Drought and re-watering modify ethylene production and sensitivity, and are associated with coffee anthesis

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May 18, 2020

Abstract

Coffee flowering requires a period of water deficit followed by rainfall to break flower bud dormancy and promote anthesis. Since drought followed by re-watering can increase shoot ethylene production, we investigated changes in root, leaf and flower bud ethylene production and expression of genes within the ethylene biosynthesis and signalling pathways and their relationship to coffee flowering. Drought decreased foliar and flower bud ethylene production without changing root ethylene production, even though all tissues likely accumulated the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid), since ACS gene expression was maintained while ACO gene expression decreased. The ethylene receptor CaETR4-like was not differentially expressed in leaves under water deficit, but it was downregulated in roots. Re-watering restored shoot ethylene production, which seems important in promoting anthesis. 1-MCP, an ethylene action inhibitor, triggered coffee anthesis without re-watering the plants, which hitherto was considered essential to allow flowering. 1-MCP positively regulated ethylene biosynthesis genes (CaACS1-like and CaACO1-like), similar to re-watering, and downregulated CaETR4-like, suggesting that changes in ethylene levels and sensitivity are required to promote coffee anthesis. Thus, drought and re-watering-induced changes in ethylene levels and sensitivity allow coffee flowering, while the growth regulator 1-MCP can potentially regulate anthesis time and intensity.

Acknowledgments:

We thank the "Instituto Nacional de Ciencia e Tecnologia do Cafe (INCT-Cafe)", the "Coordenacao de Aperfeicoamento de Pessoal de nivel Superior (CAPES)", and the "Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq)" for the financial support, the Procafé Foundation for providing the coffee seedling for the Greenhouse experiment, and the company AgroFresh, specially Felipe Terra, for kindly providing us the Harvista used in the experiments of this study.

Main Document

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flower bud ethylene production without changing root ethylene production, even though all tissues likely accumulated the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid), since ACS gene expression was maintained while ACO gene expression decreased. The ethylene receptor *CaETR4-like* was not differentially expressed in leaves under water deficit, but it was downregulated in roots. Re-watering restored shoot ethylene production, which seems important in promoting anthesis. 1-MCP, an ethylene action inhibitor, triggered coffee anthesis without re-watering the plants, which hitherto was considered essential to allow flowering. 1-MCP positively regulated ethylene biosynthesis genes (*CaACS1-like* and *CaACO1-like*), similar to re-watering, and downregulated *CaETR4-like* , suggesting that changes in ethylene levels and sensitivity are required to promote coffee anthesis. Thus, drought and re-watering-induced changes in ethylene levels and sensitivity allow coffee flowering, while the growth regulator 1-MCP can potentially regulate anthesis time and intensity.

Keywords: Flowering, ACC (1-aminocyclopropane-1-carboxylic acid), RT-qPCR, 1-Methylcyclopropene (1-MCP), Break-Thru.

1 Introduction

Flowering is one the most important developmental process of the plant life cycle, required for the reproductive success of a species and directly associated with the yield and quality of several agricultural commodities. Even though coffee is classified as the second most valuable traded commodity worldwide, little is known about the factors that control its flowering. A period of water deficit followed by rainfall is considered essential to trigger coffee anthesis (Alvim 1960), and other endogenous and environmental factors, such as gibberellins (Schuch, Fuchigami & Nagao 1990a, 1992), temperature and photoperiod (Schuch, Fuchigami & Nagzao 1990b; Drinnan & Menzel 1995; Javier *et al.* 2011), can also affect floral transition and development. However, the physiological and molecular changes involved in flower buds acquiring the competence to flower and during anthesis promotion are still mostly unknown.

Coffee flowering is known as an asynchronous process, which can greatly affect the final product (or coffee cup) quality due to the presence of fruits at different ripening stages at harvest time. In countries where coffee plantations are situated in regions with a well-defined long dry season, such as the main production areas of Brazil, the world's largest coffee producer, coffee anthesis occurs when rainfall returns, with coffee trees usually showing two to four anthesis events (Barros, Maestri & Coons 1978; Rena & Barros 2004). In contrast, in places without a pronounced or extended dry period, as in equatorial regions comprising the important coffee producing countries of Colombia, Vietnam, Indonesia and Ethiopia, anthesis can occur throughout the year. In these equatorial countries, although the main flowering events take place from January to April, anthesis may be triggered every time that a dry period is followed by rainfall. This pattern of flowering also leads to fruits at different ripening stages at harvest time, which directly affects coffee quality since green and over-ripened fruits change the acidity and bitterness of the beverage. Consequently, additional harvest events and/or the need to select fruits of uniform ripeness during harvest or post-harvesting processes increase the costs of coffee production (Rena & Maestri 1985; DaMatta, Ronchi, Maestri & Barros 2007). Thus, a better understanding of the control of the flowering process can therefore enhance the quality of the end product.

Coffee's asynchronous flowering pattern results from asynchronies in bud development along the branches at the vegetative and reproductive levels (Majerowicz & Söndahl 2005; de Oliveira, Cesarino, Mazzafera & Dornelas 2014) . In addition, environmental factors are also important, since a period of water deficit may stimulate anthesis, and low intensity rainfall events during the dry season, which often occurs before the wet season starts, can contribute to multiple flowering events (Guerra *et al.* 2005; da Silva, Brunini, Sakai, Arruda & Pires 2009). Once flower buds complete their differentiation, growth ceases, and buds may enter a dormant or latent state. Under tropical Brazilian conditions, coffee flower bud dormancy coincides with the beginning of the dry season in the main coffee producing regions. Moderate water deficit enhances the competence of flower buds to progress to anthesis, which is triggered by rain or irrigation after this period of water restriction (Alvim 1960; Magalhaes & Angelocci 1976; Barros *et al.* 1978; Crisosto, Grantz

& Meinzer 1992). Soil water deficit may stimulate root ACC accumulation (the ethylene precursor) while re-watering stimulates ACC transport to the shoot to induce an ethylene burst (Gómez-Cadenas, Tadeo, Talon & Primo-Millo 1996). Moreover, since phenological changes such as flower bud competence and regrowth may be regulated by the dynamics of a root-sourced signal such as ACC (Crisosto *et al.* 1992), ethylene is a good candidate to regulate coffee flowering.

Ethylene is involved in regulating several developmental processes, such as organ abscission, seed germination, growth transition from vegetative to reproductive phases, flowering, fruit ripening, senescence, and is also involved in biotic and abiotic stress responses (Abeles, Morgan & Saltveit 1992). Depending on the species, ethylene can inhibit (Arabidopsis - Achard et al., 2007; Chen et al., 2013) or promote (pineapple - Trusov and Botella, 2006; Wang et al., 2007) flowering. It can regulate pollen and ovule development (De Martinis & Mariani 1999; Holden, Marty & Singh-Cundy 2003), flower opening (Reid, Evans, Dodge & Mor 1989; Çelikel & Van Doorn 2012), and flower senescence (Shahri & Tahir 2014). In addition, a rapid and transient elevation in ethylene production, upon re-watering after a period of water stress, promoted rose (*Rosa hybrida*) flowers to open, by influencing the expression of a set of rehydration-responsive genes (Meng *et al.*2014). However, it is not known whether similar regulation of flowering occurs in coffee.

