EFFECT OF PHOTOPERIOD, WATER STRESS AND NITROGEN NUTRITION ON BUD PUSH, SCION GROWTH AND CYTOKININ CONCENTRATION IN CONTAINER-GROWN CITRUS NURSERY TREES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2012

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To Raman and Sukhan

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my advisor Dr. Timothy M. Spann for giving me the opportunity to study at the University of Florida. I could not have asked for a more knowledgeable, supportive and friendly advisor. I am also thankful to the members of my committee Dr. Karen E. Koch, Dr. Jeffrey G. Williamson and Dr. Arnold W. Schumann for their guidance and encouragement along the way.

I am grateful to (Late) Dr. Luis Pozo for spending hours with me in developing assays and extracting sap from nearly-dry shoots. May his soul rest in peace. I thank Dennys Cornelio for helping me in lab and greenhouse work from time to time.

I would like to thank my mom, Amarjeet Kaur, and my dad, Mangal Singh Brar, for their teachings, encouragement, aspirations and belief. A hearty thanks to my wife, Raman, for her love and support and our son, Sukhan, for being a source of inspiration. I thank my brother, Diljeet, for being my ideal in life, his wife, Navneet, and my nephew, Fateh, for their support.

During my tenure at University of Florida, I built long lasting friendships. My sincere thanks to all my friends who filled my life with love and laughs.

And above all, I thank God almighty, because He is the one leading my path.

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid
BA	Benzyl adenine
CK	Cytokinin
DS	Drought Stressed
ET	Evapotranspiration
LD	Long Day
MPA	Megapascals
PGR	Plant Growth Regulator
SD	Short Day
SD-NI	Short Day Night Interrupt
WW	Well Watered

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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December 2012

Chair: Timothy M. Spann
Major: Horticultural Sciences

In Florida, the slow growth of citrus nursery trees on rootstocks with trifoliate orange (*Poncirus trifoliata*) parentage especially during winter is a concern of economic importance. These observations started coming after the shift from open field nursery production to greenhouses occurred following a new legislation. We studied the effect of several factors on the bud push and scion growth of citrus nursery trees. The first objective was to determine the effect of photoperiod on growth of container grown trees of two trifoliate-type rootstocks, 'Carrizo' citrange and 'Swingle' citrumelo with and without non-trifoliate scions. All trees, budded or not, had reduced growth under short days (SD 10h), whereas short days-night interrupt (SD-NI 10h + 1h night-interrupt) trees grew similar to long days (LD 14h). Net CO_2 assimilation was higher under SD and SD-NI treatments than LD, with no differences in whole-plant total nonstructural carbohydrates, indicating that the growth difference is a phytochrome-mediated response. The second objective was to study the effect of drought stress on cytokinin concentration in xylem sap. In the trees grown under three water stress treatments 100% ET (Control), 50% (Mild) and 20% (Severe stress) cytokinin concentration initially started to increase with increasing water stress but later decreased with severe stress.

The foliar BA application did not have any significant effect. In the third experiment, buds taken from budwood trees were inserted into rootstock seedlings grown under same well watered/drought stress conditions and BA @ 500 ppm was applied to buds. BA significantly enhanced bud-break in well-watered and in trees moved to well-watered regime. In drought stressed, two BA applications resulted in 36 % total bud break indicating an interaction between BA and water stress. The fourth experiment shows that Nitrogen deprivation decreased leaf chlorophyll concentration and whole plant nitrogen content (% dry weight) resulting in lower photosynthetic rate. The bud survival, budbreak and scion growth, all were higher in trees under N application. The N sufficient trees had higher endogenous cytokinin levels before and after budding but not after unwrapping. The trees showed no significant changes in endogenous cytokinin levels with N application over 5 days.

CHAPTER 1
INTRODUCTION

Florida's citrus nursery industry has suffered major blows in recent years. First, the devastation by four major hurricanes during 2004 and 2005 and then the canker eradication program implemented to eradicate affected trees in groves and nurseries. The hurricanes caused extensive damage to citrus nurseries and groves and the Department of Primary Industries (DPI) original budwood foundation at Dundee, FL was destroyed. In the following year, citrus greening disease (Huanglongbing) was found in Florida and the nurseries were hit with citrus greening outbreaks. In 2006, an eradication program was started and 62% of nursery stock was eradicated as a phyto-sanitary measure. In just a year and a half, 5.4 million trees were eradicated including 7,951 budwood source trees. The state of Florida enacted Citrus Budwood Protection Rule 5B-62 late in year 2006, a legislated mandate that effective January 1, 2007, all citrus nursery propagations must occur in enclosed greenhouses, which must be insect proof, have double entry ways and the new nurseries must be one mile from citrus groves. Field grown nursery stock could no longer be sold as of January 1, 2008.

Traditionally, citrus nursery plants were produced in field nurseries, and greenhouse-grown containerized trees accounted for only 35 % of total production in the state (Davies and Zalman, 2008). However, with the recent developments, citrus nurseries started shifting to greenhouses. This shift from traditional field to greenhouse container-grown systems has given rise to problems of bud failure. The problem of inconsistency in the percentage of bud break has been previously reported for field-grown (Orillos, 1954 and Halim et al., 1990) and container-grown citrus nurseries (Maxwell and Lyons, 1979, Nauer et al. 1979, Poll, 1991; Williamson and Maust, 1996).

However, this did not receive much attention as field-grown traditional systems were not cost-intensive and the nursery businesses were still profitable even with losses of 10% or more due to bud failure because space, soil and other resources were not limiting factors. This changed dramatically when the nurseries switched over to container-grown greenhouse environments. The bench space in a greenhouse comes at a premium and the overall cost of production went up exponentially, given the cost of newly built high-tech greenhouses and other required facilities. The grower observation started coming in that in several trees, the buds won't push or the scion will not grow after bud-push. Extensive visits and surveys of the citrus nurseries in Central and North Central Florida were conducted. The search for literature revealed that little research had been directed toward elucidating the causes and factors affecting bud failure in citrus nurseries, and most of the earlier research efforts were devoted to field grown nurseries, which were not applicable to the greenhouse environments.

In the current scenario, Florida citrus nurseries have increased their inventories steadily over the past four years. As of 2010, there are 45 citrus nurseries in Florida, which are producing more than 3.1 million trees annually (FDACS Bureau of Citrus Budwood Registration Annual report, 2010).

The major factors associated with poor bud take may include type of rootstock, nutrition of rootstock and/or budwood trees, photoperiod, irrigation, soil temperature, method of bud-forcing and endogenous plant growth regulators.

Trifoliate-type rootstocks (*Poncirus trifoliata* hybrids) are used commonly in citrus propagation. In Florida, 'Swingle' citrumelo (*Citrus* ×*paradisi* ×*P. trifoliata*) is the most commonly used citrus rootstock followed by 'Carrizo' citrange (*C. sinensis* ×*P. trifoliata*)

(FDACS Bureau of Citrus Budwood Registration Annual report, 2010). Trifoliate trees are deciduous and have been known to be responsive to photoperiod. Piringer et al. (1961) reported that growth of trifoliate orange slowed markedly under short day conditions (8 h photoperiod). Warner et al, (1979) reported that some rootstocks of trifoliate parentage such as 'Carrizo' citrange responded positively to long days. Although these reports indicated that growth in trifoliate trees slowed during short days, no evidence was found whether this effect is truly due to phytochrome mediated photoperiodic response or may just be a photosynthetic growth response as in the short day the trees receive fewer number of light hours to photosynthesize.

Drought stress is another major factor that may influence budbreak and scion growth. Drought stress has been reported to cause reduction in leaf number and size in walnut (Yadollahi et al., 2010), reduction in shoot growth in maize (Sangakkara et al., 2010) and decrease in new vegetative flushes as well as new leaves and root growth in mango (Tahir et al., 2003). Cellular growth is extremely sensitive to drought stress; therefore, the low or no availability of water during active cell division and expansion stages must be affecting plant growth at both cellular and whole plant levels. The formation of the bud union, bud break and scion growth are a few of such stages in plant growth and development that are very sensitive to availability of water. Drought stress has also been reported to be affecting cytokinin synthesis and transport via the transpiration stream through xylem. Cytokinins are plant growth regulators known to have an active role in cell division and growth. Therefore, any factor affecting cytokinin availability within the plant system is bound to influence bud union formation and bud break. Drought stress seems to be one such factor.

Results of several studies suggest that cytokinin accumulation is closely correlated with plant nitrogen status (Wagner and Beck, 1993; Samuelson and Larsson, 1993; Takei et al., 2001), and that cytokinin metabolism and translocation could be modulated by the N status. Nitrogen is the second most abundant and important element in plants after carbon. Nitrogen availability is known to affect many physiological processes, including cytokinin synthesis and delivery in plants.

All these factors have been the focus of broader research in plant growth and development; however, the effect of these factors on bud break and scion growth in container-grown citrus nursery trees has not been elucidated. Based on our hypotheses regarding photoperiod, water stress and nitrogen nutrition influencing bud push and scion growth, we developed the following objectives:

1. To test the effects of photoperiod on the growth and carbohydrate partitioning of trifoliate-type rootstocks with and without sweet orange scions.
2. To study the effect of drought stress on the xylem sap cytokinin concentration in young citrus nursery trees and the role of exogenous cytokinin application in stimulating a recovery thereafter.
3. To study the effect of drought stress on bud take and scion growth as compared to well watered conditions in young citrus trees in containerized nurseries.
4. To study the effect of levels of nitrogen application on bud take and scion growth; and to quantify the effect of nitrogen application on the biosynthesis and translocation of endogenous free cytokinins in shoots of trifoliate orange rootstocks.

CHAPTER 2
REVIEW OF LITERATURE

Florida citrus nurseries produce more than 3million trees annually (FDACS Annual report, 2010). Traditionally, citrus nursery plants were produced in field nurseries, and greenhouse-grown containerized trees accounted for only 35 % of total production in the state (Davies and Zalman, 2008). However, with the outbreak of citrus greening disease, citrus nursery trees must be grown in greenhouses as per the newly instituted legislation. This shift from traditional field to greenhouse container-grown systems has given rise to problems of bud failure. The problem of inconsistency in the percentage of bud break has been previously reported for field (Orillos, 1954 and Halim et al., 1990) and container-grown citrus nurseries (Maxwell and Lyons, 1979, Nauer et al. 1979, Poll, 1991; Poll, 1993; Williamson and Maust, 1996). However, little research has been directed toward elucidating the causes and factors affecting bud failure.

The major factors associated with poor bud take may include type of rootstock, nutrition of rootstock and/or budwood trees, photoperiod, irrigation, soil temperature, method of bud-forcing and endogenous plant growth regulators.

Bud Forcing Methods

To push the bud and thereby enhance budbreak, the apical dominance of the rootstock seedling needs to be overcome through bud forcing. Common bud forcing methods are bending, lopping and topping (cutting off). In bending, the portion of rootstock seedling above the bud union is bent over in a loop and is tied to the base of the rootstock stem. In lopping, the upper portion is cut, leaving it only partially attached, and it is tied as in bending. In topping, the entire portion of rootstock stem above the bud union is cut off and removed. Another method, notching, is also practiced in many

field nurseries. In this method, two parallel cuts are made in the cambium of the rootstock stem just above the bud union and a small portion of bark is removed, creating a notch in the rootstock stem.

In general, greater scion growth has been reported using bending as compared to topping (Williamson et al., 1992; Samson, 1986; Rouse, 1988). Williamson et al. (1992) reported that greater scion growth resulted from lopping or bending, compared with topping, and this may be due to translocation of photosynthates from the bent rootstock shoots to the roots and scion. In another study, Al-Jaleel and Williamson (1993) found that scion bud break following bending + benzyladenine (BA) treatments in Swingle rootstock seedlings was significantly affected by soil temperature, with high soil temperature (25^0 C) increasing bud break to 100 %. In a similar bud forcing study using sweet orange and mandarin scions budded on Carrizo and Swingle rootstocks, bending was found to be more effective than topping in all rootstock/scion combinations (Bowman, 1999).

A study by Rouse (1988) showed that among the four bud forcing methods, bending produced the highest percent bud break when the seedling leaves below the inserted bud were not removed. In this combination, scion growth was greatest which in turn lead to a greater number of leaves per shoot and greater total leaf area compared to lopping and topping treatments. In addition, bending is also advantageous because it is easier to re-bud the seedlings in the case of bud failure as compared to when the seedling top is cut off. Similar results are reported by Samson (1986) from a study of bud forcing methods in Surinam where it was found that bending and lopping gave significantly better results than topping. Half ringing enhanced the effect of bending.

However, the effect of defoliation varied depending upon the age and position of the leaves removed.

In a survey conducted in 1988, Williamson and Castle (1989) found that lopping was the most common bud forcing method in Florida field nurseries while cutting off was preferred in container nurseries. However, the nurserymen indicated inadequate scion growth following topping in container nurseries and as a result, a significant number of nurseries had started bending the seedlings to enhance bud break and increase scion length. Williamson and Maust (1996) reported the findings of forcing treatments on different scion/ rootstock combinations and revealed that both the method of bud forcing and the rootstock variety have an effect on the growth of trees in containerized citrus nurseries. In their experiments, Hamlin orange was budded to Carrizo, Swingle and Cleopatra rootstocks. Their results indicated that for all rootstocks whole plant dry weight was greater for plants forced by bending and lopping than for plants forced by cutting off.

Photoperiod and Temperature

Plants can be categorized by their response to photoperiod. Short day plants (SD) respond to photoperiods shorter than some critical day length, and long day plants (LD) respond when the photoperiod is greater than some critical day length; plants unresponsive to day length are categorized as day neutral. Although much attention has been directed toward flowering responses to photoperiod, some research has investigated the vegetative growth and bud break responses to photoperiod. Piringer et al. (1961) conducted studies on effects of photoperiod on four citrus species, *Citrus aurantifolia*,*C. limonia*,*C. paradise* and *Poncirus trifoliata*. Seedling plants of these species were subjected to three photoperiod treatments, 8, 12 and 16 hours, for 38

weeks. After 38 weeks, plants under 16-hour photoperiod were shifted to natural photoperiod plus a 3-hour interruption in the middle of the night. These plants were observed for an additional 8 months. They reported that the growth of trifoliate orange (*P. trifoliata*) slowed down markedly under the 8-hour photoperiod. The rate of new flush production of budded grapefruit (*C. paradisi*) was also affected by photoperiod, with the interval between flushes being longer under short days. The responses of all the plants to the night interrupt treatment were similar to the ones in long day treatments. Nauer et al. (1979) reported that fall-budded navel orange plants receiving longer day length (15 hours) exhibited significantly greater growth (average 73.8 cm) as compared to growth of plants (average 58.7 cm) under short days (10 hour). However, higher greenhouse temperature (33.3°C) did not have a significant effect on plant growth as compared to relatively cool temperature (25°C) both under normal and extended day length.

Inoue (1989) studied the effects of day length and temperature on the vegetative growth of one-year-old Satsuma mandarin budded on trifoliate orange rootstocks. It was reported that shoot growth decreased under short days in spring and summer while the long day treatment (16 hour photoperiod) increased the shoot growth. The long days also resulted in higher fresh weight compared to the plants under short days. Warner et al. (1979) studied the effect of photoperiod on different citrus rootstocks and found that rootstocks such as Rubidoux, Yamaguchi, Christianson and Pomeroy of trifoliate parentage, as well as Carrizo and Savage citranges and Hawaiian sweet orange strongly responded to LD photoperiod treatments. On the contrary, Cleopatra mandarin, Troyer citrange, Swingle citrumelo and *Citrus volkameriana* were found to be less responsive to LD and exhibited better growth under SD conditions.

Researchers have long been delving into the molecular aspects of photoperiodic responses of plants. Among the earlier reported studies, Warner and Upadhya (1968) studied the effect of photoperiod on isoenzymatic composition of four citrus cultivars including tangerines, tangelos and trifoliate varieties. They observed that all of the cultivars had greater stem cross-sectional area (mm^2), greater linear growth (cm) and more branches under long day conditions. They concluded that the highly significant growth responses were accompanied by differences in the activity of enzyme systems. The number of esterase isoenzymes in trifoliate varieties and leucine amino-peptidase activity and counts in tangerine and trifoliate were higher under long day conditions. The increase in the activity of isoenzymes may be regulated by the enhanced levels of growth regulating substances, which may, in turn, be influenced by the photoperiod eventually determining the degree of plant growth.

In most deciduous tree species, short days induce bud dormancy and growth cessation, which are photoperiod-controlled responses. According to Koornneef *et al* - (1991) as cited in Bohlenius *et al.*, (2006), the genes CONSTANS (CO) and (FT) are responsible for daylength regulation of flowering in *Arabidopsis.* These genes are known to induce flowering as a response to long days. In the case of, the PtFT1 gene (*Populus trichocarpa* FLOWERING LOCUS T ortholog) was found to be an inhibitor of dormancy induction and growth cessation under short day conditions (Bohlenius *et al.*, 2006). Over-expression of PtFT1 resulted in plants not exhibiting growth cessation and dormancy under SD. PtFT1 expression was itself regulated by $PtCO_2$, a *Populus trichocarpa* CONSTANS ortholog, which is controlled by day length.

Drought Stress

Cellular growth is known to be extremely sensitive to availability of water. Effects of water deficit on cellular processes, cellular growth and vegetative growth in various trees and other crop species have been extensively reported. It has been well documented that water deficits can limit vegetative growth by inhibiting cell division and expansion and also by reducing photosynthesis as stomata close (Taiz and Zeiger, 2006; Zhang and Davies 1990; Hutton et al., 2007; Mullet and Whitsitt, 1996; Bray, 1997). Yaddollahi et al. (2010) reported from a study in walnuts that water stress caused reduction in number and size of nuts, while research in maize (Sangakkara et al., 2010) showed reduced shoot growth and in mango (Tahir et al., 2003) showed decrease in new vegetative flushes and reduction in new leaf number and shoot growth.

Role of PGRs

One of the major factors affecting bud take is the altered balance of plant growth regulators (PGRs). Cytokinins and auxins are the two PGRs known to play a vital role in growth and development of lateral buds. Cytokinins are particularly important in bud grafting since they enhance cell division, callus formation, and the eventual differentiation of vascular strands, hence playing a role in growth of grafted buds (Hartmann, 2002). Furthermore, these phytohormones are transported to shoots and lateral buds from the roots through the xylem sap (Faiss *et al.,* 1997; Emery and Atkins, 2002) and contribute to shoot branching. Therefore, the factors that can affect the synthesis and transport of cytokinins within the plant, can also affect the growth of grafted buds.

The current status of related research is reviewed here, focusing on i) the role of cytokinins in plant growth and development, and ii) the factors affecting synthesis, transport and lateral bud concentration of cytokinins.

Role of Cytokinins in Plant Growth and Development

Cytokinins are plant growth regulators known to promote cell division and differentiation. The first cytokinin identified was kinetin by Miller and co-workers (1955). Since then, a number of natural and synthetic cytokinins have been characterized. The role of cytokinins in plant development has been extensively studied, revealing the major functions of cytokinins to be as promoters of cell division, callus formation and cell differentiation (Skoog and Miller*,* 1957). Cytokinins are mobile phytohormones that regulate plant growth and development by affecting leaf senescence (Kim *et al.,* 2006); apical dominance (Tanaka *et al.,* 2006); root proliferation (Werner *et al.,* 2001); phyllotaxis (Giulini *et al.,* 2004); and nutritional signaling (Takei *et al.,* 2001, 2001a). In plants and other organisms, cytokinins are found bound to the tRNA. However, free cytokinins are also abundant in plants. The most abundant free species are the isoprenoid-type, but many plant species also contain adenine derivatives. Research on the structure of cytokinins has revealed that the naturally occurring cytokinins are N^6 substituted adenine derivatives that usually contain an isoprenoid or aromatic derivative side chain. The structure of the side chain directly relates to the activity of a particular cytokinin. This is evident from the case of zeatin. Trans-zeatin, which is most abundant in higher plants, shows a high activity in bioassays, while the cis-isomer displays a significantly lower activity (Haberer and Kieber, 2002; Sakakibara and Takei, 2002).

Much debate has focused on the synthesis and translocation of cytokinins (Emery and Atkins, 2002; Takei et al., 2001; Morris et al., 2001 and Schmulling, 2002).

However, root tips have now been established as the primary sites of cytokinin synthesis. The shoot meristems and other primordial organs are the most typical targets of cytokinins, which regulate cell cycles at these sites. Thus, these phytohormones are a limiting factor for cell division in the shoot, and also have a negative regulatory effect on the root (Schmulling, 2002). The recent advances in the understanding of cytokinin roles and activity within plants include the identification of genes encoding ATP/ADP isopentenyl transferases (Martin *et al.,* 1999, 2001) and the molecular analysis of cytokinin receptors and cytokinin catabolism (Werner *et al,* 2001). A major advance was achieved when a gene encoding IPT (ATP/ADP isopentenyl transferase) was identified in Arabidopsis. IPT is the major enzyme instrumental in cytokinin biosynthesis. There are two types of isopentenyl transferases involved in cytokinin production. One type of IPT modifies tRNA and is known as tRNA IPT. Another type of IPT is an iPMP-(isopentenyladenosine 5 monophosphate) forming enzyme, known as an adenylate IPT (Sakakibara and Takei, 2002). The expression analysis of the IPT gene showed that the expression was strongest in the root cap columella (Takei *et al.,* 2001). No IPT expression was observed in the apical meristem of the shoot (Miyawaki *et al.,* 2004), consistent with evidence for cytokinin synthesis primarily in the roots. Mahonen *et al.* (2000) identified a cytokinin receptor gene and observed that the earliest detectable expression of this gene was localized to the four vascular precursor cells innermost in the embryo of Arabidopsis*.* During post-embryonic development, expression of the cytokinin receptor gene was also detected in shoot tissue. Mutants lacking a functional gene for this receptor showed reduced cell division in the embryonic axis, which in turn led to fewer vascular initials (Scheres *et al.,* 1995; Mahonen *et al.,* 2000). Introgression

of a wild type receptor gene into the mutant plant increased the number of cells in the embryonic axis and restored the phenotype. These studies provided clear evidence for a cytokinin receptor role in vascular morphogenesis.

