DRINNAN AND MENZEL, 1994; MES, 1957; REDDY, 1979). Interestingly, only buds at the G4 stage seem to be able to respond to this stimulus (CRISOSTO et al., 1992). However, the intensity of the water stress required for making coffee flower buds competent to restart their growth is still controversial. In addition, apparently, dormant and non-dormant buds cannot be distinguished from each other at the morphological or anatomical points of view until the third or fourth day after precipitation or irrigation (MES, 1957).

Crisosto et al (1992) have shown that coffee anthesis can be induced with a relatively short period of a severe water deficit, as long as predawn leaf water potential is below -0.8 MPa. In this same study, it was show that more negative leaf water potential did not promote an enhancement in flowering stimulation, and a similar flowering stimulation could be induced with less severe, but more prolonged, water deficit. Magalhães & Angelocci (1976) found that the critical value of predawn leaf water potential for triggering flower opening was approximately -1,2 MPa, and Schuch et al (1992) showed that flower bud dormancy was broken only when leaf water potential reached -2.6 MPa (in this study, it was not informed if this value refers to predawn leaf water potential).

The studies above mentioned also have shown that if these threshold leaf water potentials are not reached, flower opening does not take place, flower buds reach stage G4, and eventually decline in number due to bud senescence. However, these studies were conducted under greenhouse conditions and bud senescence is not usually seen under field conditions. Some authors attribute this to the fact that the cumulative exposure to short water deficit periods under field conditions, during hot days, would be enough to make flower buds competent to flower (ASTEGIANO, 1984). In a practical point of view, the longer period of

dormancy imposed by the dry period allows that flower buds at different developmental stage reach stage G4, enabling a more uniform flowering upon soil rewetting (RENA & MAESTRI, 1985). Controlled water deficit, imposed on irrigated crops, has been undertaken in Brazil for coffee blossom concentration (GUERRA et al. 2005; SILVA et al. 2009). Guerra et al. (2005) have shown that withholding irrigation for about 70 days, stage at which predawn leaf water potential reached -2.0 MPa, an uniform and unique flowering event could be achieved.

Rainfall during the latter stages of flower development can negatively affect the flowering process causing an increase in the number of blossoming times, which consequently affects ripening uniformity, however, this is not always the case. Soares et al (2005) and Rezende et al (2009) have found that coffee trees continuously irrigated or exposed to some period of water deficit, showed the same number of flowering events, emitting flowers in a similar extent. In terms of productivity, non-irrigated plantations usually show a decrease in productivity, however a less severe dry period (irrigation suspension for up to 60 days) can show similar, or even higher, productivity levels compared to well-watered plants, with the advantage of displaying a higher number of fruits at the ideal ripening stage for harvest, the cherry stage (GUERRA et al. 2005; REZENDE et al. 2009; SILVA et al. 2009; SOARES et al. 2005). The higher productivity level found in plants exposed to a dry period may be attributed to the enhanced growing upon rewatering (GUERRA et al. 2005), or an intrinsic effect of water stress on flower competence, as the study of Crisosto and co-workers suggests (CRISOSTO et al. 1992). However, climatic conditions among the different coffee production regions greatly vary, and due to this the timing and extension of water deficit implementation, with no negative effects on the productivity levels, also vary.

Thus, although coffee flowering under well-watered conditions may show contradictory patterns in some cases, and the intensity of water stress required to make flower buds competent to respond the stimulus for regrowth is still not defined, a period of water deficit seems to be essential, not only to permit a synchronization of flower bud development, but also for making flower buds competent to flower (CRISOSTO et al., 1992; GUERRA et al., 2005). However, how water stress turns coffee flower buds competent to flower, and the mechanism through which flower opening is triggered, are still poorly understood.

1.2.4 Coffee flower dormancy breaking

The mechanism that controls anthesis in coffee trees has been attributed to environmental cues, such as precipitation, temperature and Vapour Pressure Deficit (VPD), and also to internal signals, such as developmental stage and alterations in plant hormone levels, or both.

Magalhães & Angelocci (1976) showed that flower bud dormancy is broken only when predawn leaf water potential is below -1.2 MPa, and flower opening would be triggered by a rapid influx of water into the flower buds, in response to a rapid absorption of water by leaves.

Some authors suggest that a rapid temperature drop, which often occurs after precipitation in the tropics, display an essential role in coffee flower dormancy breaking (BROWING, 1977; MES, 1957; SOARES et al. 2005), and alterations in flower bud development were only observed when the decrease in temperature was accompanied with a decrease in the VPD (SOARES et al. 2005).

Flower bud developmental stage also plays a central role on the responsiveness to the stimulus for regrowth. The stimulatory effect of water

deficit and subsequent irrigation on flowering is restricted to buds at the G4 stage (CRISOSTO et al. 1992). Buds at previous developmental stages do not progress to anthesis, even if plants are exposed to relatively severe water stress conditions (predawn leaf water potential of -1.9 MPa) (SOARES et al., 2005). This restriction of sensitivity to a single bud stage seems to occur due to anatomical differences between stage G4 and stages G2 and G3. G2 and G3 buds display a low number of small, dispersed primary xylem elements, whereas by stage G4 a well-defined vascular cylinder, containing secondary xylem, has developed (CRISOSTO et al., 1992).

Although the scarcity of studies relating the involvement of plant hormones in coffee flowering as a whole, they are certainly involved in coffee flower dormancy breaking as well. Gibberellin (GA3) application induced anthesis of flower buds larger than 4 mm in length in the absence of precipitation (SCHUCH et al., 1990). This effect in anthesis promotion was present only in plants exposed to some level of water deficit, and under relatively mild water stress conditions, exogenous GA application was mandatory for triggering anthesis (SCHUCH et al., 1992). Gibberellin concentration appears to increase upon rewatering before buds gain fresh weight, and since GA biosynthesis inhibitors did not prevent dormancy release, it is supposed that GA is converted from a bound form to a free, active form at the time of release from dormancy (BROWNING, 1973a). Although coffee flower bud dormancy is suggested to be maintained by a balance between GA and Abscisic acid (ABA), ABA levels seem not to be different between dormant and non-dormant buds (BROWNING, 1973a). Cytokinins also have their levels increased upon rewatering (BROWNING, 1973b).

Auxin (IAA) levels in non-stressed and water stressed coffee flower buds were relatively similar before rewatering and slightly decreased 24 hours after rewatering in water stressed buds. An increase in free and conjugated bud

IAA levels was observed three days after rewatering, which was associated with a rapid increase in water uptake at this point, remaining stable until flower opening (SCHUCH et al., 1994).

Ethylene evolution of dormant coffee flower buds was always higher than in buds that dormancy had been broken by more severe water stress levels. Increased ethylene levels were also found at anthesis, probably caused by pollination (SCHUCH et al., 1992).

Crisosto et al (1992), using a root-split system, have shown that coffee flowering can be induced, without the decline of predawn or midday leaf water potential observed in plants under water stress, as long as part of the root system is exposed to a period of drought. Furthermore, plants that had one root system exposed to drought showed the same flowering stimulation observed in plants with both root systems exposed to drought. This means that a change in the biosynthesis and/or transport of one or more signal (s) in the droughted root system, and/or a change in the biosynthesis or sensitivity to this or these signal (s) in the flower buds (or shoot system) during water deficit conditions, might be responsible for making flower buds competent to flower, and might also be responsible for triggering anthesis upon rewatering.

Considering the requirement of abiotic-induced drought followed by rainfall, a series of events well-known to induce a burst in ethylene production in woody species (GOMEZ-CADENAS et al., 1996), and the fact that coffee flower competence and/or opening stimulation seems to be controlled by changes in root-to-shoot signaling (CRISOSTO et al., 1992), ethylene is a good candidate in participating of coffee flowering promotion.

1.2.5 Dehydration, rehydration, and flowering: a focus in ethylene

1.2.5.1Hormonal control of flower development and flower opening

Floral formation is a result of sequential events involving different genetic networks (SRIKANTH; SRIKANTH, 2011). First, floral induction, which corresponds to the phase change from vegetative to reproductive growth, is regulated by an integrated network of flowering-time genes that have their expression regulated by different environmental and endogenous factors. These genes converge on the activation of meristem identity genes which confer floral meristem (FM) identity, before floral organs are initiated by the coordinated spatial and temporal expression of transcription factors. Finally, a raft of downstream target genes of the floral identity genes coordinates floral organogenesis (CHANDLER, 2011).

Growth of floral organs is mediated by a combination of cell proliferation and cell expansion (DE VEYLDER; BEECKMAN, 2007; KRIZEK, 2009). Plant hormones display a central role in flowering by regulating flowering time identity genes and the growth of floral organs. Plant hormones can be growth-retarding (ABA, ethylene, jasmonic acid) or growth-promoting (auxin, gibberellic acid, cytokinin). In addition, brassinosteroids and strigolactones two recently discovered plant hormones, act in conjunction with auxins and can be classified as growth promoting hormones (DOLFERUS, 2014).

Auxin determines the formation of floral primordia and plays an essential role in specifying floral organs, and determining the patterns of formation within a floral organ (YAMAGUCHI et al., 2013; CHENG; ZHAO 2007). Mutations in any aspects of the auxin pathways including biosynthesis, polar transport, and signaling, can cause profound defects in flower development

(CHENG; ZHAO 2007). For instance, mutations in the auxin response factor *ARF3* affect the development of all four types of floral whorls (sepals, petals, stamen, and carpels) (SESSIONS et al., 1997; SESSIONS; ZAMBRYSK, 1995), indicating that this gene affects flower meristem, floral organ initiation, and boundaries between floral organs. Other members of the ARF family have been related to other aspects of flower development (ELLIS et al. 2005; NAGPAL et al. 2005; TABATA et al., 2010; YANG et al., 2013). The auxin biosynthetic *YUC* genes also have profound effects on flower development, with double, triple and quadruple *yuc* mutants showing floral patterning defect in both organ initiation and ontogeny (CHENG et al., 2006), indicating that local auxin biosynthesis is essential for floral organ development. In addition to local auxin biosynthesis, polar auxin transport (PAT) is also required for the normal floral organ development since pin1 mutants have flowers lacking stamens and have petals with abnormal shapes or fused petals (BENNETT et al., 1995; GALWEILER et al., 1998; OKADA et al., 1991;). PAT has also been shown to be important for stamen primordial formation, anther dehiscence, pollen maturation, and pre-anthesis filament elongation (CARDARELLI et al., 2014).

Gibberellin (GA) also plays an important role in flower development promoting the elongation of plant organs, such as petals, stamens, and pistils, by opposing the function of DELLA proteins (CHENG et al., 2004). GA is necessary for fertility in flowering plants and GA deficient plants have their stamen and pollen development blocked (SUN et al., 1992; CHENG et al., 2004). Less severe GA deficient mutants have shown that GA also regulates final phases of stamen development such as filament elongation and anther dehiscence (HU et al., 2008; RIEU et al., 2008).

Cytokinins are also involved in flower development being required for pollen development (HUANG et al., 2003) and its signaling is essential for

female gametophyte development and organ size (BARTRINA et al., 2011; CHENG et al., 2013).

Jasmonic acid is a major regulator stamen development and JA biosynthesis or perception deficiencies attenuate stamen development, disrupt male fertility, and abolish seed production in different species (ACOSTA et al., 2009; CAI et al., 2014; FEYS et al., 1994).

Brassinosteroids affects organ initiation and formation contributing to the number of ovule primordia on the female side (HUANG et al., 2013), and leading to sterility on the male side due to abnormal tapetal development, reduced pollen number per locule, and defect in pollen release after anther dehiscence (YE et al., 2010). Brassinosteroids also affect pollen germination and pollen tube growth (VOGLER et al., 2014).

Studies comprising ABA effects on flowering are mainly related to its influence in flowering time and on its role in the regulation of flower identity genes. Based on the early flowering phenotype of ABA deficient mutants, ABA has been regarded as a repressor of flowering in Arabidopsis (DOMOGALSKA et al., 2010; MARTINEZ-ZAPATER et al., 1994). ABA exerts its repression role by modulating DELLA signaling (ACHARD et al., 2006), and also up-regulating the one of the central flowering repressor integrators, FLOWERING LOCUS T (FLC) (WANG et al., 2013). However, a positive effect of ABA on flowering has been reported in response to drought, where flowering was induced by the up-regulation of florigen genes through an ABA and photoperiodic-dependent manner (RIBONI et al., 2013).

Ethylene is involved in different aspects of flower development, such as floral transition (ACHARD et al., 2007) and flower senescence (reviewed in SHAHRI; TAHIR, 2013). Ethylene is commonly associated with growth inhibition, but it can also act as a growth-promoting agent, at relative low concentrations in most cases, but also at high concentrations,

frequently found in flooded habitats (PIERIK et al., 2006). Although little is known about the mechanisms through which ethylene exerts its effects on cell expansion, some studies suggest that ethylene regulation of genes related to cell-expansion and cell turgor, such as genes related to cell wall degradation, sugar transport, and aquaporins, may be involved (LIU et al., 2013; MA et al., 2008; PEI et al., 2013). Furthermore, ethylene may also regulate growth by controlling the biosynthesis or transport of other plant hormones, such as auxin (RŮŽIČKA et al., 2007).Ethylene has been shown to delay flowering in Arabidopsis by reducing bioactive GA levels and enhancing DELLAs protein accumulation, which in turn negatively regulates the floral meristem identity genes *Leafy* and *Supressor of Overexpression of Constans 1* (ACHARD et al., 2007). In relation to organ development, ethylene is required during pollen and ovule development (DE MARTINS; MARIANI, 1999; HOLDEN et al., 2003). Furthermore, ethylene regulates floral sex determination in cucumber, promoting femaleness and inhibiting maleness (DUAN et al., 2008; KAHANA et al., 1999).Flower opening is also influenced by ethylene, and it has been shown that ethylene can promote flower opening in different species such as carnation (JONES; WOODSON, 1997), *Phalaenopsis* orchids (BUI; O'NEILL, 1998), and petunia (TANG; WOODSON, 1996). In rose, ethylene usually inhibits flower opening, however, promotion of flower opening has also been observed (REID et al., 1998; ACNISH et al., 2010).

1.2.5.2 Dehydration, Rehydration and ethylene regulation

Water stress, also refereed as drought or water deficit, is a major abiotic stress that affects crop productivity nearly as much as all environmental factors combined (SAINI; WESTGATE 1999; SHARP et al., 2004). Plants facing water-deficit conditions experiment alterations in an array of processes at the physiological, biochemical and molecular levels, such as photosynthesis, nutrient uptake, and modifications in gene expression, which may ultimately lead to growth arrest and yield reduction (FAROOQ et al., 2009).

Plants in natural conditions, rained or irrigated agriculture, are exposed to fluctuating soil water availability, characterized by repeated soil drying and re-wetting cycles (DODD et al., 2015). Some phenological stages are more sensitive to water deficit, such during reproductive development (SU et al., 2013; DE STORME; GEELEN, 2014), and some plants evolved mechanisms to cope with temporary water limitations in order to ensure their survival and reproduction. Plant resistance to drought can be subdivided into escape, avoidance, and tolerance strategies. Escape strategies may rely on successful reproduction before the onset of severe stress, by means of a short life cycle, a higher rate of growth or the efficient storage and use of reserves for seed production. Dehydration avoidance, that is, the maintenance of a high (favourable) plant water status during stress, may be the result of minimised water loss (e.g. caused by stomatal closure, trichomes, reduced leaf area, senescence of older leaves, etc.) or maximised water uptake (e.g. by increased root growth). Finally, tolerance to low water potential (the maintenance of plant function at limited water availability and/or the recovery of plant water status and plant function after stress) may involve osmotic adjustments, but may also

be the result of rigid cell walls or small cells (CHAVES et al., 2003; LUDLOW, 1989). However, water-deficit may also be important for breaking bud dormancy in some species, such as coffee, and trigger flowering (ALVIN, 1960; KOZLOWSKI, 2002; OPLER et al., 1976).

As sessile organisms, plants responses to external stimuli are mediated by plant growth regulators (Phytohormones), which are essential for the ability of plants to adapt to abiotic stresses by mediating a wide range of adaptive responses (PELEG; BLUMWALD, 2011). It is generally believed that ABA and ethylene are two major plant hormones linking plant responses to stress (ALBACETE et al., 2014; DOLFERUS, 2014; YANG et al., 2007; KOHLI et al., 2013). Although ABA has long been recognised as a plant hormone that is up-regulated in response to soil water deficit around roots (DAVIES et al., 2002; WILKINSON; DAVIES, 2002; WILKINSON et al., 2012), ethylene responses to water deficit are quite variable depending on the species, genotype, organ, and water deficit intensity. Several studies have shown that ethylene biosynthesis and signaling genes are up-regulated, and ACC and/or ethylene levels are increased, under water-deficit conditions (APEBAUM; YANG, 1981; BALOTA et al., 2004; BELIMOV et al., 2009; BELTRANO et al., 1997; CHEN et al., 2002; EL-BELTAGY; HALL, 1974; LIU et al., 2013; SCHUCH et a., 1992; YANG et al., 2007; YANG et al., 2014; ZHANG et al., 2015). On the other hand, different studies have shown the opposite effect (MORGAN et al., 1990; NARAYANA et al., 1991; SCHUCH et al., 1992; LARRAINZAR et a., 2014). In addition, ethylene levels may remain unaltered under water deficit (MUNNÉ-BOSCH et al., 2002; VOISIN et al., 2006), or show different patterns according to water stress intensity (BELTRANO et al., 1997; KALANTARI et al., 2000; YANG et al., 2004).

It is well-known that water deficit can promote changes in the synthesis of plant hormones in both root and shoot systems of plants, however, signal

transport is also affected. Water deficit decrease water uptake from roots, and sap flow from roots in drying soil can entirely cease (KHALIL; GRACE, 1993; STOLL et al., 2000). Re-wetting the soil promotes the recovery of leaf water potential, growth, and gas-exchange parameters, and also promotes changes in root-to-shoot chemical signaling (GOMEZ-CADENAS et al., 1996; HANSEN; DORFFLING, 2003; TUDELA; PRIMO-MILLO, 1992). For instance, rewatering caused an exponential decline and a rapidly increase of xylem ABA and cytokinin, respectively, in sunflower plants (HANSEN & DORFFLING, 2003). An accentuated decrease in ABA levels was also observed in poplar (LOEWENSTEIN; PALLARDY, 2002), and mandarin seedlings (GOMEZ-CADENAS et al., 1996) after rewatering. Leaf and root ABA declined upon rewatering of papaya seedlings and auxin levels did not alter under water deficit recovery (MAHOUACHI et al., 2007).