Once produced, the ethylene gas can easily diffuse between intercellular spaces and adjacent tissues, and both local ethylene concentrations and cellular sensitivity to ethylene are important in mediating cellular response (Alonso & Ecker 2001). Ethylene exerts its action via the ethylene signalling pathway, where it is perceived by a family of receptors and the signal is mediated downstream by members of different gene families (Chang 2016). Among these components, ethylene receptors are key regulators of ethylene sensitivity, acting as negative regulators (Hua & Meyerowitz 1998), meaning that increases in their levels decrease ethylene sensitivity. Drought conditions can positively or negatively regulate the expression of different ethylene receptors (Arraes et al., 2015; Hopper et al., 2016; Ren et al., 2017), but there is limited information on their regulation in coffee species. Modifications in ethylene sensitivity may contribute to coffee floral buds acquiring the competence to flower in response to soil water deficit and re-watering.

Thus, we proposed that drought and re-watering could stimulate flowering of coffee trees by enhancing ethylene production and/or sensitivity. To verify this hypothesis, greenhouse-based and field experiments with soil drying and re-watering events determined the temporal changes in ethylene evolution of different plant organs, including flower buds, and the expression patterns of ethylene biosynthesis and signalling genes. Moreover, applying the growth regulator 1-Methylcyclopropene (1-MCP - an ethylene action inhibitor) to field-grown plants prior to the start of the wet season, was also used to analyze ethylene's role in coffee flowering. We show that soil drying and re-watering induces complex changes in ethylene-related gene expression linked to changes in ethylene biosynthesis and signalling, and that 1-MCP application provided a new approach to stimulate flowering in the absence of a rainfall event.

2 Materials and Methods

2.1 Greenhouse experiment - design and plant material

To determine if soil water dynamics modify ethylene production, gene expression and physiology, 6-month-old coffee (*Coffea arabica* cv.*Catuaí Vermelho*) seedlings (kindly provided by the Procafé Foundation, Varginha - Minas Gerais – Brazil) were first evaluated. Since coffee takes about three years to flower, this experiment assessed plant responses to 3 different watering treatments: well-watered (WW) plants, water-deficit (WD) plants, re-watered (RW) plants (plants submitted to water deficit followed by irrigation). The experiment was conducted in a semi-controlled greenhouse at UFLA, Brazil, in April 2017, with a day-length of 12 h (Sunrise 06:10 / Sunset 17:53) and day and night mean temperatures of 24.6°C and 22.8°C, respectively. The daily maximum temperature in the greenhouse was 34.3°C and the minimum temperature at night was 16.8°C. Mean relative humidity was 77% and it varied from 91% to 42% during the experiment. Each treatment comprised 21 plants, allowing tissue (leaves and roots) sampling from three biological replicates on seven occasions. Plants were grown in one-litre plastic bags filled with a mixture of soil, sand, and cattle manure (3:1:1, v/v/v). For the WW treatment, plants were watered to field capacity every two days, while

watering of the WD and RW treatments was suspended until predawn leaf water potential (Ψ_{pd}) declined from -0.2 MPa to -2.0 MPa. Since harvesting leaves to measure Ψ_{pd} wounds the plants thus affecting ethylene production, a preliminary experiment determined the relationship between soil moisture (measured with a ML2x ThetaProbe, Delta-T Devices, Burwell, UK) and Ψ_{pd} (measured by a Scholander-type pressure chamber) using a different set of plants (Supporting information Figure S1). Thus, Ψ_{pd} was inferred from predawn soil moisture measurements. For the RW treatment, plants were re-watered sufficiently to re-establish drained capacity.

2.1.2 Physiological analyses and tissue sampling

Plants were measured and tissues sampled at 0, 2, 4, 6, 12, 24, and 48 h after re-watering, corresponding to these times: 08:00, 10:00, 12:00, 14:00, 20:00; 08:00, and 08:00h, respectively. Only the WW and WD treatments were sampled on the first occasion, since RW plants were re-watered at 08:00. Physiological measurements included instantaneous gas-exchange variables, determined with a portable infrared gas analyser (LI-6400XTR Li-Cor, LINCOLN, NE, USA) and leaf water potential (Ψ_{leaf}), determined using a Scholander-type pressure chamber. This chamber was lined with moistened filter paper and Ψ_{leaf} was measured in two to three leaves from each plant (averaged as one biological replicate) and three replicates of each treatment. Carbon assimilation rate (A) and stomatal conductance (g_s) were evaluated in one young and fully expanded leaf from each plant with six replicates per treatment. At each sampling time, the same leaves used for IRGA measurements were immediately immersed in liquid nitrogen and roots were rapidly washed, dried using paper towels and, then frozen in liquid nitrogen. Root preparation required *circa* two minutes per sample. Plant material was stored at -80°C prior to gene expression studies.

2.1.3 Gene expression analysis

Selected genes related to ethylene biosynthesis and signalling pathways were analyzed 0, 2, 6, and 24 hours 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) (Ságio *et al.* 2014), were analyzed in leaves and roots of WW, WD, and RW plants.

2.1.3.1 RNA extraction, cDNA synthesis and RT-qPCR assay

Total RNA from leaves and roots was extracted using the ConcertTM Plant RNA Reagent (Invitrogen) according to manufacturer's protocol, with minor alterations. RNA samples (5 µg) were treated with DNase I using the Turbo DNA-free Kit (Ambion) to eliminate residual DNA contamination. RNA integrity was visually analyzed in 1% agarose gel, and RNA content, as well as quality, were accessed by spectroscopy ($OD_{260/280}$ and $OD_{260/230} > 1.8$) (NanoVue GE Healthcare, Munich, Germany). One µg of the total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, USA), according to manufacturer's protocol, and subsequently stored at -20 ºC. Real-time quantitative PCR was performed using 15 ng of cDNA in a 15 µL reaction volume with Rotor-Gene SYBR® Green PCR Kit (Qiagen), on a Rotor Gene-$Q^{(R)}$ thermocycler (Venlo, Netherlands). Reactions were carried out in 15 µL reaction volume: 7.5 µL of SYBR-green (QuantiFast SYBR Green PCR Kit - Qiagen), 0.3 µL of forward and reverse gene-specific primers (see Table 1 for primer sequences and amplification efficiencies), 1.5 µL of cDNA at 10 ng/µL, and 5.4 of RNase-DNase-free water. Three biological replicates were used, reactions were run in triplicate, and amplification 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 assess 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 ΔΔCT method (Pfaffl 2001), using *AP47*(accession no. DV690764.1) and *RPL39* (accession no. GT720707.1) (see Table 1 for primer sequences and amplification efficiencies) as reference genes (Fernandes-Brum *et al.* 2017). Supporting information (Supporting information Table S1), for this Greenhouse experiment and for the Field experiment III (described below), shows the RT-qPCR

parameters according to the minimum information for publishing quantitative real-time PCR experiments (MIQE) guidelines (Bustin*et al.* 2009).