Recent loss-of-function studies in transgenic plants have also shed light on the role of these PGRs in plant development. Endogenous hormone levels were altered through over-production of cytokinins by controlling IPT gene expression by a dexamethasone (dx) inducible and teteracycline (tc)-repressible promoter (Bohner and Gatz, 2001). Plants induced systemically for enhanced cytokinin synthesis by dx exhibited outgrowth of all lateral buds, whereas growth suppression of the lateral buds was observed under treatment with anti-inducer tc. In a study of *in vitro* shoot proliferation in citrus plants, two cytokinins, benzylaminopurine (BAP) and kinetin, were found to stimulate shoot proliferation (Al-Bahrany, 2002). Shoot multiplication was rare when BAP was absent, but maximum numbers of shoots developed with the interaction of the two cytokinins and an auxin. The processes influenced by exogenous cytokinin application are believed to be influenced by changes in endogenous levels of these phytohormones.

Signals other than root-produced cytokinins are also reported to influence branching based on studies using branching mutants (Morris *et al*., 2001). Previous work has also shown that the ratio of auxins and cytokinins plays a vital role in shoot branching (Stafstrom, 1993). Recently, the analysis of *rms* increased-branching mutant of pea has revealed that additional factors are responsible for the regulation of branching. These increased-branching mutants have reduced cytokinin concentration in the root xylem sap. This suggests that the branching in pea is correlated with the down-

regulation of cytokinin export from the roots. However, the reduced cytokinin movement from the roots did not decrease the cytokinin concentration of leaves or enhance leaf senescence. This suggests either non-involvement of root-born cytokinins or local biosynthesis of the cytokinins (Morris *et al.,* 2001).

In contrast, transgenic tobacco plants expressing the cytokinin oxidase/ dehydrogenase gene had a decreased concentration of cytokinins and showed developmental alterations in the root and shoot (Werner *et al.,* 2001). These alterations included short internodes, dwarfing, late flowering, less profuse flowering, decreased leaf surface area and a small vascular system. In addition, there were fewer new leaf primordia and/or new leaf cells formed. The same study further revealed that the growth of lateral buds slowed in the transgenic plants.

McKenzie *et al.* (1998) used a root-specific, copper-inducible gene expression system to regulate IPT gene transcription in transgenic Tobacco (*Nicotiana tabacum* L. cv tabacum**)**. When copper was applied, lateral bud growth was observed in the whole plant. These results, together with the study above, provide strong evidence for the role of cytokinins in regulating plant growth, especially that of branches, vascular system and lateral buds. It is well documented that exogenous application of cytokinins also induces growth in the lateral buds. The growth promotion of the quiescent buds by cytokinin application has been reported earlier in *Macadamia tetraphylla* and *Citrus reticulata* (Boswell *et al.,* 1981), *Citrus sinensis* (Nauer *et al*,.1979), and apple (Kender and Carpente, 1972). Clearly, cytokinins play a vital role in the growth and elongation of lateral buds.

Genetic approaches have also provided evidence for roles of cytokinins, not only in cell division and expansion, but also in light responses, nutrient metabolism, differentiation and functioning of chloroplasts, leaf senescence, and source-sink relations (Mok and Mok, 2001, Kiba *et al.,* 2005). Evidently, transgenic plants over expressing cytokinin oxidase reduce cytokinin concentration and exhibit retarded shoot development and enhanced root growth. Similar responses have been observed in tomato plants under partial root-zone drying (Mingo *et al.,* 2004; Sobeih *et al.,* 2004). Therefore, it is possible that soil drying-induce physiological changes are due to reduced cytokinin concentration in aboveground plant parts (Kudoyarova *et al.,* 2007). Previous work has also shown that reduced cytokinin delivery to the shoots is an important root to shoot signal of soil drying (Davies *et al.*1986). Cytokinins are positive regulators of cell division in the shoot apical meristem, while they are the negative regulators of cell division in the root apical meristem (Schmulling, 2002).

Cytokinin Synthesis, Transport and the Control of Shoot Branching

The main forms of cytokinins found in xylem sap are the tZ-type, such as tZ Riboside (tZR). Analysis of gene expression indicates that roots are major sites of tZ production, and that tZR acts as a root-to-shoot acropetal signal (Hirose *et al.,* 2007). In a study of movement by root-synthesized cytokinins to aerial parts of plants, distribution patterns were analyzed using free-cytokinin-responsive ARR5::GUS transformants of Arabidopsis (Aloni *et al,* 2005). In the plants exposed to wind, transpiration increased, causing enhanced movement of the transpiration stream. This resulted in significantly increased expression of the cytokinin-responsive ARR5::GUS in the shoots. Strongest labeling was in the vascular bundles of stems, leaves and buds. This finding suggests

that root-synthesized cytokinin is exported through the xylem and accumulates at the sites of highest transpiration.

Root-born cytokinins move to shoots (Letham, 1994) where they act as functional counterparts to auxin in regulation of shoot branching (Li and Bangerth, 2003; Beveridge, 2006). Auxins promote apical dominance, and therefore, have an inhibitory effect on lateral bud growth. In contrast, cytokinins have stimulatory effects on bud growth (Wickson and Thimann, 1958). Strafstrom (1993) proposed that shoot growth was regulated by the ratio of auxins to cytokinins in a gradient along the shoot axis. Bangerth (1994) maintained that if the shoot apex is removed, the export of cytokinins from roots to shoots increases, thereby enhancing lateral bud growth. Apical dominance, which is in turn, linked to auxins, is also believed to play a role in the upward movement of cytokinins.

The extent and direction of cytokinin movement has been studied in a number of plants including citrus. Benzyladenine, a naturally occurring endogenous cytokinin, and its metabolites move acropetally through the xylem and basipetally through the phloem (Friedric *et al.* 1970). According to Hirose *et al.* (2007), the transfer of cytokinin nucleosides across membranes occurs via an equilibrative nucleoside transporter (ENT). Evidence indicates that an OsENT expressed in leaf vascular bundles and phloem tissue mediates transport of adenosine and other nucleosides.

Cytokinin levels in the whole plant and in the xylem correlate positively with soil minerals (Goring and Mardanov, 1976; Salama and Wareing*,* 1979; Takei *et al.,* 2001; 2001a), especially mineral nitrogen. This finding indicates that cytokinin signals can provide information on nutrient availability. Cytokinin-mediated signaling in the plant is

effective in the control of development, protein synthesis and the acquisition of the macronutrients (Sakakibara *et al.,* 2006). The presence of cytokinins in the xylem sap and the phloem sap (Goodwin *et al.* 1978; Kamboj *et al., 1998*), further establishes the capacity for cytokinins to act as systemic mediators within the plant system. Beveridge (2006) described hormonal analyses and grafting studies with an *rms1* branching mutant of garden pea that indicated a long distance “Shoot-Multiplication Signal” (SMS) was responsible for the branching phenotype. A proposed scenario for how this signal, along with cytokinins and auxins, could regulate shoot branching is illustrated in Figure 2-2.

Domagalska and Leyser (2011) reviewed the hormonal signal integration in controlling the shoot branching where they discussed the second messenger model for bud activation (Figure 2-3). According to this model, the auxins in the apical meristem, regulate the cytokinin biosynthesis by downregulating the IPT. Therefore, when apical meristem is removed, this results in increase in the level of cytokinins in the bud promoting bud growth. There is another class of endogenous hormones called strigolactones which were discovered recently and have been demonstrated play an important role in inhibition of bud outgrowth. According to the second messenger model, auxins also upregulate the biosynthesis of strigolactones through MAX3 and MAX4 genes in *Arabidopsis thaliana* which encode CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7) and CCD8, respectively, resulting in increased production of strigolactones.

Cytokinins and Nitrogen

Results of several studies suggest that cytokinin accumulation is closely correlated with plant nitrogen status (Wagner and Beck, 1993; Samuelson and Larsson,

1993; Takei et al., 2001), and that cytokinin metabolism and translocation could be modulated by the N status. A significant finding from these studies is that an increase in cytokinin concentration occurred immediately after the N status changed from deficient to sufficient. In maize, within 1 hour of the addition of nitrate to N-deprived plants, isopentenyladenosine- 59-monophosphate (iPMP) started to accumulate in roots, followed by increases in levels of trans-zeatin riboside-59- onophosphate (ZMP), trans-zeatin riboside (ZR) and trans-zeatin (Z) (Takei et al., 2001a). Since, iPMP is the first molecule synthesized in cytokinin metabolism, results indicate that cytokinin was synthesized anew in response to nitrate supply. After the application of nitrate, both the exudation rate and the concentration of the cytokinins increased in the xylem, with ZR being the dominant cytokinin in this fluid. The spatial and temporal changes in the molecular species, and the extent of accumulation, strongly suggest a N-dependent movement of cytokinins from roots to shoots.

A NO_3-dependent movement of cytokinins has not been tested in woody species like citrus. This has two-fold implications, the first being that NO_3 is typically reduced in roots of woody plants rather than moving upward to leaves in the xylem stream (unless NO_3 assimilation in roots is overwhelmed). The second is that, in citrus seedlings, the mediation of cytokinin translocation by NO_3 can in turn influence the bud break and growth of lateral branches in the budded seedlings. Therefore, keeping in view the above correlation, we hypothesize that the application of NO_3 to N-starved seedlings will increase the cytokinin concentration of roots and xylem sap in the seedlings of citrus rootstocks.

Nitrogen nutrition of the plant may also influence cytokinin formation by altering expression of the IPT gene, which encodes a key enzyme in cytokinin biosynthesis (Hirose *et al.,* 2007). Several studies (reviewed by Domagalska and Leyser, 2011) have reported the interaction between nitrogen and cytokinins, proposing that nitrogen availability upregulates cytokinin biosynthesis genes IPT3, IPT5 and CYP735A2 in the roots which leads to increased CK transpostation via xylem stream. Cytokinins thus translocated promote bud growth (Figure 2-4). Among the members of the IPT gene family, AtIPT3 is up-regulated by nitrate (Miyawaki *et al.,* 2004; Takei *et al.,* 2004). In an *ipt3* mutant, the nitrate-dependent accumulation of cytokinin was considerably reduced, emphasizing that AtIPT3 is mainly responsible for nitrate-dependent cytokinin biosynthesis. Apart from nitrogen, other macronutrients like sulphur and phosphorous also regulate transcription of AtIPT3. In response to environmental factors, there is typically a complementary regulation between macronutrients and cytokinins for nutrient acquisition and distribution (Franco-zorilla *et al.,* 2002, 2004, 2005; Maruyama-Nakashita *et al.,* 2004). The apical meristems of shoots and lateral buds are not the primary sites of cytokinin synthesis. Cytokinins reportedly down-regulate the expression of IPTs, indicating a role for root-to-shoot cytokinin mass transport in regulating shoot synthesis. This implies that shoot synthesis of cytokinins can serve the purpose during an emergency, such as nitrogen deficiency.

To test the extent of root-born cytokinin movement to aerial parts through the transpiration stream, Kudoyarova *et al.* (2007) imposed partial root-zone drying (PRD) treatments and quantified cytokinin concentration in the xylem sap and leaves of tomato. Zeatin-type cytokinins were immunoassayed and the cytokinin concentration of

fully expanded leaves was considerably reduced as a result of partial root-zone drying. Although the cytokinin concentration of the xylem did not change significantly in PRD, the cytokinin concentration of leaves was reduced by 46%. No increase in xylem cytokinin suggested that with decreasing transpiration, loading and movement of the cytokinins from the roots into the xylem decreased considerably as the soil dried. It is well documented that water stress increases the ABA concentration of aerial parts in many plant species (Naqvi, 1994; Dodd, 2005). In the drying soils, decreased delivery of cytokinins to the shoot as a root to shoot signal has also been reported (Davies et al., 1986; Bano et al., 1994; Trejo and Davies, 1991; Naqvi, 1994). Pospisilova (2005) studied the interaction between abscisic acid and cytokinins in four different crops during water stress and subsequent rehydration. In *Phaseolus vulgaris* and *Zea mays* the ABA accumulation induced by water stress was inhibited by BA application. Also, after rehydration, in plants of beans, maize and sugar beet pre-treated with BA, the ABA concentration was lower than the control plants that were not treated with BA. It was concluded that cytokinins could partially inhibit the water-stress-induced accumulation of ABA.

Stomatal closure in response to stress-induced ABA accumulation has been reported by a number of researchers (Reviewed by Dodd, 2003). Interactions of cytokinins and ABA with regards to their effect on stomatal closure have also been observed. Das et al. (1976) reported that incubation of *Comelina* epidermis in 50µM solution of BA had an antagonistic effect on ABA-induced stomatal closure. Blackman and Davies (1985) found similar results in *Zea mays* where incubation of leaf pieces in 10 µM or 100 µM zeatin or kinetin reversed stomatal closure induced by ABA.

Recent work has identified many vital elements that are functional in the biosynthesis, metabolism, and translocation of cytokinins. The genetic and molecular analysis of mutants has elucidated the roles of these phytohormones and their underlying mechanisms in the plant system. The expression patterns and controls for many of the identified genes are yet to be determined and mutant analyses are in progress to reveal the complex interactions of various components of cytokinin signaling. The active role of cytokinins in the growth of lateral buds is supported by the studies reviewed here. Evidence includes data for the synthesis of cytokinins in the roots, their subsequent movement to the aerial parts, and their effect there on enhancement of bud growth and branching. Data also show that the transpiration stream directly influences cytokinin levels in aboveground organs, since these PGRs are transported in the xylem fluid. Therefore, to enhance the up-stream flow of these phytohormones, the understanding of environmental factors regulating transpirational flow is necessary. Factors such as root zone drying have a negative effect on the movement of xylem sap. In addition, nitrogen nutrition can affect cytokinin biosynthesis in the roots. This research will guide the proposed study, which is focused on factors affecting bud take and scion growth as well as synthesis, transport and bud concentration of cytokinins in sweet orange.

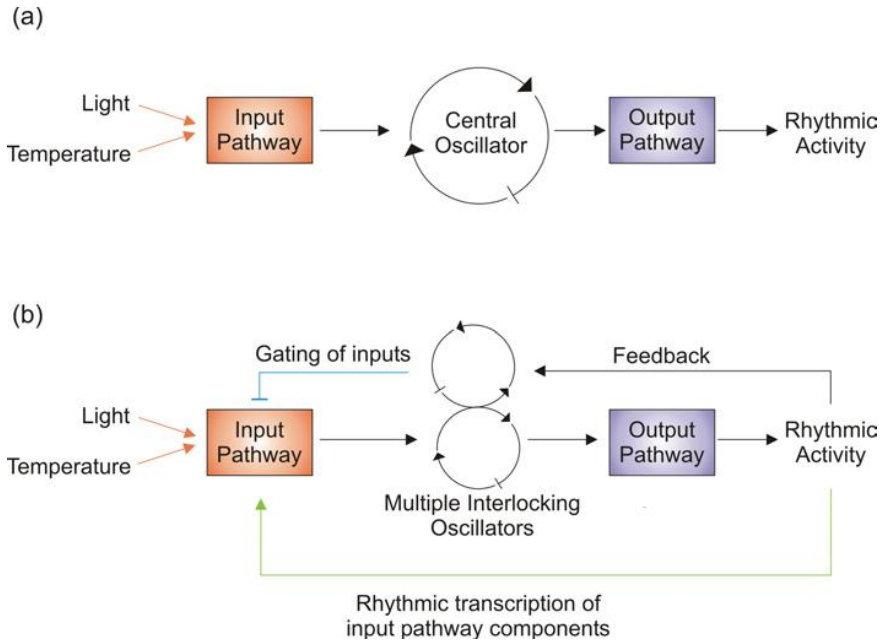


Figure 2-1. Schematic representation of circadian clock structures (a) A model depicting division of the clock into an input pathway, a central oscillator and an output pathway. (b) An elaborated description of the clock, consisting of multiple core oscillators, gated input pathways and outputs which feed back into the central oscillator. Arrows are positive arms and perpendicular lines represent negative arms of the pathway. (Figure and description: Gardner et al., 2006)

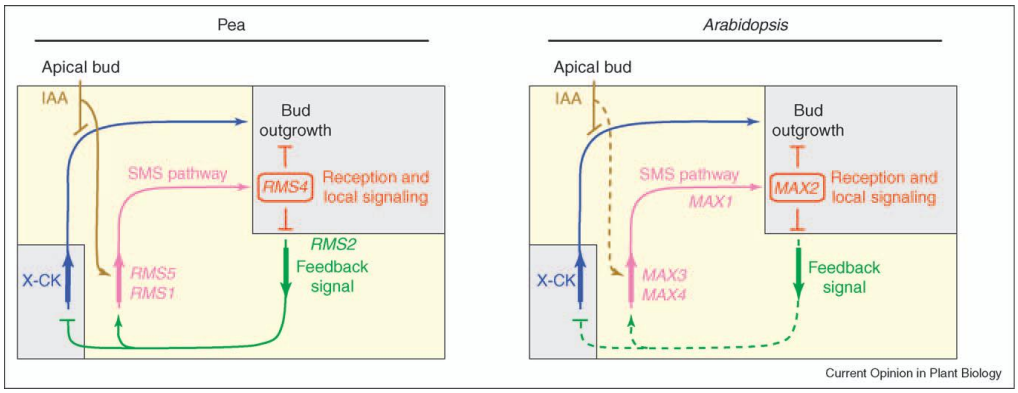


Figure 2-2. Model of branching control in Arabidopsis and pea. (Beveridge, 2006)

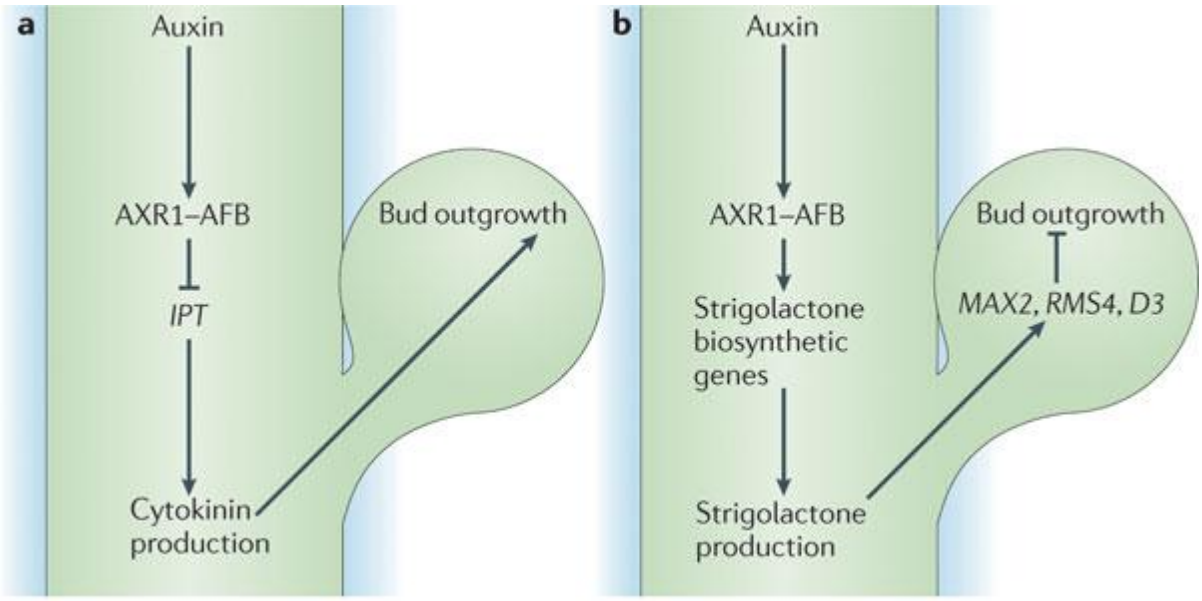


Figure 2-3. The second messenger model for bud activation. In this model, auxin regulates the production of a second messenger, which moves directly into the bud to control its activity. Cytokinins and strigolactones are candidates to serve as second messengers, as auxin regulates both biosynthesis pathways through the classical AXR1–AFB) (AUXIN RESISTANCE PROTEIN 1–AUXIN SIGNALLING F-BOX PROTEIN)-dependent auxin signalling pathway. a. Auxin regulates the biosynthesis of cytokinins by downregulating *ADENYLATE ISOPENTENYLTRANSFERASE* (*IPT*) family members at the node. Levels of cytokinins at the node and in the bud increase when the source of apical auxin is removed (decapitation), suggesting that newly synthesized cytokinins at the node are transported to the bud. b. Auxin also upregulates strigolactone in biosynthetic genes. These are *MORE AXILLARY GROWTH 3* (*MAX3*) in *Arabidopsis thaliana*, *RAMOSUS* 5 (*RMS5*) in pea, *DWARF 17* (*D17;* also known as *HIGH TILLERING DWARF* (*HDT1*)) in rice and *DECREASED APICAL DOMINANCE* (*DAD3*) in petunia, which encode CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), and *MAX4* in *A. thaliana*, *RMS1* in pea, *D10* in rice and *DAD1* in petunia, which encode CCD8. This probably leads to increased strigolactone levels. Strigolactones can prevent bud activation. (Figure and description Domagalska and Leyser, 2011)

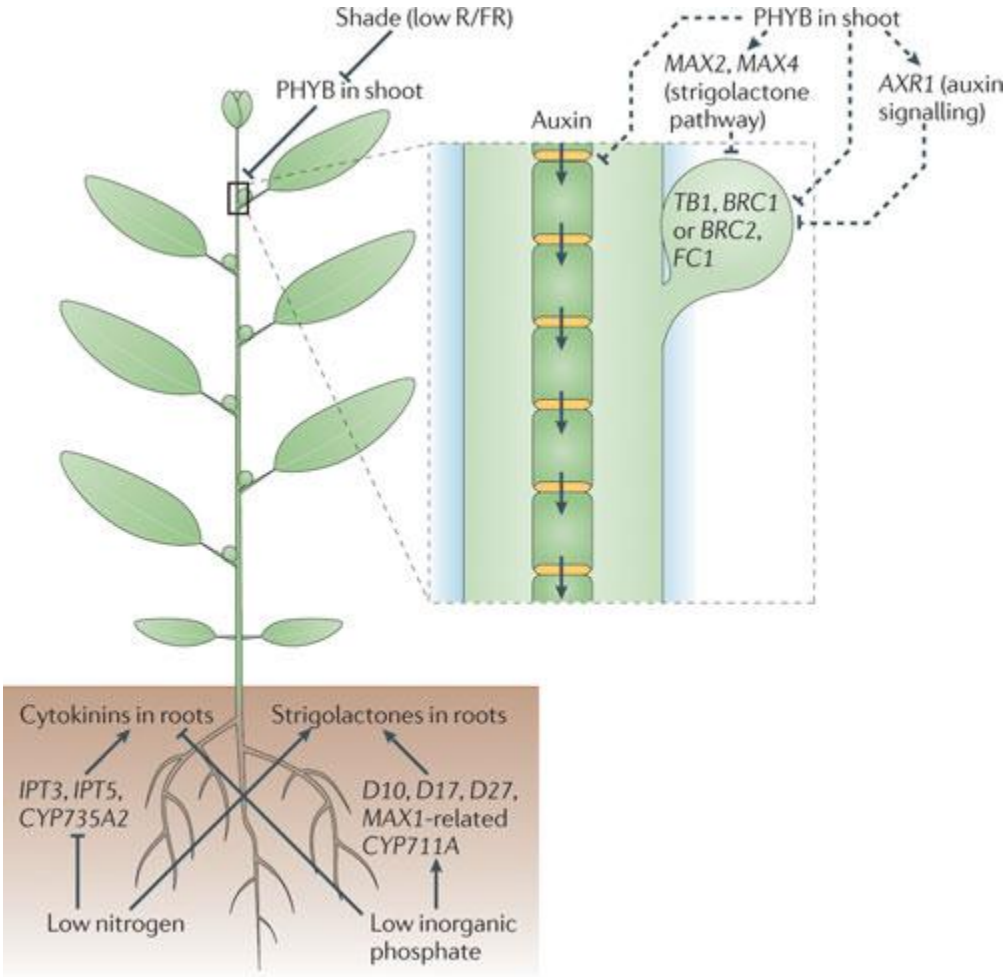


Figure 2-4. Role of nutrients in branching control. Low levels of nitrogen in soil represses shoot branching through systemic signalling by cytokinins and probably by strigolactones. Production of cytokinins in roots is controlled by nitrogen levels in the soil, which regulate the expression of cytokinin biosynthetic genes. In addition, levels of cytokinins in the roots are decreased by low levels of inorganic phosphate. (Figure and description Domagalska and Leyser, 2011)

