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Environmental Stress in Avocado (*Persea americana* Mill.): Flowering and Physiology

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by

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DEDICATION

To my parents, Amilcar and Milagros, who always believed in me and formed my values.

To my sister Martha and my brothers Fernando, Ariel and Mauricio, who shaped my character and share my most meaningful early stories.

To my siblings, Carlos, Alejandro, Gabriel, Mauricio and Monica, who represent the joy of all my family.

ABSTRACT OF THE DISSERTATION

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The challenge to produce more food under sustainable agricultural practices is to develop more efficient systems in the use of natural resources, avoiding agrochemical contamination, and prediction of yield under a changing environment. This dissertation has focused on providing information of the physiological and floral gene expression behavior of avocado trees under different environmental conditions building upon three different chapters: (1) the identification of water-use efficient avocado varieties using leaf carbon isotopic composition ($\delta^{13}C$), (2) the analysis of the physiological behavior of salinity-tolerant avocado rootstocks, and (3) the analysis of the effects of temperature, soil moisture, and light intensity on the temporal pattern of floral gene expression and floral intensity in avocado.

In the first chapter, we found carbon isotope composition measurements to be useful for identifying water-use efficient plants. In the second chapter, the relationship between the physiological performance, health and productivity of avocado exposed to a salinity treatment is described. Low photosynthetic rate and canopy damage describe the effect of salinity on avocado yield. In the third chapter, we successfully identified the

gene expression profile of putative floral genes when avocado flowering is promoted by low temperature. The results from the floral gene expression analysis in avocado suggest that the significantly greater bud expression levels of *LFY* and *AP1/FUL* promoted by low temperature were sufficient to confer bud determination, since transferring the trees from low to warm temperatures did not prevent flowering. Overall, the first two chapters successfully allowed the identification of trees or cultivars that are saline tolerant or water-use efficient while the gene expression analysis showed how the expression of putative floral genes are coordinated with bud determination, suggesting a role during the reproductive development in *P. americana*.

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Introduction

Avocado (*Persea americana* Mill.) is a crop tree known worldwide for the nutritional value of its fruit. This species is native to Meso-American montane rainforests, characterized by a warm, wet summer, and a cold dry winter. However, avocado has spread across tropical and subtropical areas around the world, growing in a wide range of climates and soil types. It has three natural races: Mexican (from sub-tropical and semi-tropical highlands), Guatemalan (from tropical high land) and West Indian (from tropical low-land). The two highland races, Mexican and Guatemalan, grow between 1400–2350 meters above sea level (m.a.s.l.), with mean monthly temperature averages of 9.7 and 21.3 ˚C for the coldest and hottest months respectively, the annual rainfall from ranges 665–1562 mm, and a pronounced warm wet summer/autumn period and cold dry period in winter/spring. The low-land race, West Indian, grows at 100–400 m.a.s.l., with mean monthly temperature averages are 26.9–29.2 ˚C for the coldest and hottest month respectively, rainfall ranges from 1100-1500 mm annually, and a cold dry period in winter/spring. 'Hass', the most important commercial variety, is a hybrid between Mexican and Guatemalan (M: 42%, G: 58% of sequence diversity) and flowers in cool (12.7–21 ˚C) dry seasons (Chen et al., 2009; Welstenholme, 2013). The interracial hybridization of these three ecotypes has resulted in modern varieties that are complex, abundant (more than 200 varieties) and often uncharacterized (Ashworth and Clegg, 2003; Schnell et al., 2003; Scora et al., 2002).

By 2014, avocado global yield represented more than 5 million tons from 547,849 ha across 70 countries (FAO, 2017). 'Hass' avocado is the most popular variety worldwide given its richness in fat, high quality lipids, proteins, vitamins, minerals, carotenoids, and antioxidants. California produces nearly 95% of the 'Hass' avocado fruit produced in USA with a market of $346 million (California Avocado Commission, 2017). However, the USA imports more 'Hass' avocado fruit than it produces and the majority of which come from Mexico to meet the US demand. Avocado production is very variable, ranging between 5–25 ton/ha and this difference is caused by the many intrinsic and environmental factors (climate). The intrinsic factors rely on differences among varieties, for example, phenology, crop load, carbon assimilation and storage, hormonal regulation, nutrient assimilation, and water-use efficiency (Garner and Lovatt, 2008; Nevin and Lovatt, 1989; Salazar-García and Lovat, 1998; Salazar-García et al., 1998, 2006).

Despite the differences among varieties, the broad requirements for avocado trees include: mild climates and low wind, poor soils with good drainage and aeration; proper temperature, rainfall, and altitude according to the variety. This species is particularly susceptible to waterlogging (hypoxia), salinity stress, and freezing temperatures (Bernstein, 2001; Garbanzo, 2010; Granados, 2013; Salazar-García et al, 1999; Wolstenholme and Whiley, 1999). Moreover, 'Hass' avocado experiences alternate bearing (AB), production of high yield in one year followed by a year with low production. AB is modulated by climate, agricultural practices, yield and endogenous factors like starch and hormone concentrations. Others factors are related with

agricultural practices like grafting, where the combination of rootstock and scion are made to acquire desirable fruit characters from the first one, evading the juvenile phase of the crop and encouraging early production. Previous studies on avocado breading programs had focused on the identification of rootstocks with *Phytoptora* resistance or salinity tolerance (Ben-Ya'acov and Michelson. 1995; Crowley, 2008).

Regarding floral morphology, avocado produces two types of floral shoots: determinate and indeterminate. In determinate floral shoots, the meristem of the primary axis forms a true inflorescence and once the fruits are harvested the inflorescence dies (Salazar-García and Lovatt, 2002). In indeterminate floral shoots, the apex of the primary axis terminates in a vegetative bud which starts growing prior to, during or after anthesis and continues the growth of the shoot after harvest. Determinate inflorescences are more productive than indeterminate floral shoot due to competition between the setting fruit and developing shoot. Avocado flowers are perfect and are grouped into clusters of sub-terminal branches that may contain up to 450 flowers (Cossio-Vargas et al., 2007; Salazar-García and Lovatt, 1998). They have synchronized dichogamy (protogynous), characterized by flowers with male and female organs that function at different times (Wolstenholme and Whiley, 1995). The total number of flowers per tree is related with yield. 'Hass' avocado usually produces a large number of flowers over the year that can reach up to 2 million flowers per tree, although only a small amount of these (usually less than 0.001%) set fruit that mature to harvest (Cameron et al., 1952; Garner and Lovatt, 2008; Slabbert, 1981).

This document is organized in three chapters based on three main research goals: *first*, the necessity to save water in agriculture has made it imperative to identify water-use efficient crops. The use of leaf carbon isotope composition ($\delta^{13}C$) is presented here as a tool to identify water-use efficient avocado varieties when a large number of plants needs to be analyzed. *Second*, with the new challenge that salinity represents for agriculture, a study of the physiological behavior of 'Hass' avocado scions grafted to different rootstock varieties growing under saline conditions is presented here. And *third*, with the need to ensure adequate flowering to sustain yields in a changing climate, the expression sequence of eight classic genes putatively regulating floral timing (induction), floral meristem identity (determinacy) and floral organ identity genes (flower formation) was quantified in buds of 'Hass' avocado trees in response to several environmental stresses. The research successfully identified several MADS-box genesthat function as putative floral meristem identity or floral organ identity genes that play a key role in regulating floral development in *P. americana* and provide a possible failsafe mechanism to synchronize flowering with the warm temperatures of spring.

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Chapter 1

Evaluation of leaf carbon isotopes and functional traits in avocado reveals water-use efficient varieties

Abstract

Plant water-use efficiency (*WUE*) describes the ratio of carbon gain to water loss during photosynthesis. It has been shown that *WUE* varies among crop genotypes, and crops with high *WUE* can increase agricultural production in the face of finite water supply. We used measures of leaf carbon isotopic composition to compare *WUE* among 24 varieties of *Persea americana* Mill. (avocado) to determine genotypic variability in *WUE,* identify potentially efficient varieties, and to better understand how breeding for yield and fruit quality has affected *WUE*. To validate carbon isotope measurements, we also measured leaf photosynthetic gas exchange of water and carbon, and leaf and stem functional traits of varieties with the highest and lowest carbon isotope composition to quantify actual *WUE* ranges during photosynthesis. Our results indicate large variation in *WUE* among varieties and coordination among functional traits that structure trade-offs in water loss and carbon gain. Identifying varieties of subtropical tree crops that are efficient in terms of water use is critical for maintaining a high level of food production under limited water supply. Plant functional traits, including carbon isotopes, appear to be an effective tool for identifying species or genotypes with particular carbon and water economies in managed ecosystems.

Introduction

Functional traits have now been used extensively in ecological studies as easy-to-measure proxies for more complex processes (Cornelissen, 1999; Westoby, 1998). The study of functional traits arose from earlier efforts to place numerous species into fewer and more tangible functional groups (Grime, 1977, 1974; Smith et al., 1997), or to place species along axes of ecological strategy variation (Reich et al., 1997; Westoby, 1998; Westoby et al., 2002). Functional traits have mostly been used to advance plant biology through simplified representations of complex processes and their use in managed systems is increasing in importance for identifying differences among species, genotypes or documenting responses to environmental change (Gleason et al., 2016; Vitoria et al., 2016; Wood et al., 2015). This trend coincides with the expansion of managed and human impacted terrestrial ecosystems across the globe. For example, at large scales, functional traits have important linkages to ecosystem and landscape processes, such as water and carbon exchange between vegetated surfaces and the atmosphere (Ainsworth and Long, 2005; Baldocchi et al., 2004), or nutrient absorption and cycling by crop plants (Chapin, 1980; Wendling et al., 2016). As climate change proceeds and the growing range of crops changes (Challinor et al., 2015; Kenny and Harrison, 1992; Lobell et al., 2006), functional traits may offer a way forward to organizing crop species along axes of trait variation that also reflect habitat suitability. Functional traits may also be applied to crop varieties to select for particular traits that promote efficiency of resource use (Farquhar and Richards, 1984; Lauteri et al., 1997; Zhang et al., 2009). All of these applications benefit from emphasizing the main strengths of trait approaches, which

center on quantifiable traits that are continuous and comparable across plant species or genotypes (Westoby, 1998).

In agriculture, functional traits that relate to water consumption are increasingly important because water is a major limiting resource for agriculture. In many parts of the world, the water resources available for agriculture are declining in availability or quality, or increasing in expense due to droughts, floods, or political disagreements (Fu et al., 2013; Lenihan et al., 2003; Mendelsohn and Dinar, 2003; Rosegrant et al., 2009). Thus, there is great interest in identifying varieties with high water-use efficiency, which is normally expressed as yield or productivity divided by water consumed in the process (Cernusak et al., 2007). At the leaf scale, photosynthetic water-use efficiency is expressed as photosynthetic rate, divided by stomatal conductance (Richards et al., 2002). A proxy for long-term time-integrated plant water-use efficiency can be obtained by the measurement of bulk leaf carbon isotope composition ($\delta^{13}C$). This relationship exists because conditions that cause the plant to reduce stomatal aperture cause an increase in water-use efficiency and also a reduction of CO_2 concentration at the site of carboxylation, forcing Rubisco to assimilate more $^{13}CO_2$ (Farquhar et al., 1982; Farquhar and Richards, 1984a). Thus, a significantly larger $\delta^{13}C$ value is interpreted as greater water-use efficiency (Cernusak et al., 2013). Furthermore, water supply to leaves by stems creates coordination of leaf traits with stem traits, such as wood density (Santiago et al., 2004). Plants often achieve low-density wood through construction of large xylem vessels, which tend to have high water transport capacity and can sustain high rates of transpiration at the leaf level, but also tend to be more vulnerable to drought-induced

xylem cavitation (Gleason et al., 2016; Pockman and Sperry, 2000; Wheeler et al., 2005).Therefore, further information about the regulation of leaf carbon and water economy can be obtained by considering water relations, and certain stem traits, along the transpiration pathway.

Stable isotope analysis of carbon has been used extensively as a key functional trait in agricultural and forestry systems to provide information on long-term time-integrated water-use efficiency (Brendel et al., 2002; Brugnoli et al., 1988; Farquhar and Richards, 1984a; Lauteri et al., 1997; Monclus et al., 2005). This has allowed researchers to identify water-use efficient crop varieties (Brugnoli et al., 1988; Farquhar and Richards, 1984a; Hubick et al., 1986), investigate relationships between *WUE* and productivity (Marguerit et al., 2014; Martin and Thorstenson, 1988; Monclus et al., 2005), and link genotypic and phenotypic responses to water deficit by experimentally mapping quantitative trait loci (Brendel et al., 2008, 2002; Brugnoli et al., 1988; Hausmann et al., 2005; Marguerit et al., 2014). Such studies that provide a long-term integrated signal for *WUE* are important because they differ from short-term traits associated with photosynthetic carbon assimilation. In agroecosystems, information on both short- and long-term traits associated with carbon assimilation and water-use efficiency, as well as knowledge of the relationships among them, is critical for crop selection and crop breeding. Thus studies on crop varieties, $\delta^{13}C$ have contributed to identification of water-use efficient varieties of wheat, peanut, tomato, barley, cowpea, coffee and rice (Farquhar and Richards, 1984a; Hall et al., 1990; Hubick et al., 1986; Hubick and Farquhar, 1989; Martin and Thorstenson, 1988; Meinzer et al., 1990; Zhao et

al., 2004). The combined analysis of $\delta^{13}C$ with leaf and stem functional traits has emerged as a useful tool to identify water-use efficient crop genotypes.

We investigated the use of leaf $\delta^{13}C$ in combination with leaf functional traits in *Persea americana* (avocado), a Meso-American tree species with a global yield of 5,028,756 kg from 547,849 ha across 70 countries in 2014 (FAO, 2017). Like many crops, avocado varieties have been selected for yield or fruit quality but not for being water-use efficient or having other physiological traits that allow survival and production under scarce resources. There is a growing interest in water-use efficient avocado varieties because of reduced water quality and availability. For example, the most recent California drought lasted four years and was the driest three-year record in California history (Department of Water Resources, 2015), putting half of the state in a category of exceptional drought (US Drought Monitor, 2015). This situation increased water supply costs and reduced water quality for food producers throughout California. Cultivation of citrus, avocado and other subtropical tree crops have been especially impacted (Campbell, 2011; Spann, 2014). With further scarcity of water resources predicted, crop varieties or varieties that are especially efficient in water use may play an increasing role in securing food production in the future. Although there are seven avocado varieties grown commercially in California, about 95% of California avocado production is based on a single variety, 'Hass'. This study aims to identify water-use efficient avocado varieties using an integrated trait analysis of leaf carbon isotope composition and leaf and stem functional traits across 24 varieties. Our main objectives were to: 1) analyze the variation in $\delta^{13}C$ among avocado varieties; 2) use measurements of instantaneous water-

use efficiency to determine what physiological factors are related to $\delta^{13}C$ in avocado leaves; 3) evaluate relationships between physiological and proxy traits; and 4) describe relationships among leaf and stem traits.

Materials and methods

Study site and plant material

The study was conducted in the University of California South Coast Research and Extension Center (REC), Irvine, California, United States (33°41'18" N, 117°43'20" W), at an elevation of 124 m. The site has a mean annual precipitation of 165 mm with 56% of rainfall occurring from November to February and an average daily temperature range of 29–17 °C in July and 18–7 °C in January over the past three years (CIMIS, 2016). Sample collection and measurements were conducted between June and September 2016 on 24 varieties of *P. americana* (avocado) that are part of the Avocado Breeding Program of the University of California and the California Avocado Commission. Irrigation rates were determined using an irrigation scheduling calculator (Hofshi and Hofshi, 2007), that is based on the Irvine 75 CIMIS station (CIMIS, 2016). Fertilizer was applied twice per year with a granular application of 21:7:14 NPK in April and a liquid application of 17:0:0 NPK in November. Trees were not pruned. The trees came from two experimental plots, an established 40-year old plot and a newer 5-year old plot. The plots are located in an open flat (0–2° slope) area and share the same deep, moderately sloped, alluvial fan soil. All trees were randomly planted at 6 m row spacing and 4.5 m tree spacing. The 5-year old trees were grafted onto ‘Dusa’ rootstocks and the

40-year old trees were grafted onto 'Thomas' rootstocks, except 'Floccosa', which was not grafted, and there were five additional varieties with unknown rootstocks (Table 1.1). All trees were physiologically mature during sample collection and measurements.

Table 1.1 Scion and rootstock varieties and number of individuals sampled for avocado study trees at South Coast Research and Extension Center, Irvine, California, USA.

Scion	Rootstock	Sample size
'UC05-1'	'Dusa'	6
'UC99-1'	'Dusa'	6
'UC99-2'	'Dusa'	6
'UC99-3'	'Dusa'	6
'UC99-4'	'Dusa'	3
'UC00-1'	'Dusa'	6
'UC00-2'	'Dusa'	6
'Flavia'	'Dusa'	6
'Eugenin'	'Dusa'	6
'AO.48'	'Dusa'	6
'UCBL'	'Dusa'	6
'Carmen'	'Dusa'	6
'Fairchild'	'Dusa'	3
'Floccosa'	'Dusa'	2
'Gem'	'Dusa'	6
'Hass'	'Dusa'	6
'LT01'	'Thomas'	6
'Mother Hass'	'Dusa'	6
'XX3'	'Dusa'	6
'Walden'	Unknown	3
'Nahlat'	Unknown	2
'Maoz'	Unknown	2
'Thomas'	Unknown	6
'Simmons'	Unknown	2

Leaf functional traits

The leaf carbon isotope composition ($\delta^{13}C$) of 24 varieties was determined using five newly formed mature leaves from the top of the canopy per individual to control for leaf variation. The number of individuals used per variety varied from 2–7 (Table 1.1). Leaves of each individual tree were pooled, dried at 65 °C for at least 48 h, ground and homogenized to a fine powder with a mill (3383L10 Wiley Mini-Mill, Swedesboro, New Jersey, USA). Leaf $\delta^{13}C$ was measured with an isotope ratio mass spectrometer (Delta *V* Advantage; Thermo Scientific, Bremen, Germany), interfaced with an elemental analyzer (ECS4010; Costech, Valencia, CA, USA) and reported in delta notation (‰) relative to the Pee Dee Belemnite standard. Isotope measurements were done at the University of California, Riverside Facility for Isotope Ratio Mass Spectrometry (FIRMS). Values of leaf $\delta^{13}C$ of the 24 varieties were used to choose a subset of eight varieties ('UC05-1', 'UC99-1', 'UC99-3', 'UC00-1', 'Carmen', 'Hass', 'Gem', 'XX3') for further detailed functional trait measurements. This subset included varieties with the highest and lowest $\delta^{13}C$ values, a range of intermediate $\delta^{13}C$ values, and varieties that are particularly important in agriculture.