Table. 1

2.2 Field experiment I

To determine if a drought and re-watering cycle altered ethylene production of field-grown plants, four different coffee cultivars, three from *Coffea arabica* (cv. *Acauã* , *IPR100* , and*Oeiras*) and one from the *Coffea canephora* (cv.*Conilon 213*) species, were evaluated for root, leaf and flower bud ethylene production during six months (from May to October). The experiment was conducted in a coffee plantation at the Department of Agriculture of UFLA, in a randomized block design with three biological replicates, each one comprising ten plants. Roots (15 to 25 cm deep), leaves (young and fully expanded at the third or fourth node from plagiotropic branches) and flower buds (G2 buds with a broad and flat apex, G3 buds up to 3 mm in length, and G4 buds ranging from 3.2 to 6 mm in length – Morais et al., 2008, according to their representation at each sampling time) from the three biological replicates were sampled monthly, from 8:00 am to 10:00 am, usually towards the end of each month. At the last sampling occasion, flower buds that progressed to anthesis in response to a rain event (3 consecutive days from 25th September 2017) were sampled on 5th October 2017, while leaves and roots were sampled on 30th October 2017. For this reason, flower buds from this sampling time were represented by G5 (flower buds ranging from 6.1 to 10 mm in length displaying a light green colour) and G6 (flower buds bigger than 10 mm in length displaying a white colour) (Morais et al., 2008) developmental stages. Tissues were immediately incubated in vacutainer glass tubes of 10 mL, sealed with serum caps and with a moist tissue placed on the bottom of each vial for 24 hours. Ethylene was quantified from the headspace gas using the F-900 Portable Ethylene Analyzer (Felix Instruments, USA). Plant material from each biological replicate was incubated in two separate vials, and the headspace gas withdrawn from the vials with a 10 mL plastic syringe. Samples, comprising 2.5 mL from each vial, were extracted using the same syringe and subsequently injected into the F-900, operating under the GC Emulation Mode. After ethylene measurement, plant material was weighed, to express ethylene evolution rate as ppm $g^{-1}h^{-1}$. Plant water status was assessed by measuring pre-dawn (between 03:30 and 05:30h) leaf water potential using a Scholander-type pressure chamber, following the same procedure for the Greenhouse experiment. Water status was assessed at the beginning (20th May 2017) and end (20th September 2017) of the dry season in the Brazilian region where the experiment was carried out, and at the end of October (30th October 2017) following 128.6 mm of rain (Supporting information Figure S2).

2.3 Field experiment II

To better understand ethylene's role in coffee flowering and evaluate a chemical treatment that could concentrate coffee anthesis and prevent its occurrence in response to low intensity rains during the dry season, the effects of exogenous 1-MCP application on coffee flowering were evaluated. The experiment was conducted in a different coffee plantation at the Department of Agriculture (UFLA), on adult, eight-year-old coffee (*C. arabica* cv. *Acaiá Cerrado*) trees. Since plants were pruned two years before the experiment, the new branches were in their first production year and had flower buds at most nodes. Treatments were implemented on 20th of August of this second year of analysis, before the start of the rainy season (Supporting information Figure S3).

The experiment was carried out in a randomized block design with 7 treatments and five replicates per treatment, arranged in three different lines (block) of plants, with each line grouping two or three treatments, at least 20 meters distant from each other to avoid any cross contamination, and being separated by a line of plants (borders). Treatments comprised five different 1-MCP concentrations (MCP1 to MCP5), a control for these treatments named BTH, which consisted of a sprayable solution containing only water and Break-Thru, an organosilicon surfactant (S240, Evonik Industries AG, Essen, Germany) present in the sprayable 1-MCP solution, and an unsprayed control (C) to all treatments. Each treatment comprised five biological replicates (one tree per replicate). A sprayable formulation of 1-MCP (3.8 % of active ingredient (a.i.) named Harvista^{tm} (AgroFresh Inc., Spring House, PA) was applied to whole trees at 2 mg a.i. L^{-1} (MCP1), 5 mg

a.i. L^{-1} (MCP2), 25 mg a.i. L^{-1} (MCP3), 50 mg a.i. L^{-1}(MCP4), and 100 mg a.i. L^{-1} (MCP5), using 800 mL per plant. The Harvista formulation at each of the five concentrations, and the BTH treatment, comprised Break-Thru at 0.035 % of the final volume. Spray solutions were prepared thus: 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 mixed; Harvista powder at the given concentration was added and the solution gently swirled until the powder completely dissolved; the remaining water was added and the solution gently stirred for about two minutes. Since 1-MCP is released as a gas, the entire foliage, adaxial and abaxial leaf surfaces, branches, and flower buds, from the coffee trees were immediately sprayed after preparing the solutions. 1-MCP application was made to the point of runoff, using a 12 L backpack sprayer (S12 - Brudden Sprayers), on a sunny day between 08:00 and 10:00 to maximize 1-MCP penetration. Coffee flowering was later evaluated by calculating the percentage of flower buds at the G4 stage (Flower buds ranging from 3.1 to 6 mm in length) (Morais et al., 2008), from three nodes, containing young and fully expanded leaves, of four plagiotropic branches at the middle third of the plants. These progressed to anthesis after rainfall that occurred 15 days after applying the treatments (Supporting information Figure S3). Predawn leaf water potential was assessed on the day before the start of the experiment using Scholander-type pressure chamber and following the same procedure described in Field experiment II.

2.4 Field experiment III

Physiological and molecular changes triggered by applying 1-MCP were assessed by analysing instantaneous gas-exchange variables, including carbon assimilation rate (A) and stomatal conductance (g_s), and the expression patterns of ethylene biosynthesis and signalling genes, in leaves and flower buds, from field-grown eight-year-old coffee (*C. arabica* cv. '*Acaiá Cerrado'*) trees. This experiment, conducted on a different coffee plantation from Field experiment II, was designed in randomized blocks, comprising three treatments and six biological replicates, arranged in three different lines (block) of plants, with each line grouping two replicates from each treatment (distant at least 20 meters from each other to avoid any cross contamination) and being separated by a line of plants (borders). Treatments consisted of plants sprayed with Harvista at the concentration of 50 mg of a.i. L^{-1}(concentration chosen based on Field experiment II), plants treated with a solution composed by water and Break-Thru (BTH), which acted as a control for the 1-MCP treatment, and a control treatment (C), with plants not sprayed with any solution. Harvista application was performed following the same procedure described for Field experiment II. Leaf gas-exchange was measured 2, 24, and 48 hours after 1-MCP application. Gene expression patterns of *CaASC1-like* , *CaACO1-like* ,*CaACO4-like* and *CaETR4-like* were evaluated by RT-qPCR in leaves and flower buds, from G4 stage (Morais *et al.* 2008), sampled 2, 6 and 24 hours after imposing the treatments, following the same procedure described for the Greenhouse experiment.

2.5 Statistics

Differences between watering (Greenhouse experiment) and 1-MCP (Field experiments II, III) treatments, in predawn leaf water potential (Field experiments I, II), and in the anthesis percentage (Field experiments II, III), were accessed by one-way ANOVA. Two-way ANOVA assessed differences in ethylene production between different tissues and over time for each coffee cultivar. When ANOVA was significant, means were discriminated using Tukey's multiple comparison test at P [?] 0.05. All statistical analyses were performed by the R software (Team 2017).

