© 2017 Mun Wye Chng
To the Moore Lab Group
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LIST OF ABBREVIATIONS

µg  Microgram
µL  Microliter
ABA  Abscisic acid
DI  De-ionized water
GA3  Gibberellic Acid
GC-MS  Gas Chromatograph-Mass Spectrometry
H  Height of plant
HCl  Hydrochloric Acid
L  Liter
LD  Long Day
LDP  Long Day Plant
Pfr  Phytochrome far-red
PGR  Plant Growth Regulator(s)
Pr  Phytochrome red
SD  Short Day
SDP  Short Day Plant
TMSD  Trimethylsilyldiazomethane
VCT  Volatile Compound Trap
VPE  Vapor-Phase Extraction
W1  Width at widest part of plant
W2  Width perpendicular to W1
WS  Water Stress
FLOWERING OF BOUGAINVILLEA ‘AFTERGLOW’: ENVIRONMENTAL TRIGGERS AND THEIR HORMONAL RESPONSES

By

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Bougainvillea cultivars are widely used in tropical ornamental landscapes for their floral displays. Flowering response in bougainvillea is typically photoperiod dependent. However, in tropical climates, photoperiod variation may be insufficient to stimulate uniform flowering response in the landscape, resulting in patchy floral displays. This study seeks to identify the mechanism behind apparent environmental triggers that have been observed to trigger flowering in the absence of photoperiod variation.

Bougainvillea ‘Afterglow’ cuttings were grown under various treatments within an open-sided greenhouse in Davie, Florida. Environmental conditions such as photoperiod, water availability and temperature were controlled in various experiments. Additional treatments were the application of exogenous the plant hormones ethylene in the form of ethephon (2-chloroethylphosphonic acid), and abscisic acid (ABA). Endogenous levels of ABA were determined using gas chromatography to separate trimethylsilylated esters of ABA from leaf tissue extracts, and measuring the ion fragments using mass spectrometry. Endogenous gibberellic acid (GA) was measured using ultra-violet spectrophotometry of filtered leaf tissue extracts. Growth and number
of inflorescences were recorded for each experiment, which ran on average for thirty days. Statistical analyses were performed using the R software package.

*Bougainvillea* ‘Afterglow’ was found to be a strongly photoperiodic short-day (SD) plant, with an inductive photoperiod of 8 hours, and an inhibitory long-day (LD) photoperiod of 14 hours. Application of ethephon to plants under SD improved flowering response, and was less effective on plants under LD. Application of ABA to plants under SD improved flowering response as well, but had no effect on plants under LD. GA was found to decline in plants that flowered, regardless of whether they were induced by photoperiod or by exogenous hormones. ABA also declined in plants that flowered. Ethephon appeared to promote flowering in *Bougainvillea* by regulating both ABA and GA levels. GA regulation of flowering response in *Bougainvillea* appeared to contradict the DELLA-protein dependent pathway described for the LD plant, *Arabidopsis thaliana*. 
CHAPTER 1
RATIONALE

Introduction

Scientific studies related to the flowering of horticultural plants in tropical and subtropical zones of the world, including the United States, have largely focused on the economically important tropical fruit crops of several key genera, namely *Mangifera* (mango) (Núñez-Elisea and Davenport, 1994; Ramírez and Davenport, 2010), *Citrus* (Koshita and Takahara, 2004) and *Litchi* (lychee) (Menzel, 1983), but less attention has been paid to woody ornamentals.

Ornamental crops in the tropics, as in any climate, can be generally classified into foliage crops and floral crops. The difference between tropical/sub-tropical and temperate floral crops from a landscape design point of view is the seasonality of flowering, and the respective environmental stimuli that trigger flowering. In high latitudes, day length (photoperiod) and temperature (vernalization) are the predominant cues that signal plants to enter reproductive phase. At lower latitudes, especially in areas with distinct wet/dry seasonal variations, the trigger is often water availability. In areas without significant variation in water availability, intensity of solar radiation has been suggested as the trigger for some species to flower (Yeang, 2007).

One of the most commonly found landscape shrubs planted in Singapore is bougainvillea (*Bougainvillea* spp.). It is an evergreen perennial native to tropical South America. It was named for Louis Antoine de Bougainville, a French navigator and military commander who was the first European to take note of the plant, in Brazil, in 1768 (Kobayashi et al., 2007). It has small, rather insignificant flowers typically subtended by three colorful bracts, borne in apical panicles on the current year wood
It is a very widespread and popular landscape ornamental plant in tropical and sub-tropical zones around the world, greatly valued for its vigor and relative resistance to pests, disease and drought, bright floral display of colorful bracts, and variability in form as it can be planted as a shrub, standard, espaliered, or trained onto a trellis.

Singapore has an equatorial climate which is characterized by consistently high temperatures (average night/day temperatures of 25/31°C) and high rainfall (average total annual rainfall of 2342.5mm out of 178 rain days evenly distributed over the year) (National Environment Agency, 2017). It also has an annual day length variation of roughly 9 minutes, although sunrise (and concurrently sunset) shift roughly 30 minutes throughout the year (Yeow, 2002). Because of this, many tropical flowering trees and shrubs planted in the city do not flower as predictably as they do in climates with more pronounced seasonality.

In Singapore’s climate, *Bougainvillea* exhibits an unpredictable and sporadic flowering pattern due to the lack of distinct seasonality. In sub-tropical climates like Florida, it flowers in fall to late spring, when the daylength is relatively short, night-time temperatures are above 21°C, and rainfall is reduced. One horticulturally significant characteristic of *Bougainvillea* is its tendency to flower more profusely when under a moderate level of drought stress. Drought stress has been successfully deployed to induce flowering in tropical and subtropical fruit trees such as mango (*Mangifera indica*) (Núñez-Elisea & Davenport 1994), and lime (*Citrus latifolia*) (Southwick & Davenport 1986). Some *Bougainvillea* growers employ this technique to encourage flowering. Most growers rely on trial and error to figure out the practical level of drought stress that suits
the purposes of their crop production (Jamie Modlin, grower - personal communication).

In the case of *Bougainvillea*, the general practice is to allow the planting media to dry out in between watering. Growers understand that there is a correlation between drought stress and bud initiation in these plants, but the underlying reason for this physiological response has not been explored in the literature.

**Objectives**

The fundamental goal of this project was to identify a common denominator linking the various environmental cues to flowering in *Bougainvillea*, and to determine the dominant environmental factors required for flowering to occur. A secondary objective was to use different methods to induce flowering under sub-optimal environmental conditions.
CHAPTER 2
LITERATURE REVIEW

Photoperiod and Flowering

Many flowering plants use environmental cues to regulate the transition from vegetative growth to sexual reproduction (Yeang, 2007). Multiple individuals of the same species need to flower at the same time for pollination to occur with the highest possible rate of outcrossing to ensure genetic diversity. To do so, environmental cues are taken to signal optimal times for flowering. The major variables that serve this function are photoperiod, temperature, and water availability (Rivera and Borchert, 2001; Yeang, 2007).

Photoperiodism is defined as "...the ability of an organism to detect day length..." (Taiz and Zeiger, 2010). Plants that have evolved to grow at latitudes where there are annual variations in daylength may exhibit the ability to detect these variations in daylight hours through the year, if the completion of their reproductive cycle is dependent on time-sensitive flowering, (during periods with high pollinator activity).

Plants can be classified in terms of their photoperiodic responses. Short-day plants (SDP) flower during short days (qualitative SDP), or their flowering may be increased or expedited by short days (quantitative SDP). Long-day plants (LDP) flower only during long days, or flowering is increased by longer days. Day-neutral plants flower equally regardless of daylengths.

The ability of plants to sense daylength is dependent on two components – the ability to detect light, and the ability to “keep time” internally (circadian cycle). The first component is photosensitivity, which is enabled by the presence of phytochromes in plant leaf cells. Several different phytochromes form a family of protein-pigment
photoreceptors. Phytochromes exist in two forms. Phytochrome red (Pr) is located in the cytoplasm and is inactive, and Phytochrome far-red (Pfr) is located in the nucleus and is active (see Fig. 2-1). Phytochromes toggle between both forms depending on the amount and wavelength of light they receive. Pr is sensitive to red light (650 – 680nm), and upon perceiving it, changes conformation and migrates to the nucleus as Pfr. Likewise, Pfr is sensitive to far-red light (710 – 730nm), and upon perception, undergoes conformational change and dephosphorylation, and converts to Pr.

Phytochrome-Interacting Factors (PIFs) are transcription factors that are associated with phytochromes. They have been found to act as repressors of light-activated genes, and promoters of dark-regulated genes (Taiz and Zeiger, 2010). The current understanding, is that when Pf responds to red light during the day and converts to Pfr, the Pfr induces phosphorylation and degradation of the PIFs, which in turn remove the suppression of light-activated genes, thus allowing the physiological responses induced by light exposure, such as shade avoidance, elongation, and flowering (Schwechheimer and Willige, 2009; Taiz and Zeiger, 2010). (Fig. 2-1)
Figure 2-1. Phytochrome Interaction Factors (PIF) under far-red light (710-730 nm) or dark conditions (above), and red light (650-680 nm) or bright conditions (below). Drawing by Mun Wye Chng.
Flowering in *Bougainvillea* has been studied for more than fifty years. Earlier research had established that photoperiod was an important determinant in flowering time (Joiner et al., 1962; Staden and Dimalla, 1980). Light intensity, cytokinin levels, and their involvement in diverting metabolic assimilates away from shoots tips to axillary buds have also been implicated in the flowering process of *Bougainvillea* (Even-Chen and Sachs, 1980; Hackett and Sachs, 1984.; Ramina et al., 1979; Staden and Dimalla, 1980). Most *Bougainvillea* varieties in the literature have been found to be quantitative SDP. These include *B. sanderiana*, *B. glabra*, *B. spectabilis*, ‘Rainbow Gold’, ‘San Diego Red’, and ‘Raspberry Ice’ (Even-Chen and Sachs, 1980; Joiner et al., 1962; Ma and Gu, 2010; Steffen et al., 1988; Tse et al., 1974).

**Phytohormones and Flowering**

Photoperiod induction of flowering causes dramatic and complex alterations of the long-distance signaling system in plants. Plants that do not require a photoperiod or temperature to flower (so-called “autonomous flowering” plants) are usually sensitive to irradiance. Photoperiod and irradiance are usually perceived by mature leaves in intact plants (Rivera and Borchert, 2001). Temperature on the other hand, is perceived by all parts of the plant, although vernalization or low temperature is usually perceived by the shoot apex. Water availability is perceived by the root system (Bernier et al., 1993). Plant response to environmental triggers are signaled by molecules known as phytohormones, of which the classical ones are auxins, gibberellins, abscisic acid, cytokinins and ethylene. The hormones thought to be related to the flowering response are gibberellic acid, abscisic acid and ethylene.
**Gibberellic Acid (GA)**

Gibberellic acid (GA) has been implicated in the flowering process. GA has been known to play an important role in cell elongation since being discovered from the spindly rice plants infected with *Gibberella fujikuroi* fungus. Besides stem elongation, it also affects fruit and flower formation, dormancy of vegetative organs, and along with ABA, the control of seed germination. Staden and Dimalla (1980) subjected *Bougainvillea* ‘San Diego Red’ to three treatments: short day (SD) of 8h light and 16h of uninterrupted dark, long day (LD) of 8h light and 16 dark interrupted by 4h of incandescent light, and SD+GA, where plants were sprayed with 10 mg/L of gibberellic acid (GA) before being subjected to the SD treatment. They found that LD treatment suppressed flowering, and was correlated with high levels of cytokinin in the mature leaves and shoot tips. The SD treatment enhanced floral development, and was correlated with a lowered cytokinin level in the mature leaves and shoot tips, except for the shoots where flower bud initiation had occurred. These shoot tips had the same levels of cytokinins as the LD treatment plants. The combination of SD and GA application yielded similar results as the LD treatment. The application of exogenous GA overcame the restriction of cytokinin production in the roots, leading to the plants exhibiting the same profile as the LD plants. The authors concluded that GA may play a role in preventing the development of flower buds in *Bougainvillea*. This was supported by other applied experiments using GA inhibitors. Paclobutrazol and dikegulac promoted flowering under LD conditions in *B. spectabilis* (Karagüzel, 1999), daminozide and chlormequat increased the number of flowers of ‘Raspberry Ice’ under LD conditions (Ma and Gu, 2010), and dikegulac promoted flowering in ‘Rainbow Gold’ under decreasing daylengths (Norcini et al., 1992).
GA is also involved in circadian responses and photoperiodism. Spinach (Spinacia oleracea) is a LDP that changes from juvenile to reproductive phase when plants are moved from SD to LD. Application of exogenous GA under SD was shown to simulate the LD effect (Zeevaart, 1971a). However, endogenous levels of GA were similar under both SD and LD. This led to the suggestion that the SD inhibition of flowering was due to other signaling molecules. However, recent studies have shown that GA does play a role in regulating responses to light and photoperiod. The GA-signaling in relation to light works via the suppression or activation of DELLA proteins (Fig. 2-2). DELLA proteins are so named due to the D-E-L-L-A amino acid chain is conserved in them. They are proteins that inhibit numerous GA-regulated responses. In the absence of GA, DELLA proteins prevent PIFs from binding to their cognate promoters and thereby preventing PIF-dependent transcriptional activation (Alabadí and Blázquez, 2009; Lau and Deng, 2010; Schwechheimer and Willige, 2009). In the presence of GA, the DELLA proteins are degraded, allowing the PIFs to activate the related growth responses like germination, stem elongation and flowering. In this way, GA appears to regulate flowering response - at least in LD plants - by releasing the suppressive effect of DELLA proteins on PIFs (Cheng et al., 2004; Schwechheimer and Willige, 2009).

Figure 2-2. Gibberellic acid closes the 'lid' of the GID1 receptor, causing DELLA protein degradation and releasing transcription factor (Hedden, 2008).
However, the relationship between GA, DELLA, PIFs and flowering in SD plants is not as well described. The GA-DELLA pathway described above relates to *Arabidopsis thaliana*, which is a LD plant. In that pathway, an increase in GA levels would promote flowering, instead of inhibiting flowering like in *Bougainvillea*. The GA-DELLA pathway an important nexus point between the signaling pathways of other hormones, especially abscisic acid and ethylene (Colebrook et al., 2014). Therefore, it is quite likely that the signaling pathways in SD plants between these three major hormones is different but equally important. The first other major hormone to look at is abscisic acid.

