Enhancing Plant Tolerance to Cold, Heat and Drought Through the Use of Selected Plant Health Protectants

By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Agricultural Science
in the Department of Plant and Soil Sciences

Mississippi State, Mississippi

December 2012

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Title of Study: Enhancing Plant Tolerance to Cold, Heat and Drought Through the Use of Selected Plant Health Protectants

Pages in Study: 237

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Studies were conducted to evaluate the effects of plant health protectants Pageant (pyraclobstrobin + boscalid), Regalia (extract of *Reynoutria sachalinensis*) and MBI-501 (an anti-transpirant) on drought, heat and cold tolerance. To measure effects on drought tolerance, Pageant, Regalia or MBI-501 were foliar applied to impatiens at four rates (0.0×, 0.5×, 1.0×, and 1.5×) based on the label rates of 0.228 $g \cdot L^{-1}$ (Pageant), 10 $mL \cdot L^{-1}$ (Regalia) and 2 $mL \cdot L^{-1}$ (MBI-501) and to tomato plants at two rates (0.0× and 1.0×) based on the label rates of 0.559 $g \cdot L^{-1}$ (Pageant), 10 $mL \cdot L^{-1}$ (Regalia) and 2 $mL \cdot L^{-1}$ (MBI-501) grown with different target substrate volumetric water contents (TVWC). Pageant applied at the 1.0× rate to well-watered impatiens, had greater shoot dry weight compared to water stressed plants. Regalia application increased root dry weight, leaf chlorophyll content and photosynthetic rate of impatiens and tomato plants. However, results tended to be in the higher TVWC (Pageant and Regalia) to moderately stressed conditions (Regalia). To evaluate heat tolerance in *Impatiens walleriana* 'Super Elfin XP White' (impatiens), Pageant (0.228 $g \cdot L^{-1}$), Regalia (10 $m \cdot L^{-1}$) or MBI-501 (2 $mL \cdot L^{-1}$) were

applied prior to the heat event. Photosynthetic rate was less with impatiens exposed to the heat event compared to plants not exposed to the heat event. However, there was no indication Pageant, Regalia or MBI-501 improved heat tolerance. To evaluate heat tolerance in *Solanum lycopersicum* 'BHN 640' (tomato) plants, Regalia was foliar applied at the 1.0× rate at 24 h or 1 h before the heat event. There was no indication Regalia improved heat tolerance. *Fragaria ×ananassa* 'Camarosa' (strawberry) plants were evaluated for chilling tolerance following application of Regalia at the 1.0× rate in a growth chamber. Results indicated no increase in chilling tolerance of strawberry plants compared to plants receiving no Regalia or chilling treatments. *Citrus unshiu* 'Owari' (satsuma) leaves were evaluated for freeze tolerance after application of Regalia at 1.0× (10 $mL \cdot L^{-1}$) rate in a programmable ultra-low freezer. Results indicated no increased freeze tolerance in satsuma leaves compared to leaves from plants receiving no Regalia or freezing treatment.

ACKNOWLEDGEMENTS

When I started down this path I knew it wasn't going to be an easy task but I didn't know how much support from GOD, my parents, my committee, my friends, and my fellow graduate students it would take.

Three years ago I started a project that tested every part of my well-being. And, to thank everyone who contributed along the way is no easy task. Everyone of my committee members, Dr. Richard Harkess, Dr. Tricia Knight, Dr. Eugene Blythe, Dr. Maria Tomaso-Peterson and last but not least Dr. Charles Gilliam, all contributed to my success. Through the good and the bad, I learned more from them then they will ever know. I don't think that anyone contributed any more or any less than another; however, I do want to express my extreme gratitude to Tricia Knight for not only mentoring me through this process but also opening up a world of opportunities.

I think the biggest obstacle I had to face was running enzyme assays. And, if it wasn't for Dr. Brian Baldwin for helping me find supplies and space, to Dr. Maria Tomaso-Peterson for letting me occupy space, Daniel Barnes for having the patience to explain step by step the ins and outs of enzyme assays, Candace Williams for introducing me to Leigh Williams whose help was unprecedented in getting the assays ran, and to my good friend Dr. Natalie Calatayud for reminding me that it is just another hurdle.

To Drs. Brian Trader and Mengmeng Gu, who started down this path with me, only to take there on path when the time came. They have both continued to be extremely supportive of me and my future. I don't think I will ever be able to fully thank Dr. Gu for allowing me the opportunity to visit China. It was an unbelievably, humbling experience that I will never forget. Not to mention, Drs. Jim Robbins and Curt Rom, two of the wonderful people I was able to get to know during the three week trip.

To my parents, Dale and Judy Hopper and Herb and Nancy Cochran, for accepting my dedication to my research, while putting up with all my uncertainty and absentness from family gatherings. To Helen, Scott, Margaret, and Jennifer who never let distance keep us from growing closer. To my nieces and nephews, Cody, Gradie, Kaylie and Davis, for the countless times I told them my career was not "being a student". To the two little ones, Liam and Alex, I am just glad you are not old enough to enquire. To my grandmother's, Mary-Margaret Cochran and Angela Burkett…thanks for everything!

To everyone else who at one time or another has leant a hand, an ear, a shoulder or a much needed distraction, Natalie Calatayud, Rebecca Munshaw, Meghan Johnston, Kyle Briscoe, Cecelia Langhorne, Jon Carson, Anna Horton, Mark McLaughlin, Ken Hubbard, Tyson Raper, Kevin Dillon, Dr. Rocky Lemus, Dr. Paul Meints, Dr. Christine Coker, Kathy Johnson and Shane Huff, I will forever by grateful!

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CHAPTER I

INTRODUCTION

A topic gaining popularity in agronomic and horticultural crop production is the promotion of plant health and stimulation of plant immunity. At the American Phytopathological Society meeting in 2009 one of the hot topic sessions was "The use of fungicides to promote plant physiological benefits in crops" (American Phytopathological Society, 2009). This was one of the first forums to discuss the registration of strobilurin fungicides for uses other than disease management in crops and opened the floor to other plant health protectants.

Strobilurins (conventional fungicides) have been shown to increase yields through direct effects on photosynthetic efficiency and transpiration rate (BASF, 2009). Additionally, an extract of giant knotweed (nonconventional fungicide) has been reported to increase chlorophyll values, increase the activity of peroxidases, polyphenoloxidases, and Phe ammonia-lyase (Daayf et al. 1997). Other plant protectants, such as antitranspirant compounds, have been shown to increase water use efficiency in plants through reduced transpiration (MacDonald et al., 2009).

Though it is known some fungicides stimulate growth and may improve plant health (Balba, 2007), little research has evaluated these compounds for increasing ornamental plant tolerance to cold, heat or drought.

Pyraclostrobin + boscalid (Pageant: BASF, Research Triangle Park, N.C.), an extract of *Reynoutria sachalinensis* (Regalia: Marrone Bio Innovations, Inc., Davis, CA), and an antitranspirant based on a long chain fatty alcohol (C8-C18) (MBI-501: Marrone Bio Innovations, Inc., Davis, CA) were evaluated for increasing *Citrus unshui* 'Owari', *Fragaria ×ananassa* 'Camarosa', *Solanum lycopersicum* 'BHN 640', and *Impatiens walleriana* 'Super Elfin XP White' tolerance to cold, heat, or drought. The objectives of this research were to:

1. Investigate the effect of plant protectant applications and timing, on overall health of cold and heat sensitive plants.
2. Determine drought tolerance and water use efficiency of drought sensitive ornamentals after applications of plant protectants.
3. Detect enzymatic activity in ornamental and specialty crops following plant protectant application to determine the physiological processes leading to increased plant immunity.

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CHAPTER II

LITERATURE REVIEW

Plant Stress

Water (drought) and temperature (cold or heat) are two of the major abiotic stress factors affecting plant growth (Schulze et al., 2005). Some of the injury symptoms and tolerance mechanisms associated with them are similar. For instance, plant water status can be affected by drought and low temperatures through partial to complete dehydration of plant cells (Schulze et al., 2005; Verslues et al., 2006). Drought conditions cause a chain of events starting with a decreased soil water potential which limits water uptake by the plant, eventually causing cell dehydration. Low temperatures cause extracellular ice formation and, through plasmolysis, the water within the cell is lost resulting in a dehydrated cell (Mengel et al., 2001; Verslues et al., 2006). Additionally, these environmental stress factors have been linked to increased reactive oxygen species (ROS) (Gulen and Eris, 2004). ROS are byproducts of plant metabolism and are vital for plant growth, even though they are highly toxic due to their oxidative abilities (Robert et al., 2009). Formation of ROS begins with the excitation of triplet ground state oxygen (O_2) to form singlet oxygen ($^1O_2$), or reduction of one electron to form superoxide radical ($O_2^{\cdot-}$), reduction of two electrons to form hydrogen peroxide (H_2O_2), or the reduction of three electrons to form a hydroxyl radical ($HO^{\cdot}$) (Mittler, 2002). Chloroplasts,

mitochondria, plasma membrane and apoplastic space are all sources of ROS in plants (Mittler et al., 2004; Rio et al., 2002; Robert et al., 2009). Since ROS are highly reactive, plants have developed protective mechanisms against oxidative damage in the form of antioxidant enzymes. These antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate-peroxidase (APX), glutathione reductase (GR) and glutathione-*S*-transferase (GST), scavenge the plant for excited oxygen species caused by abiotic stress (Mittler et al., 2004; Wu and von Tiedemann, 2002; Zhang et al., 2010).

Once a plant becomes stressed, normal growth and development ceases. The ability to overcome such stress is referred to as stress tolerance (Luan, 2002). The level to which a plant can tolerate stress depends upon the plant's physiology and biochemistry (Pagter et al., 2008a). For instance, Yamada et al. (2002) conducted a study with tropical and subtropical species and reported that *Saintpaulia* leaves were seriously injured within 15 min, whereas orchid leaves exposed to -2 °C resulted in no injury (Yamada et al., 2002). Furthermore, *Hydrangea macrophylla* stem hardiness is limited to -18 °C, whereas *H. paniculata* 'Grandiflora' clones of different origin are hardy to -36°C to -37 °C (Pagter et al., 2008b).

Plant Health Protectants

Conventional Fungicide: Strobilurins

Strobilurins were first evaluated as natural products isolated from *Strobilurus tenacellus*, *Oudemansiella mucida*, and *Myxococcus fulvus* (Bartlett et al., 2002). Strobilurins have activity against the four major fungi groups (Ascomycetes,

Basidiomycetes, Deuteromycetes and Oomycetes) and account for one-fifth of the fungicide market (Sauter, 2007). As of 2007, there were nine strobilurin fungicides on the market: kresoxim-methyl, azoxystrobin, metominostrobin, trifloxystrobin, picoxystrobin, pyraclostrobin, fluoxastrobin, dimoxystrobin, and orysastrobin. Although strobilurins were derived from natural substances, they are sold in synthetic forms due to their highly photo-degradable nature (Balba, 2007).

Classified as complex III inhibitors, the strobilurins act as mitochondrial respiration inhibitors (Sauter, 2007). The fungicide targets the electron transport chain in the mitochondria of the fungus, specifically, the quinol oxidation site of the bc_1 complex binding site (Balba, 2007), preventing electron transfer between the Q_o site of cytochrome b and cytochrome c_1, disrupting the production of ATP, and stopping all respiration (Bartlett et al., 2002). In the plant, the fungicide is a translaminar type with only limited translocation through the leaf. The target site in the plant is the electron transport chain in the mitochondria. The fungicide inhibits mitochondrial respiration triggering positive changes in the plant such as increased growth efficiency, increased stress tolerance, and disease control (BASF, 2009).

Non-conventional Fungicide: Extract of *Reynoutria sachalinensis*

Extract of *Reynoutria sachalinensis* (giant knotweed), also known as Regalia, is distributed by Marrone Bio Innovations as an organic fungicide with activity against powdery mildew, gray mold, and blights (Marrone Bio Innovations, 2011). Regalia's indirect mode of action is seen through the increased production of phytoalexins which strengthen the plant's immune system (Konstantinidou-Doltsinis and Schmitt, 1998).

After a plant has been affected by a biotic or abiotic agent, phytoalexins (antimicrobial compounds) are synthesized as a defense mechanism (Vasconsuelo and Boland, 2007). Some of these phytoalexins are lytic enzymes, such as chitinases and glucanases, oxidizing agents, cell wall lignifications, pathogenesis-related proteins, and transcripts of unknown functions (Mert-Türk, 2002). Additionally, Regalia has been reported to increase chlorophyll values and the activity of peroxidases, polyphenoloxidases, and Phe ammonia-lyase (Daayf et al., 1997). Peroxidases, are involved in lignin polymerization, cross-linkage of cell wall constituents, catabolism of auxin, formation of ROS and defense against pathogenic organisms (Bakalovic et al., 2006). Lignin polymerization provides rigidity and structural support to cell walls (Kärkönen and Koutaniemi, 2010). Under water stress, plant species with more elastic cells have relatively no change in cell water potential as water is removed; however, the more rigid a cell wall is, the greater the loss in water potential with minimal loss in water. Therefore, the more rigid cell walls are, the easier it is for water uptake without severe dehydration (Mengel et al., 2001). Thus, if application of Regalia increases peroxidases, it could result in heightened lignin polymerization and result in a more rigid cell wall, preventing extreme cell dehydration.

Antitranspirant: MBI-501

MBI-501 is a reflective type antitranspirant based on a long-chain fatty alcohol. When sprayed on leaves, reflective anti-transpirants reflect back a portion of radiation, decreasing leaf temperature and reducing transpiration (Goreta, et al., 2007; Patil and De, 1976). Although the mode of action for MBI-501 is not completely understood, reports

indicate a greater translocation of photosynthates and photosynthetic activity (personal communication, Marrone Bio Innovations).

Cold Stress

In 2007, the southeastern U.S. had abnormally warm temperatures in the month of March and April experienced record lows (NOAA, 2007). Due to the mild temperatures in March, many ornamental plants throughout the southeast initiated bud break leading to significant crop losses when the temperatures dropped below freezing in April. The damage was so extensive the browning of vegetation could be seen by space satellites. Under low temperatures, the ability of a plant to take up and conduct water is slowed, resulting in plant stress (Bray, 1997). If crops acclimate to cooler temperatures and then a heat event occurs, the cold acclimation is canceled and new growth is stimulated (Ferguson, 1995). Once new growth is initiated, high temperatures can reduce the overall flowering of cool season crops (Warner and Erwin, 2006) such as *Viola ×wittrockiana* Gams. (pansy) (Niu et al., 2000).

One of the major stress factors affecting plant growth and productivity is chilling or freezing injury. Chilling injury occurs when temperatures are low, but not below freezing (Zhang et al., 2009) and freeze injury occurs below 0°C (Jan et al., 2009). Injury usually occurs with the formation of ice on the outside of the plant with ice formation progressing into the plant cells through diffusion (Uemura and Steponkus, 1999). A plant's response to cold stress depends upon its physiology and biochemistry (Pagter et al., 2008a), which can be related to its origin (Jan et al., 2009). Temperate region plants can increase their freezing tolerance when exposed to low non-freezing

temperatures, whereas tropical and subtropical species are more sensitive to chilling and typically lack the ability to acclimate to cold temperatures (Jan et al., 2009).

Chilling and Freeze Injury

Chilling injury inhibits or slows growth, whereas freeze injury can cause discoloration and/or death. The first signs of cold injury are seen in the cell's inability to increase membrane fluidity, causing membrane leakage (Verslues, et al., 2006). As temperatures continue to decrease, injury gradually becomes more severe because of extracellular ice formation due to the lower extracellular solute concentration compared to the solute concentration inside the cell (Jan et al., 2009). When ice forms in the extracellular spaces there is a drop in water potential outside the cell, which causes the water from the cytoplasm to move through the plasma membrane by osmosis causing cellular dehydration (Xin and Browse, 2000). Dehydration is a common injury symptom associated with freeze injury in plants due to the formation of ice within the cell membranes (Thomashow, 2001). This osmotic dehydration subsequently triggers a response in the hexagonal II phase associated within the plasma membrane (Uemura et al., 2006; Kawamura and Uemura, 2003). During this reaction, there is an increase in the cryostability of the plasma membrane (Uemura et al., 2006) likely due to the accumulation of cold-induced proteins (Uemura et al., 2006; Kawamura and Uemura 2003). Injury related to rapid freezing often occurs in the cell and is a result of rapid temperature decrease. Moreover, intracellular ice formation causes puncture wounds in the plasma membrane due to water expansion (Hoshino et al., 1999; Joiner, 1958.).

As temperature decreases, water from within the cell continues to move into the extracellular spaces until total dehydration occurs. However, before total dehydration occurs, equilibrium between the extracellular and intracellular spaces may be reached (Yelenosky and Guy, 1989). Thus, damage may be limited and reversible with no injury to the cell. Extent of damage is dependent upon exposure time, how quickly the extracellular ice thaws, and the rigidity of the cell (Joiner, 1958). With highly elastic cell walls, as compared to more rigid cells, there is a greater reduction in cell volume therefore disrupting the protoplast as water moves back into the cell with warming temperatures (Xin and Browse, 2000).

In many instances injury related to low temperatures may take several hours or days before injury is evident. For instance, African violet leaves exposed to -2 °C were seriously injured within 15 min, whereas mungbean seedlings exhibited only 30% injury after 1 hour (Yamada et al., 2002).

Cold Acclimation

Many plant species have adopted cold acclimation mechanisms that allow them to survive freezing or chilling temperatures with minimal damage (Xin and Browse, 2000). Plants acclimate to cold temperatures naturally under shortened day lengths (Pagter et al., 2008b; Xin and Browse, 2000) and repeated exposure to low temperatures (Jan et al., 2009). During cold acclimation, plants respond by decreasing tissue water content and accumulating soluble carbohydrates, amino acids, and proteins (Pagter et al., 2008a). These compounds protect the cells from freezing and/or dehydration (Pagter, 2008a; Li et al., 2004). Additionally, the lipid composition of the plasma membrane is altered thereby

serving as a protective barrier against seeding of the supercooled cytosol by the extracellular ice (Uemura and Steponkus, 1999).

Heat Stress

It is inarguable that high temperatures can reduce plant growth (Wise et al., 2004). In 2007, the Intergovernmental Panel on Climate Change predicted an increase of 1.8 to 4.0 °C over the next 100 years (Xu et al., 2009). Subsequently, the higher temperatures will increase atmospheric CO_2 concentrations, alter rainfall regimes, and indirectly affect respiration and photosynthesis of crop species (Hedhly et al., 2008). These high temperatures could cause a decline in photosynthesis due to increases in photorespiration, resulting in heat stressed plants (Sharkey, 2005). Heat stress limits plant biomass production and productivity through physiological and metabolic processes (Wahid et al., 2007; Allakhverdiev et al., 2008). With the predicted temperature increases associated with global warming, heat stress will become an increasingly important issue for crop production (Asthir et al., 2009).

Wahid et al. (2007) defined heat stress as the plant's response to a rise in temperature (usually 10 to 15 °C above ambient, for an extended time) causing irreversible damage to plant growth whereas, heat tolerance is the plant's ability to survive high temperatures. Furthermore, the extent of the damage and response of the plant are dependent upon species and climatic zone, which may also determine the threshold temperature. Threshold temperature refers to the low and high temperatures a plant can tolerate and still experience normal growth (Wahid et al., 2007). High temperatures causing heat stress can have a negative impact on growth and productivity,

particularly in the warm summer months and in temperate climatic regions (Huang and Xu, 2008). Temperate plants usually have lower threshold temperatures compared to tropical plants. Wheat, a temperate crop, experiences a 4% decrease in yield for every 1 °C increase higher than the high threshold temperature (25 °C) (Asthir et al., 2009). However, threshold temperature varies among species, so determining specific threshold temperatures is difficult (Wahid et al., 2007). For example, *Brassica* will see adverse affects in flowering when threshold temperature reaches 29 °C, whereas cowpea can withstand temperatures up to 41 °C (Morrison and Stewart, 2002; Wahid et al., 2007). Furthermore, it has been reported that, once temperatures reach 30 °C, photosynthesis peaks and for every 1 °C increase above 30 °C, assimilation declines (Wise et al., 2004). Subsequently, even a brief exposure to high temperatures can cause damage to a plant by diverting its energy away from photosynthesis (Siddique et al., 1999).

Heat Injury

Under high temperatures, photosynthesis in plants is affected, specifically the photosynthetic activity of chloroplasts (Wise et al., 2004; Allakhverdiev et al., 2008). Under normal conditions, photosynthesis converts light energy into chemical energy for plant use. Photosynthesis takes place in the leaves, specifically in the chloroplasts using chlorophyll as the receptor/trapping molecules. In heat stressed plants, photosynthesis is altered and plant growth is affected. There are many processes involved in photosynthesis and it only takes alteration of one of those processes to affect plant growth (Wahid et al., 2007). There are at least three sites reported to be stress sensitive for the mechanism of photoinhibition: 1) ROS inhibit the repair of photosystem II (PSII) causing

photodamage in the oxygen-evolving complex, 2) ATP generating, and 3) the carbon assimilation process (Murata et al., 2007; Allakhverdiev et al., 2008). The damage at these sites depends on stress and the equilibrium between the damage and repair processes (Allakhverdiev et al., 2008). In potato leaves, the failure of photosynthetic electron transport at elevated temperatures affects the thermolability of PSII (Ogweno et al., 2009). Under high temperatures, PSII activity is slowed or inhibited, which can lead to separation of the oxygen evolving complex (OEC) or inhibition of OEC. Furthermore, high temperatures alter the energy distribution of photosynthesis, changing carbon metabolism enzymes, disrupting the electron transport, and inactivating the oxygen evolving enzymes of PSII (Wahid et al., 2007).

High temperatures can also induce oxidative stress. Protection against oxidative stress is essential for plant survival. Oxidative stress resulting from high temperature, can activate cell signaling pathways to produce stress proteins (Bajguz and Hayat, 2009). In response to oxidative stress, plants have developed enzymatic and non-enzymatic detoxification systems to protect against cell damage. When plant cells are injured due to high temperatures, they will generate ROS (Asthir et al., 2009). Chloroplasts are the main intracellular ROS source in plants (Robert et al., 2009) and the most heat sensitive cell function due to their photosynthetic activity (Allakhverdiev et al., 2008). During photosynthesis and respiration, the plant is steadily producing ROS and the state of the cell is controlled by protective mechanisms (Bajguz and Hayat, 2009). If these protective mechanisms are disturbed, oxidative damage can result in death of the cell. Under regular growth conditions, ROS production is very low; however, under heat stress the production is increased. This increased production of ROS causes lipid peroxidation,

protein denaturation, and DNA damage (Asthir et al., 2009). Since ROS are highly reactive, plants have developed protection mechanisms against oxidative damage in the form of antioxidant enzymes. These antioxidant enzymes, such as SOD, catalase (CAT), peroxidase (POX), ascorbate-peroxidase (APX), glutathione reductase (GR) and glutathione-*S*-transferase (GST) scavenge the plant for excited oxygen species caused by stress (Mittler et al., 2004; Wu and von Tiedemann, 2002; Gill and Tuteja, 2010; Zhang et al., 2010). The searching of O_2^- by SOD produces H_2O_2 which is then removed by APX or GR in the ascorbate-glutathione cycle (Çiçek and Çakurlar, 2008).

Heat Tolerance

When plants are exposed to high temperatures, heat shock proteins (HSP) are produced to protect proteins, membranes, and other cellular components (Barua et al. 2008; Queitsch et al., 2000). Plants from arid and semiarid regions can produce and collect significant amounts of HSP (Wahid et al., 2007). These HSP protect cells against high temperatures (Barua et al., 2003), conferring heat tolerance in the photosynthetic electron transport chain in isolated chloroplasts (Allakhverdiev et al., 2008). HSP have been correlated with the organism under stress, having extremely fast and intensive biosynthesis and their induction into a diversity of cells and organisms (Wahid et al., 2007). Furthermore, HSP are differentiated into 3 classes: HSP90, HSP70, and low molecular weight proteins of 15 – 30 kDa. Low molecular weight proteins are programmed by six nuclear gene families targeted at different proteins in separate cellular compartments: cytosol, chloroplast, endoplasmic reticulum, mitochondria, and membranes (Wahid et al., 2007). Low molecular weight proteins have also been shown

to connect with thylakoids protecting the O_2 evolution and oxygen-evolving complex proteins from heat stress (Allakhverdiev et al., 2008). Since HSP increase in the plant during heat stress, they are thought to be essential in enhancing thermotolerance (Singh and Shono, 2005).

Heat tolerance directly affects plant growth by regulating leaf gas exchange (Wahid et al., 2007). Under moderate heat stress, stomatal conductance and net photosynthesis can be slowed due to the reduction in rubisco activation. Stomata allow CO_2 entry for photosynthesis and regulate water loss by signalling the guard cells (Acharya and Assmann, 2009). Stomata also help to control leaf temperature by regulating water loss through transpiration.

Several plant hormones aid in stomatal function: auxins, abscisic acid (ABA), salicylic acid, cytokinins, ethylene, jasmonates, and brassinosteroids. Snyman and Cronje (2008) reported salicylic acid enhanced heat shock response by increasing the levels of HSP70. Brassinosteroids promote growth and are polyhydroxylated steroidal plant hormones (Acharaya and Assmann, 2009) that have been reported to protect against heat stress and other environmental stresses (Kagale et al., 2007; Symons et al., 2008; Xia et al., 2009). Confraria et al. (2007) reported 24-epibrassinolides (EBR) protected in in-vitro grown potato plants from heat stress. Additionally, brassinosteroids have been shown to work with ABA to regulate stomatal development and function (Acharya and Assmann, 2009). Dhaubhadel et al. (1999) reported brassinosteroids, specifically EBR, confer heat tolerance in plants and increase basic thermotolerance of *Brassica napus* and tomato seedlings. Additionally, after exposure to elevated temperatures, concentrations of HSP were increased in treated seedlings (Dhaubhadel et al., 2002). Kagale et al.

(2007) reported EBR enhanced the basic thermotolerance of *Arabidopsis thaliana* seedlings exposed to 43 °C. They came to the conclusion that EBR enhances a plant's reaction to heat, increasing the plant's tolerance. Anuradha and Rao (2007) reported brassinosteroids protected radish seedlings from Cd-induced oxidative stress by weakening the impact of ROS. Bajguz and Hayat (2009) reported the use of exogenously applied brassinosteroids enhanced antioxidant enzymes such as SOD, CAT, and POX under high temperatures in tomato leaves. Singh and Shono (2005) reported 1 μM EBR applied to tomato plants increased survival when plants were exposed to 45 °C for 3 hours compared to nontreated plants. These reports suggest brassinosteroids do in fact play a vital role in protecting plants from heat stress.

Heat stress can cause non-threatening injury or can be detrimental to a crop, depending on the species. Plants with a lower threshold temperature will be most sensitive to global warming. For example, if we take the reported decrease in wheat (a temperate crop) yield of 4% with every 1 °C increase in temperature (Asthir et al., 2009) and factor it into the predicted temperature increase (Xu et al., 2009), we could see a decrease in yield of 7.2% to 16% over the next century.

In oxidative stressed plants, production of stress proteins is essential to avoid death. Furthermore, the production of HSP are essential for increasing thermotolerance in plants by protecting cells from high temperatures. Biological changes can also have an effect on plant aesthetics. A decline in photosynthesis can lead to wilting, stunting, and necrosis of the plant. A plant injured due to oxidative stress would also show symptoms of wilting, desiccation, and necrosis, which are similar to symptoms of drought stress.

Plant Water Relations

Under ideal growing conditions, there is adequate water available in the soil for plant uptake. For uptake to occur there must be available water in close proximity to the roots and the water potential must be less in plant roots than in the soil. Water potential is defined as the free energy of water. Movement of water in plants is governed by diffusion from high concentration to low concentration (Mengel et al., 2001).

Water status in crop production is a recognized problem worldwide (Passioura, 2007; Farooq et al., 2009). Prolonged drought can cause serious problems, especially in poor countries where it can lead to social upheaval, mass migration, and desertification (Passioura, 2007). In order to minimize the effect of drought conditions and to stabilize crop production, we need to understand how plants respond to drought (Chaves et al., 2009).

Drought

By definition, drought is a period in which rainfall is below average or altogether absent, resulting in inadequate amounts of water for human use, agriculture, vegetation, and fauna. Initial symptoms of drought stress are visible in actively growing plant shoots and also roots to a lesser degree (Neumann, 2008).

Drought Injury

Availability of soil water is the first limiting factor associated with drought stress (Verslues et al., 2006). Decreasing soil available water decreases soil water potential, resulting in less uptake by the plant ultimately affecting plant growth through partial or complete stomatal closure, reduced transpiration and photosynthesis, and decreased

nitrate assimilation (Davies et al., 2002; Neumann, 2008; Sairam et al., 1997). Under prolonged drought, if supplemental water is not supplied, plant growth can be affected with premature leaf drop, wilting, desiccation and/or death (Neumann, 2008).

Transpiration is regulated through openings in the leaf surface known as stomata, which are controlled by guard cells. Under ideal growing conditions, the stomatal aperture is opened by increased solutes (such as potassium and chlorine) in the guard cells, which cause the guard cells to swell as a result of full turgor pressure (Luan, 2002). When turgor pressure declines and the solutes leak out; the stomatal aperture closes (Chaves et al., 2009). Additionally, plants regulate water loss by minimizing the stomatal aperture through increased production of abscisic acid (ABA) (Zhang et al., 2006). Foliar-applied abscisic acid (s-ABA) has been shown to reduce water loss and extend shelf life in bedding plants. Waterland et al. (2010) reported delayed wilting symptoms in impatiens, seed geranium, petunia, marigold, salvia, and pansies following application of s-ABA.

Drought Tolerance

In plant roots, ABA synthesis increases in response to soil water deficits leading to transport through the xylem to the shoot (Comstock, 2002). ABA in the shoot controls the stomata by signalling the guard cells (Kondo et al., 2009) to release potassium and H^+ ions reducing the osmotic potential of the guard cells leading to a decrease in water content thus reducing turgor pressure and closing the stomatal aperture (Sirichandra, et al., 2009). Once the guard cells close the stomatal opening, there is a decrease in stomatal conductance (Liu et al., 2005) and a decrease in carbon dioxide concentration

inside the leaf and chloroplasts (Yordanov et al., 2003). Photosynthetic carbon reduction and carbon oxidation cycles are the primary sink for PSII during mild drought (Cornic and Fresneau, 2002; Yordanov et al., 2003).

In addition to physiological symptoms, water-stressed plants also undergo morphological changes by adaptation of leaf surfaces and chloroplasts to high light (sun) and low light (Yordanov et al., 2003). By minimizing surface area and orienting the leaf surface perpendicular to the ground, plants minimize light exposure reducing photosynthesis and the need for chlorophyll, thus conserving energy.

Methods of Measuring Plant Water Status

Researchers typically measure plant water potential using one of four instruments: a psychrometer, a pressure chamber, a cryocopic osmometer, or a pressure probe. Psychrometers measure vapor pressure of a plant sample, pressure chambers force water out of the plant sample through pressurization, cryocopic osmometers measure the osmotic potential of a plant sample by determining its freezing point, and pressure probes measure plant cell water potential by maintaining turgor pressure of the cell and preventing the cytoplasm from entering the microcapillary (Taiz and Zeiger, 2010; Boyer, 1995). Pressure chambers are more commonly used to measure plant water status because they are light weight and easy to use in the field. When using a pressure chamber, different methods can be used to determine plant water status: pre-dawn leaf water potential (Ψ_{Pre}), mid-day leaf water potential (Ψ_{Mid}), and stem water potential (Ψ_{Stem}) (Chone et al., 2001). Before dawn, plant water status is in equilibrium with the soil; Ψ_{Pre} determines the root zone soil water potential (Williams and Araujo, 2002).

Ψ_{Mid} estimates water stress at maximal photosynthetic rates and water demand (Carroll et al., 2001; McCutchan and Shackel, 1992). Ψ_{Stem} is a measure of soil and leaf water potential showing the whole level of stress the plant is under (Santesteban et al., 2010).

Leaf relative water content (RWC) refers to how much water a leaf can hold. RWC is the ratio of the water content in the leaf at sampling compared to fully turgid (Smart and Bingham, 1974).

Crop Health

Some research has evaluated how applications of pesticides impact overall plant health. Reports have shown an increase in net photosynthesis and growth of maize seedlings by soaking the seed in 150 μM of hydrogen peroxide for 24 hours prior to germination and exposing the seedlings to 107.6°F (Wahid et al., 2008). They attribute this increase to the hydrogen peroxide pretreatment inducing defense genes to offset oxidative damage. Additionally, it has been reported that chilling tolerance in cucumbers can be increased by suppressing the hydrogen peroxide production in the leaves with exogenous application of polyamines (Zhang et al., 2009). Furthermore, BASF has recently added plant health to their Headline fungicide label with approval from the EPA (BASF, 2009). The active ingredient in Headline is pyraclostrobin, a strobilurin fungicide.

Multiple reports have indicated pyraclostrobin increases nitrate reductase activity, increases antioxidant enzymes, increases stress tolerance, reduces the amount of CO_2 lost to the atmosphere (BASF, 2009; Kohle et al., 2002; Nason et al. 2007), and increases the overall green color of plants (Balba, 2007). Increased growth efficiency is seen in a

variety of ways, such as improved nitrogen use through increased nitrate reductase (NR) activity (Bartlett et al., 2002). NR activity is the first step in nitrate-assimilation with reduction of nitrate to nitrite (NO_3^- to NO_2^-) (Kohle et al., 2002). Plants can take up nitrogen in the form of nitrate, but nitrate must be reduced to nitrite and then ammonium before it can be used. Under normal growth conditions, NR is regulated by translation and transcription, activated in light, and deactivated in the dark (Glaab and Kaiser, 1999). This cycle is mediated by a reversible phosphorylation mechanism in which an inhibitor protein binds to NR causing deactivation and, after dephosphorylation, the inhibitor releases the NR resulting in activation (Glaab and Kaiser, 1999). Therefore, the increased activity of NR is more than likely associated with acidification of the cytoplasm and blocking degradation of the NR protein (Glaab and Kaiser, 1999).

With increased levels of NR, a plant can move more nitrite through the plant to the chloroplast. Once in the chloroplast, nitrite is reduced to ammonium then synthesized to amino acids, aiding in leaf development and photosynthesis (Dechorgnat et al., 2011). Thus, increased nitrate reductase activity results in faster nitrogen assimilation, improving nitrogen use (BASF, 2012).

Nitrate reductase under abiotic stress conditions has been shown to be an important supplier of nitric oxide, which expresses plant defense mechanisms (Rio et al., 2004). This can be seen through inhibition of ACC synthase and ACC oxidase, key enzymes involved in the production of ethylene (Kohle et al., 2002). Ethylene is a phytohormone produced in all parts of the plant and increased activity is often seen in a plant under physiological stress. Increased activity in nitrate reductase increases nitric oxide which inhibits ethylene production and the result is a healthier plant.

SOD is an antioxidant enzyme produced to quench ROS. ROS are always present in the plant; however, under stressed conditions (chilling, heat or drought) they are increased. When a plant is under ideal growing conditions, there is an even balance of ROS (Bajguz and Hayat, 2009). However, once a plant is exposed to stress there is an increased production of ROS and, in order for the plant to protect itself; it must produce antioxidant species like SOD. Environmental stresses can enhance the production of ROS leading to pathogen growth (Barna et al., 2003; Kohle et al., 2002). In order for plants to protect themselves from pathogens, such as those causing leaf spot or necrosis, production of antioxidant species (AOS) must be increased (Barna et al., 2003). In disease resistant varieties, this can be done internally; however, fungicides can also be used to increase AOS. Kohle et al. (2002) reported increased levels of peroxidase in winter barley treated with F 500 (a strobilurin-type fungicide) compared to nontreated plants infected with leaf spot. Zhang et al. (2010) reported increased SOD, CAT, and POD in flag leaves of winter wheat treated with azoxystrobin (strobilurin).

Recent reports indicate exogenously applied strobilurins can increase the overall green color of plants (Balba, 2007). The significance behind this research is that exogenously applied substances, such as certain fungicides, are capable of increasing plant health. The green industry could benefit significantly by increasing crop health in changing environments where cold, heat and drought stresses are prevalent.

Conversely, there are multiple reports indicating the strobilurin fungicides do not impact yield or water use efficiency. Schnabel and Crisosto (2008) used a premix of pyraclostrobin and boscalid on peaches and concluded neither fruit development nor fruit qualities were improved. Additionally, picoxystrobin, pyraclostrobin, azoxystrobin,

kresoxim-methyl and trifloxystrobin increased the water use efficiency in well-watered wheat, but not in water-stressed wheat (Nason et al., 2007).

Plant Material Used in This Study

Cold Stress Experiments

Citrus unshiu 'Owari' (satsuma) and *Fragaria ×ananassa* 'Camarosa' (strawberry) were selected as two species commonly damaged during the growing season due to late or early season freeze/frost events. *Citrus unshiu* (satsuma mandarin) is one of 17 *Citrus* species in the Rutaceae (USDA, 2012). They are evergreen shrubs, primarily grown for their edible fruit. California is the leading state in satsuma mandarin production followed by the southeastern U.S. (Fadamiro et al., 2007). Commercial production is currently seen in Alabama, California, Florida, Louisiana, Mississippi, and Texas. California has approximately 3,000 acres of satsuma mandarins in production, followed by Louisiana (300 acres) and Alabama (100 acres). Generally, satsuma mandarin is a cold-tolerant citrus species tolerating temperatures as low -11 °C (Nesbitt et al., 2008), whereas *Citrus grandus* and *Citrus paradisi* can be damaged when temperatures border 0 °C (Champ et al., 2007). Additionally, *Citrus* species are prone to disease problems; therefore, they are usually grafted onto a less susceptible species such as *Poncirus trifoliata* (trifoliate orange). Trifoliate orange belongs to the same family as *Citrus,* but can withstand temperatures as low as -20 °C (Champ et al., 2007).

Fragaria ×ananassa (garden strawberry) is the most commercially known strawberry available (Hancock et al., 2010; Potter et al., 2000). Generally, *Fragaria* spp. are grown in temperate climates where temperatures range from 12 °C (53.6°F) to 26 °C

(78.8°F) (Ledesma et al., 2008). Once temperatures drop below 12 °C, growth of strawberry plants and fruit development begins to slow down, and once temperature drops below 4.4 °C, growth is subdued (Rowley et al., 2010). However, there are some species that, once acclimated, can tolerate -2.2 °C (Warmund and English, 1998). Nevertheless, the plants are still susceptible to chilling damage, especially if flowers and or fruit are present (Rowley et al., 2010). Traditionally, commercial strawberries are field-grown; however, they can be container-grown for winter production (Paranjpe et al., 2003). Additionally, Kadir et al. (2006), reported enhanced early production growing 'Chandler' and 'Sweet Charlie' strawberries in high tunnels in Wichita, KS.

Heat and Drought Stress Experiments

Solanum lycopersicum 'BHN 640' (tomato) and *Impatiens walleriana* 'Super Elfin XP White' (impatiens) were evaluated for heat sensitivity and water use efficiency. 'BHN 640' tomato is a determinate field variety grown commercially for the fresh market. Tomatoes are perennials; however, they are cultivated as annuals (Tigchelaar, 1986). The garden tomato is self-pollinated and has been cultivated for years around the globe for its fresh market value and for processing (paste, juice, sauce, powder, or whole) (Barone et al., 2009). Tomatoes are valuable not only nutritionally, but have also been linked to protection against diseases such as cancer and cardiovascular disease because of lycopene and its antioxidant properties (Barone et al., 2009). Tomatoes are considered the second most popular vegetable crop in the world. They are native to South America but have adapted to very diverse environments (Barone et al., 2009). While tomatoes

will grow in high temperatures, fruit production has been shown to decrease in temperatures over 32.2 °C (89.6°F) and below 21 °C (69.8°F) (Lin et al., 2006).

Impatiens is an annual in the Balsaminaceae. Of the many species belonging to the genus, *I. walleriana* is one of only two species commonly found in the industry, with the other being *I. hawkeri* (Armitage, 2004). Impatiens is a spring to fall blooming annual that requires part to full shade, a moist fertile soil, and copious amounts of water. Under dry conditions, impatiens will have wilted leaves, a common symptom of water stressed plants.