For the gene expression analysis, the expression rate and the confidence intervals were calculated according to the method proposed by Steibel (2009), which considers the linear mixed model given by the following equation:

$$y_{ijklm} = \mu + TG_{ijk} + I_l + e_{ijklm}$$

where y_{ijklm} is the Cq (Quantification cycle) obtained from the thermocycler software for the kth gene (reference or target) from the mth well, corresponding to the lth plant subject to the$i$ th treatment (WW, WD, and RW) at the *jth* time (0, 2, 6, and 24 h) for the Greenhouse experiment; TG_{ijk} is the effect of the combination of the *ith* treatment (WW, WD, RW) at the *jth* time (0, 2, 6, 24 h), except for time 0 where

only the treatments WW and WD were considered, in the expression of the gene k (reference or target). Field experiment III utilized the same model, differing from the Greenhouse experiment in having only three evaluation times (2, 6 and 24 h) instead of four.

3 Results

3.1 Greenhouse experiment

3.1.1 Physiological analysis

Leaf water potential (Ψ_{leaf}) of well-watered (WW) plants did not fall below -0.7 MPa throughout the experiment, whereas Ψ_{leaf} of plants exposed to water deficit (WD) was between -2.3 MPa and -4.0 MPa (Figure 1A). Re-watering significantly increased Ψ_{leaf} within 2 hours, and Ψ_{leaf} recovered 74% of its maximum value within 4 h (Figure 1A). However, it took 12 hours before Ψ_{leaf} of re-watered plants was similar to WW plants.

Carbon assimilation rates were highly coupled with stomatal conductance (Figures 1B, C). Stomatal conductance (g_s) of WW plants fluctuated between 70 and 120 mmol $m^{-2}s^{-1}$ during the day, and was close to zero at night (Figure 1B). Stomatal conductance of WD plants declined throughout the day having peaked at 25 mmol m^{-2} s^{-1}at 10:00h (measured 2 h into the experiment). In contrast, re-watering increased g_s within 2 h and values were 3-fold higher than WD plants throughout the day. Interestingly, stomata of re-watered plants did not close at night (measured 12 h into the experiment). Re-watered and WW plants had a similar g_s24 h after re-watering (Figure 1B). Recovery of stomatal conductance to WW values was slower than recovery of leaf water potential (cf. Figures 1A, B).

Carbon assimilation rates (A) of WW plants fluctuated between 6 and 9 μmol m^{-2} s^{-1} during the day, and was negative (indicating respiration) at night (Figure 1C). Plants exposed to water deficit had A below 2 μmol $m^{-2}s^{-1}$ throughout the experiment, with A ceasing at noon and 14:00 h (4 and 6 hours in Figure 1C). In contrast, re-watering increased A within 2 h and these plants maintained positive carbon assimilation rate at those times. All treatments had negative carbon assimilation at night (12 hours after re-watering). Carbon assimilation rates were slower to recover after re-watering, achieving 67% of WW plants after 24 hours and complete photosynthetic recovery after 48 h (Figure 1C). Thus, carbon assimilation was slower to recover than either $\Psi_{\lambda\epsilon a\varphi}$ org_s .

Figure 1.

3.1.2 Gene expression analysis

The coffee ACC synthase and ACC oxidase homolog genes,*CaACS1-like* and *CaACO4-like* respectively, had similar expression levels under well-watered and water-deficit conditions in leaves and roots, and did not respond to re-watering (Figures 2A, B, E, F). However, water-deficit down-regulated another putative coffee ACC oxidase gene (*CaACO1-like)* in both leaves and roots (Figures 2C, G). In leaves, water deficit decreased *CaACO1-like* expression by 11 and eight times at the beginning of, and 6 hours into, the experiment, respectively (Figure 2C). In roots, water deficit decreased*CaACO1-like* expression by four to 24 times, depending on the time of measurement (Figure 2G). Re-watering released *CaACO1-like*expression from water deficit inhibition within 2 hours in the roots, and within 6 hours in the leaves. Of the ethylene biosynthesis genes analyzed, only *CaACO1-like* declines in both leaves and roots under water deficit conditions, with re-watering returning expression levels to those observed in well-watered plants.

Figure 2.

The putative coffee ethylene receptor *CaETR4-like* was not differentially expressed in leaves, except 24 h into the experiment, where it was 24 times more expressed in WD plants than WW plants (Figure 2D). In roots, *CaETR4-like* was significantly more expressed in WW than WD plants at two of the four measuring times, being 5 and 4.7 times more expressed in WW plants than WD plants 2 and 6 h into the experiment (Figure 2H). Re-watering up-regulated root *CaERT4-like*expression, returning expression levels to those of WW plants within 2 hours of re-watering, achieving 5-fold higher expression than WD plants. Such differential

gene expression between re-watered and water-deficit plants was maintained 4 and 24 h into the experiment. Thus, soil water dynamics regulated *CaETR4-like* expression in the roots.

3.2 Field experiment I

During the dry season and averaged across all cultivars, predawn leaf water potential (Ψ_{pd}) declined from -0.2 MPa in May to -2.3 MPa in September (Figure 3). Following significant rainfall (145 mm) from September to the end of October (Supporting information Figure S2), Ψ_{pd} recovered to -0.5 MPa by 30th October 2017 (Figure 3). Genotypic differences in Ψ_{pd} were detected only in September, with cultivars *Acauã* and *Conilon 213* having lower values (by -0.6 and -1.3 MPa) than the other cultivars (*Oeiras* and *IPR100*) (Figure 3). Thus, significant soil drying occurred as the dry season progressed.

Ethylene levels in leaves and flower buds generally decreased throughout the season in all cultivars analyzed, while roots maintained relatively constant ethylene production (Figure 4). Foliar ethylene production showed genetic variation in May, with *IPR100* plants producing 10 times more ethylene than *Acauã* . From May to June, leaf ethylene production strongly decreased in all cultivars analyzed, by 89% in *Conilon 213* but only 22% in *Acauã* . Ethylene production in flower buds displayed a similar pattern to that found in leaves, with ethylene production decreasing by 42% (*Oeiras*) to 72% (*Conilon 213*) from May to September. However, most cultivars showed increased floral bud ethylene production in July. Rainfall over three consecutive days at the end of September (Supporting information Figure S2) promoted anthesis in all cultivars and increased floral bud ethylene production prior to flower opening. In October, after 145 mm of rainfall and plant rehydration, confirmed by Ψ_{pd} measurements (Figure 3), leaf ethylene production was 4 to 12 times higher than in September (although statistically higher only in *Oeiras*) (Figure 4). In roots, re-watering did not promote significant changes in ethylene production when compared to root ethylene production before and at the end of the dry season (Figures 3,4). Thus shoot, but not root, ethylene production was responsive to plant water status.

Figure 3.

Figure 4.

3.3 Field experiments II and III

To determine whether manipulating ethylene sensitivity could induce coffee flowering, well-watered plants (as indicated by Ψ_{pd} values from -0.26 MPa to -0.49 MPa - Table 2) were sprayed with the ethylene action inhibitor 1-MCP before the beginning of the rainy season in Field experiment II. Any variation in Ψ_{pd} (prior to imposing treatments) was attributed to spatial variation in soil water availability within the coffee plantation. Harvista (the commercial formulation of 1-MCP) application significantly promoted anthesis at the two highest concentrations (MCP4 and MCP5), had minimal effects ($< 10\%$ anthesis) at the two intermediate concentrations (MCP2 and MCP3), whereas the lowest concentrations did not induce anthesis (Figure 5) (Table 2).