**Abscisic Acid (ABA)**

Water stress is another abiotic factor that has been found to promote flowering in *Bougainvillea* (Kobayashi, et.al., 2007; Ma and Gu, 2010). This implicates the abiotic-stress-response hormones ethylene and abscisic acid (ABA) in the flowering process.

Abscisic acid was named because it was originally thought to play an inhibitory role in plant growth, induce abscission of plant parts, and control dormancy. While exogenous application of ABA does sometimes inhibit growth, it also seems to promote certain types of growth, such as in the protein synthesis in seeds. Therefore, its role is likely to be much larger than previously thought (Mansfield, 1987).

ABA is found in all vascular plants. Within the plant, ABA has been found in all major organs and living tissue from root cap to apical meristem. It is synthesized in almost all cells that contain chloroplasts or amyloplasts (Taiz and Zeiger, 2010). It is synthesized from mevalonic acid in mature leaves in response to water or drought stress. It is also found in high concentrations in seeds, where it may have been transported from other parts of the plant, or been synthesized in situ (Walton, 1987).
ABA and transpiration: In general, the known effects of ABA are the closure of stomata, induction of transportation of photosynthetic products towards developing seeds and uptake by embryos, induction of the synthesis of storage proteins in seeds, and possibly affecting the induction and maintenance of dormancy in seeds and buds (Powell, 1987).

When plants, especially smaller herbaceous plants, are under water stress, ABA levels can rise from 10- to 50-fold within 4 to 8 hours. When the plants are watered, ABA levels drop to pre-stress levels within 4 to 8 hours. In seeds, ABA levels can rise 100-fold within a few days and decline to low levels as the seeds mature and desiccate (Walton, 1987). Synthesis and metabolism, and transport or import through the xylem and phloem, are involved in the changing of ABA levels. The most apparent effect of elevated ABA levels is ABA causes the guard cells around stomata to lose turgor and thus close the stomata, reducing transpiration. Redistribution or biosynthesis of ABA is very effective in causing stomatal closure. ABA also promotes root growth and inhibits shoot growth at low water potentials (Mansfield, 1987b).

The correlation between water stress and ABA in plants has been well established in the literature (Munns and Sharp, 1993; Sharp and LeNoble, 2002; Zeevaart, 1971b). However, apart from the known effect of causing the loss of turgor in guard cells, which in turn closes stomata and reduces transpiration, much of the other possible effects of ABA on plants is less understood (Taiz and Zeiger, 2010).

ABA and shoot growth: From the 1970s to the early 2000s, it was suggested that water stress-induced increases in ABA concentrations were one of the causes of the inhibition of shoot growth (Sharp and LeNoble, 2002). Experimental applications of
ABA to well-watered plants resulted in reduced root and shoot growth. In some of these experiments, the findings suggested that the increase in endogenous ABA in water-stressed plants was enough to cause most of the inhibition of growth (Davies and Zhang, 1991).

ABA content doubled in high water-stressed Satsuma mandarin (*Citrus unshiu*) leaves, as compared to moderate water-stressed ones (Koshita and Takahara, 2004). In spinach (*Spinacia oleracea* L., cv. Savoy Hybrid 612) kept under water stress to wilting point, ABA content increased by 10 times as compared to turgid plants (Zeevaart, 1971a).

In wheat (*Triticum aestivum*), loss of turgor in the leaves due to osmotic regulation may have had an indirect negative effect on pollen and spike development because of the increase in production of ABA (Morgan, 1984). Morgan (1984) reported that the amount of grain produced was correlated with the level of water stress experienced by the plants before the onset of flowering. There was a negative correlation between wheat floret fertility and ABA concentrations, with grain yield falling as ABA concentrations increased. The flower spike could continue to expand at full turgor even under water deficit, but at the expense of leaf turgor, which fell to zero (full wilt) (Morgan 1980).

In the case of spinach plants that were subjected to limited water until wilting symptoms appeared, the ABA content in the plant (whole plant) increased 10-fold compared to turgid plants (Zeevaart, 1971a). The significant increase in ABA concentrations when spinach plants were under water stress raised the question of whether the ABA was produced by the plant when subjected to water stress, or
released from a bound form that already existed before the water stress occurred. The authors' investigation found that free ABA had increased 10-fold in wilted plants compared to turgid plants. Alkaline hydrolysis of the water residue released minimal amounts of ABA regardless of whether plants were under water stress or not. Therefore, the ABA in spinach was shown to have been synthesized by the stressed plants, and not released from a bound form.

Spinach was also subjected to short day (SD)/long day (LD) exposures to see if photoperiodism affected ABA levels (Zeevaart, 1971a). The authors found that ABA levels did not decrease when plants were exposed to LD to stimulate flowering and shoot growth. This implies that ABA alone does not have an inhibitory effect on flowering and stem growth in spinach. It was proposed that ABA might interact with other growth inhibitors such as GA in controlling flowering and stem growth. Conversely, the application of exogenous ABA to spinach under LD conditions inhibited the flowering response, but endogenous ABA levels were higher under LD than SD. Therefore, ABA did not appear to have a causal relation with the ability to flower in spinach (Metzger and Zeevaart, 1980). Only GA has been shown to induce flowering under strictly non-inductive conditions, but only for LDP or plants that require vernalization to flower. SDP and day-neutral plants tend not to be responsive to GA applications.

**Transport of ABA:** Research on water stress reactions is concerned with the sensing and signaling mechanisms of water deficits. The conventional understanding has been that as the soil dries, water uptake is reduced, and leaf water potential declines (Davies and Zhang, 1991).
Direct evidence of root signaling of water deficit can be found in plants where the root mass was split equally between two separate medium containers, one of which received adequate irrigation while the other did not. The plants showed signs of reduced growth, smaller individual leaves and fewer new leaves. When water was restored to both containers, the plants recovered leaf growth to the same level as the control plants. However, by simply cutting off the dry half of the root mass, the same effect could be achieved. Such split-root experiments show evidence that partial drying of the root system does not significantly reduce leaf conductance (water potential) even though leaf growth is reduced. Thus, it was hypothesized that the dry roots somehow chemically inhibit cell expansion and division in leaves, even though the shoots are well-supplied with water. Removing the roots removes the inhibitory effects, thus explaining why the plant recovers to well-watered status (Davies and Zhang, 1991).

In experiments analyzing the composition of xylem sap in water-stressed plants, most contents (cations, anions, pH, amino acids and plant hormones) decrease in concentration, with the clear exception of ABA (Schurr and Gollan, 1990). ABA is now thought to be synthesized in both roots and shoots. Leaves can contain a lot of ABA even when well-watered. Assuming ABA synthesized in the roots is transported to the leaves through the xylem, the leaves must be able to differentiate between the ABA that is endogenous to the leaf tissue, and that which has been delivered through the xylem sap. Shoots can do this because in well-watered plants under sufficient light, most of the ABA is sequestered in the chloroplasts. When leaf turgor is maintained, this ABA is effectively kept separate from ABA coming from the xylem sap.
External applications of synthetic ABA seem to mimic the effects of water stress on wheat leaf growth. The effects of both ABA and water stress were: smaller average cell size, smaller number of stomata on leaves, more trichomes on leaves. It was suggested that the role of ABA was intertwined with other growth regulators like cytokinins (Quarrie and Jones, 1977).

New formulations of ABA have been produced recently, and some research has been conducted on their efficacy in delaying wilting from environmental stress. s-ABA (ProTone™ from Valent BioSciences Corp.) is one such product. s-ABA is an analog of ABA, and application of s-ABA works by mimicking increased levels of ABA in the plant tissue, causing the plant to respond as if it were already under drought stress when it is still well-watered. Researchers found that exogenous applications of this product in the form of a “sprench” or spraying until runoff occurs, at 125 or 250 mg/L could significantly delay wilting caused by drought stress in several common bedding plants such as Impatiens walleriana, Catharanthus roseus, I. wittrockiana, Petunia × hybrida ‘Red and White’ (Blanchard et al., 2007) These findings were supported by other researchers (Waterland et al., 2010), who also found that there was no significant difference on the efficacy of s-ABA in delaying wilting, between the two application methods tested (spray versus drench).

**Ethylene**

Ethylene, a small molecule that exists as a gas at room temperature, is involved in stress responses, as well as developmental processes such as seed germination, senescence, and flowering time. In the LDP A. thaliana, it does in fact delay the flowering phase, and does so by reducing the abundance of activated GA in the plant, thereby increasing the concentration of DELLA proteins, which in turn delays the
flowering phase as described earlier (Achard et al., 2007). It was thought to inhibit flowering in SDP (Wilmowicz et al., 2008), but actually induces flowering in a number of plants such as pineapple (*Ananas comosus*) (Taiz and Zeiger, 2010, p. 652), and *Plumbago indica* (Nitsch and Nitsch, 1969). Wilmowicz et al (2008) studied the relation between ABA and ethylene in *Pharbitis nil*, an SDP that requires at least 16 hours of continuous darkness to induce flowering. They discovered that applying an ABA inhibitor during SD inhibited flowering, while subsequent application of ethylene inhibitors reversed this effect. The flowering responses also varied with the timing of ABA application during different phases of the inductive dark periods. These results suggest that ABA plays an important part in controlling the photoperiod flowering response of *P. nil*. The authors suggest that the inhibitory effect of ethylene may be related to its effect on ABA levels in the plant. Furthermore, application of ABA during the sub-inductive (12-hour darkness) period resulted in flowering response. Was the flowering response observed caused by ethylene having an inhibitory effect on ABA, or by direct stimulation of ABA synthesis? Since *Bougainvillea* is also an SDP, it would be interesting to apply ABA to *Bougainvillea* under the same photoperiod as described by Wilmowicz et al., and compare the responses of *Bougainvillea* to those reported for *P. nil*.

More recent research on *Bougainvillea* has found that ethylene plays an important role in flowering time. Liu and Chang (2010) found that forced bending of *Bougainvillea* shoots induced flowering and showed a correlation with elevated levels of 1-aminocyclopropane-1-carboxylate (ACC), which is a precursor of ethylene. They hypothesized that ethylene functions as a signal to flower in *Bougainvillea* shoots that
are in a vegetative state. Their subsequent experiment applying ethephon (2-chloroethylphosphonic acid), directly to Bougainvillea shoots at different stages of vegetative and reproductive growth, confirmed their hypothesis that exposing vegetative Bougainvillea shoots to ethylene would induce earlier flowering and an increased number of flowers. However, they also found that shoots in reproductive stages (with developed floral buds or flowers) reacted to ethylene by abscission of leaves and flowers/bracts (Liu and Chang, 2011). This was corroborated by Al-Qubaie, (2013) who found that 75 ppm ethephon (in conjunction with magnesium sulfate and boric acid) applied as a foliar spray to vegetative shoots accelerated flowering of B. glabra under unstated photoperiod conditions. However, applying ethephon to flowering shoots caused leaf and bract abscission (Liu and Chang, 2011).

Therefore, ethylene appears to possess bidirectional regulatory effects on flowering in Bougainvillea depending on whether the shoots are in the vegetative or reproductive phase. Since ABA and ethylene are both stress hormones closely related to water stress responses, further examination of the interaction between these two hormones in Bougainvillea could contribute to a clearer understanding of the physiological signals for flowering in Bougainvillea.

Hypotheses

Our first hypothesis was that ABA and ethylene exhibit a positive relationship in Bougainvillea under non-flowering conditions. We expect that water stress would lead to an increase in ABA levels in the leaf tissue in the short term. We expected that application of exogenous ethylene would result in an increase in ABA levels in the leaf tissue as well.
Our second hypothesis was that ABA and ethylene would exhibit a negative relationship in *Bougainvillea*. We expect that the increase in ABA due to (a) water stress, (b) application of exogenous ABA, and (c) application of exogenous ethylene would lead to a decrease in endogenous GA in the shoot tissue of *Bougainvillea*.

Our third hypothesis was that the resulting fall in GA levels in the leaf tissue under the previous treatments would result in accelerated flowering response in *Bougainvillea* even under non-inductive photoperiods.
CHAPTER 3
OPTIMAL PHOTOPERIOD FOR FLOWERING OF BOUGAINVILLEA ‘AFTERTGLOW’

Introduction

Studies related to the flowering of horticultural plants in tropical and subtropical zones of the world, including the United States, have largely focused on economically important tropical fruit crops of several key genera, namely Mangifera (mango), Citrus (citrus) and Litchi (lychee) (Koshita and Takahara, 2004; Menzel, 1983; Núñez-Elisea and Davenport, 1994; Ramírez and Davenport, 2010). Less attention has been paid to woody ornamentals in recent years as the flowering behaviors of most landscape plants are already well-understood.

Ornamental crops in the tropics can be generally classified into foliage crops and floral crops. The difference between tropical/sub-tropical and temperate floral crops from a landscape design point of view is the seasonality of flowering, and the respective environmental stimuli that trigger flowering. In high latitudes, day length (photoperiod) and temperature (vernalization) are the predominant cues that signal plants to enter reproductive phase. At lower latitudes, especially in areas with distinct wet/dry seasonal variations, the trigger is often water availability. In areas without significant variation in water availability, intensity of solar radiation has been suggested as the trigger for some species to flower (Yeang, 2007).

Bougainvillea (Bougainvillea spp.) is a widespread and common woody evergreen perennial that is used as a landscape ornamental plant in South Florida and in tropical areas around the world. It is greatly valued for its vigor and resistance to pests, disease, and drought, in addition to its bright floral display of colorful bracts, and
variability in form as it can be planted as a shrub, standard, espaliered, or trained onto a trellis (Kobayashi et al., 2007).

It is a SDP, with flowers forming in apical panicles on the current year wood (Ma and Gu, 2010). In sub-tropical/tropical climates like South Florida, Bougainvillea flowers in fall to late spring, when the daylength is less than 12 h per day and night-time temperatures are above 21°C (Schoellhorn and Alvarez, 2002). Ramina and Sachs (1979) hypothesized that flowering in Bougainvillea was a function of nutrient diversion, and in further studies Even-Chen and Sachs (1980) supported the theory that SD induction was positively correlated to photosynthetic rates in mature Bougainvillea leaves. Ma and Gu (2010) built on this theory and confirmed earlier research by Steffen et al. (1988) that flowering in Bougainvillea was controlled in some way by GA by diverting nutrient assimilates away from the apical meristem. Since GA levels are known to change in response to photoperiod, or more specifically to the effect of far-red light on phytochrome photoreceptors (Taiz and Zeiger, 2010), SD induction as in the case of 8-hour photoperiod induction of Bougainvillea ‘San Diego Red’, and 10- to 8-hour photoperiod induction for Bougainvillea glabra ‘Sanderiana’ was thought to be the result of a complex web of interactions between hormones and environmental factors (Even-Chen and Sachs, 1980; Joiner et al., 1962).