Gas exchange was measured between 1000 and 1200 h during sunny days with a portable infrared gas analyzer (Model LI-6400, Li-Cor Biosciences, Lincoln, NE, USA) on eight varieties. Two newly formed mature exposed leaves from six individual trees per variety were measured. The measurements were taken using a red/blue light source (6400-02B #SI-710, Li-Cor Biosciences) at 1,500 μmol m^{-2} s^{-1} photon flux density (PFD), 400 µmol mol^{-1} CO_2, 1.3–2.5 kPa of vapor pressure deficit, and 40–50% relative

humidity. Leaf temperature was allowed to vary naturally from 24–30 °C. Maximum rate of photosynthetic CO_2 assimilation per unit leaf area (A_{area}) and corresponding stomatal conductance (g_s) and intercellular CO_2 concentration (C_i), as well as environmental conditions inside and outside the cuvette were recorded for each measurement once stable readings were achieved after 2–5 min. Intrinsic water-use efficiency (WUE_i) was calculated as A_{area} divided by g_s. We stratified measurements across varieties by time so that one of each variety was measured in each round, before moving on to the next set of replicates. All measurements represent the maximum values that avocado varieties achieve, given the conditions provided. Leaves used for gas exchange were harvested to determine specific leaf area (*SLA*). Specific leaf area was calculated as leaf area (cm^2) measured with a leaf area meter (LI-3100; Li-Cor Biosciences) divided by dry mass (g), after drying leaves at 65 °C for 48 h. *SLA* was used to calculate maximum rate of CO_2 assimilation per unit mass (A_{mass}).

Stem functional traits

Wood density (*WD*) was determined for the eight chosen varieties by collecting six 1-cm diameter and 1-cm long stems per variety and separating sapwood from bark to measure its volume using the displacement method (Chave, 2005). On small diameter stems such as these, all wood, once the bark and phloem are removed, is considered to be sapwood. Then, the sapwood was dried at 65 °C for 48 h to measure dry mass, and *WD* was calculated as dry mass (g) divided by sapwood volume (cm^3). Leaf:sapwood area ratio (*LA:SA*) was measured on three terminal branches on each study individual as an

index of hydraulic supply relative to transpiring area (Pivovaroff et al., 2014). Six branches from each of the eight varieties were collected and transported to the laboratory where sapwood diameter at the bottom of the branch was measured using a caliper to estimate sapwood area, and all leaves distal to this sapwood were removed and their total area measured using a leaf area meter (Li-3100, Li-Cor Biosciences). *LA:SA* was determined as total leaf area (cm^2) divided by sapwood area (cm^2).

Statistical analysis

We averaged trait data for each individual and used this data to calculate and compare trait means among varieties. The traits were tested for normality using a Shapiro-Wilk test, which showed that they were normally distributed ($p \leq 0.05$) or were only slightly non-normal, so no transformations were performed. Comparisons of functional traits among varieties were done with a three-way ANOVA using the *aov* function in R, with variety and time since planting as fixed factors and rootstock as a random factor. Because there were no significant effects of rootstock ($F_{2,117} = 0.2524$; $p = 0.7773$) or time since planting ($F_{1,118} = 0.6722$; $p = 0.4130$), they were removed from the model and subsequent analyses were performed with one-way ANOVA using only variety as a factor. Tests for homoscedasticity were performed with the studentized Breusch-Pagan test using the *lmtest* package in R and the data for $\delta^{13}C$ failed the test indicating heteroscedasticity ($BP = 42.152$, $df = 23$, $p = 0.008715$). Data therefore underwent a Box Cox Transformation using the *caret* package in R, which resulted in homoscedasticity. Differences among varieties were tested with Tukey's range test post-

hoc using the *agricolae* package in R. Bivariate relationships among functional traits were first evaluated with a Pearson product-moment correlation to check for significance. If significant, standard major axis estimation (Model II Regression) was used to describe relationships using the *lmodel2* package in R (Legendre, 2014). Model II regression was chosen over linear regression (ordinary least-squares regression) because all functional traits were measured with error and our objective was to describe the relationships between traits, not to predict values of one trait from another one (Falster et al., 2003).

Results

Leaf and stem functional traits

There was substantial variation in carbon isotope composition among the 24 avocado varieties ($F = 18.77$; $p < 0.0001$), with $\delta^{13}C$ ranging from -32.62 to -27.17 ‰ across the whole data set and a gradual distribution among varieties (Figure 1.1). Eight different significance groups of varieties were detected, with 'Carmen' having the highest mean $\delta^{13}C$ (-27.86 ± 0.29 ‰) and 'XX3' the lowest (-31.93 ± 0.22 ‰). There were substantial differences in all leaf functional traits among avocado varieties. Values for A_{area} were significantly greater in 'UC00-1' than 'XX3' with all other varieties showing intermediate values (Table 1.2). For A_{mass}, 'UC99-3' was significantly greater than Carmen and 'XX3' with other varieties showing intermediate values (Table 1.2). Although 'XX3' showed the lowest values for A_{area} and WUE_i, it showed the highest values for g_s and C_i. In contrast, 'Carmen' and 'UC05-1' showed the highest values for WUE_i and the lowest values for g_s and C_i (Table 1.2). This opposite behavior of *Carmen*

and ‘UC05-1’ compared to ‘XX3’ was consistent with their $\delta^{13}C$ values located in the extremes of Figure 1.1. Values for *SLA* in ‘Carmen’ were statistically indistinguishable from ‘UC00-1’ and ‘XX3’, but significantly lower compared to all other varieties (Table 1.2). There were no significant differences in *WD* among varieties, but *LA:SA* showed significant variation among varieties with ‘XX3’ showing the highest mean value and ‘Carmen’ showing the lowest (Table 1.2). This corresponds to their $\delta^{13}C$, where ‘Carmen’ had the highest value and ‘XX3’ the lowest (Figure 1.1).

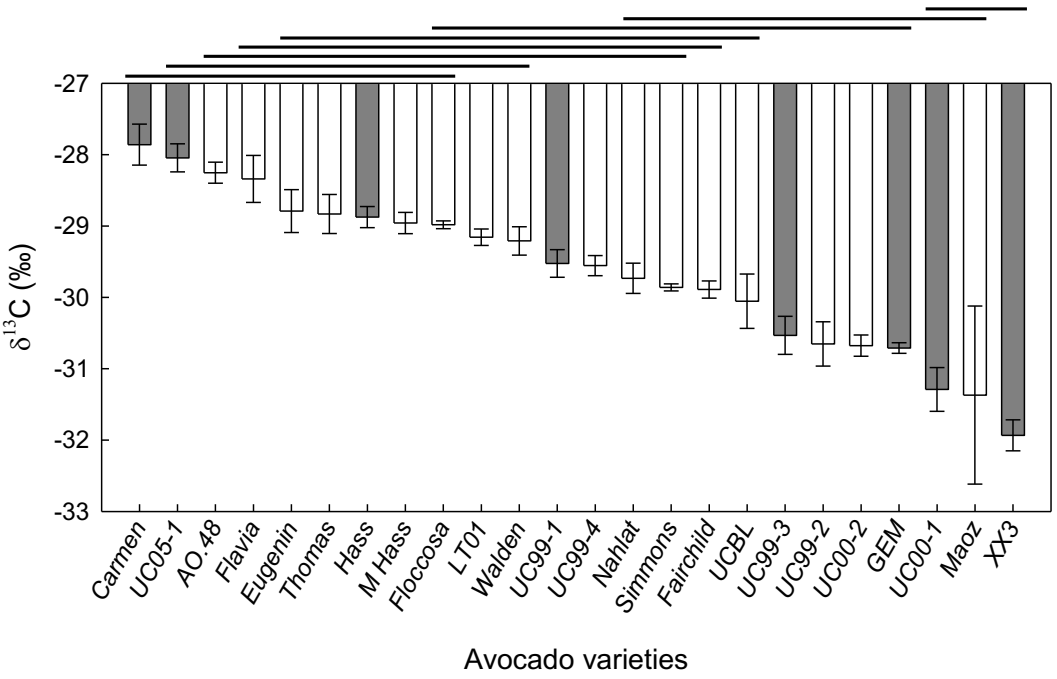

Figure 1.1. Mean ± SE leaf carbon isotope composition ($\delta^{13}C$) of 24 agricultural varieties of avocado growing at South Coast Research and Extension Center, Irvine, California, USA. There was significant variation among varieties ($F = 18.77$; $p < 0.0001$). Varieties that are overlapped by the same solid horizontal lines are not significantly different at an alpha of 0.05. Varieties with gray bars were selected for further trait measurements.

Table 1.2. Leaf functional traits of eight avocado varieties measured at South Coast Research and Extension Center, Irvine, California, USA. Values with the same superscript letter are not significantly different at an alpha value of 0.05. Units: A_{area} (μmol m^{-2} s^{-1}), A_{mass} (nmol g^{-1} s^{-1}), g_s (mol m^{-2} s^{-1}), C_i (μmol mol^{-1}), WUE_i (μmol mol^{-1}), *SLA* (cm^2 g^{-1}), *WD* (g cm^{-3}), *LA:SA* (cm^2 cm^{-2}).

Variety	A_{area}	A_{mass}	g_s	C_i	WUE_i	*SLA*	*WD*	*LA:SA*
'UC05-1'	15.2 ± 2.3^{ab}	128 ± 19^{abc}	0.12 ± 0.04^{b}	134 ± 51^{b}	141 ± 33^{a}	84.2 ± 7.8^{a}	0.31 ± 0.01	3329 ± 82^{abc}
'UC99-1'	14.1 ± 2.1^{ab}	123 ± 18^{bc}	0.13 ± 0.05^{ab}	172 ± 44^{ab}	118 ± 28^{ab}	87.5 ± 4.9^{a}	0.29 ± 0.07	3969 ± 2617^{ab}
'UC99-3'	16.3 ± 2.4^{ab}	146 ± 22^{a}	0.18 ± 0.06^{ab}	197 ± 44^{a}	99 ± 29^{b}	89.6 ± 4.5^{a}	0.32 ± 0.09	4320 ± 1869^{ab}
'UC00-1'	16.7 ± 2.2^{a}	129 ± 17^{abc}	0.17 ± 0.06^{ab}	182 ± 65^{ab}	111 ± 40^{ab}	77.1 ± 10.7^{ab}	0.36 ± 0.07	4240 ± 2090^{ab}
'Carmen'	16.1 ± 2.4^{ab}	110 ± 16^{c}	0.13 ± 0.05^{b}	135 ± 48^{b}	140 ± 32^{a}	68.4 ± 5.5^{b}	0.35 ± 0.08	1897 ± 629^{c}
'Gem'	15.1 ± 1.8^{ab}	123 ± 15^{bc}	0.17 ± 0.06^{ab}	209 ± 50^{a}	96 ± 31^{b}	81.9 ± 8.3^{a}	0.30 ± 0.04	4354 ± 1556^{ab}
'Hass'	15.7 ± 2.5^{ab}	131 ± 20^{ab}	0.16 ± 0.07^{ab}	185 ± 56^{ab}	110 ± 35^{ab}	83.4 ± 8.8^{a}	0.32 ± 0.07	2696 ± 665^{bc}
'XX3'	13.6 ± 1.3^{b}	109 ± 11^{c}	0.19 ± 0.09^{a}	234 ± 49^{a}	82 ± 30^{b}	80.7 ± 6.4^{ab}	0.36 ± 0.01	4672 ± 2283^{a}
F-value	3.04	6.12	3.42	7.22	6.44	5.80	1.24	2.26
p-value	<0.01	<0.0001	<0.005	<0.0001	<0.0001	<0.0001	0.302	<0.05

Trait relationships

There was a strong relationship between $\delta^{13}C$ and WUE_i demonstrating the utility of $\delta^{13}C$ as a time-integrated proxy for WUE_i (Figure 1.2). Measurements of $\delta^{13}C$ and WUE_i were significantly correlated with g_s, C_i and *LA:SA* (Figure 1.3), but not with A_{area}, or A_{mass} (Table 1.2), indicating that variability in $\delta^{13}C$ is more strongly correlated to variability in leaf water loss than photosynthetic rate (Table 1.2). Enrichment in ^{13}C resulted in varieties that were more water-use efficient whereas varieties with high values of C_i and g_s were relatively depleted in ^{13}C (Figures 1.2 and 1.3). g_s was strongly linked to $\delta^{13}C$, given its negative relationship with WUE_i. Values for *LA:SA* were strongly related to WUE_i and $\delta^{13}C$, indicating that varieties that have a greater leaf area with a given amount of sapwood are less conservative in their water use. Finally, C_i had a positive association with g_s ($r^2 = 0.87$; $p < 0.001$) and *LA:SA* ($r^2 = 0.56$; $p < 0.032$) demonstrating a relationship among gas exchange, water loss and morphology.

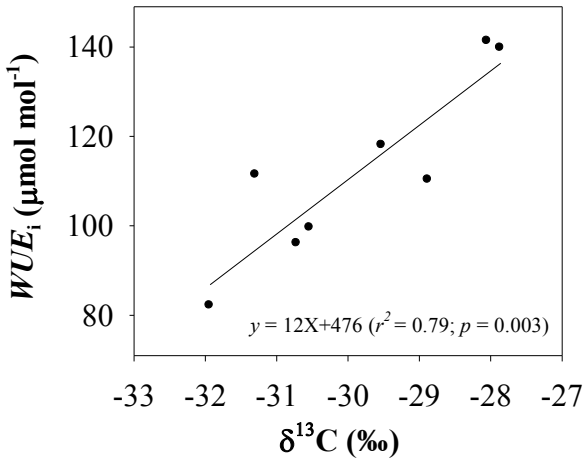

Figure 1.2 Relationship between intrinsic water-use efficiency (WUE_i) and leaf carbon isotope composition ($\delta^{13}C$) for eight avocado varieties growing at South Coast Research and Extension Center, Irvine, California, USA.

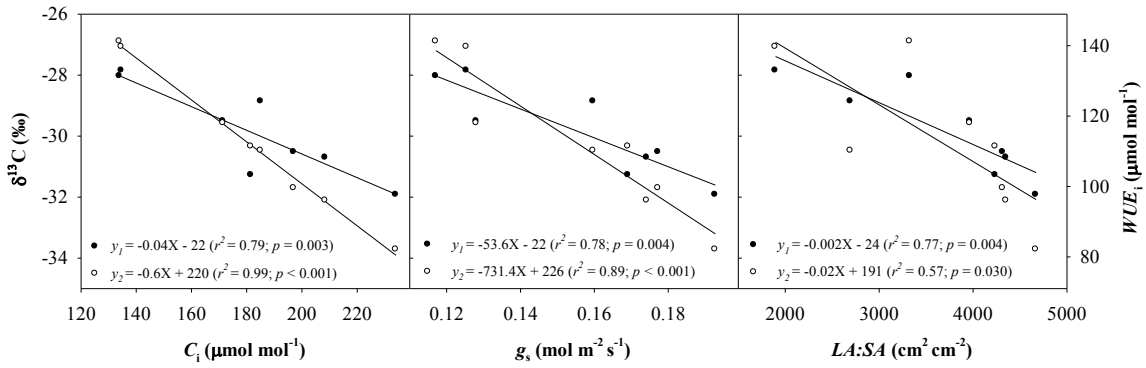

Figure 1.3 Relationships between leaf carbon isotope composition ($\delta^{13}C$) and intrinsic water use efficiency (WUE_i) with intercellular CO_2 concentration (C_i); stomatal conductance (g_s); and leaf sapwood area ratio ($LA:SA$); for eight avocado varieties growing at South Coast Research and Extension Center, Irvine, California, USA. Close symbols represent $\delta^{13}C$ values whereas open symbols represent WUE_i values.

Discussion

Our results indicate that WUE_i, as well as several key underlying functional traits, showed strong variation among varieties of avocado investigated in this study. The nature of functional trait relationships that describe photosynthetic gas exchange behavior in avocado are generally consistent with patterns found in natural vegetation at global scales (Farquhar et al., 1989; Maire et al., 2015; Wright et al., 2004). Our results also demonstrate the link between leaf $\delta^{13}C$ and WUE_i described by standard photosynthetic gas exchange measures and its utility in identifying varieties that have the potential for efficient photosynthetic productivity while conserving critical water resources (Farquhar et al., 1982; Farquhar and Richards, 1984a). Yet, we also show significant relationships of $\delta^{13}C$ with functional traits beyond the leaf, indicating that considering coordination between leaf and stem functional traits that describe hydraulic supply and transpiring area provides enhanced understanding of how stem hydraulic traits constrain leaf performance

(Pivovaroff et al., 2014). This implies that the functional trait approach is successful at evaluating the physiology and ecology of important crop species, analogous to the way that functional traits have been used to place wild species from natural ecosystems along axes of ecological strategy variation (Ackerly, 2004; Cornelissen, 1999; Westoby et al., 2002). Thus within a particular crop species, different varieties can spread out along these axes and certain traits that are relatively easily measurable can be used to inform managers where along these ecological axes different varieties fall.

Whereas most functional trait approaches to date have been directed at wild plants in natural ecosystems, it is clear that such approaches also represent a powerful tool for identifying varieties with particular behavior regarding carbon and water economy (Brugnoli et al., 1988; Farquhar and Richards, 1984a; Hubick and Farquhar, 1989; Meinzer et al., 1990). Because many of the trait relationships reflect trade-offs between high rates of resource consumption and fast growth on one end of the spectrum and low rates of resource consumption and slow growth on the other (Reich et al., 1997), it was not clear how this would play out within a species that has been subject to purposeful breeding by humans for productivity and yield. Thus, our findings that within a species, functional traits of varieties align with known biophysical and metabolic constraints on leaf physiological function to produce patterns that are broadly analogous to global patterns on wild plants, illustrate the generality of these approaches and potential for selecting nuanced tendencies in resource use in agriculture, forestry or ecological restoration.