In terms of ethylene regulation, the occurrence drought followed by rainfall or irrigation (rehydration) has been known as a series of events commonly associated with a rapid and substantial increase in ethylene production and on the transport of its precursor ACC (GOMEZ-CADENAS et al., 1996; LIU et al., 2013; TUDELA; PRIMO-MILLO, 1992).Increased ethylene levels after rewatering has also been found in wheat ears after desiccation (BALOTA et al., 2004; BELTRANO et al., 1997). These higher ethylene levels upon rewatering have been related to different processes, such as leaf abscission in citrus (GOMEZ-CADENAS et al., 1996; TUDELA; PRIMO-MILLO, 1992), and the rehydration-recovery response in rose flowers, promoting flower opening (MENG et al., 2014).

More recently, it was shown that rehydration can induce a rapid and transient increase in ethylene production in gynoecia of rose flowers, and this ethylene burst serves as a signal to ensure water recovery in flowers, and promotes flower opening by influencing the expression of a set of rehydration-

responsive genes (MENG et al., 2014).Considering that coffee flowering is induced by rain or irrigation after a period of water deficit, a drought-rewatering induced ethylene burst would be the basis of the drought-rewatering flowering in coffee. Thus, the inhibition of ethylene responses, which can be achieved through the use ethylene action inhibitors, such as 1-Methylcyclopropene (1-MCP), would help to inhibit coffee flowering upon low intensity rains, helping to reduce the number of flowering events and thus promoting blossoming concentration, as long as it does not promote increased ethylene levels in response to a loss of the negative feedback regulation of ethylene biosynthesis, as observed in some studies (ELLA et al., 2003)

1.2.5.3 Ethylene biosynthesis and signaling

The plant hormone ethylene is involved in many aspects of plant life cycle, including organ abscission, seed germination, growth transition from vegetative phase to reproductive phase, flowering, fruit ripening, senescence, and is also involved in biotic and abiotic stress responses. Ethylene production is tightly regulated by internal and external signals during development and varies according to the tissue or organ and its developmental stage, with meristematic tissues, stress conditions, and fruit ripening displaying the highest ethylene production rates (ABELES et al., 1992).

Once produced, considering its gaseous nature, ethylene easily diffuses between the intercellular spaces and adjacent tissues. Without the possibility of controlling its transport, sensitivity control is a critical factor on response limitation to target cells (ALONSO; ECKER, 2001). Ethylene action takes place via the ethylene signaling pathway. Genetic studies of ethylene action in model plants, especially in Arabidopsis (Arabidopsis thaliana) and tomato (*Solanum lycopersicum*), have established an ethylene signal transduction model, in which

ethylene is perceived by a receptor family and the signal is mediated downstream by members of different gene families (ETHERIDGE et al., 2005).

1.2.5.3.1 Ethylene Biosynthesis

Ethylene is formed from methionine via S-adenosyl-L-methionine (AdoMet) (KENDE, 1993; YANG & HOFFMAN, 1984). Approximately 80 % of all cellular methionine is converted to S-AdoMet, which is a major methyl donor in plants and is used as a substrate for many biochemical pathways, including polyamines and ethylene biosynthesis (RAVANEL et al., 1998). On the basis of the Yang cycle, the first committed step of ethylene biosynthesis is the conversion of S-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by the action of ACC synthase (ACS) (Figure 1.4) (KENDE, 1993; YANG & HOFFMAN, 1984). In addition to ACC, ACS produces 5'-methylthioadenosine, which is utilized for the synthesis of new methionine via a modified methionine cycle or Yang cycle (Figure 1.4) (MIYAZAKI; YANG, 1987). This salvage pathway preserves the methylthio group through every revolution of the cycle at the cost of one molecule of ATP (Adenosine triphophate). Thus, high rates of ethylene biosynthesis can be maintained even when the pool of free methionine is small.

ACC can be conjugated into three different forms, malonyl-ACC, γ-glutamyl-ACC, and jasmonyl-ACC, which may be important in the regulation of available ACC pool and eventually ethylene levels (VAN DE POEL; VAN DER STRAETEN, 2014). Although there are no evidences that these ACC derivatives can be transported over long distances, ACC itself has been suggested to be transported from roots to the shoot when roots are exposed to stress, such as flooding (ENGLISH et al., 1995), drought (DAVIES et al., 2000; SKIRYCZ et

al., 2011; SOBEIH et al., 2004), rehydration after drought (TUDELA; PRIMI-MILLO et al., 1992).

The second step involved in ethylene biosynthesis is the oxidation of ACC, by ACC oxidase (ACO), releasing ethylene and a cyanoformate ion $[NCCO_2]^-$, which is subsequently decomposed into CO_2 and CN^- (Figure 1.4) (YANG; HOFFMAN, 1984). The reactive cyanide (CN^-) is subsequently detoxified by B-cyanoalanine synthase to produce B-cyanoalanine to prevent toxicity of accumulated cyanide during high rates of ethylene synthesis (MILLER; CONN, 1980).

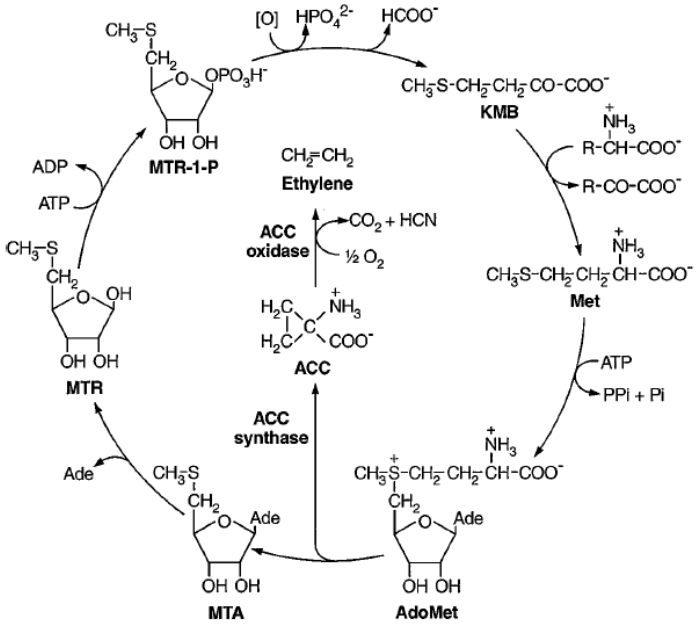

Figure 1.4 The ethylene biosynthetic pathway and the methionine cycle. ACC, 1-aminocyclopropane-1-carboxylic acid; Ade, adenine; AdoMet, *S*-adenosyl-L-methionine; KMB, 2-keto-4-methylthiobutyric acid; MET, L-methionine; MTA, 5-1-carboxylic acid; Ade, adenine; AdoMeioribose; MTR-1-P, 5ethylene synthesis (MILLER; CONN, 1980).., 1992).000, modified from MIYAZAKI; YANG , 1987).

Both steps committed to ethylene biosynthesis, performed by the ACS and ACO enzymes, are encoded by small multigene families (BABULA et al., 2006; BARRY et al., 1996; BARRY et al., 2000;LIANG et al., 1992), whose expression is differentially regulated by various developmental, environmental and hormonal signals, such as flowering (FRANKOWSKI et al., 2014; LIU et al., 2013) fruit ripening(BARRY et al., 1996; BARRY et al., 2000; SÁGIO et al., 2014), drought (LARRAINZAR et al., 2014; PADMALATHA et al., 2012), biotic stress (YIM et al., 2014), auxin (CHAE et al., 2000; TSUCHISAKA & THEOLOGIS, 2004), ABA (LUO et al., 2014; ZHANG et al., 2009a). Up until now, only one ACS and three ACO homologs have been identified in coffee and their expression patterns have been analysed during fruit ripening (SÁGIO et al., 2014).

Although the conversion of S-AdoMet to ACC by ACS was initially regarded as the rate-limiting step of ethylene synthesis and that ACO genes were originally thought to be constitutively expressed (YANG & HOFFMAN, 1984; THEOLOGIS et al., 1993), the role that ACO activity plays in the regulation of ethylene biosynthesis has become apparent in recent years. Similarly to ACS, different studies have revealed that there are differences in accumulation of specific ACO transcripts in various physiological processes, such as abiotic and biotic stresses (NIE et al., 2002), seed germination (CALVO et al., 2004), flowering (TRIVELLING et al., 2011), and fruit ripening (SÁGIO et al., 2014). This suggests that different ACOs homologs are differentially regulated during different developmental stages. The conversion of ACC into ethylene by ACO enzyme well coincides with the site of ethylene evolution so the determination of ACO transcript abundance may serve as an ethylene biosynthesis indicator (RUDUS et al., 2013). Another similarity between ACS and ACO is that both enzymes are liable and present in most tissues at very low levels, suggesting that ethylene biosynthesis is a tightly controlled process (CZARNY et al., 2006;

RUDUS et al., 2013; VAN DE POEL; VAN DER STRAETEN, 2014; WANG et al., 2002).

1.2.5.3.2 Ethylene signaling

After synthesis, ethylene is perceived and its signal is transduced through a transduction pathway to trigger specific biological responses (Figure 1.5). Ethylene is perceived by a family of membrane-localized receptors, which according to their structural similarities can be divided into two subfamilies, ETR1-like and ETR2-like (HUA et al., 1998; KLEE, 2002). Genetic studies have shown that ethylene receptors act as negative regulators (HUA; MAYEROWITZ, 1998). Thus, in the absence of ethylene, ethylene receptors are in their functionally active form, repressing ethylene responses and constitutively activating the next component of the pathway, the Raf-like serine/threonine (Ser/Thr) kinase, CTR1 (Figure 1.5). CTR1 has similarity to a mitogen-activated protein kinase kinase kinase (MAPKKK), suggesting the involvement of a MAP-kinase-like signaling cascade in the regulation of ethylene. Loss-of-function (LOF) mutations in CTR1 result in a constitutive ethylene-response phenotype, indicating that CTR1 is also a negative regulator of ethylene signaling (HUANG et al., 2003; KIEBER et al., 1993). Additional studies showed that ethylene receptors and CTR1 physically interact to each other to negatively regulate the signaling pathway (Clark et al., 1998), and its expression patterns can vary according to developmental stage, organ, and ethylene concentration (ADAM-PILLIPS et al., 2004; FERNANDEZ-OTERO et al., 2007). Further downstream of the complex receptor-CTR1, ethylene-insensitive-2 (EIN2), a membrane-integrated metal transporter-like protein, plays a major role in the ethylene response as LOF mutations result in complete ethylene insensitivity for all ethylene responses tested, indicating that EIN2 is a

positive regulator of the pathway (Figure 1.5) (ALONSO et al., 1999). EIN2 protein levels is regulated by ethylene, and its accumulation under ethylene presence correlated well with the next component of the pathway, ethylene-insensitive-3 (EIN3) (JU et al., 2012; QIAO et al., 2012; WEN et al., 2012). EIN3 belongs to a small family of transcription factors that includes EIN3 and EIN3-like proteins (CHAO et al., 1997) (Figure 1.5). LOF mutations in EIN3 cause partial ethylene insensitivity, and similarly to EIN2, EIN3 is constantly degraded in the absence of ethylene. In the presence of ethylene, its accumulation can trigger primary transcription of genes containing EN3-biding-sites in their promoter regions (GUO; ECKER, 2003). EIN3-biding-sites have been found in genes related to different process, such as senescence (ITZHAKI et al., 1994), fruit ripening (YIN et al., 2010), and also other transcription factors, like ERF1 (ethylene-response-factor 1) (SOLANO et al., 1998). ERF1 is a member of the ERF family of transcriptional factors, whose members have been shown to act as activators or repressors of additional downstream ethylene-responsive genes, regulating processes such as fruit ripening (BAPAT et al., 2010; ZHANG et al., 2009b), biotic and abiotic stress responses (THIRUGNANASAMBANTHAM et al., 2015) (Figure 1.5).Different members from every step of the ethylene signaling pathway have been identified on coffee (LIMA et al., 2011; SAGIO et al., 2014), and the expression patterns of some of them have been carried out in fruits at different ripening stages (SAGIO et al., 2014).

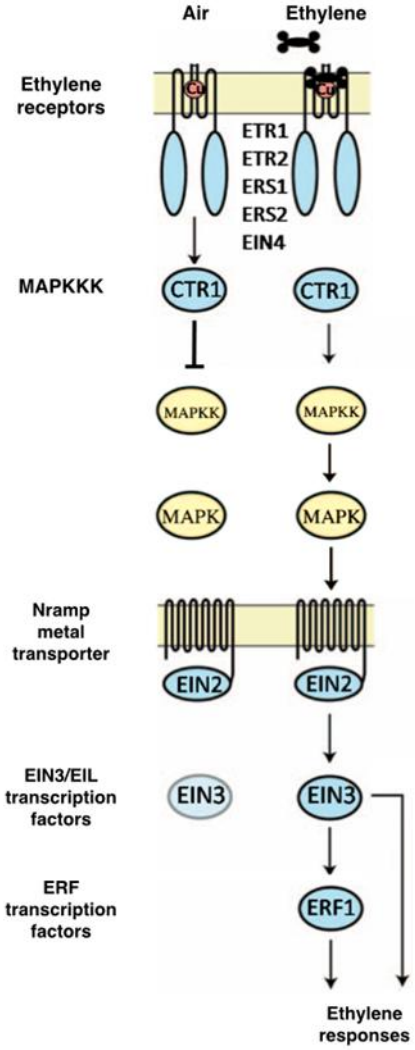

Figure 1.5 Ethylene signaling pathway in the absence (air) and in the presence of ethylene. In the absence of ethylene, ethylene receptors activate CTR1, which in turn negatively regulates the downstream response pathway, possibly through a MAP-kinase cascade. Upon ethylene biding, the complex ethylene receptor/CTR1 dissociates, and CTR1 released from the endoplasmatic reticulum membrane is inactivated, allowing EIN2 to function as a positive regulator of the pathway. EIN2 positively signals downstream to the EIN3 family of transcription factors located in the nucleus. These transcription factors can further active other transcription factors, such as ERF, and both families of transcription factors regulate ethylene responses (adapted from CHEN et al., 2005).

1.2.5.4 -1-MCP and ethylene action inhibition

1-MCP is a cyclic olefine, structurally similar to ethylene, that seemingly binds to a metal, probably copper (RODRIGUEZ et al., 1999), in the ethylene receptors and prevent ethylene from biding to the receptors, leading to an inhibition of ethylene responses. Thus, 1-MCP competes with ethylene for ethylene receptors (SISLER et al., 1996; SISLER; SEREK, 1997).

1-MCP was the first patent non-toxic ethylene action inhibitor and was developed by the American company FloralLife Inc., under the name EthylBloc. EthylBloc was registered for ornamentals in 1999 for use in the USA and in the same year a newly formed company, AgroFresh Inc., a subsidiary of Rohm and Haas (Springhouse, PA), subsequently developed 1-MCP under the trade name SmartFresh and have global use rights for edible horticultural crops. By 2005, food use registration for the chemical had been obtained for several countries, including Brazil (WATKINS, 2006)

1-MCP is commercialized as a stable powder, complexed with γ-cyclodextrin, which shows no odour, colour, and has a non-toxic mode of action, with negligible residue, and being active at very low concentrations. 1-MCP is easily released as a gas when its powder formulation is dissolved in water, meaning that plant material must be kept in enclosed areas for treatment. The extent and longevity of 1-MCP action is affected by species, cultivar, tissue and mode of ethylene biosynthesis. A 'concentration x time effect is apparent with longer exposure periods required for lower 1-MCP concentration to obtain the same physiological effects (SISLER; SEREK, 1997). Some products such as pea require higher concentration (40nL L^{-1}) than carnations (0.5 nL L^{-1}) and banana (0.7nL L^{-1}), suggesting that new receptors are produced in growing tissues or a low affinity form of the receptors is present (SILER; SEREK, 2003). Synthesis of new biding sites may be affected by temperature; in banana, temperatures

between 30 and 40°C results in faster recovery of ripening, while application of 1-MCP at 2.5°C is less effective than at 15 and 20 °C suggesting that binding of 1-MCP at low temperatures was incomplete (JIANG et al., 2004). 1-MCP is mainly used in the horticultural sector to minimise ethylene action during ripening, storage, and transport, increasing the storage and shelf life of different products, and also in the ornamental industry, increasing flower life-span (SCARIOT et al., 2014; WATKINS, 2006). In addition, 1-MCP act as a tool to better understand the involvement of ethylene in different processes where it is required, such as fruit ripening, senescence, biotic and abiotic stress responses.

Recently, AgroFresh has developed a sprayable formulation of 1-MCP (Harvista ^{TM}), for pre-harvest use, suitable for plant treatment under field conditions. In this case, the concentration of active ingredient present in the powder is much higher than the one commonly used in the ornamental and horticultural sections, 3.8% of active ingredient against 0.14% of active ingredient from the previous formulation, used for treatment under enclosed areas. Harvista have been used in different fruit trees, such as apple (ELFVING et al., 2007; YUAN; CARAUGH, 2007; MCARTNEY et al., 2008; DEELL, et al., 2010; VARANASI et al., 2013), pear (VILLALOBOS-ACUNA et a., 2010), and pitahaya (SERNA et al., 2012, 2013), and it was shown to inhibit ethylene responses by delaying fruit maturity and fruit drop, reducing fruit firmness loss, and decreasing the occurrence of low-temperatures disorders during storage.

1.3CONCLUSION

Asynchronous coffee fruit ripening is practically inevitable under Brazilian tropical conditions, and mainly on the equator region of the globe, due to asynchronies on bud development and environmental factors, such as low-intensities rains, which can lead to several blossoming events and consequently to fruits at different ripening stages at harvest time. Considering the requirement of a period of water deficit followed by rainfall to trigger coffee flowering, and the fact that this series of events is commonly associated to a rapid increase in ethylene production, ethylene may be involved in coffee anthesis induction. Thus, a better understanding of the ethylene regulation during and after water stress relief may help us to better comprehend coffee anthesis promotion or even control this process by regulating ethylene responses.

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CHAPTER 2

Ethylene regulation under different watering conditions and upon 1-MCP application

ABSTRACT

Coffee is one of the world's favourite beverage and the second most traded commodity after oil. However, coffee quality can be greatly affected by fruit ripening stage at harvest time, which is often asynchronous due to the sequential flowering in this species. Coffee sequential flowering is a result of asynchronies in bud development and also environmental factors, since anthesis is triggered by precipitation after a period of water deficit. This series of events is commonly associated to with an increase in ethylene production levels, suggesting that ethylene may be involved in the control of coffee anthesis promotion and its regulation might help to reduce the number of flowering events and thus promoting blossoming concentration. Thus, this study aimed to access the effects of exogenous 1-MCP application on flowering of coffee trees under field conditions and to study the ethylene regulation, through quantification of ethylene levels, in coffee plants under well-watered and water-deficit conditions, as well as, after plant rehydration and 1-MCP treatment. 1-MCP exogenous application, at the concentrations of 50 and 100 mg of a.i. per litre and with spray volume of one litre per plant, was able to promote coffee anthesis in trees of the cultivar ‘Acaiá Cerrado' under field conditions. Ethylene productions rates in coffee seedlings from two cultivars, ‘Bourbon Amarelo' and ‘Obatã Vermelho’, growth under greenhouse conditions, are similar under well-watered and water-deficit conditions. Plant rehydration and 1-MCP treatment does not change ethylene release rates from both cultivars when compared to plants under well-watered and water-deficit conditions.