Figure 5.

Table. 2

Fifteen days after Harvista application (5th of September), a rain event of about 17 mm (Supporting information Figure S3) promoted anthesis in all plants. Although MCP4 and MCP5 treatments had fewer flower buds at the G4 stage than the other treatments (due to Harvista application previously inducing anthesis - Table 2), there was no significant treatment difference in the percentage of G4 flower buds that progressed to anthesis. Thus, exogenous growth regulator application and endogenous physiological changes caused by rainfall had complementary effects on coffee flowering.

In Field experiment III, gas exchange analyses clearly show that Harvista (1-MCP) application significantly decreased g_s and A , with these changes being mainly caused by the surfactant Break-Thru, BTH (Figure 6). Harvista and BTH treatments decreased g_s , to values similar to plants grown under water-deficit conditions (Figure 1), within 6 hours of treatment. Stomatal conductance of both treatments recovered (Figure 6A)

within 24 h (BTH) to 48 h (Harvista). Similar to the Greenhouse experiment, A and g_s were coupled (Figures 6A, B) and A also approximately halved upon Harvista and BTH application compared to control plants, and likewise recovered within 24 h (BTH) to 48 h (Harvista) (Figure 6). Thus, foliar surfactant (BTH) application decreased leaf gas exchange, with 1-MCP prolonging the effect.

Figure 6. Stomatal conductance (A) and net carbon assimilation rate (B) of coffee plants of Field experiment III in the Control (C), BTH, and Harvista (1-MCP) treatments at 2, 24 and 48 HAT. Data are means ± 95 % confidence interval of the mean (n=6). Different letters indicate statistical difference between means within each measuring time.

In Field experiment III, Harvista application promoted coffee anthesis, while the control and Break-Thru (BTH) treatments did not flower (Table 3). Both Harvista and BTH modified foliar expression of ethylene biosynthesis genes, such as *CaACS1-like* and *CaACO1-like*(Figure 7). Within 2 hours of application, foliar expression levels of*CaACS1-like* and *CaACO1-like* increased in response to Harvista and BTH treatments. *CaACO4-like* expression was similar among treatments when measured at different times in each tissue (Figures 7B, F). Harvista (but not BTH) application decreased leaf*CaETR4-like* expression pattern throughout the experiment, while BTH decreasing expression only after 24 h (Figure 7). None of the treatments altered expression levels of the four genes in floral buds within the first 24 hours of application (Figure 7).

Table. 3

Figure 7.

4 Discussion

Soil drying and re-watering, or chemical treatment with the ethylene action inhibitor 1-MCP, induced changes in ethylene biosynthesis and related gene expression respectively, thereby promoting coffee anthesis. Soil water deficit decreased shoot ethylene biosynthesis, even though root ethylene production does not seem to change during the dry season (Figure 4). Re-watering increased shoot ethylene production (Figure 4), possibly enabled by ACC transport from roots to the shoot (Tudela & Primo-Millo 1992; Pérez-Pérez, Puertolas, Albacete & Dodd 2020), since root ethylene production was unresponsive to re-watering, despite up-regulation of a gene that encodes the enzyme responsible for converting ACC to ethylene (*CaACO1-like*). However, increased ethylene levels *per se* seem insufficient to promote coffee anthesis, since 1-MCP and its control (BTH) treatment both up-regulated ethylene biosynthesis genes in leaves and flower buds, but only 1-MCP promoted anthesis. 1-MCP application downregulated the ethylene receptor *CaERT4-like* in the shoots (Figure 7D, H) (Wu, Zhang, Wang, Guo & Dong 2017; Mata, Van de Poel, Hertog, Tran & Nicolai 2018; Ha, Lim & In 2019) (potentially enhancing ethylene sensitivity momentarily) and since ethylene receptors act as negative regulators, anthesis was triggered. To our knowledge, this is the first report that 1-MCP treatment can overcome the requirement for soil drying and re-watering in inducing anthesis in a commercially important woody species.

Although rains at the end of the drying cycle can trigger flowering of woody species (Opler, Frankie & Baker 1976; Reich & Borchert 1982; Borchert 1983, 1994), our study is the first to associate these with changes in ethylene biosynthesis and the expression of regulatory genes. Both leaf and flower bud ethylene production decreased as plants advanced through the dry season (Figure 4), consistent with soil drying decreasing foliar ethylene production of rose and other herbaceous species (Morgan, He, De Greet & De Proft 1990) and flower bud ethylene production of coffee (Schuch et al., 1992) and rose (Andersen et al., 2004). Decreased shoot ethylene production was consistent with downregulation of the *CaACO1-like* gene (Figure 2C), and the activity of ACO enzymes (Andersen *et al.* 2004; Larrainzar*et al.* 2014; Song *et al.* 2016; Rickes, Klumb, Benitez, Braga & Bianchi 2019). Significant ABA accumulation occurs in coffee during the dry season in response to leaf water deficit (Silva *et al.* 2018), which may limit expression of ACO genes (Cheng, Chiang, Hwang & Lin 2009; Linkies *et al.* 2009) or ACO activity (Bailly, Corbineau & Come 1992; Linkies *et al.* 2009; Marino *et al.*2017). Nevertheless, not every gene involved in ethylene biosynthesis responded to water deficit (e.g. *CaACS1-like* and*CaACO4-like* expression were similar between well-watered and water deficit plants - Figure 2A, B, E, F) and the multi-gene nature of both ACS and ACO genes in coffee (Ságio *et*

al. 2014) indicates fine regulation of specific enzyme isoforms in response to water deficit (Wang, Shih & Li 2005; Song *et al.* 2016; Montilla-Bascón*et al.* 2017; Dalal, Sahu, Tiwari, Rao & Gaikwad 2018). While regulation of ethylene biosynthesis genes represents one mechanism of controlling ethylene levels in the shoot, conjugation of the precursor ACC can also be important (de Poel & Van Der Straeten 2014). Indeed, soil water deficit increased 1-malonyl-ACC concentrations in rose roots (Andersen *et al.* 2004). Thus soil (and leaf) water deficit can alter both shoot gene expression and precursor levels, thereby regulating shoot ethylene production.

In contrast, there was a much weaker relationship between gene expression and ethylene production in the roots. Root ethylene production was independent of soil watering deficit (Figure 4) consistent with *CaACS1-like* expression (Figure 2E) even though*CaACO1-like* expression was strongly downregulated by soil drying (Figure 2G). This pattern of gene expression suggest that ACC probably accumulates in coffee roots in drying soil, as in citrus (Tudela & Primo-Millo 1992) via an ABA-mediated process (Gómez-Cadenas *et al.* 1996). Chemical inhibition of ABA biosynthesis prevented ACC accumulation in Citrus roots in dry soil, while exogenous ABA induced ACC accumulation (Gómez-Cadenas *et al.* 1996). In addition, decreased levels of root ACO activity (Andersen *et al.* 2004) under water deficit may also contribute for ACC accumulation under water deficit. While further measurements of ACC levels seem necessary, root ACC accumulation during the dry season could be important in regulating plant response to re-watering.