Because WUE_i is a composite variable based on both photosynthetic income (A) and rates of concomitant water loss (g_s), it is important to consider what factors control $\delta^{13}C$ to improve isotopic interpretation in other systems. We found that $\delta^{13}C$ was strongly correlated with g_s, but not A_{area}. There was 1.2-fold variation in values for A_{area} and 1.6-fold variation in g_s, suggesting that the greater range in stomatal behavior contributed to its strong relationship with $\delta^{13}C$ (Figure 1.2). These findings are critical for calibration and interpretation of $\delta^{13}C$ values in other studies, as carbon isotopes are used to re-construct past climates and vegetation types and as monitors of ecological change (Dawson and Siegwolf, 2007; Graham et al., 2014). Thus, incorporating leaf $\delta^{13}C$ into trait assemblages for determining aspects of carbon and water economy represents a promising avenue for comparisons within a species, consistent with the original use for comparing WUE_i among wheat genotypes (Farquhar and Richards, 1984a).

One of the most striking results of our study is that varieties with high *LA:SA* are less water-use efficient. This was shown with both long-term integrated and instantaneous measurements of *WUE*, yet this is somewhat counterintuitive because varieties with more leaves supplied by a given xylem area show more profligate water use. This result is likely related to varieties with a high *LA:SA* supporting high rates of water transport through high stem sapwood-specific hydraulic conductivity, as shown in woody plants from natural ecosystems (Pivovaroff et al., 2014). High values for *LA:SA* can also signify greater self-shading by leaves, which can affect radiation load and boundary layer conditions influencing photosynthesis and evaporative demand and

therefore rates of leaf water loss (Ackerly, 1999). Yet, even with greater self-shading, there is still significantly lower WUE_i in varieties with high *LA:SA*.

All of the traits measured have potential to be used in screening and selection of varieties of avocado that use water efficiently and can withstand drought. Traits that are relatively easy to measure, as $\delta^{13}C$, *SLA* and *WD*, would be of great aid in assessing large accessions of varieties, consistent with their use to characterize physiological processes for wild plants. Although the way these traits perform in predicting physiological processes varies across sites and species (Wright et al., 2005), our results indicate that within a single site and species they have potential to characterize nuanced physiological variation, and place varieties or genotypes along known axes of ecological strategy variation for resource-based comparison. With such large variation in key ecological traits in relatively limited environmental conditions, we also expect that these traits show greater variation among more wide-ranging environmental conditions than within a single managed agroecosystem. The importance of variation in these traits in identifying suitable varieties is likely more critical across sites than within this common garden experiment.

We found that variation in *WUE* exists across varieties of avocado and that certain varieties such as 'Carmen', 'UC05-1' and 'AO.48' are particularly water-use efficient. This knowledge can be used to improve efficiency of water use as water resources decline in quantity, quality, or become more expensive, and is promising to practitioners, but also raises further questions about its implementation in managed plant systems. First, though $\delta^{13}C$ is good indicator of long-term integrated *WUE*, short-term climatic

perturbations such as the El Niño Southern Oscillation or episodic drought may produce effects that must be quantified using short-term physiological measurements, such as photosynthetic gas exchange. Thus, functional trait approaches that bridge disparate time scales are most likely to reveal key processes. Second, whereas high *WUE* with respect to carbon gain per unit water loss is relatively straightforward to evaluate using functional traits, the more important metric may be fruit yield per unit water loss, so combining short-term functional trait campaigns with long-term data on yield or growth from growers or land managers has the potential to add other dimensions to conclusions based on functional traits alone. Finally, because many agricultural species have been strongly bred for high productivity, in many cases it is not clear what stress-tolerance or efficiency traits are still fixed within the organism (Milla et al., 2014). Therefore, striking a tailored balance between efficiency, stress tolerance, productivity and yield appears to be a critical challenge for agriculture as availability of water and nutrient inputs decline.

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Chapter 2

The response of ‘Hass’ avocado to salinity as influenced by rootstock

Abstract

With increasing population demands for water, drought, and extreme temperatures worldwide, agricultural production is challenged with reduced water availability and lower water quality. Salinity, which is associated with low water quality is a critical issue for California avocado growers and, coupled with avocado root rot, threatens the long-term sustainability of the industry since avocado (*Persea americana* Mill.) is known to be extremely sensitive to salinity. Salt tolerance of the 'Hass' variety, the most commonly grown scion in California, is influenced by rootstock. We investigated 'Hass' scions grafted onto three different avocado rootstocks under control (0.50 dS/m) and salinity (1.5 dS/m) conditions. Results indicated that salinity affected survival, productivity and physiological performance of avocado trees. Survival rate was 100% in the control treatment, but varied in the salinity treatment from 43% to 67%. After 13 months, fruit yield was greater in control trees than for trees in the salinity treatment, with yield responses to salinity varying among rootstock varieties. Salinity affected the efficiency of photosystem II and caused reductions in photosynthetic rate and water-use efficiency. Leaf water potential was not affected by salinity treatment confirming that poor performance of treated trees was attributable to chloride accumulation reported in previous studies and not physiological drought. Overall, our results show a coordination between the physiological performance, health and productivity of the 'Hass' scion and how the negative effects of salinity on these parameters is influenced by rootstock.

Introduction

Water deficit limits plant growth and crop yield more than all other stresses combined (Kramer, 1983). The freshwater resources needed for agricultural irrigation are limited in some areas, and availability is expected to decrease with predicted drying trends associated with climate change (Field, 2014). Agriculture is a major consumer of water throughout the world (Mendelsohn and Dinar, 2003; Viala, 2008), and during water shortages, supply of high quality water for agriculture cannot always be guaranteed (Gordon et al., 2010; Rosegrant et al., 2009). When water is in short supply, the use of water with increased dissolved solids is often the only option for continued irrigation, but salinity in irrigation water is known to reduce crop growth and yield, or cause outright mortality of crops (Maas and Hoffman, 1977; Munns and Tester, 2008). Thus drought and salinity represent challenges for agriculture that are linked; both are natural phenomena and their intensity is worsened by human activities (McWilliam, 1986). As the human population is expected to surpass nine billion by 2050, combined with the declining availability of new agricultural land, it is critical to both understand the mechanisms of salinity responses of crops and to evaluate new varieties for increased salinity tolerance.

There is great variation in the types of salts that produce salinity, but the general physiological effects in plants are well documented (Allakhverdiev et al., 2000; Munns and Tester, 2008; Shabala and Munns, 2012). Much of the study of salt stress has been conducted on plants by considering aspects of plant performance such as height or yield as a function of environmental salinity concentration (Maas and Hoffman, 1977).

However, it is important to consider that the effects of salinity within the plants Salt affects metabolism through uptake, transmembrane movement, compartmentalization, and feedbacks to growth and carbon assimilation, plant nutrient status and hormone homeostasis (Cheeseman, 1988). Among the many responses, osmotic imbalance and ion toxicity due to the accumulation of Na^+ and Cl^- are the first signs of salt stress, however, it is becoming clear that osmolyte biosynthesis and function, water flux control, and membrane transport of ions are critical components of maintenance and re-establishment of ionic balance (Hasegawa et al., 2000). In *Arabidopsis* the isolation and molecular characterization of genes involved in plant salt stress responses have been elucidated, especially in the identification of genes that regulate ion selectivity, transport and accumulation of Na^+, H^+, K^+, Cl^- and Ca^+. There is also increasing evidence that stress sensing and signaling components play important roles in regulating plant salinity stress responses, as well as novel ion transport, detoxification pathways, and the impact of epigenetic chromatin modifications on salinity tolerance (Deinlein et al., 2014; Golldack et al., 2011; Hanin et al., 2016; Yang et al., 2009). This information is increasingly being used to develop salinity tolerant varieties of key crops for incorporation into agriculture. Potassium ion accumulation in roots, for example, has been shown to increase salt tolerance in wheat and barley (Cuin et al., 2012; Wu et al., 2015) and a Na^+ transporter gene can improve grain yield in wheat (Munns et al., 2012). Germplasm screening for salt tolerance, as well as crop improvement programs using marker assisted selection as a breeding tool are part of the approaches to address the salinity problem in agriculture (Ashraf and Foolad, 2013).

Traditionally, the sensitivity of crop plants to salinity has been measured as the relative yield as a function of the electroconductivity (EC) measured in deciSiemens per meter (dS/m). In agriculture, the highest quality of water has EC values lower than 0.25 dS/m however, farmers commonly use water with EC values within a range of 0–3 dS/m (Ayers and Westcot, 1985). Avocado is known to be extremely sensitive to salinity; yield begins to decline at 0.75 dS/m with chloride concentrations > 100 ppm and the general recomendation is to maintain a 10%-20% leaching fraction to keep EC_{sw} lower than 2.0 dS/m (Crowley, 2008; Maas, and Hoffman, 1977; Mickelbart et al., 2007). With increasing water demands and droughts, avocado growers are faced with both reduced water availability and lower water quality. Salinity is a critical challenge for avocado growers and, coupled with avocado root rot, threatens the long-term sustainability of the industry. For example, since the 2009 California water management regulations (California Department of Water Resources, 2009; State of California, 2009), water allocated to agriculture has been reduced or become more expensive, affecting water supply to avocado orchards in Orange, Riverside, Santa Barbara, San Bernardino, San Diego and Ventura Counties. Since avocado is not adapted to very hot and dry climates, avocado growers have sometimes "stumped" or completely removed groves (Medellín-azuara et al., 2012; Spann, 2014). In other cases, growers were supplied with low quality or reclaimed water with EC ranging 1–2 dS/m, associated with increased concentrations of dissolved solids, which can cause accumulation of toxic elements in soil, leading to accumulation in leaves, stomatal closure, reduced productivity and soil salinization (Branson and Gustafson, 1971; Grattan et al., 1997).

Specific studies on salinity responses of avocado have shown that salt sensitivity of avocado is influenced by rootstock selection. Oster and Arpaia (1992) found that rootstock affects fruit weight and health of 'Hass' avocado trees exposed to saline water. Chloride toxicity is correlated with reduction in yield and survival rates and the ability of the rootstock to exclude Cl^- and/or Na^+ from stems and leaves in avocado, such that new growth becomes the primary mechanism of salinity tolerance (Celis, 2016; Mickelbart and Arpaia, 2002; Oster et al., 2007). Rootstock also affects leaf area and biomass accumulation in leaves and stems of avocado trees exposed to a salinity treatment, which suggests that leaf biomass production per branch could be a good predictor of salinity tolerance on avocado (Bernstein et al., 2001). Mickelbart et al. (2007) analyzed the effect of salinity in the tissue-ion concentration of 'Hass' avocado trees grafted onto different rootstocks. Low Cl^- concentration and reduced Na^+:K^+ ratios in old leaves represent good markers to identify the ion exclusion ability of avocado rootstocks as well as salinity tolerance (Mickelbart et al., 2007). Little is known regarding the relationship between physiological performance, survival and yield in avocado trees under salinity conditions and how rootstock influences these factors. We investigated the physiological performance of 'Hass' avocado scions grafted onto a select group of rootstocks. Our main objectives were to: 1) evaluate the relative effects of salinity on productivity of selected rootstocks grafted with 'Hass'; 2) determine effects of salinity on photosynthesis and plant water relations; and 3) identify the effect of salinity on leaf-scale metrics of water-use efficiency.

Materials and Methods

Study site and experimental design

This study was conducted at the University of California Agricultural Experiment Station-Citrus Research Center, Riverside, California, USA (Parcel 13-C; 33.9737°N, 117.3281°W), administered by the Department of Agricultural Operations. The mean annual temperature is 18.6 °C and ranges from a monthly mean of 11.7 °C in January to 24.9 °C in August. Mean annual precipitation is 280 mm and the soil is classified as an Arlington fine sandy loam, Haplic Durixeralf (Saito et al., 2006).

In April 2011, rootstocks from California, USA and South Africa with purported resistance to Phytopthora (*Phytothphora cinnamomi* Rands) were grafted with 'Hass' scions and planted at a spacing of 3.4 × 6.4 m (11 × 21 ft.) and allowed to grow for 2 years and 8 months. The trees were randomly assigned a position and planted in 18 rows. Beginning in November 2013, selected rows were transitioned to salinity incrementally until the full salinity treatment was implemented in January 2014. The rows were randomly selected with salinity treatments originating from a 10X tank of saline water that was diluted using a Mazzei injector (Mazzei, Backersfield, California). The remaining rows were irrigated with standard well water from the Gage Canal. The salinity treatment recipe used the standard well water as a base and added salts that mirrored the composition of Colorado River water, a typical source of water for some areas of California. The recipe was as follows: $CaCl_2$ (1.738 g/L), $MgCl_2$ (1.517 g/L), $NaSO_4$ (4.965 g/L), KNO_3 (0.063 g/L), KCl (0.008 g/L) and NaCl (0.241 g/L). A 3,000-gallon tank was used for making and storing the 10X saline water. An EC meter was used

to adjust the electrical conductivity (EC) which ranged 0.5–0.67 dS/m with chloride at 175 ppm. The water for the control treatment was from the Gage Canal, which serves as the irrigation water for the research station. The EC of standard (control) irrigation water ranged 0.5–0.67 dS/m with chloride at ~40 ppm. The salinity treatment started with a 75% dilution of salts and increased incrementally until it reached the full salinity treatment in January 2014. The application of the salinity treatment lasted until summer 2015. Trees were irrigated using 24 L/hour micro-sprinklers with two micro sprinklers per tree. Output and EC concentration for the salinity treatment was measured for each irrigation using and EC meter. Both salinity and control treatments were irrigated simultaneously two times per week during the cool season (October–April) and three times per week from May through September. We calculated the duration of irrigation based on the crop coefficient ($K_c = 0.55$) (Allen et al., 2005), daily evapotranspiration (ET_o) and leaching fraction (LF) data obtained from the California Irrigation Management System (CIMIS, http://www.cimis.water.ca.gov/), and an online Irrigation Scheduling Calculator (http://www.avocadosource.com/).

In this study, we reported data collected during 2015 even though the salinity treatment began in November 2013. During December 2014, the leaching fraction, EC and chloride concentration rose in places where control trees were planted, permitting salt accumulation into the root zone (Celis et al., 2016). In 2014, leaching fraction for November and December were ~18% and 73% respectively. EC was 4.33 dS/m and 8.22 dS/m in control and salt treated rows, respectively, and finaly, chloride concentration was 4.46 mmolc L^{-1} and 11.55 mmolc L^{-1} for the control and the saline treatment,

respectively. In 2015, corrections in the leaching fraction were made in order to reduce ion accumulation in the soil caused by leaching problems. In June 2015, leaching fraction-$EC_{1:1}$ was 10% and 22% and EC was 3.2 dS/m and 4.1 dS/m for control and saline treated trees. Chloride concentration was 6 $mmoL_c\ L^{-1}$ and 10.07 $mmolL_c\ L^{-1}$ for soils where control and saline treatment trees were maintained, respectively. (Celis et al., 2016).

This study was focused on three rootstocks. Avocado rootstocks 'R0.05' (experimental) and 'Dusa' (commercially available) are from South Africa, whereas 'PP40' is an experimental selection from the University of California rootstock breeding program. Sample sizes varied between six to nine individual trees per rootstock in the salinity treatment and between five to eight individual trees per rootstock in the control treatment, depending on variety.

Plant survival and fruit production

Survival of experimental trees was calculated as the number of surviving individuals divided by the number planted × 100% for each rootstock variety 18 months after the start of the treatment in 2013. At the same time, canopy damage was measured as percent of canopy with necrotic leaves. In February 2015, trees were harvested and total number of fruit and yield (kg fresh weight of all fruit) per tree were recorded. Using control trees as a reference, percent reduction in number of fruit and yield, and percent of canopy damaged by salinity was calculated for trees irrigated with saline water. To determine the average fresh weight of individual fruit (Fruit-FW), total yield was divided

by the total number of fruit per tree. Effects of salinity on fruit maturity were tested by determining fruit dry weight (Fruit-DW) according to Arpaia et al. (2001). Fruit-DW was determined post-harvest by coring the flesh from the fruit, recording fresh mass and then evaporating water from the cut pieces in a microwave oven until constant mass.

Physiological measurements

All physiological measurements were performed in summer 2015. Photosynthetic gas exchange was measured using a portable photosynthesis system (LI-6400, Li-Cor Biosciences, Lincoln, Nebraska, USA), on mature, fully expanded leaves of the most recent flush for each experimental tree. In the control treatment, we measured two leaves per tree. In the salinity treatment, we separated leaves into classes based on leaf burn, a brown region of dry necrotic leaf tissue emanating from the leaf tip, and measured two leaves per tree that were fully green (FG) and two leaves per tree that were partially burned (PB). We measured and compared physiological traits in both types of leaves to identify differences between them and compared them with the leaves from the control trees. Photosynthetic measurements were taken at 25 °C controlled by integrated Peltier plates, 1500 μmol m^{-2} s^{-1} of photosynthetically active radiation supplied by a red/blue light source (Li-Cor 6400-02B, Li-Cor Biosciences), with CO_2 concentration maintained at 400 μmol mol^{-1}. Measurements were conducted from shortly after sunrise until 10:00 h, before stomatal closure, so that maximum rates of photosynthetic CO_2 assimilation per unit leaf area (A_{area}), transpiration (E), stomatal conductance to water vapor (g_s) and internal CO_2 concentration (C_i) could be determined. Intrinsic water-use efficiency

(WUE_i) was calculated as A_{area}/E. Leaves measured with the portable photosynthesis system were harvested to determine specific leaf area (*SLA*). *SLA* was calculated as leaf area (cm^2) measured with a leaf area meter (LI-3100; Li-Cor Biosciences) divided by dry mass (g), after drying leaves at 65 °C for 48 h. *SLA* was used to calculate maximum rate of CO_2 assimilation per unit mass (A_{mass}). Leaf mass per area (*LMA*) was calculated as 1/*SLA*. The dark-adapted maximum quantum yield of photosystem II (F_v/F_m) was measured to monitor photosynthetic energy conversion, using a portable pulse amplitude modulated fluorometer (*Mini-PAM*, Heinz Walz GmbH, Effeltrich, Germany) at predawn on three leaves on each experimental tree. Leaves were exposed to modulated weak far-red irradiance, followed by exposure to a 0.8-s saturating flash (2,000 - 3,000 $\mu mol\ m^{-2}\ s^{-1}$) of actinic white light. To maintain a constant distance and angle (60°) relative to the leaf plane, the fiber-optic probe that delivered the measuring beam and saturating pulse was mounted above the leaf with a leaf clip holder (2030-B, Heinz Walz GmbH). Leaf water potential at predawn (Leaf $\Psi_{predawn}$) and midday (Leaf Ψ_{midday}) was measured with a pressure chamber (1001, PMS Instruments, Albany, Oregon, USA). Leaves were cut and immediately placed inside the chamber with the cut end exposed. Pressurized N_2 gas was gradually added to the chamber until sap exited the cut end as viewed with a lighted magnifying glass. This balancing pressure was taken as equal to bulk leaf water potential.