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CHAPTER III

EVALUATING PAGEANT (PYRACLOSTROBIN + BOSCALID) TO IMPROVE WATER USE EFFICIENCY OF *IMPATIENS WALLERIANA* 'SUPER ELFIN XP WHITE' AND *SOLANUM LYCOPERSICUM* 'BHN 640'

Abstract

A strobilurin fungicide, pyraclostrobin (Headline: BASF, ResearchTriangle Park, N.C.) has been reported to increase net photosynthesis and drought tolerance in wheat and corn. However, little research has evaluated strobilurin fungicides in ornamental crops. Experiments using Pageant (pyraclostrobin + boscalid, BASF Corporation, Florham Park, NJ) as a foliar spray were conducted to evaluate its physiological benefits on *Impatiens walleriana* 'Super Elfin XP White' ('Super Elfin XP White' impatiens) and *Solanum lycopersicum* 'BHN 640' ('BHN 640' tomato) under water stress. In Expts. 1 and 2, Pageant was applied to impatiens at four rates based on 3.04 oz per 100 gallons: 0.0×, 0.5× (0.114 $g \cdot L^{-1}$), 1.0× (0.228 $g \cdot L^{-1}$), and 1.5× (0.342 $g \cdot L^{-1}$). In addition, five water treatments based on target substrate volumetric water content (TVWC) in Expt. 1a [85% (well-watered), 70%, 55%, 40% or 25% TVWC] and three water treatments in Expt. 1b [85% (well-watered), 55% or 25% TVWC]. In Expt. 2, water treatments were based on 1, 3, 6, 9 or 12 days between watering (DBW) (Expt. 2a) and 1, 3 or 6 DBW (expt. 2b), maintaining 85% TVWC on days of watering. In Expt. 3, Pageant was applied to tomato plants at 2 rates based on 8 oz per 100 gallons [0× and 1.0× (0.599 $g \cdot L^{-1}$)] and

maintained at 85% (well-watered) or 55% TVWC. Shoot dry weight was greatest with application of Pageant at the 1.0× rate to well-watered (85% TVWC) impatiens. Under the DBW treatments, root dry weight was greater after the 0.5× rate application, compared to the nontreated at 6 DBW. There were no effects of different TVWC levels or Pageant rates on tomato growth. Overall, Pageant applied to well-watered impatiens enhanced shoot growth. Application of Pageant did not increase water use efficiency in either impatiens or tomato. However, after four applications of Pageant at the 0.5× rate, impatiens at 6 DBW (on average 58% TVWC) had greater root mass compared to the nontreated. While there were indications Pageant enhanced growth of impatiens, the results were not consistent within all water treatments. Since multiple reports indicate yield increases in agronomic crops after applying a strobilurin fungicide, further research is warranted in ornamentals, specifically to investigate metabolic functions.

Introduction

At the American Phytopathological Society meeting in 2009, one of the hot topic sessions was "The use of fungicides to promote plant physiological benefits in crops" (American Phytopathological Society, 2009). This forum opened the floor for discussion on how fungicides are now being registered in crops for uses other than disease management. Some fungicides, such as the strobilurins, have been shown to increase yields through direct effects on photosynthetic efficiency and transpiration rate (BASF, 2009).

Strobilurins were first evaluated as natural products isolated from *Strobilurus tenacellus*, *Oudemansiella mucida,* and *Myxococcus fulvus* (Bartlett et al., 2002).

Strobilurins have activity against the four major fungi groups (Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes) and account for one-fifth of the fungicide market (Sauter, 2007). The strobilurins target the electron transport chain in the mitochondria of the fungus (Balba, 2007) ultimately causing death by ceasing all respiration (Bartlett et al., 2002). Conversely, inside the plant the fungicide inhibits mitochondrial respiration, triggering positive changes in the plant such as increased growth efficiency, increased stress tolerance, stress management, disease control (BASF, 2009), and increased overall green color of plants (Balba, 2007). In 2009, BASF added "plant health" to their Headline fungicide (pyraclostrobin) after approval by the environmental protection agency (EPA) (BASF, 2009). Additionally, in 2010 they launched Intrinsic™ brand fungicides into the turf and ornamental market, not only for protection against fungi, but also for added plant health benefits. This brand of fungicide includes two separate brands: Honor® SC Intrinsic™ (pyraclostrobin + boscalid) and Insignia® SC Intrinsic™ (pyraclostrobin). Honor® SC Intrinsic™ includes two fungicides with two target sites: complex III of fungal respiration (pyraclostrobin) and complex II in fungal respiration (boscalid). BASF reported improved turf health after application of Honor Intrinsic by alleviating drought/moisture and temperature extremes (BASF, 2010). Other research has shown increases in antioxidant enzymes after application of strobilurin fungicides to winter wheat (Zhang et al., 2010) and to spring barley (Wu and von Tiedemann, 2002). More recently, the application of ketoconazole to *Catharanthus roseus* alleviated drought stress by enhancing antioxidant potential (Jaleel et al., 2007). However, application of picoxystrobin, pyraclostrobin, azoxystrobin,

kresoxim-methyl and trifloxystrobin increased the water use efficiency of well-watered wheat, but not of water-stressed wheat (Nason et al, 2007).

By increasing crop health, the green industry could benefit significantly by having options in changing climates where drought is prevalent. Although it is known that some fungicides stimulate growth and may improve plant health (Balba, 2007), little research has evaluated strobilurin compounds for increasing water use efficiency in ornamental plants. Pyraclostrobin is one of the strobilurin fungicides reported by BASF to improve drought tolerance in corn and wheat (BASF, 2009). Therefore, we investigated the potential for Pageant (pyraclostrobin + boscalid) (BASF Corporation, Florham Park, NJ) to improve plant water use efficiency in impatiens and tomato plants.

Materials and Methods

Plant material and culture

Experiments 1 and 2

Impatiens walleriana 'Super Elfin XP White' (impatiens) were potted on 5 May 2010 (Expt. 1) and 23 June 2010 (Expt. 2) from 288-plug flats (6 cm^3/cell) into 15-cm (1.85 L) containers with Sunshine Mix 1 (SunGro Horticulture, Bellvue, WA) used as the potting substrate. All containers were filled with substrate to the rim and lightly tapped twice on a hard surface to reduce air pockets. After potting, impatiens were watered thoroughly and placed in a controlled environment greenhouse located on Mississippi State University's main campus and grown for 4 weeks to become established in the container. On 4 June 2010, impatiens were moved to a double-layer inflated polyethylene covered greenhouse located on Mississippi State University's R.R. Foil

Plant Sciences Research Facility in a controlled environment with 60% shade and 24.4 °C/18.3 °C (day/night) set point temperatures.

Experiment 3.

Solanum lycopersicum 'BHN 640' (tomato) seed were sown on 17 May 2011 (Expt. 3a) and the 24 June 2011 (Expt. 3b), in 72-cell pack liners (41-mL) in Sunshine Mix 1 potting substrate. Three weeks later, seedlings (10.2-cm to 15.2-cm tall) were transferred into 15-cm (1.8 L) containers and allowed to grow for two weeks before initiating the experiment. Venting temperatures inside the greenhouse were set to 18.3/15.5 °C day/night (actual greenhouse temperature on average was 27.5 °C day and 24.0 °C night). Experiments were repeated (twice) in time and conducted in a similar manner.

Determining substrate volumetric water content

Physical properties tests as described previously by Hidalgo (2001) were conducted on Sunshine Mix 1 giving 90.9% total porosity, 28.3% air space, 62.6% water holding capacity, and 0.11 g/cc bulk density. Substrate volumetric water content (VWC) was determined according to the WATERSCOUT SM100 Soil Moisture Sensor instructions (Spectrum Technologies, Inc, Plainfield, IL) and fit to a regression model: VWC = 0.00076503*MW – 0.79736 (MW represents target mass wetness defined as a percentage).

Water stress and fungicide treatments

Experiment 1

On 14 June 2010, Expt. 1a was initiated by recording VWC and watering each container according to its target VWC (TVWC): 85% (control), 70%, 55%, 40%, or 25%. There were four rates of Pageant [boscalid (0.06 g ai·L^{-1} + pyraclostrobin (0.03 g ai·L^{-1}) (3.04 oz per 100 gallon)]: 0.0×, 0.5× [0.114 g·L^{-1} (0.015 oz·gal^{-1})], 1.0× [0.228 g·L^{-1} (0.03 oz·gal^{-1})], and 1.5× [0.342 g·L^{-1} (0.045 oz·gal^{-1})]. Pageant was applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) once a week three hours after watering containers to the designated TVWC; nontreated (0.0×) received water. The experiment was conducted using a split plot (Pageant rate as the main plot factor) in a randomized complete block design with a 5 × 4 factorial treatment design and 6 single pot replications.

On 27 July 2010, Expt. 1b was initiated and conducted in a similar manner to Expt. 1a with the following exceptions: based on results from Expt. 1a, only three VWC levels (85%, 55%, and 25%) were included. Expt. 1b was conducted using a split plot (Pageant rate as the main plot factor) in a randomized complete block design with a 3 × 4 factorial treatment design and 6 single pot replications.

Experiment 2

On 14 June 2010, Expt. 2a was initiated and materials and methods were similar to Expt. 1 with the following exceptions. Instead of TVWC, containers were watered based on days between watering (DBW): 1, 3, 6, 9 or 12 DBW. At each watering, containers were watered to reach 85 % VWC. The experiment was conducted using a

split plot (Pageant rate as the main plot factor) in a randomized complete block design with a 5 × 4 factorial treatment design and 6 single pot replications.

On 27 July 2010, Expt. 2b was initiated and materials and methods were similar to Expt. 2a with the following exceptions. After screening data and visual observations from Expt. 2a, 9 and 12 DBW proved to be detrimental to impatiens; therefore, only 3 DBW levels were used: 1, 3, and 6 DBW. Expt. 2b was conducted using a split plot (Pageant rate as the main plot factor) in a randomized complete block design with a 3 × 4 factorial treatment design and 6 single pot replications.

Experiment 3

Expt. 3 was designed similar to Expt. 1; however, there were only 2 TVWC levels (85 and 55%) and 2 fungicide rates based on the label rate (boscalid 0.15 g ai·L^{-1}+ pyraclostrobin 0.08 g ai·L^{-1}), 0.0× and 1.0× (0.599 g·L^{-1}). The experiment was conducted using a split plot (Pageant rate as the main plot factor) in a randomized complete block design with a 2 × 2 factorial treatment design and 6 single pot replications.

Expt. 3b was the same as Expt. 3a, except it was conducted the following month. The experiment was conducted using a split plot (Pageant rate as the main plot factor) in a randomized complete block design with a 2 × 2 factorial treatment design and 6 single pot replications.

Plant Growth

To determine physiological benefits of foliar applied pageant, initial growth indices (IGI), final GI [FGI = (height + width + perpendicular width) ÷ 3], shoot dry weight (SDW), root dry weight [RDW (in Expts. 1 and 2 only)] and total growth (TG was

determined by subtracting IGI from FGI) (Expt. 3 only) were collected at the close of the study. Shoots were harvested by cutting the entire plant at the soil line, removing the entire upper portion of the plant. Roots were harvested by first soaking the whole 15-cm container with the substrate and roots in a 17.7-L container filled with tap water. After soaking for a minimum of 8 h, substrate was washed from the roots over a screen to catch all roots. Shoots and roots were oven dried in a forced air drier at 65 °C for 72 h before obtaining dry weights. Water use efficiency (WUE), was determined as previously described (Burnett and van Iersel, 2008) using shoot and root dry weight [WUE = (SDW + RDW) ÷ total water applied].

Statistical Analysis

Data were analyzed using linear models with the GLIMMIX procedure of SAS (SAS Institute Inc, Cary, NC). Pairwise treatment differences were obtained using the LSMEANS statement for main effects with mean separation according to the Holm-Simulation method, alpha = 0.05. When there was a significant interaction (rate×TVWC or rate×DBW) the SLICEDIFF option was used to examine the pairwise comparisons using an adjusted *P* value for multiple comparisons with the SIMULATE option.

Results

Experiment 1a

Based on actual VWC (AVWC), well-watered (85%) containers were watered the same day after initial application of Pageant (DAIP), whereas 70% and 55% TVWC were

watered 3 and 6 DAIP, and 40% and 25% were watered 9 and 11 DAIP, respectively (Fig. 3.1).

Differing rates of Pageant had no effect on TG, SDW, RDW, WUE or TWA (Table 3.1). TVWC effects were seen in TG, SDW, RDW, WUE and TWA; as TVWC level dropped, impatiens average growth was less by the end of the experiment. It was visibly noted that as the duration of the experiment progressed, plants in the lower TVWC treatment exhibited wilt, leaf drop, and reduced leaf area, resulting in a reduction in TG. WUE decreased with increasing TVWC; 25% or 40% TVWC treatments were greater than impatiens at 85% TVWC. Additionally, WUE was similar in impatiens at 85%, 70% or 55% TVWC; however, TWA was different among 85%, 70%, or 55% TVWC. Over the duration of the experiment impatiens at 25% TVWC impatiens received only 0.2 L of water compared to 3.6 L at 85% TVWC treatment. There was no significant rate ×TVWC interaction. Based on visual observations, there was no indication Pageant had an effect on enhancing growth of impatiens grown under water-stress (Fig 3.2)

Experiment 1b

Similar to Expt. 1a, well-watered (85% TVWC) containers were watered the same DAIP whereas 55% and 25% TVWC were watered 5 and 10 DAIP, respectively (Fig. 3.3).

Weekly application of Pageant did not have an effect on TG, SDW, RDW, WUE or TWA (Table 3.2). Conversely, TVWC did have an effect on TG, SDW, and RDW with plants showing greater growth in association with higher TVWC. Similar to Expt.

1a, plants watered to 25% TVWC showed signs of wilting and leaf drop, resulting in reduced TG. In Expt. 1b, there was a significant rate × TVWC effect on SDW. After four 1.0× applications of Pageant, impatiens in containers maintained at 85% TVWC had greater SDW compared to nontreated impatiens (Fig. 3.4). However, when Pageant was applied to impatiens at the 1.0× rate to plants watered at 55% or 25% TVWC there was no differences in SDW compared to the nontreated plants. WUE, increased as TWA decreased. Based on visual observations, there was no indication Pageant had an effect on enhancing growth of impatiens grown under water-stress (Fig 3.5).

Experiment 2a

AVWC recorded daily for impatiens were on average: 75.0% (1 DBW), 68.2% (3 DBW), 60.0% (6 DBW), 59.5% (9 DBW), and 50.5% (12 DBW) (Fig. 3.6).

Similar to Expt. 1a, TG, SDW, RDW, WUE and TWA were not greater after the application of Pageant compared to nontreated plants (Table 3.3). DBW did have an effect on TG, SDW, and RDW of impatiens, indicating substantial loss in growth as the number of DBW increased. Additionally, impatiens at 6 DBW had a higher WUE compared to the 12 DBW treatment; however, TG was less in impatiens at 12 DBW. Similar to Expt. 1a, there was no rate × TVWC interaction, indicating Pageant applied to impatiens with various DBW did not result in improved water use efficiency or growth. Based on visual observations, there was no indication Pageant had an effect on enhancing growth of impatiens grown under water-stress (Fig. 3.7).

Experiment 2b

AVWC recorded for impatiens with 1, 3 or 6 DBW on average was 75.6% (1 DBW), 62.2% (3 DBW), and 57.9% (6 DBW) (Fig. 3.8).

Similar to Expt. 2a, TG was not greater after the application of Pageant (Table 3.4). There was a reduction in TG as DBW increased. Additionally, there was a significant rate × TVWC interaction effect on both SDW and RDW of impatiens. However, when analyzed by DBW, SDW was similar across all rates of Pageant within each DBW (Fig. 3.9). Furthermore, at 1 or 3 DBW, rates of Pageant had similar effects within DBW. Whereas, impatiens treated with Pageant at the 0.5× rate at 6 DBW resulted in greater RDW compare to the nontreated at 6 DBW (Fig. 3.10). Similar to SDW, WUE was similar across all rates of Pageant within DBW (Fig. 3.11). TWA was similar among rates of Pageant; however, decreased as DBW increased. Based on visual observations, there was no indication Pageant had an effect on enhancing growth of impatiens grown under water-stress (Fig 3.12).

Experiment 3a

Similar to the previous experiments, AVWC was monitored in tomato plants watered daily based on either 85% or 55% TVWC (Fig. 3.13). In this experiment, AVWC appeared to steadily decrease as the experiment progressed. However, AVWC was recorded between 0600 HR and 0730 HR every morning to determine how much water was needed to bring container to TVWC. As the experiment progressed and the tomato plants matured they used more water daily, indicating an increase in water applied, reported as cumulative water use (Fig. 3.14).

Applications of Pageant to tomato plants did not have a significant effect on TG, SDW, or WUE (Table 3.5). TVWC did affect TG and SDW, resulting in less growth when TVWC was maintained at 55%. Additionally, WUE was lower in plants maintained at 85% TVWC compared to 55% TVWC. Conversely, TWA was greater in 85% TVWC. There was no rate × TVWC interaction, regardless of parameter measured. Based on visual observations, there was no indication Pageant had an effect on enhancing growth of tomato plants grown under water-stress (Fig 3.15).

Experiment 3b

Similar to Expt. 3a, AVWC was monitored (Fig. 3.16) and cumulative water use was recorded (Fig. 3.17).

Pageant applied to tomato plants did not have an effect on TG, SDW, or WUE (Table 3.6). Plants maintained at 85% TVWC had greater TG and SDW compared to the 55% TVWC treatment. WUE decreased as TWA increased. There was no rate×TVWC interaction. Based on visual observations, there was no indication Pageant had an effect on enhancing growth of tomato plants grown under water-stress (Fig 3.18).

Discussion

There are multiple reports indicating strobilurin fungicides, such as Pageant, either increase yield, drought tolerance, or both in field-grown crops (BASF, 2010; Zhang et al., 2010). Conversely, there are reports indicating increased water-use efficiency after application of pyraclostrobin to well-watered wheat, but not water-stressed wheat (Nason et al. 2007). This is similar to the results in Expt. 1b with

enhanced shoot growth after application of Pageant at the 1.0× rate to well-watered (85% TVWC) impatiens. However, Pageant applied to containers maintained at 55% or 25% TVWC (Expt. 1) did not appear to increase drought tolerance or enhance growth of impatiens. In Expt. 2, the 0.5× rate at 6 DBW increased RDW compared to the nontreated at 6 DBW. Although there was a significant interaction between rate of Pageant and TVWC or DBW, TG was not significant, indicating that the application of Pageant may not be the contributing factor for the increased SDW or RDW (Brosnan et al., 2010). Furthermore, maintaining impatiens at 25% TVWC was too low for impatiens crossing the permanent wilting point (Blanusa et al., 2009). Additionally, as TWA increased impatiens had lower WUE, which is consistent with reports by Burnett and van Iersel, (2008).

Based on the results of these experiments, water use efficiency of neither impatiens nor tomatoes was increased by the use of Pageant. There were indications Pageant enhanced growth in well-watered or moderately stressed impatiens; however, results were not consistent within water treatments. Since there are contradicting reports about the use of strobilurins in regards to plant health, further research with ornamentals is needed. In particular, few if any studies have had success with strobilurins in controlled environment studies.

Table 3.1 Growth and water use efficiency (WUE)[z] of *Impatiens walleriana* 'Super Elfin XP White' following four foliar applications of Pageant based on the 1× rate (0.228 $g \cdot L^{-1}$) to plants grown in containers maintained at 85%, 70%, 55%, 40%, or 25% target substrate volumetric water content (TVWC) (Expt. 1a).

Rate	TG[y] (cm)	SDW[x] (g)	RDW[w](g)	WUE ($g \cdot L^{-1}$)	TWA[v] (L)
0.0×	3.3 a[u]	5.5 a	0.43 ab	4.0 a	1.8 a
0.5×	3.1 a	4.7 a	0.35 c	3.9 a	1.7 a
1.0×	4.1 a	5.4 a	0.47 a	4.2 a	1.6 a
1.5×	2.8 a	4.5 a	0.40 bc	4.2 a	1.5 a
TVWC					
85%	9.0 a	9.2 a	0.70 a	2.7 c	3.6 a
70%	6.4 b	6.9 b	0.50 b	3.1 c	2.3 b
55%	3.8 c	4.9 c	0.42 b	3.6 bc	1.5 c
40%	0.3 d	2.9 d	0.30 c	4.3 b	0.7 d
25%	-3.0 e	1.2 e	0.15 d	6.6 a	0.2 e
Effects					
rate	0.1153[t]	0.1116	0.1323	0.7717	0.1961
TVWC	<.0001	<.0001	<.0001	<.0001	<.0001
rate×TVWC	0.9582	0.2937	0.5048	0.4470	0.6223

[z]WUE = ((SDW + RDW) ÷ total water applied).

[y]TG: total growth = initial growth indices (GI) - final GI [GI = (height + width + perpendicular width) ÷ 3].

[x]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[w]RDW: root dry weight, oven dried for 72 h at 65 °C.

[v]TWA: average total water applied per plant.

[u]Means (within a column) with the same letters within moisture level or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[t]*P* value.

Table 3.2 Growth and water use efficiency (WUE)[z] of *Impatiens walleriana* 'Super Elfin XP White' following four foliar applications of Pageant based on the 1× rate (0.228 g·L^{-1}) to plants grown in containers maintained at 85%, 55%, or 25% target substrate volumetric water content (TVWC) (Expt. 1b).

Rate	TG[y] (cm)	SDW[x] (g)	RDW[w](g)	WUE (g·L^{-1})	TWA[v] (L)
0.0×	4.0 a[v]	4.6 a	0.42 a	5.5 a	1.5 a
0.5×	5.2 a	5.8 a	0.48 a	7.2 a	1.3 a
1.0×	5.9 a	6.5 a	0.50 a	7.4 a	1.4 a
1.5×	5.3 a	5.4 a	0.51 a	7.3 a	1.4 a
TVWC					
85%	11.7 a	9.5 a	0.68 a	3.6 c	2.8 a
55%	6.9 b	5.4 b	0.51 b	4.6 b	1.3 b
25%	-3.3 c	1.8 c	0.26 c	12.3 a	0.2 c
Effects					
rate	0.5776[u]	0.1794	0.7749	0.4464	0.1911
TVWC	<.0001	<.0001	<.0001	<.0001	<.0001
rate×TVWC	0.4688	0.0056	0.1266	0.1232	0.5671

[z]WUE = ((SDW + RDW) ÷ total water applied).

[y]TG: total growth = initial growth indices (GI) - final GI [GI = (height + width + perpendicular width) ÷ 3].

[x]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[w]RDW: root dry weight, oven dried for 72 h at 65 °C.

[v]TWA: average total water applied per plant.

[u]Means (within a column) with the same letters within moisture level or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[t]*P* value.

Table 3.3 Growth and water use efficiency (WUE)[z] of *Impatiens walleriana* 'Super Elfin XP White' following four foliar applications of Pageant based on the 1× rate (0.228 g·L^{-1}) to plants grown in containers at 1 (daily), 3, 6, 9, or 12 days between watering (DBW) (Expt. 2a).

Rate	TG[y] (cm)	SDW[x] (g)	RDW[w] (g)	WUE (g·L^{-1})	TWA[v] (L)
0.0×	3.7 a[u]	5.2 a	0.36 a	3.0 a	1.9 a
0.5×	3.9 a	5.5 a	0.4 a	3.3 a	1.9 a
1.0×	3.8 a	5.3 a	0.39 a	3.4 a	1.8 a
1.5×	3.6 a	5.4 a	0.41 a	3.3 a	1.9 a
DBW					
1	7.6 a	9.3 a	0.56 a	2.7 c	3.8 a
3	5.0 b	6.4 b	0.44 b	3.3 ab	2.1 b
6	3.7 c	4.6 c	0.42 b	3.7 a	1.3 c
9	2.0 d	3.8 c	0.29 c	3.4 ab	1.2 c
12	0.4 e	2.7 d	0.23 c	3.2 b	0.9 d
Effects					
rate	0.9713[t]	0.9496	0.3879	0.5454	0.5818
DBW	<.0001	<.0001	<.0001	<.0001	<.0001
rate×DBW	0.1211	0.9431	0.0811	0.3442	0.6937

[z]WUE = ((SDW + RDW) ÷ total water applied).

[y]TG: total growth = initial growth indices (GI) - final GI [GI = (height + width + perpendicular width) ÷ 3].

[x]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[w]RDW: root dry weight, oven dried for 72 h at 65 °C.

[v]TWA: average total water applied per plant.

[u]Means (within a column) with the same letters within moisture level or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[t]*P* value.

Table 3.4 Growth and water use efficiency (WUE)[z] of *Impatiens walleriana* 'Super Elfin XP White' following four foliar applications of Pageant based on the 1× rate (0.228 g·L^{-1}) to plants grown in containers at 1 (daily), 3, or 6 days between watering (DBW) (Expt. 2b).

Rate	TG[y] (cm)	SDW[x] (g)	RDW[w] (g)	WUE (g·L^{-1})	TWA[v] (L)
0.0×	9.5 a[u]	7.3 a	0.22 b	4.0 a	1.9 a
0.5×	10.8 a	8.4 a	0.62 a	4.8 a	1.9 a
1.0×	10.0 a	8.2 a	0.62 a	4.7 a	1.9 a
1.5×	9.5 a	6.9 a	0.62 a	4.5 a	1.8 a
DBW					
1	13.5 a	9.8 a	0.64 a	3.9 b	2.6 a
3	9.6 b	7.6 b	0.51 b	4.3 b	1.9 b
6	6.8 c	5.6 c	0.41 c	5.3 a	1.2 c
Effects					
rate	<0.9240[t]	0.8928	0.0029	0.7276	0.8155
DBW	<.0001	<.0001	<.0001	0.0003	<.0001
rate×DBW	0.8310	0.0480	0.0233	0.0114	0.0853

[z]WUE = ((SDW + RDW) ÷ total water applied).

[y]TG: total growth = initial growth indices (GI) - final GI [GI = (height + width + perpendicular width) ÷ 3].

[x]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[w]RDW: root dry weight, oven dried for 72 h at 65 °C.

[v]TWA: average total water applied per plant.

[u]Means (within a column) with the same letters within moisture level or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[t]*P* value.

Table 3.5 Growth and water use efficiency (WUE)[z] of *Solanum lycopersicum* BHN 640' tomato plants grown at 85 % or 55 % target substrate volumetric water content (TVWC) following weekly foliar applications of Pageant based on the 1× rate (0.599 $g \cdot L^{-1}$) (Expt. 3a).

Rate	TG[y] (cm)	SDW[x] (g)	WUE ($g \cdot L^{-1}$)	TWA[w]
0.0×	24.9 a[v]	60.7 a	13.2 a	5.1 a
1.0×	24.2 a	63.2 a	13.4 a	5.2 a
TVWC				
85%	28.8 a	65.9 a	9.8 b	6.8 a
55%	20.3 b	58.0 b	16.8 a	3.5 b
Effects				
rate	0.8328[u]	0.1368	0.7050	0.6940
TVWC	0.0086	0.0001	<.0001	<.0001
rate×TVWC	0.7529	0.2879	0.7541	0.7549

[z]WUE = (SDW ÷ total water applied).

[y]TG: total growth = initial growth indices (GI) - final GI [GI = (height + width + perpendicular width) ÷ 3].

[x]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[w]TWA: average total water applied per plant.

[v]Means (within a column) with the same letters within moisture level or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[u]*P* value.

Table 3.6 Growth and water use efficiency (WUE)[z] of *Solanum lycopersicum* BHN 640' tomato plants grown at 85 % or 55 % target substrate volumetric water content (TVWC) following weekly foliar applications of Pageant based on the 1× rate (0.599 $g \cdot L^{-1}$) (Expt. 3b).

Rate	TG[y] (cm)	SDW[x] (g)	WUE ($g \cdot L^{-1}$)	TWA[w]
0.0×	18.9 a[v]	24.2 a	3.4 a	7.2 a
1.0×	20.4 a	23.9 a	3.3 a	7.2 a
TVWC				
85%	23.8 a	30.5 a	3.3 a	9.2 a
55%	15.5 b	17.5 b	3.4 a	5.2 b
Effects				
rate	0.3227[u]	0.6692	0.6091	0.8205
TVWC	<.0001	<.0001	0.4802	<.0001
rate×TVWC	0.9856	0.5634	0.4259	0.9383

[z]WUE = (SDW ÷ total water applied).

[y]TG: total growth = initial growth indices (GI) - final GI [GI = (height + width + perpendicular width) ÷ 3].

[x]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[w]TWA: average total water applied per plant.

[v]Means (within a column) with the same letters within moisture level or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[u]*P* value.

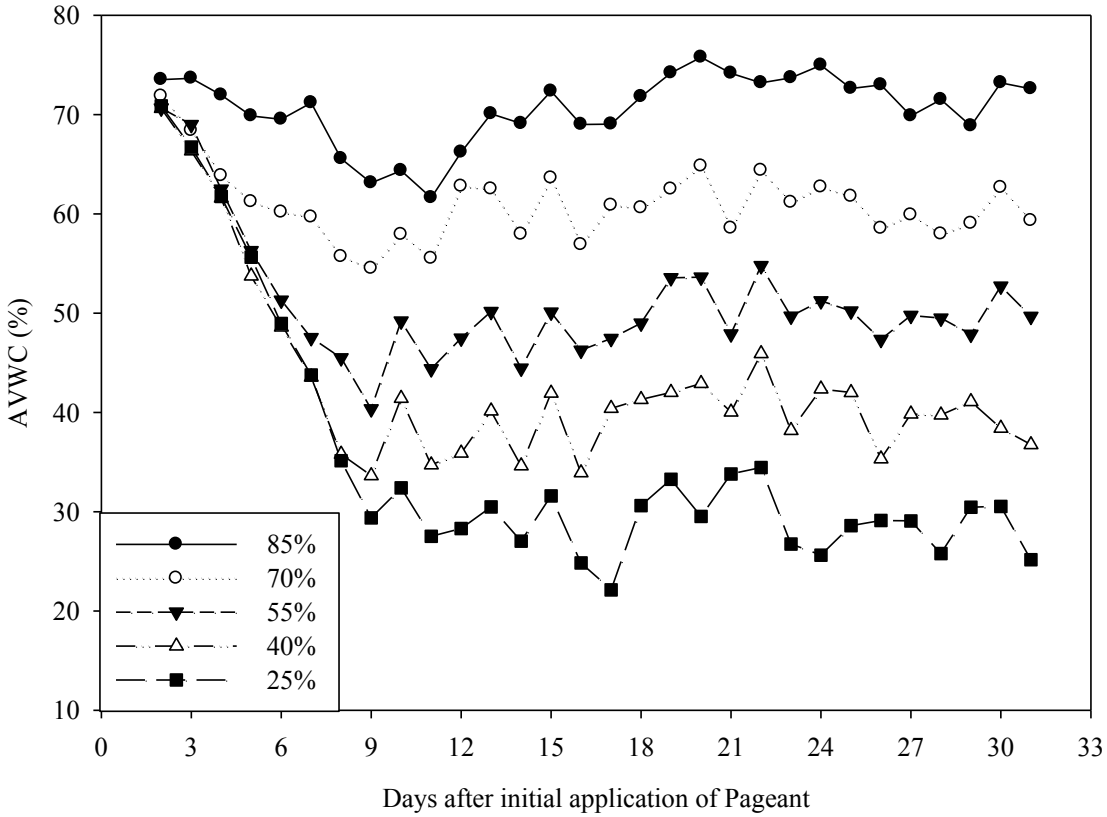

Figure 3.1 Actual substrate volumetric water content (AVWC) following foliar applications of Pageant based on the 1× rate (0.228 g·L^{-1}), to *Impatiens walleriana* 'Super Elfin XP White' grown under different target substrate volumetric water contents (TVWC): 85%, 70%, 55%, 40%, or 25%. Data points represent daily average pooled across all rates (Expt. 1a).

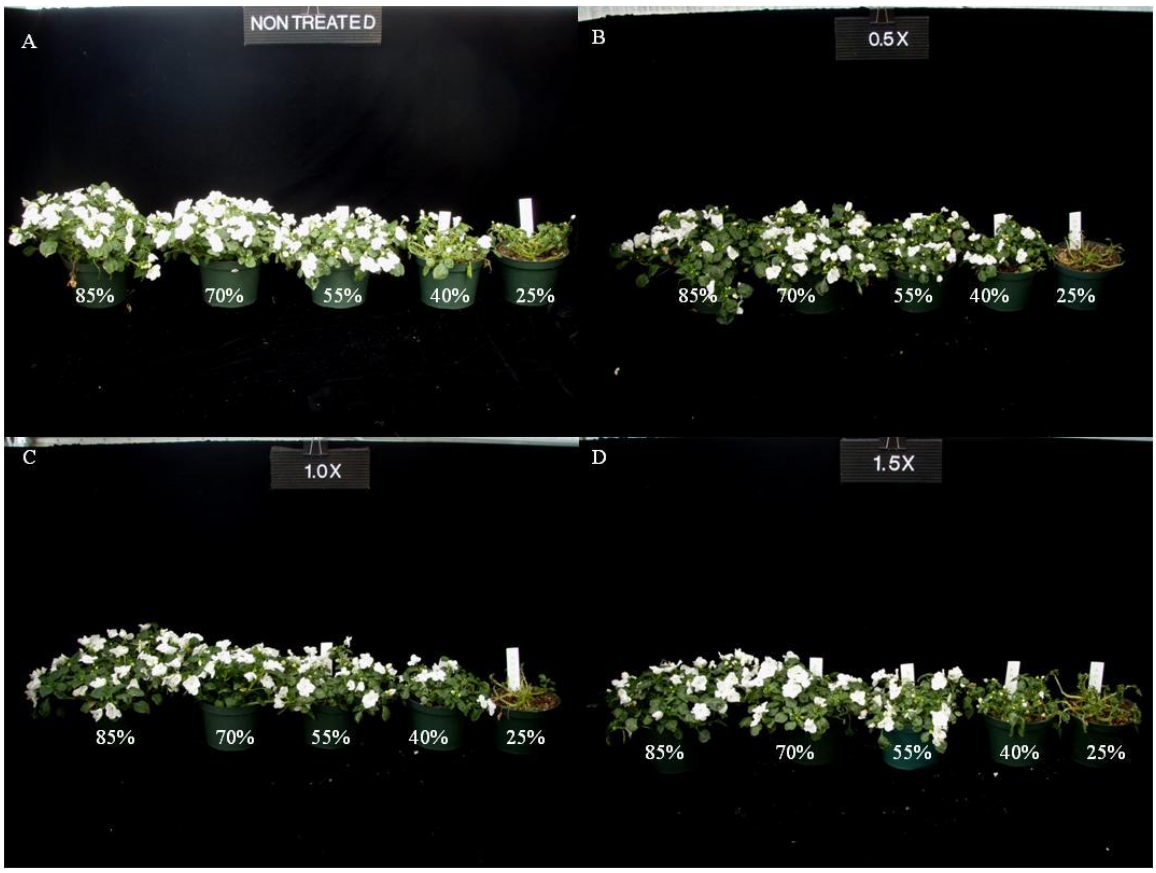

Figure 3.2 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown for four weeks at 85%, 70%, 55%, 40% or 25% target substrate volumetric water content following weekly foliar applications of Pageant: a. nontreated control (0.0× rate), b. 0.5× (0.114 g·L^{-1}), c. 1.0× (0.228 g·L^{-1}), and d. 1.5× (0.342 g·L^{-1}) (Expt. 1a).

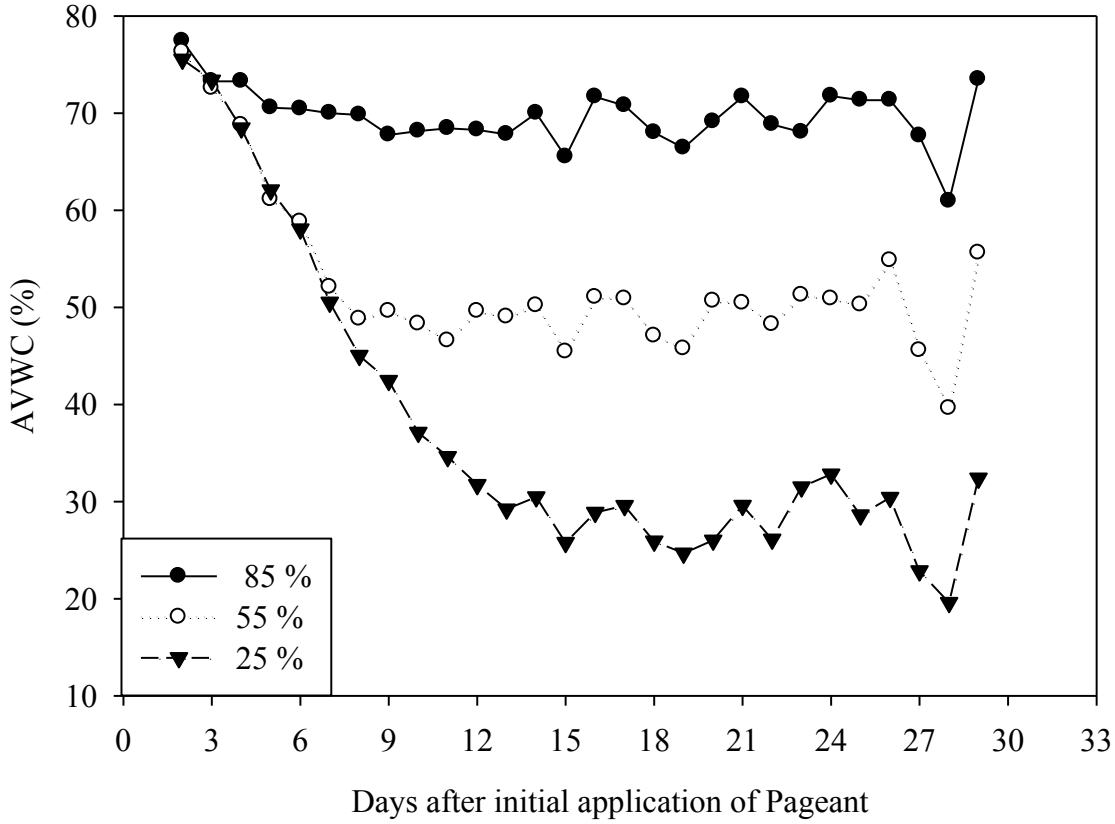

Figure 3.3 Actual substrate volumetric water content (AVWC) following foliar application of Pageant based on the 1× rate (0.228 $g \cdot L^{-1}$), to *Impatiens walleriana* 'Super Elfin XP White', grown under different target substrate volumetric water contents (TVWC): 85%, 55%, and 25%. Data points represent daily average pooled across all rates (Expt. 1b).

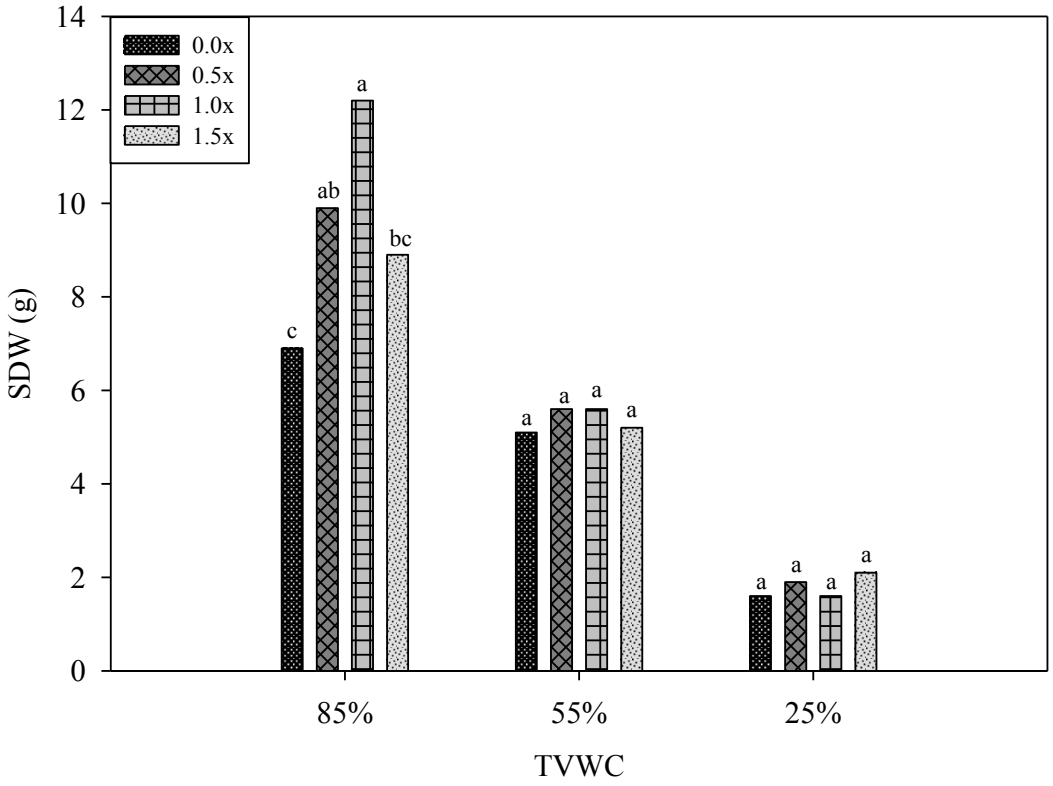

Figure 3.4 Shoot dry weight (SDW) of *Impatiens walleriana* 'Super Elfin XP White' grown under 85%, 55%, or 25% target substrate volumetric water content, following four weekly foliar applications of Pageant based on the 1.0× rate (0.228 g·L^{-1}). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, α = 0.05 (Expt. 1b).

Figure 3.5 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown for four weeks at 85%, 55%, or 25% target substrate volumetric water content following weekly foliar applications of Pageant: a. nontreated control (0.0× rate), b. 0.5× (0.114 g·L^{-1}), c. 1.0× (0.228 g·L^{-1}), and d. 1.5× (0.342 g·L^{-1}) (Expt. 1b).