CHAPTER 3
PHOTOPERIODIC PHYTOCHROME-MEDIATED VEGETATIVE GROWTH RESPONSES OF CONTAINER-GROWN CITRUS NURSERY TREES

Chapter Summary

In Florida, most citrus trees are grown on rootstocks with trifoliate orange (*Poncirus trifoliata*) parentage. Nurserymen have long noted that these rootstocks exhibit much slower growth during the winter than their non-trifoliate counterparts (e.g., *Citrus volkameriana* 'Volkamer' lemon, *C. aurantium* 'sour orange'). Since 2007 citrus nursery trees in Florida must be grown in greenhouses to protect them from the asian citrus psyllid, the vector of huanglongbing (citrus greening). This requirement has greatly increased production costs and the desire to determine why trifoliate-type rootstocks grow poorly during the winter. We hypothesized that trifoliate-type rootstocks, because of their deciduous habit, respond to photoperiod and exhibit slow growth under short days (photoperiods <12 h). Our objective was to determine the effect of photoperiod on the growth of container grown trees of the two most common trifoliate-type rootstocks, 'Carrizo' citrange and 'Swingle' citrumelo with and without non-trifoliate 'Hamlin' sweet orange scions. Three weeks after budding, all trees (budded and non-budded) were placed in growth chambers under three different photoperiods, short days (SD - 10 h photoperiod), long days (LD - 14 h) and short days + night interrupt (SD-NI - 10 h photoperiod + 1 h night interrupt) for 14 weeks, and maintained at 28/21 °C day/night temperature. All trees, regardless of being budded or not, had reduced growth under SD conditions, whereas the trees under SD-NI grew similar to those under LD. Average tree growth during the 14-weeks was 19 cm, 52 cm and 55 cm, across all rootstock × scion combinations for SD, LD and SD-NI treatments, respectively. The difference in growth between budded and non-budded trees in the SD treatment was

not significant, but was highly significant in LD and SD-NI. Across rootstock/scion combinations, the average number of new nodes produced per tree was 13, 30 and 32 in the SD, LD and SD-NI treatments, respectively, indicating that the increased growth was not just a result of internode elongation. Net CO_2 assimilation was higher under the SD and SD-NI treatments than LD, but there were no significant differences in whole-plant total nonstructural carbohydrate concentrations as a result. The ability of a 1 h night interrupt to overcome the SD response indicates that the photoperiod effect observed is a phytochrome-mediated response.

Background

Florida citrus nurseries produce more than three million trees annually (FDACS, 2010). Traditionally, citrus nursery trees were produced in field nurseries, and greenhouse-grown containerized trees accounted for only 35% of total production in the state (Davies and Zalman, 2008). However, as of Jan. 2007, all citrus nursery trees in Florida must be grown in containers in greenhouses that meet specific state requirements for pest and disease exclusion (Florida Department of State, 2010). This shift from traditional field to greenhouse container-grown systems has dramatically increased production costs and limited propagation space. In addition, production problems that were previously viewed as minor are now seen as major, chief among them being erratic, uneven scion growth particularly during the winter on trifoliate orange-type (*Poncirus trifoliata* and its hybrids) rootstocks. Grower observations indicate that budded trees on all rootstocks grow more slowly during the winter months, but that this problem is exacerbated on trifoliate-type rootstocks, to the point that many nurseries cannot propagate trees on these rootstocks for several months each year. Understanding why trifoliate-type rootstocks in particular have slow and uneven growth

during winter is critically important since these rootstocks account for >80% of all citrus tree propagations in Florida (FDACS, 2010).

Photoperiod is one of the major abiotic factors affecting the growth of trees especially during winter months (Callaham, 1962). Low temperatures coupled with short photoperiods are known to enhance dormancy and reduce vegetative growth in many tree species, while high temperatures and long days promote vegetative growth (Kozlowsky and Pallardy, 2002; Nelson and Dickson, 1980). Effects of low temperature on assimilate partitioning, vegetative growth and photosynthesis have been well documented in woody species (Greer, 1983; Greer & Warrington, 1982; Howell & Weiser, 1970; Sirtautas et al., 2011; Ushio et al., 2008). However, for photoperiod most research has focused on flowering responses, with relatively few studies on vegetative growth responses. Piringer et al. (1961) found that growth of trifoliate orange slowed markedly under short day conditions (8 h photoperiod). Fall budded 'Washington' navel orange trees are also reported to exhibit significantly greater growth under long day conditions provided by supplemental light from dusk to 2200 HR (Nauer et al., 1979). Vegetative growth of satsuma orange grown under 16 h photoperiod (Inoue, 1989) and some rootstocks of trifoliate parentage such as 'Carrizo' citrange (Warner et. al, 1979) responded positively to long days. These results shed some light on how the day length influences growth in citrus trees, but none of these studies indicate whether these growth responses were photosynthetic (i.e., carbohydrate related) or phytochrome-mediated photoperiodic effects. To improve nursery management recommendations it would be beneficial to know whether trifoliate orange rootstocks are truly responsive to

photoperiod via phytochrome and whether this response is imparted to non-trifoliate scions budded onto these popular rootstocks.

Photoperiod has also been reported to influence carbohydrate partitioning in many species, which may be related to vegetative growth changes. Higher soluble sugar content was observed in the shoot apices of wheat (Mohapatra et al. 1983) and barley (Cottrell and Dale, 1986) under short photoperiods (8 h). Arabidopsis plants under very short photoperiods (2, 3, 4 and 8 h) showed an increase in their rate of starch synthesis and a decrease in starch degradation (Gibon et al., 2009). In citrus, soluble sugar levels generally increase and starch levels decrease in winter (Dugger and Palmer, 1969); although, this relationship has not been shown to be in response to photoperiod and may be related to fruit maturation during winter.

We hypothesized that the vegetative growth of trifoliate orange-type rootstocks is responsive to photoperiod and this response is mediated by phytochrome, but sweet orange (*Citrus sinensis*) scion varieties are insensitive to photoperiod and will not respond to photoperiod when grafted on trifoliate-type rootstocks. This experiment was conducted to test the effects of photoperiod on the growth and carbohydrate partitioning of trifoliate-type rootstocks with and without sweet orange scions.

Material and methods

Plant Material

A total of 144 trees were used in the experiment, 72 of ‘Carrizo’ citrange (*C. sinensis* ×*P. trifoliata*) and 72 of ‘Swingle’ citrumelo (*C.* ×*paradisi* ×*P. trifoliata*). Half of the trees on each rootstock (36) were budded with ‘Hamlin’ sweet orange scions. This resulted in four rootstock/scion combinations: ‘Carrizo’ non-budded (Car), ‘Swingle’ non-budded (Sw), ‘Hamlin’ on ‘Carrizo’ (Ham/Car) and ‘Hamlin’ on ‘Swingle’ (Ham/Sw). All of

the trees of each rootstock, regardless of budding or not, were grown from seed germinated at the same time, therefore, were of a uniform age. The trees were obtained from a commercial citrus nursery approximately 1-month after budding when the success of the bud could be assured, but when scion growth was still <3 cm. All trees were grown in 0.95-L pots (MT38; Stuewe and Sons, Tangent, OR). All rootstock sprouts, if present, on the budded trees were removed. In an effort to produce trees of similar initial size and growth habit, the non-budded trees were pruned to approximately 15 cm (the height if the inserted bud on the budded trees) at the time they were obtained from the nursery and a single lateral bud was allowed to grow. No attempts were made to remove any lateral branches from the new growth on either the budded or non-budded trees during the experimental period.

Experimental Conditions

Twelve trees of each rootstock/scion combination were grown under each of the following three photoperiod treatments: short days (SD) – 10 h photoperiod, long days (LD) – 14 h photoperiod, and short days + night interrupt (SD-NI) – 10 h photoperiod + 1 h night interrupt in the middle of the dark period. These photoperiods were chosen to approximate the longest and shortest natural day lengths in Polk County, FL where >40% of nursery propagations occur (FDACS, 2010). The plants of each treatment were placed in growth chambers (Conviron model E15; Controlled Environments, Ltd., Winnipeg, Manitoba, Canada) set to maintain the experimental conditions. All chambers were set to maintain 28/21 °C day/night temperature. Photosynthetic photon flux (*PPF*) at plant height was maintained at 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a red to far red ration of 4:1 during the day, and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a red to far red ration of 3:1 during the night interrupt using a combination of fluorescent and incandescent lamps. This provided a

daily light integral (DLI) of 16.2 $mol \cdot m^{-2} \cdot d^{-1}$ under SD, 22.68 $mol \cdot m^{-2} \cdot d^{-1}$ under LD and 16.6 $mol \cdot m^{-2} \cdot d^{-1}$ under SD-NI conditions. Plants were grown under the experimental conditions for 14 weeks.

Data Collection

At the beginning of the experiment and weekly throughout, total shoot length (length of main stem plus all lateral branches), and number of nodes were recorded. Net CO_2 assimilation was measured during week 7 and 14 of the experiment on six plants (the same plants were measured each time) within each rootstock/scion combination in each photoperiod treatment using a portable photosynthesis system (LI-6400XT; LI-COR, Lincoln, NE) fitted with a 2 cm^2 fluorescence chamber (6400-40; LI-COR). The fluorescence chamber's light source was set to match the growth chamber light level (450 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Net CO_2 assimilation data were collected from one recently expanded, mature leaf per tree (the same leaf was used for both measurements) at least one hour after the beginning of the light period to ensure the photosynthetic rate was equilibrated for the given conditions.

At the end of the experiment, plants were destructively harvested and separated into roots (washed clean of potting media), old stems (existing at the start of the experiment), new stems (growth produced during the experiment), old leaves and new leaves. Fresh weights of all tissues were recorded and summed to determine whole-plant fresh weights. Tissues were dried to a constant weight at 65 °C, dry weight was then recorded for each tissue. Dried tissues were ground to pass a 40-mesh (0.422 mm) screen. Soluble sugars were extracted by shaking a 50 mg sample of tissue in 2 mL 80% ethanol for 20 min. Samples were centrifuged, the supernatant decanted, and the tissue reextracted twice. The supernatants were combined and the total volume

determined. Pigment was removed from the tissue extracts by adding 25 mg of activated charcoal. Soluble sugars were assayed using the phenol-sulfuric acid assay (Dubois et al., 1956; Buysse and Merckx, 1993) as modified by Chaplin and Kennedy (1994).

Tissue starch concentration was determined by suspending the insoluble fraction from the 80% ethanol extraction in 2 mL 0.2 N KOH and boiling for 30 min. After cooling to room temperature, the pH was adjusted to 4.5 by adding 1 mL 1 M acetic acid. Starch was digested by adding 118 units (1 mL) amyloglucosidase (from *Aspergillus niger*; Sigma, St. Louis, MO) and 4 units (1 mL) α-amylase (from *A. oryzae*; Sigma), each dissolved in 0.2 M calcium acetate buffer (pH 4.5). Samples were incubated for 24 h at 37 °C. After incubation, samples were centrifuged, the supernatant decanted and volume recorded. The pellets were digested a second time to determine the efficiency of the first digestion. Pigment was removed from the samples by adding 25 mg activated charcoal. The glucose liberated during each digestion was assayed using the phenol-sulfuric acid assay described earlier. The assay results from each digestion were summed for analysis.

Data Analysis

The experiment was designed as a 4 × 3 factorial with tree type and photoperiod as two factors, having 4 and 3 levels, respectively. The data were analyzed by analysis of variance using Prism 5.0 (GraphPad Software, La Jolla, CA). Differences between treatment means were tested for significance with Tukey's honestly significant difference (HSD) test (P = 0.05).

Results

Growth and Physiological Parameters

Within each tree type, those grown under LD and SD-NI had similar growth and had significantly greater shoot growth compared with trees under SD conditions (Table 3-1, Figure 3-1). All trees under SD conditions, regardless of type, grew similarly. However, under LD and SD-NI conditions, Car trees grew significantly more than the other three tree types. Ham/Car and Ham/Sw trees grew less under LD and SD-NI conditions compared with non-budded trees of each rootstock; although, this difference was only statistically significant for Ham/Car *vs*. Car. The number of new nodes per tree formed during the experiment followed a similar pattern as shoot growth, with plants under LD and SD-NI conditions producing significantly more nodes than those under SD conditions for all tree types (Table 3-1). Also, the number of new nodes was similar for all trees under SD conditions, but Car trees produced significantly more nodes under LD and SD-NI conditions compared with Sw, Ham/Car or Ham/Sw trees.

There was a significant interaction between tree type and photoperiod for leaf, stem, root and whole-plant dry weights (Table 3-2). Leaf, stem and whole-plant dry weights were significantly greater for all trees under LD and SD-NI conditions compared with SD conditions. However, root dry weight was only significantly affected by photoperiod for Ham/Car and Ham/Sw trees. There was no photoperiod response for root dry weight on Car or Sw trees. Stem dry weight followed a pattern similar to shoot growth, with Car and Sw trees having greater stem dry weight under LD and SD-NI conditions compared with Ham/Car and Ham/Sw trees (Table 3-3). This pattern was reflected in the whole-plant dry weight data (Figure 3-2).

There was no significant interaction of tree type and photoperiod on root to shoot ratio (total above ground dry weight/root dry weight; $P = 0.8576$), but the main effects of both tree type and photoperiod were significant (Figure 3-2). Trees grown under LD and SD-NI conditions had significantly lower (0.64 and 0.63, respectively) root to shoot ratio compared with trees under SD conditions (0.86; $P < 0.0001$). Ham/Car and Ham/Sw trees had significantly higher root to shoot ratios compared to Car and Sw trees (0.72 Ham/Car, 0.58 Car, 0.86 Ham/Sw, 0.70 Sw; $P < 0.0001$), with Ham/Sw having the highest root to shoot ratio and Car the lowest.

There was no significant interaction of tree type and photoperiod on net CO_2 assimilation at week 7 (Table 3-3). However, photoperiod main effects were significant with trees under both SD and SD-NI conditions having higher net CO_2 assimilation rates (12.52 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ and 14.12 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$, respectively) compared with those under LD conditions (8.60 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$; $P < 0.0001$) across all tree types (Table 3). Tree type was marginally significant ($P = 0.0514$), with Ham/Car and Ham/Sw trees having lower CO_2 assimilation rates (11.23 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ and 11.03 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$, respectively) compared with Car and Sw trees (12.35 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ and 12.36 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$, respectively) across photoperiods. At week 14, all trees grown under SD-NI conditions had significantly higher rates of net CO_2 assimilation than those under LD and SD conditions (Table 3-3).

Carbohydrates

With the exception of leaf starch, there were no significant interactions of tree type and photoperiod on individual tissue (leaf, stem, root) soluble sugar, starch or total nonstructural carbohydrate (TNC) concentrations, and tree type and photoperiod main effects for sugar, starch and TNC concentrations of the different tissues were reflected

in the whole-plant data (data not shown). Whole-plant soluble sugar concentrations were significantly affected by photoperiod only (Table 3-4). Trees grown under LD conditions had the highest soluble sugar concentration [88.78 mg/g dry weight (DW)], followed by those under SD-NI conditions (79.34 mg/g DW) and trees under SD conditions had the lowest concentration (69.58 mg/g DW; $P < 0.0001$). There was no interaction of tree type and photoperiod, nor was photoperiod significant, for whole-plant starch (Table 3-5) and TNC concentration (Table 3-6); however, tree type was significant for both. Starch concentrations were significantly greater in Sw trees (260.4 mg/g DW) across photoperiods compared with Ham/Car (229.1 mg/g DW), Ham/Sw (227.5 mg/g DW) and Car (229.8 mg/g DW; $P = 0.0004$) trees, which were all similar. Likewise, whole-plant TNC concentrations were significantly greater in Sw trees (344.4 mg/g DW) across photoperiods compared with Ham/Car (306.8 mg/g DW), Ham/Sw (302.9 mg/g DW) and Car (309.7 mg/g DW; $P = 0.0003$) trees, which were all similar.

Discussion

Vegetative development of the four tree types studied was profoundly influenced by photoperiod, similar to previous studies with trifoliate orange rootstocks (Nauer et al., 1979; Piringer et al., 1961; Warner et al., 1979). Vegetative growth is positively correlated with increasing photoperiod in many tree species (Downs and Borthwick, 1956a; Nelson and Dickson, 1980; Olesen, 1995) as well as other woody perennials such as *Vaccinium* spp. (Hall et al., 1963; Spann et al., 2003) and *Weigela florida* (Downs and Borthwick, 1956b). In the present study, the increase in vegetative growth associated with the SD-NI treatment for all tree types indicates that the photoperiod effect on the vegetative growth of trifoliate orange hybrids and sweet orange is a phytochrome-mediated response. This is in contrast to *Vacciunium corymbosum*

interspecific hybrids, which did not respond to a SD-NI treatment and the response to day length was proposed to be carbohydrate-mediated (Spann et al., 2003). In addition, although net CO_2 assimilation was elevated under SD-NI conditions relative to SD conditions at both measurement times, it does not appear to have been biologically significant since SD-NI grown plants had similar soluble sugar, starch and TNC concentrations as those grown under SD conditions. This further supports the conclusion that the greater growth observed under SD-NI conditions was a phytochrome-mediated response and not a carbohydrate response.

We hypothesized that only the trifoliate orange hybrid rootstocks would be responsive to photoperiod and that when budded with sweet orange scions the photoperiod response would be lost. This was not the case as demonstrated by shoot growth and tissue dry weight data. However, there was a significant interaction between tree type and photoperiod for shoot growth, and stem and whole-plant dry weight, such that Ham/Car and Ham/Sw trees did not respond to the same degree as Car and Sw. This suggests that sweet orange was less sensitive to photoperiod compared with trifoliate orange. Leaf dry weight data appear to be in conflict with this conclusion since the ‘Hamlin’ budded trees had the highest leaf dry weight under LD and SD-NI conditions. However, this is likely an artifact in the data due to the fact that the simple sweet orange leaves are much larger than the trifoliate leaves on the trifoliate orange hybrids as can be seen in Figure 3-1. This is supported by the evidence that the Ham/Car and Ham/Sw trees actually had fewer new nodes initiated during the experimental period under LD and SD-NI conditions compared with the trifoliate hybrids.

The lower root to shoot ratio of trees grown under LD and SD-NI conditions suggests a shift in resource allocation from root to shoot growth under these photoperiod treatments. However, there were no significant differences in root sugar, starch or TNC concentrations due to photoperiod (data not shown). The dry weight data indicate that root growth increased under LD and SD-NI conditions concomitant with shoot growth for all trees, although only significantly so for Ham/Car and Ham/Sw. However, shoot growth increased proportionately more than root growth, thus resulting in the decrease in root-to-shoot ratio with increasing photoperiod. These results are similar to findings in groundnut (*Arachis hypogaea*) (Nigam et al., 1998) and chrysanthemum (*Chrysanthemum morifolium*) (Kurilčik et al., 2008) where root and shoot growth were both found to increase with increasing photoperiod.

This work has demonstrated that the slow growth of trifoliate orange-type rootstocks observed by many citrus nurserymen during winter is a true short day phytochrome-mediated response. Furthermore, we presented evidence that sweet orange is also sensitive to photoperiod, although to a lesser degree than trifoliate orange. This is important information for making recommendations for improving nursery cultural practices. However, the generalization has been made that it is rare to find a species in which photoperiod responses are independent of temperature (Rees, 1987), as recently demonstrated for the vegetative growth of *Prunus* spp. (Heide, 2008). Whether temperature interacts with photoperiod, and if so to what extent, in controlling citrus vegetative growth is unknown. Future work should aim to determine the interaction of temperature and photoperiod in citrus, and to screen a wider range of rootstock and scion varieties for their response to photoperiod.

Table 3-1. Effect of photoperiod on the total new shoot growth and number of new nodes per tree for four different tree types grown under three different photoperiod treatments for 14 weeks (n = 12)

	Tree type			
	Ham/Car[z]	Ham/Sw	Car	Sw
Photoperiod	Total new shoot growth per tree (cm)			
LD[y]	36.9 bc[x]	35.2 bc	84.3 a	53.0 b
SD	17.6 d	15.8 d	26.3 d	16.6 d
SD-NI	40.2 bc	25.9 c	100.6 a	53.4 b
	New nodes per tree (no.)			
LD	18.6 b	16.8 b	53.5 ab	31.8 b
SD	10.6 c	9.0 c	20.1 c	10.0 c
SD-NI	19.1 b	12.8 b	62.7 a	33.7 b

[z]Ham/Car = ‘Hamlin’ sweet orange on ‘Carrizo’ citrange rootstock; Ham/Sw = ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock; Car = ‘Carrizo’ citrange rootstock; Sw = ‘Swingle’ citrumelo rootstock.
[y] LD = long-day (14 h) photoperiod; SD = short-day (10 h) photoperiod; SD-NI = short-day + 1 h night interrupt in the middle of the dark period.
[x]Means separation by Tukey’s HSD test, $P < 0.05$.