We hypothesized that all other environmental factors being equal, Bougainvillea ‘Afterglow’ would exhibit the same SD inductive response as ‘San Diego Red’, with an inductive photoperiod of between 8 and 10 hours. We also sought to clarify the most inductive daylength for this cultivar. The objective of experiment 1 was to verify that Bougainvillea ‘Afterglow’ was a quantitative short-day plant (SDP), and that flowering
could be suppressed by night interruption or daylight extension. The objective of experiment 2 was to determine the length of photoperiod that was most inductive to flowering for this cultivar.

**Materials and Methods**

Experiment 1 was conducted in December 2015, and Experiment 2 was conducted in March 2016. Established rooted cuttings of *Bougainvillea* ‘Afterglow’ were used in both experiments. They were transplanted into 10 cm pots filled with 100% 5 mm coarse washed aquarium zeolite (Pentair Aquatic Eco-systems Inc., Apopka, FL, USA). Plants were sprayed with 30 µL of ethephon (25 mL of 1200 ppm concentration; Southern Agricultural Insecticides, Inc., Hendersonville, NC) to induce leaf and inflorescence senescence, then pruned to remove apical buds, and as far as possible reduced to a length with 7 visible lateral nodes. They were then kept vegetative under 14-hour photoperiod, consisting of daylight supplemented with white LED lights supplying 70 µmol m⁻¹s⁻¹ photosynthetic photon flux (PPF). To prevent nutrient deficiencies, plants were fertilized with Peters Professional Bloom Booster (10N-30P₂O₅-20K₂O; JR Peters, Allentown, PA) at 9.4 µg total N (nitrogen) once per week starting three weeks prior to the start of the experiment. We continued to apply the fertilizer at the same rate once per week through the remainder of the experiment. In addition to fertilizer, plants were watered with 50mL of tap water (EC = 516 µS, pH = 8.3) every two days.

For Experiment 1, thirty plants (six treatments with five replicates each) were arranged in a completely randomized design in an open sided greenhouse exposed to ambient air temperatures (25.0/18.8°C day/night) and 81% relative humidity, at the University of Florida, Fort Lauderdale Research and Education Center in Davie, Florida.
Photoperiod treatments were created using a 5-gallon black plastic pot inverted over each plant to block out light. All plants were covered at 6pm and uncovered at 8am. A single 5-watt LED bulb providing 35 µmol m\(^{-1}\) s\(^{-1}\) PPF suspended inside each pot and set on a timer provided night interruption or daylength extension. There were three continuous photoperiod treatments – 14 (control), 11 and 8 hours, and three night-interruption treatments: 8+3-hours, 8+6-hours, and 11+3-hours. Night interruptions occurred after 3 hours of dark. Night interruption treatments were designed to match the number of daylight hours of the continuous photoperiod treatments. Prior experiments (data not shown) suggested that short night interruptions (5 to 30 mins of light) were insufficient to inhibit flowering.

Root zone temperature (RZT) was monitored using two dataloggers (HOBOWare Pro U12, Onset Computer Corporation, Bourne, MA), with sensors inserted into seven random replicate pots. Temperatures were logged in °C at 30 min intervals.

Plant size was recorded at the start and end of the experiment to calculate growth. Plant size was determined by the formula

\[
\text{Size} = H \times W1 \times W2
\]

(3-1)

Where H = height, rounded to the nearest cm, W1 = maximum width of the plant to the nearest cm, and W2 = width of the plant perpendicular to W1, to the nearest cm.

Growth was defined as the difference between the plant size at the end of the experiment and at the start of the experiment. Relative growth was calculated as the percentage ratio of growth over initial size.
The number of inflorescences on each plant was counted at the end of day 30. One inflorescence was defined as an individual thorn-inflorescence axil, regardless of how many florets were attached to the peduncle.

Experiment 2 was a repeat of Experiment 1 with the same preparation but with continuous photoperiod treatments without night interruption. The treatments were 14- (control), 12-, 11-, 10-, 9- and 8-hour photoperiods respectively.

Analysis of variance (ANOVA, α=0.05) was performed using R statistical analysis program, with number of inflorescences as the dependent variable and photoperiod treatment and relative growth as the independent variables. Data for experiment 1 and experiment 2 were analyzed separately. Root-zone temperature was identical across all treatments so this data was omitted from ANOVA. The average daily temperature range was 24°C to 32°C. In experiment 1, mean separation was conducted using paired t-tests to identify which treatments were significantly different. In experiment 2, Tukey’s Honestly Significant Difference was used for means separation to identify which treatments were significantly different.

**Results**

The number of inflorescences was not significantly affected by the growth of the plants, and there was no interaction between relative growth and photoperiod treatment on number of inflorescences (Table 3-1).

In experiment 1, plants grown under 8-hour and 11-hour photoperiods produced significantly more inflorescences than all other treatments (Table 3-2). All night interruption treatments inhibited flowering response. Among the three continuous photoperiod treatments, the control plants (14-hour) had the fewest flowers, while those under 8-hour grew the least but had the most number of flowers. Since night interruption
effectively inhibited flowering, the treatments for Experiment 2 omitted night interruption and focused on narrowing the range of photoperiod treatments.

In Experiment 2, control plants remained completely vegetative under 14-hour photoperiod (See Table 3-3). Plants grown under 8-hour photoperiod had the greatest number of inflorescences (13.6).

Plants grown under 9-hour photoperiod had the second highest mean number of inflorescences (11.2). There was no significant increase in the number of inflorescences produced under 12-, 11- and 10-hour photoperiods, compared to controls (Table 3-3). Linear regression analysis was performed on number of inflorescences as a response to photoperiod. The coefficient for photoperiod was -2.15, which showed that inflorescences had a negative linear relationship with photoperiod.

**Discussion**

Previous studies indicated that *Bougainvillea* ‘San Diego Red’ flowered under SD conditions (Even-Chen et al., 1979; Even-Chen and Sachs, 1980). The results of experiment 1 appear to support the hypothesis that the cultivar ‘Afterglow’ is also a SD plant that requires photoperiods of less than 12 hours to induce flowering, while the results from experiment 2 indicate that 8 hours of daylight was the most inductive photoperiod for ‘Afterglow’. These results concur with previous research which suggested that 8 hours was the optimal photoperiod for flowering of *Bougainvillea* (Schoellhorn and Alvarez, 2002; Singh et al., 2013). In addition, flowering of ‘Afterglow’ was completely inhibited by extending the photoperiod to 14 hours, either continuously or as night interruption. This result was interesting as early studies suggested that there was no clearly defined critical photoperiod to induce floral initiation in *Bougainvillea* (Joiner et al., 1962). However, these results suggest that there may be a threshold
photoperiod to inhibit flowering altogether. In addition, the inability of short night interruptions of 5 to 30 minutes to inhibit flowering would be of interest for further research.

*Bougainvillea* is an important landscape shrub in the tropics and subtropics, where annual variation of daylength is between 10 ½ hours and 13 ½ hours. In these areas, seasonal variation in daylength creates alternating inductive and sub-inductive photoperiods. We designate the latter sub-inductive rather than non-inductive because the photoperiod does not reach or exceed 14 hours, which would completely inhibit flowering. In South Florida, the inductive season would correspond to mid-October to mid-February, and the sub-inductive period would be from March through September. The tropics also encompass equatorial areas that have a constant year-round 12-hour daylength. In these places, we could consider the entire year as sub-inductive, so flowering can take place sporadically year-round in response to other factors such as microclimate, light intensity, nutrient availability, and environmental stresses. In particular, the water deficit and physical stress have been found to induce flowering in *Bougainvillea* (Liu and Chang, 2010, 2011; Ma and Gu, 2010; Schoellhorn and Alvarez, 2002). Since the stress hormones ethylene and abscisic acid (ABA) both have complex relations with GA pathways, further investigation on the interactions between ethylene and ABA levels on GA in *Bougainvillea* in relation to flowering responses under sub-inductive conditions should be taken.
Table 3-1. Effect of photoperiod and relative growth on number of inflorescences on *Bougainvillea* ‘Afterglow’ in December 2015. n=30. (*) indicates significant difference at $\alpha=0.05$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F-Value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photoperiod</td>
<td>5</td>
<td>755.5</td>
<td>151.10</td>
<td>3.235</td>
<td>*0.0295</td>
</tr>
<tr>
<td>Relative Growth</td>
<td>1</td>
<td>42.5</td>
<td>42.51</td>
<td>0.910</td>
<td>0.3527</td>
</tr>
<tr>
<td>Treatments × Relative Growth</td>
<td>5</td>
<td>267.4</td>
<td>53.48</td>
<td>1.145</td>
<td>0.3732</td>
</tr>
</tbody>
</table>

Table 3-2. Effects of photoperiod on number of inflorescences and relative growth of *Bougainvillea* ‘Afterglow’ in December 2015. Means in the same column followed by the same letters are not significantly different at $\alpha=0.05$. n=30.

<table>
<thead>
<tr>
<th>Treatment (hours)</th>
<th>Mean Number of Inflorescences</th>
<th>Standard Error</th>
<th>P-value</th>
<th>Relative Growth (%)</th>
<th>Standard Error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.4 b</td>
<td>0.000</td>
<td>na</td>
<td>262.8 a</td>
<td>107.616</td>
<td>na</td>
</tr>
<tr>
<td>11</td>
<td>12.2 a</td>
<td>5.903</td>
<td>0.09</td>
<td>253.0 a</td>
<td>142.269</td>
<td>0.99</td>
</tr>
<tr>
<td>8</td>
<td>10.2 a</td>
<td>3.680</td>
<td>0.22</td>
<td>190.7 a</td>
<td>70.292</td>
<td>0.97</td>
</tr>
<tr>
<td>8+3</td>
<td>0.0 b</td>
<td>0.000</td>
<td>1.00</td>
<td>216.8 a</td>
<td>82.451</td>
<td>0.99</td>
</tr>
<tr>
<td>8+6</td>
<td>0.0 b</td>
<td>0.000</td>
<td>1.00</td>
<td>144.4 a</td>
<td>34.371</td>
<td>0.98</td>
</tr>
<tr>
<td>11+3</td>
<td>3.0 b</td>
<td>3.000</td>
<td>0.98</td>
<td>346.9 a</td>
<td>518.011</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 3-3. Effects of photoperiods on number of inflorescences of *Bougainvillea* ‘Afterglow’ grown in February 2016. Means in the same column followed by the same letters are not significantly different at $\alpha=0.05$.

<table>
<thead>
<tr>
<th>Treatment (hours)</th>
<th>Mean Number of Inflorescences</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.0 b</td>
<td>0.000</td>
<td>na.</td>
</tr>
<tr>
<td>12</td>
<td>5.8 b</td>
<td>1.462</td>
<td>0.343</td>
</tr>
<tr>
<td>11</td>
<td>8.0 b</td>
<td>1.871</td>
<td>0.085</td>
</tr>
<tr>
<td>10</td>
<td>8.4 b</td>
<td>2.857</td>
<td>0.064</td>
</tr>
<tr>
<td>9</td>
<td>11.2 a</td>
<td>2.577</td>
<td>0.006</td>
</tr>
<tr>
<td>8</td>
<td>13.6 a</td>
<td>1.860</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
CHAPTER 4
EFFECT OF VARYING ETHEPHON RATE APPLICATIONS ON FLOWERING OF BOUGAINVILLEA ‘AFTERGLOW’

Introduction

Flowering of *Bougainvillea* ‘Afterglow’ was triggered in response to an 8-hour photoperiod. This cultivar, like several others including ‘Raspberry Ice’, ‘San Diego Red’, and ‘Rainbow Gold’ are quantitative short-day (SD) plants that flower more readily under SD conditions. Other environmental factors, such as drought stress, high light intensity and low nighttime temperatures might also enhance flowering (Hackett and Sachs, 1985; Joiner et al., 1962; Jeffrey G. Norcini et al., 1992; Norcini et al., 1994).

Other researchers have found that ethylene, applied in the form of ethephon (2-chloroethylphosphonic acid) is effective in promoting flowering of *Bougainvillea glabra* and *Bougainvillea* ‘Taipei Red’, but these experiments were conducted under shortening daylengths, and not under non-inductive photoperiods of more than 13-hour daylengths (Al-Qubaie, 2013; Liu and Chang, 2011). The application rate of 75 µL/L ethephon applied as a foliar spray was found to be effective under SD on these plants.

To find out if a different rate would be able to break the LD inhibition of flowering in *Bougainvillea* ‘Afterglow’, a rate study of ethephon was conducted. If a rate was found to effective, then the effective rate would be used in further experiments to compare the effect of ethephon application against the effect of SD photoperiod on *Bougainvillea* ‘Afterglow’.

**Materials and Methods**

Thirty rooted cuttings of *Bougainvillea* ‘Afterglow’ were grown in 10 cm plastic pots with 100% 5 mm coarse washed aquarium zeolite (Pentair Aquatic Eco-systems Inc., Apopka, FL, USA). They were kept under 14-hour photoperiod (natural daylight
with supplemental white LED lights providing 70 µmol·m⁻¹·s⁻¹ photosynthetic photon flux, to inhibit photoperiod-induced flowering. To prevent nutrient deficiencies, plants were fertilized with Peters Professional Bloom Booster (10N-30P₂O₅-20K₂O; JR Peters, Allentown, PA) at 9.4 µg total (N) nitrogen once per week starting three weeks prior to the start of the experiment. We continued to apply the fertilizer at the same rate once per week through the remainder of the experiment. Five concentrations (75, 150, 300, 600 and 1200 µL/L) of ethephon (Florel® brand, Southern Agricultural Insecticides, Inc., Hendersonville, NC) were prepared at the start of the experiment, and each plant was sprayed with 25 mL of the PGR solution on the foliage. The actual amount of ethephon applied under each treatment was thus 1.875 µL, 3.75 µL, 7.5 µL, 15 µL, and 30 µL per plant respectively. The control plants received no PGR applications. All plants were hand watered with 100 mL of tap water every 3 days. Plants were arranged in a complete randomized design in an open-sided greenhouse exposed to ambient air temperatures (28.3/20.8°C day/night) and 71% relative humidity, at the University of Florida Fort Lauderdale Research and Education Center in Davie, Florida.