Key words: Rewatering. 1-MCP. Well-watered. Water-deficit.

RESUMO

O café caumas bebidas favoritas do mundo e a segunda commodity mais comercializadas no mundo após o petróleo. Entretanto, a qualidade do caféepode ser grandemente afetada pelo estádio de maturação dos frutos na época de colheita, se mostrando frequentemente bastante desuniforme devido ao florescimento sequencial nessa espécie. O florescimento sequencia do cafeeiroéiresultante do desenvolvimento desuniforme de suas gemas e e também devido a fatores ambientais, tendo em vista que a antese éadesencadeada por precipitação após um período de restrição hídrica. Esta série de eventoséscomumente associada com um aumento nos níveis de etileno, sugerindo que o etileno possa estar envolvido no controle da promoção da antese do cafeeiro e a regulação de seus níveis poderia auxiliar na redução do número de floradas a assim promover a concentração das floradas. Assim, este estudo objetivou em avaliar os efeitos da aplicação exógena de 1-MCP no florescimento do cafeeiro em condições de campo e em estudar a regulação do etileno, por meio da quantificação de seus níveis, em plantas mudas de cafeeiro bem-irrigadas e sob déficit-hídrico, assim como após a reidratação e aplicação do 1-MCP. A aplicação exógena de 1-MCP, nas concentrações de 50 e 100 mgofa.i. perlitre e com um volume de aplicação de um litro por plantas, foi capaz de induzir a antes do cafeeiro em plantas da cultivar ‘Acaiáccerrado’eem condições de campo. As taxas de produção de etileno em mudas de cafeeiro de duas cultivares, ‘Bourbon amarelo' e ‘Obatãbvermelho’, mantidas em casas de vegetação, foi similar em plantas bem-irrigadas e sob déficit-hídrico. A reidratação das plantas e o tratamento com 1-MCP não promoveu mudanças nas taxas de produção de etileno em ambas as cultivare quando comparado com os níveis de etileno produzido em plantas bem-irrigadas e sob déficit-hídrico.

Palavras chave: Reidratação. 1-MCP. Bem-irrigado. Déficit-hídrico.

2.1 INTRODUCTION

Coffee is one of the world's favourite beverage and the second most traded commodity after oil. World exports in 2013/14 reached more than 100 million bags (60 kg) and accounted for an estimated US$16.4 billion (ICO). Coffee consumption increases year by year (around 10 % in the last five years) (ICO) and good quality coffee is more and more on demand, what is confirmed, for example, by the increase on the market for coffee capsules, which has increase 1000% in the United States, the largest world coffee consumer, in the last six years (2009 to 2015), representing 15 % of the American coffee market nowadays (CBS&A-Volcafé). However, coffee quality can be greatly affected by fruit ripening stage at harvest time, which is often asynchronous due to the sequential flowering in this species.

Coffee sequential flowering is almost inevitable due to asynchronies in bud development (DE OLIVEIRA et al., 2014; MAJEROVICZ & SONDAHL, 2005), and longer periods of water-deficit, extending the period of flower bud dormancy, permits that a higher number of buds reach the developmental stage capable of responding to the stimulus for regrowth, triggered by plant rehydration, promoting blossoming concentration (DRINNAN; MENZEL, 1994; MES, 1957; REDDY, 1979). Water stress conditions seems to have a positive effect in flower bud competence acquisition (CRISOSTO et al., 1992; SCHUCH et al., 1992), but little known about the factors that control bud competence and growth resumption after dormancy breaking. Some studies suggest that anthesis stimulation occurs in response to an increase in flower bud water potential following plant rehydration (MAGALHAES; ANGELOCCI, 1976), and changes in temperature and VPD may also contribute for anthesis (SOARES et al. 2005). However, Crisosto and co-workers (1992), using a split-root system, have shown that flowering can be stimulated in plants with water status similar to well-watered plants, as long as part of the root system is exposed to a period water

deficit. This finding suggests that flower bud competence and growth stimulation upon rewatering are mediated by changes in root-to-shoot chemical signaling (CRISOSTO et al., 1992).

Although plant hormones are intrinsically involved in flowering regulation, from flower induction to flower senescence, little is known about their role in coffee flowering. Considering the requirement of water deficit conditions for flower bud competence and blossoming concentration, changes in the regulation of plant hormones involved in drought stress responses, such as ABA and ethylene, suggest that these hormones may play an important role in flowering regulation.

ABA is known to inhibit flowering transition (ACHARD et al., 2006), but under drought conditions a positive effect in flowering induction has been observed (RIBONI et al., 2013). Ethylene can inhibit (ACHARD et al., 2007) or promote (TRUSOV; BOTELA, 2006; WANG et al., 2007) flowering depending on the species. Although ABA levels is known to increase and decrease during water stress conditions and after plant rehydration, respectively, ethylene regulation under water deficit conditions have been shown to greatly vary, increasing, decreasing, or remaining stable, according to the organ, genotype, species, and water stress intensity (MORGAN et al., 1990; MUNNÉ-BOSCH et al., 2002; YANG et al., 2004; YANG et al., 2014). In addition, rapid and transient increases in ethylene levels, due to root-to-shoot ACC transport (TUDELA; PRIMO-MILLO, 1992; GOMEZ-CADENAS et al., 1996) or local organ-specific changes in ethylene production (LIU et al., 2013; MENG et al., 2014), may occur after water stress relief, affecting processes such as leaf abscission and rehydration-recovery response in flowers (MENG et al., 2014; GOMEZ-CADENAS et al., 1996; TUDELA; PRIMO-MILLO, 1992).

Considering the requirement of water deficit conditions followed by rain or irrigation for coffee anthesis promotion, an ethylene burst triggered by this

series of events would possibly act as one of the signals necessary for flower bud growth resumption in coffee trees. If this is the case, ethylene inhibition through the use of chemicals that inhibit its action, such as 1-Methylcyclopropene (1-MCP), would help to inhibit coffee flowering upon low intensity rains, contributing to reduce the number of flowering events and thus promoting blossoming concentration, as long as it does not promote a loss of the negative feedback regulation of ethylene biosynthesis (ELLA et al., 2003), which could induce, instead of inhibiting, flowering. In addition, the analysis of ethylene regulation under well-watered and water stress conditions could provide some clue whether ethylene has any role in flower bud competence acquisition.

Thus, this study aimed to access the effects of exogenous 1-MCP application on flowering of coffee tress under field conditions, and to study the ethylene regulation, through quantification of ethylene levels, in coffee plants under well-watered and water-deficit conditions, as well as, after plant rehydration and 1-MCP treatment.

2.2 MATERIAL AND METHODS

2.2.1 Field trial

In order to better understand the effects of exogenous 1-MCP application on coffee flowering, a field experiment was conducted in one of the coffee plantations from the coffee experimental area of the Department of Agriculture of Federal University of Lavras (Lavras, Minas Gerais, Brazil - 21°14' S latitude and 45°00' W longitude at an average altitude of 915 m). Climate conditions in this region is classified as the Cwa type, according to Koppen, with two different seasons: dry (April to September) and rainy (October to March).

Eight years-old coffee trees, which had been pruned (pruning method: 'recepa' (consists of cutting off the orthotropic branch at 0.8 m above ground) two years before the beginning, from the cultivar 'Acaiá Cerrado' (*C. arabica*) were used in this experiment, which was carried out in the end of August 2013, before the start of the rainy season (see Appendix A for total precipitation levels from June to September of 2013).

Experimental design consisted of six treatments (five different 1-MCP concentrations + control), with five replicates for each one (one tree per replicate). The water status from the plants, at predawn and midday conditions, was accessed on the day before the start of the experiment using Scholander-type pressure chamber. The chamber was lined with moistened filter paper, and measurements were made between 03:30 and 05:30 (predawn) and 11:30 and 13:30 (midday). One leaf, young and fully expanded (third or fourth node) at middle third of the plant, per plant was used to evaluate the water potential from each plant.

Treatments comprised five different concentrations (C1 to C5) of a sprayable formulation of 1-MCP (3.8 % of active ingredient (a.i.); Harvista^{tm};

AgroFresh Inc., Spring House, PA), which was applied to whole trees at 2 mg a.i. L^{-1} (C1); 5 mg a.i. L^{-1} (C2); 25 mg a.i. L^{-1} (C3); 50 mg a.i. L^{-1} (C4); 100 mg a.i. L^{-1} (C5), using an application volume of 1L per plant. The Harvista formulation was supplemented with Break-Thru, an organosilicone surfactant (S240, Evonik Industries AG, Essen, Germany) at 0.035 % of the final volume. The control treatment (C) consisted of a sprayable solution containing only water and Break-Thru. Preparation of the spray solution was as follow: the spray tank was filled with two-thirds of the total volume of water required to spray five plants; Break-Thru was added and the solution was mixed; 1-MCP powder at the given concentration was added and the solution was gently swirled until the powder was completely dissolved; the remaining water was added and the solution was gently stirred for about two minutes. Since 1-MCP is given off as a gas, the entire foliage, adaxial and abaxial leaf surfaces, branches, and flower buds, from the coffee trees were immediately sprayed after the preparation of the solution. Different treatments were distant at least 20 meters from each other to avoid any cross contamination. 1-MCP application was made to the point of runoff, using a 12 L backpack sprayer (S12 - Brudden Sprayers), in a sunny day between 08:00 and 10:00 am to maximize 1-MCP penetration.

2.2.1.1 Statistics

The experiment was carried out in a randomized block design with 6 treatments and five replicates per treatment. Differences between treatments were accessed by one-way ANOVA at $P < 0.05$. When ANOVA was significant, means were discriminated using Turkey's multiple comparison test at $P \leq 0.05$.

2.2.2 Greenhouse Experiment

2.2.2.1 Plant material and culture

Coffee (*C. arabica*) plants 10 months-old from two different cultivars, 'Bourbon Amarelo' (Ba) and 'Obatã Vermelho' (Ov), were used in this experiment. Seedlings were grown in three litre plastic bags filled with an organic loam-based substrate (John Innes No 2, J Arthur Bowers, Lincoln, UK). All plants were initially watered to run-off and left to freely drain overnight, before being weighted to establish drained capacity. Plants from the well-watered (WW) treatment were maintained well-watered by replacing full evapotranspiration (*ET*) daily. For plants from the water deficit (WD) treatment, water was completely withheld until stomatal conductance (g_s) reached around 30 % of the stomatal conductance from WW plants. The experiment lasted for nine days and was conducted in a semi-controlled naturally lit glasshouse compartment (5 x 3 m), with supplementary lighting, provided with sodium lamps (Osram Plantastar (Munich, Germany) 600 W), supplying lighting for a 12-h day photoperiod (6:00 - 18:00) when ambient photosynthetically active radiation was less than 500 μmol m^{-2} s^{-1}. During the experiment the daily maximum temperature in the greenhouse was 29.8 °C, and the minimum temperature at night was 14.8 °C. Mean relative humidity was 34.8 % and it varied from 64 % to 14.8 % during the experiment.

Every three days (at day 0, 3, 6, and 9), stomatal conductance, soil moisture, leaf growth, and leaf ethylene evolution were measured for plants under WW and WD conditions. Ethylene evolution rate was also evaluated after plant rewatering and 1-MCP application. After 3, 6, and 9 days under WD conditions groups of three plants from each cultivar were rewatered or submitted to 1-MCP treatment (Plants from the Ov cultivar only underwent 1-MCP

treatment since the number of plants available was not enough for both treatments to be carried out). Plants were rewatered with the amount of water required for re-establishing drained capacity. 1-MCP treatment was performed using the same procedure described for the field experiment, except that for this experiment a hand sprayer was used instead of a backpack sprayer system, and the surfactant Silwet L-77 was used instead of Break-Thru. In order to avoid contamination of other plants in the greenhouse, plants were put in a controlled environment room (temperature of 22 °C; relative humidity 42 %; light intensity between 400 and 640 µmol m^{-2} s^{-1} PPFD) during 1-MCP application. An application volume of 30 mL per plant was used, and control plants were sprayed with a solution made of only water and Silwet L-77. Once plant surfaces were dry (around 45 minutes after application), plants were moved back to the greenhouse. For both treatments, rewatering and 1-MCP application, leaf samples for ethylene measurement were collected 48 h after treatment implementation. Ethylene evolution rate from 1-MCP control plants (plants sprayed with a solution containing only Silwet L-77 and water) was accessed only at day 0 for both coffee cultivars.

2.2.2.2 Physiological measurements

During the experimental period, plants were weighted every day, using a balance with 0.1 g resolution (Scout Pro Portable balance; Ohaus, Greifensee, Switzerland), to calculate daily *ET*. Weighting and water replacement for well-watered plants were conducted at the end of the day (18:00 h).

Stomatal conductance (g_s) was measured using a porometer (Model AP4; Delta- T Devices, Cambridge, UK). Two readings of g_s were taken either side of the mid-rib on young fully expanded abaxial side of the leaf per plant (g_s was

calculated from the mean of nine different plants). g_s measurements were made between 8:00 and 10:00 h.

Right after measuring g_s, soil moisture content (θ) was measured using a ML2x ThetaProbe interfaced with HH2 moisture meter (Delta -T Devices). Two readings, in each of the same nine plants used for g_s measurement, were performed and readings were converted from microvolts to volumetric soil moisture content (θ), based the two point calibration (field capacity and oven-dry soil) for organic soils, according to the ML2x ThetaProbe manual (Delta -T Devices). Leaf growth was accessed by measuring leaf length of young developing leaves (one leaf per plant, from nine different plants), using a Perspex ruler, and expressed as relative elongation rate (RER) at each sampling time.

Leaf ethylene evolution rate was measured using a laser-based photoacoustic ethylene detector (type ETD-300; Sensor Sense BV, Nijmegen, the Netherlands) in combination with a gas handling system, the valve control box (type VC-6, Sensor Sense (B.V., Nijmegen, the Netherlands) (see Appendix B for a more detailed description of the equipment). Ethylene evolution rate from three different plants for each treatment was evaluated, with two samples per plant. Each sample was composed by one leaf that was immediately placed in 28 mL transparent glass vial with moist tissue, put on the bottom of each vial, to avoid desiccation of the plant material. Vials were then sealed with a rubber puncture cap (Subseal, Sigma Aldrich, Dorset, UK). After incubating for 60 minutes under the same environment conditions of the plants from which the leaves were sampled, vials were directly attached to the ethylene detector for ethylene measurement. Ethylene emission for each sample was alternatively monitored by ETD-300, with a running time of 7 minutes (5 s per acquisition point) and at a flow rate of 4 L h^{-1}. Leaves were sampled from 13:00 to 15:00 h.

After ethylene measurement, leaves were weighted using a balance and ethylene evolution rate was given as nL mg-1 h-1.

2.2.2.3 Statistics

Differences between irrigation treatments, cultivars, and evaluation times (days) were accessed by one-way ANOVA at $P < 0.05$. When ANOVA was significant, means were discriminated using Turkey's multiple comparison test at $P \leq 0.05$. All statistical analysis were performed by the R software (R DEVELOPMENT CORE TEAM, 2013).

2.3 RESULTS

2.3.1 Field trial

Predawn leaf water potential was slightly lower in plants from the control treatment and ranged from -0.26 MPa to -0.49 MPa, indicating that coffee trees were not facing water stress conditions by the time of 1-MCP exogenous application (Table 2.1). Midday leaf water potential showed a higher variation among the different treatments, ranging from -1.27 MPa to -2.00 MPa, with treatments C3 and C5 displaying the lower values among the treatments (Table 1).

Table 2.1 Predawn and midday leaf water potential (MPa) for 'Acaiá Cerrado' plants from the six different treatments of the field trial.

	Treatments					
Time of the day	Control	C1	C2	C3	C4	C5
Predawn	-0.49 ± 0.09 b	-0.31 ± 0.08 a	-0.27 ± 0.09 a	-0.26 ± 0.04 a	-0.32 ± 0.06 a	-0.37 ± 0.08 ab
Midday	-1.27 ± 0.51 a	-1.42 ± 0.27 ab	-1.61 ± 0.09 ab	-2.00 ± 0.10 b	-1.42 ± 0.33 ab	-1.88 ± 0.28 b

Different letters represent statistical significance between means within each time of the day. Each value represents the mean ± 95% confidence interval of the mean (n=5).

1-MCP exogenous application showed that 1-MCP, unexpectedly, promoted anthesis at the two highest concentrations C4 and C5. A substantial flowering promotion could be observed in all five plants from C4 treatment and in two plants from treatment C5. After 12 days from 1-MCP application, flower opening was observed (Figure 2.1). No changes in flower development could be observed for C, C1, C2, and C3 treatments, indicating that flowering promotion

was indeed triggered by 1-MCP, and this flowering induction apparently requires higher 1-MCP concentrations (Figure 2.1).

After six days from 1-MCP application there was a raining event (11.6 mm) (Appendix A), and no additional flowering induction could be observed in plants from the C4 and C5 treatments. On the other hand, anthesis was promoted in plants from C, C1, C2, and C3 treatments, although flowering occurred in a patchy way, probably a result from the pruning in 2011.

Figure 2.1 Flowering induction on a plant from the control treatment (Break-Thru + water) six days after treatment implementation (A) and from plants of the C4 treatment (1-MCP applied at 50 mg a.i. L^{-1}) at six (B) and 12 (C) days after 1-MCP application. No flower induction was observed on the plant from the control treatment, while at six days after 1-MCP treatment a significant

increase in flower bud size can be observed, with flower opening taking place 12 days after 1-MCP application.

2.3.2 Greenhouse experiment

Although g_s values at day 3 were similar between well-watered and water-deficit plants, withholding irrigation significantly reduced g_s in both genotypes at days 6 and 9, being 46 % and 52 %, and 79 % and 88 %, lower in water deficit plants when compared to well-watered 'Ba' and 'Ov' plants at day 6 and 9, respectively (Figure 2.2). *ET* from 'Ba' well-watered plants was maintained under the same level until day 6, increasing at day 9. For Ov well-watered plants, *ET* continuously increased during the experiment. *ET* from water-deficit plants continuously decreased during the experiment for both cultivars, except for Ov plants at day 3, being 70 % and 81 % lower than well-watered 'Ba' and 'Ov' plants, respectively, at day 9 (Figure 2.2).