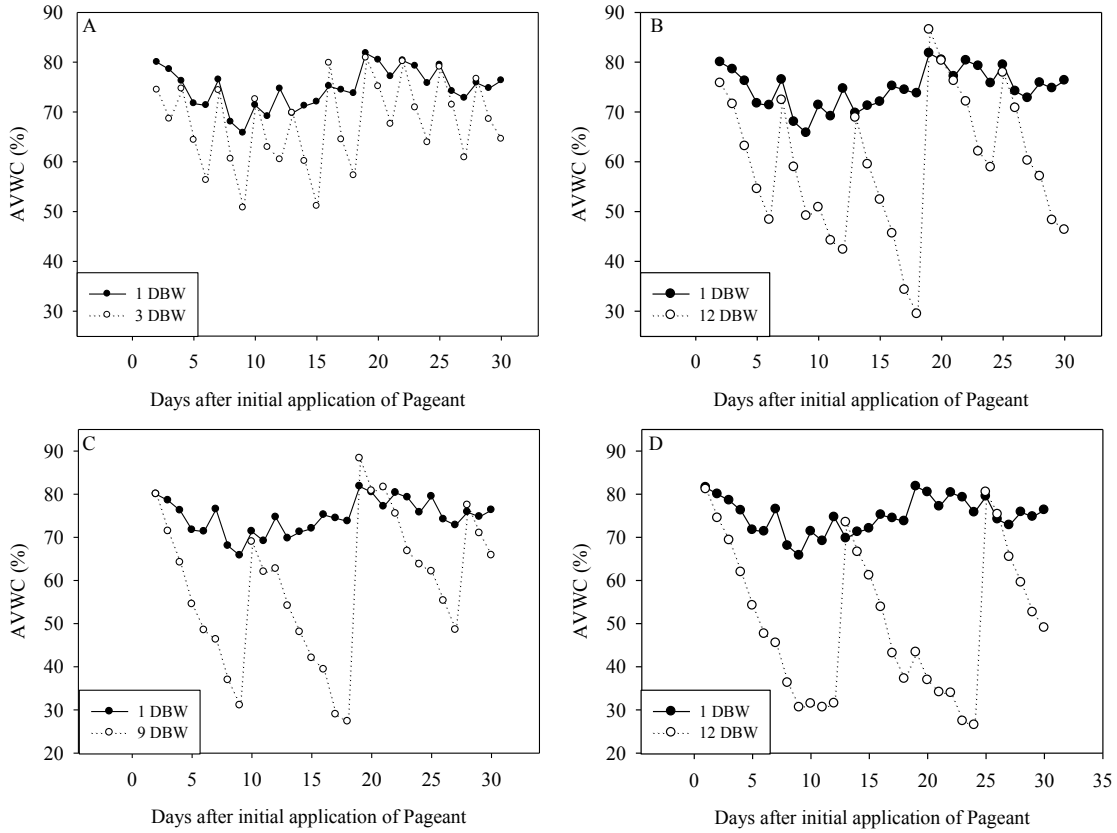

Figure 3.6 Actual substrate volumetric water content (AVWC) following initial application of Pageant based on the 1× rate (0.228 g·L^{-1}) to *Impatiens walleriana* 'Super Elfin XP White', grown in 15-cm containers in Sunshine Mix 1 and hand-watered daily (1) or at 3, 6, 9, or 12 days between watering (DBW) to raise the AVWC to 85% on the day of watering: a. 1 and 3 DBW treatments, b. 1 and 6 DBW treatments, c. 1 and 9 DBW treatments, and d. 1 and 12 DBW treatments. Data points represent daily average pooled across rates (Expt. 2a).

Figure 3.7 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown for four weeks at 1 (daily), 3, 6, 9, or 12 days between watering following weekly foliar applications of Pageant: a. nontreated control (0.0× rate), b. 0.5× (0.114 g·L^{-1}), c. 1.0× (0.228 g·L^{-1}), and d. 1.5× (0.342 g·L^{-1}) (Expt. 2a).

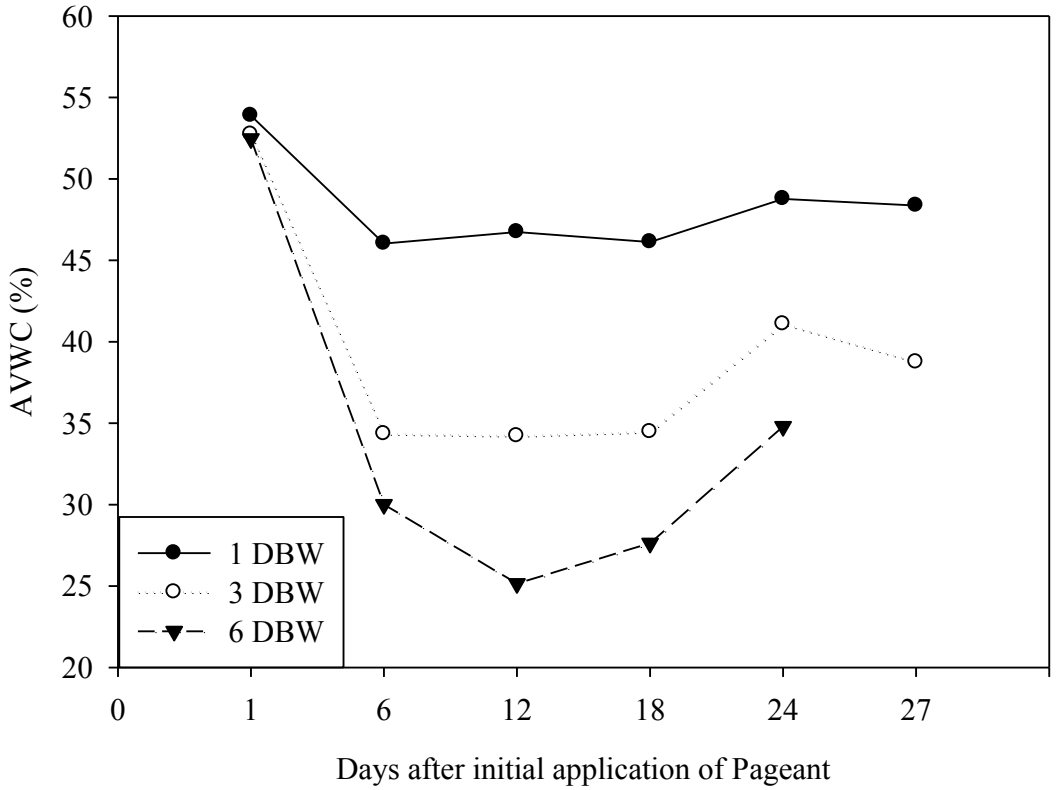

Figure 3.8 Actual substrate volumetric water content (AVWC) following the application of Pageant based on the 1× rate (0.228 g·L^{-1}), to *Impatiens walleriana* 'Super Elfin XP White' and watered at 85% target substrate volumetric water content at 1 (daily), 3 or 6 days between watering (DBW). Data points represent daily average pooled across all rates (Expt. 2b).

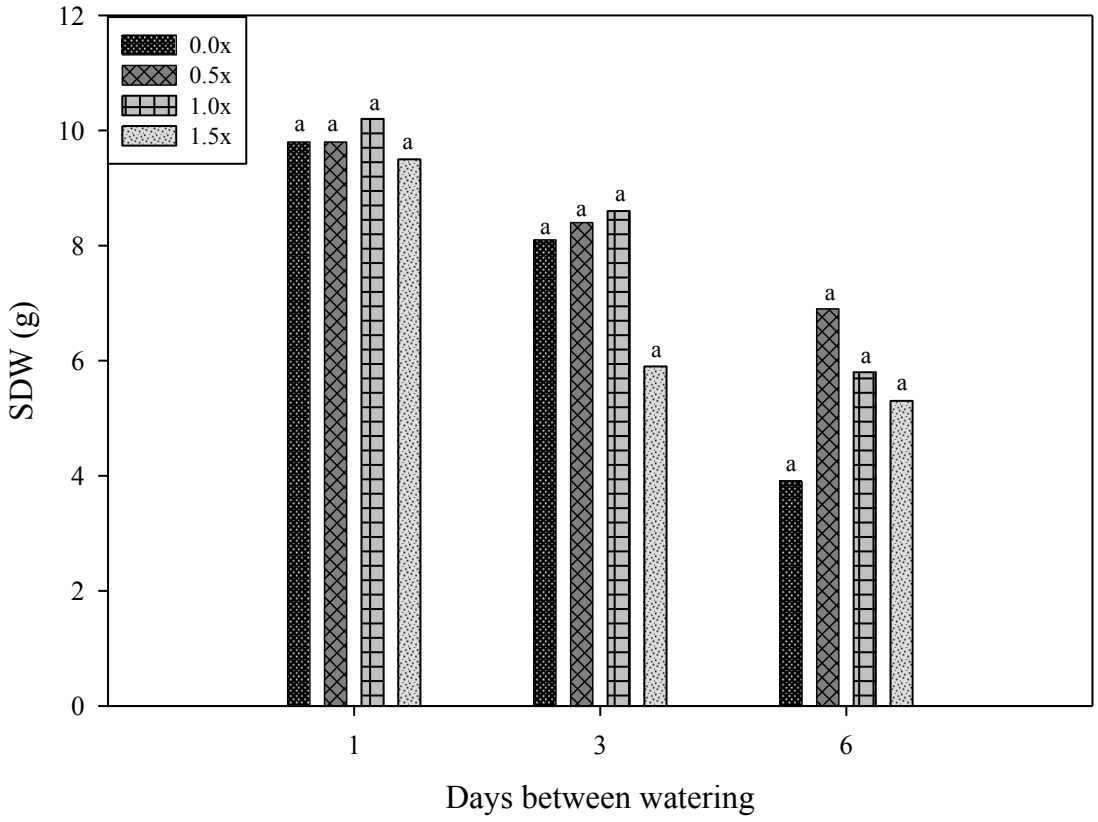

Figure 3.9 Shoot dry weight (SDW) of *Impatiens walleriana* 'Super Elfin XP White' 31 days after initial foliar application of Pageant based on the 1× rate (0.228 g·L^{-1}). Hand-watering was based on 85% target substrate volumetric water content at, 1 (daily), 3, or 6 days between watering (DBW). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, $\alpha = 0.05$ (Expt. 2b).

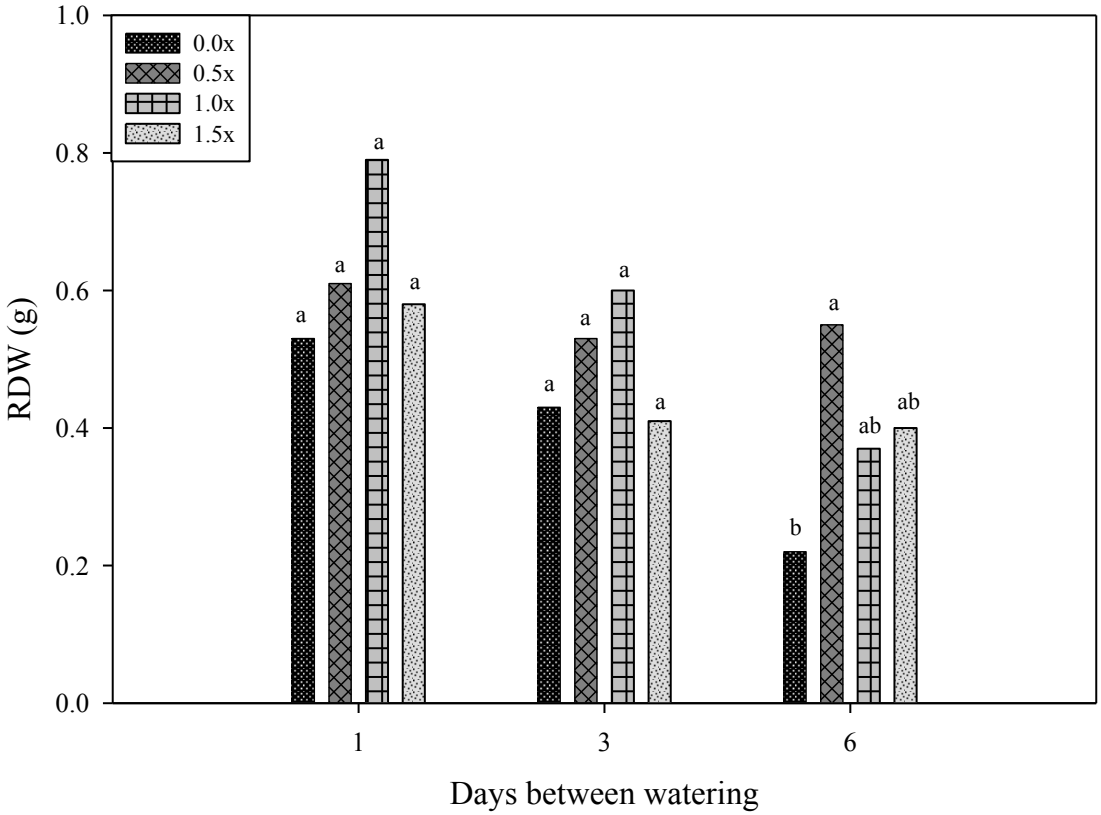

Figure 3.10 Root dry weight (RDW) of *Impatiens walleriana* 'Super Elfin XP White' 31 days after application of Pageant based on the 1× rate (0.228 $g \cdot L^{-1}$). Watering was based on 85% target substrate volumetric water content at, 1 (daily), 3, or 6 days between watering (DBW). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, $\alpha = 0.05$ (Expt. 2b).

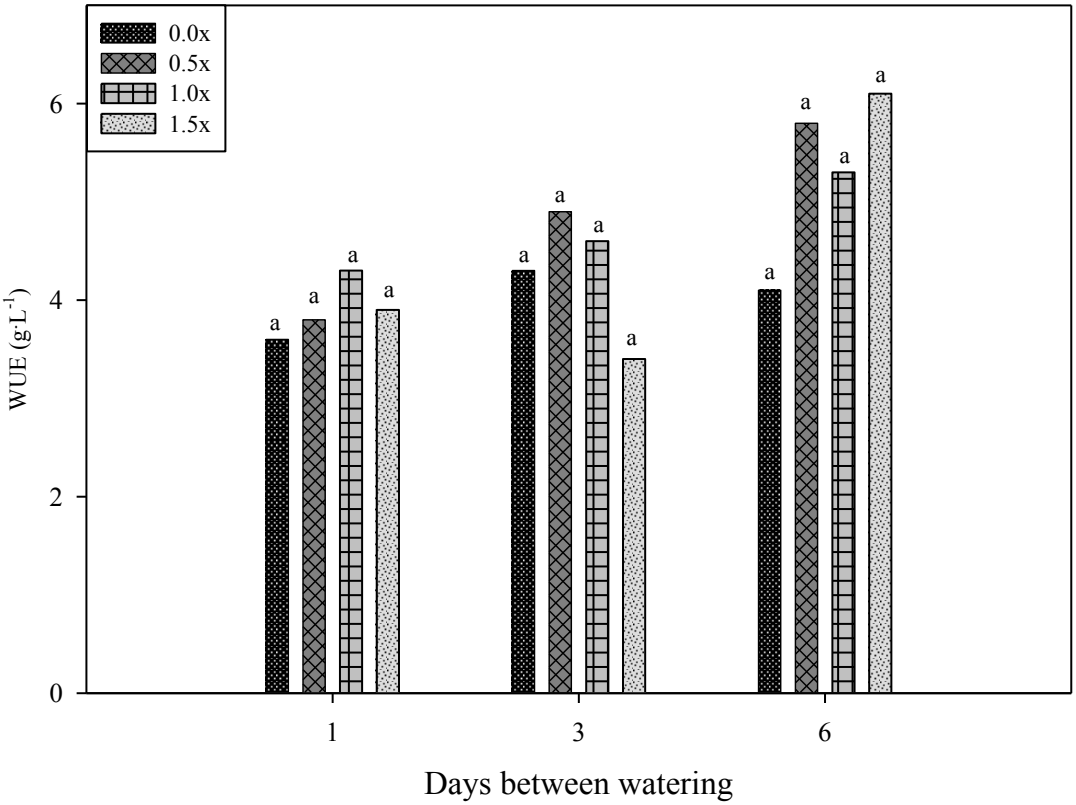

Figure 3.11 Water use efficiency [WUE = ((shoot + root dry weight) ÷ total water applied)] and mean water applied (MWA) of *Impatiens walleriana* 'Super Elfin XP White' following weekly applications of Pageant based on the 1.0× rate (0.228 g·L^{-1}). Watering was based on 85% target substrate volumetric water content at, 1 (daily), 3, or 6 days between watering (DBW). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, α = 0.05 (Expt. 2b).

Figure 3.12 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown for four weeks at 1 (daily), 3, or 6 days between watering following weekly foliar applications of Pageant: a. nontreated control (0.0× rate), b. 0.5× (0.114 g·L^{-1}), c. 1.0× (0.228 g·L^{-1}), and d. 1.5× (0.342 g·L^{-1}) (Expt. 2b).

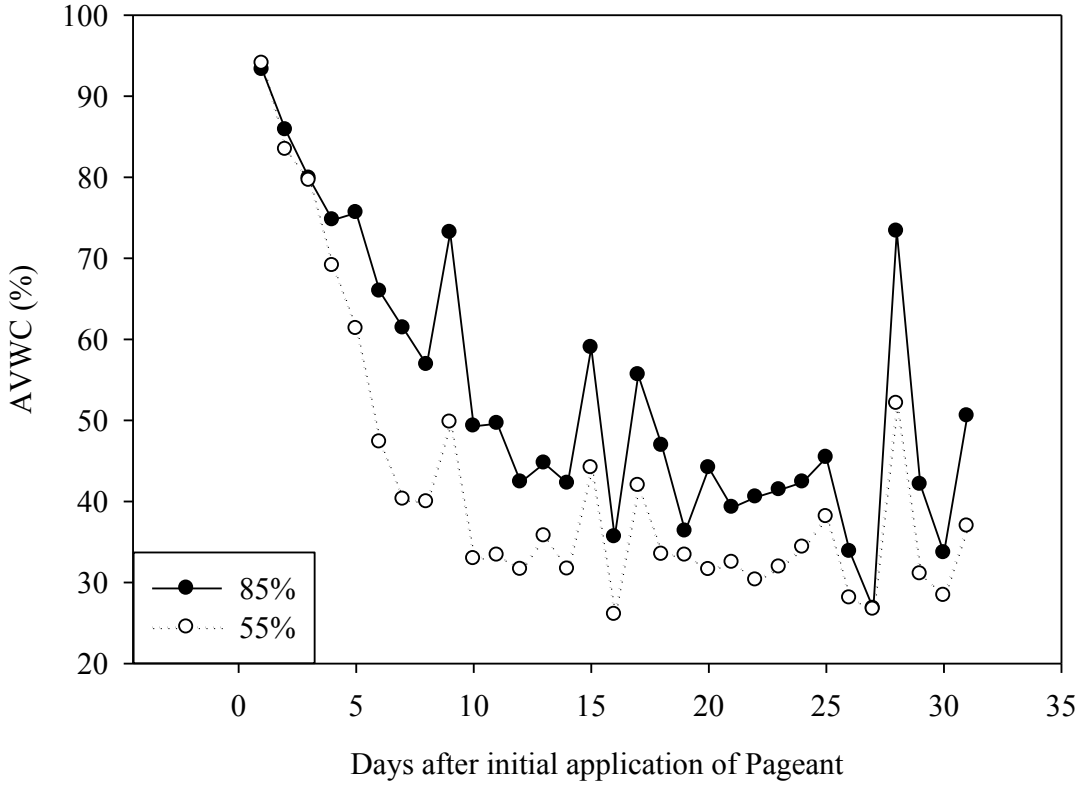

Figure 3.13 Actual substrate volumetric water content (AVWC) following weekly foliar application of Pageant to *Solanum lycopersicum* 'BHN 640' tomato plants based on the 1.0× rate (0.599 $g \cdot L^{-1}$) and hand-watered to maintain 85% or 55% target substrate volumetric water content. Data points represent daily average pooled across all rates (Expt. 3a).

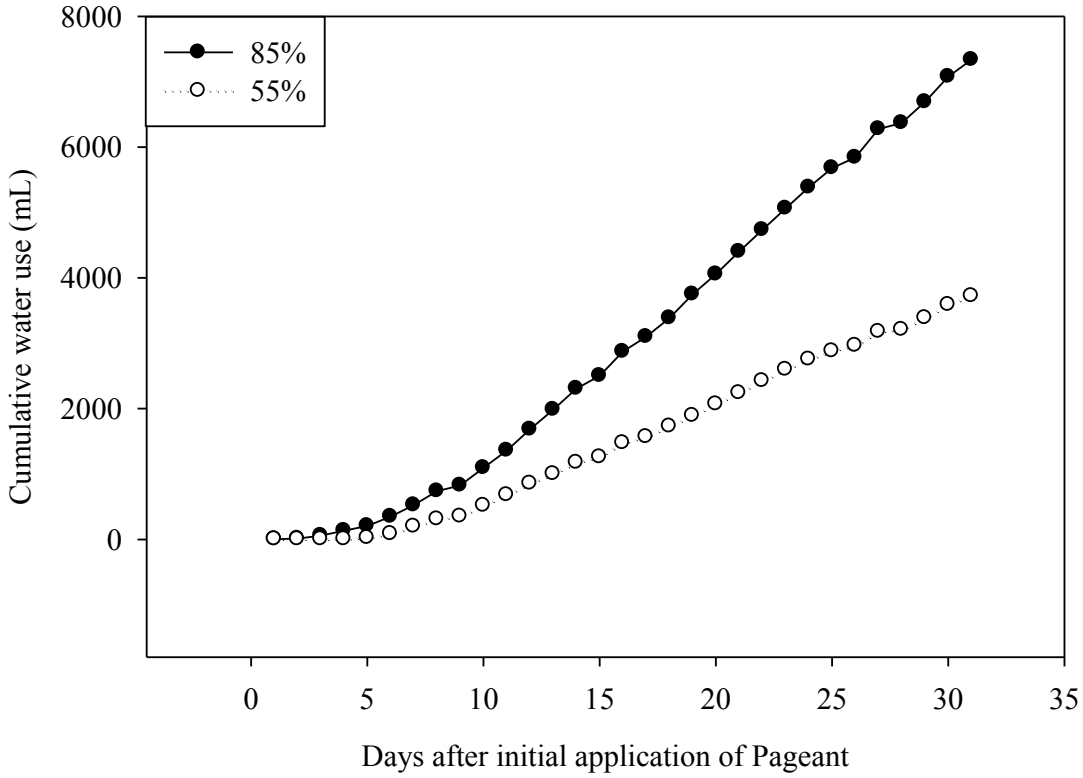

Figure 3.14 Cumulative water use of *Solanum lycopersicum* 'BHN 640' tomato plants following weekly applications of Pageant based on the 1.0× rate (0.599 $g \cdot L^{-1}$) and hand-watered to maintain an 85% or 55% target substrate volumetric water content. Data points represent daily average pooled across all rates (Expt. 3a).

Figure 3.15 Final growth of *Solanum lycopersicum* 'BHN 640' plants grown for four weeks with 85% and 55% target substrate volumetric water content (TVWC) following weekly foliar application of Pageant based on the 1.0× rate (0.599 g·L^{-1}): 1. nontreated (0.0×) with 85% TVWC, 2. nontreated (0.0×) with 55% TVWC, 3. Pageant at 1.0× with 85% TVWC, and 4. Pageant at 1.0× with 55% TVWC (Expt. 3a).

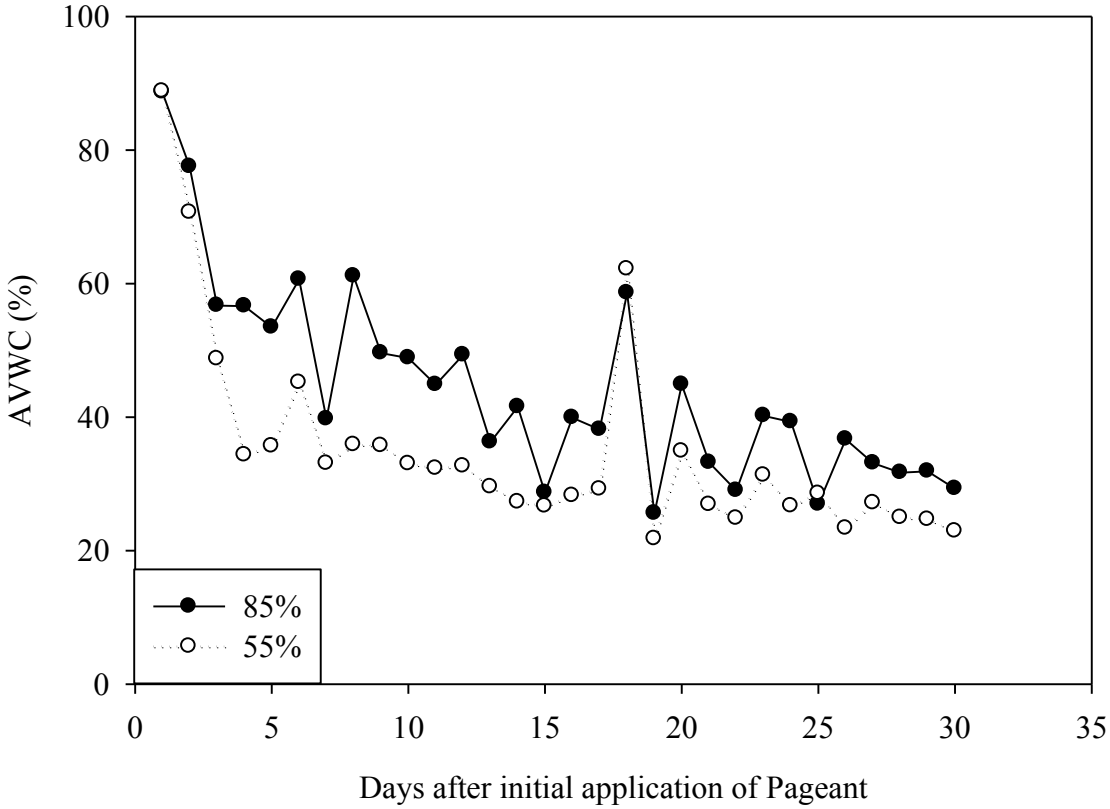

Figure 3.16 Actual substrate volumetric water content (AVWC) following weekly foliar application of Pageant to *Solanum lycopersicum* 'BHN 640' tomato plants based on the 1.0× rate (0.599 g·L^{-1}) and hand-watered to maintain 85% or 55% target substrate volumetric water content. Data points represent daily average pooled across all rates (Expt. 3b).

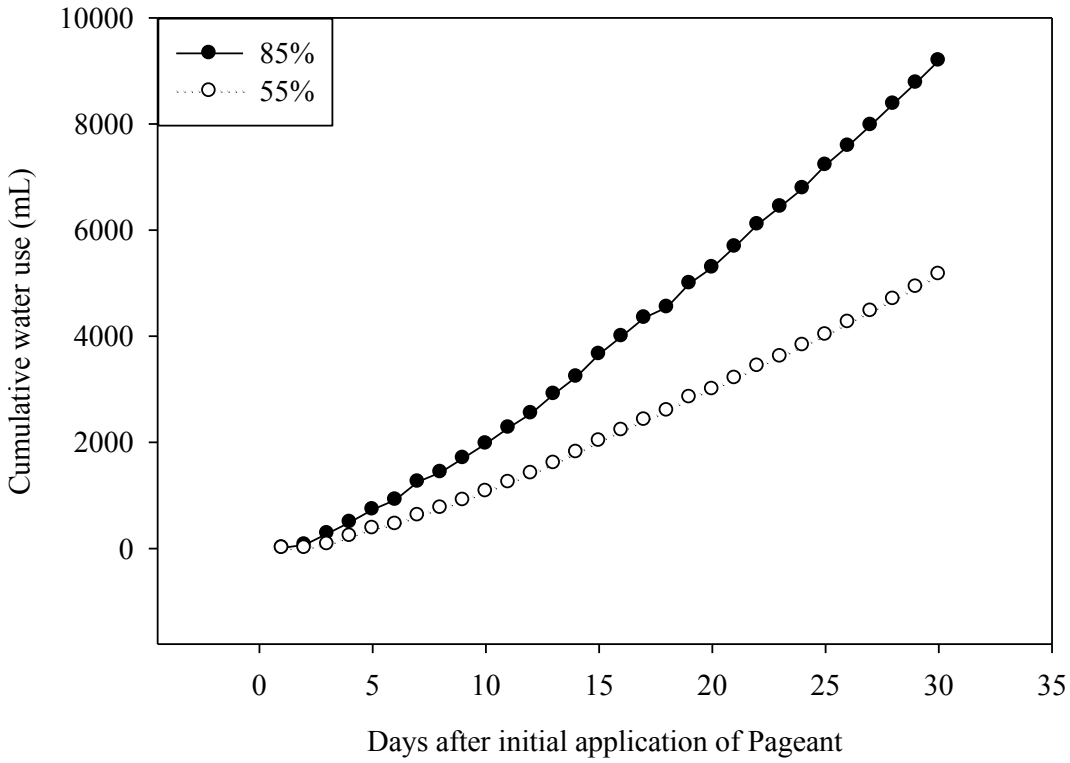

Figure 3.17 Cumulative water use of *Solanum lycopersicum* 'BHN 640' tomato plants following weekly applications of Pageant based on the 1.0× rate (0.599 $g \cdot L^{-1}$) and hand-watered to maintain an 85% or 55% target substrate volumetric water content. Data points represent daily average pooled across all rates (Expt. 3b).

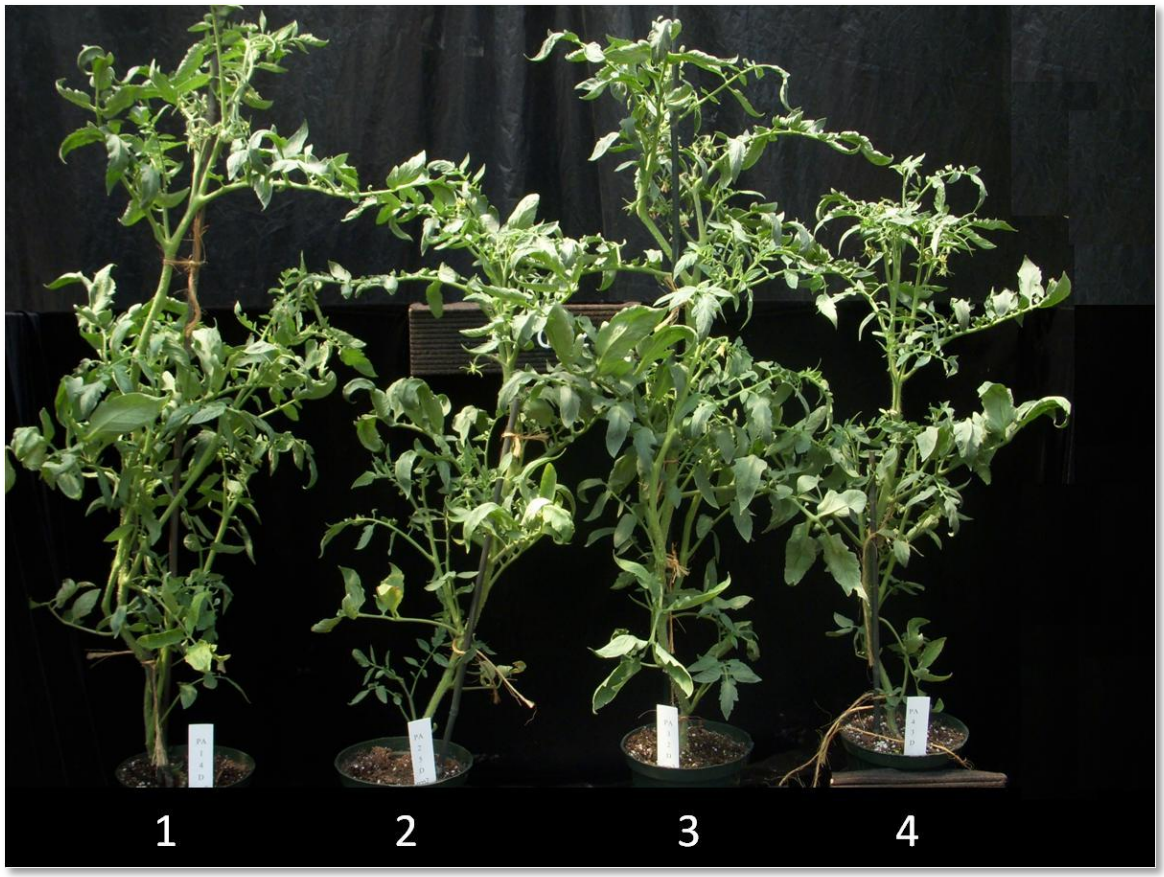

Figure 3.18 Final growth of *Solanum lycopersicum* 'BHN 640' plants grown for four weeks with 85% and 55% target substrate volumetric water content (TVWC) following weekly foliar application of Pageant based on the 1.0× rate (0.599 $g \cdot L^{-1}$): 1. nontreated (0.0×) with 85% TVWC, 2. nontreated (0.0×) with 55% TVWC, 3. Pageant at 1.0× with 85% TVWC, and 4. Pageant at 1.0× with 55% TVWC (Expt. 3b).

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CHAPTER IV

EVALUATION OF REGALIA (BIO-FUNGICIDE) AND MBI-501 (ANTITRANSPIRANT) ON DROUGHT TOLERANCE OF *IMPATIENS WALLERIANA* 'SUPER ELFIN XP WHITE' AND *SOLANUM LYCOPERSICUM* 'BHN 640'

Abstract

Regalia and MBI-501 were evaluated for their potential to enhance drought tolerance in *Impatiens walleriana* 'Super Elfin XP White' (impatiens) and *Solanum lycopersicum* 'BHN 640' (tomato). In Expts. 1 and 2, Regalia and MBI-501 were foliar applied at 0.0×, 0.5×, 1.0× or 1.5× to impatiens grown under three target substrate volumetric water contents (TVWC): 85%, 55%, or 25%. In Expts. 3 and 4, Regalia and MBI-501 were applied to impatiens watered at 1 (daily), 3, or 6 days between watering (DBW). In Expts. 5 and 6, Regalia and MBI-501 were foliar applied at 0.0× or 1.0× to tomato plants grown under 2 TVWC: 85% or 55%. Overall, in experiments using Regalia (Expts. 1,3, and 5) there were consistent results that indicated growth enhancement after application; whereas, results using MBI-501were inconsistent across experiements, suggesting water treatments may be the contributing factor. Root dry weight of impatiens was increased following the application of Regalia at the 0.5× rate. Additionally, soluble protein content was increased in impatiens and tomato plants following application of

Regalia. Regalia's mode of action is seen through enhanced natural phytoalexins, strengthening cell walls and increasing SP content, which is consistent with these results.

Introduction

Water accounts for 80 to 95% of a growing plant's tissue and is responsible for physical and biochemical reactions including translocation and distribution of nutrients and metabolites (Mengel et al., 2001). Through transpiration, plants transport minerals from the roots to the leaves. In this process, 90% of the water entering the plant is released through the stomata, controlled by guard cells. With environmental stresses, governmental regulations, and increased human populations with limited water supplies (Warsaw et al., 2009; Burnett and van Iersel, 2008; Niu et al., 2008), plant producers and landscapers have had to follow stricter water use guidelines, ultimately reducing daily irrigation.

Availability of soil water is the first limiting factor associated with drought stress (Verslues et al., 2006). Subsequently, a decrease in available soil water results in a decrease in soil water potential and less uptake by the plant, ultimately affecting plant growth through partial or complete stomatal closure, reducing transpiration and photosynthesis, with decreased nitrate assimilation (Davies et al., 2002; Neumann, 2008; Sairam et al., 1997). Under prolonged drought, if supplemental water is not supplied, plant growth can be affected with pre-mature leaf drop, wilting, desiccation and/or death (Neumann, 2008).

Antitranspirants and other exogenously applied compounds have been used to try and reduce water loss in plants since the 1950's (Biai et al., 2011; Kettlewell, et al.,

2010). Typical antitranspirants are emulsions of wax or latex which create a thin film over the surface of the plant, and kaolin clay or chitosan. Although antitranspirants may reduce water loss, there have been reports they decrease photosynthesis. del Amor et al. (2010) reported reduced photosynthesis in fully irrigated pepper plants after the use of an antitranspirant.

Reports have shown an increase in net photosynthesis and growth of maize seedlings by soaking the seed in 150 μM hydrogen peroxide for 24 h prior to exposing the seedlings to 42 °C (Wahid et al., 2008). They attributed this increase to the hydrogen peroxide pretreatment inducing defense genes to offset oxidative damage. Additionally, it has been reported that chilling tolerance in cucumbers can be increased by suppressing hydrogen peroxide production in the leaves by exogenous application of polyamines (Zhang et al., 2009). Pyraclostrobin, a strobilurin fungicide, has been shown to increase nitrate reductase activity, increase antioxidant enzymes, increase stress tolerance, reduce the amount of CO_2 lost to the atmosphere (BASF, 2009; Kohle et al., 2002; Nason et al. 2007) and increase the overall green color of plants (Balba, 2007). Furthermore, an extract of *Reynoutria sachalinensis* (giant knotweed) marketed as Regalia by Marrone Bio Innovations (Davis, CA) has been shown to increase the plant's natural defense system by increasing production of phenolics and antioxidants and by strengthening the cell walls (Marrone Bio Innovations, 2011b). Additionally, there are reports indicating MBI-501 (an antitranspirant by Marrone Bio Innovations) increases translocation of photosynthates and photosynthetic activity (personal communication Marrone Bio Innovations). The objective of these experiments was to evaluate drought tolerance of

Impatiens walleriana ‘Super Elfin XP White’ and *Solanum lycopersicum* ‘BHN 640’ after weekly applications of Regalia and MBI-501 (an antitranspirant based on a long chain fatty alcohol).

Materials and Methods

Plant material and culture

Impatiens walleriana 'Super Elfin XP White'

On 24 June 2010, *Impatiens walleriana* ‘Super Elfin XP White’ (impatiens) seedlings from a 288-plug flat were potted into 15.24-cm (1.8 L) containers with Sunshine Mix 1 (SunGro Horticulture, Bellvue, WA) potting substrate. All containers were filled to the rim of the container and lightly tapped twice on a hard surface to reduce air pockets. After potting, impatiens were watered, placed in a controlled environment greenhouse located on Mississippi State University’s main campus, and grown for 4 weeks. On 23 July 2010, impatiens were moved to an inflated double polyethylene Quonset greenhouse located on Mississippi State University’s R.R. Foil Plant Science Research Facility under 60% shade and 21.1 °C/18.3 °C (70 °F/65 °F) (day/night) set point temperatures. Experiments were repeated (twice) in time and conducted in a similar manner.

Solanum lycopersicum 'BHN 640'

On 17 May 2011, *Solanum lycopersicum* ‘BHN 640’ (tomato) seed were sown in 72-cell pack liners (41-mL) in Sunshine Mix 1 potting substrate. Three weeks later (6 June 2011), seedlings (10.2-cm to 15.2-cm tall) were transferred into 15-cm (1.8 L)

containers and allowed to grow for two weeks before initiating the experiment. Venting temperatures inside the greenhouse were set to 18.3/15.5 °C day/night (actual greenhouse temperature on average was 27.5 °C day and 24.0 °C night). Experiments were repeated (twice) in time and conducted in a similar manner.

Determining substrate volumetric water content

A physical properties test (Hidalgo, 2001) was conducted on Sunshine Mix 1: 90.9% total porosity, 28.3% air space, 62.6% water holding capacity, and 0.11 g/cc bulk density. Substrate volumetric water content (VWC) was determined according to the WATERSCOUT SM100 Soil Moisture Sensor instructions by Spectrum Technologies, Inc (Plainfield, IL) and fit to a regression model: VWC = 0.00076503*MW – 0.79736 (where MW = target mass wetness defined as a percent).

Water stress and fungicide treatments

Experiment 1

Experiment was initiated on 27 July 2012 by recording actual substrate volumetric water content (AVWC) and watering each container to the target VWC (TVWC): 85% (control), 55%, or 25%. There were four rates of Regalia, based on the recommended label rate of 0.48 g ai·L^{-1} (1.28 oz·gal^{-1}): 0.0× (nontreated), 0.5× (5 mL·L^{-1}), 1.0× (10 mL·L^{-1}), or 1.5× (5 mL·L^{-1}). Foliar applications of Regalia were applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) once per week three hours after watering containers to TVWC; nontreated (0.0×) received water. Fertilizer was applied at 200 mg N L^{-1} using

Peter's Professional 20N-8.8P-16.6K (20-10-20) Peat-Lite Special (Scotts, Maryville, OH) at each watering. The experiment was conducted using a split plot (Regalia rate as the main plot factor) in a randomized complete block design with a 3 × 4 factorial treatment design and 6 single pot replications.

Experiment 2

The experiment was initiated on 27 July 2010 and was conducted in a similar manner to Expt. 1, except four rates of MBI-501 were used based at the recommend label rate of 0.93 oz·gal^{-1}: 0.0× (nontreated), 0.5× (1 mL·L^{-1}), 1.0× (2 mL·L^{-1}), and 1.5× (3 mL·L^{-1}). MBI-501 was foliar applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) once per week three hours after watering containers to TVWC. The experiment was conducted using a split plot (MBI-501 rate as the main plot factor) in a randomized complete block design with a 3 × 4 factorial treatment design and 6 single pot replications.

Experiment 3

This experiment was initiated on 27 July 2010 and materials and methods were similar to Expt.1, except instead of maintaining daily TVWC, containers were watered based on days between watering (DBW): 1 (daily), 3, or 6 DBW. At each watering, containers were watered to 85 % TVWC. The experiment was conducted using a split plot (Regalia rate as the main plot factor) in a randomized complete block design with a 3 × 4 factorial treatment design and 6 single pot replications.

Experiment 4

This experiment was similar to Expt. 2 except instead of maintaining daily TVWC, containers were watered based on DBW: 1 (daily), 3, or 6 DBW. At each watering, containers were watered to 85 % TVWC. The experiment was conducted using a split plot (MBI-501 rate as the main plot factor) in a randomized complete block design with a 3 × 4 factorial treatment design and 6 single pot replications.