The number of inflorescences on each plant was counted at the end of day 30. One inflorescence was defined as an individual thorn-inflorescence axil, regardless of how many florets were attached to the peduncle. Plant size was recorded at the end of the experiment when flowering was observed. Plant size was determined by the formula

\[
Size = H \times W1 \times W2
\]

(4-1)

Where H = height, rounded to the nearest cm, W1 = maximum width of the plant to the nearest cm, and W2 = width of the plant perpendicular to W1, to the nearest cm.
General linear model with Poisson regression was conducted using R statistical analysis program (www.r-project.org) to identify the relationship between ethephon concentrations and number of inflorescences.

Results

There were no significant differences between treatments in terms of overall size of plants. There were significant differences in mean number of inflorescences between treatments (Table 4-1). The lowest rate (75 µL/L) and the two highest rates (600 and 1200 µL/L) resulted in flowering responses that were not significantly different from control plants at 30 days after treatment. (Table 4-1).

Among the remaining treatments that promoted flowering, 150 µL/L ethephon appeared to promote the largest number of flowers (mean = 18.0), followed by 300 µL/L (mean = 12.2) and 75 µL/L (mean = 9.8) (Table 4-1). Only the 150 µL/L treatment was significantly different from the control ($P<0.05$) (Fig. 4-1). The effectiveness of ethephon as a flowering promoter in this cultivar of Bougainvillea appeared to increase from application rates between 75 µL/L to 150 µL/L, and gradually decrease between 200 and 600 µL/L, and was not effective at higher concentrations. The highest concentration of ethephon (1200 µL/L) defoliated all plants within 72 hours of application. Therefore, at the end of the experiment, there were no flowers on plants under that treatment (mean inflorescences = 0.8) (Table 4-1).

Discussion

Under non-inductive LD conditions, control plants did not remain completely vegetative, but the number of inflorescences were not significantly greater than zero. It was clear from the results that any rate of ethephon higher than 600 µL/L would not be effective in promoting flowering in the short term (within 30 to 48 days), as it would be
likely to completely defoliate the plants. This rate (600 µL/L) can be applied if a chemical defoliant is required, without killing the plant. The practical use of such an application is to remove both foliage and inflorescences without the need for physical pruning. The new growth that comes back after defoliation tends to be from existing axillary buds, instead of the apical meristems, which creates a denser plant without increasing the overall height of the plant.

Although we found that 75 µL/L application rate did appear to promote flowering of *Bougainvillea ‘Afterglow’* under LD conditions, it was not significant. Since the Liu and Chang study was conducted in the fall of 2009 in Taipei, we could surmise that the photoperiod conditions of that experiment was SD. (Khandaker et al., 2013) found that ethylene production in *Bougainvillea* under neutral day lengths (12 hour light/dark) naturally increased with age and maturity of the shoots, leading to flowering and then abscission of the flowers. Therefore, 75 µL/L ethephon spray may promote flowering only under SD or neutral day lengths, when it constitutes a supplementary source of ethylene to accelerate the transition of shoots from the vegetative to reproductive stage. Under non-inductive LD conditions, a higher concentration of ethephon may be required to achieve the same effect. Therefore, a rate study would need to be conducted to determine the optimal concentration, if any, for application of ethephon to *Bougainvillea ‘Afterglow’* under strictly non-inductive LD conditions.

The flowering response increased at the lower application rates, and peaked between 75 µL/L and 300 µL/L, with 150 µL/L showing the only significantly positive treatment effect. A repeat of this rate including a narrower range of concentrations between 50 µL/L and 200 µL/L should be conducted to determine the most effective
rate of ethephon application for LD conditions. These results support the hypothesis that exogenous ethylene application will promote flowering of *Bougainvillea* under non-inductive LD photoperiod.

Table 4-1. ANOVA results of mean plant size and number of inflorescences at 30 days after treatment with increasing concentration of ethephon. (*) indicates statistical difference at $\alpha=0.05$. (n=30).

<table>
<thead>
<tr>
<th>Concentration of ethephon (µL/L)</th>
<th>Mean plant size (cm$^{-3}$)</th>
<th>Standard error</th>
<th>$P$-value</th>
<th>Mean number of inflorescences</th>
<th>Standard error</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2868.2</td>
<td>991.99</td>
<td>5.8</td>
<td>2.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>2883.6</td>
<td>289.24</td>
<td>0.986</td>
<td>9.8</td>
<td>1.74</td>
<td>0.149</td>
</tr>
<tr>
<td>150</td>
<td>1937.4</td>
<td>397.04</td>
<td>0.290</td>
<td>18.0</td>
<td>2.59</td>
<td>**0.003</td>
</tr>
<tr>
<td>300</td>
<td>1773.8</td>
<td>402.57</td>
<td>0.216</td>
<td>12.2</td>
<td>1.39</td>
<td>*0.090</td>
</tr>
<tr>
<td>600</td>
<td>4209.6</td>
<td>675.91</td>
<td>0.132</td>
<td>7.4</td>
<td>4.60</td>
<td>0.663</td>
</tr>
<tr>
<td>1200</td>
<td>2600.4</td>
<td>613.81</td>
<td>0.758</td>
<td>0.8</td>
<td>0.80</td>
<td>0.180</td>
</tr>
</tbody>
</table>

Figure 4-1. Effect of increasing ethephon concentration on number of inflorescences in *Bougainvillea* ‘Afterglow’ under 14-hour photoperiod. The 150 µL/L rate was significantly more effective than the control ($P=0.027$). (n=30).
CHAPTER 5
EFFECTS OF WATER STRESS, HORMONES, AND PHOTOPERIOD ON FLOWERING OF BOUGAINVILLEA ‘AFTERGLOW’

Introduction

The flowering of many ornamental Bougainvillea hybrids (Bougainvillea x buttiana) appears to have multiple environmental triggers, including photoperiod and various abiotic stress factors like wounding or drought (Hackett and Sachs, 1985; Liu and Chang, 2010, 2011; Ramina et al., 1979; Tse et al., 1974).

The results from the previous chapters showed that Bougainvillea ‘Afterglow’ is a short-day (SD) plant that flowers more profusely as the photoperiod was reduced from 12 hours to 8 hours, while a photoperiod of 14 hours effectively inhibited flowering. Therefore, in this experiment we compared the effect of the both long-day (LD) and SD, in combination with exogenous application of abscisic acid (ABA) and ethylene to simulate the effect of increase in endogenous ABA and ethylene by drought stress and physical wounding. The objectives were firstly to compare the effects of the exogenous hormone application with the actual effect of drought stress and photoperiod, and secondly to find out if one factor was more dominant than the others.

Materials and Methods

Seventy rooted cuttings of Bougainvillea ‘Afterglow’ were transplanted into 10 cm pots filled with 100% 5 mm coarse washed aquarium zeolite (Pentair Aquatic Eco-systems Inc., Apopka, FL, USA). Plants were sprayed with 30 µl of ethephon (25 mL of 1200 ppm concentration; Southern Agricultural Insecticides, Inc., Hendersonville, NC) to induce total leaf drop, then pruned to a mean height of 12 cm with no lateral shoots as much as possible. Plants were then placed under LD (14-hour photoperiod) to prevent flowering prior to the start of the experiment. This consisted of natural daylight
supplemented with white LED lights supplying 70 µmol m\(^{-1}\) s\(^{-1}\) photosynthetic photon flux (PPF). To prevent nutrient deficiencies, plants were fertilized with Peters Professional Bloom Booster (10N-30P\(_2\)O\(_5\)-20K\(_2\)O; JR Peters, Allentown, PA) at 9.4 µg total nitrogen once per week starting three weeks prior to the start of the experiment. We continued to apply the fertilizer at the same rate once per week through the remainder of the experiment. In addition to fertilizer, plants were watered with 50 mL of tap water (EC = 516 µS, pH = 8.3) every two days.

There were seven treatments, with ten replicates per treatment. There were 3 plant growth regulator (PGR) treatments under LD: 1.8 µl of ethephon (25 mL of 75 ppm concentration), 12.5 µg of ABA (25 mL of 500 ppm concentration), and a combination of both (1.8 µl ethephon + 12.5 µg ABA). Control plants were under LD with no hormones applied. There were 2 treatments under SD, which were water stress (WS) and no water stress. Plants in the water stress treatments were watered with 50 mL of water every 4 days, all other plants were watered with 50 mL of tap water every 2 days. SD treatment was an 8-hour photoperiod, achieved by covering the plants with black plastic covers between 4pm and 8am.

Plants were arranged in a complete randomized design in an open-sided greenhouse exposed to ambient air temperatures (33.3/25.2°C day/night) and 77.7% relative humidity, at the University of Florida Fort Lauderdale Research and Education Center in Davie, Florida.

Plant size was recorded at the start and end of the experiment to calculate growth. Plant size was determined by the formula below, where H = height, rounded to
the nearest cm, W1 = maximum width of the plant to the nearest cm, and W2 = width of the plant perpendicular to W1, to the nearest cm.

\[
\text{Size} = H \times W1 \times W2
\]  

(5-1)

Growth was defined as the difference between the plant size on day 1 and day 30. Relative growth was calculated as the percentage ratio of growth over initial size. The number of inflorescences on each plant was counted on day 30. One inflorescence was defined as an individual thorn-inflorescence axil, regardless of how many florets were attached to the peduncle.

The pour-through method (Wright, 1986) was used to collect leachate at the start and end of the experiment. pH and electrical conductivity (EC) were measured using a portable pH/EC meter (Hanna Instruments, Ann Arbor, MI, USA), and N-nitrate (NO$_3$), potassium (K) and calcium (Ca) levels were measured using LAQUA portable ion meters (Spectrum Technologies, Aurora, IL, USA). Phosphate (PO$_4$) levels were measured using a benchtop selective ion probe (Accumet® XL250, Fisher Scientific, Waltham, MA, USA). Results were compared using paired t-tests performed in R statistical analysis program (www.r-project.org) to check if there were any differences between treatments in terms of nutrient availability in the media.

Analysis of variance (ANOVA, \( \alpha=0.05 \)) was performed using R statistical analysis program, with number of inflorescence, growth, and change in number of leaves as the dependent variables and treatments as the independent variables, with mean separation conducted using Tukey’s HSD to identify which treatments were significantly different.
Results

ANOVA was conducted on the three growth parameters (relative growth, difference in number of leaves, and difference in number of buds) on day 30 of the experiment. There were no significant treatment effects on the overall growth (change in size) of the plants or on the change in the number of leaves from day 1 to day 30. There was a significant difference between treatments in number of buds on day 30 (Table 5-1).

Tukey’s Honestly Significant Differences (HSD) was used to identify the specific treatments that showed significant effects (Table 5-1). The combination of SD and WS induced the largest mean number of inflorescences (12.4) after thirty days ($P<0.0001$). SD without WS produced the next highest mean number of inflorescences (8.2, $P<0.05$). The other treatments were not significantly different from the control. However, the LD+WS+Ethephon treatment showed a slight positive effect on flowering even though they were not significant. Both the application of $s$-ABA alone, and in conjunction with ethephon, resulted in virtually no flowering.

Student’s t-test conducted on the NO$_3$, K, PO$_4$, pH and electrical conductivity (EC) of the leachate taken at the end of the experiment showed that there were no differences among treatments (data not shown). Therefore, nutrient availability did not contribute to any of the differences between plant size and number of inflorescences at the end of the experiment, and were excluded from the subsequent statistical analysis.

Discussion

This study was set up to identify whether one factor was more important than other factors to trigger flowering in Bougainvillea ‘Afterglow’. Two abiotic factors – photoperiod and drought stress – were used as treatments in this experiment.
Additionally, two stress hormones related to both drought stress and physical stress (abscisic acid and ethylene respectively) were also used as treatments, to see if exogenous application of these hormones could simulate the abiotic stress under LD (non-inductive) photoperiod. Liu and Chang (2011) found that exogenous foliar spray application of ethephon at 75 ppm concentration was effective in accelerating and increasing the flowering of Bougainvillea ‘Taipei Red’. We sought to replicate this finding by applying the same concentration of ethephon on Bougainvillea ‘Afterglow’ to see if the effects were the same for this cultivar.

The most salient observation from this experiment was that photoperiod was more important than all the other treatments in promoting flowering of Bougainvillea ‘Afterglow’. WS promoted flowering under both SD and LD conditions, but the effect was significant only under SD conditions. It appeared that the effect was additive to the SD conditions. The application rate of 50 mL of 75 µL/L ethephon spray was found by (Liu and Chang, 2011) to be effective in promoting flowering of Bougainvillea ‘Taipei Red’.

While water stress was found to induce some flowering in Bougainvillea ‘Afterglow’ under LD conditions, the lack of flowering response with exogenous applications of ABA suggests that exogenous ABA applied as a foliar spray did not become active in the same way that endogenous ABA within the plant tissue was. Exogenous ABA on the leaf may have remained largely on the epidermis and may not have been taken up by the plant Since endogenous ABA is both synthesized within the leaves as well as transported from the roots during a drought stress event, (Malladi and Burns, 2007; Verelst et al., 2010), application as a drench should be explored as an
alternative method. This might also suggest that the increased presence of ABA may not have been the real cause of flowering response under water stress.

ABA and ethylene have a complex interaction that is typically antagonistic, where a sharp increase in ABA usually coincides with a decrease in ethylene (Sharp and LeNoble, 2002). In this experiment, the combined application of ABA and ethephon resulted in no flowering at the end of the experiment. The question arises as to whether the concurrent application of ABA negated the mild promotive effect of ethephon on flowering. At least in the case of hibiscus flowers (*Hibiscus rosa-sinensis*), application of exogenous ABA was found to have a negative effect on the biosynthesis of ethylene in the flower tissues (Trivellini et al., 2011). The interaction between ABA and ethylene appears to take place along the gibberellic acid (GA) pathway (Sharp and LeNoble, 2002; Wilmowicz et al., 2008). Pierik et al. (2004) found that in tobacco (*Nicotiana tabacum* cv. Samsun NN), an increase in the ratio of red:far-red light (R:FR), ethylene production increased, and this coincided with the shade avoidance response (leaf elongation and leaf hyponasty). The shade avoidance response was a phytochrome-mediated function that required the presence of GA. This was further confirmed by the fact that application of paclobutrazol, a GA inhibitor, reduced the shade avoidance response in both wild-type plants and ethylene-insensitive mutants. Furthermore, it has been found that application of exogenous GA on shoot tips inhibits flowering in *Bougainvillea* 'Raspberry Ice', while application of dikegulac, another GA inhibitor, increased flowering under SD and day-neutral conditions (Jeffrey G. Norcini et al., 1992).
The shade avoidance response is a phytochrome-mediated function that requires the presence of GA. In brief, an accumulation of GA in the cell nucleus leads to the degradation of DELLA proteins, which are negative regulators of shade avoidance responses such as cell elongation. The degradation of the DELLA proteins removes their inhibitory effect, thus allowing the expression of the genes related to shade avoidance (Taiz and Zeiger, 2010).