Re-watering increased shoot ethylene production (Figure 4), consistent with the up-regulation of the *CaACO1-like* gene (Figure 2C), but no change in root ethylene production was detected (Figure 4), even though *CaACO1-like* was positively regulated (Figure 2G). Different responses between roots and shoots might be explained by increased root-to-shoot ACC transport and ACC conjugation in roots upon re-watering, as observed in other woody species such as mandarin (*Citrus reshni*) (Tudela & Primo-Millo 1992; Gómez-Cadenas*et al.* 1996), and/or with ACC release from its conjugated form, as observed in rose leaves (Andersen *et al.* 2004). Re-watering increased leaf ethylene evolution in herbaceous species such as wheat (*Triticum aestivum* – Balota et al., 2004) and tomato (*Solanum lycopersicum* - Pérez-Pérez et al., 2020) and also in rose, where it promoted flower opening by influencing the expression of a set of rehydration-responsive genes (Meng *et al.* 2014). Similarly, re-watering coffee may trigger anthesis by increasing shoot ethylene levels, but modified ethylene sensitivity may also be involved, since chemical treatment with 1-MCP also triggers anthesis.

As with re-watering (Figure 2), 1-MCP treatment up-regulated ethylene biosynthesis genes (*CaACS1-like* and *CaACO1-like*) in the shoot (Figure 7), as it (or the surfactant it is co-applied with) may impose a stress (Figure 7) and/or cause loss of the negative feedback regulation of ethylene biosynthesis (Ella, Zion, Nehemia & Amnon 2003; Trivellini, Ferrante, Vernieri & Serra 2011). However, increased ethylene biosynthesis *per se* is not enough to trigger anthesis, as suggested from the BTH treatment (Figure 7; Table 3). Alternatively, changes in ethylene sensitivity, potentially reduced by water deficit under natural conditions and increased by chemical treatment (1-MCP), seems necessary to promote anthesis. Decreased amounts of ethylene receptors enhance ethylene sensitivity (Tieman, Taylor, Ciardi & Klee 2000; Cancel & Larsen 2002; Hada *et al.* 2009), consistent with the down-regulation of *CaETR4-like* by 1-MCP application (Figure 7) (Wu *et al.* 2017; Mata *et al.* 2018; Ha *et al.*2019), thereby promoting anthesis. Furthermore, different concentrations of the ethylene releasing chemical Ethephon (data not shown) did not promote anthesis, suggesting changes in ethylene sensitivity are needed. Thus, increased ethylene levels and/or altered sensitivity are both necessary to permit coffee flower buds to progress to anthesis.

Based on the results of this and previous studies we propose a model describing the effects of water deficit and re-watering on promoting anthesis in coffee, and effects of Harvista (1-MCP) treatment (Figure 8). Soil drying increases shoot ABA levels (Silva *et al.* 2018) either due to enhanced *in situ* synthesis or ABA transport from the roots (Castro, Puertolas & Dodd 2019), thereby decreasing shoot ethylene levels (Sharp 2002), probably by downregulating*CaACO1-like* expression (Figure 2), and possibly ethylene sensitivity, considering the absence of changes in *CaERT4-like*expression. Furthermore, inhibition of ACC oxidase activity by ABA (Bailly *et al.* 1992; Linkies *et al.* 2009; Marino *et al.* 2017), and enhanced ACC conjugation under water-

deficit conditions (Andersen *et al.* 2004) contribute to decreasing shoot ethylene production. Meanwhile, ACC probably accumulated throughout the plant, since *CaACO1-like* was repressed without any change in*CaACS1-like* expression (Figure 2). Under these conditions, flower buds are maintained in a dormant state, preventing their progression from the G4 developmental stage to anthesis, but may contribute to them acquiring competency to flower.

Re-watering decreases ABA concentrations throughout the plant, with shoot ACC concentrations increasing due to ACC transport from the roots (which had accumulated during water-deficit) and/or ACC release from conjugated forms in the leaves. Lower ABA and higher ACC levels contribute to transcriptional activation of ACC oxidase genes such as*CaACO1-like* , and may enhance ACC oxidase activity. This restores shoot ethylene levels (Figure 4) and induces coffee anthesis by ensuring rehydration recovery in flowers, as observed in rose plants (Meng*et al.* 2014). However, increased ethylene levels *per se*seem insufficient to promote coffee anthesis, and changes in ethylene sensitivity may also be involved. Harvista application mimicked plant rehydration by up-regulating ethylene biosynthesis genes and potentially enhancing ethylene sensitivity by activating and inhibiting*CaACO1-like* and *CaETR4-like* expression, respectively (Figure 8).

Figure 8.

Considering the threat of extinction of several coffee species (Davis*et al.* 2019) and increasing demand for high quality coffee, a better understanding of its flowering process is of central importance. Taken together, this study suggests that re-watering droughted plants increased both shoot ethylene level and ethylene sensitivity, with both involved in promoting coffee anthesis. Demonstrating that ethylene is involved in coffee flowering opens new possibilities for coffee producers to use growth regulators, such as 1-MCP to better control the timing and intensity of flowering events. Further studies involving additional ethylene biosynthesis and signalling genes, now available with the recent release of the *Coffea arabica* genome (https://worldcoffeeresearch.org/), as well as analysing protein levels of ethylene receptors which are crucial for their activity (Kevany, Tieman, Taylor, Cin & Klee 2007), are in progress. These will help elucidate the exact mechanism(s) through which plant rehydration and Harvista (1-MCP) trigger anthesis in coffee species.

Acknowledgments:

We thank the "Instituto Nacional de Ciencia e Tecnologia do Cafe (INCT-Cafe)", the "Coordenacao de Aperfeicoamento de Pessoal de nivel Superior (CAPES)", and the "Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq)" for the financial support, the Procafé Foundation for providing the coffee seedling for the Greenhouse experiment, and the company AgroFresh, specially Felipe Terra, for kindly providing us the Harvista used in the experiments of this study. The authors declare that they have no conflict of interest.

Author Contribution

AL, AC-J, WJD, and ICD conceived and designed the study. AL and CF performed the Greenhouse experiment. ML performed the Field experiment I. AL performed the Field experiment II. IS performed the Field experiment III. AL, CF and CC performed the physiological and molecular analysis. RL and CC performed the statistical analysis. AL and ICD wrote the manuscript with editorial contributions from WJD and CF. All authors read and approved the manuscript.

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Table. 1 RT-qPCR primer sequences and amplification efficiencies.

Gene	Forward primer (5' to 3')
CaACS1-like	TCCTTACCATCCCACCAGAA
CaACO1-like	ACGTGGAAGCCAATGTTACC
CaACO4-like	CGCAACTGTTTGAGATCACG
CaETR4-like	TTGGTCCATTCAGGAACTCG
RPL39	GCGAAGAAGCAGAGGCAGAA
AP47	GGTGTACGCTCACCATTTTCATC

Table. 1 RT-qPCR primer sequences and amplificat

Table. 2 Predawn leaf water potential (MPa) of '*Acaiá Cerrado'* coffee trees from the seven treatments within Field experiment II, and treatment effects on the progression of coffee flower buds at the G4 stage to anthesis.