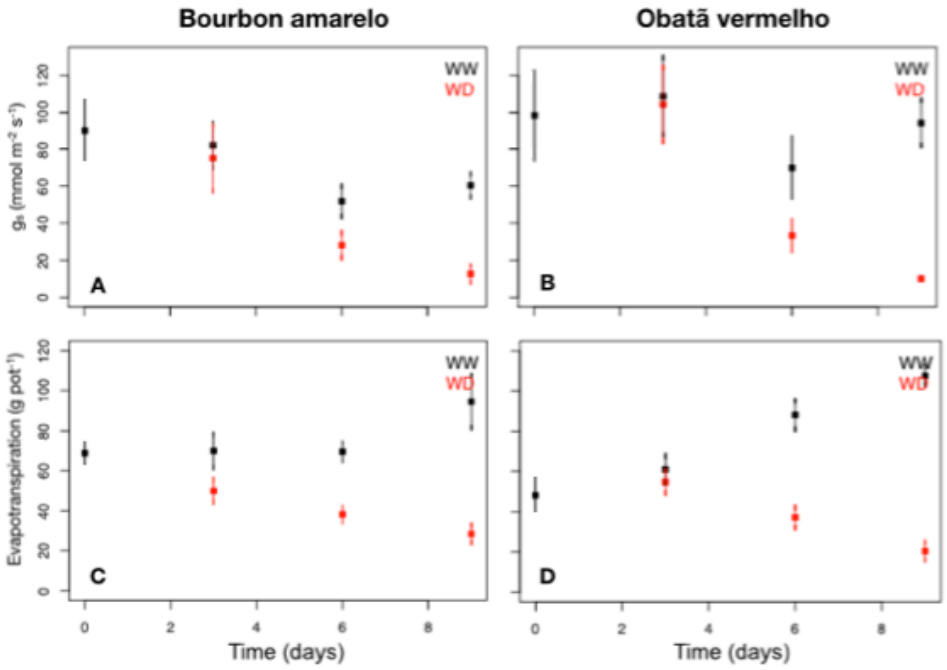

Figure 2.2 Stomatal conductance (A and B) and evapotranspiration (C and D) of 'Bourbon Amarelo' (left panel) 'Obatã Vermelho' (right panel) plants in well-watered (black circles) and water-deficit (red circles) conditions. Data are means ± 95% confidence interval of the mean (n=9).

The comparison of g_s between both cultivars studied showed that g_s levels and pattern under well-watered and water-deficit conditions were similar, with g_s from 'Ov' plants being usually higher in most sampling times, and statistically different only under well-watered conditions at day 9 (Figure 2.3). In general, *ET* also showed similar levels and pattern between both cultivars, except for the fact it continuously increased in 'Ov' plants and did not vary for 'Ba' plants under well-watered conditions until day 6. (Figure 2.3)

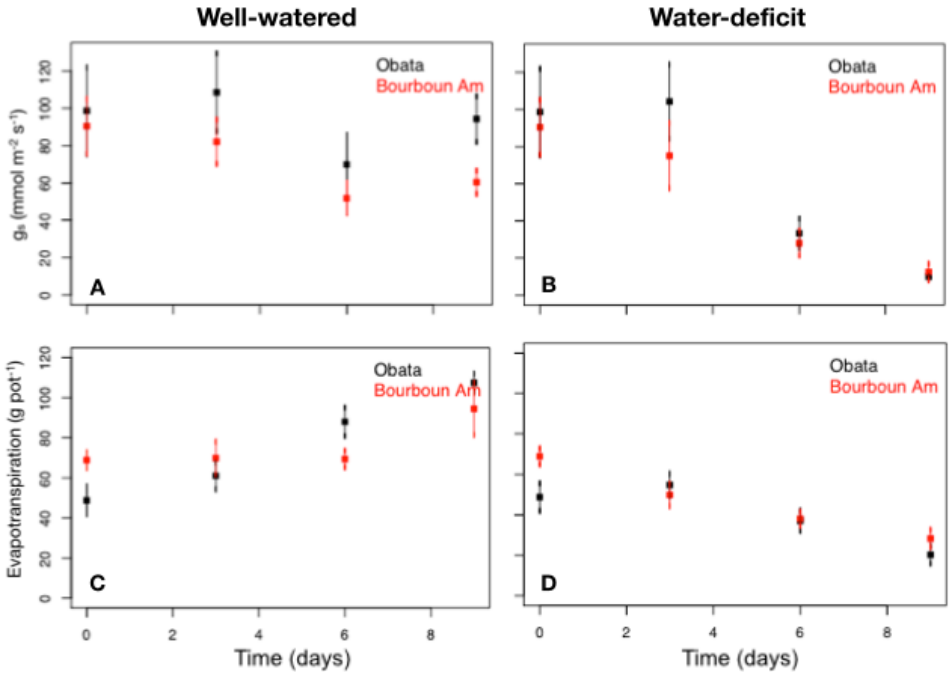

Figure 2.3 Comparison between mean stomatal conductance (A and B) and evapotranspiration (C and D) of 'Bourbon Amarelo' and 'Obatã Vermelho' plants in well-watered conditions (left panel) and water-deficit (right panel) conditions. 'Bourbon Amarelo' values are represented by red circles and 'Obatã Vermelho' values by black circles. Error bars represent 95 % confidence interval of the mean (n=9).

Soil moisture from well-watered plants was stable throughout the experiment for both cultivars and continuously reduced in water-deficit plants (Figure 2.4). θ from 'Ba' water-deficit plants reduced by 42 % after three days without watering, with further reductions of 41 % and 23 % at days 6 and 9, respectively, being 73 % lower than θ from at day 9 (Figure 2.4). For 'Ov' plants, withholding water lead to a reduction of 33 % after three days, and additional 42 % and 33 % reductions at days 6 and 9, respectively, reaching 29 % of the volumetric water content from well-watered plants at day 9 (Figure 2.4).

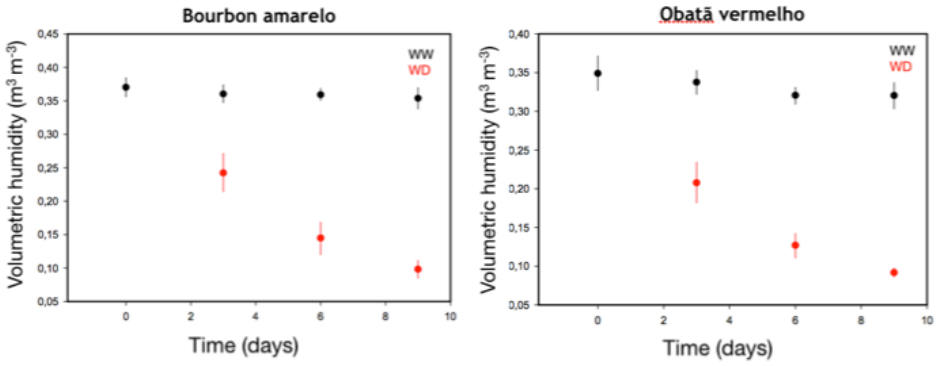

Figure 2.4 Mean volumetric humidity for 'Bourbon Amarelo' (left panel) and 'Obatã Vermelho' (right panel) plants in well-watered (black circles) and water-deficit (red circles) conditions. Error bars represent 95 % confidence interval of the mean (n=9).

Leaf relative elongation rate from well-watered and water-deficit plants were not statically different during the experiment for both cultivars, displaying values slightly higher for well-watered 'Ov' plants in every measuring time (Figure 2.5). Figure 2.5 also shows that RER occurs at similar extension rates in both cultivars under well-watered and water-deficit conditions.

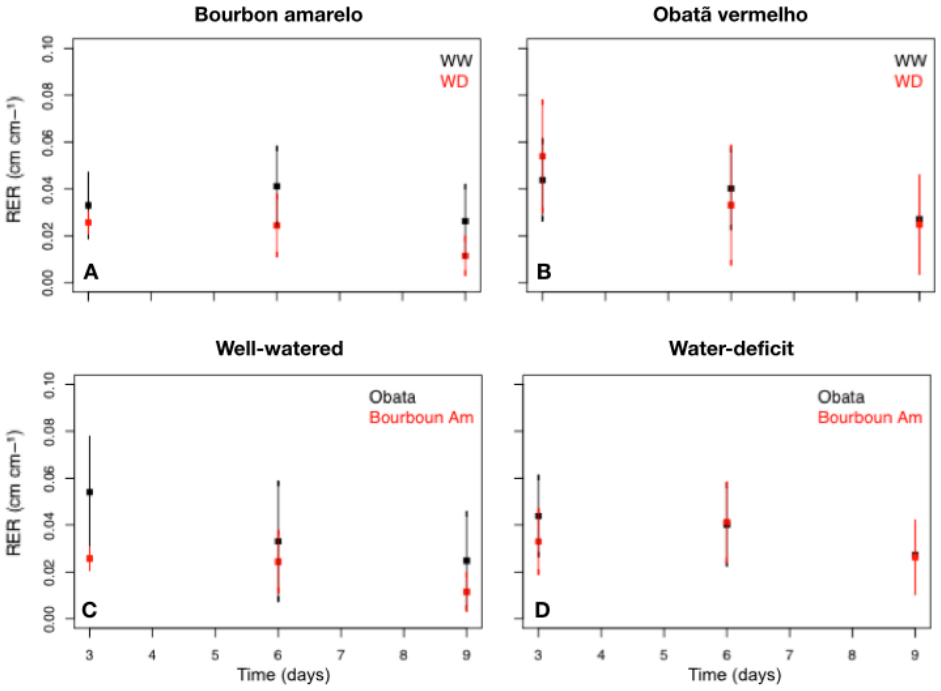

Figure 2.5 Relative elongation rate (RER) of leaves from 'Bourbon Amarelo' (A) and 'Obatã Vermelho' (B) plants in well-watered (black circles) and water-deficit (red circles) conditions. Comparison of RER in well-watered (C) and water-deficit (D) conditions between 'Bourbon Amarelo' (red circles) and 'Obatã Vermelho' (black circles) cultivars. Error bars represent 95 % confidence interval of the mean (n=9).

Leaf ethylene release rate of plants from both cultivars were not statistically different at any of the evaluated times, except for 1-MCP treated plants (Figure 2.6). In general, ethylene production rates among the different treatments and during the experiment were similar. Water deficit did not promote major changes in ethylene evolution, but an increase in 'Ov' plants could be observed at day 9, where ethylene production was 70 % higher than well-watered plants at the same sampling time and water deficit plants at day 6, though not statistically different (Figure 2.6). Apparently, Silwet L-77 does not interfere in ethylene production when compared to other treatments (Figure 2.6). 1-MCP application seems to promote a reduction in ethylene release rate,

measured at 48h after spraying the plants, from day 0 to day 6 in both cultivars. At day 9, an increase in ethylene production, 90% for Ba plants and 122 % for Ov plants, when compared to 1-MCP treated plants at day 6, could be observed for 1-MCP treated plants exposed to 9 days of water suspension, being statistically different Ba plants.

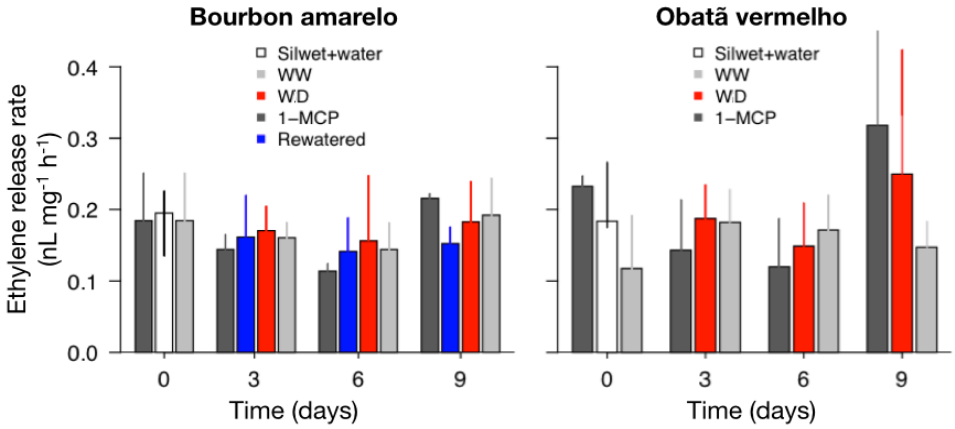

Figure 2.6 Mean leaf ethylene release rate for 'Bourbon Amarelo' (left panel) and 'Obatã Vermelho' (right panel) plants in well-watered (light grey bars), water-deficit (red bars), rewatered (blue bars), 1-MCP treated (dark grey bar), and Silwet L-77 + water treated plants at four (0, 3, 6, and 9) sampling times. Silwet L-77 + water treatment was evaluated only at day 0 for both cultivars, and ethylene release rate from rewatered plants was only measured for 'BA' plants. Leaves were sampled at the given times or after 48h of treatment implementation (Rewatering, 1-MCP, and Silwet L-77). Error bars represent 95 % confidence interval of the mean (n=3).

2.4 DISCUSSION

2.4.1 Field trial

In this study, it was first hypothesized that a rapid and transient increase in ethylene levels in the shoot, triggered by rain or irrigation after a period of water-deficit, would act as the signal that triggers coffee anthesis. Assuming the veracity of this hypothesis, the blockage of ethylene action, through the use of chemicals, such as 1-MCP, an ethylene action inhibitor, would inhibit ethylene responses and by doing so, anthesis would not occur. Often, coffee anthesis is induced by low scattered rain events, which are not sufficient to promote anthesis in a significant extent. Furthermore, the early start of the raining season, or episodic rainfall events during the dry season, at times where the majority of the flower buds have not reached the developmental stage capable of responding to the signal of regrowth, can promote anthesis in a low extent. These factors contribute to the occurrence of several blossoming periods in coffee and consequently asynchronous fruit ripening. Thus, considering the fact that 1-MCP can repress ethylene responses for relatively long periods of time (SISLER; SEREK, 2003; 1997), 1-MCP exogenous application could inhibit anthesis under the above mentioned situations, contributing for the occurrence of a main flowering event and thus leading to a higher percentage of fruits at the same ripening stage at harvest time. However, the results from the field trial obtained in this study from the 1-MCP application to coffee plants goes against this hypothesis, and instead of inhibiting coffee anthesis, 1-MCP promoted coffee anthesis at certain concentrations, acting just like if a raining event had been occurred (Figure 2.1).

1-MCP anthesis promotion could be observed only at the two highest concentrations applied, C4 and C5, with anthesis being promoted in all five

plants from the C4 treatment and only in two plants the C5 treatment. Although it could be hypothesized a possible toxic effect of 1-MCP at higher concentrations, this was probably not the case considering that the other three plants underwent through anthesis only one month later, after a 23 mm rain event at the end of September (Appendix A), suggesting that flower buds from these plants were not at the corrected developmental stage at the time of 1-MCP application. Although adjacent plants were chosen for treatment implementation, the non-anthesis from the three others plants from treatment C5 was probably caused by the fact that plants had been pruned in the previous year, a process that have been shown to alter flowering cycle, delaying harvest on weak plants (WORNER et al., 1970). This fact would suggest that the absence of anthesis on the other treatments, where 1-MCP was applied at lower concentrations, could have been caused by the same reason. However, six days after 1-MCP application, a raining event of 11.6 mm promoted anthesis in plants from these treatments, including plants from the control treatment as well, suggesting that 1-MCP concentration was indeed too low to cause any effect on flowering induction. No additional promotion of anthesis could be observed in plants from the treatments C4 or C5, suggesting that 1-MCP at these concentrations acted like the signal triggered by rain that promotes coffee anthesis. These findings suggest that the timing of application and the concentration of 1-MCP are important for triggering coffee anthesis.

The analysis of the predawn and midday water potential from the plants in the field suggest that they were not under water stress, since the mean predawn water potential levels were above -0.5 MPa for all treatments, indicating that plants were showing a good recovery in their water status compared to the lower water potential levels faced during the day (Table 2.1). Hardly predawn leaf water potential in coffee trees reaches values below -1.5 MPa. Under irrigated conditions, lower predawn leaf water potentials can be

managed to occur, and a positive effect on coffee flowering concentration, without any penalty in yield, has been observed with suspension of irrigation for around 70 days, time at which predawn leaf water potential reached -2.0 MPa (GUERRA et al., 2005).

The fact that exogenous 1-MCP application promoted coffee anthesis does not exclude the possibility that ethylene could still be acting as a signal for anthesis promotion, but this finding certainly adds some more complexity to the understanding of coffee flowering control. Since 1-MCP application promotes the inhibition of ethylene responses, by biding to ethylene receptors and thus preventing ethylene from biding to them, it can be supposed that ethylene, instead of promoting anthesis, could be inhibiting it (Figure 2.7). Following this supposition, under natural conditions, increased ethylene levels, or an increase on its sensitivity, during water-deficit conditions, would inhibit anthesis promotion. Rain or irrigation, following this period of water-deficit, would relief the water stress faced by coffee trees, decrease ethylene biosynthesis or sensitivity, allowing anthesis to take place.

Several studies in the literature suggest that ethylene levels increase under water stress conditions (APEBAUM; YANG, 1981; BALOTA et al., 2004; BELIMOV et al., 2009; BELTRANO et al., 1997; CHEN et al., 2002; EL-BELTAGY; HALL, 1974; LIU et al., 2013; SCHUCH et a., 1992; YANG et al., 2007; YANG et al., 2014; ZHANG et al., 2015). On the other hand, different studies have shown the opposite effect (LARRAINZAR et a., 2014; MORGAN et al., 1990; NARAYANA et al., 1991; SCHUCH et al., 1992). Ethylene, under water-deficit conditions, is mainly involved in growth inhibition responses, inhibiting root growth and leading reductions in the shoot/leaf expansion rates (PIERIK et al., 2006; SHARP, 2002). It is also involved in senescence and abscission processes, adjusting leaf area to water conditions and thus minimizing water loss (ABELES et al., 1992). Towards flowering regulation, ethylene is

known to promote flowering only in species from the *Bromeliaceae* family, and usually it inhibits this process in other species by reducing bioactive GA levels and enhancing DELLAs protein accumulation, which in turn negatively regulates the floral meristem identity genes. However, in both cases ethylene regulates the transition from the vegetative to the reproductive phase. In the case of coffee trees, floral transition has already occurred and flower buds have completed their differentiation. Thus, growth inhibition of flower buds by ethylene may be caused by a reduction in expansion of flower buds, through a similar mechanism by which ethylene inhibits root and shoot/leaf tissues in other species (SHARP, 2002), or it could be involved in the maintenance of the dormant state of flower buds. In this scenario, rain or irrigation would lead to decrease in ethylene levels and permit anthesis to occur.