GA has been established as an integral part of the sensing of photoperiod in plants such as the potato, which requires strict SD conditions to initiate tuberization (Malladi and Burns, 2007). The most significant observation from this experiment, and the answer to the third objective of the experiment, was that photoperiod was more important than all the other treatments in promoting flowering of *Bougainvillea* ‘Afterglow’. WS promoted flowering under both SD and LD conditions, but the effect was significant only under SD conditions. It appeared that the effect is additive to the SD conditions. This concurs with previous findings, where exogenous application of GA inhibited flowering of various *Bougainvillea* cultivars under SD photoperiod, and dikegulac, another GA inhibitor, promoted flowering under SD photoperiod (Aldrich and Norcini, 1996; Norcini et al., 1993; Tse et al., 1974).

As mentioned, GA plays an integral role in the sensing of photoperiod (Ewing, 1987; Taiz and Zeiger, 2010), while ABA and ethylene both interact at different points along the GA pathway (Alabadí and Blázquez, 2009; Koshita et al., 1999; Pierik et al., 2004). All three phytohormones appear to work differently in tissues at different stages of development. (Verelst et al., 2010) noted that while the ABA-dependent drought stress response has been repeatedly found to take place in mature leaf cells, in
meristems and dividing leaf cells of young leaves, it is GA and ethylene-dependent pathways that are activated under drought stress. To know if drought stress triggers flowering in *Bougainvillea* ‘Afterglow’ through hormonal signaling, we would need to analyze endogenous levels of ABA and ethylene under degrees of drought stress and different photoperiods, as well as the changes in GA levels in both young leaves and mature leaves under both inductive and non-inductive environmental growing conditions. If we could determine that certain fluctuations in hormonal levels in responses to these stresses are correlated to the flowering response, we could assess if the exogenous application of those hormones will simulate the same environmental stress triggers when they are absent.

Table 5-1. Tukey’s HSD Means separation of number of inflorescences under 6 different treatments and control (LD – No WS, No PGR). Treatments followed by the same letters are not significantly different at α=0.05, n=70

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inflorescences (mean)</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD + WS + No PGR</td>
<td>12.4</td>
<td>0.00002</td>
</tr>
<tr>
<td>SD + No WS + No PGR</td>
<td>8.2</td>
<td>0.045</td>
</tr>
<tr>
<td>LD + No WS + Ethephon</td>
<td>4.9</td>
<td>0.835</td>
</tr>
<tr>
<td>LD + WS + No PGR</td>
<td>3.4</td>
<td>0.998</td>
</tr>
<tr>
<td>LD + No WS + No PGR (Control)</td>
<td>2.4</td>
<td>--</td>
</tr>
<tr>
<td>LD + No WS + Ethephon + ABA</td>
<td>1.4</td>
<td>0.998</td>
</tr>
<tr>
<td>LD + No WS + ABA</td>
<td>0.9</td>
<td>0.984</td>
</tr>
</tbody>
</table>

SD = Short Day, WS = Water Stress, PGR = Plant Growth Regulators, LD = Long Day, ABA = Exogenous Abscisic Acid
CHAPTER 6
HORMONES, FLOWERING AND ABA LEVELS

Introduction

*Bougainvillea* (*Bougainvillea* spp.) is a widespread and common woody evergreen perennial that is used as a landscape ornamental plant in tropical and subtropical regions of the world. It is greatly valued for its vigor and resistance to pests, disease, and drought, in addition to its bright floral display of colorful bracts (Kobayashi et al., 2007).

It is a short day (SD) plant (Hackett and Sachs, 1985), with flowers forming in apical panicles on the current year’s wood (Ma and Gu, 2010). In sub-tropical/tropical climates like South Florida, *Bougainvillea* flowers in fall to late spring t, when the photoperiod is less than 12-h per day and night-time temperatures are above 21°C (Schoellhorn and Alvarez, 2002). Ramina and Sachs (1979) hypothesized that flowering in *Bougainvillea* was a function of nutrient diversion, and in further studies (Even-Chen and Sachs, 1980) supported the theory that SD induction was positively correlated to photosynthetic rates in mature *Bougainvillea* leaves. Ma and Gu, (2010) built on this theory and confirmed earlier research by Steffen et al (1988) and (Staden and Dimalla, 1980) that flowering in *Bougainvillea* was controlled in some way by gibberellic acid (GA) by diverting nutrient assimilates away from the apical meristem through the restriction of cytokinin production in the roots.

Water deficit stress (WDS) has been used to promote flowering and control size in nursery production settings (Kobayashi et al., 2007; Ma and Gu, 2010). Two classical plant hormones, abscisic acid (ABA) and ethylene are associated with abiotic stress responses (Taiz and Zeiger, 2010). Ethylene has been associated flowering of
Bougainvillea under physical stresses such as shoot bending and by exogenous application of the plant growth regulator ethephon or 2-chloroethylphosphonic acid (Liu and Chang, 2010, 2011). ABA is responsible for inducing the loss of guard cell turgor and thereby closing of stomata and reduction of evapotranspiration in plants that are under medium to severe WDS (Mansfield, 1987; Walton, 1987). However, no research has been published on the role it may play in the flowering response of Bougainvillea, given the apparent effect that mild WDS appears have on triggering of flowering.

We have found in earlier experiments that Bougainvillea ‘Afterglow’ is a SDP that flowers best under SD conditions of 8-hours, and flowering was suppressed under LD (14-hours), which is consistent with other cultivars of Bougainvillea × buttiana such as ‘San Diego Red’ (Even-Chen and Sachs, 1980; Joiner et al., 1962). Our first hypothesis was that increased levels of ABA and ethylene would increase the number of inflorescences in ‘Afterglow’ under SD and induce flowering under LD conditions. The first objective of this study was to examine the effect of three rates of exogenous ABA and ethylene on the flowering response of under both SD and LD photoperiod. We also hypothesized that endogenous ABA levels would be positively correlated with the flowering response. The second objective was to analyze the ABA content in the leaves under both SD and LD, to verify that endogenous ABA content in Bougainvillea was different under different photoperiods.
Materials and Methods

Fifty-six rooted cuttings of Bougainvillea ‘Afterglow’ were transplanted into 10 cm pots filled with 100% 5mm coarse aquarium zeolite (Pentair Aquatic Eco-systems Inc., Apopka, FL, USA). Plants were sprayed with 30 µl of ethephon (25 mL of 1200 µL/L concentration; Southern Agricultural Insecticides, Inc., Hendersonville, NC) to defoliate them completely, then pruned to a mean height of 12.7cm (+/- 2.8cm). Plants were placed under long days (LD) 14-hour photoperiod to prevent flowering prior to the start of the experiment. This was done by supplementing the natural daylight (around 13 hours) with white LED lights supplying 70 µmol m-1s-1 photosynthetic photon flux (PPF), measured using a handheld quantum meter (Apogee Instruments, Logan, UT).

To prevent nutrient deficiencies, plants were fertilized with Peters Professional Bloom Booster (10N-30P2O5-20K2O; JR Peters, Allentown, PA) at 9.4 µg N (nitrogen) once per week starting three weeks prior to the start of the experiment. We continued to apply the fertilizer at the same rate once per week through the remainder of the experiment. In addition to fertilizer, plants were watered with 50 mL of tap water (EC = 516 µS, pH = 8.3) every two days.

The experiment was set up in an open-sided greenhouse exposed to ambient air temperatures (33.0/25.8°C day/night) and 78.6% relative humidity, at the University of Florida Fort Lauderdale Research and Education Center in Davie, Florida. The plants were arranged in a complete randomized block design, with two photoperiod treatments being the block factor (SD=8-hour photoperiod, LD=14-hour photoperiod). Within each block there were six treatments and one control. Treatments were three rates (75 µL/L, 150 µL/L, 300 µL/L in deionized water) of ethephon (Florel®; Southern Agricultural
Insecticides, Inc., Hendersonville, NC) and ABA (ProTone®; Valent Biosciences, Libertyville, IL). Controls had no plant hormones applied. SD treatments were considered started on day 1, being the first afternoon when the plants were covered at 4pm. Hormone treatments on SD plants were applied on day 0, along with LD plants. Each treatment rate had four replicates, each replicate being a single plant.

All hormone treatments were applied as a foliar spray of 50 mL per plant at the start of the experiment. Plant size, was determined by the equation below, where \( H = \) height, rounded to the nearest cm, \( W_1 = \) maximum width of the plant to the nearest cm, and \( W_2 = \) width of the plant perpendicular to \( W_1 \), to the nearest:

\[
\text{Size} = H \times W_1 \times W_2
\]

Measurements were recorded on day 1 and day 28 of the experiment. Relative growth was calculated by dividing the difference between plant sizes on day 1 and day 28 by initial plant size on day 1.

The number of inflorescences on each plant was counted on day 30. One inflorescence was defined as an individual thorn-inflorescence axil, regardless of how many florets were attached to the peduncle.

**ABA determination:** Leaf tissue from the youngest fully expanded leaves were collected at 1, 3, 7 and 14 days after treatment. Phytohormone abundance was determined using gas chromatography – mass spectrometry (GC-MS) adapted from (Engelberth and Engelberth, 2009; Schmelz et al., 2004).

Analytical grade abscisic acid (ABA) was obtained from Sigma-Aldrich (St. Louis, MO) to be used as internal standards. Solvents used in this procedure were methanol, methylene chloride (dichloromethane), and 1-propanol. Reagents were hydrochloric
acid (Fisher Scientific, Hampton, NH) and trimethylsilyldiazomethane (TMSD) 2.0 M in hexanes (Sigma-Aldrich, St. Louis, MO).

The procedure used 200 mg of fresh tissue from each replicate was measured out and placed in a 2 mL homogenizer vial containing 300 µL of an extraction solution (1-propanol/deionized water/hydrochloric acid in 2:1:0.002 ratio) and 2.8 mm ceramic homogenizer beads. The samples were then homogenized in a Bead Ruptor 12 bead mill (Omni International, Kennesaw, GA) for 30 seconds on high, then 1mL of methylene chloride was added to each vial and the solution homogenized for another 30 seconds. The vials were then centrifuged at 6000 g for 10 mins. The organic phase (bottom layer) was carefully extracted with a glass syringe and all-metal hypodermic needle, and transferred into a 4 mL screw-capped glass vial equipped with a silicone septum. The extract was then dried under a low air flow in a fume hood until only a thin film remained. Two hundred µL of methanol was added back to each sample, along with an internal standard solution of ABA (10 ng in 10 µL of methanol). Five µL of TMSD was added to derivatize the phytohormones by trimethylsilylation. The samples were incubated at 45°C for 30 mins. They were then immediately subjected to vapor-phase extraction (VPE).

VPE was conducted by inserting a Volatile Collection Trap or VCT (Analytical Research Systems, Gainesville, FL) into the septum of each vial, along with a 20-gauge hypodermic needle. The VCT was attached to a Tygon R-3603 tube connected to a vacuum pump operating at -20kPa. The vials were first placed in a heating block at 80°C for 3 min, then moved to another heating block at 240°C for 2 min under vacuum. After VPE was completed, the vials with the VCTs still attached were removed from the
heating block to cool down for 1 min. VCT were then detached and the analytes were eluted into 2 mL GC-MS analysis vials containing 300 µL capacity glass inserts using 200 µL of methylene chloride, flushed with air. The VCTs were rinsed twice with 200 µL of methylene chloride and set aside for reuse.

The GC-MS (Agilent 7890B GC connected to 5977A MS, Quantum Analytics, Inc., Foster City, CA) was equipped with a HP-5ms (30 m × 250 µm × 0.25 µm) column with helium carrier gas at a constant flow rate of 0.7 mL/min. Ionization was carried out using an electron impact ionizer. The GC was set up to run in splitless mode, with injection temperature at 250°C and injection volume of 1 µL. The column was held at 40°C for 1 min after injection, then increased by 15°C/min until 250°C and then held at that temperature for 10 min.

Total Ion Count (TIC) was measured, and ABA abundance was indicated by the abundance of the signature ion fragments of methyl ester of ABA (mass given in m/z), which has a molecular mass of 190.1, coming off the column at 15.204 min. As we were not able to obtain an external standard of known quantity of methylated ABA, the absolute amount of ABA was not quantified. We compared the relative abundance of the methyl ester of ABA across treatment and control groups.

Analysis of variance (ANOVA) was performed to analyze differences between treatments, with post-hoc means separation using Tukey’s Honestly Significant Differences. Polynomial regression was performed to fit explanatory models for changes in ABA abundance over time. All statistical analysis was performed using the R statistical software package (www.r-project.org)
Results

Flowering and Growth

There was no difference in relative growth due to photoperiod treatments or hormone treatments alone. However, there was an interactive effect between photoperiod and ABA treatments on relative growth. Under LD, relative growth increased with increasing concentrations of ABA application from 0 to 150µL/L, and declined from 150 to 300µL/L, while under SD, relative growth increased continually with increasing concentrations of exogenous ABA (Fig. 6-1). Changes in growth due to ethephon were similar in LD and SD plants. Growth decreased at higher rates of ethephon application (Fig. 6-2).

There was a significant difference in number of inflorescences on control plants due to photoperiod, and slight differences due to treatments. Control plants grown under SD had more inflorescences than plants under LD. Plants grown under SD with ABA075 and ETH150 treatments resulted in more inflorescences than control plants under SD. Control plants under SD had significantly more flowers than control plants under LD, which remained completely vegetative (Table 6-1).

Under LD, ABA300 and ETH150 resulted in some flowering. On day 30, ETH300 plants were still recovering vegetative growth, and had no inflorescences.

Although ABA300 and ETH150 did not result in significantly more inflorescences than controls under LD, they did promote some flowering to a limited extent under LD.

ABA Levels

For plants grown under SD, exogenous ABA treatments showed a direct relation between the amount of exogenous ABA applied and the overall abundance of ABA. On
day 1, ABA300 plants had the greatest abundance of ABA, followed by ABA150, and ABA07, and control plants. All plants showed a general decrease in ABA levels from day 1 to day 7 after treatment. ABA300 plants showed the steepest decline in ABA levels, followed by ABA150, and ABA075. By day 14 after treatment, there were no significant differences in ABA abundance among all treatments (Fig. 6-3).