Experiments 5 and 6

Experiments were conducted similar to Expt. 1 and 2 except in Expt. 5, Regalia was applied to 'BHN 640' tomato plants at 2 rates 0.0× (nontreated) or 1.0× (10 $mL \cdot L^{-1}$) and in Expt. 6, MBI-501 was applied to tomato plants at 2 rates 0.0× or 1.0× (2 $mL \cdot L^{-1}$). Additionally, in both Expt. 5 and 6, three TVWC levels were reduced to two, 85% or 55%. The experiments were conducted using a split plot (Regalia or MBI-501 rate as the main plot factor) in a randomized complete block design with a 2 × 2 factorial treatment design and 6 single pot replications.

Plant Growth

At initiation of the experiments, initial growth indices [IGI = ((height + width + perpendicular width) ÷ 3)] were measured. At the end of the experiments, final growth indices [FGI= ((height + width + perpendicular width) ÷ 3)], shoot dry weight (SDW), root dry weight [RDW (Expts. 1 thru 4 only)], flower number and total growth [TG (Expts. 5 and 6 only)] were measured. Shoots were harvested by cutting the entire plant at the soil line removing the entire upper portions of the plant. Roots were harvested by

first soaking the whole container with the substrate and roots in a 17.7-L container filled with tap water. After soaking for a minimum of 8 h, the substrate was washed from the roots over a screen to catch all fallen roots. Shoots and roots, were oven dried in a forced air drier at 65 °C (149 °F) for 72 h before obtaining dry weights. TG was determined by subtracting IGI from FGI (TG = FGI – IGI) (Expt. 5 and 6 only).

Plant Water Status

Actual substrate volumetric water content (AVWC) was measured daily (between 0600 and 0800 HR) for each container using a SM100 Soil Moisture Sensor attached to a handheld FieldScout Soil Sensor Reader (Spectrum Technologies, Inc., Plainfield, IL). Daily reading was fit to the soil moisture curve and containers were hand watered to TVWC. Amount of water applied per day, per container, was used to determine cumulative water use and total water applied.

Water use efficiency (WUE), was determined as previously described (Burnett and van Iersel, 2008) using shoot and root dry weight [WUE = (SDW + RDW) ÷ total water applied].

Mid-day leaf water potential (Ψ_{stem}) (-Mpa) was measured using a Scholander type Pressure Chamber according to Kjelgren et al. (2009); leaves were wrapped in plastic wrap, followed by aluminum foil for at least one hour prior to measurement.

Relative leaf water content [RWC (Expts. 5 and 6 only)] was recorded at 14 and 28 days after initiation of experiments. RWC was calculated as [(fresh weight - dry weight) ÷ (turgid weight - dry weight) × 100%], and determined as previously described by Abreu and Munné-Brosch (2008) with modifications. Leaves were excised from the

plant and fresh weight was recorded and then placed in water for 24 h in the dark at 4 °C before measuring turgid weight. To determine dry weight, leaves were oven dried at 65 °C for 48 h.

Photosynthesis

Leaf photosynthetic rate (Pn), was recorded at 14 and 28 days after initiation of experiments using a CIRAS-2 portable photosynthesis system (PPSystems, Amesbury, MD) on the most recent mature leaf. Parameters were set at 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PPF* (using a tungsten halogen light attachment), ambient temperature, a relative humidity of 50% of ambient, and a CO_2 concentration of 400 $\mu mol \cdot mol^{-1}$.

Leaf Chlorophyll Content

Leaf chlorophyll content (LCC) was measured using a handheld 502 SPAD chlorophyll meter, (Konica Minolta, Osaka, Japan) at 1, 8, 16, 22 and 29 days after initiation of experiments.

Antioxidant Enzyme Extractions and Assays

To evaluate metabolic changes induced after application of Regalia or MBI-501, leaf samples were taken at the end of the experiments (placed in Kraft #1 coin envelopes, Quality Park Products, Minneapolis, MN) and immediately frozen with liquid nitrogen and stored at -80 °C (-112 °F) until analyzed for glutathione-*S*-transferase (Expt. 1, 3, and

5 only). Two replications were pooled to make a total of 3 samples per treatment (López-Carbonell and Jáuregui, 2005).

Crude enzyme (0.2g frozen tissue) was extracted with 1 mL of a 50 mM sodium phosphate buffer (pH 7.5) as previously described by Venisse, et al. (2001), then centrifuged at 14,000g at 4 °C until plant tissue was clearly separated from the 1 mL of extraction buffer (20 to 40 minutes) (Appendix A and B.1).

Protein content was determined for each sample according to Bradford (1976) using a Quick Start Bradford Protein Assay Kit #1 (500-0201, Bio-rad Laboratories Headquarters, Hercules, CA) (Appendix B.2).

Glutathione-*S*-transferase (GST) was assayed as previously described by Venisse et al. (2001) with some modifications. Samples were analyzed using an ELx808 Absorbance Microplate Reader with a UV filter (BioTek Instruments, Inc.) at 340 nm for 10 min. Each well contained 20µL of plant sample and 230µL of reaction buffer [0.1 M potassium phosphate buffer (pH 6.5), 3.6 mM reduced glutathione (M.W. 307.3), 100 mM 1-chlor-2,4-dinitrobenzene (CDNB M.W. 202.6)]. Activity was determined by following the formation of the conjugate of 1µmol of CDNB with reduced glutathione per min at pH 6.5 at 25 °C (extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$) (Appendix B.4). Specific activity of GST was expressed as $\mu units \cdot mg^{-1}$.

Statistical Analysis

Data were analyzed using linear models with the GLIMMIX procedure of SAS (SAS Institute Inc, Cary, NC). Pairwise treatment differences were obtained using the LSMEANS statement for main effects with mean separation according to the Holm-

Simulation method, alpha = 0.05. When there was a significant interaction (rate×TVWC or rate×DBW) the SLICEDIFF option was used to examine the pairwise comparisons, using an adjusted *P* value for multiple comparisons with the SIMULATE option.

Results

Experiment 1

At initiation of the experiment, containers were determined to be at 100 % AVWC prior to the first application of Regalia. Based on AVWC, containers maintained at 85% TVWC were not watered again until 3 days after initial Regalia application (DAIR), whereas, containers watered at 55% or 25% TVWC were not hand watered until 8 (55%) or 15 DAIR (25%), respectively (Fig. 4.1).

Application of Regalia did not affect FGI or SDW compared to the 0.0× (nontreated) (Table 4.1). RDW was 26% greater in impatiens following application of Regalia at the 0.5× rate compared to the 0.0×. Additionally, there was a TVWC effect seen in all parameters measured indicating less growth with decreasing TVWC. There were no interaction effects. WUE was unaffected by application of Regalia; however, WUE decreased with increasing TVWC (Table 4.2). MWA was similar among rates but increased with increased TVWC. These results suggest TVWC was the main factor limiting growth of impatiens after weekly applications of Regalia.

LCC in impatiens was greater using the 0.5× rate at 16 DAIR compared to the 0.0× (nontreated) (Table 4.3). However, at the close of the experiment LCC was similar among the 0.0× (nontreated), 0.5×, and 1.0× rates. At 16, 22, and 29 DAIR, LCC was

greater with increasing TVWC. Pn was similar for impatiens treated with Regalia compared to the nontreated at 14 and 28 DAIR (Table 4.4). Pn at 14 and 28 DAIR was greater at higher TVWC. Ψ_{stem} was similar among all Regalia rates at 14 and 28 DAIR. However, at 28 DAIR Ψ_{stem} was lower (more negative) in the 25% TVWC plants compared to plants watered at 85% or 55% TVWC.

GST activity was similar in leaves of impatiens after the application of Regalia compared to the 0.0× rate; however, GST did increase with decreased TVWC (Table 4.5). There was no rate×TVWC effect on GST activity. Conversely, there was a rate×TVWC effect on SP content in leaves of impatiens. SP content was greater in impatiens treated with the 1.5× rate compared to the 0.5× rate of Regalia at 85% TVWC (Fig. 4.2). Additionally, impatiens treated with all rates of Regalia (0.5×, 1.0×, or 1.5×) at 55% TVWC had greater SP content compared to the 0.0× (nontreated) at 55% TVWC.

Although visual observations did not indicate improved growth (Fig. 4.3), Regalia did improve RDW, LCC, and SP content of impatiens under moderately stressed (55% TVWC) conditions (Fig. 4.3).

Experiment 2

At initiation of the experiment containers were determined to be at 100 % AVWC before initial application of MBI-501. Based on AVWC, containers watered to 85% TVWC were not hand watered until 2 days after initial MBI-501 application (DAIM), whereas containers maintained at 55% or 25% TVWC had 8 days of dry down (55%) or 15 days of dry down (25%) respectively (Fig. 4.4).

Differing rates of MBI-501 did not affect FGI, SDW or RDW (Table 4.6). FGI, SDW, and RDW were greater at 85% TVWC treatment compared to the impatiens maintained at 55% or 25% TVWC treatments. There was no rate × TVWC interaction affecting FGI, SDW, or RDW.

WUE of impatiens was similar among rates; however, as TVWC decreased WUE increased (Table 4.7). MWA was unaffected by rate of MBI-501 but was greater at 85% TVWC compared to 55% or 25% TVWC. LCC was greater after application of the 0.5× rate of MBI-501 at 29 DAIM compared to the nontreated (Table 4.8). Subsequently, at 16, 22, and 29 DAIM, LCC content decreased with decreasing TVWC. There was no significant rate × TVWC effect. Rate of MBI-501 did not affect Pn at 14 or 28 DAIM (Table 4.9). Plants grown in substrate maintained at 85% TVWC exhibited increased Pn compared to those in substrate maintained at 25% TVWC at 14 and 28 DAIM. Fourteen DAIM, there was a rate × TVWC effect on Ψ_{stem} of impatiens. Results indicated nontreated impatiens in containers maintained at 25% TVWC had 84% and 90% lower (more negative) Ψ_{stem} compared to the 1.0× and 1.5× rates (Fig. 4.5). However, MBI-501 applied at the 1.0× and 1.5× rates to impatiens watered at 25% TVWC had similar Ψ_{stem}. At 28 DAIM, the 25% TVWC severely reduced growth of impatiens; therefore, this treatment was excluded from the data analysis. There was no rate, TVWC, or rate × TVWC effect at 28 DAIM on Ψ_{stem} of impatiens. Based on visual observations, there was no indication that MBI-501 improved growth of impatiens (Fig. 4.6)

Experiment 3

At initiation of the experiment, containers were at 100% AVWC prior to initial application of Regalia (Fig. 4.7). During the experiment, AVWC was 80% (1 DBW), 69% (3 DBW), or 61% (6 DBW).

FGI, SDW, nor RDW of impatiens were affected by rate of Regalia (Table 4.10). However, FGI and SDW were less with 3 and 6 DBW compared to watering daily (1 DBW). Withholding water for 3 or 6 days resulted in lower SDW compared to hand watering every day (1 DBW). RDW of impatiens was similar among all rates of Regalia; however, RDW was lower for plants at 3 and 6 DBW compared to 1 DBW. There was no rate × DBW effect on FGI, SDW, or RDW.

In Expt. 3, WUE was similar in impatiens treated with Regalia compared to the 0.0× (nontreated) (Table 4.11). WUE, was greater at 1 DBW compared to 3 DBW. Additionally, MWA decreased as DBW increased. There was no significant rate effect on LCC at 1, 8, 16, or 22 DAIR; however, plants receiving the 0.5× rate of Regalia had greater LCC compared to the 0.0× and 1.5× rates at 29 DAIR (Table 4.12). Additionally, there was a DBW effect seen at all DAIR where 1 and 3 DBW had greater LCC compared to plants watered at 6 DBW. Pn was not affected by rate of Regalia at 14 DAIR; however, at 28 DAIR impatiens treated with the 1.0× rate of Regalia resulted in greater Pn compared to the nontreated (Table 4.13). At 28 DAIR, 1 and 3 DBW resulted in greater Pn compared to the 6 DBW treatment. There was no significant rate × DBW effect. Impatiens treated with Regalia at the 1.5× rate had 48% and 46% lower (more negative) Ψ_{stem} compared to the 0.0× and 0.5× rate at 14 DAIR. However, at 28 DAIR Ψ_{stem} was similar among all rates. In Expt. 3, AVWC on average was 80% at 1 DBW,

69% at 3 DBW and 61% at 6 DBW; therefore, the similarity in Ψ_{stem} among varying DBW is likely due to the AVWC staying within a 20% range.

GST activity decreased in leaves treated with Regalia (0.5× 1.0× and 1.5×) (Table 4.14). Whereas, leaves of impatiens at 3 or 6 DBW had similar GST activity compared to the 1 DBW treatment. There was no rate×DBW effect on GST. However, there was a rate×DBW effect on SP content. SP content was 86%, 65%, and 84% greater in leaves of impatiens treated with the 1.5× rate at 1 DBW compared to the 0.0×, 0.5× and 1.0× treatments with 1 DBW (Fig. 4.8). Additionally, Regalia applied at the 0.5× rate increased SP content by 77% compared to the 0.0× and 1.0× treatments at 3 DBW. Visually, there was no indication Regalia improved growth of impatiens, even though LCC and Pn were greater in plants treated with Regalia (Fig. 4.9).

Experiment 4

Similar to the previous experiments, at initiation of the experiment, containers were at 100% AVWC. Substrates watered at 1, 3, or 6 DBW on average maintained an AVWC of 79%, 67%, or 60% (Fig. 4.10).

FGI, SDW, and RDW were similar among all rates of MBI-501 (Table 4.15). Impatiens at 3 and 6 DBW resulted in less shoot growth compared to the 1 DBW treatments. There was no rate × DBW effect on FGI or SDW. RDW was greater in impatiens watered at 1 DBW compared to 3 or 6 DBW.

There was a rate × DBW interaction in WUE of impatiens at 28 DAIM (Table 4.16). WUE was less in impatiens treated with the 1.5× rate of MBI-501 at 3 DBW compared to the 0.0×, 0.5× and 1.0× treatments (Figure 4.11), indicating MBI-501

applied at the 1.5× rate enhanced WUE in impatiens with 3 DBW. MWA to impatiens treated with MBI-501 were similar to the 0.0× (nontreated) and decreased with increasing DBW. There was no rate× DBW effect on MWA.

LCC was similar among all rates through the duration of the experiment (Table 4.17). There was no difference in LCC when plants were watered at 1 DBW compared to 3 or 6 DBW through the duration of the experiment; however, watering at 6 DBW resulted in lower LCC compared to 1 and 3 DBW with the exception of 1 DAIM.

Pn was not affected by application of MBI-501 (Table 4.18). Pn was higher when impatiens were watered at 1 DBW compared to 3 or 6 DBW at 28 DAIM. There was a rate × DBW interaction on Ψ_{stem}, indicating the 1.5× rate of MBI-501 adversely affected impatiens, resulting in 78% lower Ψ_{stem} compared to the 0.0× rate (Fig. 4.12). However, there were no differences in Ψ_{stem} among differing DBW using the lower rates of MBI-501.

Based on visual observations, there was no indication MBI-501 enhanced growth of impatiens (Fig. 4.13).

Experiment 5

At initiation of the experiment, substrate was ≥ 85% AVWC before the first application of Regalia (Fig. 4.14). AVWC was measured between 0600HR and 0800HR every day for the duration of the experiment and plants were hand watered to bring the substrate to the TVWC. As the experiment progressed and the tomato plants matured they used more water daily, indicating an increase in water applied, reported as cumulative water use (Fig. 4.15).

Application of Regalia did not enhance FGI, SDW, or TG (Table 4.19). There was a TVWC effect indicating FGI, SDW, and TG were greater with increasing TVWC. There was no rate × TVWC effect for FGI, SDW, or TG of tomato plants.

WUE was similar among rates of Regalia; however, WUE increased as TVWC decreased from 85% to 55% (Table 4.20). MWA was similar among rates of Regalia, but decreased with decreasing TVWC. Rate of Regalia did not affect RWC of tomato plants at 17 DAIR (Table 4.21). At the close of Expt. 5 there was a rate × TVWC effect on RWC. Nontreated (0.0×) tomato plants at 85% TVWC had greater RWC compared to the 1.0× with 85% TVWC; however, substrate maintained at 55% TVWC had similar RWC in the 0.0× and 1.0× rate (Fig 4.16). LCC, was similar among all treatments at 17 DAIR. However, at 31 DAIR LCC was greater in the 1.0× rate compared to the 0.0× rate of Regalia but was not affected by TVWC treatments. Flower number was greater at 85% compared to 55% TVWC and was not affected by rate of Regalia. Pn, was similar in all treatments regardless of Regalia rate or TVWC, at 17 and 31 DAIR (Table 4.22). Ψ_{stem} was not affected by rate, but was greater (less negative) in the 85% TVWC at 17 DAIR; however, by the end of the experiment there were no differences.

There was a rate×TVWC effect on GST activity (Table 4.23). GST was greater in the 0.0× with 85% TVWC compared to the 1.0× at 85% TVWC (Fig. 4.17). Additionally, the 1.0× rate at 55% TVWC had greater GST activity compared to the 0.0× rate at 55% TVWC. There was a rate×TVWC effect on SP content. SP content was greater in leaves treated with the 1.0× rate at 85% TVWC compared to the 0.0× (nontreated) at 85% TVWC; whereas, the nontreated at 55% TVWC had greater SP

content compared to the 1.0× rate at 55% TVWC (Fig. 4.18). Similar to Expt. 3, Regalia seems to have a positive effect on impatiens even though visual observations do not indicated enhanced growth of tomato plants (Fig. 4.19).

Experiment 6

At initiation of the experiment, AVWC measurements were similar to Expt. 5 (Fig. 4.20). AVWC was measured between 0600HR and 0800HR every day for the duration of the experiment and plants were hand watered to bring the substrate to the TVWC. As the experiment progressed and the tomato plants matured they used more water daily, indicating an increase in water applied, reported as cumulative water use (Fig. 4.21).

MBI-501 applied to tomato plants did not enhance FGI, SDW or TG (Table 4.24). Similar to the results using Regalia, TVWC was the limiting factor affecting growth. Tomato plants grown at 85% TVWC had greater FGI, SDW and TG compared to 55% TVWC. There was no rate × TVWC effect.

WUE of tomato plants decreased as TVWC increased (Table 4.25). At 17 DAIM, the rate of MBI-501 did not affect RWC; however, at 31 DAIM the 1.0× rate resulted in greater RWC compared to the nontreated (0.0× rate) (Table 4.26). Additionally, RWC was greater at 85% TVWC at 17 and 31 DAIM. LCC was greater at 17 DAIM with the 0.0× rate compared to the 1.0× rate of MBI-501 (Table 4.26). However, at 31 DAIM there was no rate effect. There was no difference in LCC due to TVW. Flower number was not affected by rate of MBI-501 or TVWC. There was no rate × TVWC effect on flower number. Pn and Ψ_{stem} were similar among all treatments regardless of rate or

TVWC (Table 4.27). There was no rate × TVWC effect on Ψ_{stem}. Visually there was no indication MBI-501 improved growth of tomato plants (Fig. 4.22).

Discussion

RDW was increased after the application of Regalia at the 0.5× rate which is consistent with data from Marrone Bio Innovations (2011a) reporting increased root growth in strawberry and tomato seedlings after application of Regalia. Growth of impatiens was significantly less as TVWC decreased due to reduced CO_2 uptake, and greater Ψ_{stem} (Burnett and van Iersel, 2008). In the 25% TVWC treatment, there was substantially less MWA over the duration of the experiment resulting in lower WUE and reducing stomatal conductance ultimately affecting biomass production. At the close of Expt. 1, impatiens maintained at 25% TVWC were showing signs of water stress injury and as a result were producing more GST then impatiens maintained at 85% or 55% TVWC (Gill and Tuteja, 2010). Regalia applied at the 1.5× rate (85% TVWC) and the 0.5×, 1.0×, or 1.5× rate (55% TVWC) produced or enhanced SP content compared to the nontreated, which is typical of Regalia application (Marrone Bio Innovations, 2012).

LCC was greater after application of MBI-501 at the 0.5× rate compared to the 0.0× (nontreated). Previous research has shown reduced transpiration with a decrease in Pn after the application of an antitranspirant (del Amor, 2010); however, rate of MBI-501 did not adversely affect Pn, regardless of rate, 28 DAIM. These results agree with Goreta et al., (2007) indicating application of an antitranspirant did not increase or enhance Pn, Ψ_{stem}, or WUE of impatiens. TVWC appeared to be the limiting factor affecting growth of impatiens, with reduced shoot growth in plants maintained at 55 or 25% TVWC

compared to 85% TVWC. Additionally, impatiens at 85% TVWC received 94% more MWA, resulting in increased Pn (Niu et al., 2008; Stewart et al., 2007). By the end of Expt. 2, impatiens at 25% TVWC had passed the permanent wilting point (Blanusa et al., 2009).

Growth of impatiens was not enhanced by Regalia application; however, increasing TVWC did increase growth. Typically, WUE is greater with decreasing moisture content, resulting in less growth (Burnett and van Iersel, 2008); however, the 1 DBW treatment had greater FGI and greater WUE compared to the 3 DBW treatment with similar MWA applied between treatments. Moreover, WUE of impatiens was similar between the 1 and 6 DBW treatments yet MWA was 20% less with the 6 DBW treatment compared to the 1 DBW treatment. These results are more than likely due to the AVWC remaining within a 20% range throughout the experiment, which could also explain the similarity in Ψ_{stem} between all TVWC. Pn was greater in impatiens treated with Regalia at the 1.0× rate compared to the nontreated at the close of Expt. 3. Unlike Expt. 1, the antioxidant enzyme GST was less in leaves treated with Regalia compared to the nontreated. ROS are always present in the plant; however, only under elevated levels do they stimulate the production of antioxidant enzymes. Furthermore, several fungicides (carbendazim, tebuconazole, azoxystrobin, and JS399-19) have shown a decrease in 0_2^- levels in flag leaves of winter wheat (Zhang et al., 2010). Therefore, Regalia may have protected against the formation of ROS, reducing the need for antioxidant enzymes. Conversely, SP content was greater in leaves treated with the 1.5×

rate at 1 DBW compared to the 0.0×, 0.5× and 1.0× rate at 1 DBW. Additionally, Regalia applied at the 0.5× rate at 3 DBW resulted in greater SP content compared to the 0.0× and 1.0× rate at 3 DBW.

Results from Expt. 4, indicated water-stress treatments appear to be the contributing factor affecting shoot growth of impatiens. These findings were similar to Blanusa et al., (2009) showing reduced growth of impatiens and petunia under water stress. Previous reports have indicated reduced photosynthesis after the use of antitranspirants (del Amor et al., 2010); however, the results reported in this paper indicate MBI-501 did not reduce Pn. WUE was less in impatiens treated with the 1.5× rate of MBI-501, indicating improved WUE at 3 DBW compared to the nontreated at 3 DBW. At 1 and 3 DBW, Ψ_{stem} was similar among all rates. These findings are similar to Goreta et al., (2007) who reported no significance in gas exchange or leaf water potential in plants treated with film-forming materials compared to nontreated. However, there was a negative effect on Ψ_{stem} after the 1.5× rate compared to the nontreated at 6 DBW, indicating the water stress treatment and high rate of MBI-501 were both contributing factors. Overall, WUE was improved after the 1.5× rate; however, this was the only indication that MBI-501 positively influenced drought tolerance of impatiens.

Application of Regalia did not enhance growth (Expt. 5); however, shoot growth of tomato plants was reduced in the 55% TVWC compared to the 85% TVWC treatment, which is consistent with previous studies (Rahman et al., 2004). As reported by Burnett and van Iersel (2008), WUE decreases with increased TVWC which was seen through less biomass production of tomato per L, with increasing MWA. Regalia application did

increase LCC compared to the nontreated, which is similar to Daayf et al. (1997). The increase in LCC could also explain the increase in Pn after Regalia application, since under drought stress, plants will conserve energy by reducing photosynthesis thus, the need for chlorophyll; however, application of Regalia increased LLC in tomato leaves, increasing Pn.

RWC decreases in plants grown under water-stress (Yuan et al., 2010) which was indicated by the lower RWC in tomato plants at 55% TVWC compared to 85% TVWC, respectively. GST activity was increased in leaves treated with the 1.0× rate at 55% TVWC compared to the nontreated at 55%. Additionally, the 0.0× rate at 85% TVWC had greater GST activity compared to the 1.0× rate at 85% TVWC. Several factors could have contributed to these results. First, the increased levels of LCC and Pn in leaves treated with Regalia at the 1.0× rate may have provided some form of protection; however, TVWC was also a contributing factor as seen through reduced growth. Thus, in the 1.0× at 85% TVWC Regalia may have enhanced production of phytoalexins (Daayf et al., 1997); yet, with the high TVWC the plants did not need to increase production of antioxidant enzymes. Furthermore, nontreated leaves at 55% TVWC had reduced GST activity compared to the 1.0× at 55%. Thus, both the nontreated and the 1.0× treatment at 55% TVWC were affected by the low TVWC with the nontreated not enhancing its own defense mechanisms; whereas, application of Regalia at the 1.0× rate to plants at 55% TVWC, increased the production of GST. Subsequently, the nontreated at 85% TVWC had greater GST activity, expressing the need for protection against ROS even though it had a high TVWC. Conversely, Regalia has been reported to increase natural

proteins in plants (Marrone Bio Innovations, 2012); which coincide with the results presented indicating increased SP content in leaves treated with the 1.0× rate at 85% compared to the 0.0× at 85%. Furthermore, there was a decrease in SP content in leaves treated with the 1.0× at 55% compared to the 0.0× rate at 55% TVWC; suggesting decreased TVWC was the contributing factor (Rahman, et al., 2004). While all efforts were made to control for confounding variables, temperature in the greenhouse on several occasions climbed well above the set points which could have caused adverse effects on plant growth; however, all plants were exposed to the same conditions.

MBI-501 applied to water-stressed tomato plants did not enhance growth (Expt. 6). As with many other crops, growth of tomato was suppressed with decreasing TVWC (Rahman, et al., 2004). WUE decreased with increasing MWA, which is consistent with Burnett and van Iersel (2008). RWC was greater in leaves treated with the 1.0× rate of MBI-501 compared to the nontreated; however, rate of MBI-501 did not enhance or increase LCC, Pn, nor Ψ_{stem}, which is consistent with previous research (Goreta, et al., 2007; McKenney and Kamp-Glass, 1990). Additionally, the 55% TVWC treatment decreased RWC (Liu et al., 2005), indicating the water-stress treatment appears to be the limiting factor and not the rate of MBI-501.

In conclusion, the objective of these experiments was to determine whether drought tolerance was enhanced in 'Super Elfin XP White' impatiens or 'BHN 640' tomato plants following the application of Regalia or MBI-501. While growth was

unaffected by rate of Regalia, impatiens and tomato plants treated with Regalia did have increased chlorophyll content, a higher photosynthetic rate and greater soluble protein content in moderately stressed (55% TVWC) plants.

Table 4.1 Growth of *Impatiens walleriana* 'Super Elfin XP White' after four weekly foliar applications of Regalia based on the 1× rate (10 $mL \cdot L^{-1}$), to plants grown in containers maintained at 85%, 55% or 25% target substrate volumetric water content (TVWC) (Expt. 1).

Rate	FGI[z] (cm)	SDW[y] (g)	RDW[x] (g)
0.0×	18.5 a[w]	4.1 a	0.42 b
0.5×	19.9 a	4.5 a	0.57 a
1.0×	19.9 a	4.5 a	0.52 ab
1.5×	17.2 a	3.6 a	0.41 b
TVWC			
85%	24.1 a	7 a	0.66 a
55%	19.9 b	4.1 b	0.48 b
25%	12.5 c	1.1 c	0.30 c
Effects			
rate	0.1509[v]	0.3437	0.0010
TVWC	<.0001	<.0001	<.0001
rate×TVWC	0.8587	0.0552	0.8915

[z]FGI: final growth indices [(height + width + perpendicular width) ÷ 3].

[y]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[x]RDW: root dry weight, oven dried for 72 h at 65 °C.

[w]Means (within a column) with the same letters within TVWC or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[v]*P* value.

Table 4.2 Water use efficiency (WUE) of *Impatiens walleriana* 'Super Elfin XP White' after four weekly applications of Regalia based on the 1.0× rate (10 mL·L^{-1}) to plants grown in containers maintained at 85%, 55% or 25% target substrate volumetric water content (TVWC) (Expt. 1).

Rate	WUE (g·L^{-1})[z]	MWA (L)[y]
0.0×	5.7 a[x]	1.2 a
0.5×	6.9 a	1.1 a
1.0×	6.5 a	1.2 a
1.5×	5.4 a	1.1 a
TVWC		
85%	3.2 b	2.3 a
55%	4.3 b	1.0 b
25%	10.8 a	0.1 c
Effects		
rate	0.2484[w]	0.7035
TVWC	<.0001	<.0001
rate×TVWC	0.9937	0.8766

[z]WUE = [(SDW+RDW) ÷ total water applied].

[y]MWA: mean water applied.

[x]Means (within a column) with the same letters within moisture level or rate are not statistically different according to the Holm-Simulation method for mean comparison, alpha = 0.05.

[v]*P* value.

Table 4.3 Leaf chlorophyll content[z] of *Impatiens walleriana* 'Super Elfin XP White' measured after four weekly foliar applications of Regalia based on the 1.0× rate (10 mL·L^{-1}) to plants grown in containers maintained at 85%, 55%, or 25% target substrate volumetric water content (TVWC) (Expt. 1).

	Days after initial application of Regalia				
Rate	1	8	16	22	29
0.0×	48.2 a[y]	47.6 a	48.0 b	52.0 a	48.0 ab
0.5×	50.8 a	48.2 a	52.2 a	54.5 a	50.7 a
1.0×	47.7 a	48.1 a	52.0 a	52.8 a	47.3 ab
1.5×	47.0 a	46.8 a	48.5 ab	51.9 a	46.3 b
TVWC					
85%	48.1 a	49.2 a	54.5 a	57.0 a	55.2 a
55%	49.0 a	47.1 ab	51.0 b	54.2 b	51.4 b
25%	48.2 a	46.7 b	45.0 c	47.2 c	38.9 c
Effects					
rate	0.2271[x]	0.7613	0.0069	0.4881	0.0495
TVWC	0.5529	0.0336	<.0001	<.0001	<.0001
rate×TVWC	0.9689	0.8192	0.3390	0.2837	0.2693

[z]Chlorophyll content determined using a handheld 502 SPAD meter (Konica Minolta Optics Inc., Minolta, Japan): 1, 8, 16, 22, and 29 days after initial application of Regalia.

[y]Means (within a column) with the same letters within TVWC or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[x]*P* value.

Table 4.4 Leaf photosynthetic rate and stem water potential of *Impatiens walleriana* 'Super Elfin XP White' grown in containers maintained at 85%, 55%, or 25% target substrate volumetric water content (TVWC), 14 and 28 days after initial application of Regalia based on the 1.0× rate (10 $mL \cdot L^{-1}$) (Expt. 1).

	Leaf photosynthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$)[z]		Ψ_{stem} (-MPa)[y]	
	Days after initial application of Regalia			
Rate	14	28	14	28
0.0×	7.4 a[x]	8.4 a	-0.079 a	-0.201 a
0.5×	11.9 a	9.0 a	-0.117 a	-0.052 a
1.0×	8.1 a	8.4 a	-0.093 a	-0.092 a
1.5×	4.9 a	7.0 a	-0.070 a	-0.185 a
TVWC				
85%	12.0 a	11.7 a	-0.073 b	-0.061 b
55%	7.3 b	9.7 b	-0.120 ab	-0.078 b
25%	4.9 b	3.2 c	-0.081 a	-0.259 a
Effects				
rate	0.4386[w]	0.7771	0.8189	0.1771
TVWC	<.0001	<.0001	0.0410	0.0272
rate×TVWC	0.9620	0.3245	0.4960	0.2104

[z]Leaf photosynthetic rate measured using a CIRAS-2 (PPSystems, Amesbury, MD) at 14 and 28 days after initial application of Regalia.

[y]Stem water potential was measured by first wrapping the leaf in plastic film then covering with aluminum foil for one hour before taking measurements.

[x]Means (within a column) with the same letters within TVWC or rate are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.5 Glutathione-*S*-transferase (GST) activity and soluble protein (SP) content in leaves of *Impatiens walleriana* 'Super Elfin XP White' affected by Regalia application based on the 1.0× rate (10 $mL \cdot L^{-1}$), to plants grown in containers at 85%, 55%, or 25% target substrate volumetric water content (TVWC) (Expt. 1).

Rate	GST ($\mu units \cdot mg^{-1}$)	SP ($\mu g \cdot mL^{-1}$)
0.0×	92.4 a[z]	1.2 b
0.5×	65.5 a	2.2 a
1.0×	33.2 a	2.6 a
1.5×	80.2 a	2.5 a
TVWC		
85%	36.5 b	2.7 a
55%	50.5 b	2.7 a
25%	116.6 a	1.0 b
Rate	0.0787[y]	0.0044
TVWC	0.0007	<.0001
Rate×TVWC	0.1606	0.0144

[z]Means with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparisons $\alpha = 0.05$.

[y]*P* value.

Table 4.6 Growth of *Impatiens walleriana* 'Super Elfin XP White' following weekly foliar applications of MBI-501 based on the 1.0× rate (2 $mL \cdot L^{-1}$) to plants grown in containers maintained at 85%, 55%, or 25% target substrate volumetric water content (TVWC) (Expt. 2).

Rate	FGI[z] (cm)	SDW[y] (g)	RDW[x] (g)
0.0×	18.9 a[w]	3.9 a	0.31 a
0.5×	18.2 a	3.9 a	0.41 a
1.0×	19.3 a	4.2 a	0.33 a
1.5×	19.4 a	4.0 a	0.36 a
TVWC			
85%	25.1 a	7.4 a	0.55 a
55%	19.3 b	3.5 b	0.32 b
25%	12.5 c	1.0 c	0.21 c
Effects			
rate	0.8635[v]	0.9712	0.4842
TVWC	<.0001	<.0001	<.0001
rate×TVWC	0.7115	0.9780	0.4716

[z]FGI: final growth indices [(height + width + perpendicular width) ÷ 3].

[y]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[x]RDW: root dry weight, oven dried for 72 h at 65 °C.

[w]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[v]*P* value.

Table 4.7 Water use efficiency (WUE) of *Impatiens walleriana* 'Super Elfin XP White' after four weekly applications of MBI-501 based on the 1.0× rate (2 mL·L^{-1}) to plants grown in containers maintained at 85%, 55% or 25% target substrate volumetric water content (TVWC) (Expt. 2).

Rate	WUE (g·L^{-1})[z]	MWA (L)[y]
0.0×	4.94 a[x]	1.22 a
0.5×	5.44 a	1.16 a
1.0×	5.05 a	1.18 a
1.5×	4.91 a	1.23 a
TVWC		
85%	3.10 a	2.48 a
55%	3.82 b	0.97 b
25%	8.34 b	0.15 c
Effects		
rate	0.8010[w]	0.9263
TVWC	<.0001	<.0001
rate×TVWC	0.8204	0.9995

[z]WUE = [(SDW+RDW) ÷ total water applied].

[y]MWA: mean water applied.

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.8 Leaf chlorophyll content[z] of *Impatiens walleriana* 'Super Elfin XP White' measured after initial foliar application of MBI-501 based on the 1.0× rate (2 mL·L^{-1}) to plants grown in containers maintained at 85%, 55%, or 25% target substrate volumetric water content (TVWC) (Expt. 2).

	Days after initial application of MBI-501				
Rate	1	8	16	22	29
0.0×	47.6 a[y]	47.7 ab	49.9 a	50.8 a	48.2 b
0.5×	49.5 a	48.5 a	52.3 a	52.1 a	52.1 a
1.0×	49.0 a	47.4 ab	51.5 a	52.2 a	49.3 ab
1.5×	48.7 a	45.6 b	51.8 a	51.5 a	49.0 ab
TVWC					
85%	48.9 a	48.2 a	55.5 a	57.9 a	58.2 a
55%	48.7 a	47.0 a	51.4 b	53.6 b	55.1 b
25%	48.5 a	46.7 a	47.3 c	43.4 c	34.9 c
Effects					
rate	0.2098[x]	0.0203	0.8269	0.8150	0.0290
TVWC	0.9014	0.1967	<.0001	<.0001	<.0001
rate×TVWC	0.7800	0.1629	0.8625	0.1633	0.4990

[z]Chlorophyll content determined using a handheld 502 SPAD meter (Konica Minolta Optics Inc., Minolta, Japan): 1, 8, 16, 22, and 29 days after initial application of MBI-501.

[y]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[x]*P* value.

Table 4.9 Leaf photosynthetic rate and stem water potential of *Impatiens walleriana* 'Super Elfin XP White' following foliar application of MBI-501 based on the 1.0× rate (2 mL·L^{-1}) to plants grown in containers maintained at 85%, 55%, or 25% target substrate volumetric water content (TVWC) (Expt. 2).

	Leaf photosynthetic rate (μmol·m^{-2}·s^{-1})[z]		Ψ_{stem} (-Mpa)[y]	
	Days after initial application of MBI-501			
Rate	14	28	14	28
0.0×	7.8 a[x]	5.5 a	-0.23 a	-0.07 a
0.5×	11.2 a	6.0 a	-0.15 ab	-0.04 a
1.0×	9.9 a	7.8 a	-0.06 b	-0.04 a
1.5×	6.7 a	6.1 a	-0.07 b	-0.12 a
TVWC				
85%	15.9 a	8.6 a	-0.05 b	-0.06 a
55%	8.7 b	8.4 a	-0.11 a	-0.09 a
25%	2.0 c	2.1 b	-0.23 a	-
Effects				
rate	0.1221[w]	0.2738	0.0855	0.2380
TVWC	<.0001	0.0002	0.0005	0.4311
rate×TVWC	0.1977	0.9831	0.0084	0.2645

[z]Leaf photosynthetic rate measured using a CIRAS-2 (PPSystems, Amesbury, MD) at 14 and 28 days after initial application of MBI-501.

[y]Stem water potential was measured by first wrapping the leaf in plastic film then covering with aluminum foil for one hour before taking measurements.

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.10 Growth of *Impatiens walleriana* 'Super Elfin XP White' following weekly foliar applications of Regalia based on the 1.0× rate (10 $mL \cdot L^{-1}$) to plants grown in containers at 1 (daily), 3, or 6 days between watering (DBW) (Expt. 3).

Rate	FGI[z] (cm)	SDW[y] (g)	RDW[x] (g)
0.0×	20.9 a[w]	5.0 a	0.36 a
0.5×	21.2 a	5.7 a	0.38 a
1.0×	22.3 a	5.9 a	0.40 a
1.5×	21.1 a	4.4 a	0.34 a
DBW			
1	23.4 a	6.6 a	0.46 a
3	21.5 b	5.2 b	0.34 b
6	19.3 c	3.9 c	0.30 b
Effects			
rate	0.6735[v]	0.9180	0.7920
DBW	<.0001	<.0001	<.0001
rate×DBW	0.9610	0.8231	0.7685

[z]FGI: final growth indices [(height + width + perpendicular width) ÷ 3].

[y]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[x]RDW: root dry weight, oven dried for 72 h at 65 °C.

[w]Means (within a column) with the same letters within DBW or rate are not statistically differnt according to the Holm-Simulation method for mean comparison,$\alpha = 0.05$.

[v]*P* value.

Table 4.11 Water use efficiency (WUE) of *Impatiens walleriana* 'Super Elfin XP White' after four weekly applications of Regalia based on the 1.0× rate (10 mL·L^{-1}) to plants grown in containers at 1 (daily), 3 or 6 days between watering (DBW) (Expt. 3).

Rate	WUE (g·L^{-1})[z]	MWA (L)[y]
0.0×	2.8 a[x]	2.13 a
0.5×	3.2 a	2.01 a
1.0×	3.5 a	1.96 a
1.5×	2.6 a	2.01 a
DBW		
1	3.4 a	2.09 a
3	2.8 b	2.22 a
6	3.0 ab	1.77 b
Effects		
rate	0.5427[w]	0.6445
DBW	0.0044	0.0007
rate×DBW	0.7743	0.9690

[z]WUE = [(SDW+RDW) ÷ total water applied].

[y]MWA: mean water applied.

[x]Means (within a column) with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.12 Leaf chlorophyll content[z] of *Impatiens walleriana* 'Super Elfin XP White' measured after initial foliar application of Regalia applied weekly, based on the 1.0× rate (10 mL·L^{-1}) to plants grown in containers at 1 (daily), 3, or 6 days between watering (DBW) (Expt. 3).

	Days after initial application of Regalia				
Rate	1	8	16	22	29
0.0×	48.7 a[y]	46.9 a	53.5 a	54.9 a	54.2 b
0.5×	49.1 a	48.2 a	53.5 a	56.8 a	57.5 a
1.0×	48.7 a	49.4 a	54.5 a	55.8 a	55.5 ab
1.5×	48.7 a	46.8 a	53.8 a	55.9 a	53.1 b
DBW					
1	49.5 a	48.7 a	55.3 a	57.5 a	56.6 a
3	49.8 a	48.3 ab	54.9 a	56.5 a	57.0 a
6	47.2 b	46.4 b	51.3 b	53.5 b	52.0 b
Effects					
rate	0.9718[x]	0.0536	0.7950	0.6243	0.0045
DBW	0.0033	0.0346	<.0001	0.0013	<.0001
rate×DBW	0.1997	0.6622	0.6583	0.0828	0.2073

[z]Chlorophyll content determined using a handheld 502 SPAD meter (Konica Minolta Optics Inc., Minolta, Japan): 1, 8, 16, 22, and 29 days after initial application of Regalia.