Table 3-2. Tissue and whole-plant dry weights for four different tree types grown under three different photoperiod treatments for 14 weeks (n = 12)

	Dry weight (g)							
	Leaves				Stems			
Photoperiod	Ham/Car[z]	Ham/Sw	Car	Sw	Ham/Car	Ham/Sw	Car	Sw
LD[y]	3.8 a[x]	3.1 b	2.8 b	2.8 b	3.8 b	3.2 b	6.7 a	7.3 a
SD	1.5 c	1.3 c	1.2 c	1.6 c	2.3 c	1.8 c	4.3 b	4.5 b
SD-NI	4.0 a	2.8 b	2.8 b	2.7 b	4.1 b	3.1 b	6.4 a	6.5 a
	Roots				Whole-plant			
LD	4.9 bc	4.8 bc	5.2 abc	6.3 a	12.5 bcd	11.1 cde	14.7 ab	16.4 a
SD	2.9 d	3.1 d	4.0 cd	5.1 abc	6.7 f	6.2 f	9.5 ef	11.2 cde
SD-NI	5.2 abc	4.5 c	4.3 c	5.8 ab	13.3 bc	10.4 de	13.3 bc	15.0 ab

[z]Ham/Car = ‘Hamlin’ sweet orange on ‘Carrizo’ citrange rootstock; Ham/Sw = ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock; Car = ‘Carrizo’ citrange rootstock; Sw = ‘Swingle’ citrumelo rootstock.
[y]LD = long-day (14 h) photoperiod; SD = short-day (10 h) photoperiod; SD-NI = short-day + 1 h night interrupt in the middle of the dark period.
[x]Means separation within a tissue type by Tukey’s HSD test, $P < 0.05$.

Table 3-3. Instantaneous net CO_2 assimilation for four different tree types grown under three different photoperiod treatments for 14 weeks. Measurements were made during weeks 7 and 14 on the same plants (n = 6)

Photoperiod	Instantaneous Net CO2 assimilation ($\mu mol\ CO_2 \cdot m^{-2} \cdot s^{-1}$) Tree type			
	Ham/Car[z]	Ham/Sw	Car	Sw
		Week 7		
LD[y]	6.97	5.78	6.16	6.36
SD	8.22	7.99	8.76	8.40
SD-NI	9.90	11.35	9.77	9.50
		Week 14		
LD	3.74 c[x]	3.48 c	4.80 c	5.01 c
SD	4.71 c	4.73 c	4.55 c	5.01 c
SD-NI	9.91 b	13.6 a	12.12 a	11.68 ab

[z]Ham/Car = ‘Hamlin’ sweet orange on ‘Carrizo’ citrange rootstock; Ham/Sw = ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock; Car = ‘Carrizo’ citrange rootstock; Sw = ‘Swingle’ citrumelo rootstock

[y]LD = long-day (14 h) photoperiod; SD = short-day (10 h) photoperiod; SD-NI = short-day + 1 h night interrupt in the middle of the dark period

[x]Means separation by Tukey’s HSD test, $P < 0.05$

Table 3-4. Whole-plant soluble-sugar concentrations for four different tree types grown under three different photoperiod treatments for 14 weeks (n = 6)

	Soluble sugar conc. (mg glu equivalents/g dry wt.)			
	Tree type			
Photoperiod	Ham/Car[z]	Ham/Sw	Car	Sw
LD[y]	85.6 ± 4.7	90.9 ± 4.3	86.8 ± 4.4	91.8 ± 4.7
SD	70.8 ± 2.7	58.6 ± 2.9	73.1 ± 7.1	75.9 ± 3.9
SD-NI	76.7 ± 10.2	76.6 ± 4.4	79.6 ± 4.1	84.5 ± 5.5

	df	F	*P*
Tree type × photoperiod	6	0.7218	0.6336
Tree type	3	1.476	0.2302
Photoperiod	2	13.24	<0.0001

[z]Ham/Car = ‘Hamlin’ sweet orange on ‘Carrizo’ citrange rootstock; Ham/Sw = ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock; Car = ‘Carrizo’ citrange rootstock; Sw = ‘Swingle’ citrumelo rootstock

[y]LD = long-day (14 h) photoperiod; SD = short-day (10 h) photoperiod; SD-NI = short-day + 1 h night interrupt in the middle of the dark period

Table 3-5. Whole-plant starch concentrations for four different tree types grown under three different photoperiod treatments for 14 weeks (n = 6)

	Starch conc. (mg glu equivalents/g dry wt)			
	Tree type			
Photoperiod	Ham/Car[z]	Ham/Sw	Car	Sw
LD[y]	226.5 ± 12.3	223.9 ±15.8	210.8 ± 11.2	249.5 ± 5.0
SD	218.0 ± 12.7	215.7 ± 8.0	245.4 ± 4.0	268.7 ± 5.6
SD-NI	242.8 ± 11.5	242.9 ± 9.6	233.4 ± 9.4	262.9 ± 11.8

	df	F	*P*
Tree type × photoperiod	6	1.388	0.2343
Tree type	3	7.060	0.0004
Photoperiod	2	2.985	0.0581

[z]Ham/Car = ‘Hamlin’ sweet orange on ‘Carrizo’ citrange rootstock; Ham/Sw = ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock; Car = ‘Carrizo’ citrange rootstock; Sw = ‘Swingle’ citrumelo rootstock

[y]LD = long-day (14 h) photoperiod; SD = short-day (10 h) photoperiod; SD-NI = short-day + 1 h night interrupt in the middle of the dark period.

Table 3-6. Whole-plant total nonstructural carbohydrate concentrations for four different tree types grown under three different photoperiod treatments for 14 weeks (n = 6)

	total nonstructural carbohydrate concn (mg glu equivalents/g dry wt)			
	Tree type			
Photoperiod	Ham/Car[z]	Ham/Sw	Car	Sw
LD[y]	312.1 ± 8.3	314.9 ± 17.6	297.6 ± 14.2	341.2 ± 7.7
SD	288.7 ± 13.0	274.3 ± 9.3	318.4 ± 10.7	344.7 ± 9.1
SD-NI	319.4 ± 20.0	319.4 ± 11.1	313.0 ± 8.3	347.4 ± 13.1

	df	F	*P*
Tree type × photoperiod	6	1.437	0.2157
Tree type	3	7.148	0.0003
Photoperiod	2	2.169	0.1232

[z]Ham/Car = ‘Hamlin’ sweet orange on ‘Carrizo’ citrange rootstock; Ham/Sw = ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock; Car = ‘Carrizo’ citrange rootstock; Sw = ‘Swingle’ citrumelo rootstock

[y]LD = long-day (14 h) photoperiod; SD = short-day (10 h) photoperiod; SD-NI = short-day + 1 h night interrupt in the middle of the dark period

Figure 3-1. Representative examples of trees of (A.) 'Hamlin' sweet orange on 'Carrizo' citrange rootstock, (B.) 'Hamlin' sweet orange on 'Swingle' citrumelo rootstock, (C.) 'Carrizo' citrange rootstock, and (D.) 'Swingle' citrumelo rootstock grown under long days (LD, 14 h), short days (SD, 10 h) and short days + night interrupt (SD-NI, 10 h + 1 h) photoperiods for 14 weeks. (Photo: Gurreet Brar)

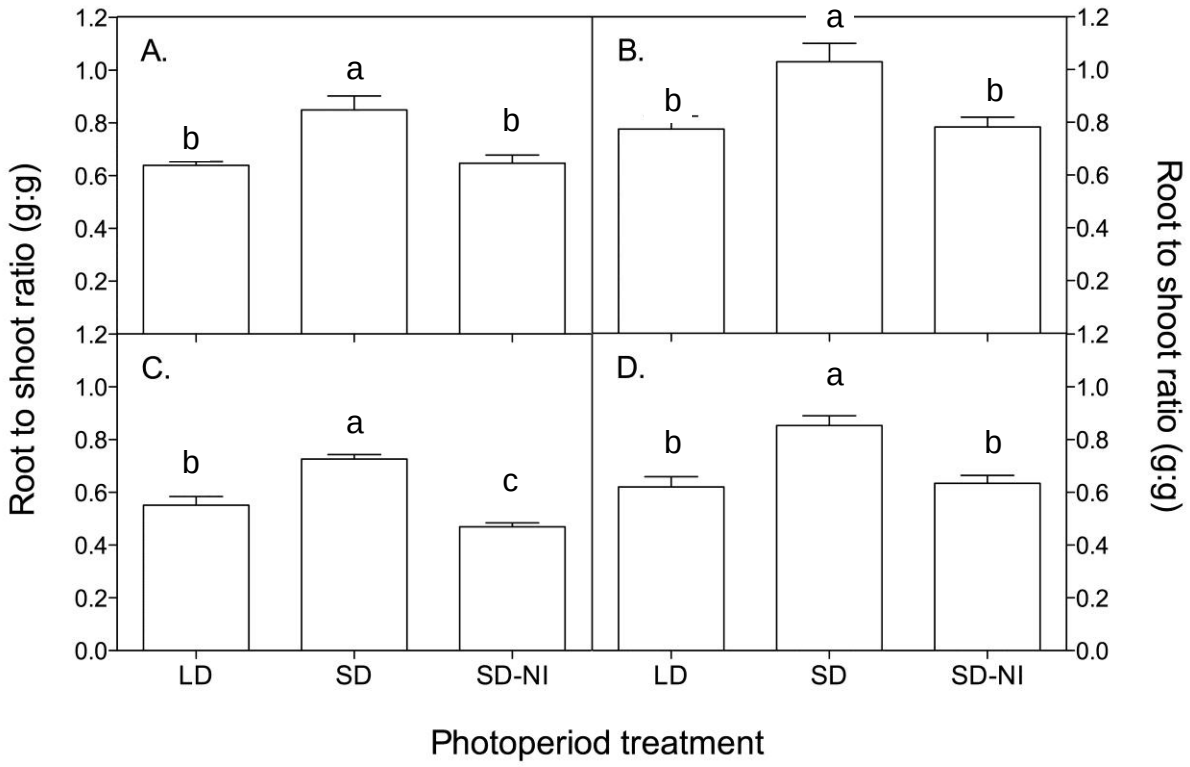


Figure 3-2. Root to shoot ratio of (A.) ‘Hamlin’ sweet orange on ‘Carrizo’ citrange rootstock, (B.) ‘Hamlin’ sweet orange on ‘Swingle’ citrumelo rootstock, (C.) ‘Carrizo’ citrange rootstock, and (D.) ‘Swingle’ citrumelo rootstock grown under long days (LD, 14 h), short days (SD, 10 h) and short days + night interrupt (SD-NI, 10 h + 1 h) photoperiods for 14 weeks. Error bars represent standard error of the mean (n=12).

CHAPTER 4
XYLEM SAP CYTOKININ CONCENTRATION AS INFLUENCED BY WATER STRESS IN CONTAINERIZED CITRUS NURSERY TREES

Chapter Summary

Water stress is known to alter the concentrations of plant growth hormones which are major root-to-shoot signals in plants under stress. This research was conducted to quantify the effect of water stress on xylem sap cytokinin concentration in container grown citrus nursery trees. Two sets of trees of ‘Hamlin’ sweet orange budded on ‘Swingle’ citrumelo rootstock, were subjected to three water stress treatments (30 trees per treatment): 100% evapotranspiration (ET) (control); 50% ET (mild stress) and 20% ET(severe stress) for 15 days. Stem water potential and net photosynthesis measurements were taken periodically. From the first set, five trees were destructively harvested every other day from each of the treatments, while to the second set, foliar application of benzyladenine (BA) was given for three consecutive days, starting at day 16 of stress treatments. The trees were destructively harvested and the xylem sap (800 µl per tree) was extracted using a Scholander-type pressure chamber. The sap samples were analyzed for dihydro-zeatin riboside (DHZR) levels by enzyme-linked immunosorbent assay. The stem water potential decreased (became more negative) with the decreasing level of irrigation and with the increasing duration of water stress. The DHZR concentration showed significant increase initially under severe water stress, while it deceased sharply as the severe water stress prolonged. The initial increase in DHZR concentrations may be attributed to a possible stimulation of cytokinin biosynthesis in the root-tips in response to the water stress. DHZR has earlier been reported to increase with water stress in other plant species. However, to determine the

implication of altered cytokinin levels on the bud push and scion growth in citrus, the effect of water stress on cytokinin export and delivery rates must be explored.

Background

Water stress is known to alter many physiological processes within the plant system. Evidently, water stress also changes the concentration of plant growth regulators, which in turn play a vital role in many physiological responses. Endogenous hormones are known to be major root-to-shoot signals in plants under stress. Many researchers have noted that abscisic acid (ABA) and cytokinins are positive and negative signals respectively, from the drought stressed roots (Davies and Zhang, 1991; Itai and Vaadia, 1965). While a lot of research work has been done on the role of ABA in stress signaling and changes in its concentration as a result of water stress, relatively few studies have been reported on water stress and cytokinins.

Cytokinins are known to enhance cell division and, thus, are important in stimulating vegetative bud and shoot growth. Therefore, any biotic or abiotic stress affecting cytokinin levels in shoots may influence bud and scion growth. However, not much work has been reported on the effect of water stress on cytokinin concentration and its subsequent effect on bud push and scion growth in citrus. In grapevines, Dry and Loveys (1999) reported that the reduction in shoot growth was due to low availability of cytokinins during moderate water stress. Satisha et al. (2007) also observed reduced xylem sap cytokinin concentration in response to moderate water stress, while Stern et al. (2003) reported increase in cytokinin levels in xylem sap of lychee trees due to water stress. Jackson (2009) and Shashidhar et al. (1996) have argued that it is the delivery rate and not the absolute concentrations of cytokinins in the xylem sap which may influence the physiological responses. In the present study we

hypothesized that the xylem sap cytokinin concentration in young citrus nursery trees will be reduced by drought stress and exogenous cytokinin application will stimulate a recovery.

Materials and Methods

Plant Material

The experiment was conducted at the University of Florida IFAS Citrus Research and Education Center, Lake Alfred, Florida. A total of 180 trees of 'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck) budded on 'Swingle' citrumelo (*C. paradisi* Macfad. × *Poncirus trifoliata*) rootstock were obtained from a commercial citrus nursery. The trees were washed of potting medium and re-potted in washed quartz sand in 2.65-L citra-pots (model CP413CH; Stuewe and Sons, Tangent, OR) and were allowed to acclimate for 8 weeks.

Experimental Conditions

The trees were grown in custom-built walk-in growth chambers for the duration of the experiment. Two sets of 90 trees each were subjected to three water stress treatments (30 trees per treatment): 100% evapotranspiration (ET) (control); 50% ET (mild stress) and 20% ET (severe stress) for 15 days. The trees under severe water stress were given a supplemental irrigation after eight days (middle of the experiment period) to prevent permanent wilting and loss of experimental material. From the first set of 90 trees, five trees were destructively harvested every other day from each treatment for xylem sap extraction. In the second set, half of the trees were changed to 100% ET watering regime while half remained under 50%. Further, in each of the 100% and 50% watering, half trees were applied with foliar application of benzyladenine (BA) for three consecutive days, starting at day 16 of stress treatments. Therfore, there were four

treatment combinations: 50%+BA, 50% + No BA, changed to 100% + BA, changed to 10% + No BA and 100 % ET (control). The trees were destructively harvested for sap extraction at the end of the three day period. The growth chamber conditions were set to maintain long day photoperiod (14 hour daylight) and 28/21 °C day/night temperature. Photosynthetic photon flux (*PPF*) at plant height was maintained at 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Stem Water Potential

Stem water potential was measured on two leaves per plant with a Scholander-type pressure chamber. The selected leaves were covered with Mylar bags for at least one hour prior to taking measurements to equilibrate the leaf and stem water potential (Begg and Turner, 1970). The measurements were taken during mornings, just before water application.

Net Photosynthetic Rate

Photosynthesis measurements were taken on four different days spread across the experimental period. Six trees were selected from each treatment and the leaves were tagged. The measurements were taken from the same leaves at each measurement time at the midpoint of the light period using an LI-6400XT portable photosynthesis system (LI-COR, Lincoln, NE).

Xylem Sap Cytokinin Analysis

The main stem of each harvested tree was cut near the soil level and placed in the pressure chamber with the cut end exposed in order to extract 800 µL of sap. Modified Beiliskey's solution was added to the sap in a ratio of 2:1 (sap:Beiliskey's solution) and the tubes were immediately frozen in liquid nitrogen. The xylem sap (800 µL per tree) was extracted using the Scholander-type pressure chamber. The sap

samples were analyzed for dihydro-zeatin riboside (DHZR) by enzyme-linked immunosorbent assay (ELISA) using Phytodetek Immunoassay Kits (Agdia, Elkhart, IN).

Statistical Analysis

The experiment was designed as a 3x5 factorial with drought stress and date of harvesting for cytokinins as two factors having 3 and 5 levels, respectively. The treatment comparisons were performed by ANOVA. The mean separations were calculated by Bonferroni posttests. The results were graphically displayed using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

Stem Water Potential (Ψ)

The different irrigation treatments had direct effects on the stem water potential (Ψ_{stem}) of the trees. The water potential decreased in proportion of the decreasing water supply (Table 4-1). The Ψ_{stem} remained between -0.77 to -0.99 MPa for control trees while it decreased gradually over time with the increasing water stress in mild (from for first day to the last day of measurements) and severe stress treatments. The Ψ_{stem} values in trees under moderate stress (50%) decreased from -0.78 MPa on day 1 to -2.11 MPa on day 10. The values for severe stress trees were -0.89 MPa on day 1, -3.36 MPa on day 7 and -2.54 MPa on day 10. After the supplemental irrigation to severe stress treatment trees (20% irrigation) at day 8, the Ψ_{stem} increased quickly (to -1.61 MPa), signifying that it is a very good indicator of water status of the tree. For the duration of the experiment, the average Ψ_{stem} in 100%, 50% and 20% treatments was -0.88, -1.62 and -2.00 MPa, respectively.

Photosynthetic Parameters

Net photosynthetic rates were measured at four different intervals during the experiment. The data shows that photosynthesis decreased according to the water stress levels in different irrigation treatments (Table 4-2, Figure 4-1). The average photosynthetic rate for well watered trees was 9.48 $\mu mol \cdot m^{-2} \cdot s^{-1}$ across all days, while it averaged 5.91 and 5.23 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for moderate and severe water stress treatments, respectively. At the start of the experiment, all three treatments did not differ in terms of net CO_2 assimilation rate. However, in the subsequent days, trees under 50% and 20% showed significant reductions in net CO_2 assimilation rate ($p<0.001$). Stomatal conductance (g_s) and transpiration also varied in accordance with the water stress of the trees (Figure 4-1).

Xylem-sap Cytokinin Concentration

The xylem sap concentration of dihydro-zeatin riboside (DHZR) was found to be influenced by the degree of water stress in the trees (Table 4-3). On day 3 of the experiment (first harvest), trees under all treatments showed similar levels of DHZR with no significant differences ($p>0.05$). The DHZR concentration for the first harvest was 17.93, 16.26 and 22.57 picomol/mL for well-watered, moderate stress and severe stress treatments, respectively. However, in the subsequent days, the DHZR concentration started to increase with the increasing levels of water stress. This increase was very steep in the case of 20% irrigation followed by 50%, with 20% having highly significant ($p<0.001$) and 50% having significant ($p<0.05$) differences from 100% at day 5 (July 8) which was the second harvest date, while the well-watered trees showed no change. At day 7 when the trees under 20% irrigation were the most stressed, xylem sap DHZR levels dropped down and were not significantly different from control trees which had

higher values than day 3. Interestingly, both 20% and 50% ET trees had their respective lowest cytokinin concentrations on the days they had lowest Ψ_{stem} and before that the cytokinin levels in each drought stressed treatment increased once.

The BA application did not have any significant effect on the xylem sap DHZR concentration (Figure 4-2). For the first two days of BA sprays, there was no significant difference among the treatments except control (100% ET throughout), which had significantly higher cytokinin concentration. However, the trees that were changed to 100% regime showed an increase in cytokinin levels on the third day of BA spray, while within those trees BA and no-BA trees did not differ significantly. This increase could be due to a change in irrigation pattern. Interestingly, in the third day of BA application, BA and no BA trees in 50% ET started showing some differences, and trees under BA application had significantly higher cytokinin concentration ($p<0.01$).

The pressure required to extract sap from the shoots also varied from 1.03 MPa for well-watered trees to 2.07 and 3.10 MPa, respectively for moderate and severe stress trees.

Discussion

The concentration of the DHZR increased in the trees under water stress treatments as compared to the well-watered trees. An increase in DHZR levels with increasing water stress was also reported in lychee trees (Stern et al., 2003). Many researchers have reported that the root-tip cytokinin production is stimulated at moderate water stress (Taylor and Klepper, 1978). In the current study, the observation that the lowest cytokinin concentrations coincided with the lowest water potential values in each drought stress treatment indicates that the xylem sap cytokinin levels actually decreased with the severe water stress ($\Psi_{stem} \leq -1.9$). Hubick et al. (1986) reported from

a study in sunflower that showed the moderate stress led to decreased cytokinin concentration in shoot xylem sap, while the cytokinin activity in roots increased significantly. They suggested that the moderate water stress reduced cytokinin transport to the shoots whereas synthesis of its storage forms increased in roots. Whether the reduction in cytokinin levels is triggered in moderate or severe water stress could be dependent on type of plant and extent of its drought tolerance.

The Ψ_{stem} data reveal that although trees under 20% ET progressed to soil drying and drought stress more quickly than those in the 50% treatment, the Ψ_{stem} of the latter decreased gradually as a result of cumulative water stress. This implies that the trees in the 50% treatment were as drought stressed at day 11 as those in the 20% treatment midway through the experiment (day 7). Looking at the DHZR concentrations from this perspective reveals that the DHZR levels rose initially during periods of moderate drought stress, but decreased sharply at the onset of severe drought stress. This is consistent with the sharp decline observed in DHZR levels pertaining to 20% treatment on July 10 and 50% treatment on July 12 and 14. The initial increase in DHZR concentration in both drought-stressed treatments may well be an artifact due to reduced sap flow and may not be an actual increase in the xylem sap cytokinin levels as discussed by Jackson (2009). Figure 4-2 reveals that the cytokinin concentration started to increase in those trees that were moved from 50% to 100% watering regime. This observation indicates that the initial reduction in cytokinin levels might be due to conversion of root-produced cytokinins to their storage forms rather than to their discontinued synthesis, and as soon as the water status of the tree improved, these storage forms start being converted to readily available active forms.