Leaf carbon isotopic composition

We used bulk leaf carbon isotopic composition ($\delta^{13}C$) as a measure of long-term integrated water-use efficiency. This technique is based on the observation that conditions causing plants to reduce stomatal aperture cause an increase in water-use efficiency and also a reduction of CO_2 concentration at the site of carboxylation, forcing Rubisco to assimilate more $^{13}CO_2$ (Farquhar and Richards, 1984b). Thus, larger $\delta^{13}C$ values are interpreted as greater water-use efficiency (Cernusak et al., 2013). $\delta^{13}C$ has become an important tool for comparing water-use efficiency among agricultural varieties, including wheat, barley and cowpea (Farquhar and Richards, 1984b; Hall et al., 1990; Hubick and Farquhar, 1989), and more recently avocado (Acosta-Rangel et al., 2018). Twenty sun-exposed and fully expanded leaves, from control trees and FG leaves from treated trees, but not PB leaves, were sampled from terminal branches that were not fruiting or flushing in October 2015. Samples were weighed, oven dried at 105 ^{o}C for 24 hours until completely dry and then ground using a mortar and a pestle. Subsamples of 0.5 mg ± 0.05 (dry wt) leaf tissue were loaded into tin capsules. Values of $\delta^{13}C$ were determined with a stable isotope ratio mass spectrometer (Isoprime Ltd., Cheadle, United Kingdom). Isotopes are reported in per mil (‰) relative to the standard VPDB (Vienna Pee Dee Belemnite), and verified with EDTA and USGS40 as working standards, which have $\delta^{13}C$ values of –32.24 and –26.39‰, respectively.

Data analysis

Statistical analysis was performed using R software. The data were tested for normality using a Shapiro-Wilk test and homoscedasticity using Levene's test. Averages of each variable were calculated to compare the effect of rootstock and salinity using one-way nested ANOVA, with Tukey post-hoc tests for parametric variables and Kruskal-wallis post-hoc tests for non-parametric variables with significance of $p < 0.05$. Pearson product-moment analysis was performed to identify correlations among all variables.

Results

Effect of salinity on the survival rate and yield of 'Hass' avocado grafted to different rootstocks

The salinity treatment produced a progressive health decline to avocado trees over time that resulted in canopy damage ($p < 0.001$) and reductions in survival rate ($p = 0.0153$) and production ($p < 0.05$) (Table 2.1). In contrast, the three rootstock varieties had no effect on survival nor health over the 'Hass' scion ($p > 0.05$). The canopy damage induced by the saline treatment increased by 42%, 48% and 43% for 'R0.05', 'PP40' and 'Dusa', respectively, compared to control trees, which also had a percent of damage inflicted by heat (Table 2.1). In terms of tree survival, 100% of the trees from the control treatment survived regardless of rootstock, whereas, 33% of trees grafted in 'R0.05' or 'PP40' and 57% of trees grafted in 'Dusa' died under the salinity treatment. Salinity also reduced the productivity of all avocado trees independently of rootstock. The number of fruit per tree ranged from 43 to 53 in control trees and decreased in all trees exposed to

salinity (11–29 fruit/tree), representing an average reduction of 63% compared to the control. Similar results were found for yield, in which control trees produced 5.9–7.3 kg of fruit/tree and trees under the salinity treatment produced 1.5–2.8 kg of fruit/tree with a mean reduction of 68% compared to the control (Table 2.1, Figure 2.1). In contrast, no effect of rootstock on fruit maturity was detected ($p > 0.05$).

Table 2.1. Canopy damage, survival rate, reduction in number of fruits/tree and yield reduction/tree of 'Hass' avocado scions grafted onto different rootstocks after 13 months of salinity treatment. All trees in control treatment survived.

Rootstock varieties	Damage in canopy (%)			Survival (%)[z]	Reduction in number of fruit/tree (%)	Reduction in kg fruit/tree (%)
	Control	Saline treatment	Difference			
'R 0.05'	15	57	42	67	45	60
'PP 40'	12	69	48	67	74	75
'Dusa'	31	74	43	43	69	68
Average	20	64	44	54	63	68

[z] From Celis (2016)

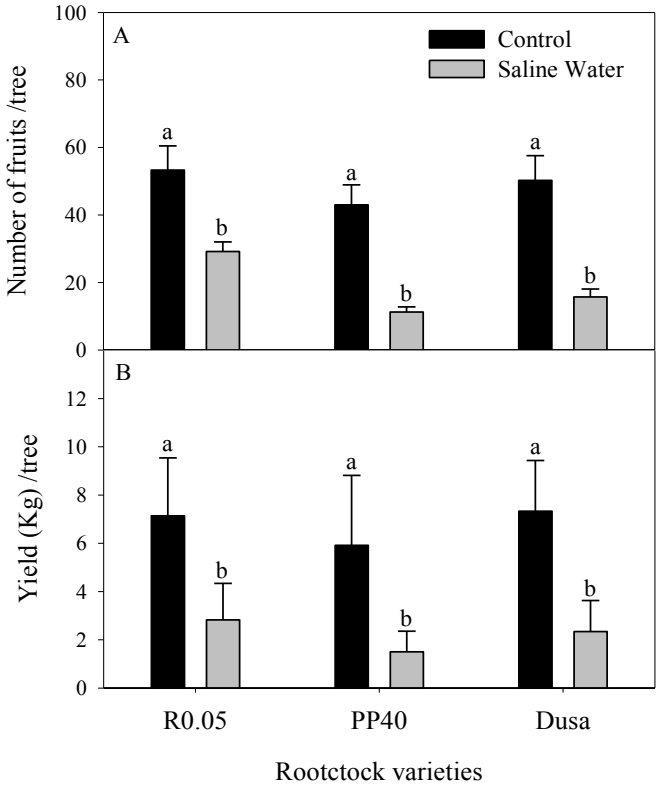

Figure 2.1. Number of fruit (A) and yield (kg) (B) produced by trees grafted onto different avocado rootstocks. Bars represent mean ± SE of control (black) treatment (EC = 0.50–0.75 dS/m) and salinity (light gray) treatment (EC = 1.5 dS/m). Different letters shared by the bars indicate significant differences within rootstocks at $p < 0.05$.

Effect of salinity on the physiological performance of 'Hass' *avocado scions grafted on different rootstocks*

Salinity negatively affected 'Hass' avocado scion physiological performance (Table 2.2). In most cases, the fully green (FG) leaves of trees in the salinity treatment showed similar physiological values as the leaves of control trees, but the partially burned (PB) leaves in the salinity treatment showed significantly reduced physiological values. Values for A_{area}, A_{mass} and F_v/F_m, were statistically similar for control and FG leaves,

whereas PB leaves had a significantly lower values ($p < 0.001$, Figure 2.2). Leaf A_{area} for the control, FG and PB leaves averaged 15.28 ± 0.92, 12.35 ± 0.74 and 5.95 ± 1.5 μmol m^{-2} s^{-1}, respectively. PB leaves had a 50% reduction of carbon uptake compared to FG leaves, which were both subjected to the same salinity treatment. Leaf A_{mass} in control, FG and PB leaves averaged 142.87 ± 17.28, 128.15 ± 8.71 and 59.11 nmol g^{-1} s^{-1}, respectively, and similar to A_{area}, PB leaves had more than a 50% reduction in A_{mass} compared to FG leaves. F_v/F_m in control, FG and PB leaves averaged 0.80 ± 0.02, 0.78 ± 0.04 and 0.53 ± 0.07, respectively. The F_v/F_m values of PB leaves were far below 0.75, considered the minimum value for healthy leaves (Figure 2.2). Avocado rootstock varieties had no significant effect on these physiological traits ($p > 0.05$, Table 2.2).

Table 2.2. Effect of salinity treatment and avocado rootstocks on 'Hass' avocado leaf physiological traits. Numbers in bold type represent *p*-values with significance at an alpha of 0.05.

Variable	Test	Normality	Homoscedasticity	Treatment	Rootstock	Interaction (Treatment x Rootstock)
A_{area}	Anova	0.676	0.860	**<0.001**	0.088	0.917
A_{mass}	Anova	0.159	0.312	**<0.001**	0.099	0.777
F_v/F_m	Kruskal-Walis	0.6243	0.018	**<0.001**	0.946	-
g_s	Anova	0.080	0.776	**0.028**	**<0.001**	**0.015**
E	Anova	0.568	0.093	**<0.001**	0.052	0.115
C_i	Kruskal-Walis	0.015	0.426	**0.010**	**<0.001**	-
WUE_i	Anova	0.983	0.328	**<0.001**	**<0.001**	0.279
$\delta^{13}C$	Anova	0.069	0.050	**<0.001**	**<0.001**	0.055
LMA	Anova	0.299	0.439	0.093	0.281	0.332
Leaf $\Psi_{predawn}$	Anova	0.514	0.492	0.212	0.417	0.652
Leaf Ψ_{midday}	Anova	0.214	0.243	0.096	0.657	0.519

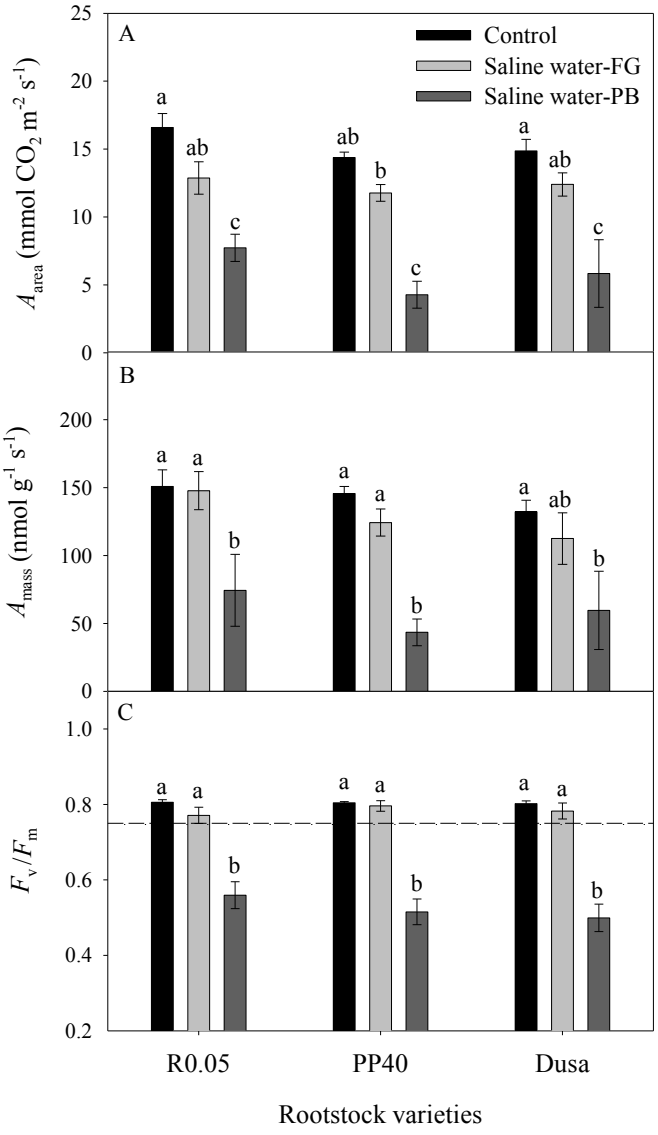

Figure 2.2. Net photosynthetic rate per unit leaf area (A), net photosynthesis per unit mass (B) and maximum quantum yield of PSII (C) in leaves of 'Hass' scion grafted onto different avocado rootstock varieties. Different colored bars represent mean ± SE of control (black) treatment (EC = 0.50–0.75 dS/m) and salinity treatment (EC = 1.5 dS/m) of both fully green (FG) (light gray) and partially burned (PB) (dark gray) leaves. Different letters shared by the bars indicate significant differences at $p < 0.05$.

Water relations in avocado leaves were also affected by the salinity treatment, however, the responses were modulated by rootstock variety (Table 2.2). For each rootstock, control and FG leaves had similar values for g_s, E, WUE_i and C_i ($p < 0.05$), whereas PB leaves had variable responses. In 'PP40', for example, PB leaves had a reduction in g_s and E greater than 50% relative to the control and FG leaves. 'R0.05' and 'Dusa' also had a significant reduction of ~50% in WUE_i for PB leaves compared to control leaves, but contrastingly ~2-fold greater values in C_i. Under the control conditions, a natural variation was found among the rootstock varieties regarding water relations. 'PP40' had ~2-fold greater rates of g_s compared to 'R0.05' and 'Dusa' (g_s = 0.29 ± 0.04, 0.147 ± 0.06 and 0.128 ± 0.06 mol H_2O m^{-2} s^{-1}, respectively). C_i was also ~2-fold greater in 'PP40' compared to 'R0.05' and 'Dusa' (C_i = 284 ± 2, 154 ± 24 and 140 ± 20 mm CO_2 m^{-2} s^{-1}, respectively) (Figure 2.3). Under salinity treatment, FG leaves from the 'R0.05' rootstock had significantly greater water-use efficiency compared to 'PP40', likely due to high rates of stomatal conductance in control and FG leaves of 'PP40'. The general low performance of PB leaves remained similar across the rootstocks.

Analysis of the effects of salinity and rootstock on other physiological traits, like LMA and leaf water potential, did not show significant differences among treatment or rootstocks varieties ($p > 0.05$) (Table 2.2). Leaf $\Psi_{predawn}$ ranged from –0.11 to –0.21 MPa and leaf Ψ_{midday} ranged from –0.92 to –1.38 MPa (Figure 2.4), showing that water was available and being used by treated trees.

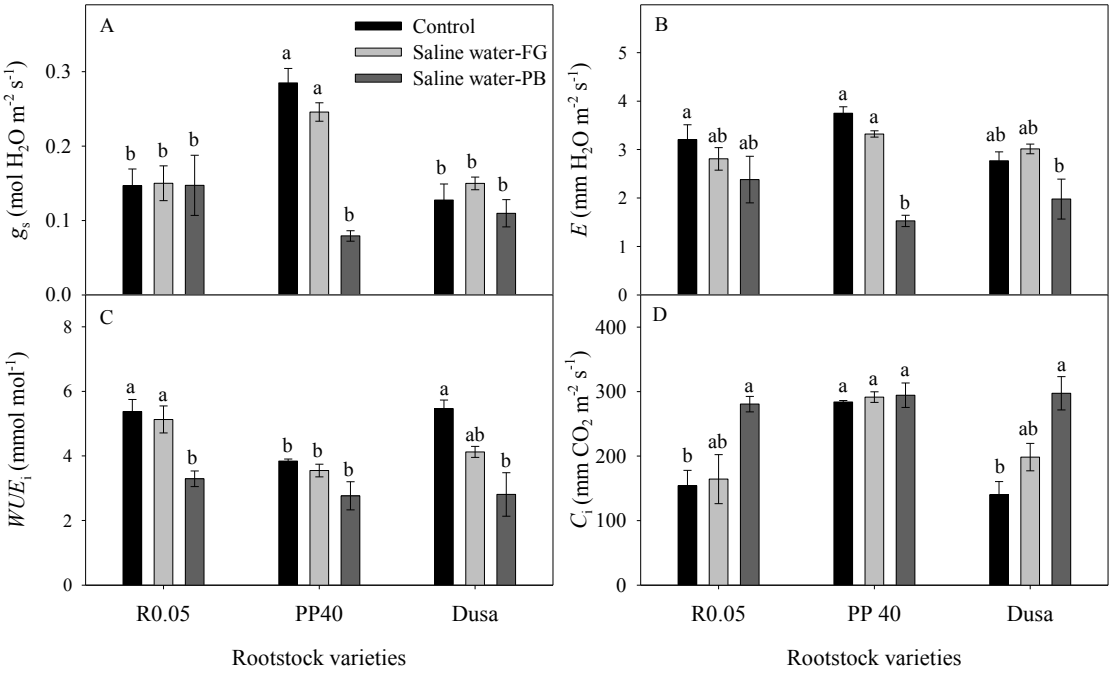

Figure 2.3. Stomatal conductance (A), transpiration (B), water-use efficiency (C) and internal CO_2 concentration (D) in leaves of 'Hass' scions grafted on different avocado rootstock varieties. Different colored bars represent mean ± SE of control (black) treatment (EC = 0.5–0.75 dS/m) and salinity treatment (EC = 1.5 dS/m) of both fully green (FG) (light gray) and partially burned (PB) (dark gray) leaves. Bars with different letters are significantly different across rootstocks at $p < 0.05$.

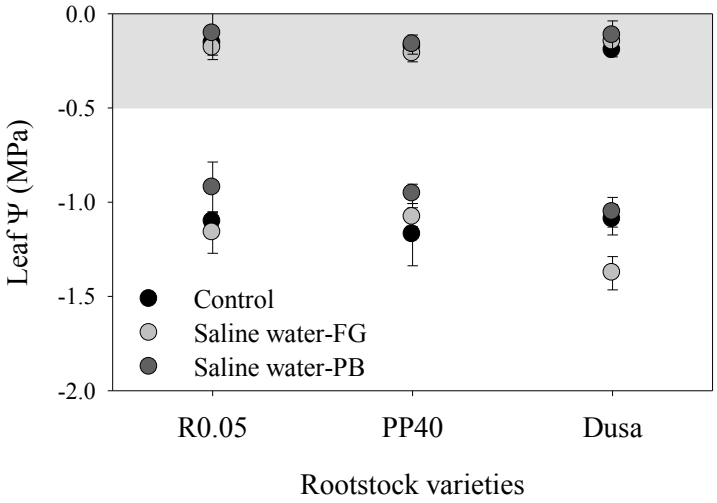

Figure 2.4. Leaf $\Psi_{predawn}$ (shaded area) and Leaf Ψ_{midday} (clear area) of 'Hass' scions grafted onto different avocado rootstock varieties. Different colored symbols represent mean ± SE of control (black) treatment (EC = 0.55–0.75 dS/m) and salinity treatment (EC = 1.5 dS/m) of both fully green (FG) (light gray) and partially burned (PB) (dark gray) leaves. No differences between treatments or among rootstocks were found ($p > 0.05$).