[y]Means (within a column) with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[x]*P* value.

Table 4.13 Leaf photosynthetic rate and stem water potential (Ψ_{stem}) of *Impatiens walleriana* 'Super Elfin XP White' measured after initial foliar application of Regalia applied weekly, based on the 1.0× rate (10 $mL \cdot L^{-1}$) to plants grown in containers at 1 (daily), 3, or 6 days between watering (DBW) (Expt. 3).

	Leaf photosynthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$)[z]		Ψ_{stem} (-MPa)[y]	
	Days after initial application of Regalia			
Rate	14	28	14	28
0.0×	11.0 a[x]	6.5 b	-0.065 b	-0.053 a
0.5×	13.6 a	9.1 ab	-0.068 b	-0.074 a
1.0×	9.8 a	9.5 a	-0.084 ab	-0.095 a
1.5×	11.1 a	9.1 ab	-0.126 a	-0.130 a
DBW				
1	13.0 a	10.6 a	-0.069 a	-0.077 a
3	10.7 a	10.1 a	-0.090 a	-0.061 a
6	10.4 a	4.9 b	-0.097 a	-0.128 a
Effects				
rate	0.9408[w]	0.0365	0.0132	0.2716
DBW	0.0442	<.0001	0.2788	0.0526
rate×DBW	0.9475	0.3078	0.3496	0.8283

[z]Leaf photosynthetic rate measured using a CIRAS-2, PPSystems, Amesbury, MD, Plainfield, IN: 14 and 28 days after initial application of Regalia.

[y]Stem water potential was measured by first wrapping the leaf in plastic film then covering with aluminum foil for one hour before taking measurements.

[x]Means (within a column) with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.14 Glutathione-*S*-transferase (GST) activity and soluble protein (SP) content in leaves of *Impatiens walleriana* 'Super Elfin XP White' affected by Regalia application based on the 1.0× rate (10 $mL \cdot L^{-1}$), to plants grown in containers at 1 (daily), 3 or 6 days between watering (DBW) (Expt. 3).

Rate	GST ($\mu units \cdot mg^{-1}$)	SP ($\mu g \cdot mL^{-1}$)
0.0×	62.4 a[z]	1.7 b
0.5×	18.9 b	4.1 a
1.0×	24.5 b	2.3 b
1.5×	23.6 b	4.5 a
DBW		
1	30.9 a	3.2 a
3	31.0 a	3.6 a
6	35.2 a	2.7 a
Rate	<.0001[y]	0.0013
DBW	0.8234	0.3434
Rate×DBW	0.0717	0.0002

[z]Means with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparisons $\alpha = 0.05$.

[y]*P* value.

Table 4.15 Growth of *Impatiens walleriana* 'Super Elfin XP White' following weekly foliar applications of MBI-501 based on the 1.0× rate (2 $mL \cdot L^{-1}$) to plants grown in containers at 1 (daily), 3 or 6 days between watering (DBW) (Expt. 4).

Rate	FGI[z] (cm)	SDW[y] (g)	RDW[x] (g)
0.0×	21.7 a[w]	5.2 a	0.42 a
0.5×	22.1 a	5.2 a	0.43 a
1.0×	22.3 a	5.8 a	0.47 a
1.5×	21.6 a	4.8 a	0.38 a
DBW			
1	24.3 a	7.0 a	0.55 a
3	21.6 b	5.0 b	0.39 b
6	19.7 c	3.8 c	0.33 b
Effects			
rate	0.9342[v]	0.3303	0.3463
DBW	<.0001	<.0001	<.0001
rate×DBW	0.6783	0.4228	0.2634

[z]FGI: final growth indices [(height + width + perpendicular width) ÷ 3].

[y]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[x]RDW: root dry weight, oven dried for 72 h at 65 °C.

[w]Means (within a column) with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[v]*P* value.

Table 4.16 Water use efficiency (WUE) of *Impatiens walleriana* 'Super Elfin XP White' following four weekly applications of MBI-501 based on the 1.0× rate (2 mL·L^{-1}) to plants grown in containers at 1 (daily), 3 or 6 days between watering (DBW) (Expt. 4).

Rate	WUE (g·L^{-1})[z]	MWA (L)[y]
0.0×	3.5 ab[x]	1.6 a
0.5×	3.5 ab	1.6 a
1.0×	3.7 a	1.7 a
1.5×	3.3 b	1.6 a
DBW		
1	3.2 c	2.3 a
3	3.4 b	1.5 b
6	3.9 a	1.0 c
Effects		
rate	0.0494[w]	0.4582
DBW	<.0001	<.0001
rate×DBW	0.0190	0.7719

[z]WUE = [(SDW+RDW) ÷ total water applied].

[y]MWA: mean water applied.

[x]Means (within a column) with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.17 Leaf chlorophyll content[z] of *Impatiens walleriana* 'Super Elfin XP White', measured after initial foliar application of MBI-501 based on the 1.0× rate (2 mL·L^{-1}) to plants grown in containers at 1 (daily), 3 or 6 days between watering (DBW) (Expt. 4).

	Days after initial application of MBI-501				
Rate	1	8	16	22	29
0.0×	48.2 a[y]	48.0 a	55.6 a	56.8 a	55.8 a
0.5×	49.5 a	49.2 a	55.8 a	57.3 a	58.0 a
1.0×	49.3 a	49.2 a	54.1 a	56.2 a	56.6 a
1.5×	49.4 a	48.0 a	52.7 a	55.0 a	54.8 a
DBW					
1	49.5 a	49.6 a	56.4 a	58.4 a	58.4 a
3	48.5 a	49.6 a	55.5 a	57.5 a	56.7 a
6	49.2 a	46.6 b	51.8 b	53.0 b	53.8 b
Effects					
rate	0.4389[x]	0.7012	0.4954	0.3374	0.1779
DBW	0.3245	0.0005	<.0001	<.0001	<.0001
rate×DBW	0.5530	0.9168	0.5432	0.7592	0.5382

[z]Chlorophyll content determined using a handheld 502 SPAD meter (Konica Minolta Optics Inc., Minolta, Japan): 1, 8, 16, 22, and 29 days after initial application of MBI-501.

[y]Means (within a column) with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[x]*P* value.

Table 4.18 Leaf photosynthetic rate and stem water potential (Ψ_{stem}) of *Impatiens walleriana* 'Super Elfin XP White' measured after initial foliar application of MBI-501 applied weekly, based on the 1.0× rate (2 mL·L^{-1}) to plants grown in containers at 1 (daily), 3, or 6 days between watering (DBW) (Expt. 4).

	Leaf photosynthetic rate (μmol·m^{-2}·s^{-1})[z]		Ψ_{stem} (-Mpa)[y]	
	Days after initial application of MBI-501			
Rate	14	28	14	28
0.0×	13.2 a[x]	5.7 a	-0.09 a	-0.04 a
0.5×	16.1 a	6.6 a	-0.07 a	-0.04 a
1.0×	13.5 a	7.6 a	-0.09 a	-0.04 a
1.5×	11.3 a	7.1 a	-0.11 a	-0.06 a
DBW[v]				
1	15.8 a	11.0 a	-0.08 b	-0.04 a
3	14.2 a	6.3 b	-0.06 b	-0.04 a
6	10.6 b	3.0 c	-0.14 a	-0.05 a
Effects				
rate	0.2309[w]	0.5532	0.631	0.0642
DBW	<.0001	<.0001	0.0023	0.4318
rate×DBW	0.2161	0.3425	0.9881	0.0346

[z]Leaf photosynthetic rate measured using a CIRAS-2 (PPSystems, Amesbury, MD) at 14 and 28 days after initial application of MBI-501.

[y]Stem water potential was measured by first wrapping the leaf in plastic film then covering with aluminum foil for one hour before taking measurements.

[x]Means (within a column) with the same letters within rate or DBW are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.19 Growth of *Solanum lycopersicum* 'BHN 640' plants grown under 85% or 55% target substrate volumetric water content (TVWC), following weekly foliar applications of Regalia at the 1.0× rate (10 $mL \cdot L^{-1}$) (Expt. 5).

Rate	FGI[z] (cm)	SDW[y] (g)	TG[x] (cm)
0.0×	51.9 a[w]	40.5 a	20.8 a
1.0×	52.5 a	40.4 a	20.4 a
TVWC			
85%	55.8 a	45.7 a	23.8 a
55%	48.7 b	35.2 b	17.4 b
Effects			
rate	0.5992[v]	0.9875	0.7180
TVWC	<.0001	<.0001	<.0001
rate×TVWC	0.7916	0.5509	0.7324

[z]FGI: final growth indices [(height + width + perpendicular width) ÷ 3].

[y]SDW: shoot dry weight, oven dried for 72 h at 65 °C.

[x]TG: total shoot growth over study (final growth indices - initial growth indices)

[w]Means (within a column) with the same letters, within rate or TVWC are not not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[v]*P* value.

Table 4.20 Water use efficiency (WUE) of *Solanum lycopersicum* 'BHN 640' after four weekly applications of Regalia at the 1.0× rate (10 mL·L^{-1}) to plants grown in containers maintained at 85% or 55% target substrate volumetric water content (TVWC) (Expt. 5).

Rate	WUE (g·L^{-1})[z]	MWA (L)[y]
0.0×	7.7 a[x]	6.0 a
1.0×	7.9 a	5.9 a
TVWC		
85%	6.1 b	7.8 a
55%	9.5 a	4.0 b
Effects		
rate	0.7155[w]	0.8484
TVWC	<.0001	<.0001
rate×TVWC	0.5768	0.7428

[z]WUE = [(SDW) ÷ total water applied].

[y]MWA: mean water applied.

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, alpha = 0.05.

[w]*P* value.

Table 4.21 Relative leaf water content[z] (RWC), leaf chlorophyll content[y], and flower number of S*olanum lycopersicum* 'BHN 640', grown under 85% or 55% target substrate volumetric water content (TVWC) following weekly foliar applications of Regalia at the 1.0× rate (10 mL·L-1) (Expt. 5).

	RWC (%)		Leaf chlorophyll content		Flower number
Rate	17 DAIR	31 DAIR	17 DAIR	31 DAIR	31 DAIR
0.0×	41.6 a[x]	45.8 a	53.6 a	58.9 b	7.1 a
1.0×	41.0 a	45.1 a	54.4 a	62.8 a	6.4 a
TVWC					
85%	43.6 a	48.4 a	54.8 a	59.4 a	8.4 a
55%	39.1 b	42.5 b	53.2 a	62.3 a	5.1 b
Effects					
rate	0.5718[w]	0.5639	0.5706	0.0430	0.4465
TVWC	<.0001	<.0001	0.2659	0.1264	0.0009
rate×TVWC	0.3560	0.0111	0.9330	0.0986	0.1148

[z]Relative leaf water content = (fresh weight - dry weight) ÷ (turgid weight - dry weight) × 100.

[y]Chlorophyll content determined using a handheld 502 SPAD Chlorophyll Meter (Konica Minolta Inc., Minolta, Japan) at 17 and 31 days after initial application of Regalia (DAIR).

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.22 Leaf photosynthetic rate and stem water potential (Ψ_{stem}) measured in *Solanum lycopersicum* 'BHN 640' grown under 85% or 55% target substrate volumetric water content (TVWC), 17 and 31 days after initial foliar application of Regalia at the 1.0× rate (10 mL·L-1) (Expt. 5).

	Leaf photosynthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$)[z]		Ψ_{stem} (-MPa)[y]	
	Days after initial application of Regalia			
Rate	17	31	17	31
0.0×	21.4 a[x]	7.3 a	-0.32 a	-0.21 a
1.0×	20.8 a	10.8 a	-0.37 a	-0.26 a
TVWC				
85%	21.6 a	8.5 a	-0.30 b	-0.23 a
55%	20.6 a	9.6 a	-0.39 a	-0.25 a
Effects				
rate	0.7891[w]	0.0551	0.2535	0.1754
TVWC	0.5554	0.5201	0.0251	0.4705
rate×TVWC	0.5495	0.1193	0.0897	0.4932

[z]Leaf photosynthetic rate measured using a CIRAS-2 (PPSystems, Amesbury, MD) at 17 and 31 days after initial application of Regalia.

[y]Stem water potential was measured by first wrapping the leaf in plastic film then covering with aluminum foil for one hour before taking measurements.

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.23 Glutathione-*S*-transferase (GST) activity and soluble protein (SP) content in leaves of *Solanum lycopersicum* 'BHN 640' affected by Regalia application at the 1.0× rate (10 mL·L^{-1}) to plants grown in containers with 85% or 55% target substrate volumetric water content (TVWC) (Expt. 5).

Rate	GST (μunits·mg^{-1})	SP (μg·mL^{-1})
0.0×	55.6 a[z]	3.7 b
1.0×	49.2 a	6.6 a
TVWC		
85%	47.1 a	6.3 a
55%	57.7 a	4.0 a
Rate	0.2391[y]	<.0001
TVWC	0.0549	<.0001
Rate×TVWC	<.0001	<.0001

[z]means with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, α = 0.05.

[y]*P* value.

Table 4.24 Growth of *Solanum lycopersicum* 'BHN 640' plants grown under 85 % or 55% target substrate volumetric water content (TVWC), following weekly foliar applications of MBI-501 at the 1.0× rate (2 $mL \cdot L^{-1}$) (Expt. 6).

Rate	FGI[z] (cm)	SDW[y] (g)	TG[x] (cm)
0.0×	51.6 a[w]	40.5 a	19.6 a
1.0×	50.5 a	40.2 a	18.8 a
TVWC			
85%	55.2 a	45.3 a	23.3 a
55%	46.9 b	35.5 b	15.1 b
Effects			
rate	0.3542[v]	0.6229	0.5765
TVWC	<.0001	<.0001	<.0001
rate×TVWC	0.2649	0.8164	0.3408

[z]FGI: final growth indices [(height + width + perpendicular width) ÷ 3].

[y]SDW: shoot dry weight, oven dried for 72 h at 65°C.

[x]TG: total shoot growth over study (final growth indices - initial growth indices).

[w]Means (within a column) with the same letters, within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[v]*P* value.

Table 4.25 Water use efficiency (WUE) of *Solanum lycopersicum* 'BHN 640' after four weekly applications of MBI-501 at the 1.0× rate (2 mL·L^{-1}) to plants grown in containers maintained at 85% or 55% target substrate volumetric water content (TVWC) (Expt. 6)

Rate	WUE (g·L^{-1})[z]	MWA (L)[y]
0.0×	8.0 a[x]	5.8 a
1.0×	7.8 a	5.8 a
TVWC		
85%	6.1 a	7.7 a
55%	9.7 b	4.0 b
Effects		
rate	0.6836[w]	0.9144
TVWC	<.0001	<.0001
rate×TVWC	0.6551	0.3143

[z]WUE = [SDW ÷ total water applied].

[y]MWA: mean water applied.

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.26 Relative leaf water content[z] (RWC), leaf chlorophyll content[y], and flower number of S*olanum lycopersicum* 'BHN 640', grown under 85% or 55% target substrate volumetric water content (TVWC) following weekly foliar applications of MBI-501 at the 1.0× rate (2 mL·L^{-1}) (Expt. 6).

	RWC (%)		Leaf chlorophyll content		Flower number
Rate[x]	17 DAIM	31 DAIM	17 DAIM	31 DAIM	31 DAIM
0.0×	41.0 a[w]	45.3 b	57.3 a	60.4 a	6.0 a
1.0×	41.8 a	48.1 a	55.5 b	61.8 a	5.9 a
TVWC					
85%	43.7 a	48.4 a	56.3 a	59.7 a	6.8 a
55%	39.1 b	45.0 b	56.5 a	62.6 a	5.0 a
Effects					
rate	0.3842[v]	0.0095	0.0311	0.3469	0.9646
TVWC	<.0001	0.0023	0.8694	0.0531	0.0743
rate×TVWC	0.5572	0.1702	0.8797	0.0669	0.6928

[z]Relative leaf water content = (fresh weight - dry weight) ÷ (turgid weight - dry weight) × 100.

[y]Chlorophyll content determined using a handheld 502 SPAD Chlorophyll Meter (Konica Minolta Inc., Minolta, Japan) at 17 and 31 days after initial application of Regalia.

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

Table 4.27 Leaf photosynthetic rate and stem water potential (Ψ_{stem}) measured in *Solanum lycopersicum* 'BHN 640' grown under 85% or 55% target substrate volumetric water content (TVWC), 14 and 28 days after initial foliar application of MBI-501 at the 1.0× rate (2 mL·L^{-1}) (Expt. 6).

	Leaf photosynthetic rate (μmol·m^{-2} s^{-1})[z]		Ψ_{stem} (-MPa)[y]	
	Days after initial application of MBI-501			
Rate	14	28	14	28
0.0×	18.8 a[x]	4.0 a	-0.35 a	-0.20 a
1.0×	19.3 a	5.4 a	-0.36 a	-0.22 a
TVWC				
85%	20.5 a	4.3 a	-0.32 a	-0.20 a
55%	17.7 a	5.1 a	-0.39 a	-0.22 a
Effects				
rate	0.8721[w]	0.2764	0.7596	0.4121
TVWC	0.3847	0.5250	0.1942	0.3802
rate×TVWC	0.5319	0.0587	0.0620	0.5078

[z]Leaf photosynthetic rate measured using a CIRAS-2 (PPSystems, Amesbury, MD) at 17 and 31 days after initial application of MBI-501

[y]Stem water potential was measured by first wrapping the leaf in plastic film then covering with aluminum foil for one hour before taking measurements.

[x]Means (within a column) with the same letters within rate or TVWC are not statistically different according to the Holm-Simulation method for mean comparison, $\alpha = 0.05$.

[w]*P* value.

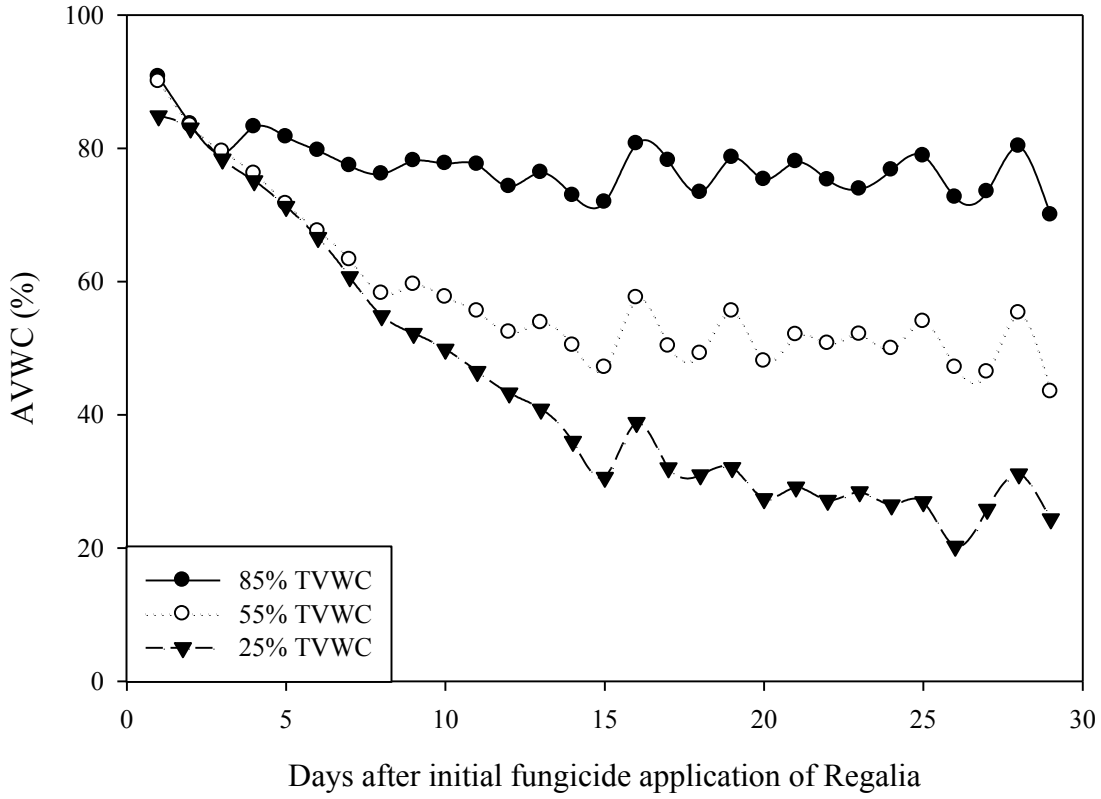

Figure 4.1 Actual substrate volumetric water content (AVWC) following foliar applications of Regalia based on the 1× rate (10 mL·L^{-1}) to *Impatiens walleriana* 'Super Elfin XP White' grown at three different target substrate volumetric water contents (TVWC): 85%, 55%, and 25%. Data points represent daily average pooled across all rates (Expt. 1).

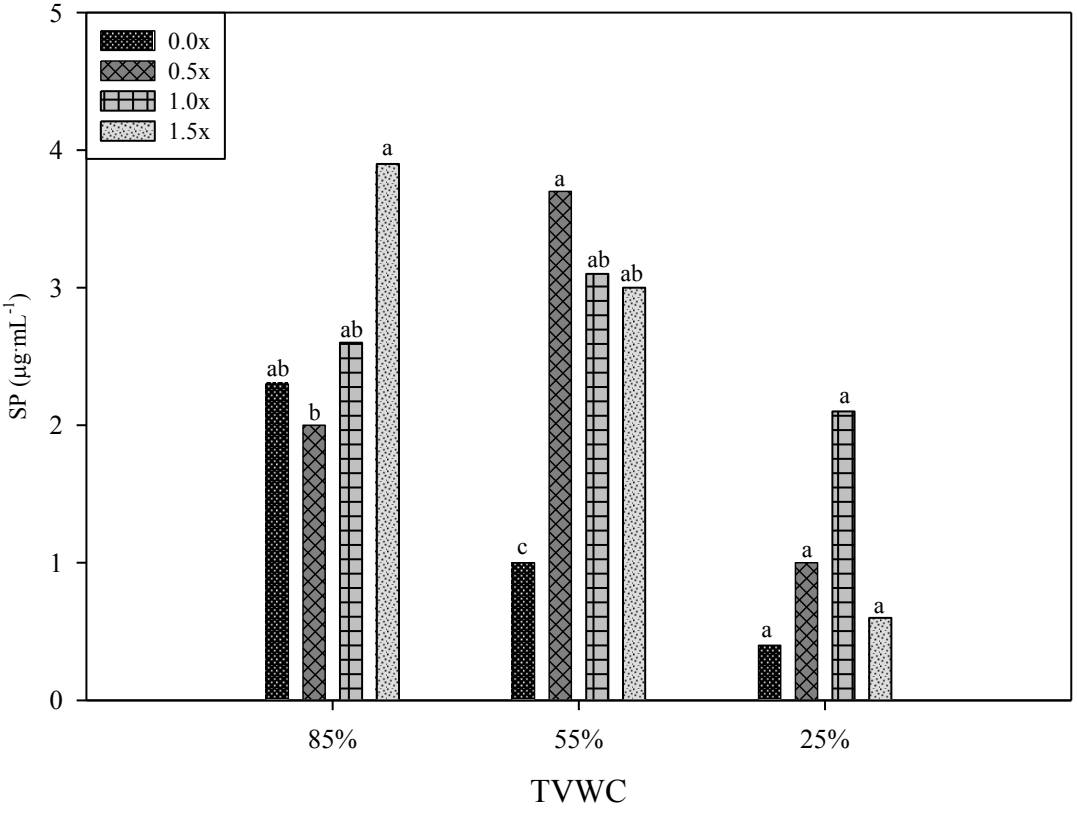

Figure 4.2 Soluble protein (SP) content in leaves of *Impatiens walleriana* 'Super Elfin XP White' following foliar application of Regalia based on the 1× rate (10 mL·L^{-1}) to plants grown in containers with 85%, 55%, or 25% target substrate volumetric water content (TVWC). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, $\alpha = 0.05$ (Expt. 1).

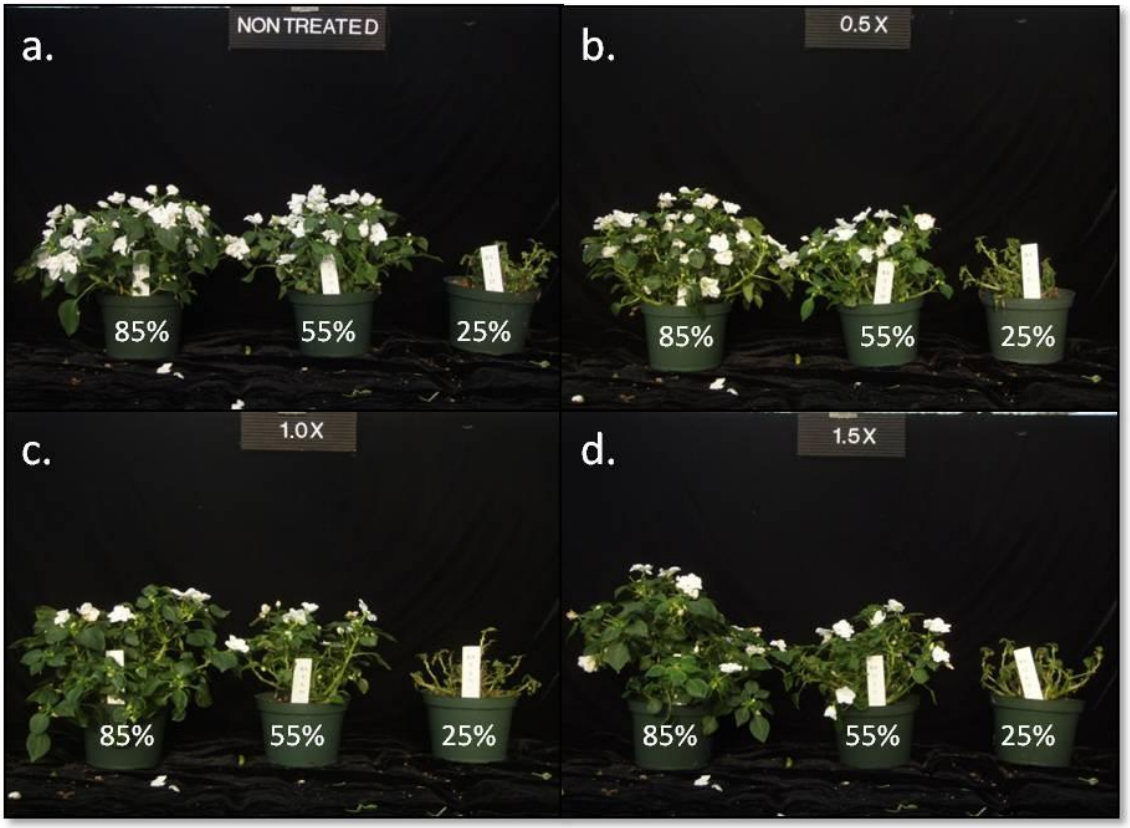

Figure 4.3 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown at 85%, 55% and 25% target substrate volumetric water content for four weeks following weekly foliar application of Regalia: a. nontreated control (0.0× rate), b. 0.5× rate (5 mL·L^{-1}), c. 1.0× rate (10 mL·L^{-1}), d. 1.5× rate (15 mL·L^{-1}) (Expt. 1).

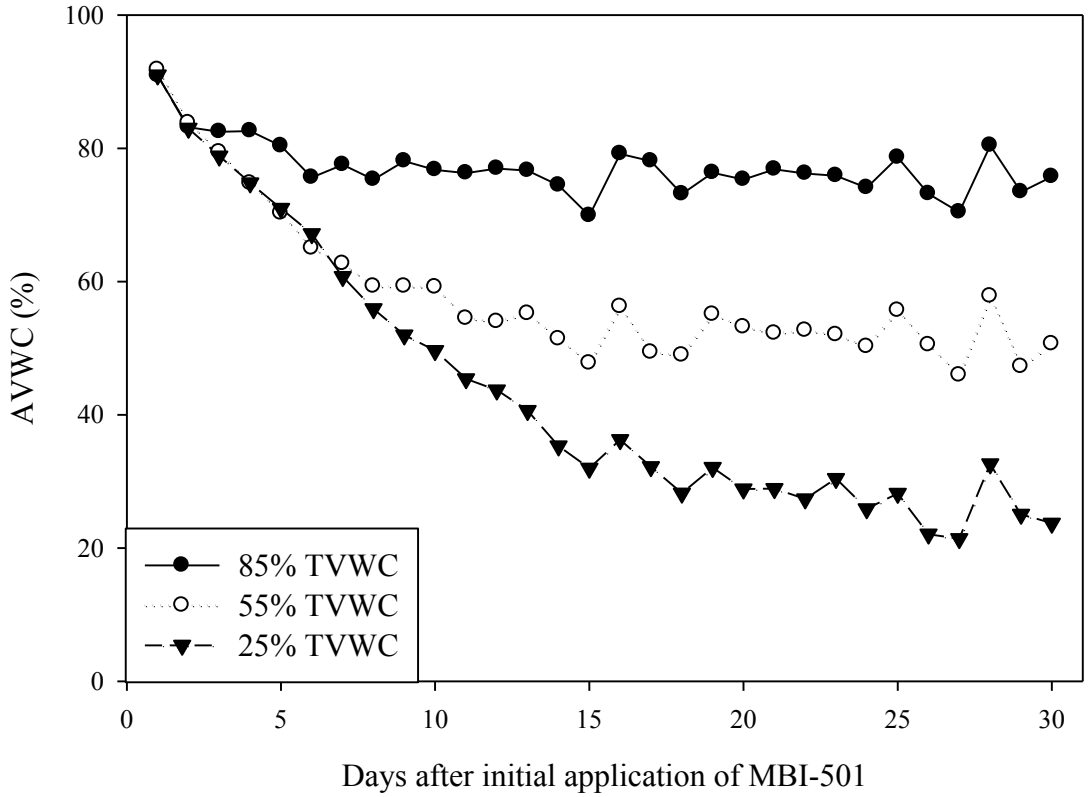

Figure 4.4 Actual substrate volumetric water content (AVWC) after weekly, foliar applications of MBI-501 based on the 1× rate (2 mL·L^{-1}) to *Impatiens walleriana* 'Super Elfin XP White', and grown with different target substrate volumetric water content (TVWC): 85%, 55%, and 25%. Data points represent daily average pooled across all rates (Expt. 2).

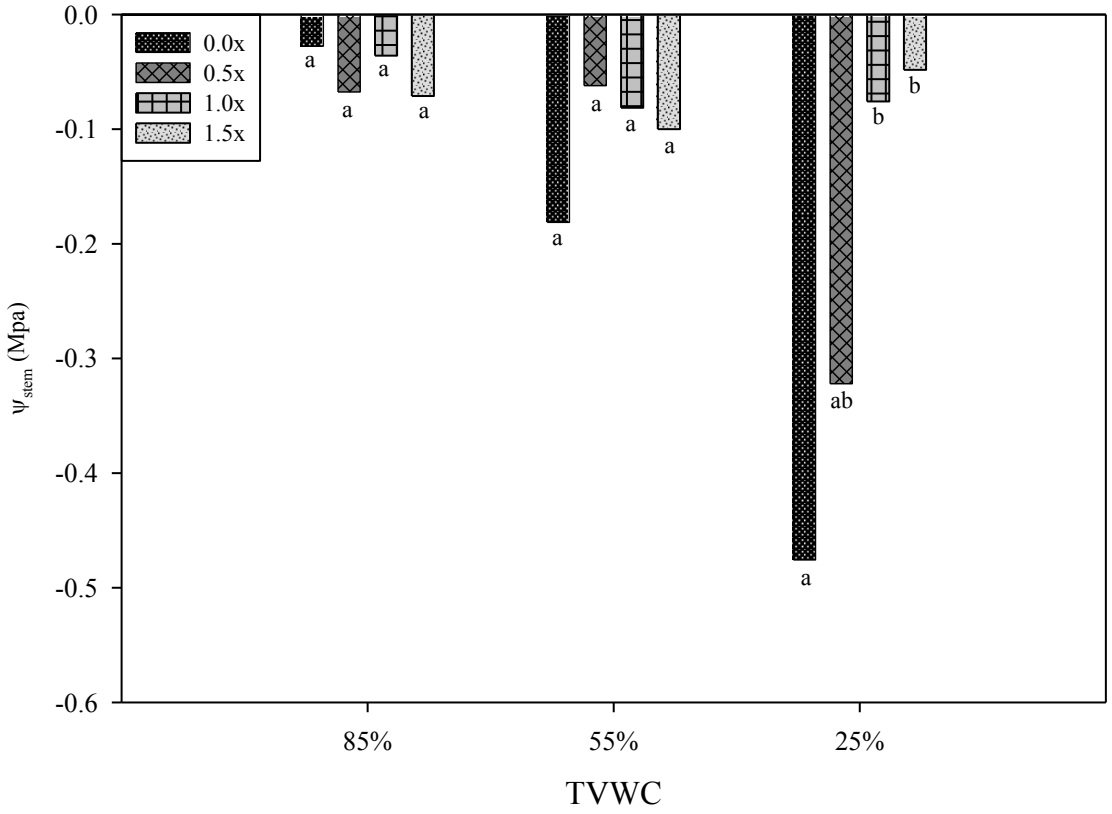

Figure 4.5 Mid-day stem water potential (Ψ_{stem}) of *Impatiens walleriana* 'Super Elfin XP White' following four weekly foliar applications of MBI-501 based on the 1× rate (2 mL·L^{-1}). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, $\alpha = 0.05$ (Expt. 2).

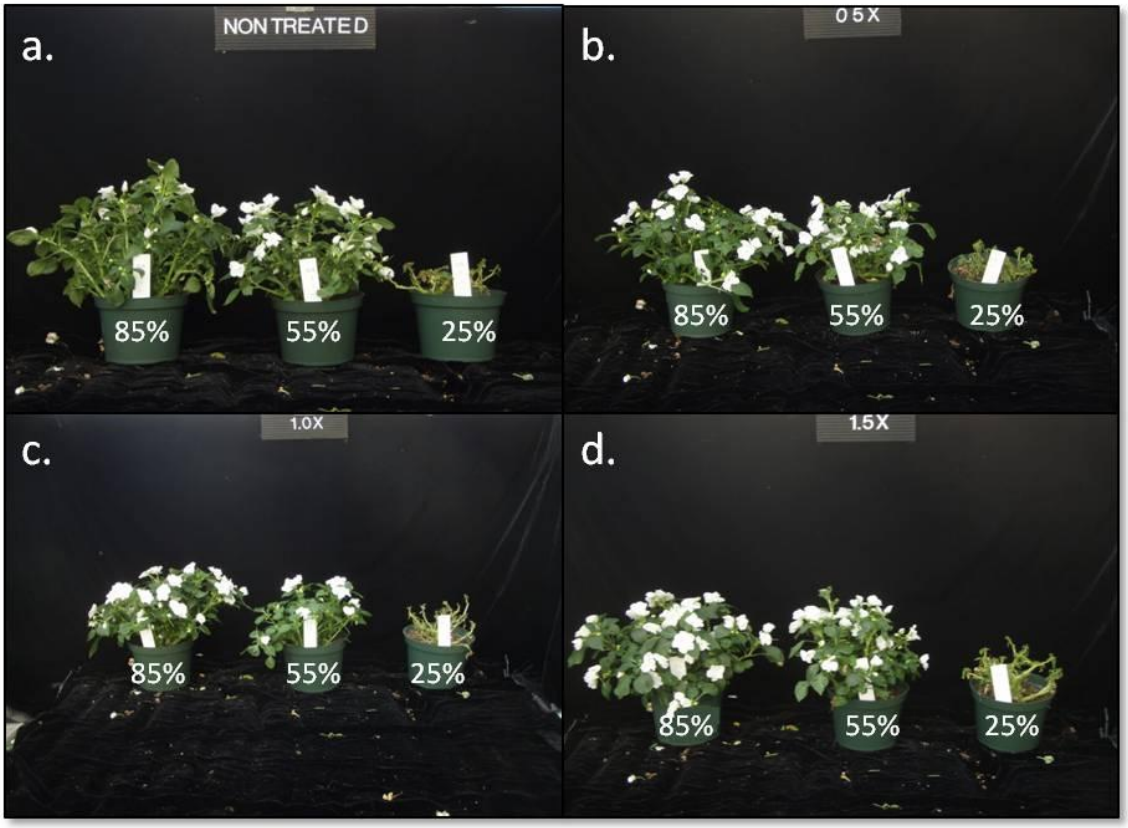

Figure 4.6 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown for four weeks at 85%, 55% and 25% target substrate volumetric water content following weekly foliar applications of MBI-501: a. nontreated control (0.0× rate), b. 0.5× (1 mL·L^{-1}), c. 1.0× (2 mL·L^{-1}), and d. 1.5× (3 mL·L^{-1}) (Expt. 2).

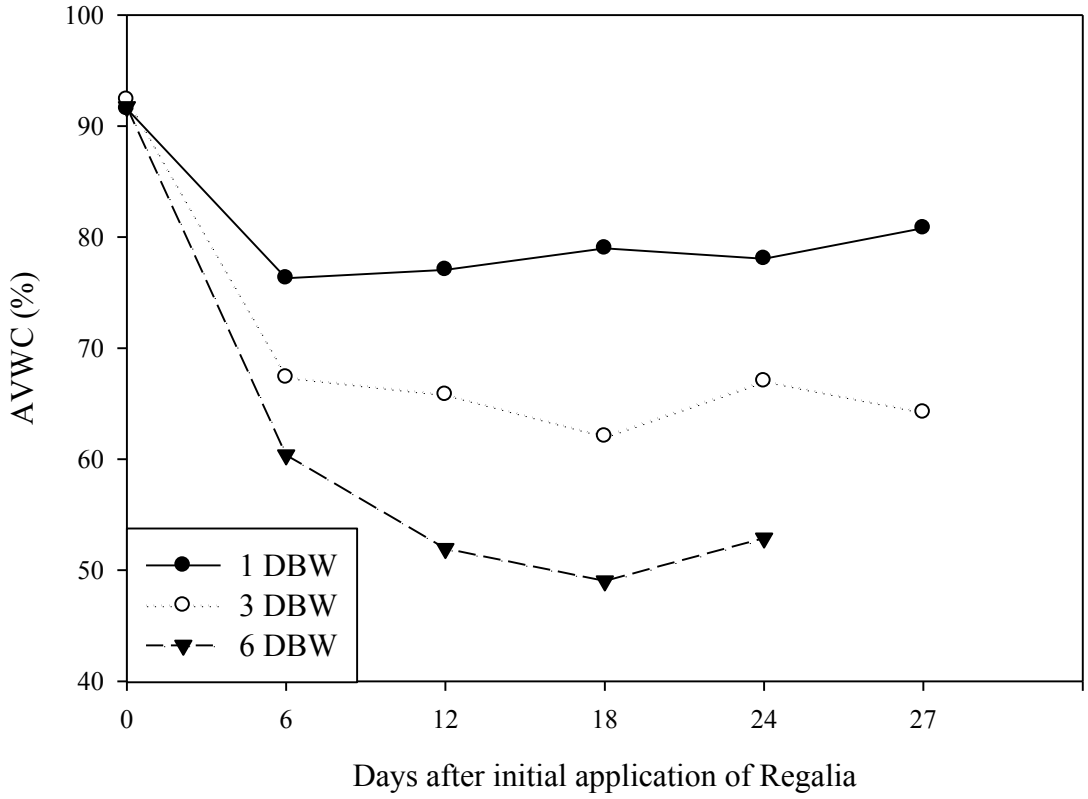

Figure 4.7 Actual substrate volumetric water content (AVWC) following foliar application of Regalia based on the 1.0× rate (10 mL·L^{-1}), to *Impatiens walleriana* 'Super Elfin XP White', with 1 (daily), 3 or 6 days between watering (DBW). On each day of watering, containers were watered at 85% target substrate volumetric water content. Data points represent daily average pooled across all rates (Expt. 3).