The BA spray did not have any effect on cytokinin levels. This may be attributed to low concentration (100 ppm) of BA solution and in future studies higher BA concentrations may be tried. Secondly, on the third day, trees under 50% ET+BA began to show a slight increase, which indicates that the continuous application of BA for several days may be helpful in achieving significant results towards recovery.

The requirement for greater pressure to draw the xylem sap may indicate that the initial increase in cytokinin levels observed in stressed trees was due to a reduction in the transpiration stream as opposed to actual increase in cytokinin levels. Consequently, there is very little or no availability to the leaves and shoots. Kudoyarova et al. (2007) reported an increase in cytokinin concentration during initial periods of drought stress, but that these levels decreased with prolonged soil drying prolonged and progressive severity of drought stress. In two separate studies in grapevines, Dry and Loveys (1999) reported that the reduction in shoot growth was due to low availability of cytokinins due to moderate water stress and Satisha et al. (2007) observed reduced xylem sap cytokinin concentration in response to moderate water stress.

Conclusion

The effect of drought stress on xylem sap cytokinin concentration in container grown citrus nursery trees was quantified. The cytokinin concentration decreased with severe drought stress and the water status of the trees appears to be associated with the availability of free-cytokinins in the xylem stream. Also, foliar sprays of 100 ppm BA could not bring about a recovery in cytokinin levels. Further studies to explore delivery of cytokinins via the xylem stream are suggested in order to more clearly understand the underlying mechanisms of transport of this PGR under conditions of drought stress.

Table 4-1. Midday stem water potential (Ψ_{stem}) of container grown citrus trees (cv. Hamlin) under well-watered and drought stress conditions.

Drought Stress[z]	Midday stem water potential (MPa)					
	[y]Days into drought stress					
	July 4	July 6	July 8	July 10	July 12	July 14
100%	-0.77 ± 0.06	-0.84 ± 0.05	-0.90 ± 0.02	-0.94 ± 0.05	-0.99 ± 0.04	-0.85 ± 0.08
50%	-0.78 ± 0.05	-1.17 ± 0.14	-1.74 ± 0.12	-1.92 ± 0.10	-1.98 ± 0.08	-2.11 ± 0.05
20%	-0.90 ± 0.02	-1.49 ± 0.25	-2.11 ± 0.11	-3.36 ± 0.09	-1.61 ± 0.11	-2.54 ± 0.22

	df	F	P
Days X Drought stress	10	17.75	<0.0001
Drought stress	2	162.3	<0.0001
Days into drought stress	5	51.51	<0.0001

[z]100% = well-watered control; 50% = moderate stress; 20% = severe drought stress.
[y]The measurement dates were day 1(July 4), day 3 (July 6), day 5 (July 8), day 7 (July 10), day 9 (July 12) and day 11 (July 14)

Table 4-2. Instantaneous net CO_2 assimilation for container grown citrus nursery trees under three different drought stress treatments. Measurements were made at four different dates on the same plants (n = 5)

Treatment[y]	Instantaneous net CO2 assimilation ($\mu mol\ CO_2 \cdot m^{-2} \cdot s^{-1}$)			
	Date			
	July 6	July 8	July 12	July 16
100%	[z]10.35a	8.63a	9.54a	9.43a
50%	9.29a	5.63b	5.08c	3.66d
20%	9.23a	3.73d	4.69cd	3.30d

[y]100% evapotranspiration (well-watered control), 50% (moderate stress) and 20% evapotranspiration (severe drought stress)
[z]Means separation by Tukey's HSD test, $P < 0.05$. Different letters show significant differences

Table 4-3. Concentration of dihydro-zeatin riboside (DHZR), a cytokinin in the xylem sap of drought stressed and well watered container-grown citrus nursery trees

Drought Stress[y]	Conc. of dihydro-zeatin riboside (DHZR) (picomoles/ml)				
	[x]Sampling Date				
	July 6	July 8	July 10	July 12	July 14
100%	22.33 ± 1.52	17.68 ± 0.59	45.85 ± 4.02	35.24 ± 2.70	49.53 ± 4.13
50%	16.26 ± 4.21	38.44 ± 4.33	77.82 ± 4.77	44.73 ± 2.18	39.83 ± 3.48
20%	22.56 ± 1.14	59.29 ± 4.75	51.37 ± 7.34	65.41 ± 9.08	74.26 ± 8.04

	df	F	P
Sampling date X Drought stress	8	7.599	<0.0001
Drought stress	2	25.15	<0.0001
Sampling date	4	33.42	<0.0001

[x]The sampling dates were day 3 (July 6), day 5 (July 8), day 7 (July 10), day 9 (July 12) and day 11 (July 14)

[y]100% evapotranspiration (well-watered control), 50% (moderate stress) and 20% evapotranspiration (severe drought stress)

Table 4-4. Transpiration data for container grown citrus nursery trees under three different drought stress treatments. Measurements were made at four different dates on the same plants (n = 5)

Treatment[y]	Leaf transpiration (mmol H2O/m2/s) Date			
	July 6	July 8	July 12	July 16
100%	[z]1.025a	1.027a	1.118a	1.094a
50%	1.032a	0.679a	0.831a	0.844a
20%	0.970a	0.446b	0.900a	0.591a

[y]100% evapotranspiration (well-watered control), 50% (moderate stress) and 20% evapotranspiration (severe drought stress)
[z]Means separation by Tukey's HSD test, $P < 0.05$. Different letters (a,b) show significant differences

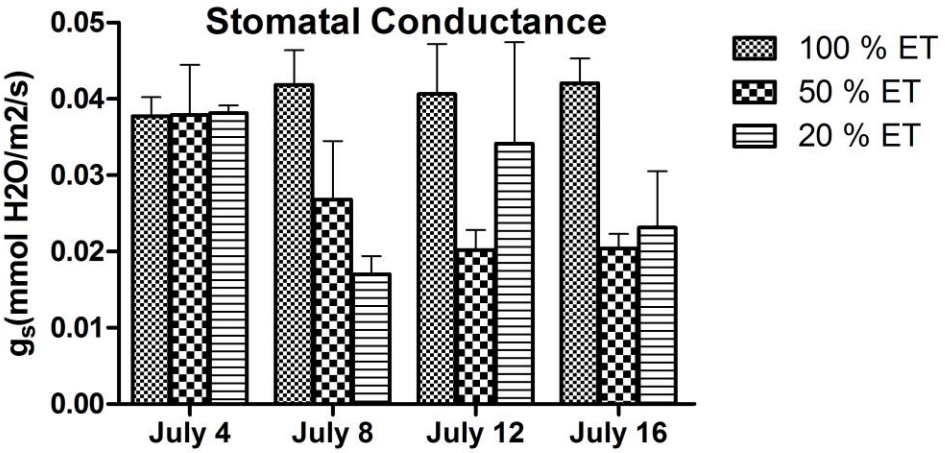


Figure 4-1. Stomatal conductance of container grown citrus trees (cv. Hamlin) under well- watered and drought stress conditions. Measurements were taken on four intervals during the experimental period.

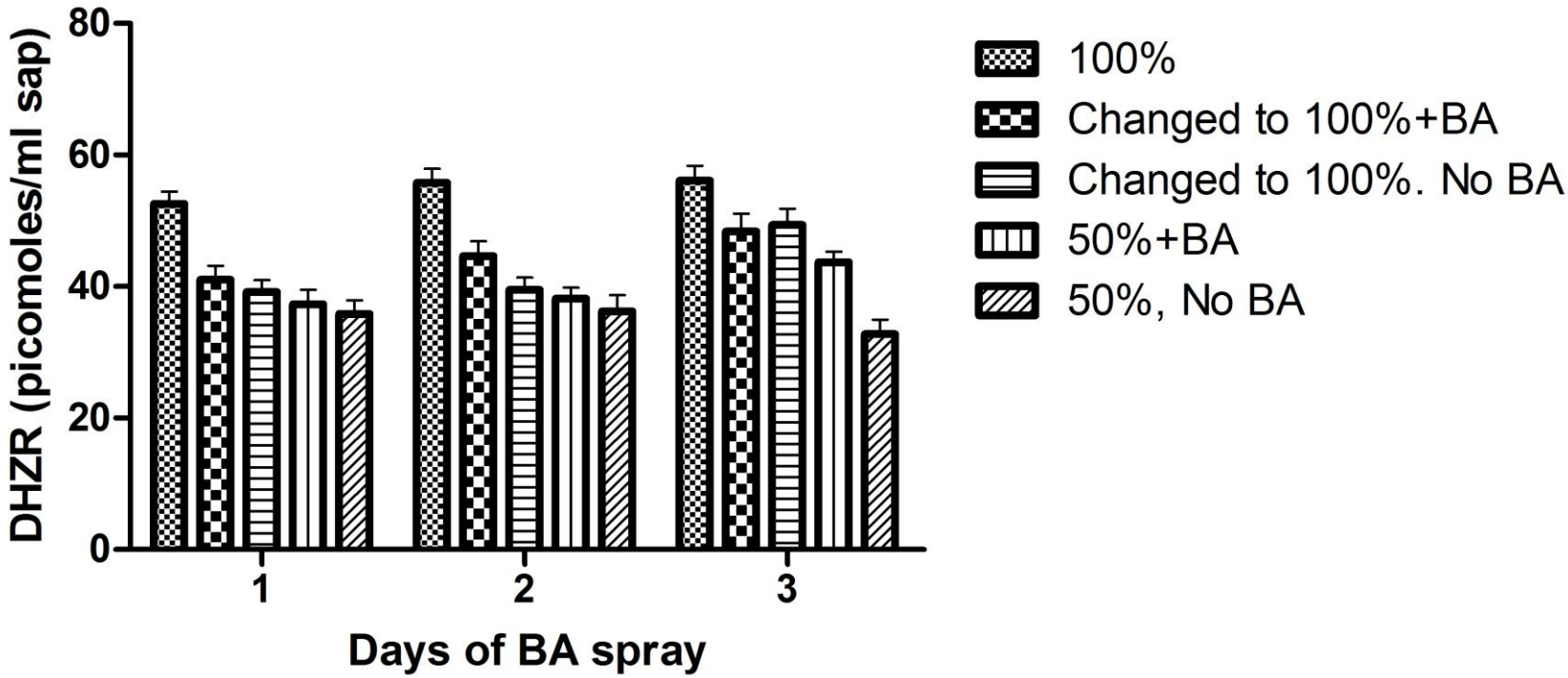


Figure 4-2. The concentration of zeatin-type cytokinin dihydro-zeatin-riboside (DHZR) in the xylem sap after the trees were shifted to well-watered conditions and sprayed with 100 ppm BA.

CHAPTER 5
BUD-TAKE AND SCION GROWTH FOR BUDS TAKEN FROM DROUGHT STRESSED BUDWOOD TREES AND RESPONSE OF BUDS TO BA APPLICATION

Chapter Summary

We grew budwood source trees (Hamlin sweet orange) and rootstock seedling trees (Swingle citrumelo) under well watered (100% ET) and drought stress (50% ET) conditions to determine if the water status of the budwood and/or rootstock affected bud live and growth. We hypothesized that the survival and growth of buds harvested from drought stressed source trees would be negatively affected compared with buds from well-watered trees. One container-grown budwood tree was grown under each watering treatment so as to minimize variation among buds due to tree differences. After three weeks, 24 buds were harvested from each budwood tree (drought stressed and well watered). During the same three week period, 48 rootstock seedlings were grown under the same well watered or drought stress conditions (24 trees each). The harvested buds were inserted into the rootstock seedlings creating 12 trees of each budwood/rootstock water stress combination. The respective drought stress treatments were continued post-budding. The bud live and scion growth were measured over time. Seven weeks after budding, 500 ppm benzyladenine (BA) solution was applied to the buds followed by a repeat application two weeks later to the buds that did not break. Just before the second application of BA, half of the trees from drought stress treatment were moved to 100% watering regime. The bud break was generally poor (<5%) in all the treatments until 6 weeks after budding. However, BA application significantly enhanced bud break (66%) in well-watered trees, but for drought stressed trees a single application of BA failed to promote bud break. Within a week after the second BA application, 100% bud break was observed in case of well watered trees and in the trees that were moved from

drought stress to well watered regime. In the drought stressed trees, two BA applications resulted in a total bud break of 36%. The results pertaining to bud break and scion growth indicate that there is an interaction between BA application and water stress and the well-watered trees showed greater bud break success rate with BA application as compared to the drought stressed trees.

Background

Cellular growth is extremely sensitive to drought stress. Availability of water in the soil is therefore a major limiting factor in growth and development of trees. Effects of water deficit on cellular processes, cellular growth and vegetative growth in various trees and other crop species have been extensively reported (Mullet and Whitsitt, 1996; Bray, 1997). Drought stress has been reported to cause reduction in leaf number and size in walnut (Yadollahi et al., 2010), reduction in shoot growth in maize (Sangakkara et al., 2010) and decrease in new vegetative flushes as well as new leaves and root growth in mango (Tahir et al., 2003).

Formation of bud union and subsequent scion growth in citrus is a critical period in citrus nursery production. However, the effect of drought stress during this phase of nursery production has not been documented. Little research work has been done on the water relations of citrus nursery trees and the effects of water deficit on the bud push and scion growth. The present study was conducted to find out the implications of low soil water availability during this critical phase of development. It was hypothesized that the drought stress has significant negative effects on bud take and scion growth as compared to well-watered conditions in young citrus trees in containerized nurseries.

Materials and Methods

Overall Approach

Drought stress treatments were given to citrus rootstock seedlings as well as budwood trees. When the trees attained levels of drought stress (monitored by midday water potential), buds taken from drought stressed and well-watered trees were budded onto drought stressed and well-watered rootstock seedlings. Observations regarding percent bud break and scion growth were recorded. Trees were also sampled for determining the cytokinin concentrations at four different times. Midday water potential and net CO_2 assimilation rate were recorded periodically throughout the experimental period and temperature and relative humidity were monitored continuously.

Plant Material

The experiment was conducted at the University of Florida IFAS Citrus Research and Education Center, Lake Alfred, Florida. A total of 84 liner trees of 'Swingle' citrumelo (*Citrus paradisi* Macfad. × *Poncirus trifoliata*) were obtained from a citrus nursery and two pot-grown budwood trees of cv. Hamlin were used from the research center collection. The liner trees were re-potted in washed quartz sand in 2.65 L citra-pots (model CPOT-5H, Stuewe and Sons, Tangent, OR) and were acclimated for 8 weeks before the treatments started. For well watered and drought stress treatments one budwood tree was used to minimize the variability among buds in each treatment.

Experimental Conditions

After re-potting, the plants were moved to a custom-built walk-in growth chamber. Day and night temperatures were set at $28^{o}/21^{o}C$ and the photoperiod was set to long day (14 hours daylight). Photosynthetic photon flux (*PPF*) at plant height averaged at 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The plants were grown in these conditions in the growth chamber for

17 weeks. The liner as well as budwood trees were grown under two conditions: well-watered (100% ET) and drought stress (50% ET). Daily water use by trees was determined gravimetrically and used to calculate the amount of water required to maintain drought stress levels. Water status of the tress was monitored daily by recording midday water potential.

Budding and Bud Forcing

Three weeks after the start of the treatments, when the midday water potential reached pre-determined levels for drought stress based on previous research (Brar ch 4, 2012), the liner trees under both treatments were budded with the buds taken from well-watered and drought stressed budwood trees. In all, there were four treatment combinations as shown in the Table 5-1.

Budding was performed on the 1/4 to 3/8 inch thick seedlings by making inverted T cut 15 cm above the sand level. Mature angular shoots were selected for taking buds and the terminal buds were discarded on each shoot where the buds were taken. Inserted buds were tightly wrapped with a budding tape for three weeks. After three weeks, the buds were unwrapped and were forced by bending the rootstock stem above the bud union. Two weeks after unwrapping, the portion of the rootstock above the bud union was removed Figures 5-5 to 5-7 show the pictures of the budding procedure while figure 5-8 shows pictures of the bud break and growing scions.

Benzyl Adenine Application

After observing the buds for budbreak for four weeks after unwrapping, a 500 ppm solution of benzyladenine was prepared and was applied on the buds by dabbing with a cotton swab twice- at 4 and 6 weeks after unwrapping. The solution was prepared afresh minutes before each application.

Midday Water Potential

Stem water potential (Ψ) was recorded weekly by using Scholander type pressure chamber. Water potential measurements were taken in rootstock trees each week for 17 weeks (total duration of the experiment) while it was monitored for three weeks (before budding) in case of budwood trees. The measurements were taken at noon and the leaves were covered with aluminum foil bag for 15 minutes before taking the readings, to achieve equilibrium between leaf and stem water potential. The budding operations were performed when the water potential values in drought stressed treatment approached -2 MPa.

Bud Break and Scion Length

Bud break and scion length were recorded weekly for each tree. Cumulative scion length is reported here by totaling the length of scions in each treatment for each week.

Sap Collection and Analysis

The main stem of each harvested tree was cut at the bottom and placed in the pressure chamber in order to extract 800 µL sap into a 1.5 mL Eppendorf tube. Modified Beiliskey's solution was added to the sap in a ratio of 2:1 (sap:Beiliskey's solution) and the tubes were immediately frozen in liquid nitrogen. The sap samples were analyzed for dihydro-zeatin riboside (DHZR) by enzyme-linked immunosorbent assay (ELISA) using Phytodetek Immunoassay Kits (Agdia Inc.).

Statistical Analysis

The experiment was designed as a factorial over time with the four levels of budding combinations and 10 levels of weeks after unwrapping of buds. The data pertaining to water potential, photosynthesis and cytokinin concentration were analyzed

by analysis of variance and using Tukey's HSD for comparison of means. The cumulative bud break and scion growth during successive weeks were compared arithmetically to determine rate of bud break and growth.

Results

Midday Water Potential

The midday water potential (Ψ_{stem}) in the well-watered liner trees averaged between -0.92 and -0.85 MPa for the 17-week period (Table 5-2). In the drought stressed liner trees, the water potential ranged between -2.14 and -2.01 MPa during the period after the trees were budded which was significantly lower that the well-watered liners ($p<0.001$). In case of well-watered budwood trees (Table 5-3), the water potential values were between -0.78 and -0.68 MPa while it went down from -1.16 to -1.91 MPa from week 1 to week 3 in drought stressed budwood trees ($p<0.001$).

Percent Budbreak

In total 82 out of 84 trees showed formation of a successful bud union (budding success of 97.7%). While all the trees in well-watered treatment formed successful bud-union, 2 out of 46 buds on the drought stressed trees failed. Although this bud failure could be due to the water status of the trees, the overall budding success was quite positive even in the case of drought stressed trees. The percent budbreak was recorded starting the week of unwrapping the buds. During the first four weeks after unwrapping, only one bud started growing in WW/WW treatment and two buds started in WW/DS treatment while there was no bud break in DS/WW and DS/DS trees during first 4 weeks (Figure 5-1). However, the two scions in WW/DS started wilting soon after emerging and died within a week after budbreak. The major change in budbreak was observed after first application of 500 ppm BA. In just a week after first BA application

on buds, the WW/WW and DS/WW (both well-watered treatments) trees showed 50% and 58.33 % budbreak, respectively. However, the two sets of trees on drought stressed liners (WW/DS and DS/DS) showed 16.66% and 0% budbreak in the same period. WW/WW and DS/WW trees attained 100% and 91.66% budbreak two weeks after second BA application while WW/DS and DS/DS trees reached only 80% and 83.33% budbreak until the end of the experiment (four weeks after the second BA application).

After the second BA application, half of the trees in each drought stressed treatment (WW/DS and DS/DS) were moved to well-watered conditions (100% ET). It was observed that in both these treatments, the trees which were moved to 100% ET treatment, attained 100% budbreak in just a week following BA application and their subsequent shifting to well-watered regime.

Scion Growth (cm)

The total scion growth showed a trend similar to percent budbreak in all the treatment combinations (Figure 5-2). Due to high variability in scion length within each treatment, the total scion length for all the trees in every treatment are shown. At the end of 6 weeks after unwrapping(second BA application) the total scion length in WW/WW and DS/WW trees was 17.2 cm and 9.4 cm, respectively, while it was 4.7 cm and 0 cm in WW/DS and DS/DS treatments. However, at the end of the experiment (10 weeks after unwrapping), the scion growth was greatest in DS/WW (69.2 cm) followed by WW/WW (55 cm), DS/DS changed to WW at week 6 (50.2 cm), WW/DS changed to WW at week 6 (34.8 cm), DS/DS (5.2 cm) and WW/DS (4.7 cm).

Cytokinin Concentration

Figure 5-3 shows that the DHZR concentration of the well-watered trees was significantly higher than for the drought stressed trees at the time of budding ($p<0.05$), 4 weeks after unwrapping ($p<0.001$) and at harvest ($p<0.05$). Within the drought stress treatment, a significant reduction in cytokinin concentration was observed at 4 weeks after budding as compared to budding and unwrapping, suggesting that the cytokinin concentration in the drought stressed trees decreased gradually over time as the duration of stress extended. However, at the time of unwrapping the buds, differences between cytokinin concentration of well watered and drought stressed trees were not significant. In the trees which were changed from drought stress to the well watered regime, the cytokinin concentration was significantly higher than drought stressed trees ($p<0.01$) and did not differ significantly from well watered trees.

Discussion

Moderate drought stress did not influence budding success. However, in the initial weeks after unwrapping, the only two buds that pushed and grew soon wilted and died. It can be inferred that although the formation of bud union was not affected and the buds stayed alive throughout, drought stress affects bud push and scion growth early on. For the first two weeks after unwrapping, DS/WW had a marginally higher bud break while WW/WW had no bud break as did the DS trees. It was not until the first BA application that the WW trees started having significant bud break. This suggests that watering alone does not overcome the problem of poor bud break and the plant growth regulator (BA) plays significant role in enhancing bud break. The quick increase in the percent bud break curve and the scion growth curve immediately after the first BA application seconds that. However, the PGR application seems to be effective only in

the well-watered trees while the drought stressed trees did not show much improvement in percent bud break even after one BA application. This suggests that there is an interaction between water status of the tree and the application of BA on the buds. A number of researchers have found the correlation of increased water status of tree with the increase in cytokinin uptake and delivery within the xylem stream of the tree (Aloni et al., 2005; Kudoyarova et al., 2007). The cytokinin applied externally on the buds might well be supplementing the already enhanced cytokinin levels within the plants in the well-watered regime, while in the drought stressed trees, one application of the limited quantity of cytokinin may not be sufficient to achieve desired results on its own. Secondly, it is well documented that in the drought stressed trees, levels of Abscisic Acid (ABA) increase manifold as compared to well watered trees and that ABA acts as a root-to-shoot signal in drought stressed trees to regulate stomatal movements (Bano et al., 1993). ABA is known to inhibit cell expansion and lateral growth activity within the plant. Therefore, the negative effect on the bud break and scion growth in the drought stressed trees may well be due to an ABA-cytokinin interaction. We infer that the exogenous cytokinin application overrides ABA induced inhibition, thereby stimulating bud break.