Effect of salinity on leaf carbon isotopic composition

The analysis of carbon isotopic composition showed significant differences among treatments and rootstock varieties ($p < 0.001$, Table 2.2). The salinity treatment increased $\delta^{13}C$ in FG leaves compared to leaves from control trees in 'Dusa' and 'PP40', but not in 'R0.05' (Figure 2.5).

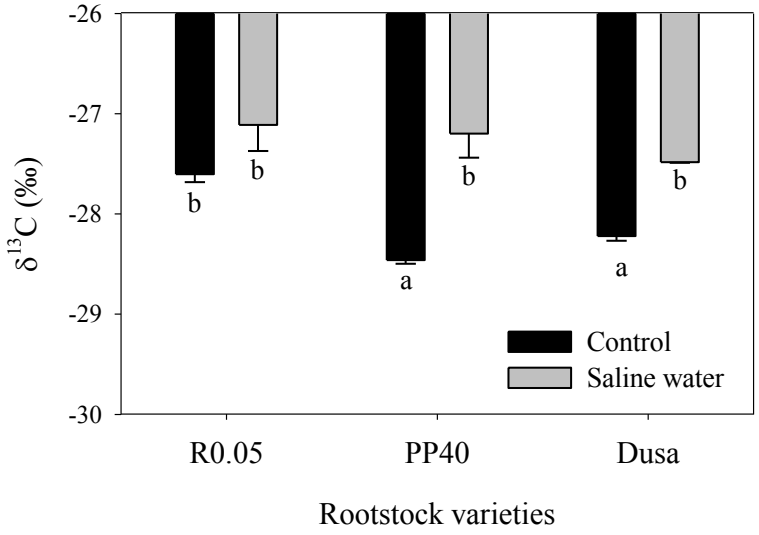

Figure 2.5. $\delta^{13}C$ in leaves of 'Hass' scion grafted on different avocado rootstock varieties. Different colored bars represent mean ± SE of control (black) treatment (EC = 0.505–0.75 dS/m) and salinity treatment (EC = 1.5 dS/m) of fully green (FG) leaves (light gray). Bars with different letters are significantly different across rootstocks at $p < 0.05$.

Integration of traits that promote scion productivity

Physiological traits in avocado trees were correlated with survival rates and production. A_{mass} was positively correlated with survival ($r = 0.84$, $p < 0.038$). High A_{mass} values belonged to leaves from the control treatment, in which all trees survived, and low values of A_{mass} belonged to leaves from trees under salinity treatment, in which the survival rate was reduced to nearly 60% on average. A_{area} values were positively correlated with greater production in terms of number of fruit ($r = 0.95$, $p < 0.004$) and yield ($r = 0.94$, $p < 0.005$). The correlation between physiological traits showed a coordination between carbon assimilation and water movement where high photosynthetic rates and photosynthetic yield were associated with higher transpiration (*r*

= 0.87, $p < 0.002$) and water-use efficiency ($r = 0.82$, $p < 0.015$), and low internal CO_2 concentration ($r = –0.73$, $p < 0.026$). Water potential was not correlated with any other traits and Pearson correlation values ranged from –0.64 to 0.44 ($p > 0.05$). Surprisingly, $\delta^{13}C$ was not associated to WUE_i or any other physiological trait.

Discussion

This field-based salinity trial on established avocado plants identified varying levels of salinity tolerance in rootstocks when plants were irrigated with 1.5 EC water as compared to the control (0.57 EC). Survival and yield were related to photosynthetic rate, suggesting that the reduction in carbon assimilation from leaves in salinity treatments contributed to reductions in yield and increases in mortality. In general, rootstock varieties showed similar behavior with regards to carbon uptake but 'PP40' had poor stomatal control that reduced its water-use efficiency. Physiological results indicated that damaged partially burned (PB) leaves present in the canopies of 'Hass' scions on all rootstocks had a reduction in carbon assimilation and a loss of stomatal control under salinity treatment. Because all three rootstock varieties investigated herein have been previously verified as root rot tolerant the survival and yield of 'Hass' scions in this study should make these three rootstocks key candidates for further trials and incorporation into commercial growing operations. Overall, the results are promising for identifying potential germplasm material for future breeding projects and further investigations of the underlying mechanisms and points of control for genetic improvement of avocados in California.

Previous studies on avocado and citrus traits have shown variation in the performance of scions grafted with different rootstock varieties (Bañuls et al., 1990; Mickelbart and Arpaia, 2002). Celis (2016) measured leaf ion concentrations in the same trees as in the present study, finding that avocado trees with the highest levels of survival rate and yield also had the lowest leaf sodium and chloride concentrations. The most vigorous rootstock varieties were chosen for testing salinity tolerance in this study to understand what characteristics potentially contribute to maintaining output in salinity conditions. The results of this study indicate that reduced canopy damage and good stomatal control mitigate the effect of salinity on avocado production.

One of the most striking results of our study is the consistent statistical relationship that A_{area} showed with survival and yield. These data suggest that the carbon income from photosynthesis promotes survival and yield and that the reduction in photosynthetic carbon income, due to canopy damage in the salinity treatment, contributes to poor performance. The reduction in photosynthetic rate in the scions of rootstock varieties that performed relatively well in salinity treatments is consistent with a number of studies on reduced gas exchange under salinity conditions (Ball and Farquhar, 1984; Ishikawa et al., 1991). In PB leaves, there was a strong reduction in F_v/F_m, below the 0.75 value that is considered healthy for leaves (Bolhar-Nordenkampf et al., 1989). Therefore, damage to photosystems II is a component of the reduction in carbon gain of PB leaves. However, for FG leaves, F_v/F_m values were similar to that of control leaves, indicating that any reduced photosynthetic rates resulted from tighter stomatal control of gas exchange rather than damage to the photosystem or a change in

carboxylation efficiency (Santiago et al., 2000). Therefore, if it is a consistent pattern that FG leaves in salinity treatment are as healthy as they look, it may be possible to visually assess the degree of salinity stress and potential effects on survival and yield by measuring the percent of damage in the canopy. Furthermore, leaf water potential was not affected by the treatment and the stomatal closure in the salinity treatment probably helped to conserve the plant water status and reduce ion accumulation. The role of changing water status in salinity-induced mortality could have had a greater role in varieties that suffered complete mortality that were outside of the vigorous varieties chosen in this study.

Because nearly all commercial production of avocados in California uses 'Hass' scions, there have been relatively few recent studies on variation in scion physiological performance. However, one recent study showed that among 24 avocado scions, there was 2-fold variation in WUE_i (Acosta-Rangel et al., 2018), and that much of this variation was related to differences in leaf sapwood area ratio (*LA:SA*), g_s and C_i. *LA:SA* ratio varied up to 2.5-fold in the amount of leaf area supported by a given cross-sectional area of sapwood. Plant species or varieties that tend to maintain lower *LA:SA* ratio, g_s and C_i tend to be more conservative and show higher WUE_i, indicating that there may be further ways to overcome salinity by combining successful rootstocks with the right scion.

Overall, 'R0.05', 'PP40' and 'Dusa' performed well considering the conditions of the experiment. These three rootstocks had the highest survival rate, yield and toxic ion exclusion among 13 avocado rootstocks reported by Celis (2016), in a study performed simultaneously to the present study. During the first year of the saline treatment, there

was a significant salinity stress due to accumulation of salts and a weather event that resulted in salts moving into the root zone rather than being leached past the root zone. During the time of these measurement, leaching fraction had been adjusted; however, the trees had not fully recovered, which could explain low yield in salinity treated rows despite of canopy recovery. Taken together, the results suggest that 'Hass' scions on 'R0.05', 'PP40' or 'Dusa' would perform uniformly under the levels of salinity currently encountered in avocado-growing areas in California and globally and may be able to tolerate anticipated near-term increases in salinity in irrigation and reclaimed water available to growers. With the appropriate leaching fraction, these rootstocks could outperform other rootstocks grown under saline conditions.

To conclude, an average of 60% loss in productivity using irrigation water with 1.5 dS/m reveals the sensitivity of avocados to salinity. The physiological responses of the trees under salt stress provide an indication of how well the trees might do as the quality of water for agriculture worsens. Currently, California growers use water with EC > 0.75 dS/m, but the threshold of water quality to prevent yield reduction in avocado is considered to be EC = 0.75 dS/m (Oster et al., 2007). Future screenings for salinity tolerant rootstocks are required to improve yield when poor quality soil or water is used.

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Chapter 3

Effects of temperature, soil moisture and light intensity on the temporal pattern of floral gene expression and flowering in avocado (*Persea americana* cv. 'Hass')

Abstract

Low temperature stress is well known to promote flowering in adult (competent) *Persea americana* trees. Other environmental stresses, such as reduced soil moisture and limited light intensity, are suggested to have a similar effect. However, documentation of environment-floral gene interactions associated with floral development in *P. americana* is limited. 'Hass' avocado trees (3.5 years from budding) were maintained under optimal growing conditions (OGC) for 5 months before the experiment was initiated in mid-July. Subsets of trees were subjected to three different environmental stresses, low temperature (LT), the positive flowering control, low soil moisture (LSM) or low light intensity (LLI) for 8 weeks followed by OGC for 6 weeks or maintained exclusively under OGC for 14 weeks. Only LT-treated trees flowered (week 14). Bud expression profiles of orthologs of *Arabidopsis thaliana* flowering genes, *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), *APETALA1/FRUITFUL* (*AP1/FUL*), *APETALA2* (*AP2*), *APETALA3* (*AP3*), *PISTILLATA.1* (*PI.1*), *AGAMOUS.1* (*AG.1*) and *AGAMOUS.3* (*AG.3*), were quantified in avocado trees over time. At the start of the experiment, *LFY*, *AP1/FUL*, and *AP2* were strongly expressed in buds of all trees, whereas transcripts of *FT* and the floral organ identity genes *AP3, PI.1* and *AG.1* were at detectable levels, and those of *AG.3* were not detected. By week 8 of LT treatment, bud expression of *AP1/FUL* and *LFY* increased to levels significantly greater than those of trees in all other treatments, with the exception that LSM also increased *LFY* expression, but not *AP1*/*FUL*, to that of LT-treated trees and greater than that of LLI- and OGC-treated trees. Two weeks after transfer of LT-treated trees to OGC, *FT* expression increased relative to week 8 to a level significantly

greater than trees in all other treatments, followed by activation of the downstream floral organ identity genes *AP3*, *PI.1, AG.1* and *AG.3* by week 12 (2 weeks before flowering). In contrast, for trees in the LSM, LLI and OGC treatments, bud expression of *FT*, *AP3*, *PI.1* and *AG.1* remained low or at the limit of detection, with *AG.3* below the limit of detection through week 12; these trees did not flower. Taken together, the results suggest that the floral induction process was initiated by July, but only LT up regulated bud expression of both *LFY* and *AP1/FUL*, and subsequently *FT*, sufficiently to activate the downstream floral organ identity genes and result in flowering. The results further demonstrated that the significantly greater bud expression levels of *LFY* and *AP1/FUL* in week 8 of LT treatment were sufficient to confer bud determination, since transfer of trees from LT to the warm temperature of the OGC did not prevent flowering. The fact that bud expression of *FT*, *AP3*, *PI.1*, *AG.1* and *AG.3* did not occur until after transfer of the LT-treated trees to OGC suggests a possible failsafe mechanism to synchronize flowering with the warmer temperatures of spring.

Introduction

The ‘Hass’ variety, a Mexican x Guatemalan hybrid of *Persea americana* (Mill.), dominates the worldwide avocado industry (Chanderbali et al., 2008). Flowering is the critical first step in fruit production. For ‘Hass’ avocado, yield is directly proportional to floral intensity; the more flowers, the more fruit (Garner and Lovatt, 2008). Thus, understanding the regulation of floral development in ‘Hass’ avocado trees might lead to strategies for improving yield and mitigating alternate bearing (Chaikiattiyos et al., 1994; Nevin and Lovatt, 1989; Ziv et al., 2014). Under the tropical conditions of its origin (Berg and Ellstrand, 1986; Scora et al., 2002; Smith, 1966), flowering in adult (competent) *P. americana* trees is day neutral, low intensity, and sporadic, being largely dependent on shoot age through a presumed endogenously (developmentally) regulated autonomous flowering pathway (Salazar-García et al., 1998). Fruit developing from multiple blooms vary in age, making it difficult to harvest a large number fruit of uniform maturity at any one time. In semi- and subtropical ‘Hass’ production areas, winter environmental conditions, which include low temperature, reduced soil moisture content and/or low light intensity, synchronize flowering into a single, high intensity spring bloom, resulting in uniform fruit maturity and a condensed harvest period (Buttrose and Alexander, 1978; Chaikiattiyos et al., 1994; Nevin and Lovatt, 1989).

Low temperature is well documented to promote flowering in *P. americana* (Buttrose and Alexander, 1978; Nevin and Lovatt, 1989; Salazar-García et al., 1999), but the potential roles of reduced soil moisture and low light intensity stress in avocado flowering, individually or as factors supplementing low temperature, remain unclear

(Buttrose and Alexander, 1978; Chaikiattiyos et al., 1994). In horticultural woody evergreen species, like *Citrus* spp*,* coffee (*Coffea arabica*), mango (*Mangifera indica*), loguat (*Eriobotrya japonica*), carambola (*Averrhoa carambola*) and Litchi (*Litchi chinensis*), water stress promotes flowering (Crisosto et al., 1992; Fernández et al., 2009; Nakajima et al., 1992; Núñez-Elisea and Davenport, 1994; Southwick and Davenport, 1986; Stern et al., 1993; Wu et al., 2017). However, in an experiment testing the effect of reduced soil moisture content on avocado flowering, water-deficit stress stopped vegetative shoot extension growth but failed to promote flowering (Chaikiattiyos et al., 1994). As a native cloud montane forests species (Williams, 1976), it was hypothesized that low light intensity during winter conditions might contribute to flowering in *P. americana*, although there was no evidence to support this idea (Butrose and Alexander, 1978). Reducing light intensity by 50% combined with a low temperature treatment did not significantly affect flowering (Buttrose and Alexander, 1978). However, the independent effect of low light intensity stress on flowering of 'Hass' avocado has not been tested. Given the limited number of reports in the literature investigating the role of environmental factors in promoting flowering in 'Hass' avocado, it is clear that further research is required, especially research to identify environment-floral gene interactions that regulate avocado floral development.

The influence of endogenous versus environmental factors on the transition of the shoot apical meristem (SAM) from vegetative to reproductive development and the time at which this occurs in 'Hass' avocado remains equivocal. Under California growing conditions, weekly anatomical analysis of apical buds in 'Hass' avocado trees provided

evidence that the transition from a vegetative to floral SAM started at the end of vegetative shoot elongation (July-August). The time of phase transition was identified by the change in the shape of the primary axis meristem (PAM) from convex to flat to convex in the apical buds (Salazar-Garcia et al., 1998). In another study, foliar-applied gibberellic acid (GA_3) was used to elucidate 'Hass' avocado bud anatomy associated with bud determination (irreversible committment to floral development) (Salazar-García et al., 1999). When applied before the SAM is determined, GA_3 reduces flowering by sustaining continued vegetative development of the SAM. However, after the SAM is determined, GA_3 has no effect on floral development. The results using GA_3 documented that buds committed to floral development can be recognized when three or more secondary axis inflorescence meristems have developed, making this criterion an effective biomarker for bud determination. Using this biomarker, irreversible commitment to floral development was documented to occur from the end of October through November in California (Salazar-García and Lovatt, 1998, Salazar-García et al., 1999), suggesting the low temperatures of autumn into winter might regulate bud determination.

Similarly, the expression patterns of *FT, LFY* and *AP1/FUL* genes in buds of 'Hass' avocado growing in Israel provided evidence that *LFY* and *AP1/FUL* were expressed at low levels in August, with significantly increased expression during late October through November, concurrent with increased *FT* expression in leaves (Ziv et al., 2014). The anatomical analysis showed the formation of two secondary axis inflorescence meristems in apical buds occurred at the end of November and preceded the

accumulation of *FT* transcripts in buds, which began in mid-December and peaked in late January (Ziv et al., 2014). Results of additional research appeared to rule out the potential transport of *FT* RNA from leaves into the buds. The authors concluded that the low temperatures of early winter committed 'Hass' avocado buds to floral development (Ziv et al., 2014), suggesting a potential role for *LFY*, *AP1/FUL* and/or *FT* in bud determination.

The function of floral genes has been extensively studied in the model plant *A. thaliana*, but orthologs in other species may or may not have the same function. In *A. thaliana*, *FT* is the key gene regulating phase transition (Müller-Xing et al., 2014), whereas the subsequent expression of the down stream floral meristem identity genes, *LFY* and *AP1*, targets of *FT*, is the first indication that the SAM has been successfully induced to flower (Blazquez et al., 2006; Sablowsky, 2007). As a member of the Lauraceae, *P. americana* is a basal angiosperm (noncore eudicot) documented to have *AP1/FUL* gene(s) that is/are orthologous to both: *AP1* and *FUL* genes of species from the core eudicots group (Litt and Irish, 2003). Whether the function of these genes in *P. americana* is conserved remains to be determined. The *AP1/FUL* family includes examples of both gene functional conservation and divergence. In the genus *Papaver*, for example, *AP1/FUL* (reported as *FUL*-like) genes have functions of core eudicot *AP1* and *FUL*, whereas *AP1/FUL* (reported as *FUL*-like) genes in *Aquilegia* species (Ranunculales) exhibit some but not all functions of the eudicot *AP1* and *FUL* genes (Pabón-Mora et al., 2013). In *P. americana*, *AP1/FUL* is interesting in that it is expressed in the carpel. This suggsets that at least some functions of avocado *AP1*/*FUL* might be

similar to *A. thaliana,* but also suggest functions different from other basal angiosperms, in which *AP1/FUL* is expressed in the carpel (Chanderbali et al., 2006).