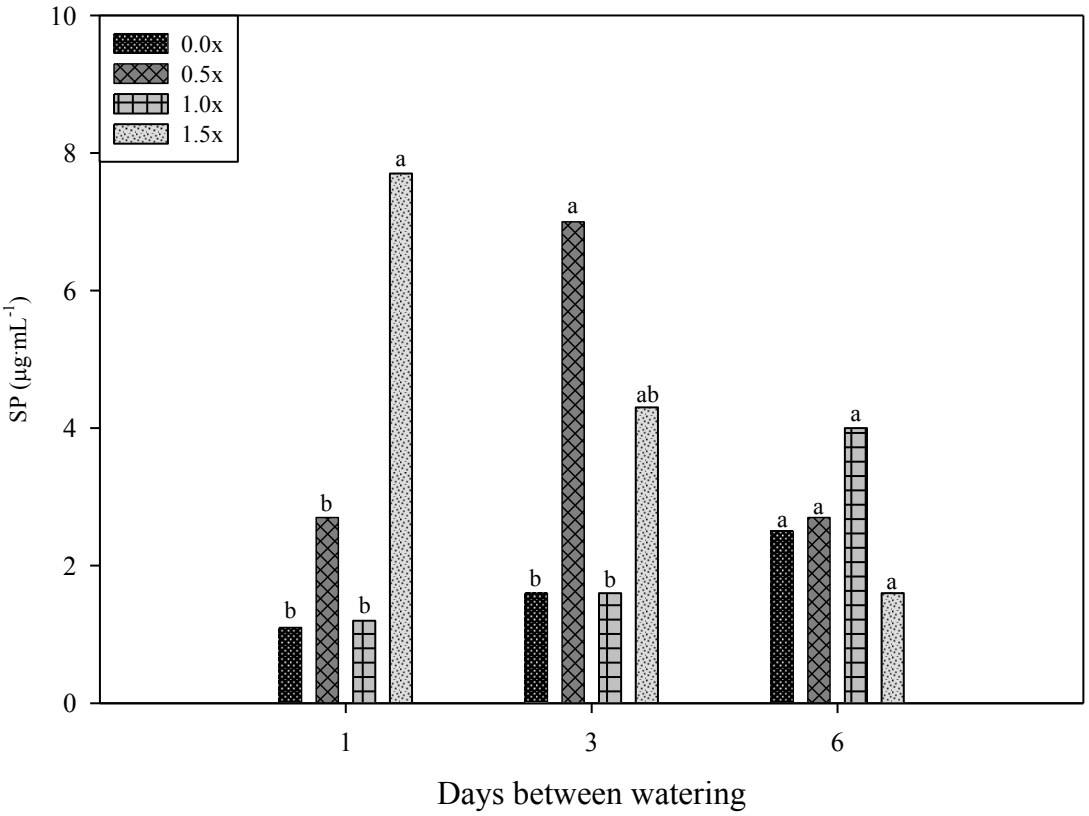

Figure 4.8 Soluble protein (SP) content in leaves of *Impatiens walleriana* 'Super Elfin XP White' following application of Regalia based on the 1.0× rate (10 mL·L^{-1}) to plants grown in containers with 1 (daily), 3 or 6 days between watering (DBW). Watering was based on 85% target substrate volumetric water content at 1, 3, or 6 days between watering. Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, α = 0.05 (Expt. 3).

Figure 4.9 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown for four weeks with 1, 3 or 6 days between watering (DBW) following weekly foliar applications of Regalia: a. nontreated control (0.0× rate), b. 0.5× rate (5 mL·L^{-1}), c. 1.0× rate (10 mL·L^{-1}), d. 1.5× rate (15 mL·L^{-1}) (Expt. 3).

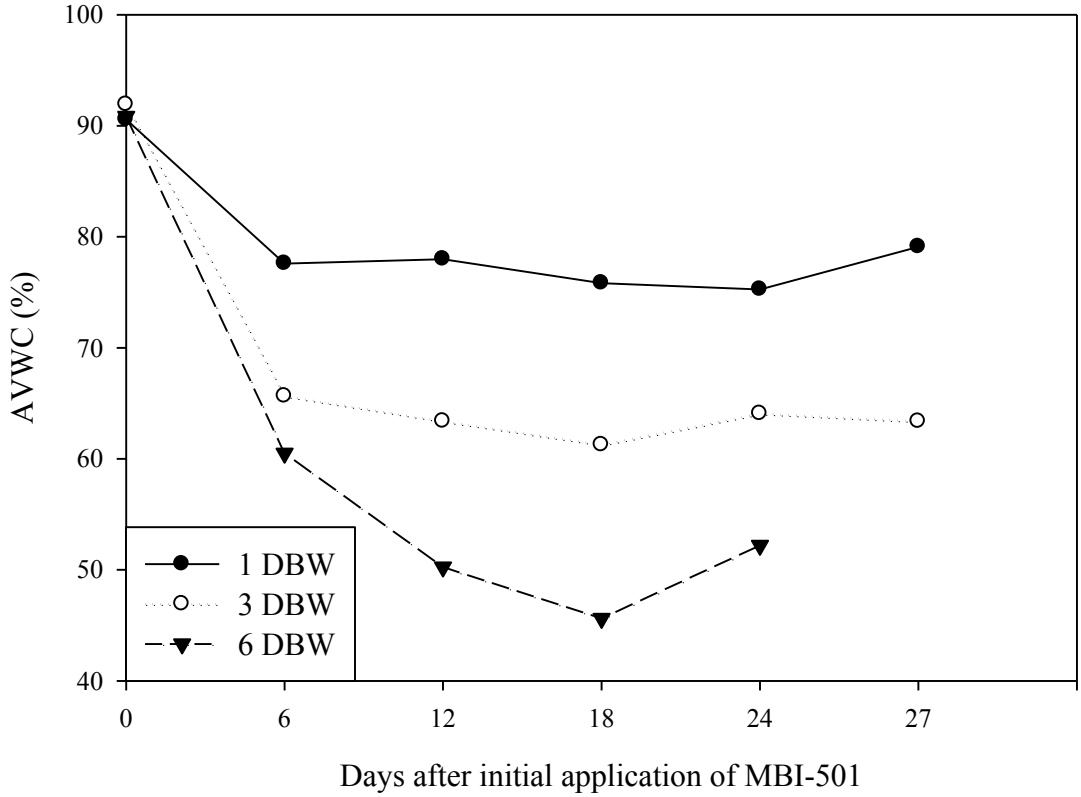

Figure 4.10 Actual substrate volumetric water content (AVWC) following foliar application of MBI-501 based on the 1.0× rate (2 mL·L^{-1}) to *Impatiens walleriana* 'Super Elfin XP White', with 1 (daily), 3 or 6 days between watering (DBW) based on 85% target substrate volumetric water content. Data points represent daily average pooled across all rates (Expt. 4).

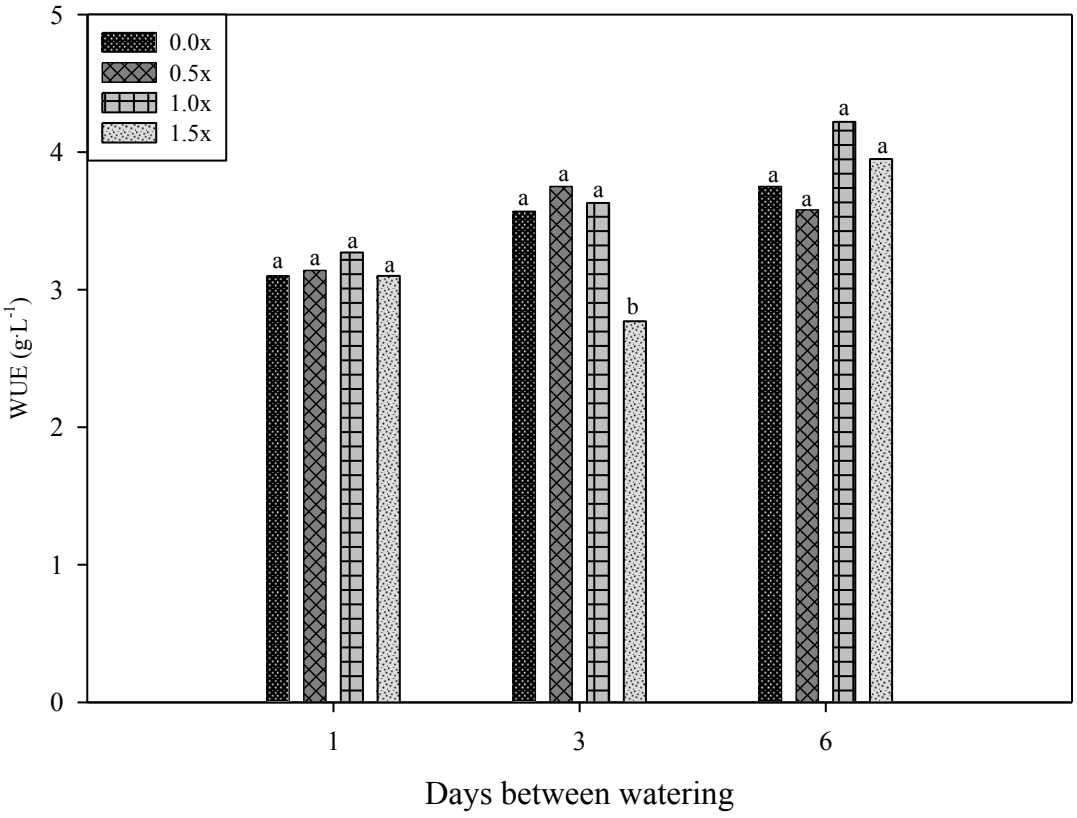

Figure 4.11 Water use efficiency [WUE = ((shoot + root dry weight) ÷ total water applied)] of *Impatiens walleriana* 'Super Elfin XP White' after weekly applications of MBI-501 based on the 1× rate (2 mL·L^{-1}). Watering was based on 85% target substrate volumetric water content at 1, 3, or 6 days between watering. Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, α = 0.05 (Expt. 4).

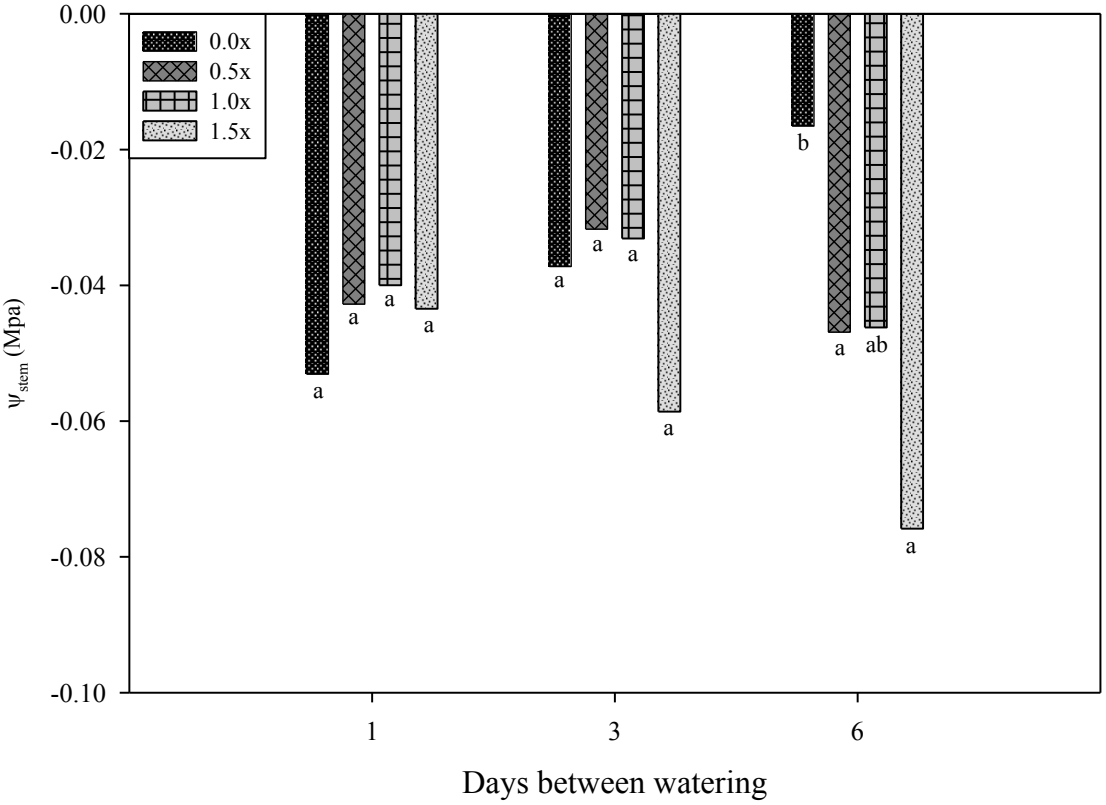

Figure 4.12 Stem water potential (Ψ_{stem}) of *Impatiens walleriana* 'Super Elfin XP White' following foliar application of MBI-501 based on the 1.0× rate (2 $mL \cdot L^{-1}$), at 1, 3, or 6 days between watering (DBW). Watering was based on 85% target substrate volumetric water content at 1, 3, or 6 days between watering. Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, $\alpha = 0.05$ (Expt. 4).

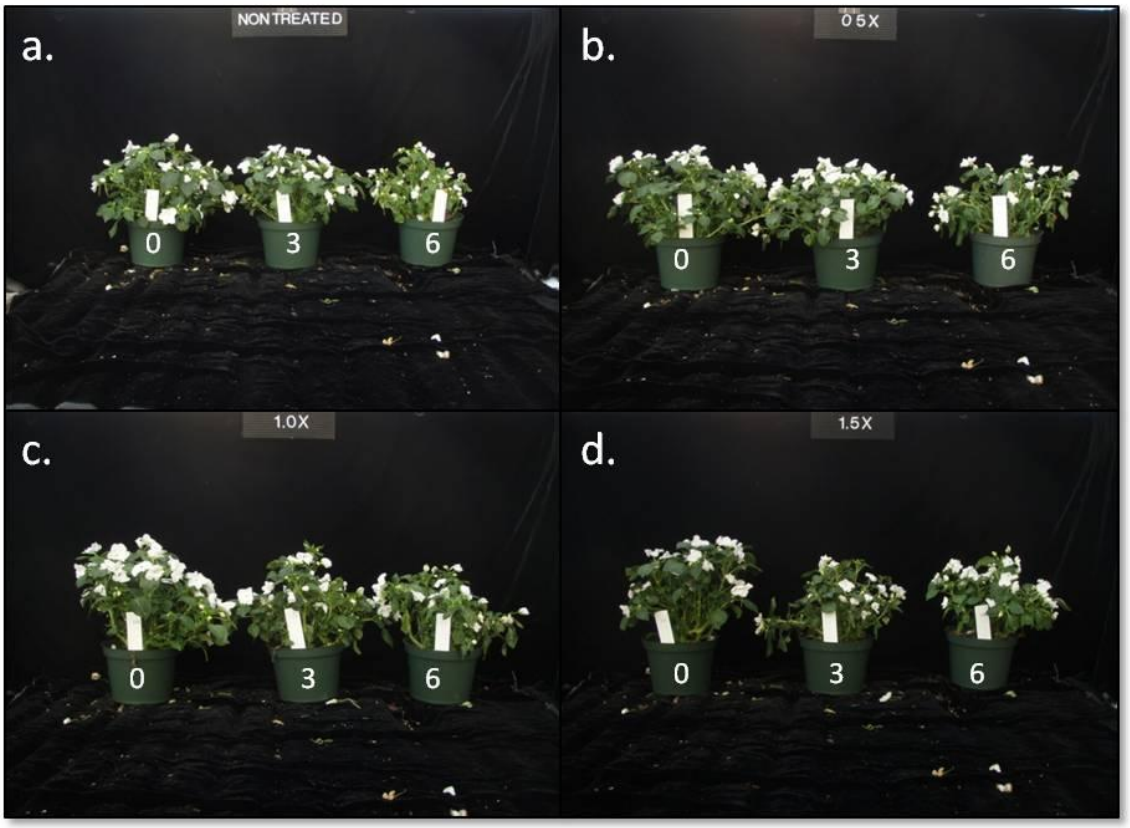

Figure 4.13 Final growth of *Impatiens walleriana* 'Super Elfin XP White' grown for four weeks with 1, 3 or 6 days between watering following weekly foliar applications of MBI-501: a. nontreated control (0.0× rate), b. 0.5× rate (1 $mL \cdot L^{-1}$), c. 1.0× rate (2 $mL \cdot L^{-1}$), and d. 1.5× rate (3 $mL \cdot L^{-1}$).

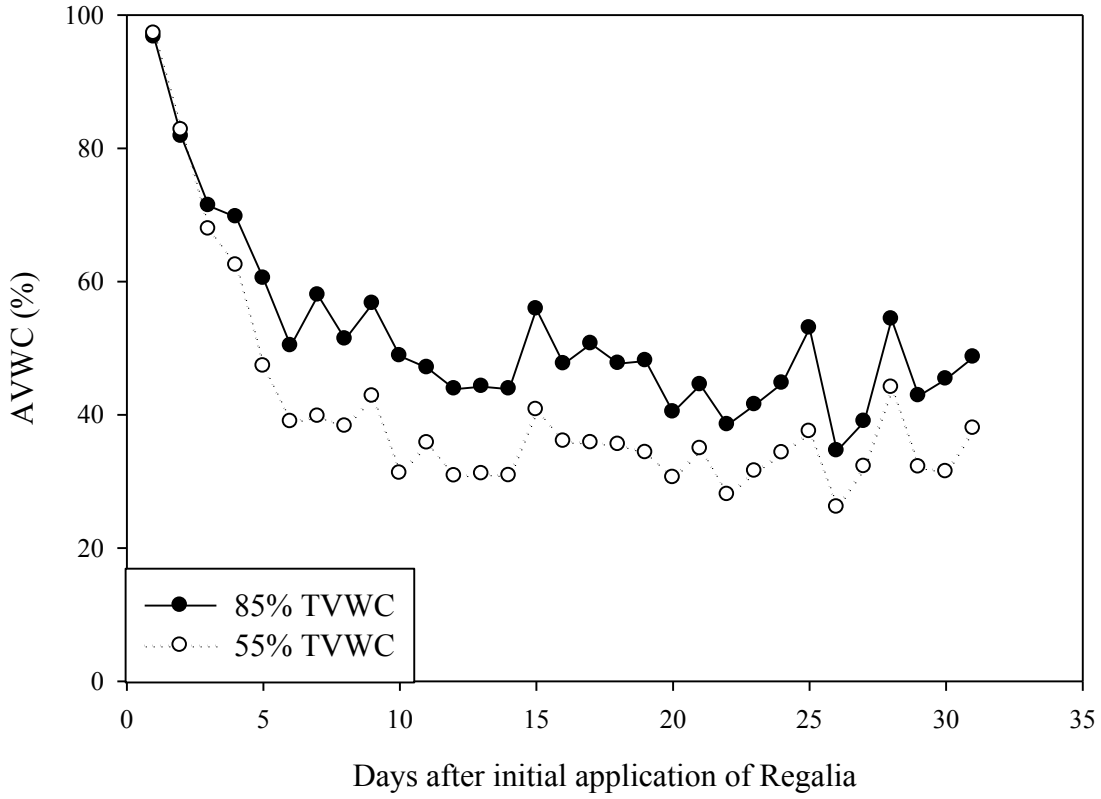

Figure 4.14 Actual substrate volumetric water content (AVWC) following weekly application of Regalia at the 1.0× rate (10 mL·L^{-1}) to *Solanum lycopersicum* 'BHN 640' plants, grown under two different target substrate volumetric water contents [TVWC (85% and 55%)]. Data points represent daily average pooled across all rates (Expt. 5).

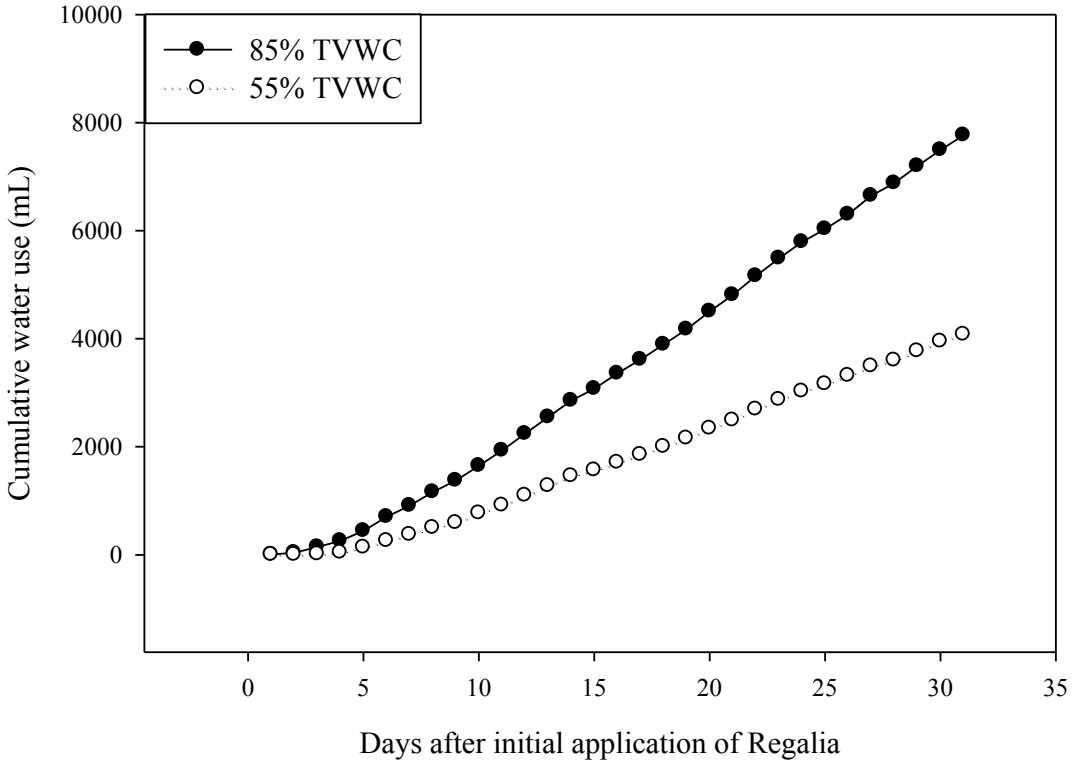

Figure 4.15 Cumulative water use of *Solanum lycopersicum* 'BHN 640' plants following weekly foliar applications of Regalia at the 1.0× rate (10 mL·L^{-1}), grown under two different target substrate volumetric water contents [TVWC (85% and 55%)]. Data points represent daily average pooled across all rates (Expt. 5).

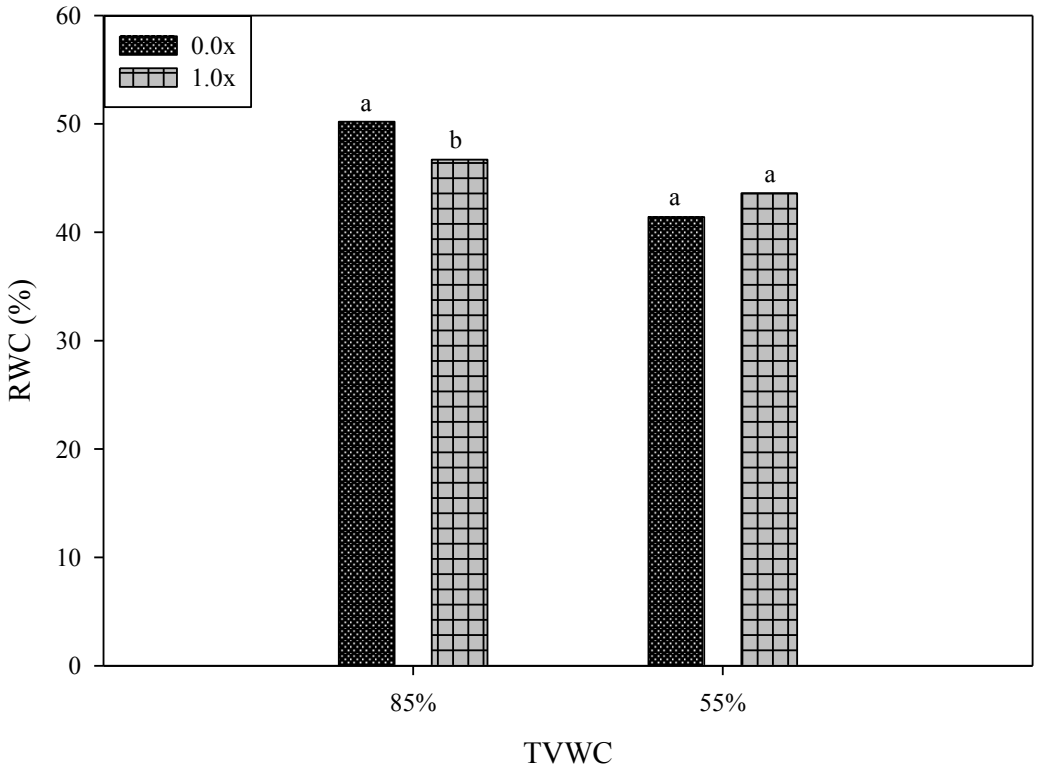

Figure 4.16 Relative leaf water content [RWC = (leaf fresh weight - leaf dry weight) ÷ (leaf turgid weight - leaf dry weight)×100] of *Solanum lycopersicum* 'BHN 640', grown with 85% and 55% target substrate volumetric water contents (TVWC), following foliar application of Regalia at the 1.0× rate (10 mL·L^{-1}). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, α = 0.05 (Expt. 5).

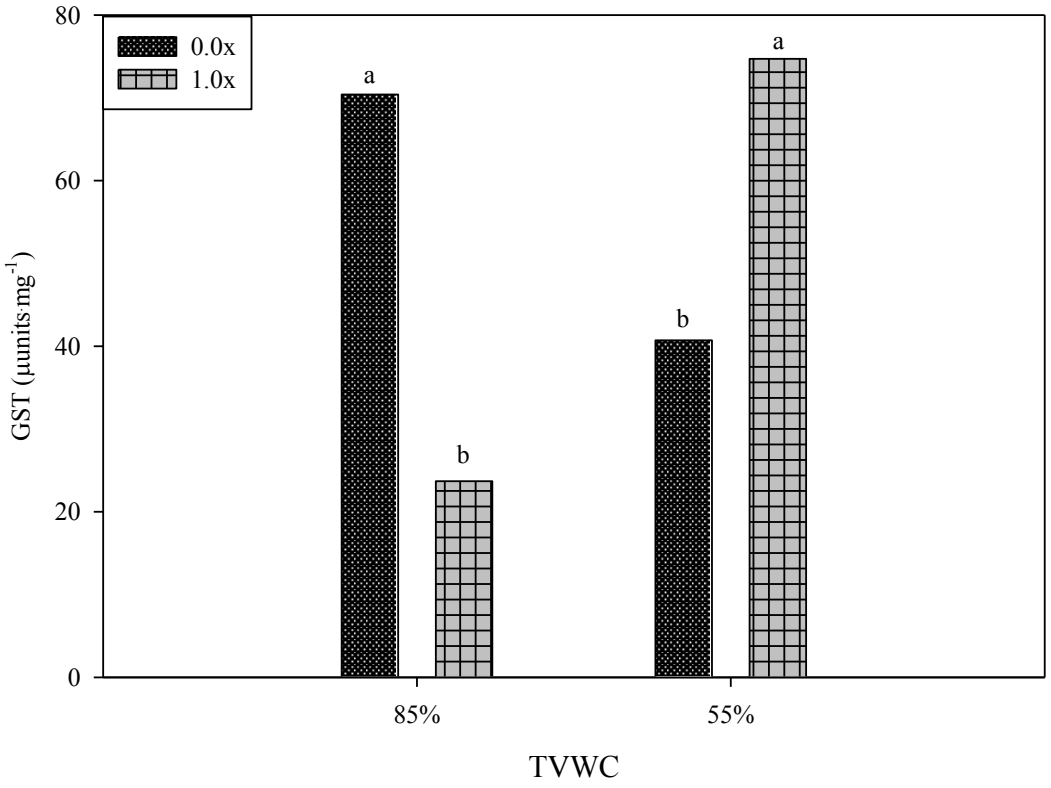

Figure 4.17 Glutathione-*S*-transferase activity in *Solanum lycopersicum* 'BHN 640' leaves following foliar application of Regalia at the 1.0× rate (10 mL·L^{-1}) to plants grown in containers with 85% or 55% target substrate volumetric water content (TVWC). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, $\alpha = 0.05$ (Expt. 5).

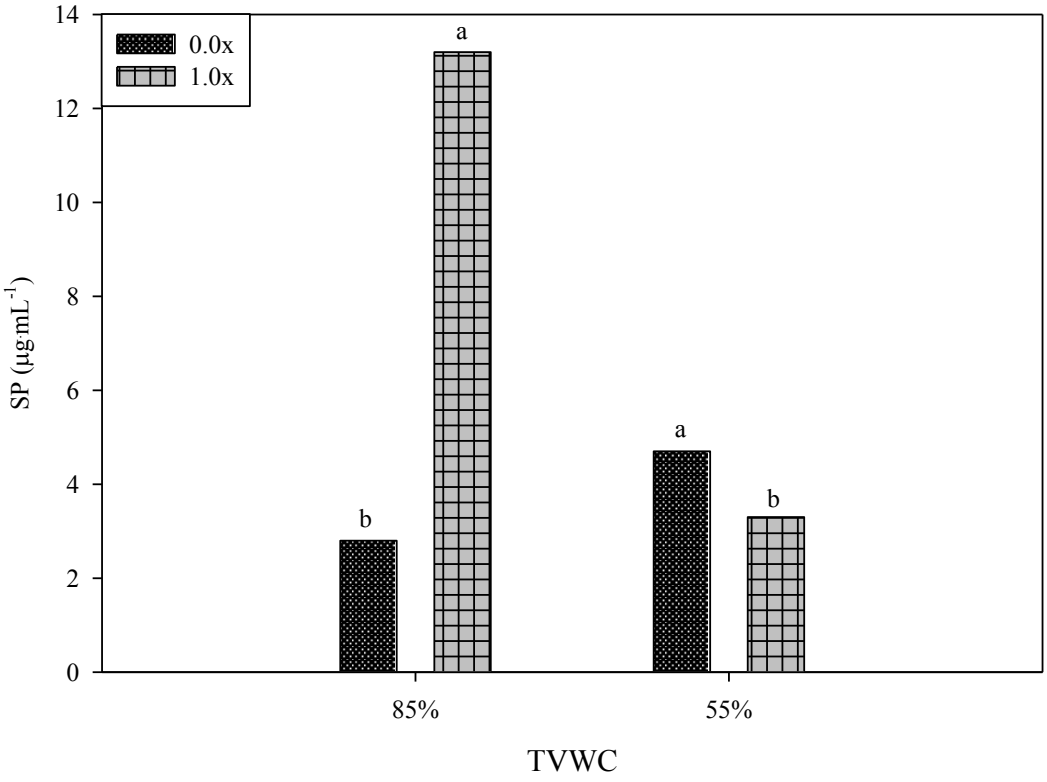

Figure 4.18 Soluble protein (SP) in *Solanum lycopersicum* 'BHN 640' leaves following foliar application of Regalia at the 1.0× rate (10 mL·L^{-1}) to plants grown in containers with 85% or 55% target substrate volumetric water content (TVWC). Means with the same letters are not statistically different according to the SLICEDIFF option of GLIMMIX using adjusted *P* values obtained from the Simulation method, $\alpha = 0.05$ (Expt. 5).

Figure 4.19 *Solanum lycopersicum* 'BHN 640' plants grown for four weeks with 85% and 55% target substrate volumetric water content (TVWC), following weekly foliar application of Regalia at the 1.0× rate (10 $mL \cdot L^{-1}$): 1. Nontreated (0.0×) at 85% TVWC, 2. nontreated (0.0×) at 55% TVWC, 3. Regalia at 1.0× at 85% TVWC, and 4. Regalia at 1.0× at 55% TVWC (Expt. 5).

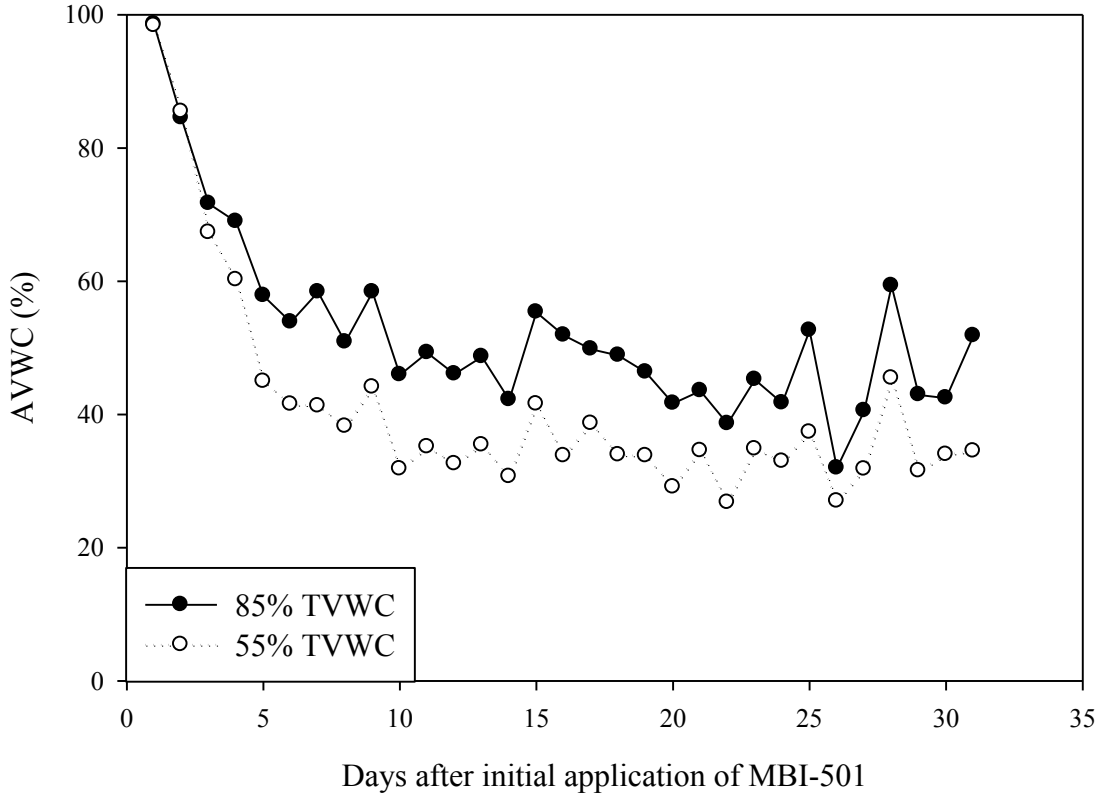

Figure 4.20 Actual substrate volumetric water content (AVWC) after weekly foliar applications of MBI-501 at the 1.0× rate (2 mL·L^{-1}) to *Solanum lycopersicum* 'BHN 640', plants grown under two target substrate volumetric water contents [TVWC (85% and 55%)]. Data points represent daily average pooled across all rates (Expt. 6).

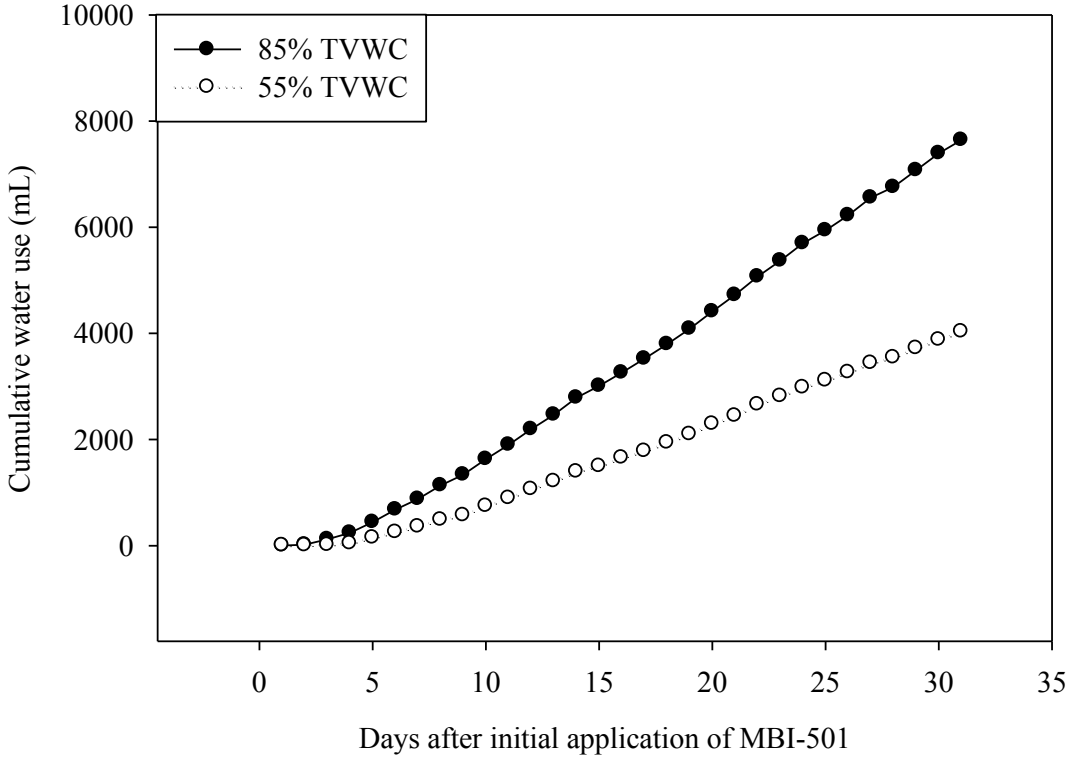

Figure 4.21 Cumulative water use of *Solanum lycopersicum* 'BHN 640' after weekly foliar applications of MBI-501 at the 1.0× rate (2 mL·L^{-1}), grown under two target substrate volumetric water contents [TVWC (85% and 55%)]. Data points represent daily average pooled across all rates (Expt. 6).

Figure 4.22 Final growth of *Solanum lycopersicum* 'BHN 640' plants grown for four weeks with 85% and 55% target substrate volumetric water content (TVWC) following weekly foliar application of MBI-501 at the 1.0× rate (2 mL·L^{-1}): 1. nontreated (0.0×) with 85% TVWC, 2. nontreated (0.0×) with 55% TVWC, 3. Regalia at 1.0× with 85% TVWC, and 4. Regalia at 1.0× with 55% TVWC (Expt. 6).

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CHAPTER V

HEAT TOLERANCE OF *IMPATIENS WALLERIANA* 'SUPER ELFIN XP WHITE' AFFECTED BY APPLICATION TIMING OF PAGEANT, REGALIA, OR MBI-501

Abstract

Plant health protectants are widely used on turf and ornamentals for protection against biotic and abiotic stresses. Some have been reported to increase production of antioxidant enzymes, increase root growth and increase photosynthesis in crops. In Expt. 1, Pageant (pyraclostrobin + boscalid), a strobilurin fungicide labeled for disease control and plant health, was applied as a foliar spray at 0× (nontreated) or 1× (0.228 $g \cdot L^{-1}$) to *Impatiens walleriana* 'Super Elfin XP White' 72, 48, 24, or 1 h before exposing plants to three 24-h periods with 12-h day at 32.2 °C and 12-h night at 28.3 °C. In Expt. 2 Regalia (extract of *Reynoutria sachalinensis*) was applied as a foliar spray at 0× (nontreated) or 1× (10 $mL \cdot L^{-1}$) to impatiens 24 or 1 h before exposing plants to three 24-h periods with 14-h day at 38.8 °C and 10-h night at 32.2 °C. In Expt. 3, MBI-501 (reflective type antitranspirant) was applied as a foliar spray at 0× (nontreated) or 1× (2 $mL \cdot L^{-1}$) to impatiens 24 or 1 h before exposing plants to three 24-h periods with 14-h day at 38.8 °C and 10-h night at 32.2 °C. There were two control treatments in each experiment, one with plants exposed to the heat event and one with plants maintained at 21.1 °C /18.3 °C (day/night): heat event was expressed over three 24-h periods at 32.2 °C/28.3 °C with a

12-h day/night (Expt. 1) and over three 24-h periods at 38.8 °C/32.2 °C 14-h day/10-h night (Expts. 2 and 3). Photosynthesis (Pn), stomatal conductance (Gs), and specific leaf weight [SLW (leaf dry weight per leaf area as $g \cdot cm^2$)] were measured before, during, and the day after the heat event (Expt. 1). Leaf surface temperature was measured during the heat event and electrolyte leakage (EL) was measured immediately following the heat event and every 3 days after for a total of 4 times. Leaf samples were collected at the end of the heat event, immediately frozen in liquid nitrogen, and stored in a -80 °C freezer until determination of antioxidant enzymes. After the heat event, Pn and Gs were similar among all treatments, whereas SLW was greater in impatiens sprayed with Pageant 48 or 24 h before the heat event compared to the nontreated plants receiving no heat event. EL was greater at 6 days after heat event (DAH) in no spray no heat impatiens (NSNH) compared to no spray heat impatiens (NSH); however 9 DAH, treatments were similar. Based on these results, Pageant, Regalia nor MBI-501 applied to impatiens increased heat tolerance.

Introduction

It is inarguable that high temperatures can reduce plant growth (Wise et al., 2004). In 2007, the Intergovernmental Panel on Climate Change predicted an increase of 1.8 to 4.0 °C over the next 100 years (Xu et al., 2009). The higher temperatures are predicted to increase atmospheric CO_2 concentrations, alter rainfall regimes, and indirectly affect respiration and photosynthesis of crop species (Hedhly et al., 2008). These high temperatures could cause a decline in photosynthesis due to an increase in photorespiration resulting in heat stressed plants (Sharkey, 2005). Heat stress limits plant

biomass production and productivity through physiological and metabolic processes (Wahid et al., 2007; Allakhverdiev et al., 2008). With the predicted temperature increases associated with global warming, heat stress will become an increasingly important issue for crop production (Asthir et al., 2009).

Wahid et al. (2007) defined heat stress as the plant's response to a rise in temperature (usually 10 to 15 °C above ambient for an extended time) causing irreversible damage to plant growth, whereas heat tolerance is the plant's ability to survive high temperatures. Furthermore, the extent of the damage and response of the plant are dependent upon species and climatic zone which may also determine the threshold temperature; the low and high temperatures a plant can tolerate and still experience normal growth (Wahid et al., 2007). High temperatures causing heat stress can have a negative impact on growth and productivity (Huang and Xu, 2008). Temperate plants usually have lower threshold temperatures compared to tropical plants. Wheat, a temperate crop, experiences a 4% decrease in yield for every 1 °C increase over the high threshold temperature (25 °C) (Asthir et al., 2009). However, threshold temperatures vary among species so determining specific threshold temperatures is difficult (Wahid et al., 2007). For example, brassica will see adverse effects in flowering when threshold temperature reaches 29 °C whereas cowpea can withstand temperatures up to 41 °C (Morrison and Stewart, 2002; Wahid et al., 2007). Furthermore, it has been reported once temperatures reach 30 °C photosynthesis peaks and for every 1 °C increase

above 30 °C, assimilation declines (Wise et al., 2004). Even a brief exposure to high temperatures can cause damage to a plant by diverting energy away from photosynthesis (Siddique et al., 1999).