After the second BA application (two weeks after the first application), the WW trees continued to exhibit increased bud break and achieved 100 % bud break within the next two weeks. However, the DS trees could attain only 80% bud break with two BA applications. The effects of drought stress become more visible if we look at the total scion growth (Figure 5-4). Even after two BA applications and 10 weeks after unwrapping, the scions in DS trees grew very little. The close observations of the trees

revealed that the buds, although started growing, but were sitting just there at the bud break stage and the scions were growing minimally. This was found to be in correlation with the water status of the trees as the well-watered trees were exhibiting excellent and significantly higher scion growth in comparison. Interestingly, the trees that were moved from drought stress to a well-watered regime quickly attained 100% bud break within a week and started showing significantly higher scion growth. This observation makes the interaction between plant growth regulator (PGR) application and water status of the tree even clearer.

Table 5-1. Drought stress treatment combinations in Container-grown citrus nursery trees

Treatment	Budwood		Rootstock	Symbol used
Treatment 1 (control)	well watered	on	well watered	WW/WW
Treatment 2	well watered	on	drought stress	WW/DS
Treatment 3	drought stress	on	well watered	DS/WW
Treatment 4	drought stress	on	drought stress	DS/DS

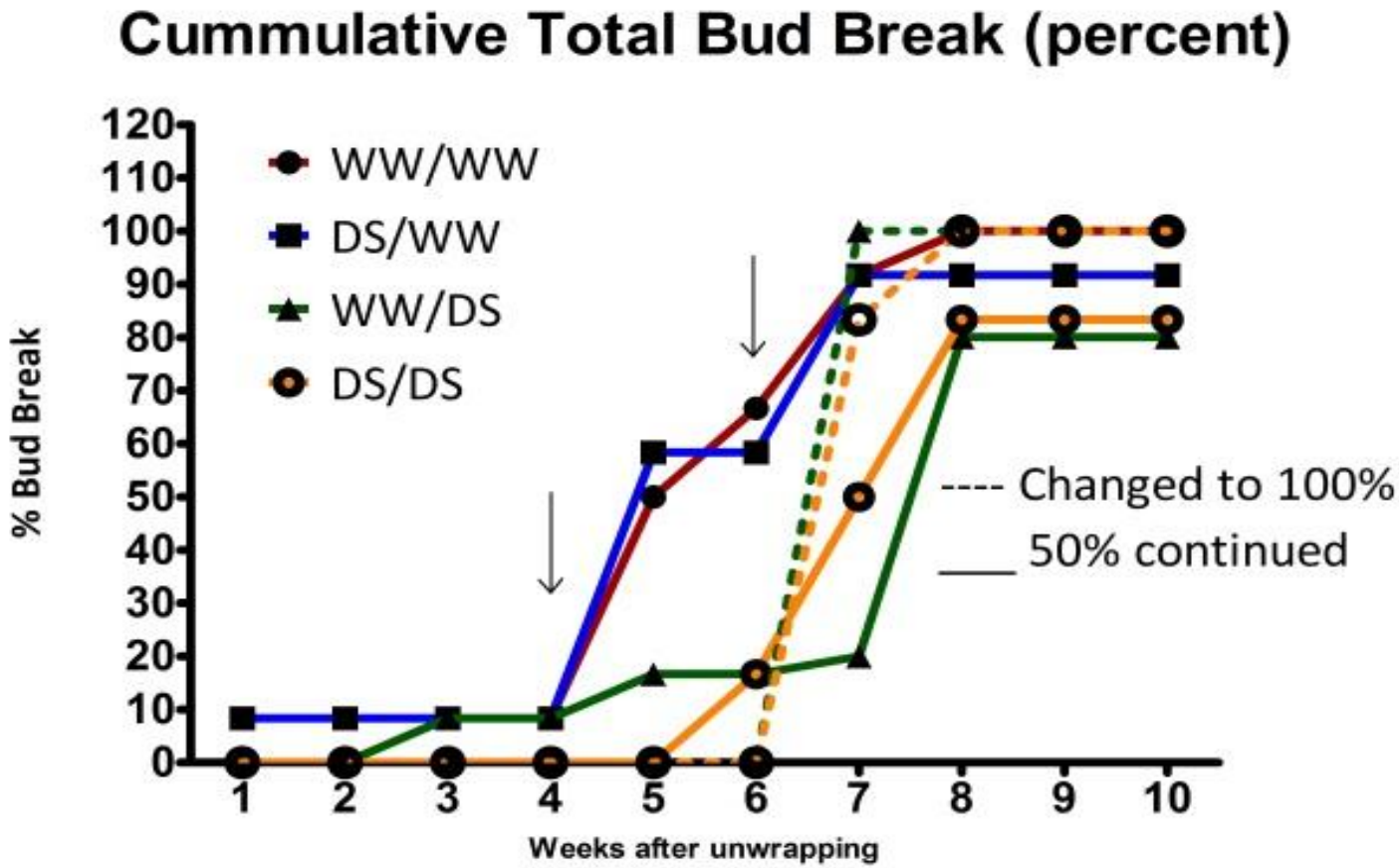


Figure 5-1. Cumulative total percent bud break for budded citrus nursery trees (Hamlin sweet orange on Swingle citrumelo rootstock). Arrows show timing of application of Benzuyl adenine @ 500 ppm.

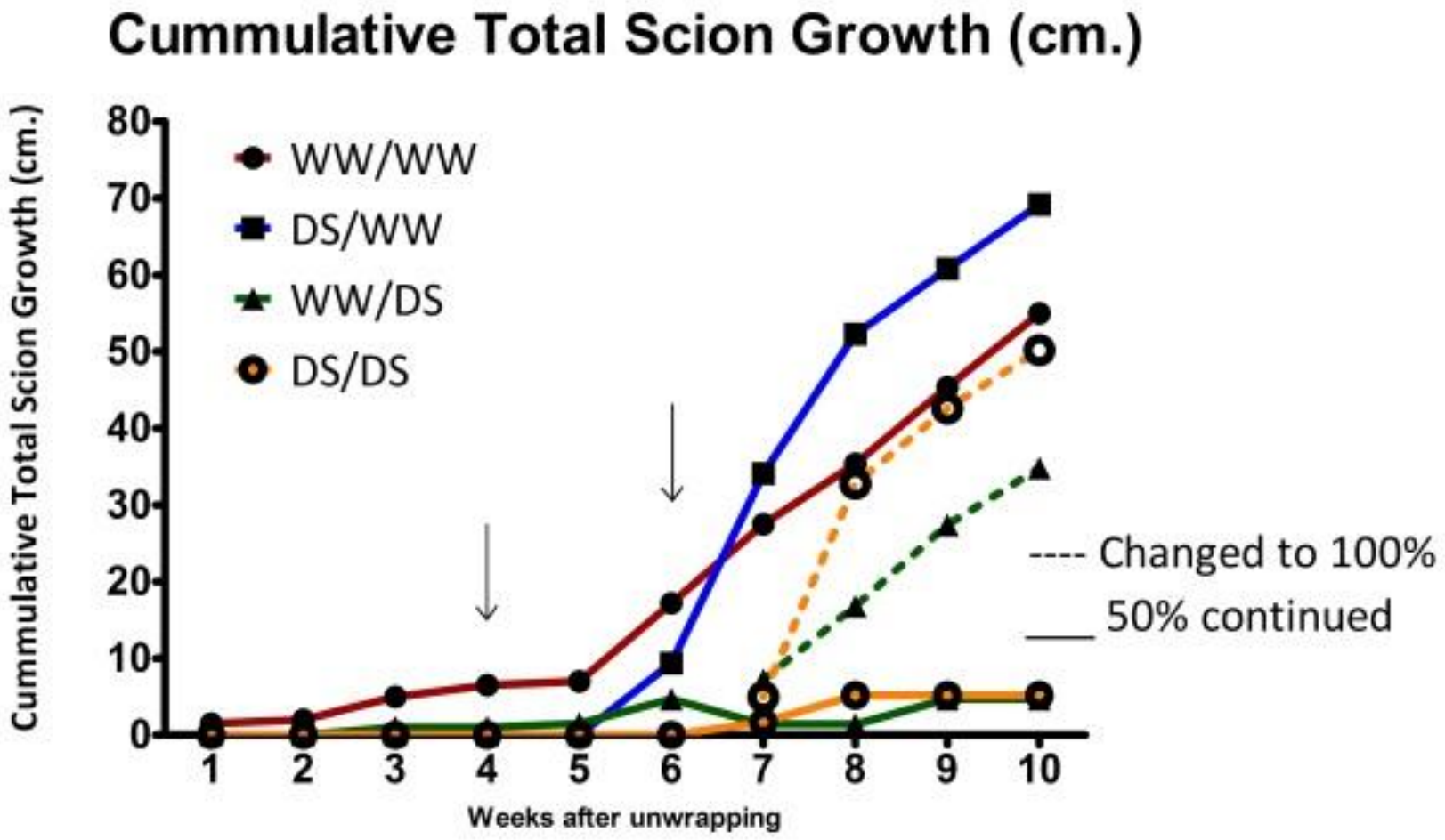


Figure 5-2. Cumulative total percent bud break for budded citrus nursery trees (Hamlin sweet orange on Swingle citrumelo rootstock). Arrows show timing of application of Benzuyl adenine @ 500 ppm.

Table 5-2. Average midday stem water potential of well watered and drought stressed liner trees in container-grown citrus nursery over 17 weeks (n=12)

Week	Well Watered[x]	Drought Stressed[y]	Difference	t	P value
1	-0.8647	-0.8245	0.04022	0.6792	P > 0.05
2	-0.8475	-1.615	-0.7670	12.95	P<0.001
3	-0.9021	-1.879	-0.9768	16.49	P<0.001
4	-0.8532	-1.948	-1.095	18.48	P<0.001
5	-0.925	-2.137	-1.212	20.47	P<0.001
6	-0.8532	-2.074	-1.221	20.62	P<0.001
7	-0.8733	-2.109	-1.235	20.86	P<0.001
8	-0.8762	-2.04	-1.163	19.65	P<0.001
9	-0.8733	-2.126	-1.253	21.15	P<0.001
10	-0.8963	-2.114	-1.218	20.57	P<0.001
11	-0.8532	-2.068	-1.215	20.52	P<0.001
12	-0.8475	-2.063	-1.215	20.52	P<0.001
13	-0.8532	-2.011	-1.158	19.55	P<0.001
14	-0.8532	-2.068	-1.215	20.52	P<0.001
15	-0.8532	-2.054	-1.201	20.28	P<0.001
16	-0.8705	-2.086	-1.215	20.52	P<0.001
17	-0.8676	-2.114	-1.247	21.05	P<0.001

[x]100% evapotranspiration (well-watered control)
[y]50% (drought stressed)

Table 5-3. Midday stem water potential of budwood trees for three weeks prior to budding (n=6)

Week	WW[x]	DS[y]	Difference	t	P value
1	-0.68	-1.161	-0.483	8.845	P<0.001
2	-0.78	-1.643	-0.856	15.69	P<0.001
3	-0.77	-1.908	-1.138	20.85	P<0.001

[x]WW-Well Watered control (water applied @ 100% Evapotranspiration)
[y]DS-Drought stressed (water applied @ 50% Evapotranspiration)

Table 5-4. Instantaneous Net CO2 assimilation for well watered and drought stressed container-grown citrus nursery trees (n=12)

Week[x]	Instantaneous Net CO2 assimilation ($\mu mol\ CO_2 \cdot m^{-2} \cdot s^{-1}$) Treatment	
	Well Watered[z]	Drought Stressed
1	7.97 ± 0.58	8.03 ± 0.43
3[y]	7.50 ± 0.41	2.79 ± 0.20
5	8.10 ± 0.32	6.11 ± 0.32
7	8.70 ± 0.44	5.04 ± 0.44
9	8.46 ± 0.42	5.15 ± 0.31
11	9.09 ± 0.52	5.65 ± 0.39
13	9.37 ± 0.32	7.22 ± 0.42

	Df	F	p
Week x Treatment	6	7.269	P<0.0001
Treatment	1	160.4	P<0.0001
Week	6	12.73	P<0.0001

[x]Measurements were taken once every two weeks,
[y]The trees were budded just before week 3 measurements
[z]Well Watered control (water applied @ 100% Evapotranspiration) and Drought stressed (water applied @ 50% Evapotranspiration)

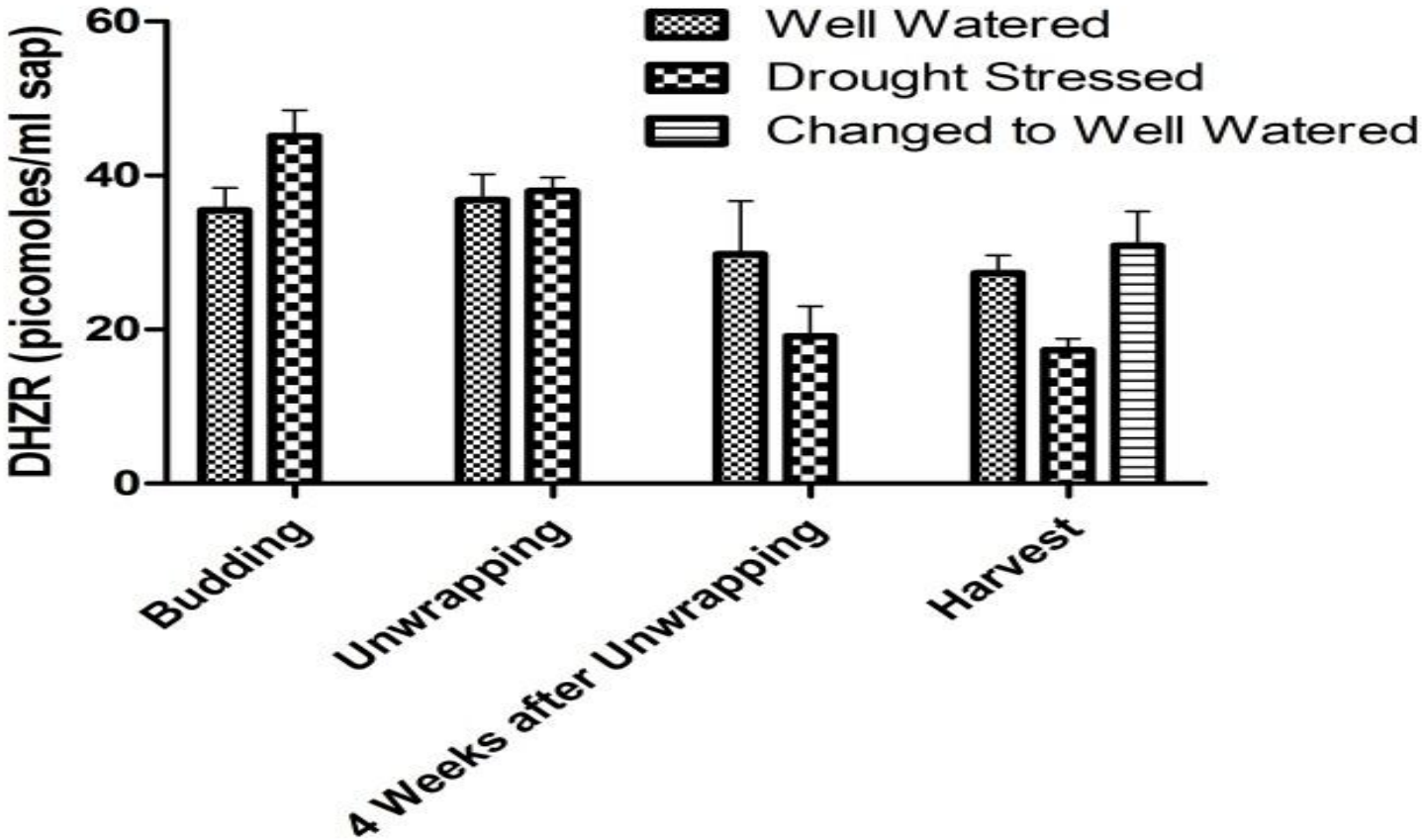
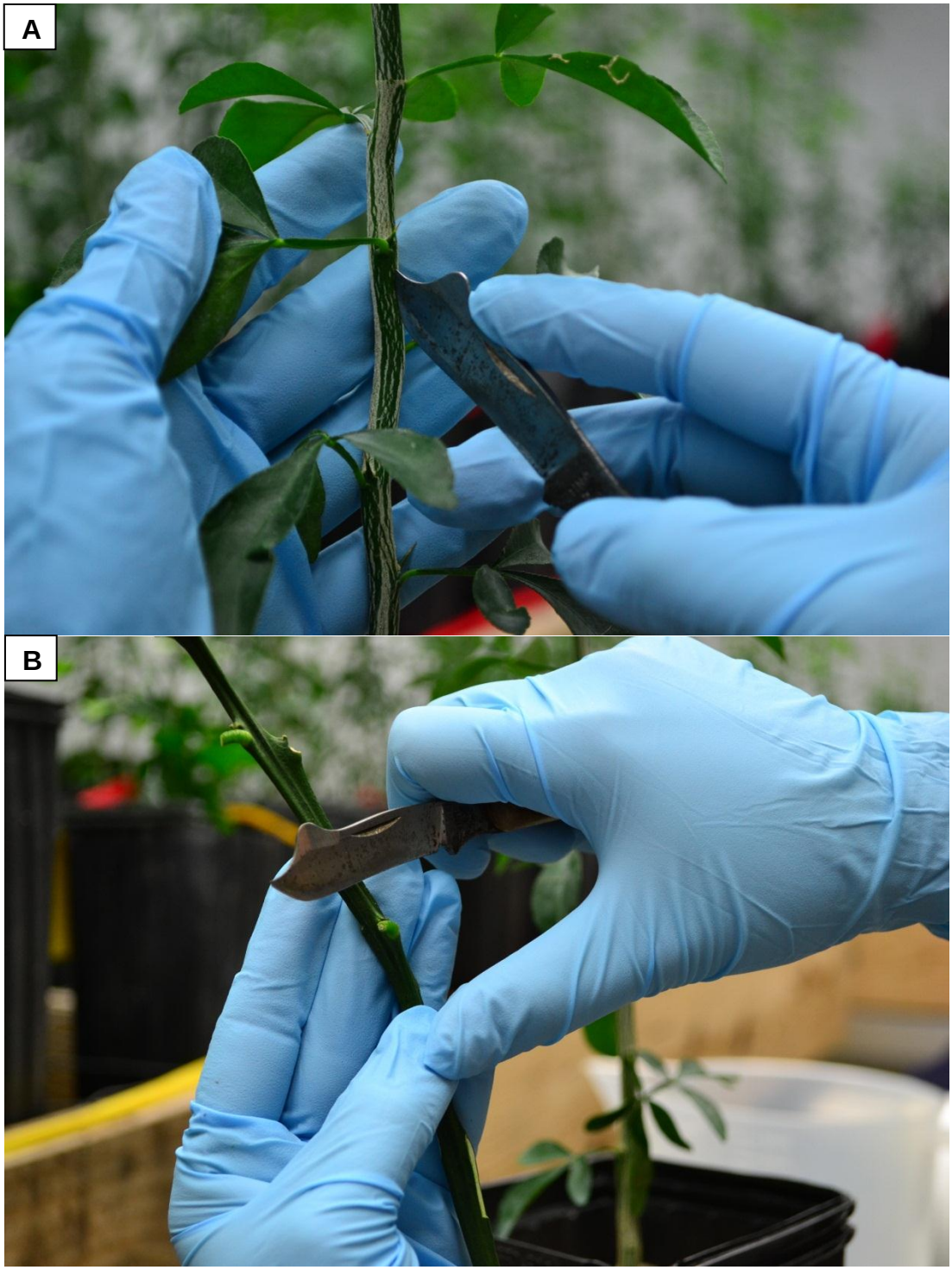


Figure 5-3. Cytokinin (Dihydro-zeatin ribside) concentrations in container grown citrus trees under well watered and drought stress treatments at four different times during the experiment.

Figure 5-4. A budded lot of container-grown citrus trees in growth chamber (Photo: Gurreet Brar).