The flower of *P. americana* is characteristic of basal angiosperms, with the first and second whorls comprising an undifferentiated perianth of similar petaloid tepals and multiple whorls of stamens, including staminodes, which surround a single carpel (Blanke and Lovatt, 1992; Chanderbali et al., 2006, 2009). The expression patterns of the genes that specify floral organ development in *P. americana* are broad and overlapping compared to the highly specific floral organ identity programs of *A. thaliana* described by the ABC model of floral organ specification. In the ABC model, A function genes *AP1* and *AP2* specify sepals, A function genes plus the B function genes *AP3* and *PI* specify petals, B function genes plus the C function gene *AG* (antagonistic to the A-function genes) specify stamens, and the C function gene alone specifies the carpel (Bowman et al., 1991; Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). In the *P. americana* flower, *AP1/FUL* is expressed in both whorls of tepals and in the stamens, with homologs of *AP3* and *PI.1* (B function genes) also expressed in the two whorls of tepals and the stamens (Chanderbali et al., 2006, 2009; Soltis et al., 2007). Three *AG* homologs (putative C function genes) were identified in *P. americana*, with *AG.1* and *AG.2* expressed in outer and inner tepals, stamens and the carpel and *AG.3* expression restricted to stamens and the carpel (Chanderbali et al., 2006, 2009). In *P. americana*, the very thorough analysis of orthologs of *A. thaliana* floral organ identity genes associated with the avocado flower (Chanderbali et al., 2006, 2008, 2009; Soltis et al., 2007a, b, 2009) has yet to be integrated with the expression of the upstream genes with floral timing

(promoter, integrator) function, *FT* and *LFY* (Lee and Lee, 2010; Moon et al., 2005; Parcey, 2005), or that function in floral mersitem identity, *LFY* and *AP1/FUL* (Bowman et al., 1991; Parcey, 2005; Ratcliffe et al., 1999; Siriwardana and Lamb, 2012), and no studies have examined the environmental regulation of the expression of avocado floral organ identity genes.

The overall goal of the research presented herein was to identify the potential regulatory role of known flowering genes in floral development in *P. americana*. The first objective was to quantify the temporal pattern of expression of *FT*, *LFY*, *AP1/FUL*, *APETALA* 2 (*AP2*), and floral organ identity genes, *AP3, PI.1, AG.1*, and *AG.3*, in buds of 'Hass' avocado trees under (1) low temperature stress conditions known to promote significant flowering and (2) under optimal growing conditions that sustain vegetative shoot growth. Using low temperature stress as the positive control, the second objective was to quantify interactions between the environment and floral gene expression in buds of 'Hass' avocado trees subjected to two additional environmental stresses, low soil moisture content and low light intensity, relative to the capacity of each stress to promote flowering. The results reported herein are the first to quantify the effects of different environmental factors (stresses) on floral gene expression in *P. americana* related to inflorescence number and thus, are the first to report environment-floral gene interactions that do and do not result in flowering. The results of this research provide new insight into the sequence and timing of floral gene activity leading to flower formation in *P. americana*.

Materials and methods

Plant material and treatment conditions

Adult 'Hass' avocado trees (3.5 years from budding onto clonal Duke 7 [Mexican race] rootstocks), produced by Brokaw Nursery, Ventura, California, and grown in 12-liter plastic tubes containing steam-sterilized University of California soil mix (Baker, 1957), with all flowers and fruit removed to prevent negative effects on floral gene expression and flowering (Ziv et al., 2014) were used in this research. For the 5 months prior to the initiation of the experiment, the trees were maintained under optimal growing conditions (30 °C 14-h day/20 °C 10-h night photosynthetic active radiation [PAR] > 900 μmoles m^{-2} sec^{-1}, irrigated with 1200 mL/day to maintain a soil volumetric water content [VWC] between 20% to 25%, relative humidity was approx. 80%) in a temperature/humidity controlled glasshouse, with supplemental lighting to maintain 14-h days, at the Citrus Research Center and Agricultural Experiment Station of the University of California, Riverside. The experiment was initiated on 15 July, during the flush of summer vegetative shoot growth and buds were vegetative (Stage 1 of the developmental scale of Salazar-García et al., [1998]). At this time, trees were randomly assigned to four treatments, which included three different environmental stresses imposed for 8 weeks (other than the factor changed to create a stress, the growth conditions were same as OGC), followed by transfer of the trees from the stress condition to OGC for the final 6 weeks of the 14-week experiment: (1) low temperature (LT) control, 14 °C 10-h day/10 °C 14-h night (Percival PGW growth chamber; 2.3 x 1.5 x 2.0 m; Percival, Boone, IA) (Nevin and Lovatt, 1989); (2) low soil moisture (LSM), soil volumetric water content 8%

to 12% maintained by deficit irrigation (600 mL water every 3 days); (3) low light intensity (LLI), PAR < 130 μmoles m^{-2} sec^{-1} using black net shade cloth to reduce light intensity 85%); and (4) optimal growth conditions (OGC) (no stress) for 14 weeks (Table 3.1). All trees were fertilized equally for the 5 months prior to and during the 14-week experiment. Data loggers (Campbell Scientific CR1000, Logan, UT) were used to monitor environmental conditions. To determine treatment effects on tree water status, midday leaf water potential (Ψ_{midday}) was measured every 2 weeks with a pressure chamber (1001, PMS Instruments, Albany, OR).

Table 3.1. Growth conditions for 'Hass' avocado trees subjected to 8 weeks of low temperature (LT), low soil moisture (LSM), or low light intensity (LLI) and then transferred to optimal growing conditions (OGC) for 6 weeks or maintained under OGC for 14 weeks.

Treatment	Temperature day/night (C°)	Photoperiod day/night (hours)	Soil moisture (% VWC)	Light intensity (μmol m^{-2} s^{-1})
Low temperature (LT)	14/10	10/14	20–25	> 900
Low soil moisture (LSM)	30/20	14/10	8–12	> 900
Low light intensity (LLI)	30/20	14/10	20–25	< 130 (~85% light reduction)
Optimal growing conditions (OGC)	30/20	14/10	20–25	> 900

Bud sample collection, RNA isolation and cDNA synthesis

For each treatment, the distal five buds from 10 nonbearing shoots from three trees (replications) per sample date were collected at week 4, 8, 10 and 12 from a total of 12 trees per treatment; a composite sample (three biological replications) was collected from all trees at the start of the experiment (week 0). Collected samples were placed

between moistened paper towels inserted into aluminum bags inside labeled plastic bags, which were sealed, placed in a cooler box and transported to the laboratory (~5 minutes). In the laboratory, the shoots, with leaves removed, were immediately frozen in liquid nitrogen and stored at -80 ^{o}C until used for RNA extraction. Total RNA was extracted from bud tissue, previously ground in liquid nitrogen, using Isolate Plant RNA Mini Kit (Bioline USA Inc., Taunton, MA). The RNA quality and quantity were analyzed by spectroscopy using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA). For cDNA synthesis, 1 µg of total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) to eliminate any DNA contamination. First-strand cDNA synthesis was performed using a Tetro cDNA Synthesis Kit (Bioline USA Inc., Taunton, MA) with oligo (dT) primer in a 30-µL reaction at 42 ^{o}C for 60 min according to the protocol of the manufacturer.

PCR primer design and amplification efficiency

The sequences of *A. thaliana* homologs *FT*, *LFY*, *AP1/FUL*, *AP2*, *AP3*, *PI*, *AG* and *ß-ACT* in *P. americana* were obtained from the reference sequence database of the National Center for Biotechnology Information ([NCBI, http://www.ncbi.nlm.nih.gov), except *AP2* in *P. borbonia*, which was obtained from the 1,000 Green Plant Transcriptome Project, University of Alberta, Canada (ONEKP, http://www.onekp.com) (Table 3.2). Two sets of primers for *P. americana FT* (Ziv et al., 2014) and *LFY* (Chanderbali, personal communication) were used in this research. The seven additional

primer sets were designed using the website PrimerQuest Tool from Integrated DNA Technologies Company (http://www.idtdna.com/primerquest/Home/Index). For primer design, the following filters were used: melting temperatures (Tm) of 60 to 62 °C; primer lengths of 18 to 24 bp; and amplicon lengths of 150 to 297 bp. The annealing temperature and concentration of the primer sets were optimized for Quantitative Real-time PCR (qPCR) to efficiencies within the range 87% to 115%. The size and sequence of the amplicon products were verified by 2% (w/v) gel electrophoresis and sequence analysis provided by the Institute for Integrative Genome Biology, University of California, Riverside. The DNA sequence of each amplicon was compared with its respective target gene sequence in *P. americana* using BLAST (NCBI web page) and ClustalW (Geneious Software, version 10.2.3) (Table 3.2).

Quantitative real-time PCR analysis

Quantitative real-time PCR was carried out using a C1000 Touch™ thermal cycler (Bio-rad Laboratories, Hercules, CA) with the CFX96 Touch™ real-time PCR detection system. The final reaction volume was 18 µL containing 100 ng of RNA in 2 µL, 0.6 µL of gene-specific forward and reverse primer mix (10 nM), 9 µL of SensiMix™ SYBR & Fluorescein (2X) mix (Bioline USA Inc., Taunton, MA), and 6.4 µL of PCR-grade water. Each reaction was run at 95 ˚C for 10 minutes followed by 40 cycles of 95 ˚C for 10 seconds and 60 ˚C for 1 minute. Melt-curve analysis ranging from 60 to 95 ˚C was performed at the end of each qPCR run to confirm that nonspecific products were not formed. Only Cq values less than 35 were used to calculate the relative

expression levels (fold change) of the target genes using the Pfaffl method (Pfaffl, 2001) with 'Hass' avocado flowers collected from orchard trees at full bloom as the control (expression level of 1) and *β-Actin* (*ACT*) (Table 3.2) as the reference gene (endogenous control). Relative expression values reported herein for *FT*, *AP3*, *PI.1*, *AG.1* and *AG.3* are low due to the significant expression of these genes in the avocado flowers used as the control (expression level of 1); average Cq values for these genes averaged between 23.8 and 31.0. Gene expression data for each treatment were the means of three biological replications; each biological replication was the mean of three qPCR technical replications.

Treatment effects on bud development

The fate of the distal five buds from six (nonbearing) shoots per tree (30 buds/tree) for each of six trees (replications) for each of the four treatments was quantified at weeks 0, 12 and 14. The developmental stage of each bud was identified according to the classification of Salazar-García et al. (1998). No shoots were collected from these trees to prevent changes in the fate of the bud. Results for the five distal buds on six shoots per tree were averaged for the six individual trees (replications) per treatment and reported as the average value per tree.

Statistical analysis

All statistical analysis was performed using R software version 3.4.3 (The R Foundation, Vienna, Austria). All data were tested for Linear model assumptions using

Kolmogorov–Smirnov and Levene tests. For variables related to bud fate, a general linear model with Poisson correction was used to determine the effect of treatments for a given week on the stage of bud development and number of floral shoots, vegetative shoots and inactive (quiescent) buds per tree. Significant differences were considered with a family error rate of $\alpha \leq 0.05$. Post-hoc comparisons were performed using a pairwise t-test with Bonferroni adjustment. Relative expression data were transformed using $\log_{10}$ function in order to obtain a symmetrical distribution. Analysis of variance (ANOVA) was used to compare the effect of treatments within a week and across time (weeks) for a given treatment. When ANOVA testing indicated significant differences at $\alpha \leq 0.05$ for equal variances, Duncan's multiple range test (DMRT) was performed to identify differences between treatments and weeks, respectively. When ANOVA testing indicated significant differences at $\alpha \leq 0.01$ for unequal variances, the non-parametric Kruskal-Wallis test was used to identify differences between treatments and weeks, respectively. Data were back transformed for presentation in Tables 3.4 and 3.5. Relative gene expression levels < 0.005 are report as detected (D) in Table 3.5. These data were transformed using $\log_{10}$ function and included in the statistcial analysis presented in Table 3.4 and Table 3.5. Pearson's product-moment correlation coefficients were calculated to identify significant relationships ($r > 0.5$, $p < 0.05$) between floral shoot number and the relative expression level of each gene in a given week, as well as the relationships among the relative expression levels of all genes over time.

Table 3.2. Forward and reverse primers for target floral genes and *ß-ACT* of 'Hass' avocado used in the quantitative real-time PCR (qPCR) assay

Annotation	Accession number (*Species*)	Source	Forward primer (5' to 3') Reverse primer (5' to 3')	Product size (bp)	PCR product sequence blast against target gene sequence		Primer Efficiency
					E-value	Identity	
FT	GenBank[z]: KM023154.1 (*P. americana*)	Ziv et al., 2014	TCCGGGGTGGCGTCAGAACT TCTCCGGCTGTCGTCGGACT	142	5E-50	98%	1.99
LFY	GenBank: FD502004.1 (*P. americana*)	DePamphilis et al., 2008 AGGB[y]	GCAGCGTGAACATCCCTTCATTGT TGGATCAAGAACTCCCTGCACTGT	114	5E-60	100%	1.97
AP1/FUL	GenBank: DQ398019.1 (*P. americana*)	Chanderbali et al., 2006	CATTCACCATCCTTGCTACTG GAGCACCTACTTCCTCTTCT	105	9E-21	100%	1.99
AP2	ONEKP[x]: WIGA-2009052 (*P. borbonia*)	Matasci et al., 2014	GGCCCAAGTAGACGTATTTC TCGACAAAGTACCGGATTTC	122	5E-27	97%	2.06
AP3	GenBank: AY337748.1 (*P. americana*)	Kim et al., 2004	TGCGAGCATTGGAAGGAA GCATGGTTGGATGCAGAAAG	130	1E-13	90%	2.01
PI.1	GenBank: AY337738.1 (*P. americana*)	Kim et al., 2004	CAGATGGAGTTCTTAAGGGCACTC GATATTTGCTGCTGATGCAA	88	4E-38	99%	1.97
AG.1	GenBank: DQ398021.1 (*P. americana*)	Chanderbali et al., 2006	AGAACGCAAACAGGCATCTG CTACTGATGCCTTTCTCCAATCT	98	1E-13	87%	1.97
AG.3	GenBank: DQ398023.1 (*P. americana*)	Chanderbali et al., 2006	GCACTCCAGCTAGGATGATAAA CTAGGAACTGCAGCCTTCAA	109	4E-13	95%	1.99
ß-ACT	GenBank: GU272027.1 (*P. americana*)	Dahan et al., 2010	AACATTGTGCTTAGCGGTGGTTCC TCCACATCTGTTGGAAGGTGCTCA	183	3E-78	96%	1.99

[z] NCBI GenBank and Reference Sequence databases (www.ncbi.nlm.nih.gov).
[y] Ancestral Angiosperm Genome Project (AGGB).
[x] 1000 Green Plant Transcriptome Project (ONEKP) (www.onekp.com).

Results

Effects of temperature, soil moisture and light intensity on floral development

At the start of the experiment (week 0), all apical and axillary buds were vegetative (Stage 1 of the developmental scale of Salazar-García et al. [1998]) (Fig. 3.1A). During the first 4 weeks of the experiment, shoot terminal buds of trees in all treatments continued the extension growth of vegetative shoots. Differences due to treatment effects on shoots and leaves that developed during this period were visible (Fig 3.2). Shoot extension and leaf expansion was slower under low temperature (LT) and leaves were small and less mature than leaves of trees in the three other treatments at week 4 (Fig. 3.2A). Leaves of trees under LLI were larger than those of trees in the other treatments (Fig. 3.2C). Developing shoots and young leaves of trees in the LSM treatment showed symptoms of water-deficit stress (Fig. 3.2B). At week 4, the terminal buds of trees in all treatments remained vegetative (Stage 1) (see inserts in Fig. 3.2). Prior to week 8, vegetative shoot extension growth had ceased for trees in all treatments. By week 8, the end of the stress treatments, terminal buds of LT-treated trees were at Stages 4 to 5 of inflorescence development, with separated bud scales evident; the four proximal axillary buds were at earlier stages of floral development (Fig. 3.1 and 3.3A). For 8-week LSM-treated trees, terminal and axillary buds were vegetative (Stage 1) (Fig. 3.3B). For the 8-week LLI- and OCG-treated trees, terminal buds were at Stages 1 to 2; axillary buds remained closed, pointed, vegetative and quiescent (inactive) (Fig. 3.3C-D). By week 12, four weeks after transfer of the trees from the stress treatments to OGC, the majority of the terminal buds of LT-treated trees were at the cauliflower stage of

inflorescence development (Stage 8); axillary buds were at Stages 5 and 6 (Fig 3.1. and 3.4A and B). The terminal buds of LSM-, and LLI- and OGC-treated trees were at Stages 1 to 2; axillary buds were quiescent (Fig. 3.4C-E). Maximum bloom occurred in week 14 only on trees subjected to 8 weeks of LT treatment; the trees from the other treatments never flowered. By week 14, 78% of the 30 buds analyzed per LT-treated tree produced floral shoots, all of which were indeterminate, 6% less than in week 12 due to abscission of inflorescences primary and secondary axes between weeks 12 and 14. In addition to producing floral shoots, 0.03% of the four proximal axillary buds produced vegetative shoots and 4.7% remained quiescent (inactive). For LSM-, LLI- and OGC-treated trees, terminal buds remained quiescent at Stages 1 to 2; proximal axillary buds were small and quiescent. In addition, buds that failed to produce floral shoots did not undergo bud break and did not produce new vegetative shoot growth (Table 3.3).

Consistent with previous reports that the low temperature treatment used in this research promoted floral development (Nevin and Lovatt, 1989; Salazar-García et al., 1999), flowering in LT-treated trees was independent of water-deficit stress. For LT-treated trees, soil volumetric water content (VWC) was approximately 20% from weeks 2 through 8 of treatment, resulting in leaf midday water potentials > –1.0 MPa during this period, likely due to reduced transpiration under low temperature (Fig. 3.5A-B). In contrast, trees in the LSM treatment were subjected to 8% to 12% soil VWC during weeks 2 through 8 and had significantly reduced leaf midday water potential to ≤ –2.0 MPa (Fig. 3.5A-B). Although considered a moderate degree of water-deficit stress (Chaikiattiyos et al., 1994), leaf necrosis and abscission and shoot tip browning was

visible for young developing leaves and shoots of LSM-treated trees after 4 weeks of treatment (Fig. 3.3B). Mature tissues were not damaged, but abscission of these older leaves occurred. There remained, however, many shoots with healthy viable buds. For trees in LLI and OGC treatments, soil VWC was ≥ 20%, with leaf midday water potentials between –1.0 and –1.5 for weeks 0 through 10. Thus, failure of trees in the LLI and OGC treatments to flower was not due to the negative effect of water-deficit stress.