On the other hand, ethylene can still be acting as a signal for anthesis promotion. Under water stress conditions, ethylene production rates may also display no alteration (MUNNÉ-BOSCH et al., 2002; VOISIN et al., 2006), or even reduce under these conditions (MORGAN et al., 1990). In addition, major modifications in ethylene levels may be observed only when plants are rewatered, as observed in woody species such as citrus (TUDELA;PRIMO-MILLO, 1992; GOMEZ-CADENAS et al., 1992), and also in rose (MENG et al., 2014). In the case of citrus, although leaf ethylene levels did not increase during water-deficit conditions, accumulation of the ethylene precursor ACC was observed in the roots. Rewatering promoted a rapid and transient increase in ethylene levels in the shoot through the xylem transport of ACC accumulated in the roots, leading to leaf abscission. For rose plants, rewatering induced an organ-specific up-regulation of ethylene biosynthesis, which was shown to be important for the rehydration response of flowers through the regulation of rehydration responsive genes. Although several studies have shown that 1-MCP application can lead to a reduction in ACC or ethylene levels, or the down-

regulation of ethylene biosynthesis genes (BULENS et al., 2012; CHIRIBOGA et al., 2013; YANG et al., 2013), 1-MCP can also promote an increase in ethylene production rates or the up-regulation of ethylene biosynthesis genes (INABA et al., 2007; NAKATSUKA et a., 1998), probably due to the loss of negative feedback regulation of ethylene biosynthesis. Both positive and negative feedback regulation of ethylene biosynthesis have been reported in different plant species (reviewed in KENDE, 1993; NAKATSUBA et al., 1998; BARRY et al., 2000), and massive induction in ethylene biosynthesis rates have been shown to occur upon 1-MCP application (ELLA et al., 2003). Thus, 1-MCP application in coffee trees may have lead to a burst in ethylene production, which is in accordance with the initial hypothesis of this study, and this increase in ethylene levels possibly mimicked the increase in ethylene levels after rain and irrigation under natural conditions, triggering anthesis (Figure 2.7).

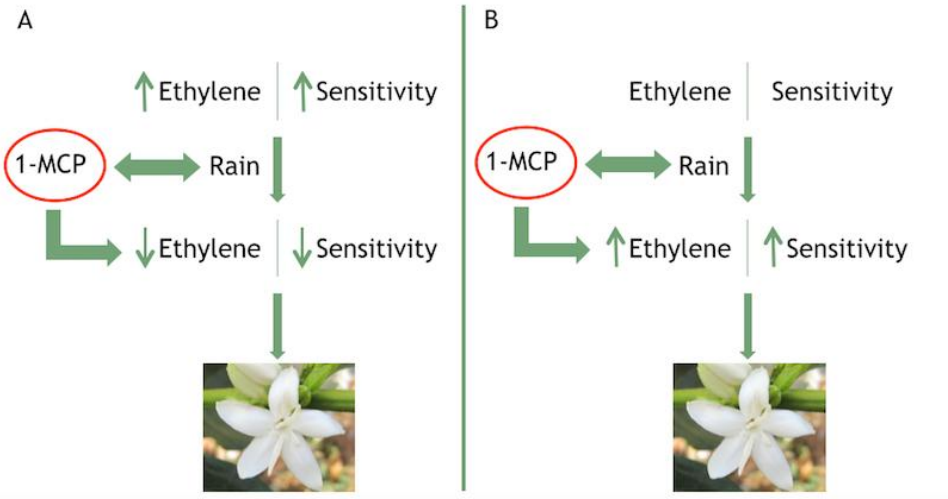

Figure 2.7 Putative mechanisms through which 1-MCP treatment may induce coffee anthesis. Ethylene levels or sensitivity may increase under water-deficit conditions and 1-MCP treatment may act similar to a raining event by promoting a reduction in ethylene levels or sensitivity, allowing anthesis to take place (A). On the other hand, ethylene levels or sensitivity may remain stable or decrease under water-deficit conditions and 1-MCP treatment may act similar to a raining event by promoting a rapid and transient increase in ethylene levels, due to the loss of the negative feedback regulation of ethylene biosynthesis, promoting coffee anthesis.

2.4.2 Greenhouse experiment

Withholding irrigation lead to significant reductions in stomatal conductance, and consequently in evapotranspiration rates, in both coffee cultivars as the water content from soil started to decrease (Figures 2 and 3). Although after three days soil volumetric water content significantly reduced, by 42 % and 33 % in Ba and Ov cultivars, respectively, only minor changes were observed in gs and E levels. This suggest that at this time, water content of the soil had not reduced at a level capable of inducing significant changes in these parameters, or in the case of g_s, changes in its levels may have occurred along

the day, since g_s was measured only during the first hours of the day. As the soil became drier, g_s and ET reduced at levels significantly lower than the ones observed for well-watered, indicating that plants were facing water stress conditions. Stomatal closure is one the first water saving strategies displayed by plants under water-deficit conditions, and the plant hormone ABA, synthesized by leaves and/or transported from roots through the xylem, promotes stomatal closure via a well-established signal transduction pathway (FAN; ASSMANN, 2004; KIM et al., 2010), decreasing plant transpiration.

Coffee stomata are strongly sensitive to both soil and atmospheric water deficits. In arabica coffee, g_s appears to be an early indicator of soil drying, showing decreases as soon as one-third of the available soil water has been depleted, although this is not effectively accompanied by proportional reductions in transpiration. For both arabica and robusta coffee, g_s has been shown to decrease curvilinearly with decreasing Ψ_{leaf}, with no apparent threshold value of Ψ_{leaf} at which stomatal closure is observed . This suggests a strong sensitivity of coffee stomata to rising internal water deficit. The positive relation between g_s and Ψ_{leaf} is expected when soil moisture changes and indirectly affects stomata through a hydraulic feedback.

Growth rates are also negatively regulated under water stress conditions, which affect cell proliferation and expansion rates leading to growth inhibition (CLAEYS; INZÉ, 2013). However, in this study, it was not observed any change in relative growth rates from plants under water-deficit conditions, nor between the two different coffee cultivars, when compared to well-watered plants (Figure 2.5). This was probably caused because water-deficit treatment was applied over a relatively short time and for this reason drought effects on growth were not significant.

The ethylene production data from plants under water-deficit suggest that ethylene biosynthesis is not altered during water stress conditions in the two

cultivars analyzed. Ethylene release rate from coffee plants under water-deficit were similar to the levels observed in well-watered plants, and some studies in different species have found the same pattern obtained in this work (MUNNÉ-BOSCH et al., 2002; VOISIN et al., 2006). Although increased levels of ethylene under water stress conditions is frequently reported in the literature, these may partially be attributed to mode of water stress imposition and also the method used to measure ethylene. Most measurements of leaf ethylene evolution reported in the literature have measured head-space ethylene accumulation from detached leaves (due to mechanical limitations) (SOBEIH et al., 2004). However detached leaves may respond differently to soil drying than intact plants and, according to Morgan et al (1990), some explanations for this is that detached leaves are subject to several stresses - excision, handling, disruption of transport systems, gravitropic disorientation, and rapid loss of water. In addition, some studies suggest that ethylene enhancement during water stress conditions may be related to its intensity (BELTRANO et al., 1997; KALANTARI et al., 2000; YANG et al., 2004), and the rate at which Ψ_{leaf} falls (MORGAN et al., 1990), with slow soil drying reducing ethylene production rates. Although in this study soil drying was rapidly imposed, due to the complete suspension of irrigation and the small pot size, no increase in ethylene was observed. However, it must be taken into account that plants used in this experiment were facing root growth restriction due to the small pot size used in this experiment. Root-restriction stress is known to enhance the concentration of ABA within the roots and in the xylem sap (HURLEY; ROWARTH, 1999; LIU; LATIMER, 1995) and ABA is known to restrict ethylene production (VOISIN et al., 2006; HUSSAIN et al., 2000; SHARP, 2002; SHARP et al., 2004). The lower g_s levels observed in well-watered ‘Ba' plants, compared to ‘Ov’ g_s values, corroborates for this fact, since ‘Bourbon Amarelo’ plants were around 20 cm higher than ‘Obata Vermelho’ plants, and thus were facing more intense root growth restriction and possibly

they were producing higher amounts of ABA. In a previous experiment, conducted five months before the experiment detailed in this chapter, using the same plants, g_s levels from ‘Ba’ plants showed higher levels than g_s from ‘Ov' plants (data not shown).

Rewatering and 1-MCP treatment also did not promote any significant change in ethylene release rates in the two cultivars studied (Figure 2.6). Although it has been reported that 1-MCP may differentially regulate ethylene biosynthesis for extended periods of time (days) (BULLENS et al., 2012; CHIRIBOGA et al., 2013), it may also promote short-time (hours) modifications in the ethylene production patterns (ELLA et al., 2003). In this study, coffee leaves from 1-MCP treated plants were sampled only 48 h after 1-MCP application and some studies suggest that changes in the ethylene production upon 1-MCP application may take place in few hours after 1-MCP application (ELLA et al., 2003). Apparently this may also be case for coffee plants, since measurement of ethylene evolution at one and four hours after 1-MCP application appears to lead to significant elevations in ethylene production, although the low number of replicates and the absence of control (Surfactant + water) plants (Appendix C). The time for ethylene measurement from plants submitted to rehydration may also not have been optimal, considering that increases in ethylene production upon rewatering have been observed in shorter measuring times than the one used in this study, such as six hours (GOMEZ-CADENAS et al., 1996; BELTRANO et al., 1997), or even after only one hour after rewatering detached rose flowers (LIU et al., 2013). Thus, further studies involving ethylene analysis at times closer to treatment implementation, 1-MCP application or rewatering, are needed in order to elucidate if there is any change in ethylene production rates at these short time periods.

2.5 CONCLUSION

1-MCP exogenous application, at the concentrations of 50 and 100 mg of a.i. per litre and with spray volume of one litre per plant, is able to promote coffee anthesis in trees of the cultivar ‘Acaiá Cerrado' under field conditions.

Ethylene production rates in coffee seedlings of the ‘Bourbon Amarelo' and ‘Obatã Vermelho’ cultivars, grow under greenhouse conditions, are similar under well-watered and water-deficit conditions. Plant rehydration and 1-MCP treatment do not change ethylene release rates in leaves sampled 48 h after treatment implementation, when compared to plants under well-watered or water-deficit conditions.

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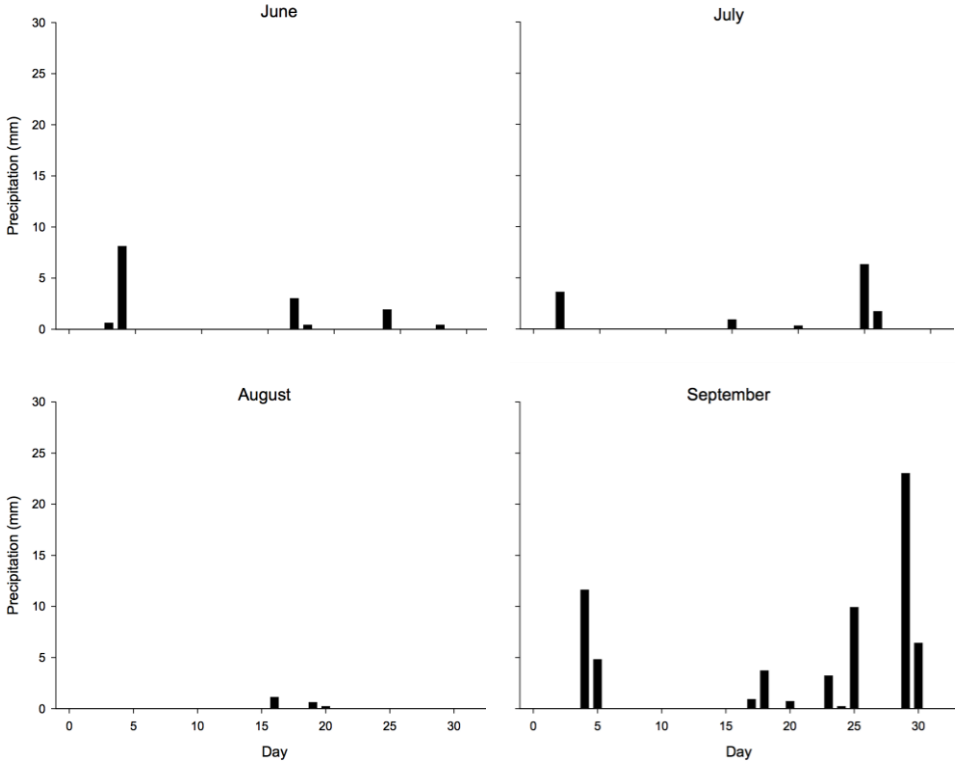
APPENDIX A

1- Total precipitation levels from June to September of 2013.



APPENDIX B

1- Ethylene measurement using the ETD-300 ethylene detector

The ethylene detector ETD-300 (Sensor Sense BV, Nijmegen, the Netherlands) is one of the first commercial ethylene detectors based in the laser sensing technique. The laser-based detectors take the advantage of using tuneable lasers to selectively emit in the absorption range of ethylene in combination with detection techniques, such as photoacoustic spectroscopy (CRISTECU et al., 2012). In this case, light is generated by a laser and directed into an absorption cell where it is absorbed by the ethylene molecules and converted into heat. By switching the laser on and off with a mechanical chopper the temperature changes periodically, giving rise to a periodic pressure change, resulting in acoustic energy detected by a miniature microphone. The intensity of the sound is proportional to the concentration of absorbing gas molecules present in the cell (CRISTECU et al., 2012). This results a high sensitivity level and a short response time, with ETD-300 showing a detection limit of 300 pL L^{-1} within a 5 s measuring time, which is the best-achieved sensitivity worldwide. For instance, the sensitivity and response time of ETD-300 is around 100 times higher and faster, respectively, than a gas chromatograph.

Both the ethylene detector and the valve control box are operated fully automatically by a computer program, allowing ethylene monitoring in three operation modes, namely continuous flow, stop-and-flow and sampling. In the continuous flow mode, up to six cuvettes with biological samples are continuously flushed with air at a constant flow rate of 1 L h^{-1}. Ethylene released in the headspace is transported to the ETD-300 alternately, allowing a succession of typically 10 min for each cuvette. In this way, the dynamics of the ethylene emission can be studied in sufficient detail over several hours or for an

even longer period. Stop-and-flow mode is used when plant biomass is very small and ethylene concentration is below the detection limit to be measured continuously, requiring a period of time (e.g. 1 h) to accumulate, before being transported to the detector. While ethylene from a cuvette is measured (typically 15 – 30 min) no air flow is applied to the other cuvettes, hence optimizing the measurement efficiency. Finally the sample mode approach is used when multiple samples need to be screened, ethylene production needs to be quantified once per sample, rather than monitored over time. For this, plant tissue is usually incubated for a certain period of time, and part of the headspace gas from the vial is transferred for an empty vial for future quantification, allowing that a large amount of samples can be collected within a short period of time. (CRISTECU et al., 2012)

Briefly, during measurement of the samples, compressed air from a cylinder is passed through a catalyser to remove the hydrocarbons and the distributed by the valve controller, at a controlled flow rate, through a needle that is inserted inside the tube, to the cuvettes enclosing the biological samples or the stored gas samples. A second needle inserted inside the vial collect all the gas inside the vial, and before being monitored by the detector, CO_2 and H_2O vapour are eliminated using a scrubber with KOH and $CaCl_2$, respectively (CRISTECU et al., 2012).

In the greenhouse experiment 1, the sample mode approach was used for measuring the ethylene release rate from coffee leaves. However, due to low amounts of ethylene produced by coffee leaves, headspace gas was not transferred for an empty vial before the measurement. After the 60 minutes of incubation, the 28 mL vial with the coffee leaf inside of it was directly coupled to the needle pair from one of the six channel of the valve controller box. Considering that the measuring time for each sample lasted seven minutes, samples were put to incubate in the vials in seven minutes intervals.

APPENDIX C

1- Ethylene production shortly after 1-MCP application

Figures 1 and 2 shows the result given by the Sensor Sense ETD-300ethylene detector. Ethylene production is estimated based on the peak areas present on graph given by the detector. Figure 2.8 shows the peaks areas (Plant 1 (black peak), Plant 2 (red peak), and Plant 3 (green peak)) from leaves of the 'Obata Vermelho' coffee cultivar, sampled one hour after 1-MCP application (the next three peaks, colours purple, blue, and pink, represent peak areas of leaves from the greenhouse experiment from Chapter 2, which were sampled after 24 h of 1-MCP application). Figure 2.9 represent the peak areas of leaves, sampled from the same plants used to analyze ethylene production one hour after 1-MCP application, sampled four hours after 1-MCP application.

Table 2.2 represents the ethylene release rate obtained from the samples displayed in figures 2.8 and 2.9, and it shows also the fold difference in ethylene release rate from these samples compared to the average ethylene production rate from the greenhouse experiment detailed on Chapter 2 of this thesis, where leaves were sampled 48 h after 1-MCP application.

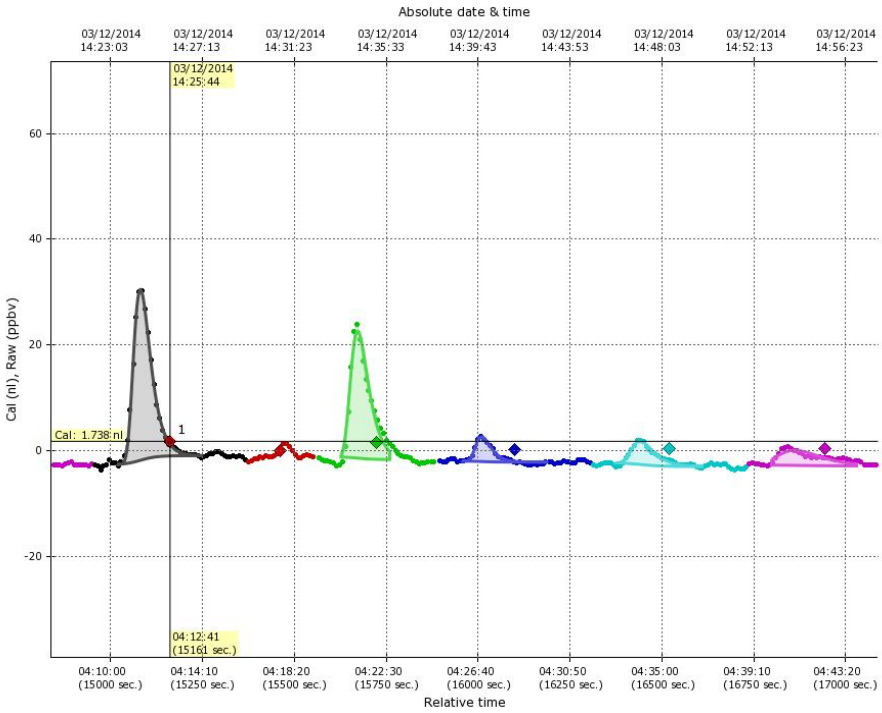


Figure 2.8 Peak areas generated by the Sensor Sense ethylene detector (ETD-300) for coffee leaves, from three different plants (Plant 1 (black peak), Plant 2 (red peak), and Plant 3 (green peak)), sampled one hour after 1-MCP application. The purple, blue, and pink peaks represent peak areas of leaves from the greenhouse experiment from Chapter 2, which were sampled after 24 h of 1-MCP application.