For plants under SD and treated with ethephon, the highest abundance of ABA was found in ETH150 plants, followed by ETH075, ETH300, and control plants. Only ETH150 treated plants showed a significantly greater abundance of ABA on the first day after treatment. All plants showed a rapid decline in ABA abundance over the fourteen days after treatment, with ETH150 plants showing the greatest decline. ABA abundance on day 14 after treatment did not differ among the treatments (Fig. 6-4).

Plants grown under LD and treated with exogenous ABA had lower abundance of ABA (about half) compared to SD plants, even on the first day after treatment, although plants under both photoperiods received the same amount of exogenous ABA (Fig. 6-5 and Fig. 6-4). Plants treated with ABA150 had the greatest abundance of ABA on day 1 after treatment, and rapidly declined over the next 13 days. ABA075 and ABA300 had the next greatest abundance of ABA, followed by control plants. All plants showed a rapid decline in ABA over the 14 days after treatment, and there were no differences among treatments at day 14.

Plants grown under LD and treated with ethephon had the lowest abundance of endogenous ABA among all groups, with detectable ABA levels at the low end of the sensitivity of the mass-spectrometer (Fig. 6-6). As in the SD plants, ETH150 plants had the highest initial abundance of ABA. However, ETH300 plants had the second-highest
abundance of initial ABA. ETH075 plants had the same amount of ABA as control plants. All plants showed a decline in ABA abundance from day 1 to day 14 after treatment.

There were significant differences in ABA abundance over time, between photoperiods for plants treated with ABA across treatment rates. Plants under SD showed significantly more ABA levels in most treatments. Plants sprayed with 300 µL/L ABA showed significantly higher initial ABA levels (day 1 and day 3) under SD as compared to LD, but ABA levels had equalized by day 7. Plants sprayed with 75 µL/L ABA showed a spike in ABA levels on day 3 under SD as compared to LD, but levels had also equalized by day 7. Plants sprayed with 150 µL/L ABA showed remarkably similar levels of ABA across both SD and LD photoperiods, with a rapid fall from day 1 to day 3. ABA levels fell to very low by day 3 and remained the same from then to day 14, and there were no differences between SD and LD.

ABA abundance in control plants showed opposing trends over the fourteen days after treatment. LD control plants started with higher ABA levels, which rapidly decreased by day 3 and were not significantly more than initial levels by day 7. SD control plants, in contrast, showed a great increase in ABA levels on day 3, which then rapidly declined through day 7.

Low concentrations of both ethephon and ABA treatments resulted in similar responses in ABA levels. 75 µL/L ethephon treatment on both SD and LD plants yielded almost identical responses, with high initial ABA in the LD plants, rapid decline over the first 3 days, then further decline until day 7. In the SD plants, ABA rose slightly from day 1 to 3, then declined to day 7 to the same level as LD plants. For 75 µL/L ABA
treatments, SD plants showed the same response, although the quantity of ABA was much higher than the ethephon-treated plants, since ABA was applied directly to the leaves. The LD plants started with less ABA on day 1 than SD plants, and the abundance declined gradually until day 7.

The medium concentration (150 µL/L) treatments caused very different responses in ABA levels, between the ABA and ethephon treatments. Ethephon 150 µL/L treatment did not increase in ABA under LD, but caused a dramatic (seven-fold) increase in ABA under SD, between days 1 and 3. The ABA levels peaked around day 3 and then rapidly declined to the same level as the LD plants by day 7. The 150 µL/L ABA treatment resulted in an unusual response – ABA levels were almost identical between LD and SD plants. Starting with very high ABA levels on day 1, which was expected, it rapidly declined to a very low level by day 3 and remained the same at day 7.

For the high concentration treatments (300 µL/L), there were differences between the hormone treatments as well as between photoperiods (Table 6-1). ABA levels were much higher in both SD and LD plants sprayed with ABA. However, there was also a significant difference in ABA levels between the SD and LD plants treated with the same amount of ABA. SD plants started with 5 times more ABA levels on day 1, gradually declined through day 3, and then declined further until they were the same as LD plants on day 7. LD plants started with much lower ABA levels even though they were sprayed with the same amount of ABA 24 hours prior to harvesting. ABA levels declined slightly and gradually over day 3 to day 7. We ran a linear regression on the ABA abundance sampled on 1, 3, 7 and 14 days after treatment. The coefficients of each regression
were plotted on a scatter plot and a polynomial regression was performed on the slopes to show the relationship between the increasing application rate of ethephon and exogenous ABA on the change in endogenous ABA levels over the first 14 days after treatment. The results showed that ABA levels in all plants, including controls, declined over the first 14 days after treatment. Plants showed greater decline in endogenous ABA levels when sprayed with between 75 to 150 µL/L of ethephon (Figure 6-7). Between 150 to 300 µL/L application rates, the decline in endogenous ABA levels was reduced.

With exogenous ABA applications, there was a similar trend in the change in total ABA levels in the leaf tissues over the same fourteen-day period. However, the amount of change in ABA levels was much greater than the ethephon-treated plants. All plants under exogenous ABA treatments showed a decline in ABA levels over the first fourteen days after treatment. Plants under LD showed an increasing decline in ABA between 0 to 150 µg/L ABA application, and a reduced decline in ABA from between 150 to 300 µg/L application. Under SD, plants showed an increasing decline in overall ABA level with increasing application rates of exogenous ABA (Fig. 6-8).

Discussion

We sought to discover if exogenous ABA and ethylene application in the form of ProTone® and Florel® foliar sprays would promote flowering under non-inductive conditions. The results from this experiment suggest that photoperiodic inhibition of flowering in this cultivar of *Bougainvillea* was the dominant factor in controlling the flowering response, and the hormone treatments did not contribute significantly to the promotion of flowering.
These results contrast with Liu and Chang (2011), who found that ETH075 could promote flowering of *Bougainvillea* ‘Taipei Red’ vegetative shoots. However, those experiments were conducted in the spring in Taipei under natural daylight conditions, which would have been SD photoperiods. We found that under sufficiently short photoperiod (8 hours), control plants with no ethephon applied flowered as much as those with ETH075.

Photoperiod appeared to be the overriding factor influencing flowering of *Bougainvillea* ‘Afterglow’, as shown by the strong inhibitory effect of LD treatment on flowering. Since ABA300 did induce a slight flowering response in both SD and LD, while lower concentrations of ABA did not, we suggest that further examination of higher application rates of ABA might be useful in determining if flowering could be promoted.

The difference in ABA levels across photoperiod was unexpected, especially under the ABA treatments, given that the amounts of exogenous ABA applied was the same across photoperiods. This suggests that endogenous ABA levels in *Bougainvillea* are different depending of photoperiod, which would be of interest to examine in further detail. Endogenous ABA in the LD spinach (*Spinacia oleracea*) was found to be higher under the inductive LD photoperiod than under non-inductive SD (Zeevaart, 1971).

Growth of *Bougainvillea* appeared to be affected by exogenous application of ABA, promoting shoot lengthening under SD, while inhibiting growth under LD. In addition, flowering was reduced under SD with increasing concentration of exogenous ABA. It has been found that gibberellic acid (GA) applied to *Bougainvillea* under SD inhibits flowering, while application of GA-inhibitors promotes flowering under SD conditions. It has been established that GA is an inhibitory hormone to flowering in *Bougainvillea*
(Hackett and Sachs, 1985.; Ramina et al., 1979; Tse et al., 1974). It is also known that
GA is a promoter of shoot elongation and cell expansion (Taiz and Zeiger, 2010; Talon
and Zeevaart, 1990). These results in *Bougainvillea* suggest that excessive ABA has a
promotive effect on endogenous GA, which would explain greater lengthening of shoots,
while suppressing flowering even under inductive SD conditions. This effect appears to
take place only if the amount of exogenous ABA applied is higher than 15 µg per plant,
or 300 µL/L in solution, which represents a significant increase over the normal
endogenous levels of ABA.

Ethylene levels corresponded with ABA levels on day 1 after treatment, with a
weak correlation ($R=0.41$, $p$-value $= 4.31\times10^{-6}$), suggesting that exogenous ABA
application does not affect ethylene functions directly.

**Summary**

In this experiment, we used exogenous ABA to simulate the sharp increase in
endogenous ABA when a plant is under WDS. However, under production
circumstances, irrigation upon the first signs of wilting would rapidly reduce the amount
of ABA in the leaves, as observed by Zeevaart (1971) in the case of spinach, whereas
exogenous ABA could be expected to remain in the leaf tissue for an extended period
regardless of the presence or absence of abiotic stresses. Therefore, for future research
it would be pertinent to observe the differences in levels of endogenous ABA in
*Bougainvillea* under WDS, as well as under SD and LD conditions. The role of
endogenous GA in *Bougainvillea* is also important to examine as the functionality of
ABA and ethephon on flowering may be regulated by the GA pathway. The next step in
the project would be to examine the effect of exogenous ABA and ethephon application
on the GA levels in flowering and non-flowering *Bougainvillea* shoots.
Table 6-1. Means and standard deviation of number of inflorescences produced by *Bougainvillea* ‘Afterglow’ under two photoperiods and six hormone treatments. N=56. Means followed by the same letter were not significantly different at α=0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>8-hour Photoperiod (SD)</th>
<th>14-hour Photoperiod (LD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of inflorescences</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Control</td>
<td>8.00 b</td>
<td>8.2</td>
</tr>
<tr>
<td>ABA075</td>
<td>12.00 a</td>
<td>5.4</td>
</tr>
<tr>
<td>ABA150</td>
<td>9.00 b</td>
<td>3.4</td>
</tr>
<tr>
<td>ABA300</td>
<td>6.25 b</td>
<td>4.6</td>
</tr>
<tr>
<td>ETH075</td>
<td>5.50 b</td>
<td>6.6</td>
</tr>
<tr>
<td>ETH150</td>
<td>10.75 a</td>
<td>3.9</td>
</tr>
<tr>
<td>ETH300</td>
<td>0.00 c</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Figure 6-1. Relative growth response of *Bougainvillea* ‘Afterglow’ to increasing exogenous ABA application rates under short-day and long-day photoperiods (n=32)
Figure 6-2. Relative growth response of *Bougainvillea* ‘Afterglow’ to increasing rates of ethephon under short-day and long-day photoperiods (n=32).
Figure 6-3. Abundance of abscisic acid (ABA) in plants grown under SD (8-hour) photoperiod, over 14 days after exogenous ABA treatment. (trt= treatment, aba=abscisic acid, 75=75 μL/L, 150=150 μL/L, 300=300 μL/L, n=32)
Figure 6-4. Abundance of abscisic acid (ABA) in *Bougainvillea* 'Afterglow' grown under SD (8-hour) photoperiod, over 14 days after ethephon treatment. (eth=ethephon, 075=75 μL/L, 150=150 μL/L, 300=300 μL/L, n=32)
Figure 6-5. Abundance of abscisic acid (ABA) in Bougainvillea ‘Afterglow’ grown under LD (14-hour) photoperiod, over 14 days after exogenous ABA treatment. (075=75 µL/L, 150=150 µL/L, 300=300 µL/L, n=32)
Figure 6-6. Abundance of abscisic acid (ABA) in Bougainvillea ‘Afterglow’ grown under LD (14-hour) photoperiod, over 14 days after ethephon treatment. (075=75 µL/L, 150=150 µL/L, 300=300 µL/L, n=32)
Figure 6-7. Change in endogenous ABA levels under increasing rates of ethephon application (n=32)
Figure 6-8. Change in endogenous ABA levels in *Bougainvillea* ‘Afterglow’ in response to increasing rates of exogenous ABA application under short-day and long-day photoperiods (n=32).
CHAPTER 7
PHOTOPERIOD, EXOGENOUS ABA AND ETHYLENE EFFECTS ON FLOWERING AND ENDOGENOUS GIBBERELLIC ACID IN BOUGAINVILLEA ‘AFTERGLOW’

Introduction

Flowering response in Bougainvillea ‘Afterglow’ was strongly influenced by photoperiod, with short-day (SD) photoperiod of 8 hours promoting early and profuse flowering, while long-day (LD) photoperiod of 14 hours effectively inhibited flowering under otherwise normal growing conditions.

Gibberellic acid (GA) is known to accumulate in leaf tissue during periods of darkness in Arabidoposis thaliana, which is a LD plant (Taiz and Zeiger, 2010). GA is also a flowering promoter in LD plants, such as A. thaliana (Wilson et al., 1992), spinach (Spinacia oleracea) (Metzger and Zeevaart, 1980), and Silene armeria (Talon and Zeevaart, 1990). However, its function in the flowering response of SD plants is not clear. Since exogenous GA has been found to be an inhibitor of flowering in Bougainvillea under SD (Ramina et al., 1979; Tse et al., 1974), and GA-inhibiting plant growth regulators (PGR) have been found to promote flowering of some Bougainvillea cultivars under SD conditions (Aldrich and Norcini, 1996; Karagüzel, 1999; Ma and Gu, 2010), we would expect that endogenous GA would be lower in Bougainvillea growing under SD and higher in plants grown under LD (Even-Chen et al., 1979). The evidence from the literature, which comprises indirect testing through GA and GA-inhibitor application, supports this hypothesis. The first objective of this study was to verify the hypothesis that endogenous GA in SD was significantly lower than that under LD, through the direct measurement of GA abundance in the leaf tissue.

In preliminary experiments, we found that some degree of water deficit stress (WDS) can promote flowering of Bougainvillea under LD conditions (Appendix A). As
ABA is known to increase dramatically within 24 hours of WDS onset (Jiang and Zhang, 2002; Zeevaart, 1971), and decrease rapidly once the stress is relieved, we expected that some level of exogenous ABA application might promote flowering of *Bougainvillea* under LD. In LD spinach, it was found that plants under SD had lower endogenous ABA levels and remained vegetative, while plants under LD had higher ABA levels, and went into the reproductive phase (Zeevaart, 1971). The second objective of this study was to examine the endogenous ABA levels under both SD and LD. We expect to find that plants under SD conditions will have higher levels of endogenous ABA. We found that exogenous ABA applied at 300 µL/L as a foliar spray had a limited effect of promoting flowering under LD conditions. As ABA is synthesized in the leaves and roots, and can be transported from the roots to the shoots when WDS was detected (Malladi and Burns, 2007), it seems likely that the effect of exogenous ABA application would differ between a foliar spray and a soil drench method. Therefore, for this study, exogenous ABA will be applied as a soil drench.