Treatment	Leaf water potential (MPa)	Anthesis percentage (%)
Control	- 0.33 ± 0.03 a	0.0 ± 0.0 b
BTH	- 0.49 ± 0.09 b	0.0 ± 0.0 b
MCP1	- 0.31 ± 0.08 a	0.0 ± 0.0 b
MCP2	- 0.27 ± 0.09 a	5.8 ± 7.22 b
MCP3	- 0.26 ± 0.04 a	8.5 ± 7.48 b
MCP4	- 0.32 ± 0.06 a	96.3 ± 1.65 a
MCP5	- 0.37 ± 0.08 ab	91.9 ± 6.63 a

Different letters represent statistical significance among treatments within each experiment. Each value represents the mean ± 95% confidence interval of the mean (n=5).**Table. 3** Effect of Harvista application on the progression of coffee flower buds at the G4 stage to anthesis upon a raining event in Field Experiment III.

Treatment	Anthesis percentage (%)
Control	0.0 ± 0.0 b
BTH	0.0 ± 0.0 b
Harvista	80.7 ± 7.28 a

Different letters represent statistical significance among treatments within each experiment. Each value represents the mean ± 95% confidence interval of the mean (n=6).

Figure 1. Leaf water potential (A), stomatal conductance (B), and net carbon assimilation rate (C) of coffee plants in the Greenhouse experiment under well-watered (WW) and water-deficit (WD) conditions, and re-watered (RW) at time 0 hours. Data are means ± 95 % confidence interval of the mean (n=6). Different letters indicate statistical difference between means within each measuring time.

Figure 2. Fold-change (FC) estimates for each contrast are presented for *CaACS1-like* (A and E), *CaACO4-like* (B and F), *CaACO1-like* (C and G), and *CaETR4-like* (D and H) in coffee leaves (left panel) and roots (right panel) within the Greenhouse experiment, sampled before (T0), and 2 (T2), 6 (T6), and 24 (T24) hours after re-watering. Segments represent the 95% confidence interval and comparisons whose confidence

intervals include the value 1 are not significant at $\alpha = 5\%$ (n = 3).

Figure 3. Predawn leaf water potential in May (20th May 2017 - before the dry season), September (20th September 2017 – end of the dry season), and October (30th October 2017 - rainy season) for the four different coffee cultivars analyzed in Field experiment I. Data are means ± 95 % confidence interval of the mean (n=6). Different letters within the same month indicate statistical ($P < 0.05$) differences between cultivars within the same month, respectively.

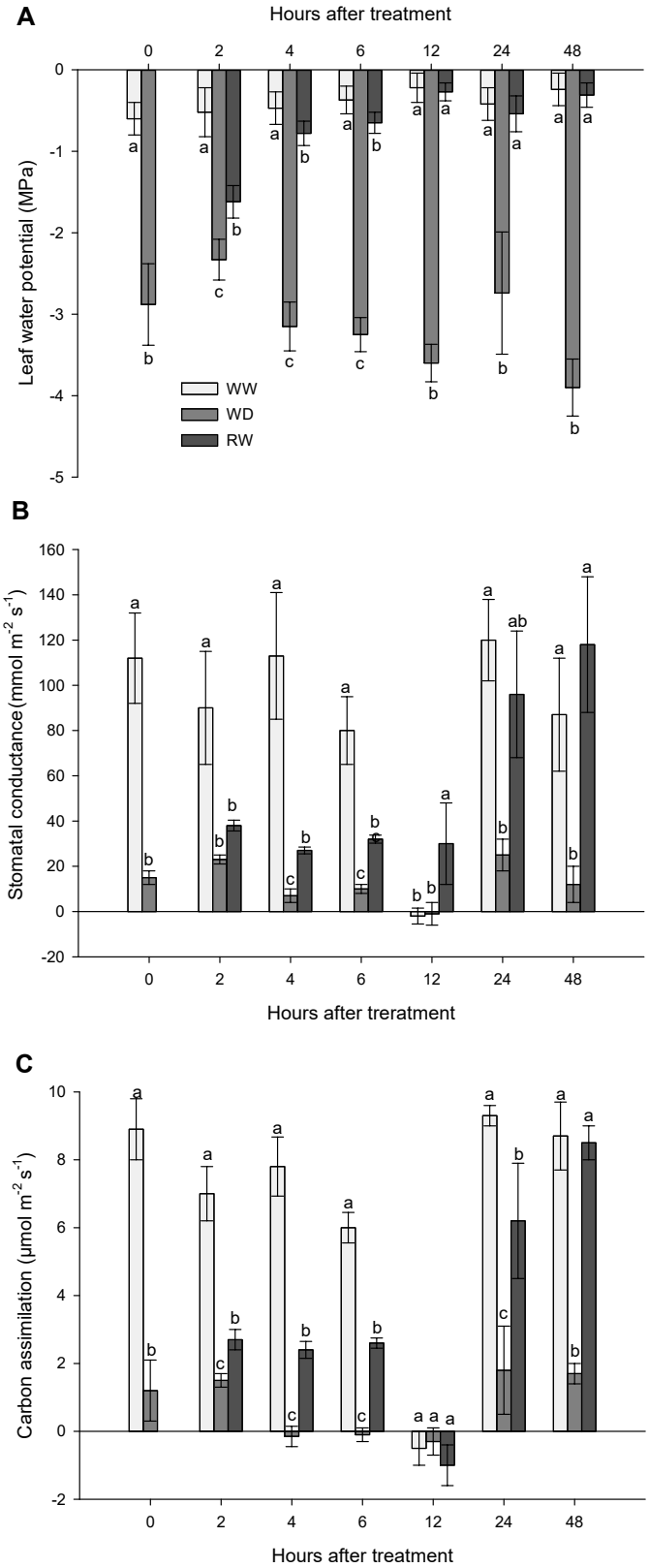
Figure 4. Ethylene production from leaves, flower buds and roots of the *Acauã* (A), *Oeiras* (B), *IPR100* (C),*Conilon 213* (D), and coffee cultivars analyzed in Field experiment I, from May (before the dry season) to October (rainy season). Data are means ± 95 % confidence interval of the mean (n=3). Different upper-case and lower-case letters indicate statistical difference of each tissue among the different months and among the different tissues within the same month, respectively. Note flower bud ethylene production was measured on 05th October 2017 in flower buds that progressed to anthesis in response to the rain event that started on 25th September 2017, while leaf and root ethylene production was measured on 31th October 2017.