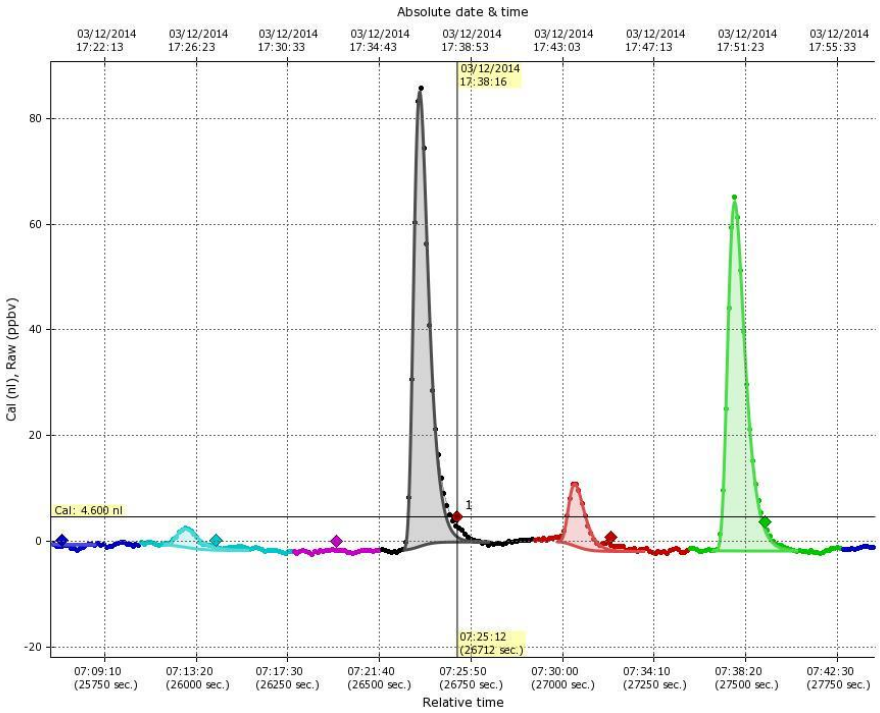


Figure 2.9 Peak areas generated by the Sensor Sense ethylene detector (ETD-300) for coffee leaves, from three different plants (Plant 1 (black peak), Plant 2 (red peak), and Plant 3 (green peak)), sampled one hour after 1-MCP application. The purple, blue, and pink peaks represent peak areas of leaves from the greenhouse experiment from Chapter 2, which were sampled after 24 h of 1-MCP application.

Table 2.2Ethylene release rate from the coffee leaves from figure 2.8 and 2.9 after one and five 1-MCP application and the fold difference in ethylene production from these leaves compared to the average ethylene production from coffee leaves from the greenhouse experiment of chapter 2 of this thesis.

	Time after 1-MCP treatment (h)	Ethylene release rate (nL mg^{-1} h^{-1})	Fold difference
Average ethylene production - Greenhouse exp. – (Chapter 2)	48	0.17	1.0
Plant 1	1	1.56	9.0
Plant 2	1	0.09	0.5
Plant 3	1	1.40	8.0
Plant 1	5	4.87	27.9
Plant 2	5	0.71	4.0
Plant 3	5	2.78	15.9

CHAPTER 3

Short-time responses in ethylene regulation upon rewatering and 1-MCP treatment

ABSTRACT

Coffee flowering usually takes place with the beginning of the rainy season, which break flower bud dormancy and allows buds to restart their growth. Flower bud dormancy permits that anthesis does not occur under unfavorable environment conditions, enabling flowering concentration, since coffee trees shows asynchronies bud development. Some studies suggest that changes in root-to-shoot signaling, during water-deficit conditions and upon its relief, are essential for flower bud competence and growth resumption. The plant hormone ethylene might be involved in this process since its levels have been show to drastically change upon plant rehydration, usually leading to increases in above ground plant organs. These changes in ethylene levels may take place rapidly, usually only a few hours, and for a short-time period. Thus, this study aimed to access the changes in ethylene regulation, through expression analysis of ethylene biosynthesis and signaling genes, at short times after plant rewatering and 1-MCP application. Coffee seedlings rehydration lead to significant increases on the expression of ethylene biosynthesis and signaling genes in leaves and roots, compared to the expression levels from plants under water-deficit conditions, few hours (2 h or 6 h, depending on the gene) after plant rewatering. 1-MCP application promoted a significant increase on the expression of two ethylene biosynthesis genes, *CaACS1-like* and *CaACO1-like*, at two hours after treatment implementation.

Key words: Gene expression. Rehydration. RT-qPCR

RESUMO

O florescimento do café normalmente ocorre com o começo da estação chuvosa, a qual quebra a dormência dos botões florais e permite o reinicio do crescimento. A dormência dos botões florais permite que a antese não ocorra em condições desfavoráveis, possibilitando a concentração do florescimento, tendo em vista que o cafeeiro apresenta um desenvolvimento das gemas de forma desuniforme. Alguns estudos sugerem que mudanças na sinalização entre as raízes e a parte aérea, durante as condições de deficit hídrico e após o seu término, são essenciais para desenvolvimento das gemas e o recomeço do crescimento. O fitormônio etileno pode estar envolvido nesse processo considerando que seus níveis podem mudar drasticamente após a reidratação da planta, geralmente aumentando em órgãos da parte aérea. Estas mudanças nos níveis de etileno ocorrem de maneira rápida, em poucas horas, e por um curto intervalo de tempo. Assim, este estudo objetivou avaliar as mudanças na regulação do etileno, pela análise da expressão de genes de sua biossíntese e sinalização, em curtos intervalos de tempo após a reidratação ou aplicação do 1-MCP. A reidratação de mudas de cafeeiro levou a um aumento significativo na expressão de genes da biossíntese e sinalização do etileno em folhas e raízes, comparados com os níveis de expressão encontrados em plantas sob déficit-hidrico, em poucas horas (2 h ou 6 h, dependendo do gene) após a reidratação. A aplicação de 1-MCP promoveu um aumento significativo na expressão de dois genes da biossíntese de etileno, *CaACS1-like* e *CaACO1-like*, após duas hora da aplicação do tratamento.

Palavras-chave: Expressão gênica. Reidratação. RT-qPCR

3.1 INTRODUCTION

Coffee flowering usually takes place with the beginning of the rainy season, which break flower bud dormancy and allows buds to restart their growth (ALVIN, 1960; BARROS et al., 1999; RENA & MAESTRI, 1985). Flower bud dormancy permits that anthesis does not occur under unfavorable environment conditions, such as water-deficit or low temperatures, which are usually present in the dry season that precedes the first rains of the wet period in most coffee production regions. This feature enables flowering concentration, since coffee trees shows asynchronies bud development (DE OLIVEIRA et al., 2014; MAJEROVICZ & SONDAHL, 2005), and allows that initial fruit expansion occurs under more favorable water conditions.

Little is known about the mechanism through which anthesis is induced, and although water is considered to be important for bud growth resumption once plants are rehydrated (MAGALHAES; ANGELOCCI, 1976), it is not the only factor that contributes for this process. Some studies suggest that changes in root-to-shoot signaling, during water-deficit conditions and upon its relief, are essential for flower bud competence and growth resumption (CRISOSTO et al., 1992).

Among the putative chemicals predicted to be involved in coffee flowering promotion, plant hormones probably play a central role in this process considering that their biosynthesis, signaling, and transport can rapidly change when plants facing water stress conditions are rewatered (GOMEZ-CADENAS et al., 1996; MAHOUACHI et al., 2005). Among these hormones, ethylene levels have been show to drastically change upon plant rehydration, usually leading to increases in above ground plant organs. This enhancement in ethylene levels may be caused by the transport of the ethylene precursor ACC from roots (GOMEZ-CADENAS et al., 1996; TUDELA; PRIMO-MILLO, 1992), or by

organ-specific up-regulation of ethylene production rates (LIU et al., 2013; MENG et al., 2014). These changes in ethylene levels may take place rapidly, usually only a few hours (often less than seven hours), and for a short-time period (often less than six hours).

If coffee flowering is indeed induced by an increase on ethylene levels upon plant rewatering, the controlling of ethylene action through the use of chemicals such as 1-MCP, an ethylene action inhibitor, could help to avoid flowering caused by the early start of rainy season or episodical raining events during the dry season, which can contribute for the occurrence of several blossoming events. However, we have found that exogenous application of 1-MCP leads to coffee anthesis promotion.

Although measurement of ethylene levels from coffee seedlings treated with 1-MCP showed no change in ethylene levels, at 48 h after 1-MCP application, when compared to non-treated plants, changes in these levels may have occurred at earlier times than 48 h after spraying the plants. Some studies show that a rapid and intense increase in ethylene biosynthesis may occur a few hours after 1-MCP application, a response probably associated to the loss of negative feedback regulation (ELLA et al., 2003).

Thus, this study aimed to access the changes in ethylene regulation, through expression analysis of ethylene biosynthesis and signaling genes, at short times after plant rewatering and 1-MCP application.

3.2 MATERIAL AND METHODS

3.2.1 Plant material and culture

Six months old coffee (*Coffea arabica*) plants from the cultivar 'Catuaí Vermelho', kindly provided by the Procafé Foundation (Varginha - Minas Gerais - Brazil) for this experiment, which consisted of five different treatments: well-watered (WW) plants, water-deficit (WD) plants, Rewatered (RWT) plants, 1-MCP treated (1-MCP) plants, and 1-MCP control (BTH) plants (Break-Thru + water treated plants). The experiment was conducted in a semi-controlled greenhouse (Federal University of Lavras - UFLA, Minas Gerais Brazil), with day and night mean temperatures of 24.6 ºC and 22.8 ºC, respectively. The daily maximum temperature in the greenhouse was 34.3 ºC and the minimum temperature at night was 16.8 ºC. Mean relative humidity was 77.3 % and it varied from 91 % to 42 % during the experiment. Plants were grown in one litre plastic bags filled with a mixture of soil, sand, and manure (3:1:1, v/v/v). Plants from the WW, 1-MCP, and BTH treatments were maintained well-watered, receiving regular irrigation, while plants from the WD and RWT treatments watering was suspended until predawn leaf water potential reached -2.0 MPa. This was performed by measuring soil water potential, since plants were only six-months old and there was no enough leaf material for measuring leaf water potential. In addition this practice would damage the plants and since we are working with ethylene, this procedure would affect our data. Thus, before starting the experiment, a model (see Appendix D) relating soil moisture, measured by using a ML2x ThetaProbe, and predawn leaf water potential (spare plants were used for this), measured by a Scholander-type pressure chamber, was performed in order to indirectly infer the soil water potential through the

voltage data obtained with the probe from plants kept in the dark, assuring that pots had around the same water content before starting the experiment.

Physiological analysis and plant material sampling were carried out at 0, 2, 4, 6, 12, 24, and 48h after plant rehydration or 1-MCP application. These times corresponded to the following day times: 08:00, 10:00, 12:00, 14:00, 20:00; 08:00, and 08:00h, respectively. At time 0, physiological analysis and plant material sampling were carried out only in plants from the WW and WD treatments, since rewatering, 1-MCP, and 1-MCP control treatments were imposed at 08:00, being assumed that plants from these treatments had similar physiological characteristics since they had the same age and were grown in the same environmental conditions. Physiological measurements included leaf water potential (Ψ_{leaf}), carbon assimilation rate (A), stomatal conductance (g_s), transpiration (E). Plant material sampling included leaves and roots for WW, WD, and RWT treatments, and only leaves for 1-MCP and BHT treatments. Leaves were immediately immersed in liquid nitrogen and roots were first washed, dried using paper towels, cut, and then frozen in liquid nitrogen. All plant material was stored at freezer -80 °C for the gene expression studies.

Rewatering treatment was carried out by submerging plant root systems in water for ten minutes. After that, plants were put back to bench for water drainage. 1-MCP application was carried out following the same procedure used for the greenhouse experiment from chapter 2. However, in this case, Break-Thru was used as a surfactant and plants were sprayed with 1-MCP outside the greenhouse under ambient conditions.

3.2.1.1 Physiological measurements

Leaf water potential (Ψ_{leaf}) was determined using a Scholander-type pressure chamber. Two to three leaves from each plant, from the three replicates

from each treatment, were evaluated and water potential was given as an average from these measurements.

Instantaneous gas-exchange variables, namely net carbon assimilation rate (*A*), stomatal conductance (g_s), and transpiration rate (*E*), were measured under artificial, saturating photon flux density (1000 µmol m^{-2} s^{-1}), at ambient CO_2 concentration and humidity, using a portable infrared gas analyzer(6400Xt Li-Cor Portable Photosynthesis System). Six measurements from six different plants from each treatment were evaluated and variable values are given as an average from these measurements.

3.2.1.1.1 Statistics

Differences between irrigation treatments and evaluation times (days) were accessed by one-way ANOVA at $P < 0.05$. When ANOVA was significant, means were discriminated using Turkey's multiple comparison test at $P \leq 0.05$. All statistical analysis were performed by the R software (R DEVELOPMENT CORE TEAM, 2013).

3.2.1.2 Gene expression analysis

Expression analysis of putative genes related to the coffee ethylene biosynthesis and signaling pathways performed in leaves and roots at four different sampling times (0, 2, 6, and 24h after treatments were imposed) through Reverse Transcription - quantitative Polymerase Chain Reaction (RT-qPCR). Gene expression levels of one ACC synthase (*CaACS1-like* - accession no. KF975694), two ACC oxidases (*CaACO1-like* and *CaACO4-like* -accession no. KF975695 and AGM48542, respectively), and one ethylene receptor (*CaETR4-like* - accession no. KF975698), were analyzed in leaves and roots

from WW, WD, and RWT plants, and in leaves from the 1-MCP and BTH treatments.

3.2.1.2.1 RNA extraction and cDNA synthesis

RNA extraction was performed according to the Concert Plant RNA Reagent protocol (Invitrogen, USA), with minor alterations. Leaf or root tissue was first ground in a mortar with liquid nitrogen. 600 µL of cold (4 °C) plant RNA reagent (Concert) was added to 100 mg of plant tissue and sample were mixed (vortex) for one minute for homogenisation. Samples were incubated for 10 minutes at room temperature and subsequently centrifuged for seven minutes at 14,800 rpm at 4 °C. Around 400 µL of the supernatant was then transferred for a new centrifuge tube and 100 µL of 5 M NaCl and 300 µL of chloroform were added. After mixing (vortex) for one minute, samples were centrifuged at 4 °C and for ten minutes at 14,800 rpm to separate the organic and aqueous phases. The top aqueous phase (around 350 µL) was transferred to a new centrifuge tube the step of adding NaCl and chloroform followed by centrifugation was repeated. Then the top aqueous phase (around 300 µL) was transferred to a new centrifuge tube and an equal volume of isopropyl alcohol was added. Tube were mixed by inversion (15 times), and for RNA precipitation, samples were put in a -20 °C freezer for one hour. Samples were then centrifuged at 4 °C for 25 minutes at 14,800 rpm and the supernatant was discarded. The pellet was washed by adding 600 µL of 75 % ethanol and after a final centrifugation step (5 minutes, at 4 °C and 14.800 rpm), ethanol was discarded and the pellet was resuspended with 20 µL of RNase-free water. Samples were then stored at -20 °C for further analysis.

After RNA extraction, samples quantity and quality were accessed by spectrocospy (NanoVue GE Healthcare, Munich, Germany), and RNA integrity

was visually analyzed in 1 % agarose gel, stained with Gel Red Nucleic Acid Gel Stain and visualized in an UV-transilluminator (UVITEC FireReader XS D-77Ls-20.M). RNA samples were then treated with DNase I using Turbo DNA-free kit (Ambion) for elimination of residual DNA contamination. 5 µg of RNA (in a volume of 22 µL) were used for DNase treatment in a 25 µL volume reaction, containing 0.1 volume of 10x Turbo DNase Buffer and 0.1 volume of Turbo DNase. After gently mixing the samples, they were incubated at 37 °C for 30 minutes. Then, DNase Inactivation reagent was added (0.1 volume) and samples were well mixed. Samples were incubated for five minutes at room temperature finally centrifuged at 10,000 g for two minutes. 15 µL of the supernatant were transferred to a new tube and samples were stored at -20 °C freezer.

After checking the quantity, quality and integrity of the DNase treated RNA by the same methods described above, cDNA synthesis were carried out following the instructions of the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). 1µg of RNA in a volume of 10 µL was used for cDNA synthesis in 20 µL volume reaction. A mix containing 2 µL of the 10x Reverse Transcriptase buffer, 2µL of 10x RT random primers, 0.8 µL dNTP mix, 1 µL of the MultiScribe TM Reverse transcriptase, and 4.2 of DNase-RNase-free water were added to each reaction. After gently mixing the samples, the RT-PCR was carried out in a thermocycler (Multigene Gradient - Labnet) with the following reaction conditions: ten minutes at 25 °C, followed by 120 minutes at 37 °C, and 5 minutes at 85 °C. Samples were then stored at -20 °C for further analysis.

3.2.1.2.2 RT-qPCR

Quantitative expression analysis of the four genes related to ethylene biosynthesis and signaling pathways were performed on a Rotor Gene-Q

thermocycler (Qiagen), using the SYBR-green detection system and the relative quantification using the $\Delta\Delta C_T$ method, as described by Pfaffl (2001). Reactions were carried out in 15 µL reaction volume: 7.5 µL of SYBR-green (QuantiFast SYBR Green PCR Kit - Qiagen), 0.3 µL (1µM final concentration on the reaction) of forward and reverse gene-specific primers (see Table 3.1 for primer sequences and amplification efficiencies), 1µL of cDNA at 10 ng/µL, and 5.4 of RNase-DNase-free water. Three biological replicates for each treatment and four (0, 2, 6, and 12 h) sampling times were used, reactions were run in triplicates, and amplification was performed with the following reaction conditions: initial enzyme activation with 5 minutes at 95 °C, then 40 cycles of 95 °C for 5 seconds, followed by 10 seconds at 60 °C, and completed by a melting curve analysis to access specificity of the reaction by raising the temperature from 60 to 95 °C, with 1°C increase in temperature every 5 seconds. Relative fold differences were calculated based on the $\Delta\Delta C_T$ method (Pfaffl, 2001), using Ubiquitin and RPL39 as reference genes (Barreto, unpublished results), and were calculated relative to a calibrator sample, which was selected based on the lower expression level, among treatments and tissues analyzed, for each gene.

Table 2.1 Predawn and midday leaf water potential (MPa) for ‘Acaiá Cerrado' plants from the six different treatments of the field trial.

	Treatments					
Time of the day	Control	C1	C2	C3	C4	C5
Predawn	-0.49 ± 0.09 b	-0.31 ± 0.08 a	-0.27 ± 0.09 a	-0.26 ± 0.04 a	-0.32 ± 0.06 a	-0.37 ± 0.08 ab
Midday	-1.27 ± 0.51 a	-1.42 ± 0.27 ab	-1.61 ± 0.09 ab	-2.00 ± 0.10 b	-1.42 ± 0.33 ab	-1.88 ± 0.28 b

Different letters represent statistical significance between means within each time of the day. Each value represents the mean ± 95% confidence interval of the mean (n=5).