Both exogenous ethylene and ABA appear to promote flowering of *Bougainvillea* at low application rates. Therefore, we may expect that these applications have the effect of inhibiting GA content in *Bougainvillea* shoots. The third objective of this study was to examine if the application of exogenous ethylene and ABA at various concentrations reduced or increased endogenous GA in *Bougainvillea*.

**Materials and Methods**

Sixty-five rooted cuttings of *Bougainvillea* 'Afterglow' were transplanted into 10-cm pots filled with 100% 5 mm coarse aquarium zeolite (Pentair Aquatic Eco-systems Inc., Apopka, FL, USA). Plants were sprayed with 30 µl of ethephon (25 mL of 1200 µL/L solution; Southern Agricultural Insecticides, Inc., Hendersonville, NC) to defoliate
them completely, then pruned to a mean height of 12.7 cm (+/- 2.8 cm). Plants were then placed under long days (LD) 14-hour photoperiod to prevent flowering prior to the start of the experiment. This was done by supplementing the natural daylight (around 13 hours) with white LED lights supplying 70 µmol m-1s-1 photosynthetic photon flux (PPF), measured using a handheld quantum meter (Apogee Instruments, Logan, UT). To prevent nutrient deficiencies, plants were fertilized with Peters Professional Bloom Booster (10N-30P2O5-20K2O; JR Peters, Allentown, PA) at 9.4 µg N (nitrogen) once per week starting three weeks prior to the start of the experiment. We continued to apply the fertilizer at the same rate once per week through the remainder of the experiment. In addition to fertilizer, plants were watered with 50 mL of tap water (EC = 516 µS, pH = 8.3) every two days.

The plants were arranged in a complete randomized design on one bench in an open-sided greenhouse exposed to ambient air temperatures (26.6/17.9°C day/night) and 76.0% relative humidity, at the University of Florida Fort Lauderdale Research and Education Center in Davie, Florida.

There were two treatments without any hormones applied – LD control, and SD control. The SD treatment was carried out by covering the plants with a black plastic 5-gallon container between 4pm and 8am the following morning, for the duration of the experiment. Two PGR treatments, ethephon (Florel®; Southern Agricultural Insecticides, Inc., Hendersonville, NC) and ABA (ProTone®; Valent Biosciences, Libertyville, IL), were applied on LD plants. Ethephon concentrations applied were 50 µL/L, 75 µL/L, 100 µL/L, 150 µL/L, 200 µL/L, applied as a foliar spray of 25 mL per plant. Ethephon treatments from hereon will be indicated by “FL” followed by the
concentration, e.g. FL50, FL75, … etc. ABA application rates were 50 µL/L, 75 µL/L, 150 µL/L, 300 µL/L, 400 µL/L, and 500 µL/L, applied as a soil drench of 100 mL per plant. ABA treatments from hereon will be indicated as PR50, PR75 … etc. Each treatment rate, including controls, contained 5 replicates, with each replicate being an individually potted plant.

The number of inflorescences on each plant was counted on day 48 after treatment. One inflorescence was defined as an individual thorn-inflorescence axil, regardless of how many florets were attached to the peduncle.

**ABA determination**

ABA abundance was determined using gas chromatography – mass spectrometry (GC-MS) adapted from Engelberth and Engelberth, (2009) and Schmelz et al., (2004).

Analytical grade abscisic acid (ABA) was obtained from Sigma-Aldrich (St. Louis, MO) to be used as internal standards. Solvents used in this procedure were methanol, methylene chloride (dichloromethane), and 1-propanol. Reagents were hydrochloric acid (Fisher Scientific, Hampton, NH) and trimethylsilyldiazomethane (TMSD) 2.0M in hexanes (Sigma-Aldrich, St. Louis, MO).

200 mg of fresh tissue from each replicate was measured out and placed in a 2 mL homogenizer vial containing 300 µL of an extraction solution (1-propanol/deionized water/hydrochloric acid in 2:1:0.002 ratio) and 2.8 mm ceramic homogenizer beads. The samples were then homogenized in a Bead Ruptor 12 bead mill (Omni International, Kennesaw, GA) for 30 seconds on high, then 1 mL of methylene chloride was added to each vial and the solution homogenized for another 30 seconds. The vials were then centrifuged at 6000 g for 10 mins. The organic phase (bottom layer) was carefully
extracted with a glass syringe and all-metal hypodermic needle, and transferred into a 4 mL screw-capped glass vial equipped with a silicone septum. The extract was then dried under a low air flow in a fume hood until only a thin film remained. 200 µL of methanol was then added back to each sample, along with an internal standard solution of ABA (10 ng in 10 µL of methanol). 5 µL of TMSD was then added to derivatize the phytohormones by trimethylsilylation. The samples were incubated at 45°C for 30 mins. They were then immediately subjected to vapor-phase extraction (VPE).

VPE was conducted by inserting a Volatile Collection Trap or VCT (Analytical Research Systems, Gainesville, FL) into the septum of each vial, along with a 20-gauge hypodermic needle. The VCT was attached to a Tygon R-3603 tube connected to a vacuum pump operating at -20 kPa. The vials were first placed in a heating block at 80°C for 3 min, then moved to another heating block at 240°C for 2 min under vacuum. After VPE was completed, the vials with the VCTs still attached were removed from the heating block to cool down for 1 min. VCT were then detached and the analytes were eluted into 2 mL GC-MS analysis vials containing 300 µL capacity glass inserts using 200 µL of methylene chloride, flushed with air. The VCTs were then rinsed twice with 200 µL of methylene chloride and set aside for reuse.

The GC-MS (Agilent 7890B GC connected to 5977A MS, Quantum Analytics, Inc., Foster City, CA) was equipped with a HP-5ms (30 m × 250 µm × 0.25 µm) column with helium carrier gas at a constant flow rate of 0.7 mL/min. Ionization was carried out using an electron impact ionizer. The GC was set up to run in splitless mode, with injection temperature at 250°C and injection volume of 1 µL. The column was held at
40°C for 1 min after injection, then increased by 15°C/min until 250°C and then held at that temperature for 10 min.

Total Ion Count (TIC) was measured, and ABA abundance was indicated by the abundance of the signature ion fragments of methyl ester of ABA (mass given in m/z), which has a molecular mass of 190.1, coming off the column at 15.204 min. As we were not able to obtain an external standard of known quantity of methylated ABA, the absolute amount of ABA was not quantified. We compared the relative abundance of the methyl ester of ABA across treatment and control groups.

**GA determination**

The abundance of GA in the leaf tissue was determined using an adapted version of the spectrophotometric method as developed by Holbrook, Edge and Bailey, (1961) and further modified by Berríos, Illanes and Aroca, (2004). Chemicals and reagents used were: Zinc acetate solution (21.9 g zinc acetate dissolved in deionized water and 1 mL glacial acetic acid, made up to 100 mL); Potassium ferrocyanide solution (10.6 g analytical grade potassium ferrocyanide dissolved in 100 mL DI water); dilute hydrochloric acid 35% (HCl); and gibberellic acid (GA3) standard (analytical grade GA3 dissolved in ethanol at various concentrations for calibration).

Fresh tissue was harvested from plants and 200 mg from each replicate was weighed out and homogenized in 0.5 mL of methanol, and then centrifuged at 6000 g for 3 mins. The extract was transferred into a glass vial, to which 1 mL of ethanol and 1 mL of deionized water was added, followed by 1 mL of the zinc acetate solution, and the mixture vigorous shaken and left to stand for 2 minutes at room temperature. One mL of the potassium ferrocyanide solution was then added to the mixture and vortexed for 10 seconds, then left to stand for 10 minutes and vortexed for another 10 seconds. The
resulting precipitate was filtered using P8 filter paper (Fisher Scientific, Waltham, MA, USA), and the clear extract retained for analysis. A 500 µL sample of the extract was transferred into a test tube. When the sample was ready to be analyzed, 800 µL of dilute HCl was added to the test tube, which was then vigorously shaken, and then pipetted into a quartz cuvette, to be analyzed in the ultra-violet spectrophotometer (Cary 100, Varian Inc, Palo Alto, CA, USA) with absorbance measured at 254 nm over 2 minutes. The differentials of the resulting curves were measured against a calibration curve constructed prior to the experiment using known concentrations of GA₃ standard solutions of 0.125, 0.25, 0.5, and 1.0 µg/L. The resulting quotient would be the concentration of GA in the sample, expressed in µg/L.

ABA and GA abundance was collected on 1, 3, 7, 28 and 48 days after treatment, when most of the plants that had inflorescences were visibly blooming.¹ Linear regression was performed on ABA and GA abundance over time to determine the rate of change in hormone levels in response to the treatments.

All statistical analysis was performed using the R statistical software package (www.r-project.org). Tests conducted include analysis of variance (ANOVA), with post-hoc means separation using Tukey’s Honestly Significant Differences, linear regression and logistic regression.

¹ All treatments showed a high percentage (between 75% to 100%) of defoliation within 72 hours of treatment, except for FL50, FL75 and PR50, which showed minimal defoliation. This resulted in difficulty having enough tissue to collect for analysis of hormones on day 7 after treatment.
Results

Flowering Response

Polynomial regression on the number of flowers in response to the increasing rate of ethephon applications showed that there was a negative relationship between ethephon rates from 50 to 200 µL/L and flowering. Further increases in rates of ethephon application resulted in reduced flowering. FL50 plants produced the highest number of flowers (mean=10.6, P<0.01), which were higher than the SD control (mean=9.6, P<0.05). Both were significantly higher than the LD control (mean=1.8). FL50 application on plants grown under LD most closely matched the natural photoperiod-induced flowering response for the SD plants. All other FL treatments resulted in some flower but the numbers of flowers were not significantly more than the LD controls (Table 7-1).

FL75 and PR50 also induced more flowering than LD control plants, but were much fewer than FL50 and SD controls. FL50 and SD plants also started flowering earlier than all other plants, starting at 35 days after treatment, while the other plants that did eventually flower only did so from day 45 to day 48.

There was no significant relationship between exogenous ABA application and flowering response. At low rates between 50 to 150 µL/L of ABA application, there was some flowering response but these were not significantly different to the control plants (Fig. 7-2).

High concentration applications of ABA (PR300, PR400 and PR500) induced a delayed senescence of foliage, leading to complete (100%) loss of leaves by 4 days after treatment and were slow to recover growth. None of the plants under these treatments produced any flowers.
ABA Levels

There was a significant difference in endogenous ABA levels between SD and LD control plants (Fig. 7-3). Plants under SD showed an increase in ABA levels over the first 7 days after treatment, but plants under LD showed no change in endogenous ABA levels.

Among exogenous ABA treated plants, PR300 plants showed a significant increase in total ABA, rising exponentially to 9 times more than other treatments at day 7 (Fig. 7-3). PR150 and PR500 plants had an increase in total ABA between day 1 and 3, and decline between day 3 and 7. PR400 plants showed an initial decrease in total ABA, followed by an increase to the same initial level at day 7. PR75 plants showed a consistent decrease in total ABA from day 1 to day 7.

Among ethephon treatments, FL100 plants showed the highest increase in endogenous ABA, followed by FL150 plants. Other ethephon treatments resulted in no significant changes in endogenous ABA (Fig. 7-5).

GA Levels

There were also differences between treatments in terms of the change in GA levels over the experimental period of 48 days. Linear regression was run on GA concentrations sampled on 1, 3, 7, 28 and 48 days after treatment. The coefficients (slope) of each sample were then plotted on a polynomial regression curve comparing the amount of change in GA levels at each application rate. Among ethephon treatments, GA levels decreased over the experimental period (showed a negative slope) between 0 to 75 µL/L application rates. GA levels increased in response to increasing ethephon application rates between 75 to 150 µL/L, and decreased in response to the 200 µL/L application rate (Fig. 7-6). Fig. 7-1 and 7-6 showed mirroring
trends. Change in GA levels corresponded with the flowering response under increasing concentration of ethephon treatments. Between 0 to 75 µL/L of ethephon, plants showed an increase in number of flowers. Between 75 to 150 µL/L, there were zero or insignificant inflorescences (P>0.1). At 200 µL/L application rate, there was some flowering response. Changes in GA levels were inversely related to flowering response.

SD and LD controls showed a significant difference in the change in GA levels, as was expected. SD control plants, had a marked decrease in GA levels, while LD control plants had a very slight decrease in GA levels (Table 7-2). More notably, all treatments that yielded exactly zero flowers (PR300, PR400 and PR500) showed an increase in GA levels over the 48-day period, instead of a decrease. Among all PGR treatments, only FL50 (mean = -0.44 µg/L) plants showed a marked decrease in GA levels compared to controls. However, this decrease was not as significant as the decrease in SD control plants (mean=-1.09 µg/L). GA levels increased in all ABA treatments, except for PR50, which had a mean decrease of -0.4 µg/L. The lowest rate of ABA drench, PR50, led to a decrease in GA levels and resulted in a positive flowering response. In treatments at rates higher than 50 µg/L, exogenous ABA caused an increase in overall GA levels, and did not lead to any significant flowering response.

Among ethephon treatments, there were significant differences in the change in GA levels in plants under FL50 and FL100 treatments. FL50 plants showed the greatest decrease in GA levels over the experiment, while FL100 plants showed the greatest increase. FL50 plants, as noted, produced the most flowers, while FL100 plants did not produce any (mean=0.5). FL150 plants showed a much smaller increase in GA levels, which also resulted in negligible flowering (mean=0.5). FL75 and FL200 plants both
showed a slight decrease in GA levels as compared to LD controls, but not as great as FL50. Both treatments resulted in slight flowering responses. Most treatments that yielded a positive flowering response (mean number of inflorescences > 1) also resulted in a decrease in GA levels between day 1 and day 48. When we considered the flowering response as a binary flower/non-flowering categorical response, a logistic regression showed that the change in GA levels was strongly predictive of the positive or negative flowering response ($P<0.01$). A decrease in GA was a strong predictor of a positive flowering response, while an increase in GA was strongly predictive of a negative flowering response. The mean number of inflorescences and the mean differences in GA levels were negatively correlated ($R = -0.83$).

**Discussion**

The first objective of this experiment was to observe the changes in GA levels in the plants under both SD and LD. Previous research had shown that GA was an important hormone in the flowering response of other *Bougainvillea* cultivars by observation of the flowering response to applications of exogenous GA, as well as GA-inhibiting PGRs (Aldrich and Norcini, 1996; Even-Chen and Sachs, 1980; Karagüzel, 1999; Norcini et al., 1994; Ramina et al., 1979). In this experiment, we sought verify this hypothesis by positive measurement of endogenous GA in the plant tissue in response to different photoperiod regimes and exogenous hormone applications.