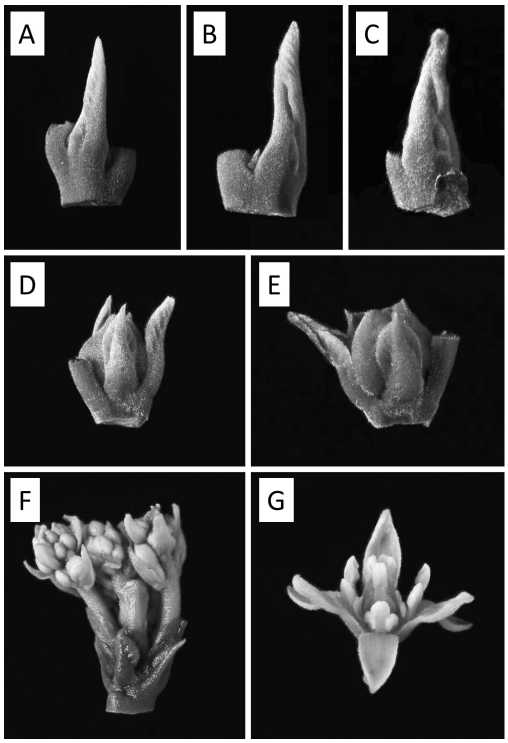

Figure 3.1. Stages of 'Hass' avocado inflorescence development: (A) Stage 1, vegetative; (B) Stage 2, primary axis meristem is flat, one to three secondary axis meristems are present; (C) Stage 3, primary axis meristem is convex, four secondary axis meristems are present, bud is determined; (D) Stage 5, tertiary axis meristems present on the oldest secondary axes, initial development of the perianth of terminal flowers of both secondary and tertiary axes; (E) Stage 6, oldest secondary axes has formed cymes of flowers, having a complete perianth, anthers with sporogenous tissue and a gynoecium at early development; (F) Stage 8, cauliflower stage of inflorescence development, all flowers are present , microspores are present, and integuments are forming on the ovule; and (G) Stage 11, flowers at anthesis. (Adapted from Salazar-García et al. [1998] with permission from the author).

Figure 3.2. ‘Hass’ avocado trees after 4 weeks of treatment: (A) low temperature; (B) low soil moisture; (C) low light intensity; and (D) optimal growing conditions; the terminal buds of trees in each treatment remain at Stage 1 (vegetative) (See insets).

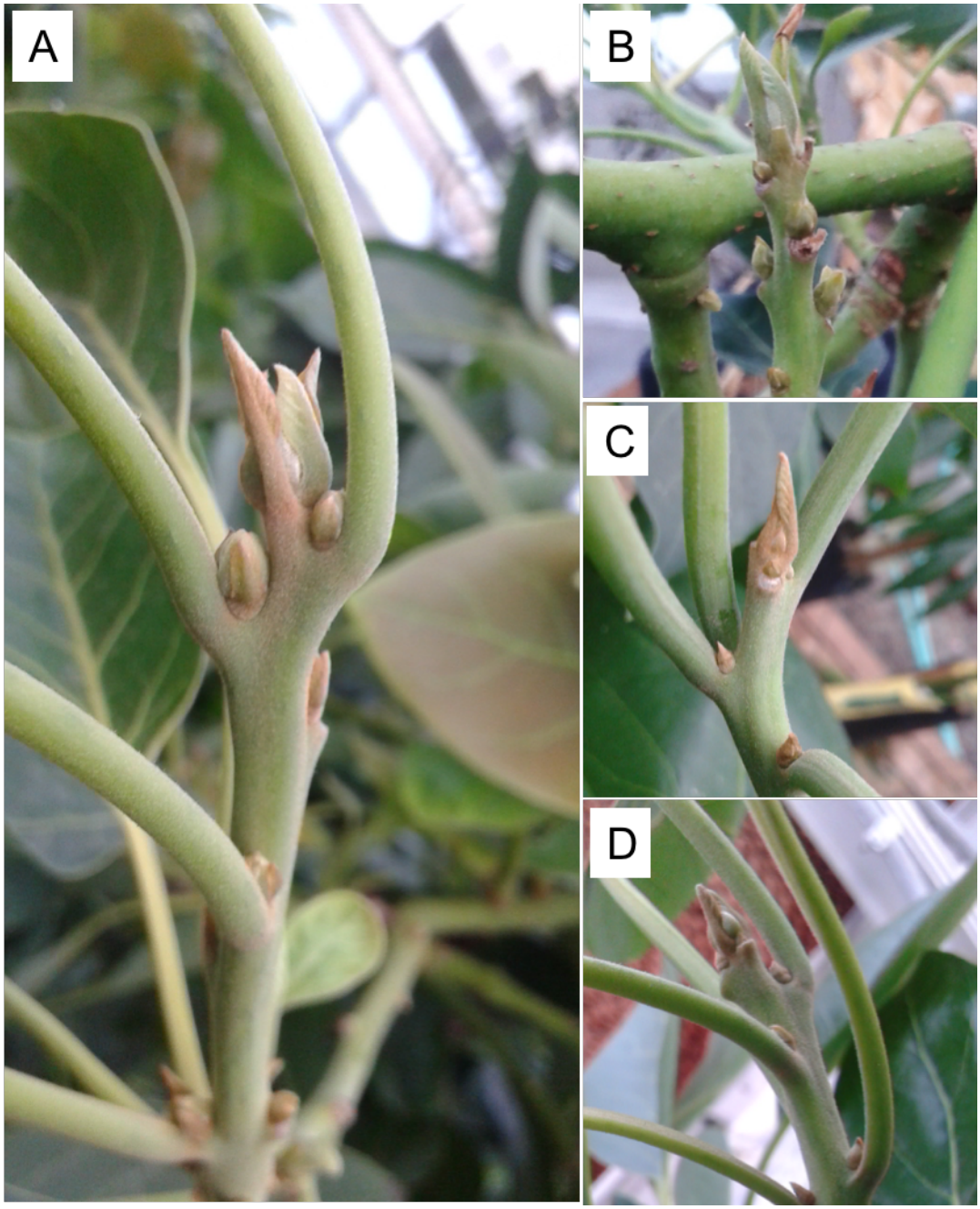

Figure 3.3. ‘Hass’ avocado trees after 8 weeks of treatment: (A) low temperature, terminal buds are at Stages 4 to 5, with proximal axillary buds at earlier stages of floral development; (B) low soil moisture, terminal buds and proximal axillary buds are vegetative; (C) low light intensity, terminal buds at Stages 1 to 2, proximal axillary buds remain closed, pointed and quiescent (inactive); and (D) optimal growing conditions; terminal buds are at Stages 1 to 2, proximal axillary buds remain closed, pointed and quiescent (See Fig. 3.1 for details of bud development).

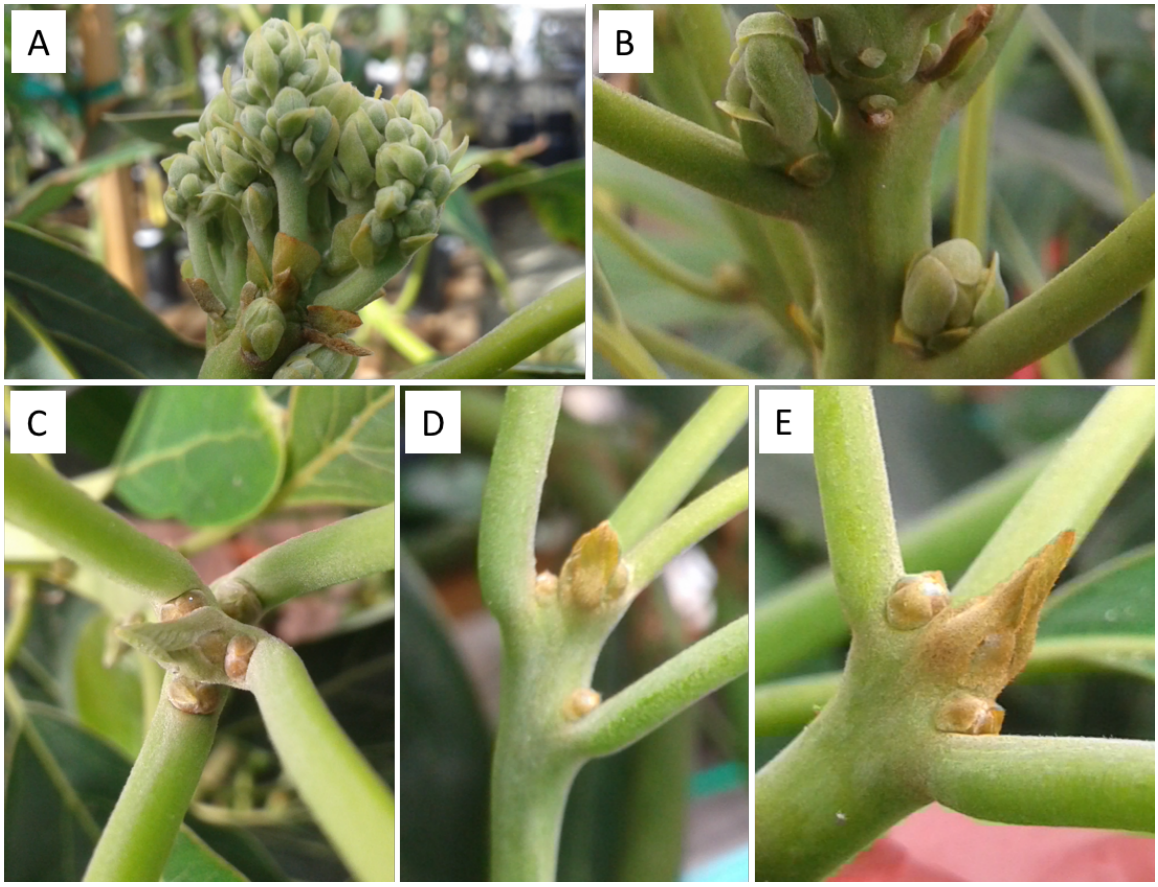

Figure 3.4. ‘Hass’ avocado trees after 12 weeks of treatment: (A) low temperature, terminal buds are at Stage 8, cauliflower stage of inflorescence development and (B) proximal axillary buds at Stages 5 to 6; (C) low soil moisture, terminal buds are vegetative (Stage 1), with quiescent proximal axillary buds; (D) low light intensity, terminal buds are at Stages 1 to 2, with quiescent proximal axillary buds; and (E) optimal growing conditions, terminal buds are at Stages 1 to 2, with quiescent proximal axillary buds. (See Fig. 3.1 for details of bud development).

Table 3.3. Developmental fate of buds of 'Hass' avocado trees subjected to 8 weeks of low temperature (LT), low soil moisture (LSM), or low light intensity (LLI) and then transferred to optimal growth conditions (OGC) for 6 weeks or maintained under OGC for 14 weeks (treatment details are provided in Table 3.1).

Treatment	Bud developmental stage/tree[z]			Floral shoots (no./tree)		Vegetative shoots (no./tree)		Quiescent buds (no./tree)	
	Week 0	Week 12	Week 14	Week 12	Week 14	Week 12	Week 14	Week 12	Week 14
LT (Control)	1.0[y]	5.8 a[Bx]	7.9 a[A]	25.2 a	23.3 a	0.2	0.3	4.7 b	4.7 b
LSM	1.0	1.0 b	1.0 b	0.0 b	0.0 b	0.0	0.2	30.0 a	29.8 a
LLI	1.0	1.0 b	1.0 b	0.0 b	0.0 b	0.0	0.0	30.0 a	30.0 a
OGC	1.0	1.0 b	1.0 b	0.0 b	0.0 b	0.0	0.0	30.0 a	30.0 a
p-value	NS	< 0.001	< 0.001	< 0.001	< 0.001	NS	NS	< 0.001	< 0.001

[z] Based on the 'Hass' avocado scale for inflorescence development by Salazar-García et al. (1998). (Developmental details are provided in Figure 3.1).

[y] Values represent the mean for 30 buds per tree for six trees per treatment and represent the average for a single terminal bud and four proximal axillary buds per shoot.

[x] Means within a vertical column with different lower-case letters are significantly different at the specified *p*-value using a pairwise t-test with Bonferroni adjustment. Means for bud developmental stage for trees in the LT treatment at week 12 and 14 followed by different upper-case letters are significantly different at $p < 0.01$ by pairwise t-test; NS refers to not significant.

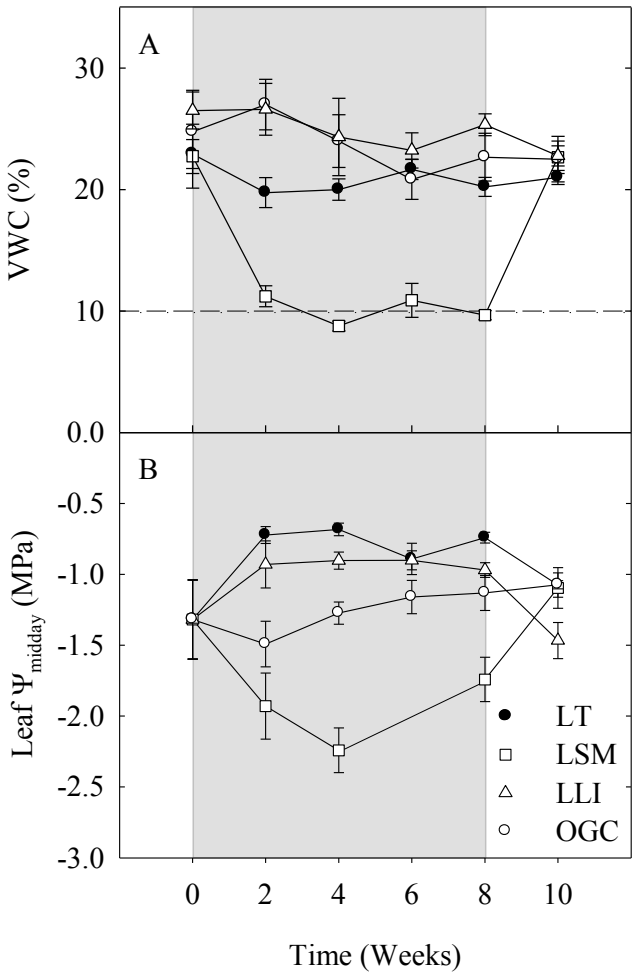

Figure 3.5. Soil moisture content reported as soil volumetric water content (% VWC) (A) and leaf midday water potential (MPa) (B) for weeks 0 through 10 for trees subjected to 8 weeks of low temperature (LT), low soil moisture (LSM), or low light intensity (LLI) and then transferred to optimal growing conditions (OGC) for 6 weeks or maintained under OGC for 14 weeks. The horizontal dashed line in figure A is the target 10% VWC set for the LSM treatment.

Effects of temperature, soil moisture and light intensity on the bud expression of putative FT, LFY, AP1/FUL and AP2 genes in 'Hass' avocado

The relative expression levels of *FT* and *LFY* were low (0.05) and high (4.61), respectively, in buds of all trees at the initiation of the experiment (week 0), following five months of growth under OGC. With the exception of LT-treated trees, bud expression of *FT* remained low for trees in all treatments with no significant change in expression through week 10 of the experiment, two weeks after the end of the stress treatments (Table 3.4). In contrast, for LT-treated trees, bud transcript levels of *FT* increased from week 8 (end of LT treatment) to week 10 ($p = 0.004$) (2 weeks after transfer to OGC) to a value greater than that of trees in all other treatments ($p = 0.005$). Moreover, additional analysis revealed that transcripts of *FT* continued to accumulate after transfer of the LT-treated trees to OGC through week 12, resulting in a 3-fold increase in *FT* transcript levels relative to week 10 ($p < 0.01$) (data not shown). In contrast, bud *LFY* expression increased over time in buds of LT- and LSM-treated trees during the 8-week treatment period to levels greater than LLI- and OGC-treated trees by week 8 ($p = 0.002$) and prior to any change in *FT* expression (Table 3.4). By week 10, two weeks after transfer of all trees to OGC, *LFY* expression was significantly greater in buds of LT-treated trees than LSM- and OGC-treated trees, with *LFY* expression in buds of LLI-treated trees intermediate to, but not significantly different from, trees in all other treatments ($p = 0.009$).

Table 3.4. Relative expression of *FT*, *LFY*, *AP1/FUL* and *AP2* in buds from 'Hass' avocado trees subjected to 8 weeks of low temperature (LT), low soil moisture (LSM), or low light intensity (LLI) and then transferred to optimal growth conditions (OGC) for 6 weeks or maintained under OGC for 14 weeks (treatment details are provided in Table 3.1).

Gene	Treatment	Week 4	Week 8	Week 10	*p*-value
	LT (Control)	0.01 a^{Bz}	0.02 a^{B}	0.08 a^{A}	0.004
	LSM	0.02 a^{A}	0.02 a^{A}	0.01 c^{A}	NS
FT	LLI	0.01 a^{A}	0.01 a^{A}	0.02 bc^{A}	NS
	OGC	0.03 a^{A}	0.02 a^{A}	0.02 b^{A}	NS
	p-value	NS	NS	0.005	
	LT (Control)	2.59 ab^{B}	17.26 a^{A}	9.39 a^{AB}	0.035
	LSM	5.51 a^{A}	12.93 a^{A}	1.60 b^{A}	NS
LFY	LLI	3.32 ab^{A}	1.65 b^{A}	4.04 ab^{A}	NS
	OGC	1.61 b^{A}	2.35 b^{A}	1.94 b^{A}	NS
	p-value	NS	0.002	0.009	
	LT (Control)	0.79 a^{A}	1.07 a^{A}	0.64 a^{A}	NS
	LSM	0.34 b^{A}	0.31 c^{A}	0.13 b^{B}	0.033
AP1/FUL	LLI	0.35 b^{A}	0.23 d^{A}	0.50 a^{A}	NS
	OGC	0.50 b^{A}	0.43 b^{A}	0.60 a^{A}	NS
	p-value	0.047	< 0.001	0.030	
	LT (Control)	0.50 a^{A}	0.87 a^{A}	0.51 a^{A}	NS
	LSM	0.73 a^{A}	0.69 a^{A}	0.29 a^{A}	NS
AP2	LLI	0.43 a^{A}	0.23 b^{A}	0.76 a^{A}	NS
	OGC	0.41 a^{A}	0.53 a^{A}	0.14 a^{A}	NS
	p-value	NS	0.001	NS	

[z] Data are the means for three trees (replications) calculated relative to the expression of each target gene in 'Hass' avocado flowers (expression level = 1; normalized with *β-ACTIN* expression) (Pfaffl, 2001); for the same week for a given gene, values in a vertical column with different lower-case letters are significantly different at the specified *p*-value according to ANOVA and Duncan's multiple range test (DMRt); across weeks within a single treatment for a given gene, values in a horizontal row with different upper-case letters are significantly different at the specified *p*-value according to ANOVA and DMRT; NS refers to not significant.