3.3 RESULTS

3.3.1 Physiological analysis

Leaf water potential Ψ_{leaf} from WW, 1-MCP, and BTH treatments were maintained above -0.7 MPa throughout experiment and plants exposed to water-deficit, water potential was always above -2.0 MPa, reaching values around -3.5 MPa and -4.0 MPa by end of the experiment (Figure 3.1). Rewatering promoted significant changes in Ψ_{leaf} by increasing it by 43 %, compared to Ψ_{leaf} from plants of the WD treatment at time 0, within 2 hours, and further increasing it by 53 % two hours later, reaching -0.75 MPa at noon (Figure 3.1). Ψ_{leaf} from rewatered plants was maintained in the same level for the next two hours and by 12 hours after plant rehydration, Ψ_{leaf} was similar to plants under well-watered conditions. 1-MCP and BTH treatments did not promote any change in Ψ_{leaf} during the experiment (Figure 3.1).

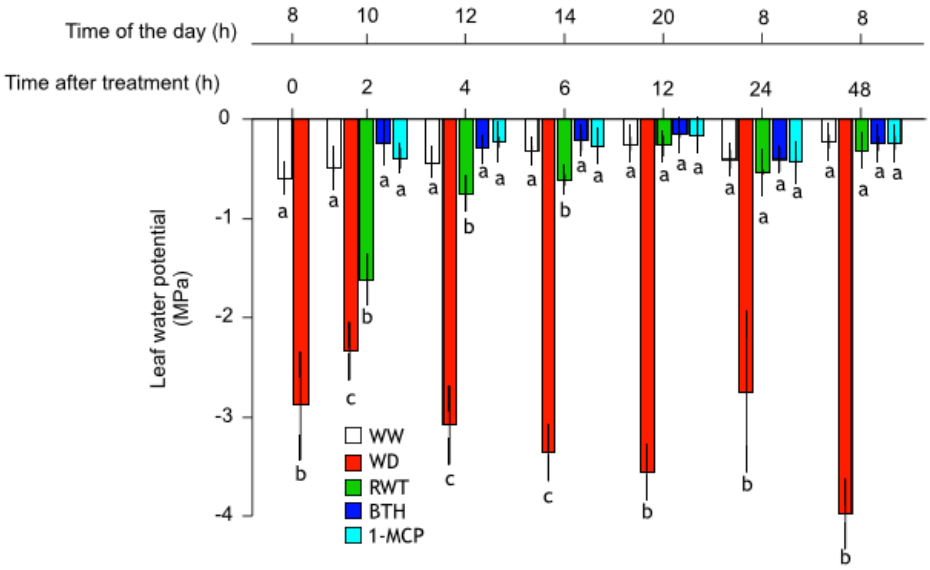


Figure 3.1 Leaf water potential from coffee plants under well-watered (WW) and water-deficit (WD) conditions, submitted to rewatering (RWT), treated with Break-Thru (BTH), or treated with 1-MCP (1-MCP). The upper row of the x axis refers to the time of the day that measurements were performed and the lower row refers to the timed passed after treatment implementation (rewatering, Break-Thru, and 1-MCP treatments). Data are means ± 95 % confidence interval of the mean (n=6). Different letters represent statistical significance between means within each measuring time.

Withholding irrigation significantly decreased g_s when compared to well-watered plants, with mean g_s from WD treatment being lower than 25 mmol m^{-2} s^{-1} throughout the experiment (Figure 3.2). Mean g_s from well-watered plants fluctuated between 70 mol m^{-2} s^{-1} and 120 mol m^{-2} s^{-1}during the day, reaching its lowest value at 14:00 h. After two hours of plant rehydration g_s levels from rewatered plants were not different from the g_s level observed in water-deficit plants, but it was around three times higher than g_s levels from plants exposed to water-deficit at times 4 and 6. After 24 h, g_s levels from rewatered were similar to the level from well-watered plants, and it slightly increased after 48 h from rewatering. Interestingly, 1-MCP and BTH treatments promoted a significant drop in g_s, and although plants were under well-watered

conditions, g_s from these plants reached values similar to the ones observed in plants under water-deficit conditions at time 2. After 24 h, g_s levels from plants treated with BTH recovered, displaying g_s levels similar to well-watered plants, and for 1-MCP treated plants this could be observed only 48 h after spraying the plants (Figure 3.2).

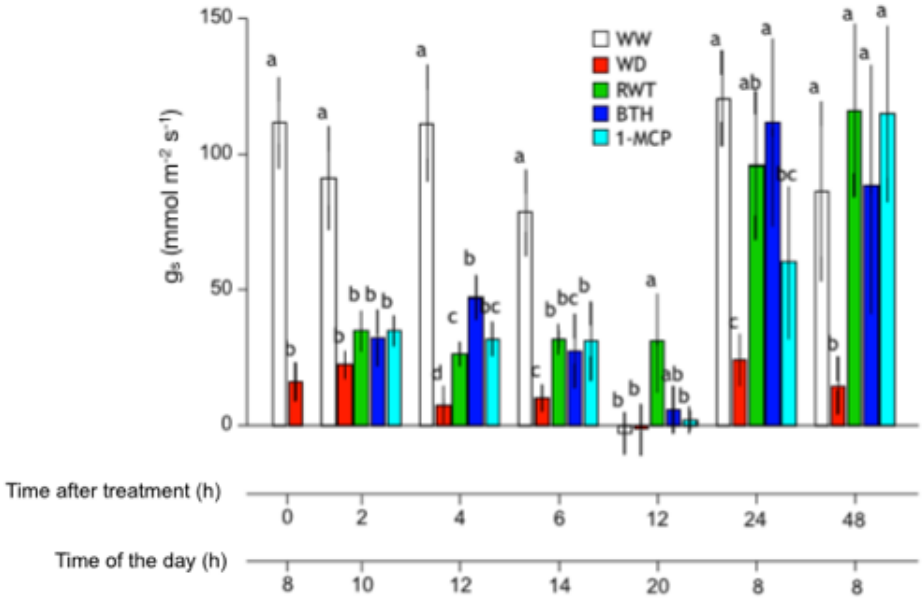


Figure 3.2 Stomatal conductance from coffee plants under well-watered (WW) and water-deficit (WD) conditions, submitted to rewatering (RWT), treated with Break-Thru (BTH), or treated with 1-MCP (1-MCP). The lower row of the x axis refers to the time of the day that measurements were performed and the upper row refers to the timed passed after treatment implementation (rewatering, Break-Thru, and 1-MCP treatments). Data are means ± 95 % confidence interval of the mean (n=6). Different letters represent statistical significance between means within each measuring time.

The analysis of *A* and *E* (Figures 3 and 4) permits the observation of a significant coupling between these parameters and g_s (Figure 3.2). *A* and *E* from plants under water-deficit were below 2 µmol m^{-2} s^{-1} and 0.7 mmol m^{-2} s^{-1}

throughout the experiment, respectively, and *A* completely ceased at noon and 14:00 h. Rewatering permitted that plants sustained a slightly higher carbon assimilation rate than water-deficit plants, even thought their g_s levels were similar at time 2, and at times 4 and 6, A was substantially higher than plants under water-deficit and transpiration was around three times higher compared to them. Similar to g_s, *A* and *E* also decreased upon 1-MCP and Break-Thru application, returning to control (WW) levels after 24 h and 48 h, respectively (Figures 3.3 and 3.4).

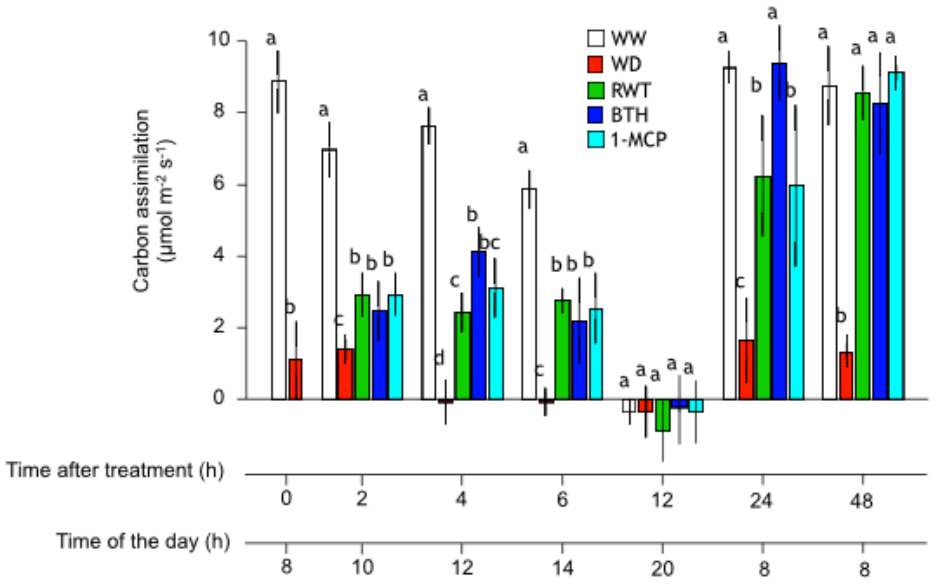


Figure 3.3 Net carbon assimilation rate from coffee plants under well-watered (WW) and water-deficit (WD) conditions, submitted to rewatering (RWT), treated with Break-Thru (BTH), or treated with 1-MCP (1-MCP). The lower row of the x axis refers to the time of the day that measurements were performed and the upper row refers to the timed passed after treatment implementation (rewatering, Break-Thru, and 1-MCP treatments. Data are means ± 95 % confidence interval of the mean (n=6). Different letters represent statistical significance between means within each measuring time.

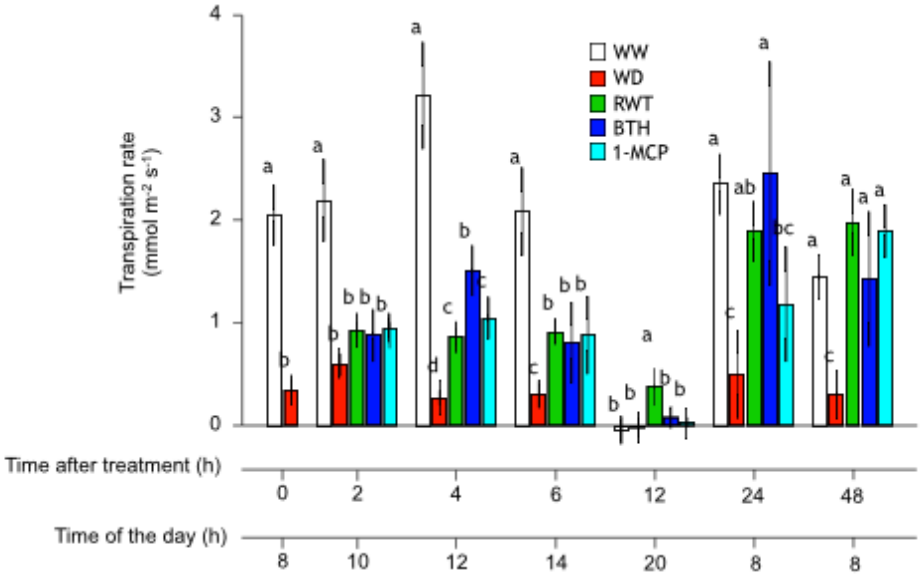


Figure 3.4 Transpiration rate from coffee plants under well-watered (WW) and water-deficit (WD) conditions, submitted to rewatering (RWT), treated with Break-Thru (BTH), or treated with 1-MCP (1-MCP). The lower row of the x axis refers to the time of the day that measurements were performed and the upper row refers to the timed passed after treatment implementation (rewatering, Break-Thru, and 1-MCP treatments. Data are means ± 95 % confidence interval of the mean (n=6). Different letters represent statistical significance between means within each measuring time.

3.3.2 Gene expression analysis

The analysis of the gene expression pattern of the coffee ACC synthase homolog gene *CaACS1-like* showed that this gene is in general much more expressed in coffee roots than in leaves (Figure 3.5). In coffee leaves, *CaACS1-like* displayed similar expression levels under well-watered and water-deficit conditions, being differentially expressed only at time 0. Rewatering and Break-Thru treatments did not promote significant changes in *CaACS1-like* expression, but a reduction on expression in Break-Thru treated plants could be observed 24 h after treatment implementation. 1-MCP application promoted an up-regulation

of *CaACS1-like* at 2 hours after spraying the plants, showing a seven fold increase in expression levels when compared to well-watered *CaACS1-like* expression levels. In root tissue, relatively similar *CaACS1-like* expression levels were found among the different treatments, except for times 0 and 2, where its expression was 6 and 2 times higher in well-watered plants when compared to *CaACS1-like* expression in plants under water-deficit.

The putative coffee ACC oxidase *CaACO4-like* was clearly more expressed in coffee leaves than in roots in most measuring times and treatments (Figure 3.5). In leaves, water-deficit seems to promote a reduction on *CaACO4-like* expression, as observed in times 0 and 6, where it was four times less expressed than its expression levels under in well-watered conditions. Rewatering promoted a five fold increase in *CaACO4-like* expression at time 6, compared to its expression level in leaves from plants under water-deficit, and 24 h after plant rehydration it was two and three times more expressed than in well-watered and water-deficit plants, respectively. 1-MCP and Break-Thru treatments did not promoted significant changes *CaACO4-like* expression, except at time 2 where 1-MCP application slightly increased *CaACO4-like* expression when compared to well-watered plants. In roots, *CaACO4-like* expression in the WW, WD, and RWT treatments was similar in all measuring times, except at time 6, where it was three times more expressed in rewatered plants, compared to water-deficit and well-watered plants.

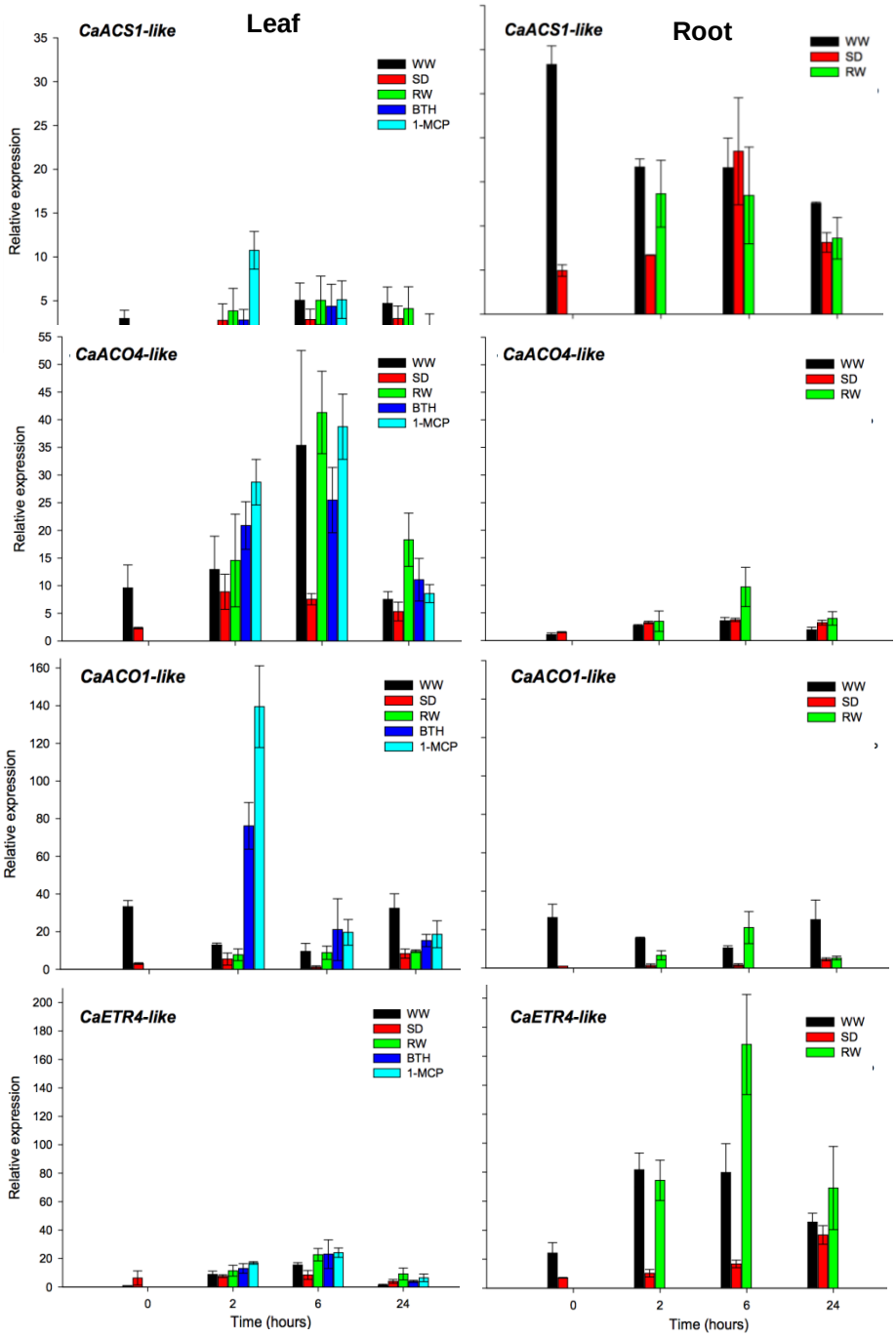

Leaf
Root
CaACS1-like
CaACO4-like
CaACO1-like
CaETR4-like
WW
SD
RW
BTH
1-MCP
Relative expression
Time (hours)
0
2
6
24

Figure 3.5 Relative gene expression profiling of *CaACS1-like*, *CaACO4-like*, *CaACO1-like*, and *CaETR4-like*, in coffee leaves (left panel) and roots (right panel) sampled at times 0, 2, 6, and 24 (times refer to the time passed after treatment implementation: rewatering, Break-Thru, and 1-MCP treatments). *Columns* represent the fold difference in gene expression for well-watered (WW), water-deficit (WD), rewatered (RW), Break-Thru (BTH) treated, and 1-MCP (1-MCP) treated plants, relative to calibrator samples for each gene (*CaACS1-like*: T0 SD leaf; *CaACO4-like*: T0 WW root; *CaACO1-like*: T0 SD root; *CaETR4-like*: T0 WW leaf). Expression values for each sample were obtained from three biological replicates and *error bars represent* standard error for them. Gene transcripts were normalized by two reference genes (*Ubiquitin* and *RPL39*).