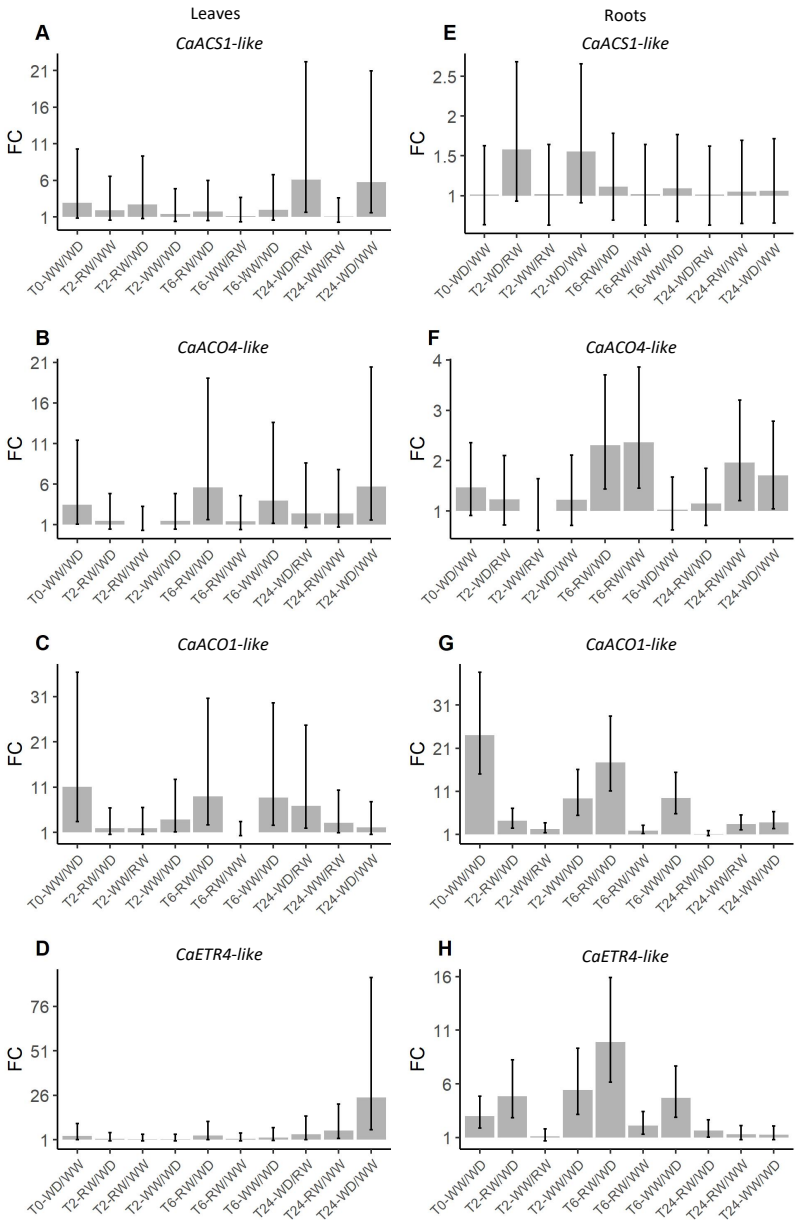
Figure 5. Representation of the anthesis induction from Field experiment II in response to Harvista application. Control plants (A) did not show anthesis induction, as with the BTH, MCP1, MCP2, and MCP3 treatments (not shown), while plants from the MCP4 and MCP5 (B) treatments showed a significant increase in flower bud size (B), with flower opening taking place 12 days after Harvista application (C).

Figure 6. Stomatal conductance (A) and net carbon assimilation rate (B) of coffee plants of Field experiment III in the Control (C), BTH, and Harvista (1-MCP) treatments at 2, 24 and 48 HAT. Data are means ± 95 % confidence interval of the mean (n=6). Different letters indicate statistical difference between means within each measuring time.

Figure 7. Fold-change (FC) estimates for each contrast are presented for *CaACS1-like* (A and E), *CaACO4-like* (B and F), *CaACO1-like* (C and G), and *CaETR4-like* (D and H) in coffee leaves (left panel) and flower buds (right panel), from plants of Field experiment III, sampled 2 (T2), 6 (T6), and 24 (T24) hours after imposing treatments. Segments represent the 95% confidence interval and comparisons whose confidence intervals include the value 1 are not significant at $\alpha = 5\%$ (n = 3).

Figure 8. Model describing the effects of water deficit, rehydration, and Harvista treatment on coffee anthesis, as discussed in the text. Solid arrows denote increases, T end to the arrows indicate decreases, and dashed arrows denote no significant changes in expression or relative amount. Bold symbols represent traits measured in this study. ABA = abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; MACC = 1-malonyl-ACC.





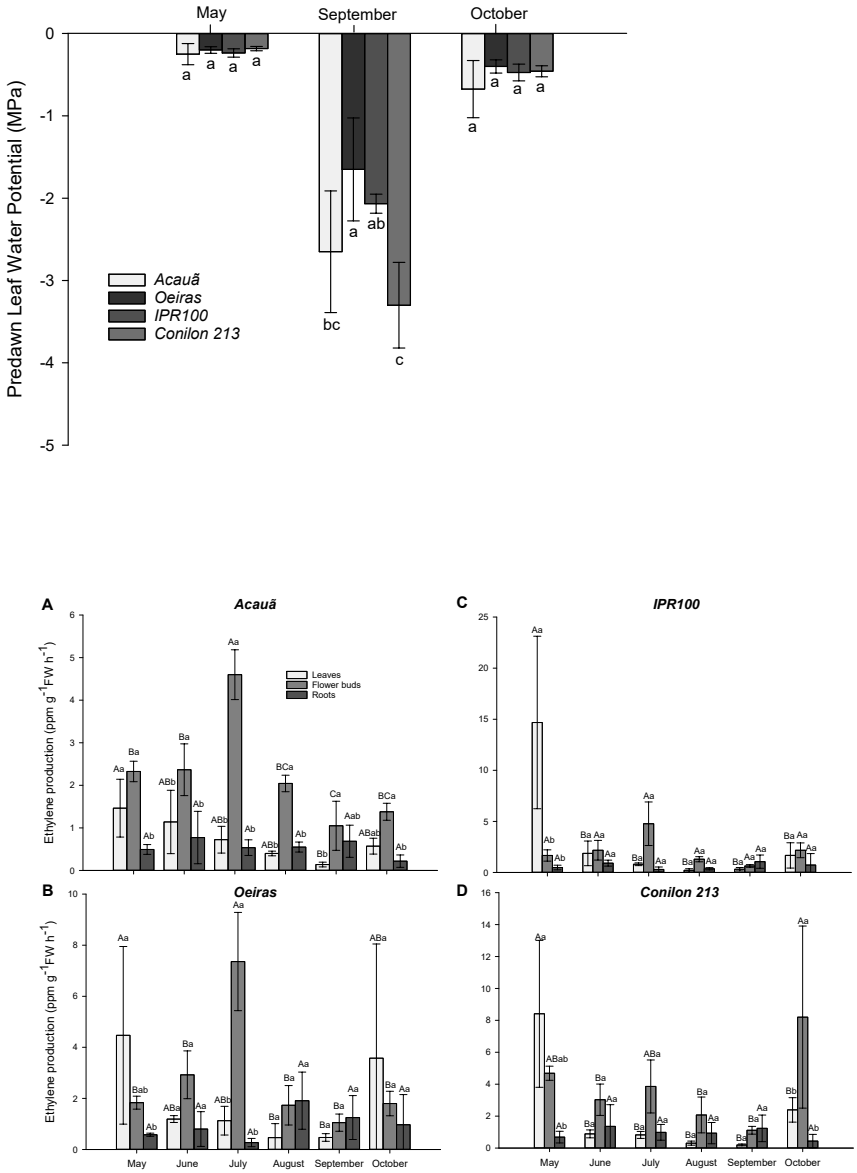


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Figure 5.pdf available at https://authorea.com/users/323588/articles/452170-drought-and-re-watering-modify-ethylene-production-and-sensitivity-and-are-associated-with-coffee-anthesis