The negative correlation between the final endogenous GA levels and the flowering response supports the hypothesis that GA is inhibitory to flowering of *Bougainvillea* ‘Afterglow’. The strong decrease in endogenous GA levels in plants grown under inductive SD photoperiod indicates that GA content in *Bougainvillea* must decline in order for flowering to occur.
The second objective of this experiment was to examine the effect of photoperiod on endogenous ABA levels in *Bougainvillea*. The results support the hypothesis that endogenous ABA is dependent on photoperiod, as SD plants contained a significantly higher amount of ABA than LD plants. The relationship between ABA and GA is complicated, but it has been suggested that the hormones are antagonistic as related to light-sensing and response (Alabadí and Blázquez, 2009; Kojima, 1996; Lau and Deng, 2010). The high endogenous levels of ABA and correspondingly low levels of GA in SD plants suggests that this is the case in *Bougainvillea*. The reverse occurs when grown under LD, as endogenous ABA is low, although in this experiment we did not find that endogenous GA was any higher than in SD plants.

The last objective of this experiment was to examine the effect of the application of low rates of ethephon and ABA to plants grown under non-inductive LD photoperiod. The two hormones were found in previous experiments to promote flowering under SD conditions and to a limited extent under LD conditions. This experiment found that application of low rates of both hormones resulted in positive flowering responses as well as a slight reduction in GA levels. However, as the rate of exogenous hormones was increased, the effect on GA level gradually reversed. The suppressive effect of ethylene and ABA on GA in *Bougainvillea* appears to be dose-dependent. In the previous chapter, it was noted that high concentrations of exogenous ABA appeared to promote shoot elongation at the expense of flowering even under otherwise-inductive SD conditions. In this experiment, we found that higher concentrations of exogenous ABA had a markedly positive effect on the total GA levels in *Bougainvillea* over the experimental period under LD. The plants treated with PR400 and PR500 were greatly
inhibited in their growth, as was found in the previous experiment with PR300 plants grown under LD.

The effect that GA has on flowering in *Bougainvillea* suggests that its function in photoperiod sensing is different from the DELLA-mediated PIF5 degradation model (Cheng et al., 2004; Li et al., 1992; Schwechheimer and Willige, 2009) constructed from the study of genetic responses in the LD plant *Arabidopsis thaliana*. Rather than promote a flowering response under long periods of darkness, GA in *Bougainvillea* appears to accumulate under long periods of light and promotes shoot growth.

**Summary**

In conclusion, the interaction between ABA and ethylene in *Bougainvillea* is still unclear, although the results from this experiment and in the previous chapter suggest that they are bi-directional controllers in terms of flowering. It is very likely that their signaling involves the GA pathway in *Bougainvillea*. Ethylene is a promoter of flowering of *Bougainvillea* under SD conditions, and can break LD inhibition if applied at a very low rate of 50 µL/L as a foliar spray. It is notable that ABA appears to be an important part of the photoperiod response. A low concentration soil drench of 75 to 100 µL/L to potted *Bougainvillea* may be able to break LD inhibition, but this still need further verification. The integration of endogenous ABA and GA signaling is probably important in understanding the control of flowering time in *Bougainvillea* ‘Afterglow’.
Figure 7-1. Number of inflorescences on *Bougainvillea* ‘Afterglow’ under increasing rates of ethephon application (n=30).
Figure 7-2. Number of inflorescences in *Bougainvillea* 'Afterglow' under increasing rate of exogenous ABA applications (n=30).
Figure 7-3. Endogenous levels of ABA in *Bougainvillea* ‘Afterglow’ under short-day and long-day photoperiods (n=10)
Figure 7-4. Change in endogenous ABA levels in *Bougainvillea* ‘Afterglow’ under increasing ABA application rates.
Figure 7-5. Change in endogenous ABA in *Bougainvillea* ‘Afterglow’ under increasing rates of ethephon application. (n=30).
Figure 7-6. Change in endogenous gibberellic acid (GA) levels in Bougainvillea 'Afterglow' under increasing rates of ethephon (n=30).

\[-0.147 - 0.0142x + 0.000302x^2 - 1.16e-06x^3 \quad R^2 = 0.32\]
Table 7-1 Means number of inflorescences. Means followed by the same letter are not significantly different to each other at α=0.05. NS=not significant N=65

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate (µL/L)</th>
<th>Mean number of inflorescences</th>
<th>Standard Error</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LD</td>
<td>0</td>
<td>1.8</td>
<td>1.32</td>
<td>NS</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>9.6</td>
<td>0.81</td>
<td>A</td>
</tr>
<tr>
<td>FL</td>
<td>50</td>
<td>10.6</td>
<td>2.27</td>
<td>A</td>
</tr>
<tr>
<td>FL</td>
<td>75</td>
<td>5.2</td>
<td>2.50</td>
<td>B</td>
</tr>
<tr>
<td>FL</td>
<td>100</td>
<td>0.5</td>
<td>0.45</td>
<td>NS</td>
</tr>
<tr>
<td>FL</td>
<td>150</td>
<td>0.4</td>
<td>0.40</td>
<td>NS</td>
</tr>
<tr>
<td>FL</td>
<td>200</td>
<td>3.9</td>
<td>1.56</td>
<td>B</td>
</tr>
<tr>
<td>PR</td>
<td>50</td>
<td>4.4</td>
<td>2.20</td>
<td>NS</td>
</tr>
<tr>
<td>PR</td>
<td>75</td>
<td>3.0</td>
<td>2.32</td>
<td>NS</td>
</tr>
<tr>
<td>PR</td>
<td>150</td>
<td>2.4</td>
<td>1.12</td>
<td>NS</td>
</tr>
<tr>
<td>PR</td>
<td>300</td>
<td>0.0</td>
<td>0.00</td>
<td>NS</td>
</tr>
<tr>
<td>PR</td>
<td>400</td>
<td>0.0</td>
<td>0.00</td>
<td>NS</td>
</tr>
<tr>
<td>PR</td>
<td>500</td>
<td>0.0</td>
<td>0.00</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 7-2 Differences in GA levels across long-day, short-day, and ethephon and ABA application rates. Means +/- standard errors followed by the same letter are not significantly different to each other at α=0.05. NS=not significant. N=65

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate (µL/L)</th>
<th>Mean Coefficient of Change in GA Levels</th>
<th>Standard Error</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LD</td>
<td>0</td>
<td>-0.09</td>
<td>NS</td>
<td>0.15</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>-1.05</td>
<td>A</td>
<td>0.29</td>
</tr>
<tr>
<td>FL</td>
<td>50</td>
<td>-0.44</td>
<td>NS</td>
<td>0.17</td>
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<tr>
<td>FL</td>
<td>75</td>
<td>-0.12</td>
<td>NS</td>
<td>0.41</td>
</tr>
<tr>
<td>FL</td>
<td>100</td>
<td>0.84</td>
<td>B</td>
<td>0.18</td>
</tr>
<tr>
<td>FL</td>
<td>150</td>
<td>0.28</td>
<td>NS</td>
<td>0.13</td>
</tr>
<tr>
<td>FL</td>
<td>200</td>
<td>-0.19</td>
<td>NS</td>
<td>0.13</td>
</tr>
<tr>
<td>PR</td>
<td>50</td>
<td>-0.4</td>
<td>NS</td>
<td>0.14</td>
</tr>
<tr>
<td>PR</td>
<td>75</td>
<td>0.05</td>
<td>NS</td>
<td>0.68</td>
</tr>
<tr>
<td>PR</td>
<td>150</td>
<td>0.57</td>
<td>NS</td>
<td>0.85</td>
</tr>
<tr>
<td>PR</td>
<td>300</td>
<td>0.29</td>
<td>NS</td>
<td>0.11</td>
</tr>
<tr>
<td>PR</td>
<td>400</td>
<td>0.35</td>
<td>NS</td>
<td>0.23</td>
</tr>
<tr>
<td>PR</td>
<td>500</td>
<td>0.77</td>
<td>B</td>
<td>0.59</td>
</tr>
</tbody>
</table>
"Bougainvillea ‘Afterglow’ was found to be a SDP, like many other cultivars previously explored in the literature, including ‘Raspberry Ice’, ‘Taipei Red’, ‘Rainbow Gold’, and ‘San Diego Red’ (Even-Chen et al., 1979; Liu and Chang, 2011; Ma and Gu, 2010; J. G. Norcini et al., 1992). Plants flowered much more readily under short days (8 hours) than long days (14 hours). Long days almost completely inhibited flowering in the absence of any other influencing factors. Photoperiod appeared to be more dominant environmental cue to flowering for this plant than drought stress, and the response is apparently mediated by both GA and ABA. Endogenous GA decreased in plants that flowered under SD, and increased in non-flowering plants under LD. Plants responded differently to exogenous ABA depending on the photoperiod they were grown in. Plants grown in SD responded to increasing rates of 0 to 100 µg/L exogenous ABA with a decrease in endogenous GA, resulting in shorter shoots and an increased flowering response. When application rates were increased from 100 to 300 µg/L, plants responded with an increase in endogenous GA, resulting in longer shoots and fewer flowers. Plants grown under LD responded to exogenous ABA applied at 0 to 75 µg/L as a drench with decreasing GA, shorter shoots and some flowering. As ABA application rates increased from 75 to 500 µL/L, endogenous GA increased, and plants did not flower.

Ethylene could overcome LD inhibition. The application of 50 µL/L ethephon as a foliar spray on plants grown under LD resulted in a flowering response. The ethephon application resulted in a decrease in both GA and ABA in treated plants, mirroring the response found in plants grown under SD. This suggests that ethylene plays a part in
the regulation of flowering response in *Bougainvillea* through the control of the relative abundance of both ABA and GA. Ethylene appeared to interact with GA differently under SD and LD. Under LD, increasing ethephon application rates from 0 to 75 µL/L led to a decrease in GA over the 30 days following treatment, while increasing rates from 75 to 300 µL/L led to an increase in GA over the same period.

The decrease in GA in plants that flowered suggested that the role of GA in signaling for the flowering response in *Bougainvillea* is not dependent on the same DELLA-protein pathway regulation of PIF5 as in LD plants such as *A thaliana*.

*Bougainvillea* grown in the true tropics and near the equator would not experience the LD inhibitory effect of 14-hour daylengths. Therefore, either ethephon applied as a foliar spray at 50 µL/L or and exogenous ABA applied as a soil drench at 75 µL/L would probably be sufficient to promote flowering of *Bougainvillea* planted in the landscape.
APPENDIX A
PRELIMINARY EXPERIMENT ON INDUCTION OF FLOWERING BY WATER STRESS

Introduction

*Bougainvillea* is one of the most common and defining landscape shrubs in tropical parts of the world. It is a short-day, flowering ornamental woody shrub that is valued for its ability to thrive under harsh urban conditions (subject to compaction, pollution, drought, etc). However, it exhibits an unpredictable and often non-synchronous flowering pattern, especially when there is a lack of distinct variation in photoperiod, such as in the true tropics. One horticultural practice used to induce flowering is to subject the plants to slight drought stress\(^2\). However, little research has been done to identify the reason why this seems to trigger flowering, or if indeed it does trigger flowering at all. The objective of this study was to evaluate the impact of different levels of drought stress on the flowering of one cultivar of *Bougainvillea*.

Materials and Methods

Thirty-five rooted *Bougainvillea x glabra* ‘Afterglow’ cuttings were grown in a 50% perlite: 50% coir media with 12g of Osmocote® 10-10-10. They were pruned to the same height and transferred to a growth chamber under 14-hour photoperiod (cool white fluorescent light on a timer) with temperature set at constant 25°C. Plants were watered every 2 days with 50 mL of tap water. After 3 weeks of growing in the growth chamber, plants were subjected to seven levels of water stress (A=50 mL upon wilting, B=25 mL/2days, C=25 /1day, D=50 mL/2days, E=50 mL/1day, F=100 mL/1day, G=standing water) where A=very high, B= high, C and D=medium, E=low, F=very low, and G=saturation. Stomatal conductance was measured using a leaf porometer (Decagon Devices, model SC-1) as a proxy for water stress. The number of flower buds
on each plant was counted at the end of 6 weeks. Pour-through leachates were analyzed for N, P, and K levels.

**Results**

Plants grown under medium water stress (50 mL per 2 days) produced more flowers than those grown under low or high water stress. These results support published findings that watering in between periods of medium water stress induces flowering despite long-day conditions.

Figure A-1. Stomatal conductance and mean inflorescences under varying levels of drought stress. Error bars indicate standard errors. (n=35)

Plants grown in standing water were expected to fail due to root rot, but surprisingly were the earliest to flower, and produced more flowers than plants grown under all the other treatments. Treatment D (50 mL water every 2 days) resulted in the highest mean N, P, and K levels and were not significantly different among treatments.
except for treatment G. There was probably higher availability of nutrients in Treatment G (standing water) due to constant wetting of the incorporated controlled-release fertilizers. All treatments resulted in some flowering response, which was not expected as 14-hour photoperiod had been previously found to be highly inhibitory to flowering. Temperature may have played a part in this response, as it has been noted that *Bougainvillea* flower in response to lower nighttime temperatures (Hackett and Sachs, 1985). The temperature in this experiment was set to 25°C, which is lower than the typical ambient temperature in the landscape in the tropics for most of the growing season (Calle et al., 2010; Rivera and Borchert, 2001). Furthermore, anaerobic stress may explain the flowering response, which warrants further investigation in relation to stress hormones and their role in flowering responses.
Figure B-1. Ethylene levels (parts per million) on day 1, day 14, day 28 after treatment, under varying rates of exogenous ABA application. There were no differences between treatments and photoperiod. ▲ = Short-day, ■ = Long-day, solid line = linear regression for short-day, dotted line = linear regression for long-day.
Figure B-2. Ethylene levels (parts per million) on day 1, day 14, day 28 after treatment, under varying rates of exogenous ethephon application. There were no differences between treatments and photoperiod. ▲ = Short-day, ■ = Long-day, solid line = linear regression for short-day, dotted line = linear regression for long-day
LITERATURE CITED


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BIOGRAPHICAL SKETCH

The author’s undergraduate majors were history and geography. He graduated with a Bachelor of Social Sciences (Hon) in geography from the National University of Singapore, where his honors thesis was on the viability of mixed-use development as a tool for increasing public transport usage. He was enrolled in the Master of Urban Horticulture degree at the University of Melbourne, where his research project was on the use of clove essential oil as a postharvest protectant against *Botrytis* rot. He received his Ph.D. in horticultural sciences with a focus on environmental horticulture, from the University of Florida in the summer of 2017.