High temperatures can also induce oxidative stress. Protection against oxidative stress is essential for plant survival. Oxidative stress resulting from high temperatures can activate plant cell signaling pathways to produce stress proteins (Bajguz and Hayat, 2009). In response to oxidative stress, plants have developed enzymatic and non-enzymatic detoxification systems to protect against cell damage. When plant cells are injured due to high temperatures they generate reactive oxygen species (ROS) (Asthir et al., 2009). ROS are byproducts of plant metabolism and are vital for plant growth even though they are highly toxic due to their oxidative abilities (Robert et al., 2009). Formation of ROS begins with the excitation of triplet ground state oxygen (O_2) to form singlet oxygen ($^1O_2$), reduction of one electron to form superoxide radical ($O_2^{\cdot-}$), reduction of two electrons to form hydrogen peroxide (H_2O_2), or the reduction of three electrons to form a hydroxyl radical ($HO^{\cdot}$) (Mittler, 2002). Chloroplasts are the main intracellular ROS source in plants (Robert et al., 2009) and the most heat sensitive cell function due to their photosynthetic activity (Allakhverdiev et al., 2008). During photosynthesis and respiration, the plant is steadily producing ROS and the state of the cell is controlled by protective mechanisms (Bajguz and Hayat, 2009). If these protective mechanisms are disturbed, oxidative damage can result in cell death. Under regular growth conditions, ROS production is very low; however, under heat stress the production is increased causing lipid peroxidation, protein denaturation, and DNA

damage (Asthir et al., 2009). Since ROS are highly reactive, plants have developed protection mechanisms against oxidative damage in the form of antioxidant enzymes. These antioxidant enzymes, such as SOD, catalase (CAT), peroxidase (POX), ascorbate-peroxidase (APX), glutathione reductase (GR) and glutathione-S-transferase (GST), scavenge the plant for excited oxygen species caused by stress (Mittler et al., 2004; Wu and von Tiedemann, 2002; Gill and Tuteja, 2010; Zhang et al., 2010). The searching of O_2^- by SOD produces H_2O_2 which is then removed by APX or GR in the ascorbate-glutathione cycle (Çiçek and Çakurlar, 2008).

During production, bedding plants often lack thermotolerance and are injured from high temperatures (Natarajan and Kuehny, 2008). The objective of these experiments was to evaluate pyraclostrobin + boscalid (Pageant; BASF Corporation, Florham Park, NJ), an extract of *Reynoutria sachalinensis* (Regalia; Marrone BioInnovations, Davis, CA), and an antitranspirant (MBI-501; Marrone BioInnovations) on improving heat tolerance in *Impatiens walleriana* 'Super Elfin XP White' (impatiens).

Materials and Methods

Plant Material and Culture

In August 2010, impatiens were potted from 288-plug trays (Germania Seed Company, Chicago, IL) into 15.5-cm (1.85 L) containers (Expt. 1). In May 2011, impatiens were potted from 288-plug trays into 10-cm (1.2 L) containers (Expts. 2 and 3). Sunshine Mix 1 (SunGro Horticulture, Bellevue, WA) was used as the potting substrate.

Fertilizer was applied with irrigation at 200 ppm N using Peter's Professional 20N-8.8P-16.6K (20-10-20) Peat-Lite Special (Scotts, Maryville, OH).

Experiment 1

Pageant ([boscalid (0.06 g ai·L^{-1}) + pyraclostrobin (0.03 g ai·L^{-1})], was applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) to impatiens as a folair spray at 0× (nontreated) or 1× (0.228 g·L^{-1}) 72, 48, 24, or 1h before exposing plants to a heat event. The heat event was expressed in a programmable growth chamber over three 24-h periods with 12-h days at 32.2 °C (90 °F) and 12-h nights at 28.3 °C (83 °F). There were two control treatments, one with plants maintained at 21.1 °C /18.3 °C day/night temperatures (NSNH) and one with plants exposed to the heat event (NSH). Photosynthesis (Pn), stomatal conductance (Gs), and specific leaf weight [SLW (leaf dry weight per leaf area as g·cm^2)] were measured prior to the heat event, the first day of the heat event, immediately after the heat event (Day 3), and the day after the heat event (DAH) as previously described by Lasseigne et al. (2007). In addition, leaf samples (20 mature leaves per plant) were collected at the end of the heat event, immediately frozen in liquid nitrogen, and stored in a -80 °C freezer until determination of antioxidant enzymes. Prior to and after the heat event impatiens were in a glass greenhouse under 21.1 °C /18.3 °C day/night set point temperatures located on Mississippi State University's on campus greenhouse facility. The experiment was conducted using a randomized complete block design and six single plant replications. Data were analyzed using linear models with the GLM procedure of

SAS (version 9.2, SAS Institute Inc, Cary, NC) with mean separation according to Tukey's studentized range test (α =0.05).

Experiment 2

Regalia (extract of *Reynoutria sachalinensis* 0.48 g ai·L^{-1}) (Marrone BioInnovations Inc., Davis, CA), was applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) to impatiens as a foliar spray at 0× (nontreated) or 1× (10 mL·L^{-1}) to impatiens either 24 (24-hH) or 1h (1-hH) before exposing plants to a heat event. The heat event was expressed over three 24-h periods with 14-h days at 38 °C (100.4 °F) and 10-h nights at 32.2 °C (90 °F). There were two control treatments, one with plants maintained at 21.1°C/18.3°C (day/night) temperatures (NSNH) and one with plants exposed to the heat event (NSH). Leaf surface temperatures at 1000HR and 1400HR were measured while plants were under heat stress (IR Crop Temperature Meter; Spectrum Technologies Inc., Plainfield, IL). Electrolyte leakage (EL%), as previously described by Liu et al. (2011) was measured immediately following the heat event and every 3 DAH for a total of 4 times. Immediately after the heat event, leaf samples (20 mature leaves per plant) were excised from the plant, frozen in liquid nitrogen, and placed in a -80°C freezer until determination of enzyme analysis. The experiment was conducted using a completely randomized design and six single plant replications. Data were analyzed using linear models with the GLM procedure of SAS (version 9.2, SAS Institute Inc, Cary, NC) with mean separation according to Tukey's studentized range test (α =0.05).

Experiment 3

Materials and methods were similar to Expt. 2 with the following exception: MBI-501 (reflective type antitranspirant) was applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) to impatiens as a foliar spray at 0× (nontreated) or 1× (2 $mL \cdot L^{-1}$), 24 h (24-hH) or 1 h (1-hH) before exposing plants to the heat event. The experiment was conducted using a completely randomized design and six single plant replications. Data were analyzed using linear models with the GLM procedure of SAS (version 9.2, SAS Institute Inc, Cary, NC) with mean separation according to Tukey's studentized range test (α =0.05).

Antioxidant enzyme extractions and assays

Crude enzyme was extracted with 1 mL of a 50 mM sodium phosphate buffer (pH 7.5) as previously described by Venisse, et al. (2001), then centrifuged at 14,000g at 4 °C until plant tissue was clearly separated from the 1 mL of extraction buffer (20 to 40 minutes) (Appendix A and B.1).

Soluble protein (SP) content was determined for each sample according to Bradford (1976) using a Quick Start Bradford Protein Assay Kit #1 (500-0201, Bio-Rad Laboratories Headquarters, Hercules, CA) (Appendix B.2).

Glutathione reductase (GR) was assayed as previously described (Esterbauer and Grill, 1978) with modifications as follows (Appendices B.3). Samples were analyzed using a PowerWave HT Microplate Spectrophotometer (BioTek Instruments, Inc. Winooski, VT) at 340 nm for 10 min. Each well contained 15µL of plant sample and 200µL of reaction buffer [0.1 mM Tris-Hydrochloride pH 7.8 (M.W. 157.6), 1%

ethylenediaminetetraacetic acid disodium salts (M.W. 372.24), 1% bovine serum albumin (Bio Rad #500-206 2mg/mL), and 8.4 mM of β-nicotinamide adenine dinucleotide phosphate (NADPH M.W. 833)]. Activity was determined following the reduction of one unit of GR which catalyzes 1µmol NADPH per minute at pH 7.6 at 25 °C (extinction coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$) (Appendix B.3). Specific activity of GR was expressed as $\mu units \cdot mg^{-1}$.

Glutathione-S-transferase (GST) was assayed as previously described by Venisse et al. (2001) with some modifications. Samples were analyzed using an ELx808 Absorbance Microplate Reader with a UV filter (BioTek Instruments, Inc.) at 340 nm for 5 min. Each well contained 20µL of plant sample and 230µL of reaction buffer [0.1 M potassium phosphate buffer (pH 6.5), 3.6 mM reduced glutathione (M.W. 307.3), 100 mM 1-chlor-2,4-dinitrobenzene (CDNB M.W. 202.6)]. Activity was determined by following the formation of the conjugate of 1µmol of CDNB with reduced glutathione per min at pH 6.5 at 25 °C (extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$) (Appendix B.4). Specific activity of GST was expressed as $\mu units \cdot mg^{-1}$.

Results and Discussion

Experiment 1

Pn and stomatal conductance were similar in impatiens before and 3 DAH (Table 5.1). On the first day of the heat event, impatiens sprayed with Pageant 1h before the heat event had a greater Pn compared to the NSNH treatment. By the third day of the heat event, all plants exposed to the heat event were photosynthesizing at a higher rate

than the NSNH treatment. Prior to the heat event Gs was similar among all treatments (Table 5.1). However, at day 1 of the heat event Gs was greater in the NSNH impatiens compared to 24-hH and 1-hH plants. Day 3, Gs was greater in NSH, 48-hH, and 24-hH impatiens compared to NSNH. There was a trend for Gs to be less in the NSNH impatiens compared to plants exposed to heat. SLW was not significant during the first day impatiens were exposed to the heat event (Table 5.1). However, by the third day of heat exposure and the DAH, SLW was greater for impatiens exposed to the heat event. Lasseigne et al. (2007) indicated lower SLW was an indication of less strain on *Salvia* taxa grown under high temperatures. Additionally, higher SLW can result in higher Pn and an indication plants are under stress (Thiaw and Hall, 2004). There were no differences in GR or GST activity in leaves of impatiens regardless of treatment (Table 5.2). However, protein content was greater in the 72-hH and 24-hH treatments compared to the nontreated (NSNH). These results contradict previous reports indicating reduced SP content after exposure to heat stress (Gulen and Eris, 2004). However, while high temperatures can reduce or cease plant growth due to inactivation of PSII (Kadir et. al., 2007) this injury can be reversed depending on temperature, exposure time, and/or plant species. Thus, the heat event did affect growth of impatiens; however, the impatiens were able to recover 3 DAH. Exposing impatiens to 12-h days at 32.2 °C (90 °F) and 12-h nights at 28.3 °C (83 °F) was not a severe heat stress.

Experiment 2

Plants exposed to the heat event had greater leaf surface temperature during the heat event (Fig. 5.1). At the end of the experiment, there were no differences in TG or

SDW regardless of treatment (Table 5.3). There were no differences in EL between Regalia treated impatiens or impatiens exposed to the heat event compared to the nontreated (NSNH) (Table 5.4). Gulen and Eris (2004), indicated similar EL between strawberry plants exposed to temperatures below 40 °C with a significant increase above 40 °C, which could explain the lack of cell membrane injury to impatiens in this experiment. GR activity was greater in impatiens exposed to the heat event (NSH, 24-hH and 1-hH) compared to the NSNH treatments (Table 5.5). SP content associated with the GR assays was greater in the NSNH treatment compared to impatiens exposed to the heat event. GST was greater in the 1-hH treatment compared to the NSNH treatment; however, all impatiens exposed to the heat event had similar GST activity. Similar to GR analysis, the SP determined from the GST assay, was greater in the NSNH treatment compared to impatiens exposed to the heat event. GR is produced under heat stress to detoxify ROS and has been shown to increase in strawberry plants after exposure to temperatures above 30 °C with a decrease in total protein (Gulen and Eris, 2004). Therefore, the heat event did affect metabolic changes but, there were no indications Regalia enhanced heat tolerance of impatiens.

Experiment 3

Leaf surface temperatures were similar to those of Expt. 2 (Fig. 5.2). At the close of the experiment there was a difference in TG and SDW of impatiens following application of MBI-501 and heat event (Table 5.6). TG was statistically greater in NSNH impatiens compared to those in the 24-hH and 1-hH treatments, yet similar to the NSH impatiens. Similarly, SDW was greater in the NSNH impatiens compared to impatiens

treated with MBI-501 (24-hH and 1-hH) but not to NSH impatiens. EL was significantly different at 3 and 6 DAH (Table 5.7). At 3 DAH EL was greater in the NSNH impatiens compared to NSH and 1-hH impatiens. Similar results were seen 6 DAH with greater EL in the NSNH compared to the NSH. However, 24-hH and 1-hH had similar EL to NSNH. Impatiens exposed to the heat event and treated with MBI-501 had similar EL compared to NSNH at close of the experiment. GR activity was greater in the 1-hH treatment compared to the NSNH and 24-hH treatments; however, SP content was similar among all treatments (Table 5.8). GST activity was greater in all treatments exposed to the heat event (NSH, 24-hH, and 1-hH) compared to the NSNH treatment. SP content was unaffected by heat treatment. These findings are consistent with previous research indicating increased antioxidant activity after exposure to high temperatures (Gulen and Eris, 2004; Du et al, 2009).

The cell membrane is one of the first sites injured due to stress (Bajji et al., 2002) and EL is a widely accepted tool to assess membrane damage and heat tolerance (Yeh and Lin, 2003). It was hypothesized MBI-501 applied to impatiens may provide limited protection from heat stress. However, there was a negative correlation between SDW and high temperature indicating injury (Haldimann and Feller, 2005). Therefore, there was not sufficient evidence indicating improved heat tolerance of impatiens following the application of MBI-501.

Table. 5.1 Photosynthesis, stomatal conductance, and specific leaf weight for *Impatiens walleriana* 'Super ElfinXP White' exposed to a heat event[z], following application of Pageant (1× = 0.228 g·L^{-1}) (Expt. 1).

	Day of measurement[y]			
	Pre	1st Day	3rd Day	Post
Treatment[x]	*Photosynthesis (μmol·m^{-2}·$^{-1}$)* [w]			
NSNH	3.9 a[v]	4.7 b	5.1 b	6.1 a
NSH	4.9 a	7.1 ab	8.6 a	6.9 a
72-hH	4.6 a	6.8 ab	8.8 a	7.5 a
48-hH	3.9 a	6.8 ab	8.8 a	6.8 a
24-hH	4.5 a	7.5 ab	8.6 a	7.2 a
1-hH	4.3 a	8.2 a	8.4 a	6.9 a
Significance[u]	NS	*	***	NS
	Stomatal Conductance (mmol·m^{-2}·s^{-1}) [w]			
NSNH	56.4 a	100.0 b	143.5 b	113.0 a
NSH	64.8 a	182.6 ab	318.1 a	129.6 a
72-hH	76.1 a	182.2 ab	245.3 ab	123.1 a
48-hH	57.4 a	187.4 ab	309.8 a	141.3 a
24-hH	61.9 a	214.6 a	293.9 a	137.5 a
1-hH	64.7 a	236.2 a	261.3 ab	137.4 a
Significance	NS	**	*	NS
	Specific leaf weight (g·cm^{2}) [s]			
NSNH	-	0.0014 a	0.0014 b	0.0015 b
NSH	-	0.0016 a	0.0019 a	0.0019 ab
72-hH	-	0.0016 a	0.0020 a	0.0019 ab
48-hH	-	0.0017 a	0.0020 a	0.0021 a
24-hH	-	0.0017 a	0.0020 a	0.0019 a
1-hH	-	0.0016 a	0.0020 a	0.0019 ab
Significance	-	NS	***	**

[z]Heat event was expressed in a programmable growth cahmber over three 24-h periods with 12-h days at 32.2°C and 12-h nights at 28.3°C.

[y]Day of measurement: pre = measurements before heat event, Day1 = measurements the first day of heat event, Day3 = measurements the third day of heat event, Post = three days after heat event.

[x]Treatments: NSNH = no spray no heat, NSH = no spray heat, 72-hH = Regalia applied at 1× 72 h before heat event, 48-hH = Regalia applied at 1× 48 h before heat event, 24-hH = Regalia applied at 1× 24 h before heat event, 1-hH = Regalia applied at 1× 1 h before heat event.

[w]Photosynthesis and stomatal conductance measurements were taken using a CIRAS-2 portable photosynthesis system, (PP Systems, Amesbury, MD), on mature leaves.

[v]Means followed by the same letters within same column for each data set are not significantly different according to Tukey's Studentized Range test, $\alpha = 0.05$.

[uNS, *, **, ***]Indicates nonsignificant or significant at $P \leq 0.05$, 0.01, 0.001, respectively.

[s]Specific leaf weight: leaf dry weight per leaf area (g·cm^2).

Table 5.2 Glutathione reductase (GR), glutathione-*S*-transferase (GST), and protein content in leaves of *Impatiens walleriana* 'Super Elfin XP White' affected by timing of Pageant application (1× = 0.228 g·L^{-1}), prior to a three day heat event[z] (Expt. 1).

Treatments[y]	GR (μunits·mg^{-1})	GST (μunits·mg^{-1})	Protein (μg·mL^{-1})
NSNH	1.20	0.57	1.4 b[x]
NSH	1.99	0.11	1.7 ab
72-hH	3.16	0.38	1.9 a
48-hH	1.51	1.07	1.5 ab
24-hH	2.90	1.99	1.8 a
1-hH	1.62	0.84	1.8 ab
Significance[w]	NS	NS	*

[z]Heat event was expressed in a programmable growth chamber over three 24-h periods with 12-h days at 32.2°C and 12-h nights at 28.3°C

[y]Treatments: NSNH = no spray no heat, NSH = no spray heat, 72-hH = Pageant at 1× 72 h before heat event, 48-hH = Pageant at 1× 48 h before heat event, 24-hH = Pageant at 1× 24 h before heat event, 1-hH = Pageant at 1× 1 h before heat event.

[x]Means (within a column) with the same letters are not statistically different according to Tukey's studentized range test for mean comparison $\alpha = 0.05$.

[wNS,*]Indicates nonsignificant or significant difference at $P \leq 0.05$.

Table 5.3 Growth of *Impatiens walleriana* 'Super Elfin XP White' after exposure to 38.8 °C/32.2 °C (12-h day/ 12-h night) for three days, following a foliar application of Regalia (1× = 10 mL·L^{-1}) (Expt. 2).

Treatments[z]	Total growth[x]	Shoot dry weight[y]
NSNH	4.2	10.1
NSH	6.0	8.1
24-hH	4.5	8.8
1-hH	4.9	9.5
Significance[x]	NS	NS

[z]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = Regalia at 1× 24 h before heat, 1-hH = Regalia at 1× 1 h before heat.

[x]Total growth: Final growth indices (GI) - initial GI [GI = (height + width + perpendicular width)÷3].

[y]Shoot dry weight oven dried for 72 h at 65 °C.

[x]NSIndicates nonsignificant at $P \leq 0.05$.

Table 5.4 Evaluating electrolyte leakage (%)[z] of *Impatiens walleriana* 'Super Elfin XP White' following application of Regalia (1× = 10 mL·L^{-1}), prior to exposure to elevated temperatures[y] (Expt. 2).

	Days after heat event			
Treatments[x]	1	3	6	9
NSNH	7.8	9.3	10.1	11.2
NSH	8.6	7.7	9.1	9.5
24-hH	9.3	7.8	8.5	9.6
1-hH	10.1	7.8	9.7	11.2
Significance[w]	NS	NS	NS	NS

[z]Electrolyte leakage (EL) was determined by taking three, 20 mm disc samples and adding 20 mL of distilled water and shaken for 20 h, before reading first electrical conductivity reading (EC_1) then autoclaved at 120 °C and cooled to room temperature before second reading (EC_2) [$EL = (EC_1 \div EC_2) \times 100$].

[y]Heat event was expressed in a programmable growth chamber over three 24-hr periods with 12-h days at 38.8 °C and 12-h nights at 32.2 °C.

[x]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = Regalia at 1× 24 h before heat event, 1-hH = Regalia at 1× 1 h before heat event.

[wNS]Indicates nonsignificant at $P \leq 0.05$.

Table 5.5 Glutathione reductase (GR), glutathione-*S*-transferase (GST), and soluble protein (SP) content in leaves of *Impatiens walleriana* 'Super Elfin XP White' affected by Regalia application (1× = 10 mL·L^{-1}), prior to a three day heat event[z] (Expt. 2).

Treatments[y]	GR (μunits·mg^{-1})	SP (μg·mL^{-1})	GST (μunits·mg^{-1})	SP (μg·mL^{-1})
NSNH	0.8 b[x]	2.2 a	26.9 b	2.1 a
NSH	2.1 a	1.8 b	75.7 ab	1.4 b
24-hH	1.6 a	1.9 b	73.3 ab	1.6 b
1-hH	2.1 a	1.8 b	97.7 a	1.5 b
Significance[w]	***	**	**	***

[z]Heat event was expressed in a programmable growth chamber over three 24-h periods with 14-h days at 38.8 °C and 10-h nights at 32.2 °C.

[y]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = Regalia at 1× 24 h before heat event, 1-hH = Regalia at 1× 1 h before heat event.

[x]Means (within a column) with the same letters are not statistically different according to Tukey's Studentized range test for mean comparison $\alpha = 0.05$.

[w]**, ***,Indicates nonsignificant or significant at $P \leq 0.01$ or 0.001.

Table 5.6 Growth of *Impatiens walleriana* 'Super Elfin XP White' after exposure to 38.8 °C/32.2 °C (12-h day/ 12-h night) for three days, following a foliar application of MBI-501 (1× = 2 mL·L^{-1}) (Expt. 3)

Treatments[z]	Total growth[x]	Shoot dry weight[y]
NSNH	8.3 a	11.0 a
NSH	5.6 ab	9.4 ab
24-hH	5.3 b	9.0 b
1-hH	3.4 b	8.1 b
Significance[x]	***	**

[z]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = MBI-501 at 1× 24 h before heat, 1-hH = MBI-501 at 1× rate 1 h before heat.

[x]Total growth: Final growth indices (GI) - initial GI [GI = (height + width + perpendicular width)÷3].

[y]Shoot dry weight oven dried for 72 h at 65 °C.

[x]**, *** Indicates significant at $P \leq 0.01$ or 0.001.

Table 5.7 Evaluating electrolyte leakage (%)[z] of *Impatiens walleriana* 'Super Elfin XP White' following application of MBI-501 (1× = 2 mL·L^{-1}), prior to exposure to elevated temperatures[y] (Expt. 3).

	Days after heat event			
Treatments[x]	1	3	6	9
NSNH	14.5	10.9 a	13.4 a	10.5
NSH	14.9	8.2 b	10.9 b	11.4
24-hH	17.3	9.7 ab	12.2 ab	11.0
1-hH	16.8	8.8 b	12.6 ab	10.2
Significance[w]	NS	**	*	NS

[z]Electrolyte leakage (EL) was determined by taking three, 20 mm disc samples and adding 20 mL of distilled water and shaken for 20 h, before reading first electrical conductivity reading (EC_1) then autoclaved at 120 °C and cooled to room temperature before second reading (EC_2) [$EL = (EC_1 \div EC_2) \times 100$].

[y]Heat event was expressed in a programmable growth chamber over three 24-hr periods with 12-h days at 38.8 °C and 12-h nights at 32.2 °C.

[x]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = Regalia at 1× 24 h before heat event, 1-hH = Regalia at 1× 1 h before heat event.

[w]NS,*,** Indicates nonsignificant or significant at $P \leq 0.05$ or 0.01.

Table 5.8 Glutathione reductase (GR), glutathione-*S*-transferase (GST), and protein content in leaves of *Impatiens walleriana* 'Super Elfin XP White' affected by MBI-501 application (1× = 2 mL·L^{-1}), prior to a three day heat event[z] (Expt. 3).

Treatments[y]	GR (μunits·mg^{-1})	Protein (μg·mL^{-1})	GST (μg·mg^{-1})	Protein (μg·mL^{-1})
NSNH	0.04 b[x]	1.8	31.0 b	2.2
NSH	0.17 ab	1.6	94.9 a	1.9
24-hH	0.08 b	1.6	147.5 a	1.9
1-hH	0.29 a	1.5	145.8 a	1.6
Significance[w]	**	NS	***	NS

[z]Heat event was expressed in a programmable growth chamber over three 24-h periods with 12-hr days at 38.8 °C and 12-h nights at 32.2 °C

[y]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = MBI-501 at 1× 24 h before heat event, 1-hH = MBI-501 at 1× 1 h before heat event.

[x]Means (within a column) with the same letters are not statistically different according to Tukey's studentized range test for mean comparison $\alpha = 0.05$.

[w]NS, **, *** Indicates nonsignificant or significant difference at $P \leq 0.01$ or 0.001.

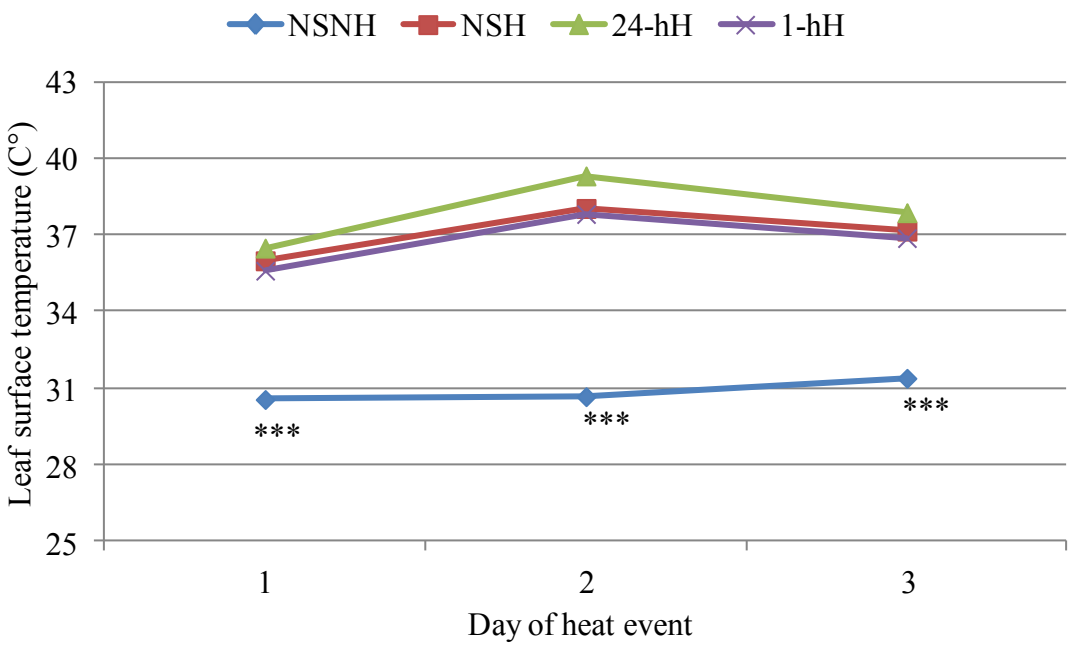

Figure 5.1 Leaf surface temperature of *Impatiens walleriana* 'Super Elfin XP White' measured using a IR Crop Temperature Meter (Spectrum Technologies Inc, Plainfiled, IL) following application of Regalia (1× = 10 mL·L^{-1}) 24 h or 1h before heat event with two nontreated control groups (0×): one kept at 21.1 °C /18.3 °C (day/night) (NSNH) and one exposed to the heat event [NSH (three 24-h periods with 12-h days at 38.8 °C and 12-h nights at 32.2 °C)]. *** Indicates significant differences between NSNH and NSH, 24-hH, and 1-hH at $P \leq 0.001$ (Expt. 2).

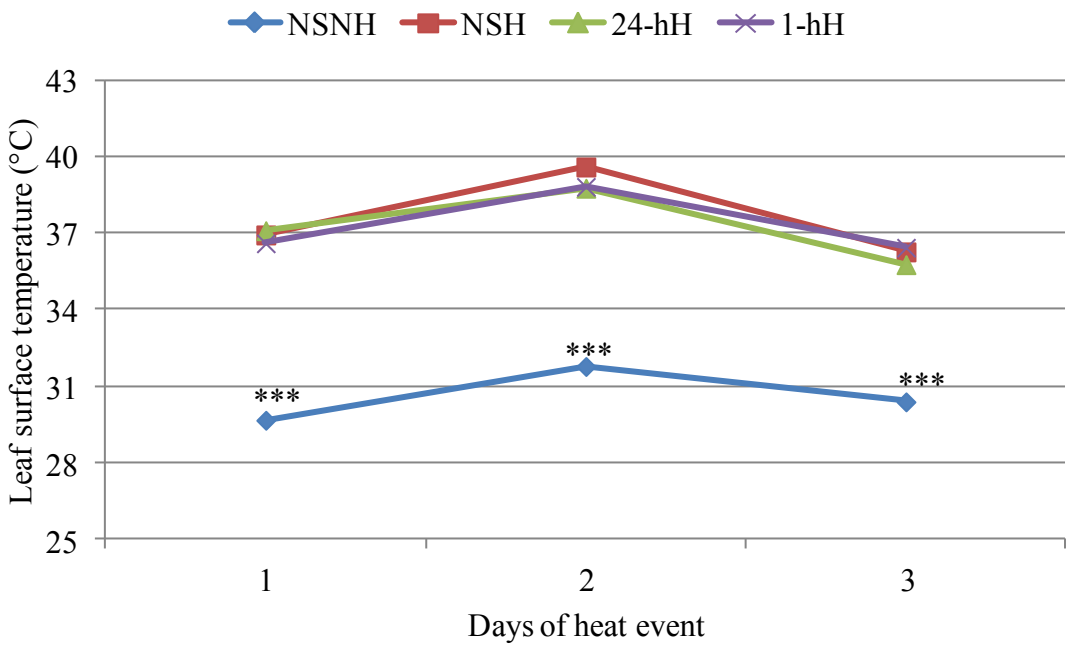

Figure 5.2 Leaf surface temperature of *Impatiens walleriana* 'Super Elfin XP White' measured using a IR Crop Temperature Meter (Spectrum Technologies Inc, Plainfiled, IL) following application of MBI-501 (1× = 2 mL·L^{-1}) 24 h or 1h before heat event with two nontreated control groups (0×): one kept at 21.1 °C /18.3 °C (day/night) (NSNH) and one exposed to the heat event [NSH (three 24-h periods with 12-h days at 38.8 °C and 12-h nights at 32.2 °C)]. ***Indicates significant differences between NSNH and NSH, 24-hH, and 1-hH at $P \leq 0.001$ (Expt. 3).

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CHAPTER VI

EVALUATING THE POTENTIAL OF REGALIA (AN EXTRACT OF *REYNOUTRIA SACHALINENSIS*) TO INCREASE HEAT TOLERANCE OF *SOLANUM LYCOPERSICUM* 'BHN 640'

Abstract

High air temperatures can kill plant cells or severely reduce photosynthetic rates, ultimately affecting plant growth. In the southeastern U.S., high air temperatures cannot be avoided, especially in the summer months. Therefore, plant protectants such as Regalia, could be beneficial if they can provide protection or heat tolerance after application. Regalia was applied at 1× (10 $mL \cdot L^{-1}$) to *Solanum lycopersicum* 'BHN 640' (tomato) plants either 24 h (24-hH) or 1 h (1-hH) before being exposed to a heat event. The heat event was expressed over three, 24-h periods with 12-h days at 44 °C and 12-h nights at 33 °C. There were two control treatments, one with plants maintained at 24 °C day and 21 °C night temperatures (NSNH) and one with plants exposed to the heat event (NSH). Total growth (TG), shoot dry weights (SDW), specific leaf weight (SLW), and number of opened flowers (F) were measured at the close of the experiment. Gross photosynthesis (Pgross) was determined by measuring photosynthesis (Pn) and respiration (Rp) before the heat event (pre), the day after the heat event (post) and 3, 6, 9 and 12 days after for a total of six measurements (post3, post6, post9, and post12). Tomato plants exposed to the heat event all had less TG, SDW, and F compared to

NSNH tomato plants. Pgross was greater in NSNH treated tomato plants when measured post and post3 compared to NSH, 24-hH, and 1-hH plants. Data did indicate differences among treatments; however, there were no differences between plants exposed to the heat event and treated with Regalia (24-hH and 1-hH) compared to the nontreated 'BHN 640' tomato plants exposed to the heat event (NSH).

Introduction

Under high temperatures, photosynthesis in plants is affected, specifically the photosynthetic activity of chloroplasts (Wise et al., 2004; Allakhverdiev et al., 2008). Under normal conditions, photosynthesis converts light energy into chemical energy for use in the plant. Photosynthesis takes place in the leaves specifically in the chloroplasts using chlorophyll as the receptor molecule. In heat stressed plants, photosynthesis is altered and plant growth is affected. There are many processes involved in photosynthesis and it only takes alteration of one of those processes to affect plant growth (Wahid et al., 2007).

In tomato leaves, the failure of photosynthetic electron transport at elevated temperatures affects the thermolability of photosystem II (PS II) (Ogweno et al., 2009). Inhibiting or reducing PS II activity can lead to separation or inhibition of the oxygen evolving complex (OEC) altering the energy distribution of photosynthesis, changing the carbon metabolism enzymes, disrupting the electron transport, and deactivating the oxygen evolving enzymes of PS II (Wahid et al., 2007).

High temperatures can also induce oxidative stress. Oxidative stress resulting from high temperature can activate plant cell signaling pathways to produce stress

proteins (Bajguz and Hayat, 2009). When plant cells are injured due to high temperatures they will generate reactive oxygen species (ROS) (Asthir et al., 2009). ROS are byproducts of plant metabolism and are vital for plant growth even though they are highly toxic due to their oxidative abilities (Robert et al., 2009). Formation of ROS begins with the excitation of triplet ground state oxygen (O_2) to form singlet oxygen ($^1O_2$), reduction of one electron to form superoxide radical (O_2^-), reduction of two electrons to form hydrogen peroxide (H_2O_2), or the reduction of three electrons to form a hydroxyl radical (HO^-) (Mittler, 2002). Chloroplasts are the main intracellular ROS source in plants (Robert et al., 2009) and the most heat sensitive cell function due to their photosynthetic activity (Allakhverdiev et al., 2008). During photosynthesis and respiration, the plant is steadily producing ROS and the state of the cell is controlled by protective mechanisms (Bajguz and Hayat, 2009). If these protective mechanisms are disturbed, oxidative damage can result in death of the cell. Under regular growth conditions, ROS production is very low; however, under heat stress the production is increased. This increased production of ROS causes lipid peroxidation, protein denaturation, and DNA damage (Asthir et al., 2009). Since ROS are highly reactive, plants have developed protection mechanisms against oxidative damage in the form of antioxidant enzymes. These antioxidant enzymes, such as SOD, catalase (CAT), peroxidase (POX), ascorbate-peroxidase (APX), glutathione reductase (GR) and glutathione-S-transferase (GST) scavenge the plant for excited oxygen species caused by stress (Mittler et al., 2004; Wu and von Tiedemann, 2002; Gill and Tuteja, 2010; Zhang et al., 2010). The searching for O_2^- by SOD produces H_2O_2 which is then removed by

APX or GR in the ascorbate-glutathione cycle (Çiçek and Çakurlar, 2008).

Solanum lycopersicum (garden tomato) is a member of the nightshade family (Solanaceae) in which there are 42 genera. *Solanum* species can be annual or short-lived perennials; however, the tomato, which is a perennial, has traditionally been cultivated as an annual (Tigchelaar, 1986). The garden tomato is self pollinated and has been cultivated for years across the globe for its fresh market use as well as for processing (paste, juice, sauce, powder, or whole) (Barone et al., 2009). They are valuable not only nutritionally, but have also been linked to protect against diseases such as cancer and cardiovascular disease because of antioxidant properties (lycopene) (Barone et al., 2009).

Tomatoes are produced globally and considered the second most popular vegetable crop in the world. They are native to South America but have adapted to very diverse environments (Barone et al., 2009). While they will grow in high temperatures, fruit production decreases in temperatures over 32.2°C (89.6°F) and below 21°C (69.8°F) (Lin et al., 2006). For many years the breeding objectives have been to increase fruit production in high temperatures (Hanson et al., 2002). Therefore, the objective of this experiment was to evaluate Regalia for improving heat tolerance of tomato plants.

Materials and Methods

Plant Material and Culture

Tomato seed ('BHN 640') were sown in 72-cell (41 mL) liners using Sunshine Mix 1 (SunGro Horticulture, Bellvue, WA) as the potting substrate on June 17, 2011. Three weeks later, liners were transferred to 15-cm containers and grown in a greenhouse located at Mississippi State University's R.R. Foil Plant Science Research Facility.

Tomato plants were grown for an additional three weeks to allow rooting and venting temperatures inside the greenhouse were set to 18.3/15.5 °C day/night (actual greenhouse temperature on average was 27.5 °C day and 24.0 °C night). Fertilizer was applied with irrigation at 200 ppm N using Peter's Professional 20N-8.8P-16.6K (20-10-20) Peat-Lite Special (Scotts, Maryville, OH).

Treatments and Heat Event

Single, foliar applications of Regalia [1× = 0.48 g ai·L^{-1} (10 mL·L^{-1})] were applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) 24 h before heat the event (24-hH) and 1 h prior to the heat event (1-hH). Heat event was expressed over three 24-h periods with 12-h days at 44 °C (111.2 °F) and 12-h nights at 33 °C (91.4 °F). There were two control treatments, one with plants maintained at 24 °C day and 21 °C night temperatures (NSNH) and one with plants exposed to the heat event (NSH). The experiment was conducted using a complete randomized design and six single plant replications.

Data Collected

To evaluate plant responses to heat treatments, initial growth indices (GI) and final GI [GI = (height + width + perpendicular width) ÷ 3] were used to determine total growth [TG = (final GI - initial GI)]. At the close of the experiment, shoot dry weight [SDW (Shoots were harvested by cutting the entire plant at the soil line removing all upper portions of plant material, then oven dried at 65 °C for 72 h)], final specific leaf weight [SLW (SLW was determined as previously described by Lasseigne et al., (2007)

as leaf dry weight per leaf area, $g \cdot cm^2$)], and number of open flowers (F) were measured or collected. Additionally, leaf samples (2 fully expanded leaves per plant were excised immediately after the heat event and frozen in liquid nitrogen before storing in a -80 °C cooler for determination of glutathione reductase, and glutathione-S-transferase) were collected at the close of the experiment. Leaf surface temperature (LST) was measured each day of the heat event using an infrared gun (IR Crop Temperature Meter, Spectrum Technologies Inc., Plainfield, IL) at 1000HR and 1400HR on 4 mature leaves. Photosynthesis (Pn) and respiration (Rp) were measured before (pre) and at the end of the heat event (post), and at 3 (post3), 6 (post6), 9 (post9) and 12 (post12) days after the heat event to determine gross photosynthesis [Pgross = (Pn + Rp)]. Pn was measured using a CIRAS-2 (PPSystems, Amesbury, MD) by placing the cuvette on the most recent mature leaf. Rp was measured on the same leaf as Pn, after exposing the plants to 30-min of dark (Gratani et al., 2011).

Antioxidant enzyme extractions and assays

Crude enzyme was extracted with 1 mL of a 50 mM sodium phosphate buffer (pH 7.5) as previously described by Venisse, et al. (2001), then centrifuged at 14,000g at 4 °C until plant tissue was clearly separated from the 1 mL of extraction buffer (20 to 40 minutes) (Appendix A and B.1).

Protein content was determined for each sample according to Bradford (1976) using a Quick Start Bradford Protein Assay Kit #1 (500-0201, Bio-Rad Laboratories Headquarters, Hercules, CA) (Appendix B.2).

Glutathione reductase (GR) was assayed as previously described (Esterbauer and Grill, 1978) with modifications as follows (Appendices B.3). Samples were analyzed using a PowerWave HT Microplate Spectrophotometer (BioTek Instruments, Inc. Winooski, VT) at 340 nm for 10 min. Each well contained 15µL of plant sample and 200µL of reaction buffer [0.1 mM Tris-Hydrochloride pH 7.8 (M.W. 157.6), 1% ethylenediaminetetraacetic acid disodium salts (M.W. 372.24), 1% bovine serum albumin (Bio-Rad #500-206 2mg/mL), and 8.4 mM of β-nicotinamide adenine dinucleotide phosphate (NADPH M.W. 833)]. Activity was determined following the reduction of one unit of GR which catalyzes 1µmol NADPH per minute at pH 7.6 at 25 °C (extinction coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$). GR specific activity was expressed as $\mu units \cdot mg^{-1}$ (Appendix B.3).