Bud expression of *AP1/FUL* at week 0 (data not shown) was equal to week 4 for trees in all treatments, except LT, where it was greater at week 4 ($p = 0.047$) and remained greater until week 8 ($p < 0.001$) (Table 3.4). By week 10, bud expression of

AP1/FUL in LT-treated trees was equal to that of trees in all other treatments, with the noted exception that buds of LSM-treated trees had *AP1/FUL* transcript levels significantly lower than those of trees in all other treatments ($p = 0.030$) (Table 3.4). The reduction in *AP1/FUL* transcript level from week 8 to week 10 ($p = 0.033$) in buds of LSM-treated trees suggests that water-deficit stress had a negative effect on *AP1/FUL* expression from which the trees did not recover after transfer to the OGC (Table 3.4). For *AP2*, bud transcript levels across treatments and over time were similar to that of week 0 (data not shown), with the exception that LLI reduced bud *AP2* expression at the end of week 8 relative to trees in all other treatments ($p = 0.001$) (Table 3.4). The number of floral shoots produced in week 14 was significantly correlated across treatments with the expression of *FUL* at week 4 ($r = 0.99$, $p < 0.0001$) and week 8 ($r = 0.98$, $p < 0.05$), during and at the end of the LT-treatment, and *FT* at week 10 ($r = 0.98$, $p < 0.05$) and week 12 ($r = 0.99$, $p < 0.0001$), after transfer from the LT to OGC.

Effects of low temperature, low soil moisture and low light intensity on bud expression of putative floral organ identity genes AP3, PI.1, AG.1 and AG.3 in 'Hass' avocado

The relative expression level of *AP3* in buds remained low in all treatments from week 0 until week 12, except for LT-treated trees. By this time, *AP3* transcript accumulation in buds of LT-treated trees was greater than in previous weeks ($p = 0.003$) and all other treatments ($p < 0.001$) (Table 3.5). For LSM-treated trees, bud *AP3* expression decreased to the limit of detection in week 12 ($p = 0.002$), a level equal to that LLI-treated trees; buds of OGC-treated trees had an intermediate level of *AP3* expression

that was lower than that of LT-treated trees at week 12 but greater than that of LSM- and LLI-treated trees ($p = 0.001$).

Relative expression of *PI.1* in buds was low (0.01) at week 0, and fluctuated between 0.01 and the limit of detection (D) in response to the stress treatments over the 12-week experiment. Only buds of LT-treated trees accumulated *PI.1* transcripts over time ($p < 0.001$) to levels greater than those of trees in all other treatments in weeks 10 ($p = 0.02$) and 12 ($p < 0.001$) (Table 3.5).

The expression pattern of *AG.1* in buds of 'Hass' avocado trees was similar to that of *PI.1*. Bud expression of *AG.1* was low (0.01) at week 0 and fluctuated between 0.01 and the limit of detection (D) in response to the stress treatments over the 12-week experiment. Only buds of LT-treated trees accumulated *AG.1* transcripts over time ($p < 0.001$) to levels greater than those of trees in all other treatments in weeks 10 ($p = 0.002$) and 12 ($p < 0.001$) (Table 3.5). In contrast, bud expression of *AG.3* was not detected (ND) at week 0 and it fluctuated between not detected and detected within treatments over the 12 weeks (Table 3.5). Only buds on LT-treated trees expressed *AG.3*. The LT treatment significantly increased bud *AG.3* expressions from the limits of detection in week 10 to low expression in week 12 ($p = 0.037$); *AG.3* was not detected in the buds of trees in all other treatments at week 12, consistent with the fact that trees did not produce flowers.

It is noteworthy that the expression of the floral organ identity genes increased only in buds of LT-treated trees and only after the LT-treated trees were transferred to OGC, which resulted in a significant increase in transcript level from week 10 to week 12

for *AP3* ($p = 0.003$), *PI.1* ($p = 0.001$), *AG.1* ($p = 0.001$) and *AG.3* ($p = 0.037$). Only the LT-treated trees flowered.

Table 3.5. Relative expression of floral organ identity genes *AP3, PI.1, AG.1* and *AG.3* in buds from 'Hass' avocado trees subjected to 8 weeks of low temperature (LT), low soil moisture (LSM), or low light intensity (LLI) and then transferred to optimal growth conditions (OGC) for 6 weeks or maintained under OGC for 14 weeks (treatment details are provided in Table 3.1).

Gene	Treatment	Week 4	Week 8	Week 10	Week 12	*p*-value
	LT (Control)	0.01 a^{Bz}	0.01 a^{B}	0.01 a^{B}	0.11 a^{A}	0.003
	LSM	0.01 a^{A}	0.01 a^{B}	0.01 a^{B}	D c^{B}	0.002
AP3	LLI	0.01 a^{A}	D a^{A}	0.01 a^{A}	D c^{A}	NS
	OGC	0.02 a^{A}	0.01 a^{A}	0.01 a^{A}	0.01 b^{A}	NS
	p-value	NS	NS	NS	< 0.001	
	LT (Control)	D a^{B}	D a^{B}	0.03 a^{B}	0.24 a^{A}	< 0.001
	LSM	D a^{A}	0.01 a^{A}	0.01 b^{A}	D b^{A}	NS
PI.1	LLI	0.01 a^{A}	D a^{A}	0.01 b^{A}	0.01 b^{A}	NS
	OGC	0.01 a^{A}	0.01 a^{A}	0.01 b^{A}	0.01 b^{A}	NS
	p-value	0.088	NS	0.002	< 0.001	
	LT (Control)	D a^{B}	0.01 a^{B}	0.01 a^{B}	0.06 a^{A}	< 0.001
	LSM	D a^{A}	0.01 a^{A}	D b^{A}	D b^{A}	NS
AG.1	LLI	D a^{A}	D a^{A}	D b^{A}	D b^{A}	NS
	OGC	D a^{A}	0.01 a^{A}	D b^{A}	D b^{A}	NS
	p-value	NS	NS	0.002	< 0.001	
	LT (Control)	ND	ND	D a^{B}	0.04 ^{A}	0.037
	LSM	D ^{A}	ND	D a^{A}	ND	NS
AG.3	LLI	ND	ND	ND	ND	NA
	OGC	ND	D	ND	ND	NA
	p-value	NA	NA	NS	NA	

[z] Data are the means for three trees (replications) calculated relative to the expression of each target gene in 'Hass' avocado flowers (expression level = 1; normalized with *β-ACTIN* expression) (Pfaffl, 2001); D refers to detected, which indicates a relative expression value < 0.005; ND, refers to not detected, which indicates the expression level of the target gene in each of the three biological replications was below the threshold value for detection (quantification cycle [Cq] in qPCR ≥ 35); for the same week for a given gene, values in a vertical column with different lower-case letters are significantly different at the specified *p*-value according to ANOVA and Duncan multiple range test (DMRT); across weeks within a single treatment for a given gene, values in a horizontal row with different upper-case letters are significantly different at the specified *p*-value according to ANOVA and DMRT for *AP3, PI.1* and *AG.1,* and according to the Kruskal-Wallis test for *AG.3*; NS refers to not significant; NA refers to not applicable.

Discussion

When successful, the floral induction process results in bud determination, irreversible commitment of the bud to floral development, and subsequent flower formation. Prior to this point, the floral induction process can be interrupted or even aborted by endogenous or exogenous environmental factors (Hong and Jackson, 2015). The results presented herein are the first to report the pattern of expression of putative key genes involved in floral development under environmental conditions that resulted in successful induction and flowering and those that did not. The 'Hass' avocado trees used in this research were grown under OGC for five months before the experiment was initiated in mid-July, prior to the completion of the summer flush of vegetative shoot extension growth. Thus, it is of interest that at the start of the experiment (week 0), *LFY* and *AP1/FUL*, classic floral meristem identity genes, and *AP2*, a floral organ identity gene, were strongly expressed in buds of all trees, with *FT*, *AP3*, *PI.1* and *AG.1* RNA at low but detectable levels, and only *AG.3* transcript levels below the limits of detection. These results are consistent with the initiation of the floral induction process, but the continuation of vegetative shoot growth over the next four weeks by trees in all treatments suggests the trees may not have been developmentally competent. These data suggest that *LFY*, *AP1/FUL* and *AP2* transcript levels at the start of the experiment and those attained by the end of 8 weeks of treatment for trees in all treatments, except LT, were insufficient to result in bud determination, or there were other factors preventing flowering.

In this research, only trees subjected to LT treatment flowered. Therefore, successful completion of the floral induction process resulting in bud determination in *P. americana* is logically related to changes in the pattern of floral gene expression that occurred exclusively in buds of LT-treated trees. Comparison of the gene expression pattern among the treatments provides further evidence that sufficient expression of both *LFY* and *AP1/FUL* is required for bud determination in *P. americana*. Buds of LSM-treated trees failed to express *AP1/FUL* and buds of LLI and OGC-treated trees failed to express either *LFY* or *AP1/FUL* at the level of LT-treated trees and did not flower. Taken together, the results of this research are consistent with a role for *LFY* and *AP1/FUL* in floral meristem identity and bud determination in *P. americana* similar to that of their *A. thaliana* counterparts. In *A. thaliana*, transition from vegetative to reproductive development is controlled by multiple environmental and endogenous signals that ultimately upregulate *LFY* and *AP1*, key regulators of floral meristem identity, and consequently determination (Blazquez et al., 2006; Sablowsky, 2007).

Two novel findings of this research were related to *FT* expression. First, was the notable absence of significant *FT* expression in week 0, despite significant levels of *LFY*, *AP1/FUL* and *AP2* transcripts. Bud expression of *FT* remained uniformly low in buds of all trees over the course of the experiment, with the exception of LT-treated trees. Second was the activation of *FT* only after transfer of the LT-treated trees to the warm temperatures of the OGC. The expression of *FT* significantly increased from week 8 to 10 and week 10 to 12 in buds of LT-treated trees, the only trees that flowered. The late expression of *FT* also occurred in buds of field-grown 'Hass' avocado trees in Israel,

AP1/FUL (reported as *AP1*) reached maximum expression in November, prior to *FT*, which reached maximum expression simultaneously with the maximum expression of *LFY* in January (winter in Israel) (Ziv et al., 2014). There is a striking similarity with regard to the sequential activation of *AP1/FUL*, *LFY* and *FT* in 'Hass' avocado buds in the two experiments.

In *A*. *thaliana,* the floral timing gene *FT* is initially expressed in leaves at the initiation of the floral induction process (Lee and Lee, 2010; Moon et al., 2005; Parcey, 2005). The protein produced from *FT* subsequently activates *LFY* and *AP1*, which reciprocally activate each other. *FT* also redundantly activates *AP1*, and consequently *LFY*, which both remain active after their earlier role in bud determination; *AP1* in the development of the perianth organs (sepals and petals) and *LFY* in the activation of the genes specifying stamen and carpel development (Sablosky, 2007). Thus, *A*. *thaliana*, *FT* has a second important role in maintaining determination, inflorescence meristem and floral meristem identity, to prevent floral reversion, a role independent of floral induction (Müller-Xing et al., 2014; Parcey et al., 2002). The observed activation of *FT* in avocado in weeks 10 through 12, after transfer of LT-treated trees to OGC, is consistent with this role for *FT*. In the avocado flower, *AP1/FUL* is expressed in the perianth organs (i.e., in both whorls of tepals) and in the stamens. A role for *LFY* in floral organogenesis has not been reported in *P*. *americana*, but bud *LFY* expression remained elevated concurrently with increased bud *FT* expression and the initial activation of the floral organ identity genes in week 10.

Maximum expression of putative floral organ identity genes was two weeks before anthesis of the LT-treated trees, consistent with the reported expression of these genes in the different floral organd of the avocado flower (Chanderbali et al., 2006, 2008, 2009; Soltis et al., 2007a, b, 2009). In *P. americana*, *AP3*, *PI.1* and *AG.1* are expressed in the outer and inner tepals and stamens; *AG.3* is expressed in the stamens and carpel (Chanderbali et al., 2006). For LSM-, LLI- and OGC-treated trees at week 12, bud transcript levels of *AP3*, *PI.1*, *AG.1* and *AG.3*, in all cases, were unchanged or lower than in week 4, remaining at low to detectable levels or below the limits of detection (*AG.3*). LSM-, LLI- and OGC-treated trees did not flower and terminal and proximal axillary buds remained at Stages 1 to 2 and quiescent (Salazar-García et al., 1998). The results establish that transcript levels that accumulated in the buds of LT-treated trees for the genes analyzed in this research were sufficient for bud determination, inflorescence development and flower formation.

The requirement for adequate transcript levels of *LFY*, *AP1/FUL*, and *FT* for successful floral development in *P. americana* is consistent with those genes that have been identified as required to confer floral identity to newly developing meristems in other angiosperm species, including *LFY*, *AP1/FUL* and *AP2,* with *FT* important for maintaining floral commitment to prevent floral reversion (Müller-Xing et al., 2014; Parcy et al., 2002). The result reported herein, to the authors' knowledge, are the first data on the expression of *AP2* in *P. americana* over time. In *A. thaliana*, *AP2* has a class A function with a role in determination and sepal development. The results of this research do not clarify the role of *AP2* in avocado floral development but also do not rule

out the possible role in bud determination. Bud *AP2* expression was correlated with bud expression of *AP1/FUL* in week 8. Maximum expression of *AP2* occurred in week 8 for buds of LT-treated trees, though it was not significantly different from that of trees in the LSM and OGC treatments. Thus, it might be assumed that this level of *AP2* expression was adequate for bud determination by week 8 of LT-treated trees and subsequent successful floral development.

In basal angiosperms (noncore eudicots), such as *P*. *americana*., the late expression of *FT*, after the increased expression of *AP1/FUL* and *LFY* in buds of LT-treated trees, seems inconsistent with a role for *FT* in the floral induction process, but it is commensurate with maintenance of the commitment to flowering after successful induction and bud determination. Further research is required to define the role of *FT* in regulating floral development in *P. americana,* including a possible role for *FT* in the activation of downstream floral organ identity genes. In addition, the temporal pattern of *FT* expression relative to that of *AP1/FUL* raises the question of how *AP1/FUL* is activated under low temperature in avocado. Relative to this question, the low transcript levels of *AP1/FUL*, *LFY* and *AP2* in buds of trees prior to the end of the summer vegetative shoot flush are of interest, with the possibility they are related to the initiation of the induction process via an autonomous (developmentally regulated) or photoperiod (leaf initiated) floral development pathway.

The results of this research provide additional evidence that low soil moisture resulting in water-deficit stress, even moderate water-deficit stress, does not induce flowering in avocado and new evidence that low light intensity stress also does not

promote flowering in avocado. The results of this research confirmed that low temperature induces flowering in avocado and identified the first pattern for the activation of putative key floral genes that culminated in determination in buds of 8-week LT-treated trees, increased expression of *AP1/FUL* and subsequently *LFY*, with basal levels of *AP2* expression not limiting to successful induction when *AP1/FUL* and *LFY* transcript levels reach threshold levels.

In conclusion, consistent with successful induction (bud determination), floral reversion did not occur upon transfer of the 8-week LT-treated trees to OGC prior to *FT* activation in week 10, suggesting that *FT* expression is not essential for bud determination. Thus, the results provided evidence suggesting a second role for *FT* in *P. americana* independent of a role in floral induction, i. e., maintenance of determination (inflorescence meristem and floral meristem identity) to prevent floral reversion. In avocado, four weeks after the LT-treated trees were transferred to OGC, bud expression of *FT* and the floral organ identity genes *AP3*, *PI.1*, *AG.1* and *AG.3* reached the maximum level observed in this research. The fact that bud expression of *FT*, *AP3*, *PI.1*, *AG.1* and *AG.3* did not occur until after transfer of the LT-treated trees to OGC suggests a possible failsafe mechanism to synchronize flowering with the warmer temperatures of spring. Transcripts of *AP1/FUL* and *LFY* would accumulate under low fall and winter temperatures to a level that confers bud determination, but *FT*, which our results suggest plays role in maintaining commitment to flowering in avocado, and the downstream floral organ identity genes *AP3*, *PI.1*, *AG.1* and *AG.3* would only be expressed when

spring temperatures were sufficiently warm, thereby preventing flowering under adverse temperature conditions.

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General conclusion

P. americana has been shown to have a phenotypic plasticity that has allowed it to overcome different environmental conditions, and this support its successful dispersion in sub-tropical, semi-tropical and tropical areas around the world. In this dissertation, a broad spectrum of leaf carbon isotopic composition among 24 avocado varieties is shown, its relationship with photosynthesis and stomatal conductance allowed the identification of water-use efficient avocado varieties. Water balance more than carbon assimilation impacts water-use efficiency and it opens a new scenario for the search of varieties or scion-rootstock combinations that could save water without losing yield.

Although the three varieties that responded best to saline treatment were chosen for this study, from a pull of 13 varieties, the results still point *P. americana* as a crop highly susceptible to salinity, but provide more tools for the identification of avocado trees with potential salinity tolerance. With the appropriate leaching fraction, these three rootstocks could outperform other rootstocks grown under saline conditions.

The results from the floral gene expression analysis in avocado suggest that the significantly greater bud expression levels of *LFY* and *AP1/FUL* promoted by low temperature were sufficient to confer bud determination, since transferring the trees from low to warm temperatures did not prevent flowering. The fact that bud expression of *FT*, *AP3*, *PI.1*, *AG.1* and *AG.3* did not occur until trees that were switched from low to warm temperatures, suggests a possible failsafe mechanism to synchronize flowering with the warmer temperatures of spring.