Figures 5-5. The process of T-budding; A) making cuts on the rootstock; B) cutting the bud from budwood shoot; C) inserting the bud on rootstock; D) the inserted bud, E) tying the inserted bud. (Photos: Gurreet Brar)

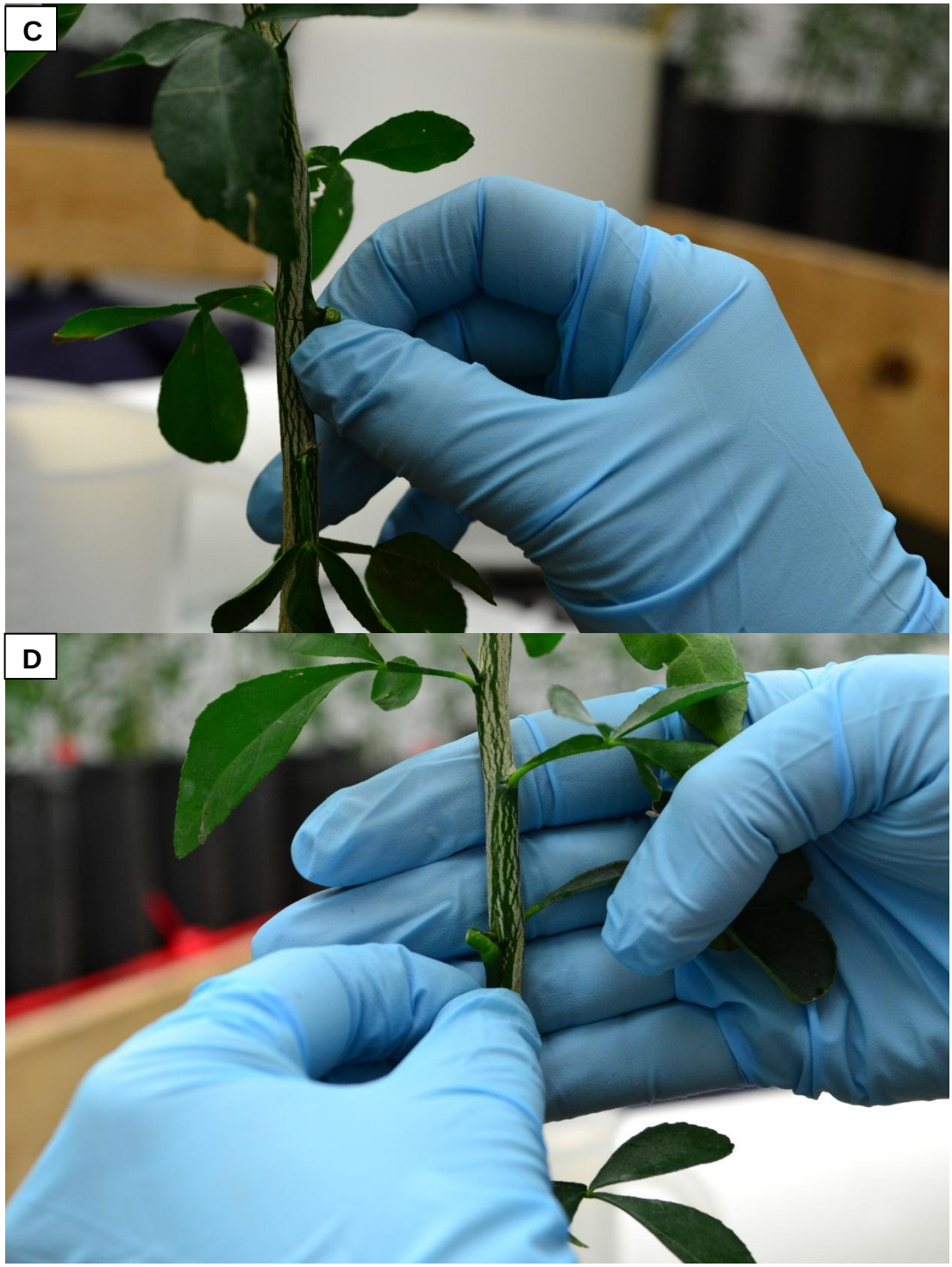


Figure 5-5. Continued. (Photos: Gurreet Brar)

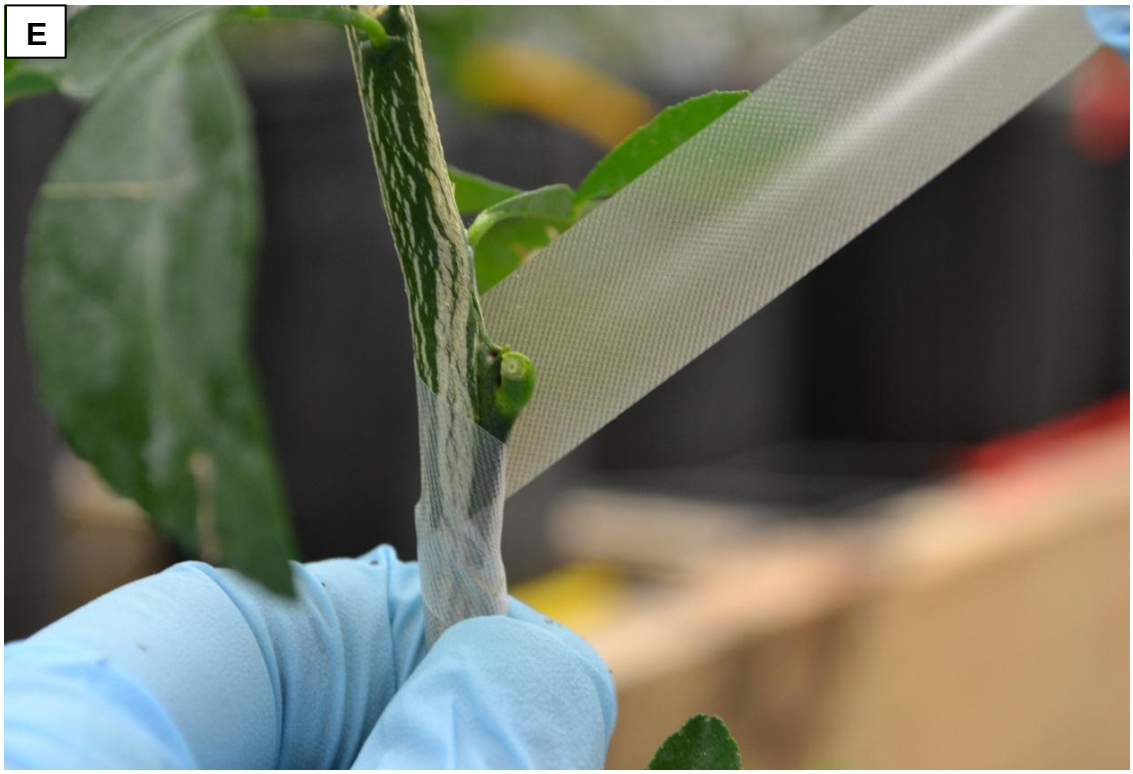


Figure 5-5. Continued. (Photo: Gurreet Brar)

Figure 5-6. The stages after unwrapping. A) Bud break; B) and C) the growing scions. (Photos: Gurreet Brar)

CHAPTER 6
EFFECT OF NITROGEN APPLICATION ON BUD TAKE, SCION GROWTH AND THE LEVEL OF ENDOGENOUS CYTOKININS IN SHOOTS OF TRIFOLIATE ORANGE ROOTSTOCKS

Chapter Summary

This research was conducted to study the effect of levels of nitrogen application on bud take and scion growth; and to quantify the effect of nitrogen application on the biosynthesis and translocation of endogenous free cytokinins in shoots of trifoliate orange rootstocks. In experiment 1, the liner trees of citrus rootstock 'Swingle' citrumelo (*Citrus ×paradisi ×Poncirus trifoliata*) and bud wood trees of sweet orange cv. Hamlin were subjected to two treatments consisting of no N application and 150 mL of 200 mg L^{-1} N solution per tree per week. Four treatment combinations were developed by budding N sufficient and N deficient buds on N sufficient and N deficient liners. In the second experiment, the trees were subjected to two treatments: to one set 150 mL of 200 mg L^{-1} N solution was applied every day for eight days, while no N was applied to the second set. After 5 days, the trees under both treatments were further subdivided into two categories: half of trees from N+ treatment were moved to N- and half remained in N+, and vice versa. Trees from each of these combinations were harvested daily for three days to extract xylem sap for cytokinin analysis. The results show that N deprivation decreased leaf chlorophyll concentration by 26%, while N application increased it by 28.6 % in respective treatments. The whole plant nitrogen content (% dry weight) was also significantly higher in N+ trees. As a result, the N-sufficient trees also had significantly higher net photosynthetic rates than the N-deprived trees. The bud survival rate, bud break, and scion growth, all were positively influenced by N application. The N sufficient trees had higher endogenous cytokinin levels before

budding, at the time of budding, and at unwrapping, but not 6 weeks after unwrapping when the scions were growing. The second experiment showed no significant changes in endogenous cytokinin levels with N application over 5 days.

Background

Plants respond to changed nitrogen status in many ways. This includes changes from gene expression to enzyme activity to the biosynthesis of various metabolic compounds. Nitrogen is the key component of essential plant pigments like chlorophyll and also of various critical enzymes essential for plant growth, and in addition to that nitrogen also affects citrus plant nutrition indirectly by affecting uptake of other nutrient elements (Chapman, 1968). In citrus much attention has been devoted to fertilization requirements of field-grown bearing and non-bearing trees and very little research work has been done in optimizing nitrogen nutrition of container-grown nursery trees. Nitrogen fertilization is critical during the nursery stages and has far reaching effects on growth and productivity of the citrus crop. Previous studies suggest that critical nitrogen concentration for relative total plant dry weight accumulation in container-grown citrus nursery trees is 16.8 mg L^{-1} (Williamson and Maust, 1994), while Omari et al. (2012) reported that 5 mM N is optimum for maintaining good growth of nursery trees.

Nitrogen application has also been reported to be crucial from cytokinin biosynthesis and the accumulation of cytokinin is closely correlated with plant nitrogen status (Wagner and Beck, 1993; Samuelson and Larsson, 1993; Takei et al., 2001), and cytokinin metabolism and translocation could be modulated by the N status. Takei et al. (2001) reported an increase in cytokinin concentration immediately after the N status changed from deficient to sufficient in maize. Within 1 hour of the addition of nitrate to N-deprived plants, isopentenyladenosine - 59-monophosphate (iPMP) started to

accumulate in roots, followed by increases in levels of trans-zeatin riboside-59-monophosphate (ZMP), trans-zeatin riboside (ZR) and trans-zeatin (Z). Since, iPMP is the first molecule synthesized in cytokinin metabolism, these results indicate that cytokinin was synthesized anew in response to nitrogen supply. The spatial and temporal changes in the molecular species, and the extent of accumulation, strongly suggest a N-dependent movement of cytokinins from roots to shoots.

An N-dependent movement of cytokinins as well as the effect of nitrogen application on bud break and scion growth has not been tested in woody species like citrus. In citrus seedlings, the mediation of cytokinin translocation by N can in turn influence the bud break and growth of lateral branches in the budded seedlings. Therefore, keeping in view the above correlation, we hypothesized that, i) the application of nitrogen to N-starved seedlings will increase the cytokinin concentration of xylem sap in the seedlings of citrus rootstocks, and ii) nitrogen application to the budwood and liner trees will increase the percent bud break and scion growth in budded citrus nursery trees as compared to the N-starved liner and budwood trees.

Materials and Methods

Experimental Conditions

The experiments were conducted at the University of Florida IFAS Citrus Research and Education Center, Lake Alfred, Florida. The plants were grown in a custom-built walk-in growth chamber. Day and night temperatures were set at $28^{o}/21^{o}C$ and the photoperiod was set to long day conditions (14 hours daylight). Photosynthetic photon flux (*PPF*) at plant height averaged at 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Experiment 1

Liner trees of 'Swingle' citrumelo (*Citrus ×paradisi ×Poncirus trifoliata*) citrus rootstock were obtained from a commercial citrus nursery near the University of Florida Citrus Research and Education Center, Lake Alfred, Florida where the experiment was done. In total 84 liner trees were obtained, repotted in 2.65 L citra-pots (model CPOT5; Stuewe and Sons, Tangent, OR) in washed quartz sand soon after they were received, and acclimated for 4 weeks before the treatments began.

The bud wood and liner trees were subjected to treatments consisting of varying levels of nitrogen application. Two treatments consisting of no N application and 150 mL of 200 mg L^{-1} N solution per tree per week were applied to liner trees for 12 weeks before budding. Hence, the total number of treatment combinations were four: N sufficient budded on N sufficient (N+/N+), N deficient budded on N sufficient (N-/N+), N sufficient budded on N deficient (N+/N-) and N deficient budded on N deficient (N-/N-). The buds were unwrapped after 3 weeks and the bud survival rate was noted. The resulting percentage bud break and scion growth was measured for 8 weeks after unwrapping. During the experiment period net photosynthetic rate was measured with a LI-6400XT portable photosynthesis system (LI-COR, Lincoln, NE). The leaves were periodically sampled for analysis of total chlorophyll content. Also, three trees from each treatment combination were destructively harvested for cytokinin analysis at four different intervals: 6 weeks before budding, at budding, at unwrapping and a week before final harvest (7 weeks after unwrapping) of the trees.

Stem water potential

Stem water potential measurements were taken on two leaves per plant with Scholander-type pressure chamber. The selected leaves were covered in Mylar bags

for one hour prior to taking measurements to equilibrate the leaf and stem water potentials. The measurements were taken during noon-time between the hours of 1200 and 1300.

Total chlorophyll content

Leaf tissue was collected monthly from 6 trees per treatment (two leaf discs per tree) to determine the concentration of chlorophyll a and b content and total chlorophyll was calculated. Two leaf discs (6.35 mm diameter) were excised from fresh leaf samples and were placed in a test tube to which 2 mL of *N,N*-dimethylformamide was added. The test tubes were covered with aluminum foil and allowed to stand in the dark at room temperature for 72 hours. Afterwards the solution was transferred to 1.5 ml quartz cuvettes, making sure to leave the leaf tissue behind. The absorbance values were read at 647 and 664 nm in a spectrophotometer (model Genesys 10S; Thermo Scientific, Madison, WI). The chlorophyll concentration (mg/L) was calculated based on the formulas of Inskeep and Bloom (1985):

$$\text{Chl } b = 20.70\, A_{647} - 4.62\, A_{664}$$

$$\text{Chl } a = 12.70\, A_{664} - 2.79\, A_{647}$$

$$\text{Total chlorophyll} = 17.90\, A_{647} + 8.08\, A_{664}$$

Where, A_{647} is absorbance at 647 nm (maximum for chl *b*) *and* A_{664} is absorbance at 664 nm (maximum for chl *a*).

The values were converted from mg/L to g/cm^2 as follows:

$$\text{mg/L} = \text{mg}/0.633\ \text{cm}^2$$

Where, 0.633 cm^2 is the total area of the two leaf discs (mg/0.633 cm^2)/ (633) = g/cm2

Whole plant nitrogen content

Trees were destructively harvested from both treatment sets at certain intervals for whole plant nitrogen content analyses. After harvesting, the trees were washed, cut into pieces and oven dried at 65 °C for 48 hours. The samples were ground and sent to a commercial laboratory for analysis (Waters Agricultural Laboratories, Camilla, GA).

Xylem sap cytokinin analysis

The main stem of each harvested tree was cut at the bottom and placed in the pressure chamber in order to extract 800 µL sap. Modified Beiliskey's solution was added to the sap in a ratio of 2:1 (sap:Beiliskey's solution) and the tubes were immediately frozen in liquid nitrogen. The xylem sap (800 µL per tree) was extracted using the Scholander-type pressure chamber. The sap samples were analyzed for dihydro-zeatin riboside (DHZR) by enzyme-linked immunosorbent assay (ELISA) using Phytodetek Immunoassay Kits (Agdia, Elkhart, IN).

Experiment 2

A total of 88 healthy trees of 'Swingle' citrumelo (*C.* ×*paradisi* ×*P. trifoliata*) were obtained from a citrus nursery. The trees were re-potted in 2.65 L citra-pots (model CPOT5; Stuewe and Sons, Tangent, OR) in washed quartz sand soon after they were received and were acclimated in the growth chambers for 8 weeks before the treatments began. The trees were subjected to two treatments: to one set 150 mL of 200 mg L^{-1} N solution was applied every day for eight days, while no N was applied to the second set. Four trees were destructively harvested for 5 consecutive days from each treatment set for xylem sap extraction. The xylem sap was then analyzed for cytokinin analysis. Midday stem water potential measurements were taken every day

while net photosynthesis was measured every other day for the duration of the experiment.

After 5 days, the trees under both treatments were further subdivided into two categories: half of trees from N+ treatment were moved to N- and half remained in N+, and vice versa. As a result, we had four different categories: N+ to N+, N+ trees moved to N-, N- to N- and N- trees moved to N+. Four trees from each of these combinations were harvested daily for three days to extract xylem sap. The sap was then analyzed for cytokinin concentration.

Statistical Analysis

The experiments were completely randomized designs with factorial arrangement, with different levels of N application and duration. Experiment 1 had four levels of rootstock-scion combinations pertaining to N application and eight levels of weeks after budding. Experiment two was done over five days with two levels of N application; therefore, it was a 2x5 factorial arrangement. The treatment comparisons were performed by ANOVA. The mean separations were calculated by Tukey's HSD.

Results

Experiment 1

Over the 17 week period, the nitrogen application to the trees (N+) resulted in significant changes in the midday stem water potential, photosynthesis, leaf chlorophyll concentration, whole plant nitrogen level, percent bud break and scion growth as compared to the trees to that no N was applied (N-).

Midday stem water potential of N-deficient and N-sufficient container-grown citrus nursery trees has been given in Table 6-1. The average stem water potential ranged between –0.75 MPa and –0.85 MPa for N- trees, while it varied from –0.75 MPa to –

1.03 MPa for the N+ trees. For the first 6 weeks, trees under both treatments did not differ with respect to the midday stem water potential ($p > 0.05$). However, at week 7 the N− trees showed slightly higher stem water potential than the N+ trees ($p < 0.05$), and at week 8 the difference was non-significant. However, week 9 and onwards until the end of experiment (week 17) both the treatments were significantly different from each other ($p < 0.001$), with N- having higher stem water potential.

The leaves were sampled and analyzed for chlorophyll concentration once every month from February to July. The trees under both the treatments (N− and N+) had somewhat similar total leaf chlorophyll concentration (chlorophyll a + b) after they were received in February (Table 6-2). The average total chlorophyll concentration on sampling date of 13 February was 0.023 $g \cdot cm^{-2}$ for N− trees and 0.028 $g \cdot cm^{-2}$ for N+ trees. However, for the sampling date of 16 July, it was 0.017 $g \cdot cm^{-2}$ for N− trees and 0.036 g/cm^2 for N+ trees. The treatments did not show significant differences ($p > 0.05$) for the sampling dates of 13 February and 16 March. However, for the subsequent sampling dates of 12 April, 18 May, 18 June and 16 July, N+ trees had significantly higher ($p < 0.001$) total leaf chlorophyll concentration than N− trees. Overall, the factor 'Sampling Date' was not significant ($p = 0.2153$) while the factor 'N application' was highly significant ($p < 0.001$). Figure 6-3 shows a visual comparison of N-starved trees with N-sufficient trees after 17 weeks under treatments.

The net photosynthetic rate (µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$) was measured every other week and the treatments started showing the effect of nitrogen application at week 5 and afterwards. The net photosynthetic rate in N− trees averaged 9.15 and 2.54 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ while it was 9.64 and 11.14 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ in case of N+ trees, for week 1

and week 17, respectively (Table 6-3). For week 1 and week 3, both the treatments did not differ significantly ($p > 0.05$), at week 5 the differences were significant ($p < 0.05$), while week 7 onwards the average net photosynthetic rates of the two treatments differed significantly from each other. Average whole plant nitrogen content (% dry weight) varied largely between treatments. At the start of the experiment in March, the average whole plant nitrogen content was 3.28% and 3.42% in N− and N+ trees, respectively, while it was 1.22% and 3.56% in July, one week before finally harvesting the trees (Table 6-4).

A significant effect of not applying any nitrogen was observed in the form of poor bud survival (Data not shown). The percentage of buds that failed in the N− trees budded with buds from N+ trees (N+/N–) was 50% while it was 58% in the case of N– trees budded with buds from N– trees (N–/N–). In the case of N+ trees budded with buds from N– trees (N–/N+), only 16% buds failed while N+/N+ did not show any bud failure. The N application further showed significant effect on bud break (Figure 6-1). The N+/N+ trees achieved 100% bud break by week 5 and N–/N+ achieved 83% bud break by the end, which consisted of 100% of the survived buds. However, the N+/N– and N–/N– trees achieved only 33% bud break by the end of the experiment. The treatments N+/N+ and N–/N+ (all N applied liner trees) and N+/N– and N–/N– (all N deprived liner trees) were not significantly different between them. The trees on N+ liners had significantly higher bud break than the trees on N– liners.

The scion growth showed the similar trend with respect to the effect of nitrogen application (Figure 6-2). The cumulative scion growth for N+/N+ trees was 81.6 cm, N–/N+ 89.5, N+/N– 11.5 and N–/N– 10.4 cm, by the end of week 8. The results show that

the budded trees on N+ liners grew more as compared to the trees on N– liners. It was observed that in some N– trees, some buds did not start growing at all after bud break; therefore the scion growth was corrected to calculate growth in cm per number of growing scions. However, it was observed that this correction also showed greater differences in N+ and N– trees. From the observation it is suggested that the N+ scions not only grew more than the N– scion, but they also grew faster.

The xylem sap concentration of dihydro-zeatin riboside (DHZR) was also found to be influenced by the degree of nitrogen application in the trees. The xylem sap was collected from the experimental trees at four intervals during the experiment: Six weeks before budding, at budding, at unwrapping the buds and at harvest. The average DHZR concentration at first sampling (six weeks before budding) was 24.1 picomoles/mL in N+ trees and 15.66 picomoles/mL in N– trees, at budding it was 47.90 and 21.40; at unwrapping 68.10 and 29.01 and at harvest it was 57.84 and 18.50 picomoles/mL, respectively in N+ and N– trees (Table 6-5). The xylem sap cytokinin concentration was not significantly different at first sampling ($p > 0.05$); however, it was highly significant in all the later sampling dates ($p < 0.001$).

Experiment 2

The midday stem water potential of the trees under both the treatments did not differ significantly during the first three days of the experiment; however, the treatments started showing some differences by day 4 ($p < 0.01$) and day 5 ($p < 0.05$). The average midday stem water potential for the N– trees was –0.79 MPa at day 1 and –0.80 MPa on day 5, while it was –0.78 MPa and –0.91 MPa at days 1 and 5 respectively for N+ trees (Table 6-6). For the second part of the experiment 2, the trees which were moved to N– from N+, did not differ in Ψ_{stem} values ($p > 0.05$) from N+ trees over three day

period. Similarly, the trees moved from N– to N+ did not differ statistically from those trees that remained under N– ($p > 0.05$). The photosynthesis data showed similar results. All the trees did not differ statistically during the full experimental period ($p > 0.05$). The average rate of photosynthesis for the N– trees ranged between 5.32 and 5.84 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ while it was between 5.24 and 7.02 µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ for N+ trees for the 8 day experiment (Table 6-7).

The average percent whole plant nitrogen content did not show significant changes with N application, except at day 2 and day 6 when there were significant differences between the two treatments ($p < 0.05$). The average N content of N– trees was 1.25% at day 1, 1.28% at day 5 and 1.29% at day 8. In the case of N+ trees, it was 1.42% at day 1, 2.72% at day 5 and 2.50% at day 8 (Table 6-8). During the second part of experiment 2, the trees that were changed from N– to N+ were not significantly different than N– trees in terms of their N concentration. Similarly, change from N+ to N– did not cause significant changes in their N level as compared to those trees that remained at the N+ level throughout the experiment (Table 6-9).