The second coffee putative ACC oxidase gene, *CaACO1-like* showed similar expression levels in leaves and roots, and it was down-regulated under water-deficit conditions in both tissues (Figure 3.5). Rewatering promoted a seven fold increase in *CaACO1-like* expression six hour after re-wetting the soil, compared to water-deficit plants, but one day after plant rehydration its expression in rewatered plants was similar to plants from the WD treatment. 1-MCP and Break-Thru treatments promoted a relatively strong up-regulation of *CaACO1-like* two hours after spraying the plants. *CaACO1-like* was approximately 11 and six times more expressed in 1-MCP and Break-Thru treated plants, when compared to well-watered plants in the same measuring time. At times 6 and 24, *CaACO1-like* expression was slightly higher and lower in 1-MCP and Break-Thru treated plants, when compared to its expression in well-watered plants, respectively. In roots, *CaACO1-like* was 26, 11, seven, and five times, more expressed in well-watered plants than in water-deficit plants at 0, 2, 6, and 24 measuring times, respectively. Rewatering caused a transient up-regulation of CaACO1-like, which was five and 13 times more expressed in roots from rewatered than roots from water-deficit at two and six hours after plant rehydration.

The expression pattern of the putative coffee ethylene receptor *CaETR4-like* shows that this gene is usually much more expressed in roots than in leaves

(Figure 3.5). In leaves, *CaETR4-like* was six times more expressed in plants under water-deficit than in well-watered plant at time 0, and it was slightly more expressed at time 24 as well. At times 2 and 6, *CaETR4-like* expression was not significantly different between WW and WD treatments, and rewatering lead to a 2.6 fold increase in its expression at time 6, compared to water-deficit plants, and this positive regulation was still present 24 h after plant rehydration, with *CaETR4-like* being six times more expressed in rewatered plants than in well-watered plants. In roots, *CaETR4-like* was significantly more expressed under well-watered conditions than under water-deficit at three of the four measuring times, being three, 8, and 4.7 times more expressed in well-watered plants than in WD plants at times 0, 2, and 6, respectively. Rewatering promoted an up-regulation of *CaERT4-like* and two hours after re-wetting it showed similar expression levels to well-watered plants and a 10 fold increase in expression, when compared to plants under water-deficit. This fold difference in expression level between rewatered and water-deficit plants was maintained during the two following hours, and at time 24 *CaETR4-like* was expressed in a similar extension among the three treatments.

3.4 DISCUSSION

3.4.1 Physiological analysis

Withholding water significantly decreased in Ψ_{leaf} from coffee plants and rewatering promoted a substantial recovery of it, since after only four hours from plant rehydration, it increased from -2.9 MPa to -0.75 MPa, presenting levels slightly lower than well-watered plants at time six, and similar levels after 12 h from rewetting the soil. This rapid recovery of leaf water potential was probably associated to the small pot size used in this experiment. In addition, it has been shown that even at low (-6.5 MPa) Ψ_{leaf}, a substantial hydraulic conductivity, ranging from 20 to 40 %, is present in different coffee cultivars (TAUSEND et al., 2000). Coffee plants may show a rapid recovery in the total hydraulic conductance of the soil-leaf pathway (G_t) once plants are rewatered suggesting that loss of xylem hydraulic conductivity may be largely reversible in coffee, lending support to the rapid recovery of Ψ_{leaf} upon rewatering found in this experiment.

Similar to the greenhouse experiment from Chapter 2, a significant coupling between g_s and E could be observed, and this also applies for A in this experiment. A significant decrease in these three parameters could be observed in plants under water-deficit, and treated with Break-Thru and 1-MCP as well. At atmospheric CO_2 concentrations and saturating light, coffee displays low A, typically in the range of 4–11 µmol $CO_2 m^{-2} s^{-1}$ (FRANCK et al., 2006; SILVA et al., 2004) which is in the lower range recorded for trees (CELEUMANS; SAUGIER, 1993). However, the maximum A values obtained in common A/Ci curves can surpass 20 µmol $CO_2 m^{-2} s^{-1}$ (ARAUJO et al., 2008), whereas the photosynthetic capacity, determined under true CO_2 saturation (50 mmol CO_2 mol^{-1} air), reaches values exceeding 30 µmol $O_2 m^{-2} s^{-1}$ (SILVA et al., 2004),

suggesting that the lower *A* levels observed in water-deficit plants in this experiment, and in coffee species as a whole, might largely result from diffusive constraints to photosynthesis. Plant rewatering recovered the g_s levels in 24 h, and after 48 h *E* and *A* showed similar levels than those observed for well-watered plants, suggesting that the water-deficit treatment did not cause any long-term effect on the photosynthetic apparatus of the plants.

Interestingly, g_s levels, along with A and E, from plants of the BTH and 1-MCP treatments showed a sharp decrease upon treatment implementation, reaching levels close to those observed for plants under water-deficit conditions after two hours from spraying the plants, even though plants from these treatments were under well-watered conditions. Decreased levels were maintained during the application day and returned to control levels after 24 and 48 h from application of Break-Thru and 1-MCP, respectively. This result suggests a possible effect of the surfactant on these parameters, which is enhanced with the addition of 1-MCP.

Surfactants are substances added to spray formulations that promote the enhancement of deposition, retention, spread, penetration and uptake of the spray droplets. These substances act as humectants and prevent spray droplets from drying too quickly, thereby enhancing the uptake of pesticides in spray droplets through the stomata and cuticular membrane (RAMSEY et al., 2005). Organosilicone surfactants, such as Break-Thru and Silwet L-77, is an unique class of surfactant capable of reducing the surface tension of aqueous solutions (approx. 73-76 mN/m) to 22 mN/m, below that of ethanol or acetone, at very low concentrations, usually around 0.025 to 0.1% (XU et al., 2011). Although most studies involving the use of surfactants do not analyze changes in gas-exchanges parameters, reductions in net carbon assimilation rates, from 4 to 24 h, have been show to occur after application of organosilicone surfactant (ORBOVIC et al., 2001). Although changes in stomatal conductance may take

place upon organosilicone surfactant application, as we have found in this work, radiolabelled samples suggest that stomatal infiltration in presence of organosilicone surfactants is a mass flow phenomenon and occurs rapidly (STOCK; HOLLOWAY, 1993). For instance, the uptake of 85 % of the glyphosate applied to beans leaves in presence of Silwet L-77 at 0.5 % occurs during the first 10 minutes from application (LIU, 2004).

Based on the responses in gas-exchange parameters observed upon Break-Thru and 1-MCP application, and making a connection with field trial from chapter two of this thesis, stomatal closure may have contributed to anthesis induction by increasing Ψ_{leaf} during the day. Although plants from the field trial were not under water stress conditions, low Ψ_{leaf} levels, ranging from 1.27 MPa to -2.00 MPa, could be observed at noon. Thus, stomatal closure during the day, in response to Break-Thru and Break-Thru plus 1-MCP treatments, could have lead to a transient increase in the water status of the shoot, since it has been observed that stomatal closure can lead to increases in Ψ_{leaf} (DODD et al., 2009; KUDOYAROVA et al. 2007), enabling a higher transport of water to flower buds and contributing to anthesis. However, it must be noted that anthesis was not observed on the control treatment, where it was applied a solution containing only water and Break-Thru, nor in the lower 1-MCP concentration treatments, C1, C2, and C3, suggesting a specific role of 1-MCP, at higher concentrations, in anthesis promotion. This effect may have been a sustained effect on stomatal closure triggered by 1-MCP, since g_s levels similar to well-watered plants were observed only 48 h after 1-MCP application (Figure 3.2), or a transient and rapid induction in ethylene production, which has been shown to be important for flower rehydration-response (MENG et al., 2014), caused by 1-MCP, or both.

The sustained effect in stomatal closure in the presence of 1-MCP, when compared to plants from the BTH treatment, may be related to effects of

ethylene in stomatal closure. Ethylene has been shown to close stomata (DESIKAN et al., 2006), and also in stomatal opening (MADHAVAN et al., 1983). Ethylene has also been shown to antagonise drought-and ABA-induced stomatal closure (TANAKA et al., 2005; WILKINSON; DAVIES, 2009). In this case, stomatal closure in response to ABA has been shown to be reduced under elevated levels of ethylene (TANAKA et al., 2005), and 1-MCP application can restore the sensitivity of stomata to drying soil in elevated ozone conditions (WILKINSON; DAVIES, 2009). Although a transient increase in ethylene production may have occurred upon 1-MCP application, ethylene insensitivity may have promoted an increase in ABA sensitivity, leading to the closure of coffee stomata for a longer period of time, which may have contributed for a deeper or sustained increase in shoot water status.

3.4.1 Gene expression analysis

Expression analysis of genes related to the ethylene biosynthesis and signaling pathways, in coffee leaves and roots from plants under different watering conditions, permitted the observation that coffee roots seems to produce higher amount of the ethylene precursor ACC, based on *CaACS1-like* expression levels, leaves may produce more ethylene than roots, considering the greater expression levels from one of the ACC oxidases analyzed, *CaACO4-like*, when compared to roots, and roots may be less sensitive to ethylene, taking into account the higher expression levels of the ethylene receptor *CaETR4-like* in this tissue compared to leaves (Figure 3.5).

Comparison of the expression levels of the four genes analyzed in this study in well-watered and water-deficit conditions, suggest that ethylene levels, in roots and leaves, may be lower under water-deficit conditions. *CaACS1-like* expression patterns under well-watered, water-deficit, and upon rewatering were

similar and relatively higher expression levels of this gene was found in roots when compared to leaves (Figure 3.5). This may be associated to the elevated expression levels of *CaETR4-like* in roots, compared to its expression in leaves. Ethylene receptors act as negative regulators, and if they are not inactivated upon ethylene biding, ethylene responses may be inhibited. Although it could be thought that ethylene responses would be repressed or activated in roots from plants well-watered or under water-deficit, respectively, these inferences must be made with caution since transcript levels may not represent the protein levels (KEVANY et al., 2007). Thus ethylene responses have been regarded to be finely tuned between ethylene and ethylene receptors levels (KEVANY et al., 2007).

Although relatively similar expression levels of the coffee ACC synthase *CaACS1-like* were found for well-watered and water-deficit plants in both leaves and roots, expression patterns from the two ACC oxidases analyzed indicate that higher ethylene production rates may be present in well-watered conditions. Expression patterns of *CaACO1-like* showed that this gene is down-regulated under water-deficit conditions in both leaves and roots. In addition, although *CaACO4-like* showed similar expression levels in both watering conditions in roots, it was higher expressed in leaves from plants under well-watered conditions in some of the evaluated times, when compared to plants under water-deficit. Other studies have shown that the expression of genes related to ethylene biosynthesis pathway may be down-regulated under drought stress (LARRAINZAR et al., 2014), and ethylene levels may also be reduced under water limited conditions (MORGAN et al., 1990).

Reduced levels of ethylene in coffee plants may display an important role in coffee flower bud competence acquisition. It has been shown that xylem connection of flower buds to the inflorescence and the branch at early developmental stages is poorly established, and only buds at the G4 (3 to 6 mm

in length) have a well-defined vascular cylinder, containing secondary xylem (CRISOSTO et al., 1992). However, in this study, it is not informed if the G4 buds used for the histological analysis were obtained from plants that had been passed through a period of water-deficit conditions. Apparently, plants had not been exposed to drought stress, since flower buds were harvest in May 1989 and water suspension treatment was imposed in June. This information is important since elevated levels of ethylene have been shown to inhibit xylem differentiation (ZOBEL; ROBERTS, 1978). Elevated levels of ethylene production from roots of well-watered plants have been suggested to inhibit xylem development, as accessed by the occurrence blossom end rot (BER) in tomato (DAVIES et al., 2000). BER is a good indicator of limitations in calcium transport and therefore provides an indirect method to for assessment of xylem connection (DAVIES et al., 2000). Plants that experienced an episode of soil drying, which may have lead to a reduction in ethylene levels, showed some protection against BER, indicating the presence of a more established xylem connection (DAVIES et al., 2000). Auxin is also essential for xylem differentiation (BISHOPP et al., 2011; FABREGAS et al 2015; FUKUDA et al., 1980), and inhibition of xylem differentiation is probably associated to changes in auxin levels and the polar auxin transport, which are both regulated by ethylene (SWARUP et al., 2007; RUZICKA et al., 2007). Although ethylene has been shown to increase auxin biosynthesis, it has also been shown to regulate auxin transport through the positive regulation of auxin efflux carriers, resulting in elevated auxin transport, which prevents localised accumulation of auxin (LEWIS et al., 2011). Thus, increased levels of ethylene in coffee plants under well-watered conditions would prevent the accumulation of auxin in flower buds, and thus xylem differentiation would be arrested in these circumstances, corroborating for the understanding of the requirement, or positive effect, of water-deficit conditions in coffee flower bud competence acquisition. The

reduction in ethylene levels under water-deficit conditions may be ABA regulated, since leaf ethylene production rates shows no increase under water-deficit conditions in wild-type maize plants, but increased levels of ethylene could be observed in ABA-deficient transgenic lines (VOISIN et al., 2006). Other studies in the literature supports the control of ethylene levels by ABA (HUSSAIN et al., 2000; SHARP, 2002; SHARP et al., 2004). In addition, ABA has also been shown to induce the expression of ethylene receptors genes, possibly reducing the ethylene sensitivity (MULLER et al., 2000), considering that ethylene receptors act as negative regulators.

Although rewatering did not promote significant changes in *CaACS1-like* expression levels in leaves and roots, it lead to an increase in expression levels of both ACOs analyzed in this study, and also in the ethylene receptor *CaETR4-like*, with their expression levels reaching similar levels to those observed in well-watered plants few hours later upon rewatering. This finding, associated to the ACC production levels and its conversion to ethylene under water-deficit conditions in roots, indicate that an increase in ethylene levels in the shoot may occur upon plant rewatering, and this increase could be associated to anthesis promotion under natural conditions. This is supported by the fact that ACC biosynthesis levels occur at similar extents under well-watered and water-deficit conditions (based on the expression levels of *CaACS1-like*). However, its conversion to ethylene under water-deficit conditions may occur in a lower rate than in well-watered conditions, due to the reduction on the expression levels of *CaACO1-like*, suggesting that an accumulation of ACC in the roots may possibly occur. When plants are rewatered, ACC from the roots may be transported to the shoot and this, associated with the increase in expression levels of ethylene biosynthesis genes in the leaves observed upon rewatering in this work, may lead to increased ethylene levels in coffee plants after rain or irrigation, inducing coffee anthesis.

The expression patterns of two ethylene biosynthesis genes in response to 1-MCP application also corroborates for the fact that increased levels of ethylene may be responsible for coffee anthesis promotion. *CaACS1-like* and CaACO1-like were significantly up-regulated right after (two hours) 1-MCP application (Figure 3.5). Break-Thru treatment has also lead to a significant up-regulation of *CaACO1-like*, and this may be probably associated to the stress faced by the plants, confirmed by the changes in gas-exchanged parameters, upon treatment implementation, but no changes in *CaACS1-like* expression were observed. The up-regulation of *CaACS1-like* and *CaACO1-like* upon 1-MCP treatment may have greatly enhanced the ethylene levels in the seedlings used in this experiment and, considering that a similar response may have occurred in the field trial detailed in Chapter 2 of this thesis, where 1-MCP exogenous application induced coffee anthesis, this finding lends supports for the fact that anthesis promotion in the field trial was probably triggered by a transient increase in ethylene levels.

Thus, the results obtained in this study support to the first hypothesis assumed in this thesis where a drought-rewatering-induced ethylene burst would be the basis of the drought-rewatering-induced flowering in coffee trees.

3.5 CONCLUSION

Coffee seedlings rehydration lead to significant increases on the expression of ethylene biosynthesis and signaling genes in leaves and roots, compared to the expression levels of these genes in plants under water-deficit conditions, few hours (2 h or 6 h, depending on the gene) after plant rewatering.

Down-regulation of *CaACO1-like* in roots under water-deficit conditions may contribute to a possible accumulation of ACC in roots.

1-MCP application promoted a significant increase on the expression of two ethylene biosynthesis genes, *CaACS1-like* and *CaACO1-like*, at two hours after treatment implementation.

3.6 CONCLUDING REMARKS

The results from this study suggest that possible elevated ethylene levels in the shoot, in response to plant rehydration, may be responsible for triggering coffee anthesis upon rain or irrigation. This is supported by the expression analysis of the coffee ethylene biosynthesis and signaling genes, in leaves and roots, upon rewatering and 1-MCP treatment.

Rewatering have shown to rapidly induce the expression of ethylene biosynthesis and signaling genes, reaching gene expression levels similar to those found in well-watered plants in few hours (less than six hours) at both leaves and roots. This finding, associated to a possible accumulation of ACC in the roots, suggested by the inhibition of one the ACC oxidases (*CaACO1-like*) under water-deficit conditions, which may be transported to the shoot upon plant rehydration, indicate that increased levels of ethylene may be present after rain or irrigation and promote coffee anthesis.

The up-regulation in the expression of two ethylene biosynthesis genes, *CaACS1-like* and *CaACO1-like*, right after 1-MCP application, lends further support for this putative effect of ethylene in anthesis promotion, since exogenous 1-MCP application under field conditions triggered coffee anthesis at certain concentrations. Thus, coffee anthesis promotion upon 1-MCP treatment possibly occurred through elevated ethylene levels caused by the positive regulation of ethylene biosynthesis genes.

Concluding, as first hypothesised in this study, a drought-rewatering-induced ethylene burst might be the basis of the drought-rewatering-induced flowering in coffee trees.

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Appendix D

1 Model psiLeaf x soil electrical conductance

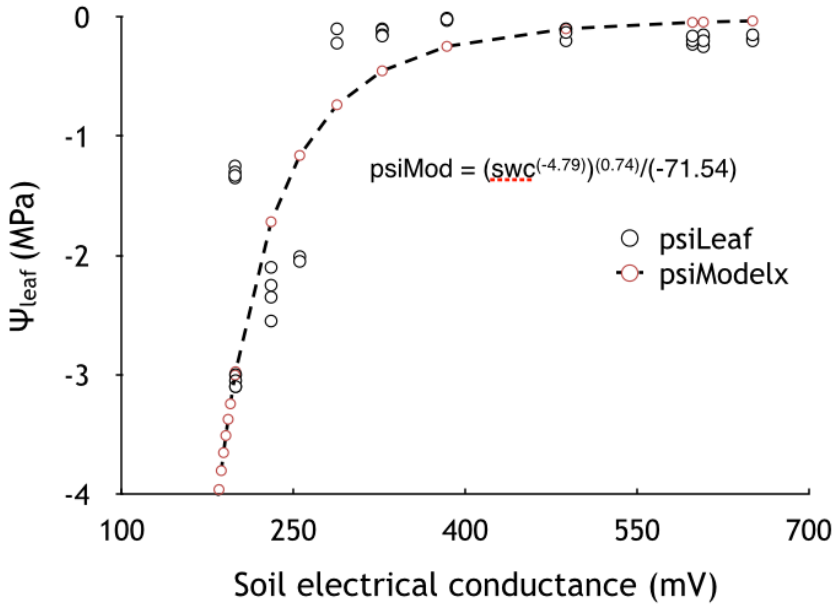

Figure 3.6 Model relating predawn leaf water potential and soil electrical conductance from coffee plants under different water-deficit levels.