The DHZR concentration over the five-day period during the first part of experiment 2 did not seem to be affected by nitrogen application and had very high variability. The average DHZR concentration for the N– trees varied between 38.69 and 29.22 picomoles/mL while it was between 42.73 and 34.48 picomoles/mL for N+ trees over the 5-day period (Table 6-10). The values pertaining to DHZR levels did not show any statistical differences among them. During the second part, half of the trees in each treatment were moved to the other treatment and half remained under same treatment. This resulted in four treatment combinations: N- changed to N+, N+ changed to N-, N+

throughout and N- throughout. The average cytokinin levels on day 1 were (Table 6-11): 34.38 picomoles/ml (in N- changed to N+), 38.08 (N+ changed to N-), 31.47 (N- throughout) and 38.10 picomoles/ml (in N+ throughout). These levels on day 3 were: 39.71 picomoles/ml (in N- changed to N+), 38.85 (N+ changed to N-), 29.43 (N- throughout) and 36.79 picomoles/ml (in N+ throughout).

Discussion

Nitrogen is an important structural component of chlorophyll. In this experiment, leaf chlorophyll content decreased in the nitrogen deficient trees as the weeks progressed. Reduction in chlorophyll concentration is one of the characteristic symptoms in N deficient trees. Also, looking at the table 6-3, it is clear that net photosynthetic rate gradually decreases with increasing nitrogen deficiency. The very first known effect of reduced N supply is on the photosynthetic capacity of the trees. Previous research in maize also suggests that several proteins in thylakoid and stroma decease as a result of N deficiency and similarly, levels of many key enzymes involved in the Calvin cycle also decrease, thereby limiting photosynthetic capabilities of the trees under N deficiency (Sugiharto et al., 1990). However, interestingly, the trees supplied with weekly N solution showed lower midday water potential compared to N-deficient trees. This might suggest that N deficiency has an effect on stomatal apparatus, maintaining higher water content and losing less water. Studies in cotton by Radin and Parker (1979) showed that N-deficient plants actually exhibited many characteristics of drought resistance and plants abundant in N lost twice the amount of water per unit change in water potential through leaves as compared to the trees deficient in nitrogen.

Nitrogen nutrition had a significant effect on survival rate of newly inserted buds and the bud break thereafter. The N- trees had 50 % and 58% bud failure respectively, in trees budded with N+ and N- buds. These data show that nitrogen deprivation has a profound effect on the formation of the bud union and survival of buds. Scion growth in the budded trees also showed a similar trend and were greatly affected by the nitrogen status of the trees. Boughalleb et al. (2011) reported that studies with greenhouse-grown lemon (*Citrus limon* cv. Eureka) and orange trees (*Citrus sinensis* cv. Maltese), showed that increasing N application increased leaf number, shoot length, total leaf area, and stem diameter, with optimum growth observed with 50 and 100 mg N per liter. Similarly, Guazzelli et al. (1993) compared growth reponses of N-sufficient and N-deficient field grown citrus nursery trees to nitogen fertilization. They observed significantly lower shoot number in case of non-fertilized trees as compared to those that were fertlized. Williamson and Maust (1994) also reported higher shoot:root ratio in container grown citrus nursery trees with increase N application.

The effect of nitrogen on plant growth in general and production of new shoots in particular has been well studied and reported by many researchers (). However, the second part of this experiment was conducted to study whether nitrogen application enhances the cytokinin concentration of the trees, which in turn might be effective in increasing bud survial (by aiding formation of bud union, a definitive role of cytokinins), bud break, and scion growth (where cytokinins are believed to be playing a major role). The results of experiment 2 indicate that the cytokinin concentration in the nursery trees was not influenced significantly by the nitrogen application over the 5 day period. The differences on cytokinin levels between N– and N+ trees were not statistically significant

due to high variability among N+ trees. In part 2, the trees moved from N- to N+ had significantly higher cytokinin concentration than N- trees on day 3, which in turn indicates that N application is responsible for enhanced cytokinin levels in the xylem stream.

Conclusion

These studies show that N deprivation decreased leaf chlorophyll content and whole-plant nitrogen content (% dry weight) in container-grown citrus nursery trees. As a result, the N-sufficient trees also had significantly higher net photosynthetic rate than the N-deprived trees. The bud survival rate, bud break and scion growth, all were positively influenced by N application. The N sufficient trees had higher endogenous cytokinin levels before budding, at the time of budding and at unwrapping, but not 6 weeks after unwrapping when the scions were growing. The second experiment showed no significant changes in endogenous cytokinin levels with N application over 5 days. Also the trees moved from N- to N+ had higher average cytokinin content over three days but were not statistically significant.

Table 6-1. Midday Stem Water Potential of N-deficient and N-sufficient citrus nursery trees.

	Midday Stem Water Potential (MPa)	
[x]Week	[y]N-	N+
1	-0.76 ± 0.017	-0.75 ± 0.025
2	-0.76 ± 0.017	-0.78 ± 0.018
3	-0.79 ± 0.019	-0.79 ± 0.019
4	-0.82 ± 0.012	-0.83 ± 0.008
5	-0.84 ± 0.012	-0.87 ± 0.012
6	-0.85 ± 0.024	-0.89 ± 0.015
7	-0.83 ± 0.012	-0.90 ± 0.014
8	-0.84 ± 0.009	-0.90 ± 0.012
[z]9	-0.83 ± 0.012	-0.93 ± 0.014
10	-0.84 ± 0.016	-0.96 ± 0.014
11	-0.81 ± 0.027	-1.02 ± 0.019
12	-0.79 ± 0.015	-1.03 ± 0.013
13	-0.78 ± 0.018	-1.02 ± 0.012
14	-0.75 ± 0.019	-0.98 ± 0.019
15	-0.77 ± 0.012	-1.01 ± 0.018
16	-0.78 ± 0.014	-0.99 ± 0.019
17	-0.76 ± 0.018	-0.98 ± 0.021

	Df	F	P
N status x week	16	18.17	< 0.001
N Status	1	454.5	< 0.001
Week	16	16.44	< 0.001

[x] The midday stem water potential was measured weekly from 20th March 2012 to 16th July 2012

[y] N- = no N applied; N+ = 200 mg $\cdot L^{-1}$ per week

[z] Budding was performed during week 9

Table 6-2. Total Leaf chlorophyll concentration of N-deficient and N-sufficient container-grown citrus nursery trees.

	Average Leaf Total Chlorophyll Concentration (mg cm^{-2})	
[x]Sampling Date	[y]N-	N+
1	0.023 ± 0.0044	0.028 ± 0.0021
2	0.020 ± 0.0025	0.025 ± 0.0019
3	0.019 ± 0.0021	0.036 ± 0.0025
4	0.018 ± 0.0013	0.036 ± 0.0018
5	0.015 ± 0.0014	0.038 ± 0.0022
6	0.017 ± 0.0013	0.036 ± 0.0007

	Df	F	P
N Status x Sampling Date	5	6.006	< 0.0001
N Status	1	130.5	< 0.0001
Sampling Date	5	1.463	0.2153

[x]Leaf tissue was samples monthly between 13th February 2012 and 16th July 2012
[y]N- = no N applied; N+ = 200 mg $\cdot L^{-1}$ per week

Table 6-3. Net photosynthetic rate for N-deficient and N-sufficient citrus nursery trees

	Net Photosynthetic Rate (µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$)		
[x]Week	[y]N-	N+	
1	9.16 ± 0.28	9.64 ± 0.25	
3	9.61 ± 0.33	9.89 ± 0.28	
5	9.22 ± 0.28	10.61 ± 0.31	
7	8.52 ± 0.20	10.86 ± 0.27	
9	6.59 ± 0.40	11.17 ± 0.32	
11	6.10 ± 0.28	10.55 ± 0.33	
13	3.26 ± 0.39	10.61 ± 0.31	
15	2.67 ± 0.26	11.08 ± 0.29	
17	2.54 ± 0.23	11.14 ± 0.29	
	Df	F	P
N status X week	8	61.02	< 0.0001
N status	1	888.5	< 0.0001
Week	8	38.34	< 0.0001

[x]Net photosynthetic rate was measured every other week over 17 week period
[y]N- = no N applied; N+ = 200 mg $\cdot L^{-1}$ per week

Table 6-4. Whole plant Nitrogen content (%) for N-deficient and N-sufficient citrus nursery trees (Experiment 1)

	N content (%)		
[x]Sampling Date	[y]N-		N+
1	[z]3.280		3.420
2	2.720		3.680
3	1.810		4.120
4	1.200		2.645
5	1.220		3.560
	Df	F	P
N status X Sampling date	4	28.21	< 0.001
N status	1	335.0	< 0.001
Sampling date	4	46.01	< 0.001

[x] Trees were harvested for N content analysis at the following dates: 14 March, 20 April, 15 May, 20 June and 26 July.
[y]N- = no N applied; N+ = 200 $mg \cdot L^{-1}$ per week
[z]Each value is the averages of two trees

Table 6-5. Concentration of dihydro-zeatin riboside (DHZR), a cytokinin in the xylem sap of N-deficient and N-sufficient citrus nursery trees; Experiment 1 (n=4)

	Conc. of dihydro-zeatin riboside (DHZR) (picomoles/ml)	
[x]Sampling Time	[y]N-	N+
1	[z]15.66a	24.10a
2	21.40a	47.90b
3	29.01a	68.10c
4	18.50a	57.84bc

	Df	F	P
N status X sampling time	3	16.53	<0.001
N status	1	250.7	<0.001
Sampling time	3	43.90	<0.001

[x] Xylem sap was extracted at four intervals- 6 weeks before budding, at budding, at unwrapping and a week before end of experiment
[y]N- = no N applied; N+ = 200 mg·L $^{-1}$per week
[z]Means separation by Tukey's HSD test, $P < 0.05$.

Table 6-6. Midday stem water potential of N-deficient and N-sufficient citrus nursery trees; Experiment 2 part 1 (n=6)

	Midday stem water potential (MPa)	
[x]Day	[y]N-	N+
1	-0.79 ± 0.02	-0.78 ± 0.03
2	-0.78 ± 0.03	-0.80 ± 0.03
3	-0.80 ± 0.03	-0.86 ± 0.02
4	-0.78 ± 0.02	-0.90 ± 0.01
5	-0.80 ± 0.01	-0.91 ± 0.01

	Df	F	P
N status x day	4	2.532	0.0518
N status	1	16.39	0.0002
Day	4	3.209	0.0202

[x]The measurements were taken over five consecutive days
[y]N- = no N applied; N+ = 200 mg·L $^{-1}$per week

Table 6-7. Net photosynthetic rate for N-deficient and N-sufficient citrus nursery trees, Experiment 2 (n=6)

	[x]Net Photosynthetic Rate (µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$)	
Days	[y]N-	N-
0	5.83 ± 0.61	5.26 ± 0.52
2	5.60 ± 0.86	5.25 ± 0.62
4	5.84 ± 0.64	5.54 ± 0.73
6	5.32 ± 0.42	6.76 ± 0.71
8	5.56 ± 0.94	7.02 ± 0.39

	Df	F	P
N status X days	4	1.182	0.33
N status	1	0.6340	0.4297
Days	4	0.5886	0.6724

[x]Net photosynthetic rate was measured every other day over 8 day experimental period.
[y]N- = no N applied; N+ = 200 mg·L $^{-1}$per week

Table 6-8. Whole plant Nitrogen content (%) for N-deficient and N-sufficient citrus nursery trees (Experiment 2, part 1)

	N content (%)	
Day	N-	N+
1	1.25d	1.42c
2	1.31cd	2.79a
3	1.31d	1.97ab
4	1.58c	2.13b
5	1.28cd	2.72a

	Df	F	P
N status x day	4	1.572	0.2556
N status	1	17.42	0.0019
Day	4	1.633	0.2409

Table 6-9. Whole plant Nitrogen content (%) for N-deficient and N-sufficient citrus nursery trees (Experiment 2, part 2)

	N content (%)			
Day	N+ to N+	N+ to N-	N- to N+	N- to N-
1	[x]1.87bcd	3.22a	1.86c	1.23d
2	3.02a	2.03cd	1.37d	1.05d
3	2.50b	2.56abc	1.41d	1.28cd

	Df	F	P
N status x day	6	2.720	0.0661
N status	3	15.65	0.0002
Day	2	0.3419	0.7171

[x]Means separation within columns by Tukey's HSD test, $P < 0.05$.

Table 6-10. Concentration of dihydro-zeatin riboside (DHZR), a cytokinin in the xylem sap of N-deficient and N-sufficient citrus nursery trees; Experiment 2, part 1 (n=4)

Day	Conc. of dihydro-zeatin riboside (DHZR) (picomoles/ml)	
	[x]N-	N+
1	38.69 ± 3.23	34.48 ± 3.14
2	31.06 ± 4.79	42.73 ± 4.30
3	29.22 ± 5.0	36.73 ± 4.18
4	38.53 ± 3.67	39.83 ± 1.44
5	29.98 ± 6.09	36.68 ± 3.40

	Df	F	P
N status X days	4	1.570	0.2079
N status	1	4.888	0.0348
Days	4	0.5176	0.7234

[x]N- = no N applied; N+ = 200 $mg \cdot L^{-1}$ per week

Table 6-11. Concentration of dihydro-zeatin riboside (DHZR), a cytokinin in the xylem sap of N-deficient and N-sufficient citrus nursery trees; Experiment 2, part 2 (n=4)

	Conc. of dihydro-zeatin riboside (DHZR) (picomoles/ml)			
Day	[x]No N to N	No N to No N	N to N	N to No N
1	[y]34.38ab	31.47b	38.10ab	38.08ab
2	33.80b	32.39ab	38.39ab	41.84a
3	39.71a	28.43b	36.79ab	38.85ab

	Df	F	P
N status X days	6	0.7050	0.6475
N status	3	5.003	0.0053
Days	2	0.1413	0.8687

[x]N- = no N applied; N+ = 200 mg $\cdot L^{-1}$per week

[z]Means separation by Tukey's HSD test, $P < 0.05$.

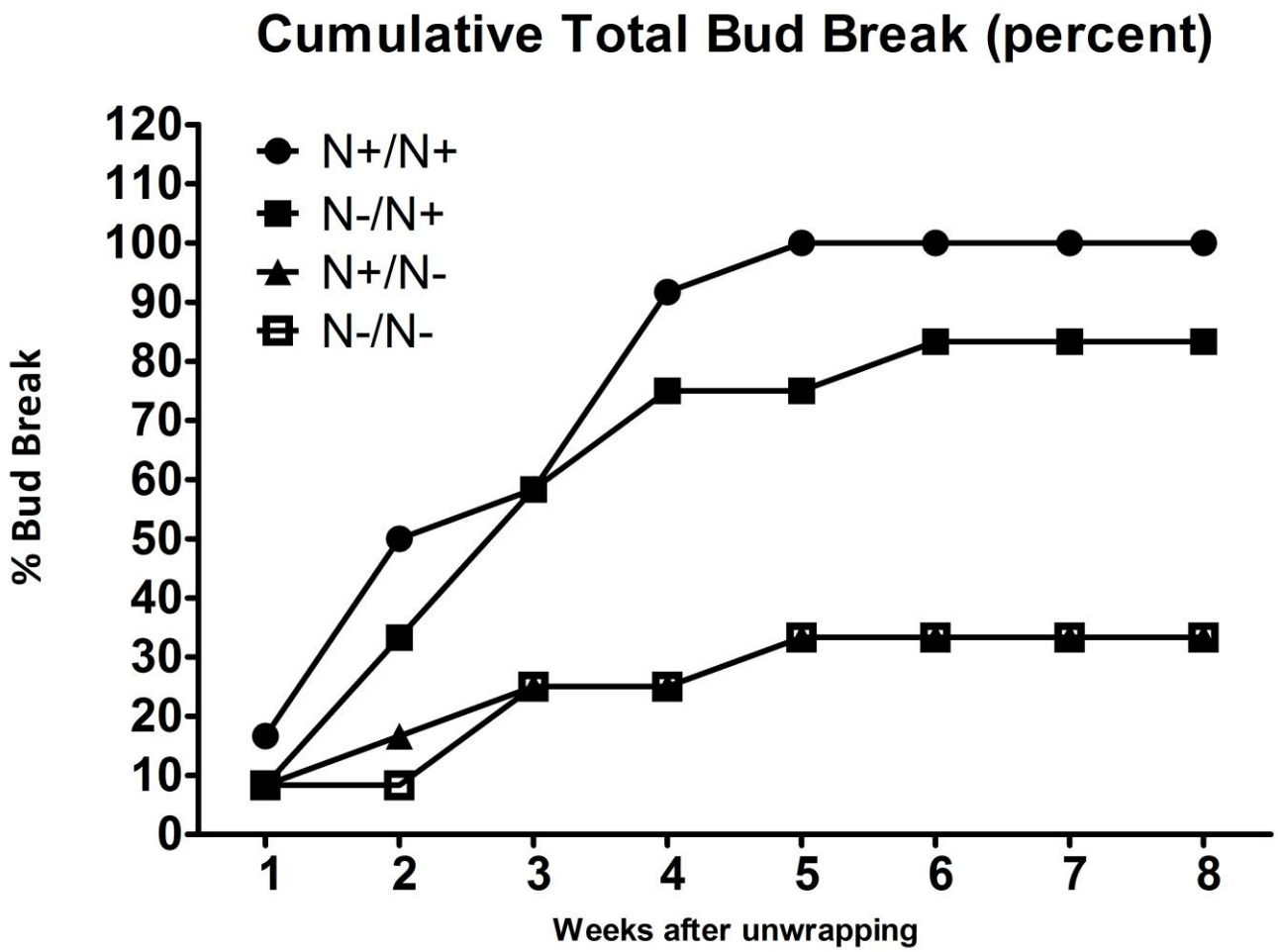


Figure 6-1. Cumulative percent bud break in N deficient and N sufficient liner trees of Swingle citrumelo rootstock budded with buds from N deficient and N sufficient budwood trees in container grown citrus nursery (n=12)

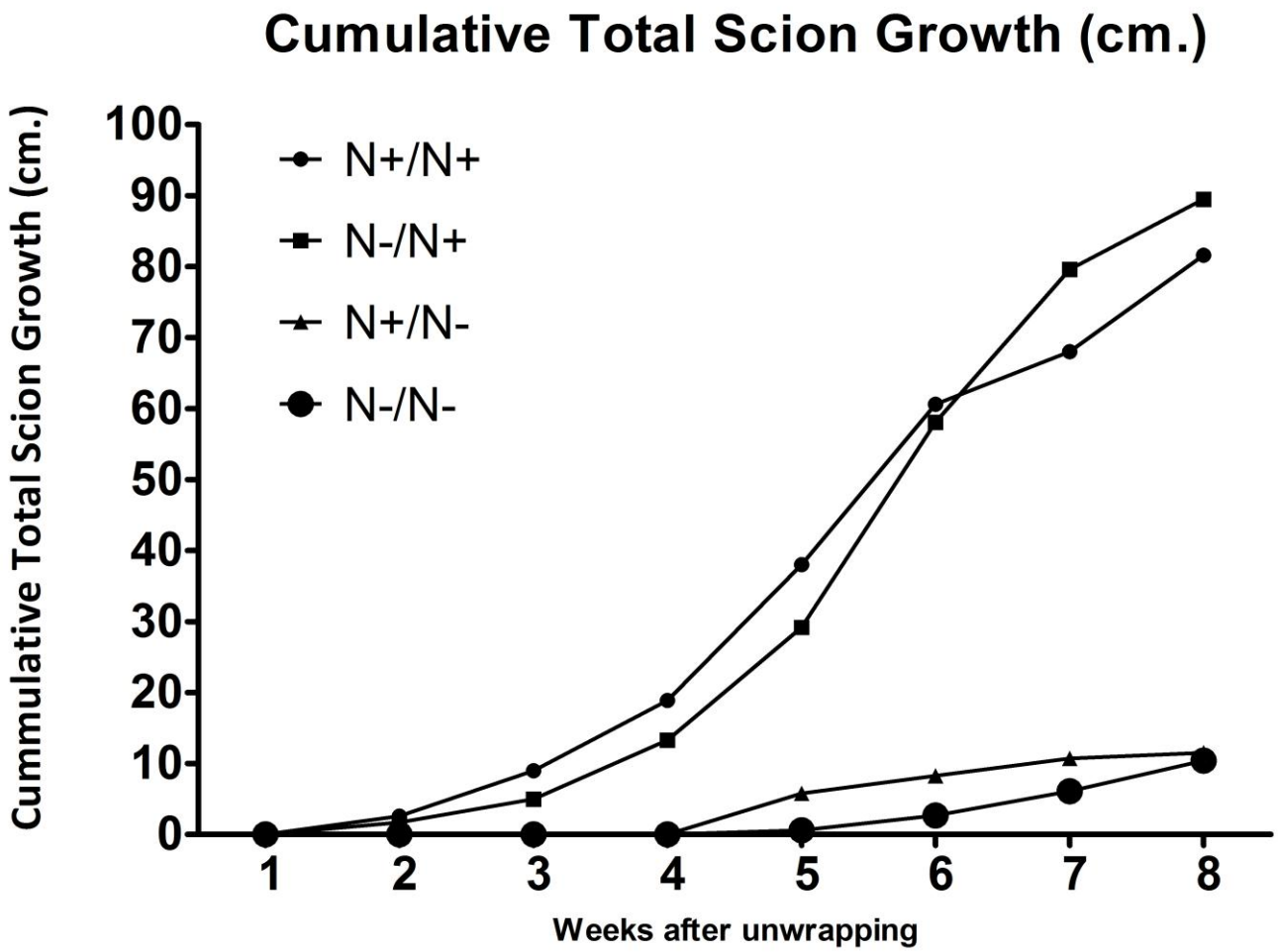


Figure 6-2. Cumulative scion growth in N deficient and N sufficient liner trees of Swingle citrumelo rootstock budded with buds from N deficient and N sufficient budwood trees in container grown citrus nursery (n=12).

Figure 6-3. A picture showing visual comparison of an N deficient tree with a tree having higher N content. The tree on the left shows yellowing of the leaves, a typical N deficiency symptom. (Photo: Gurreet Brar)

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BIOGRAPHICAL SKETCH

Gurreet Pal Singh Brar was born in Punjab, India in 1978. He attended Government High School Rupana and DAV College Chandigarh for his high school and senior secondary education, respectively. He received his BS Agriculture (Honors) at Punjab Agricultural University in 1999 and his MS Horticulture (Pomology) at the same institution. He worked on nutrient removal by pear cv. Patharnakh and graduated with MS in August 2002. After that he worked at various positions across research and extension systems in Pepsi Foods, Punjab Agricultural University (PAU) Fruit Research Station and Department of Forestry and Natural Resources at PAU. He joined Ph.D. program at University of Florida in 2008 in Horticultural Sciences. He has done extensive work in horticultural extension along with applied research. He has written three books for the farmers so far and published more than three dozen trade journal articles.