Glutathione-S-transferase (GST) was assayed as previously described by Venisse et al. (2001) with some modifications. Samples were analyzed using an ELx808 Absorbance Microplate Reader with a UV filter (BioTek Instruments, Inc.) at 340 nm for 5 min. Each well contained 20µL of plant sample and 230µL of reaction buffer [0.1 M potassium phosphate buffer (pH 6.5), 3.6 mM reduced glutathione (M.W. 307.3), 100 mM 1-chlor-2,4-dinitrobenzene (CDNB M.W. 202.6)]. Activity was determined by following the formation of the conjugate of 1µmol of CDNB with reduced glutathione per min at pH 6.5 at 25 °C (extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$). GST specific activity was expressed as $\mu units \cdot mg^{-1}$ (Appendix B.4).

Statistical Analysis

Analysis of variance (pooled across two experimental runs) was used to test differences in TG, SDW, SLW, and F (SAS 9.2; SAS Institute Inc., Cary, NC). When differences were found, the generalized linear model procedure was used with mean separation according to Tukey's Studentized range test, $\alpha = 0.05$.

Results

NSNH tomato plants had greater TG, SDW, and F compared to plants exposed to heat event (Table 6.1). However, SLW was similar in NSNH tomato plants compared to plants exposed to the heat event. LST was higher in plants exposed to the heat event compared to the NSNH treatment (Fig. 6.1); however, there was no difference in LST among plants exposed to the heat event and rate of Regalia. Pgross was similar among all treatments prior to application of Regalia (Fig. 6.2). At post and post3, Pgross was greater in the NSNH treatment compared to the NSH, 24-hH, and 1-hH treatments. Six days after (post6) the heat event, Pgross was similar among treatments. GR, GST, nor Protein was increased in 'BHN 640' tomato plants treated with Regalia prior to the heat event (Table 6.2).

Discussion

High temperature can reduce or altogether cease plant growth due to inactivation of PSII (Kadir et. al., 2007). This was evident in this experiment with a reduction in shoot growth among all plants exposed to the heat event. Initially, plants exposed to the heat event had a 62% (post) and 39% (post3) reduction in Pn likely due to a reduction in

PS II electron transport (Heckathorn et al., 1998) and higher leaf surface temperature (Haldimann and Feller, 2005); however, by the end of the experiment there were no differences in Pn. Furthermore, it was apparent plants were affected by the heat event but there were no indications an application of Regalia protected or enhanced heat tolerance in 'BHN 640' tomato plants.

Table 6.1 Total growth, shoot dry weight, specific leaf weight, and flower number of *Solanum lycopersicum* 'BHN 640' tomato plants following foliar applications of Regalia at 0× or 1× (10 mL·L^{-1}), 24 h or 1 h before exposure to elevated temperatures[z].

Treatments[y]	TG[x]	SDW[w]	SLW[v]	Flower[u]
NSNH	13.0 a[t]	16.2 a	0.0050	5.0 a
NSH	1.9 b	11.9 b	0.0053	2.0 b
24-hH	5.1 b	11.6 b	0.0058	1.3 b
1-hH	3.8 b	10.2 b	0.0053	1.4 b
Significance[s]	***	***	NS	***

[z]Heat event was expressed in a programmable growth chamber over three 24-h periods with 12-h days at 44°C and 12-h nights at 33°C.

[y]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = application of Regalia at 1× 24 h prior to heat event, 1-hH = Regalia applied at 1× 1 h before heat event.

[x]TG: total growth, final growth indices (GI) - initial GI [GI = (height + width + perpendicular width)÷3].

[w]SDW: shoot dry weight, oven dried for 72 hours at 65 °C.

[v]Specific leaf weight: leaf dry weight per leaf area (g·cm^2).

[u]Number of open flowers at the close of experiment.

[t]Means (within a column) with the same letter are statistically similar according to Tukey's Studentized range test for mean comparisons, $\alpha = 0.05$.

[s]NS, ***,Indicates nonsignificant or significant at $P \leq 0.001$.

Table 6.2 Glutathione reductase (GR), glutathione-*S*-transferase (GST), and protein content in leaves of *Solanum lycopersicum* 'BHN 640' affected by Regalia application (1× = 10 mL·L-1), prior to a three day heat event.z

Treatments[y]	GR (μunits·mg^{-1})	GST (μunits·mg^{-1})	Protein (μg·mL^{-1})
NSNH	0.94	1.23	2.1
NSH	0.63	2.30	2.5
24-hH	0.89	1.83	2.1
1-hH	1.03	2.05	2.1
Significance[x]	NS	NS	NS

[z]Heat event was expressed in a programmable growth chamber over three 24-h periods with 12-h days at 32.2°C and 12-h nights at 28.3°C.

[y]Treatments: NSNH = no spray no heat, NSH = no spray heat, 24-hH = Regalia at 1× 24 h before heat event, 1-hH = Regalia at 1× 1 h before heat event.

[xNS], Indicates non-significant difference at $P \leq 0.05$.

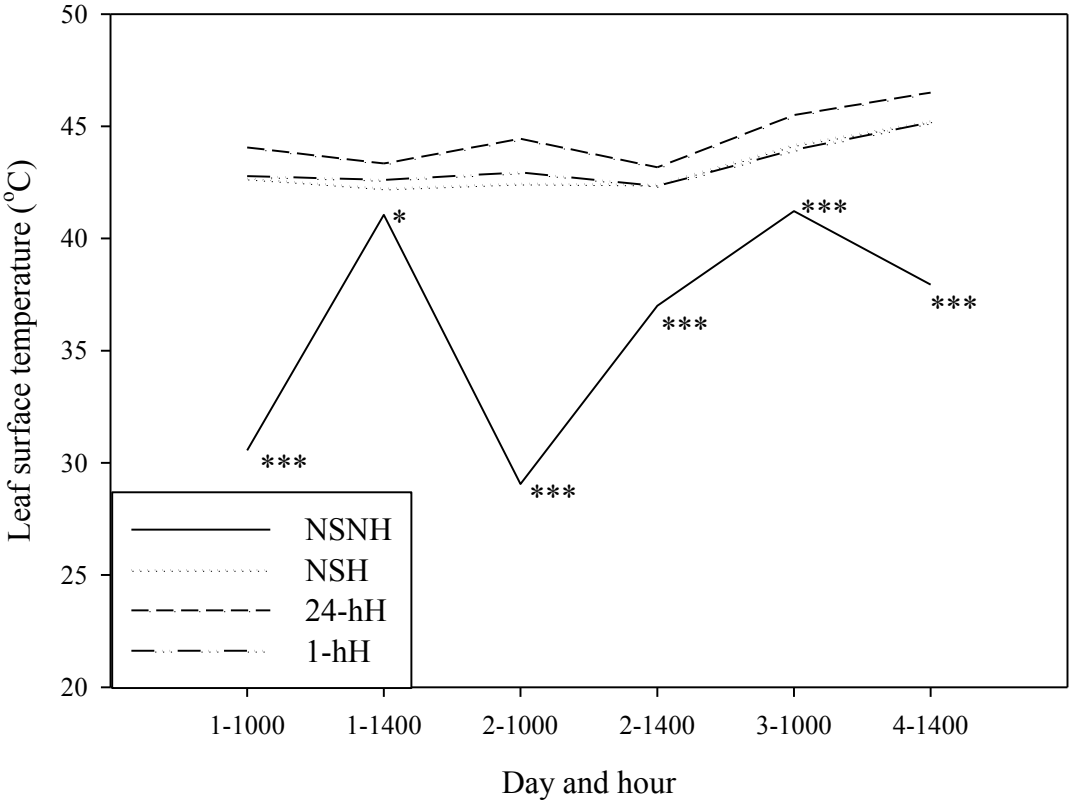

Figure 6.1 Leaf surface temperature (measured each day for 3 days during the heat event at 1000HR and 1400HR) of *Solanum lycopersicum* 'BHN 640' tomato plants measured using a IR Crop Temperature Meter (Spectrum Technologies Inc, Plainfiled, IL): NSNH = no spray no heat (kept at 24 °C /21 °C day/night), NSH = no spray heat (three 24-h periods with 12-h photoperiods at 44 °C/ 33 °C day/night), 24-hH = Regalia (1× = 10 mL·L^{-1}) 24 h before heat event, 1-hH = Regalia applied at 1× 1 h before heat. *,*** significant differences between NSNH 'BHN 640' tomato plants and NSH, 24-hH, and 1-hH at $P \leq 0.05$ or 0.001.

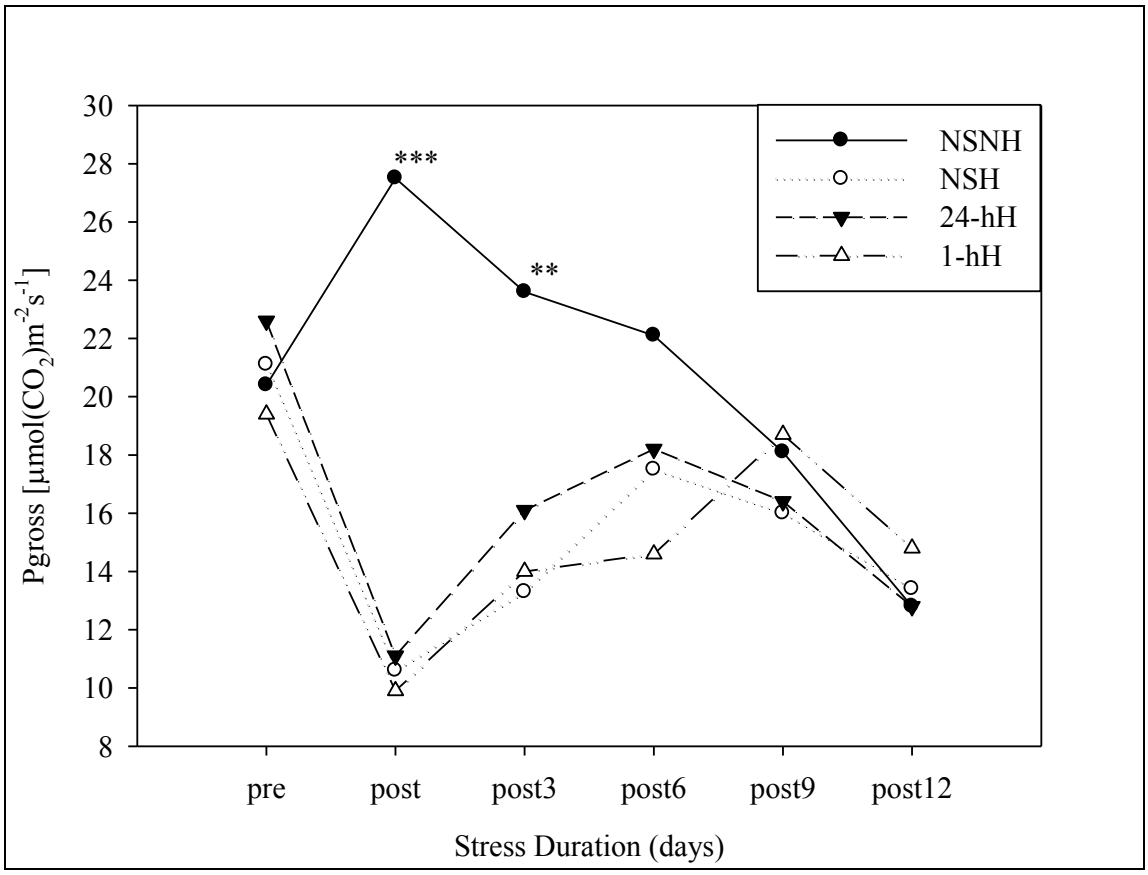

Figure 6.2 Gross photosynthetic rate [Pgross = (net photosynthesis + respiration)] of *Solanum lycopersicum* 'BHN 640' plants after exposure to heat event (H = three days at 44 °C /33 °C with 12-h day/night). There were four treatments: no spray no heat (NSNH), no spray heat (NSH), Regalia (1× = 10 mL·L^{-1}) 24 h (24-hH) or 1 h (1-hH) before heat event. Pgross was measured six times during the stress duration: pre = before H, post = immediately after H, post3 = 3 days after H, post6 = 6 days after H, post9 = 9 days after H, and post12 = 12 days after H. **,*** significant differences between NSNH plants and NSH, 24-hH, and 1-hH at $P \leq 0.01$ or 0.001.

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CHAPTER VII

EVALUATING REGALIA FOR POTENTIAL TO INCREASE COLD TOLERANCE OF *FRAGARIA ×ANANASSA* 'CAMAROSA' AND *CITRUS UNSHIU* 'OWARI'

Abstract

Fragaria ×ananassa 'Camarosa' (strawberry) plants were exposed to a chilling event (15 h with a decrease in temperature from 12 °C to 0 °C by 4 °C·h^{-1}) 24 h after a foliar application of Regalia at 0× or 1× (10 mL·L^{-1}), Expt. 1. At 4 and 0 °C, leaf samples were excised and prepared for percent electrolyte leakage (EL). Final growth indices (FGI), shoot dry weight (SDW), and fruit yield were measured to determine if the application of Regalia increased chilling tolerance. In Expt. 2, *Citrus unshiu* 'Owari' (satsuma) liners were sprayed with Regalia at 0× or 1× (10 mL·L^{-1}) 24 h prior to a freeze event: a 14 h period in a programmable freezer with a 2 °C·h^{-1} decrease in temperature. There were 5 temperature set points (4, 0, -4, -8, and -12 °C) and at each set point the temperature was held for one hour before leaf samples were pulled for EL assay. In Expt. 1, there were no differences in strawberry plants exposed to chilling versus nonchilling temperatures. In Expt. 2, EL was greater in satsuma leaves exposed to 0 °C, -4 °C, -8 °C and -12 °C compared to the nontreated (NSNF). EL injury was a good indication the freeze event was successful in testing cold tolerance; however application of Regalia did not enhance or increase cold tolerance in satsuma liners.

Introduction

In 2007, the southeastern U.S. had abnormally warm temperatures in the month of March and the following month experienced record lows (NOAA, 2007). Due to the mild temperatures in March, many ornamental plants throughout the southeast initiated bud break which led to significant crop losses when the temperatures dropped in April. The damage was so extensive the browning of vegetation could be seen by space satellites. This injury was a result of plants deacclimating with the exposure to the warmer temperatures of March, initiating new growth (Ferguson, 1995). Once the new growth was initiated, the plants were no longer acclimated to the cooler temperature. Therefore, when the temperature dropped below normal in April, the plants were injured.

One of the major stress factors affecting plant growth and productivity is chilling or freezing injury. Chilling injury occurs when temperatures are low but not below freezing (0 °C) (Zhang et al., 2009) and freeze injury occurs below 0 °C (Jan et al., 2009). Chilling injury can cause discoloration, photoinhibition, dehydration, and membrane fluidity (Solanke and Sharma, 2008; Wolfe, 1978). Freeze injury usually occurs by the formation of ice on the outside of the plant which then progresses into the cells of the plant through diffusion (Uemura and Steponkus, 1999). A plant's response to cold stress depends upon its physiology and biochemistry (Pagter et al., 2008) which can be related to its origin (Jan et al., 2009). Temperate region plants can increase their freezing tolerance when exposed to low non-freezing temperatures, whereas tropical and subtropical species are more sensitive to chilling and typically lack the ability to acclimate to cold temperatures (Jan et al., 2009).

Extract of R*eynoutria sachalinensis* (giant knotweed), also known as Regalia, is distributed by Marrone Bio Innovations as an organic fungicide with activity against powdery mildew, gray mold, and blights (Marrone Bio Innovations, 2011). Regalia's indirect mode of action is seen through the increased production of phytoalexins which strengthen the plant's immune system (Konstantinidou-Doltsinis and Schmitt, 1998). After a plant has been affected by a biotic or abiotic agent, phytoalexins (antimicrobial compounds) are synthesized as a defense mechanism (Vasconsuelo and Boland, 2007). Some of these phytoalexins are lytic enzymes, such as chitinases and glucanases, oxidizing agents, cell wall lignifications, pathogenesis-related proteins, and transcripts of unknown functions (Mert-Türk, 2002). Additionally, Regalia has been reported to increase chlorophyll values and the activity of peroxidases, polyphenoloxidases, and Phe ammonia-lyase (Daayf et al., 1997). Peroxidases are involved in lignin polymerization, cross-linkage of cell wall constituents, catabolism of auxin, formation of ROS, and defense against pathogenic organisms (Bakalovic et al., 2006). Lignin polymerization provides rigidity and structural support to cell walls (Kärkönen and Koutaniemi, 2010). Thus, if application of Regalia increases peroxidases, it could result in heightened lignin polymerization and result in a more rigid cell wall preventing extreme cell dehydration to freezing temperatures.

The objective of these experiments was to evaluate Regalia for increasing chilling and freezing tolerance of *Fragaria ×ananassa* 'Camarosa' (Expt. 1) and *Citrus unshui* 'Owari' (Expt. 2).

Materials and Methods

Experiment 1

Plant Material and Culture

On 21 October 2010, *Fragaria ×ananassa* 'Camarosa' (strawberry) (Triple J Nursery, Hayden, AL) liners were potted into 15.5-cm azalea containers with Sunshine Mix 1 (SunGro Horticulture, Bellvue, WA) potting substrate. After potting, plants were moved to a single hoop style high tunnel with straight raisable sidewalls, located on Mississippi State University's R.R. Foil Plant Science Research Facility for vernalization. Plants were hand watered as needed and fertilizer was applied with irrigation at 200 ppm N using Peter's Professional 20N-8.8P-16.6K (20-10-20) Peat-Lite Special (Scotts, Maryville, OH). During the winter months, the outside temperature was closely monitored and when temperature dropped below -2 °C, the sidewalls were lowered and temperature was monitored inside the high tunnel.

Chilling Stress and Treatments

In late February 2011 (18.7 °C/14.4 °C average high/low), the strawberry plants started flowering. On 5 March 2011 (19.8 °C/9.9 °C), plants were sprayed with Regalia at 0× or 1× rate 24-h before the chilling event (24-hC). Regalia was applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY) as a foliar spray based on the label rate of 0.48 g ai·L^{-1} (10 mL·L^{-1}). The following day chilling treatment was initiated at 1700 HR by placing plants in an environmental growth chamber (Percival Scientific Inc., Perry, IA) for a total of 15 h in

the dark. Temperature was lowered from 16 °C by 4 °C·h^{-1} until reaching 0 °C and held for 8 h. Strawberry plants exposed to the chilling event remained in the growth chamber for 1 h after the 8 h of chilling and temperature was raised at a rate of 4 °C·h^{-1}, then returned to high tunnel. There were also two nonsprayed controls: one was left in the high tunnel (NSNC) and one was exposed to the chilling treatment (NSC). Expt. 1 was conducted using a complete randomized design with 6 single plant replications.

Electrolyte leakage

Flower bud samples were taken from nontreated plants left in the high tunnel at 1730 HR for determination of electrolyte leakage as previously described by Carter et al. (1999) with modifications. After chilling treatment had initiated, flower bud samples (bud stage with visible white petals) were taken after 30 min of exposure to 4 °C and after 30 min of exposure to 0 °C for determination of electrolyte leakage (EL_{temp}). Twenty-four hours after chilling treatment, flower buds were taken for final electrolyte leakage (EL_{final}) determination. Electrolyte leakage for EL_{temp} and EL_{final} were determined as described by Nesbitt et al. (2002) with modifications. EL was determined by placing two flower buds cut in half into 50 mL vials filled with 20 mL of distilled water. Samples were then placed on a shaker for 12 h before taking the first electrical conductivity reading (EC_1) then autoclaved at 120 °C and shaken an additional 12 h before taking the second reading (EC_2) [$EL = (EC_1 \div EC_2) \times 100$].

Enzyme extractions and assays

Immediately after 8 h of chilling, leaf samples (4 mature leaves) were taken and frozen in liquid nitrogen and stored in a -80 °C freezer.

Crude enzyme (0.2 g of frozen tissue) was extracted with 1 mL of a 50 mM sodium phosphate buffer (pH 7.5) as previously described (Venisse, et al., 2001), then centrifuged at 14,000g at 4 °C until plant tissue was clearly separated from the 1 mL of extraction buffer (20 to 40 minutes) (Appendix A and B.1).

Soluble protein (SP) content was determined for each sample according to Bradford (1976) using a Quick Start Bradford Protein Assay Kit #1 (500-0201, Bio-Rad Laboratories Headquarters, Hercules, CA) (Appendix B.2).

Glutathione reductase (GR) was assayed as previously described by Esterbauer and Grill (1978) (Appendices B.3). Samples were analyzed using a PowerWave HT Microplate Spectrophotometer, BioTek Instruments, Inc. (Winooski, VT) at 340 nm for 10 min. Each well contained 15µL of plant sample and 200µL of reaction buffer [0.1 mM Tris-Hydrochloride pH 7.8 (M.W. 157.6), 1% ethylenediaminetetraacetic acid disodium salts (M.W. 372.24), 1% bovine serum albumin (Bio-Rad #500-206 2mg/mL), and 8.4 mM of β-nicotinamide adenine dinucleotide phosphate (NADPH M.W. 833)]. Activity was determined following the reduction of one unit of GR which catalyzes 1µmol NADPH per minute at pH 7.6 at 25 °C (extinction coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$). GR specific activity was expressed as $\mu units \cdot mg^{-1}$ (Appendix B.4).

Glutathione-S-transferase (GST) was assayed as previously described by Venisse et al. (2001) with some modifications. Samples were analyzed using an ELx808

Absorbance Microplate Reader with a UV filter (BioTek Instruments, Inc., Winooski, VT) at 340 nm for 5 min. Each well contained 20µL of plant sample and 230µL of reaction buffer [0.1 M potassium phosphate buffer (pH 6.5), 3.6 mM reduced glutathione (M.W. 307.3), 100 mM 1-chlor-2,4-dinitrobenzene (CDNB M.W. 202.6)]. Activity was determined by following the formation of the conjugate of 1µmol of CDNB with reduced glutathione per min at pH 6.5 at 25 °C (extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$).

Yield

On 1 April 2011, strawberry fruit were harvested every two days and rating of the strawberries was based on the USDA Strawberry Grading criteria (Table 7.1) (USDA, 2006). Fruit was collected until there was no marketable berry based on USDA criteria for marketable strawberries.

Shoot growth

Initial growth indices and final growth indices [GI = (height + width + perpendicular width) ÷ 3] were used to determine total growth [TG = (final GI - initial GI)]. Additionally, shoot dry weight [SDW (shoots were harvested by cutting the entire plant at the soil line removing all upper portions of plant material then oven dried at 65 °C for 72 h)] was determined at the close of the experiment.

Statistical Analysis

An analysis of variance procedure (data pooled across two experimental runs) was used to test the effects of chilling and Regalia application on TG, SDW, Yield, Grade,

EL_{Temp} and EL_{Final}. All statistical analysis were performed using the generalized linear model procedure of SAS (version 9.2, SAS Institute Inc, Cary, NC), $\alpha = 0.05$.

Experiment 2

Plant Material and Culture

On 26 January 2011, 60-cm bare root *Citrus unshiu* 'Owari' (satsuma) liners grafted onto *Poncirus trifoliata* rootstock (Willits and Newcomb, Bakersfield, CA) were potted into 9.6-L (2.5 gal) treepots (Stuewe and Sons, Inc. Tangent, OR). Potting substrate was a 3 pine bark: 1 sand (v:v), amended with 3.56 kg·m^{-3} of 16-6-12 Harrell's (Harrell's, Lakeland, FL) 3 to 4 month control release fertilizer with micronutrients, 3.07 kg·m^{-3} of dolomotic lime, and 0.89 kg·m^{-3} Micromax micronutrients (Micromax; The Scotts Co., Marysville, OH). At time of potting, Subdue Maxx (Syngenta, Wilmington, DE) 0.2 mL·L^{-1} was applied to the container substrate. Satsuma liners were placed in a greenhouse and maintained at 23.9/21.1 °C (day/night) to allow rooting. In June 2011, venting temperature inside the greenhouse was lowered to 18.3/15.5 °C day/night (actual greenhouse temperature on average was 27.5 °C day and 24.0 °C night from June to September) and substrate was top dressed with 3.56 kg·m^{-3} of 16N-6P-12K Harrell's. Due to the extreme heat experienced during the summer months, a 20% shade cloth was placed over the greenhouse to alleviate heat stress.

Cold Acclimation

Prior to acclimation (at least 1 week) actual greenhouse temperature averaged 22.5 °C/18.7 °C (day/night). Acclimation was conducted in an environmental growth

chamber (Percival Scientific Inc.) and modified as described by Ebel et al. (2004). At initiation of acclimation, satsuma liners had started naturally acclimating to short days. Therefore, 8 February 2012 satsuma liners were placed in the growth chamber at 20/11 °C (68/51.8°F) for 7 days under 11-h day and 13-h nights15/7 °C and on 15 February 2012 temperature was lowered to 15/7 °C (59/44.6°F) under 10.5-h day and 13.5-h nights until initiation of the experiment.

Spray Treatment

Satsuma liners were divided into nontreated versus treated with Regalia at the 1× rate [0.48 g ai·L^{-1} (10 mL·L^{-1})]: no spray and no freeze event [NSNF (kept at 15 °C)], no spray exposed to freeze event (NSF), and Regalia applied at 1×, 24 h before the freeze event (24-hF). Regalia was applied using a hand held sprayer (Model # 20010 with a 301120-4 brass nozzle, Chapin International, Inc., Batavia, NY). Expt. 2 was conducted using a complete randomized design with 4 single plant replications.

Freeze treatment

Twenty-six leaves per replication (four replications) from satsuma liners were excised from current year's growth after application of Regalia. Leaves were placed in the freezer with an initial temperature of 7 °C and lowered at a rate of 3 °C·h^{-1} until reaching 4 °C, then lowered at a rate of 2 °C·h^{-1} and held for 1 h at each temperature treatment (Hacker and Neuner, 2007, Ebel et al., 2004). Stress duration was a total of 14 h and leaves were sampled at 2 (4 °C), 5 (0 °C), 8 (-4 °C), 11 (-8 °C), or 14 h (-12 °C) before being withdrawn and placed at 4°C. All Samples remained at 4 °C until all leaves

had been sampled and thawed for 12 h (Pagter et al., 2008; Carter et al., 1999; Rajashekar et al., 1999).

Evaluation of Freeze Injury

Leaf samples were pulled after 1 h at each sample temperature for determination of electrolyte leakage (EL) as described by Ebel et al. (2004) and antioxidant enzyme analysis as described (Yang et al., 2011, Ehsani-Moghaddam et al., 2006) with the following modifications: one leaf was used per vial for electrolyte leakage analysis and 5 leaves per vial for determination of antioxidant activity (glutathione reductase and glutathione-*S*-transferase). EL was determined by first gently washing the leaves three times with double distilled water then placing two 1.5-cm leaf disks into 50 mL vials. Twenty mL of double distilled water was added to each vial and shaken for 24 h at 20 °C before measuring with a conductivity meter (Pagter et al., 2008 and Nesbitt et al., 2002). Samples were then autoclaved at 120 °C for 20 min and shaken for 24 h at 20 °C before remeasuring conductivity. In addition, EL was determined for the double distilled water to give the zero level of EC (Pagter et al., 2008). EL was determined as $EL = [(EC_{frozen}- EC_{water}) \div (EC_{autoclave}- EC_{water}) \times 100]$.

Antioxidant Enzyme extractions and assays

Leaf samples (5 mature leaves) were taken at each temperature treatment, frozen in liquid nitrogen, and stored in a -80 °C freezer.

Crude enzyme (0.2 g of frozen tissue) was extracted with 1 mL of a 50 mM sodium phosphate buffer (pH 7.5) as described by Venisse, et al. (2001), then centrifuged

at 14,000g at 4 °C until plant tissue was clearly separated from the 1 mL extraction buffer (20 to 40 minutes) (Appendix A and B.1).

Soluble protein (SP) content was determined for each sample according to Bradford (1976) using a Quick Start Bradford Protein Assay Kit #1 (500-0201, Bio-Rad Laboratories Headquarters, Hercules, CA) (Appendix B.2).

Glutathione-S-transferase (GST) was assayed as described by Venisse et al. (2001) with some modifications. Samples were analyzed using an ELx808 Absorbance Microplate Reader with a UV filter (BioTek Instruments, Inc., Winooski, VT) at 340 nm for 5 min. Each well contained 20µL of plant sample and 230µL of reaction buffer [0.1 M potassium phosphate buffer (pH 6.5), 3.6 mM reduced glutathione (M.W. 307.3), 100 mM 1-chlor-2,4-dinitrobenzene (CDNB M.W. 202.6)]. Activity was determined by following the formation of the conjugate of 1µmol of CDNB with reduced glutathione per min at pH 6.5 at 25 °C (extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$) (Appendix B.4).

Statistical Analysis

A two-factor analysis of variance procedure (data was pooled across two experimental runs) was used to test the effects of freezing and Regalia application on EL. When differences were identified, data were analyzed with linear models using the GLIMMIX procedure of SAS 9.2 (SAS Institute Inc, Cary, NC) with mean separation according to the Holm-simulation method ($\alpha = 0.05$). EL parameters were fit to polynomial curves for each treatment when significant trends were identified using linear models with the REG procedure of SAS 9.2 for each treatment.

Results

Experiment 1

Daily air temperature was monitored at the experimental site from November 2010 to June 2011 (Fig. 7.1). During the chilling event (March 2011), the deviation in air temperature for the plants exposed to the chilling event was also monitored (Fig. 7.2). After chilling in a growth chamber, strawberry plants were returned to the high tunnel. Over the next 3 weeks flowers developed into fruit and harvesting began on 1 April 2011 continuing until 17 May 2011. Peak harvest time for 'Camarosa' strawberries was between 15 April and 5 May.

There were no significant differences in TG, SDW, Yield, and Grade between treated and nontreated, or between chilled and non-chilled plants (Table 7.2). There were no differences in EL_{Temp} in strawberry plants exposed to chilling compared to no chilling (Fig. 7.3). Twenty-four hours after chilling, EL_{Final} was similar between chilled and non-chilled plants (Fig. 7.4). GR activity was similar in 'Camarosa' strawberry leaves exposed to chilling compared to nonchilling leaves (Table 7.3). GST activity was greater in the 24-hC treatment compared to NSC and NSNC leaves. SP content in leaves was similar among all treatments.

Experiment 2

There were differences in EL between treatments and temperature (Table 7.4). EL in satsuma leaves exposed to 4 °C (NSF and 24-hF) was similar to nontreated (NSNF); however, satsuma leaves exposed to 0, -4, -8, and -12 °C (NSF and 24-hF) had

greater EL compared to NSNF (Fig. 7.5). As temperature decreased, EL increased with maximum injury at -8 °C.

There were no differences in GST activity or SP content between Regalia treated and nontreated Satsuma leaves (Table 7.5).

Discussion

In Expt. 1, application of Regalia prior to a chilling event did not enhance growth or yield in 'Camarosa' strawberry plants. There were approximately 40 d between time of chilling event and peak harvest. During this period, flowers that went through the chilling event were spent; however, new uninjured flowers emerged which would explain a lack of differences in any of the parameters measured at the end of the experiment.

GST activity was similar in NSC leaves compared to NSNC leaves; however, GST was greater in 24-hC leaves compared to NSNC leaves. Since GST is known to increase in cold hardened plants (Janda et al., 2003), the application of Regalia applied 24-h before chilling may have induced GST activity.

The 15 h chilling event did not cause permanent injury to 'Camarosa' strawberry plants. Nestby and Bjøgum (1999) reported fruit yield for three strawberry cultivars exposed to 0, -8, -12, and -16 °C. These findings suggest that if 'Camarosa' plants are flowering and exposed to ≤ 15 h of chilling, there is no permanent injury and yield is not affected.

In Expt. 2, application of Regalia prior to a freeze event did not protect 'Owari' satsuma leaves from freeze injury, compared to the NSF plants. Low temperatures were the determining factor and not the application of Regalia.

GR was not analyzed for this experiment due to the lack of detection of GR as a result of the leaves being absent from light while under the freeze treatment. It was not unexpected there were no differences in GR content with freeze stress. GR is an antioxidant enzyme predominantly produced in the chloroplasts (Gill and Tuteja, 2010). While many reports have indicated increased GR activity in plants under stress, there are reports indicating no change or decreases in activity. Lu et al. (2008) reported no change in GR activity in *Eupatorium odoretum* exposed to cold stress but an increase when exposed to high temperatures. Additionally, GR activity decreased in watermelon (*Citrullus lanatus* [Thomb.] Mansf. cv. Dulce maravilla) exposed to 10 °C compared to plants maintained at 35 °C (Rivero et al., 2002). Furthermore, it has been reported the reduced form of glutathione (GSH) is light dependent (Noctor et al., 1997). GSH is involved in many metabolic regulatory and antioxidative processes (Gill and Tuteja, 2010) and in order to control, GR is produced to catalyze GSH (Karuppanapandian et al., 2011). Additionally, Robert et al. (2009) reported a direct correlation with the increase of antioxidants produced in the chloroplasts (SOD, GR, and APX) with the increase in light but a decrease in CAT, which is produced in the peroxisomes and mitochondria.

Typical label recommendation is to apply Regalia in 7 to 14 day intervals as a disease preventative. Moreover, research has shown that exogenously applied ABA or glycine betaine did not show an increase in accumulation until 42 to 72 hours after application (Rajashekar et al., 1999). Therefore, future research could evaluate a four week application of Regalia prior to exposing leaves to a freeze event. Results indicated increased GST activity in strawberry leaves following the application of Regalia

compared to the nontreated plants exposed to the same chilling conditions, which is consistent with Regalia's mode of action. While, Regalia may not be a quick response to protect 'Camarosa' strawberry plants or 'Owari' satsuma leaves from chilling or freezing temperatures, it does appear to enhance antioxidant activity in strawberry plants under moderate or chilling conditions.

Table 7.1 Strawberry grading, based on United States Standards, Expt. 1.

Average Berry Grade	Description
US#1	perfect, 100% red, size not less than 3/4"
Combined	US#1 and US#2 combined, at least 80% #1 size, no defects at least half pink/red
US#2	free from decay, not less than one-half pink or red, size not less than 5/8"
Non-marketable	anything that does not fit into one of the the above criteria

Table 7.2 Total growth, yield, and grade of *Fragaria ×ananassa* 'Camarosa' strawberry plants sprayed with Regalia (1× = 10 mL·L^{-1}) 24 h before exposing to 0 °C for 8 h[z] (Expt. 1).

Treatments[y]	TG (cm)[x]	SDW (g)[w]	Yield[v]	Grade[u]
NSNC	7.5	10.0	16.2	28.0
NSC	7.1	9.2	17.1	28.9
24-hC	6.7	8.2	16.5	27.3
Significance[t]	NS	NS	NS	NS

[z]Chilling event was expressed in a programmable growth chamber for a total of 15 h in the dark. Temperature was lowered from 16 °C by 4 °C·h^{-1} until reaching 0 °C and held for 8 h.

[y]Treatments: NSNC = no spray with no chilling ecvent, NSC = no spray with chilling event, and 24-hC = Regalia at 1× 24 h before chilling event. NSNC treatment remained in a high tunnel suitable for southeast strawberry production.

[x]TG: total growth, final growth indices (GI) - initial GI [GI = (height + width + perpendicular width)÷3].

[w]SDW: shoot dry weight, oven dried for 72 h at 65 °C

[v]Yield: average number of fruit per plant.

[u]Represents average grade given to fruit quality based on US Strawberry Grading Criteria.

[tNS],Indicates nonsignificant difference at $P \leq 0.05$.

Table 7.3 Glutathione reductase (GR), glutathione-S-transferase (GST), and protein content in leaves of *Fragaria* x*ananassa* 'Camarosa' affected by Regalia application (1× = 10 mL·L-1), prior to exposure to 0 °C for 8 h[z].

Treatments[y]	GR (μunits·mg^{-1})	GST (μg·mg^{-1})	Protein (μg·mL^{-1})
NSNC	3.02	0.19 b	0.68
NSC	1.46	0.87 b	0.57
24-hC	1.28	3.65 a	0.63
Significance[x]	NS	**	NS

[z]Chilling event was expressed in a programmable growth chamber for a total of 15 h in the dark. Temperature was lowered from 16 °C by 4 °C·h^{-1} until reaching 0 °C and held for 8 h.

[y]Treatments: NSNC = no spray with no chilling event, NSC = no spray with chilling event, and 24-hC = Regalia at 1× 24 h before the chilling event. NSNC treatment remained in a high tunnel suitable for southeast strawberry production.

[xNS,**]Indicates nonsignificant or significant difference at $P \leq 0.01$.

Table 7.4 Electrolyte leakage of *Citrus unshiu* 'Owari' satsuma leaves following application of Regalia at the 1× (10 mL·L^{-1}) rate, prior to exposure to freezing temperatures (Expt. 2).

Treatment[z]	EL (%)[y]
NSNF	8.7 b[x]
NSF	69.1 a
24-hF	66.2 a
Temperature[w]	
4 °C	7.1 c
0 °C	43.9 b
-4 °C	60.2 a
-8 °C	65.5 a
-12 °C	63.3 a
Trt	<.0001[v]
Temp	<.0001
Trt×Temp	<.0001

[z]Treatments: NSNF = no spray no freeze, NSF = no spray freeze, and 24-hF = Regalia applied at 10 mL·L^{-1} 24 h before freeze.

[y]EL:electrolyte leakage determined as $[(EC_{frozen} - EC_{water}) \div (EC_{autoclave} - EC_{water}) \times 100]$.

[x]means with the same letters within treatment or temperature are not statistically differnet according to the Holm-simulation method for mean comparisons alpha=0.05.

[w]Temperature was decreased at 2 °C·h^{-1} and held for 1 h at each set point: 4, 0, -4, -8, and -12 °C.

[v]*P* value.

Table 7.5 Glutathione-*S*-transferase (GST) activity and soluble protein (SP) content in leaves of *Citrus unshiu* 'Owari' affected by Regalia application (1× = 10 mL·L^{-1}), prior to exposure to freezing temperatures (Expt. 2).

Treatment	GST (μunits·mg^{-1})	SP (μg·mL^{-1})
NSNF	94.1 a[y]	2.8 a
NSF	139.8 a	2.5 a
24-hF	185.5 a	2.4 a
Temperature[x]		
4 °C	340.7 a	2.1 a
0 °C	111.2 a	2.4 a
-4 °C	109.0 a	3.1 a
-8 °C	69.2 a	2.6 a
-12 °C	68.8 a	2.5 a
Trt	0.6742[w]	0.2669
Temp	0.2900	0.0974
Trt×Temp	0.8962	0.7855

[z]Treatments: NSNF = no spray no freeze, NSF = no spray freeze, and 24-hF = Regalia applied at 10 mL·L^{-1} 24 h before freeze.

[y]means with the same letters within treatment or temperature are not statistically differnet according to the Holm-simulation method for mean comparisons alpha=0.05.

[x]Temperature was decreased at 2 °C·h^{-1} and held for 1 h at each set point: 4, 0, -4, -8, and -12 °C.

[w]*P* value.

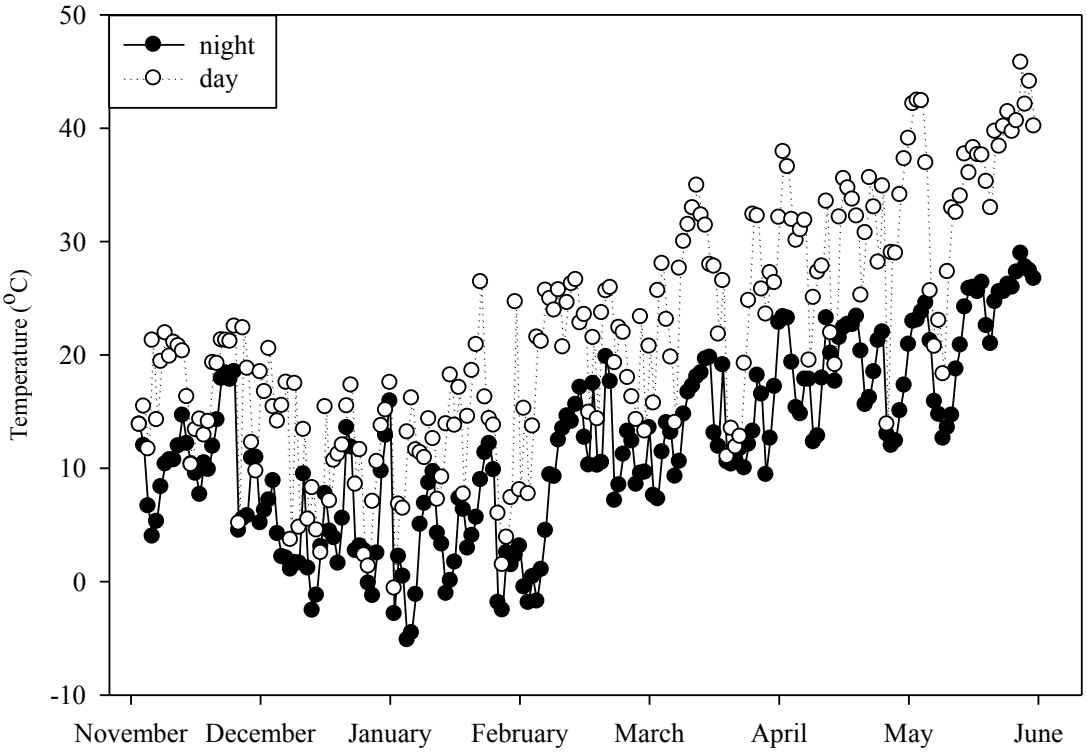

Figure 7.1 Average daily day and night air temperatures recorded in a high tunnel located at the R.R. Foil Plant Science Research Facility, Starkville, MS (33°28”09,33” N and 88°46”59,09” W), measured 15-cm from the ground, November 2010 to June 2011 (Expt. 1).

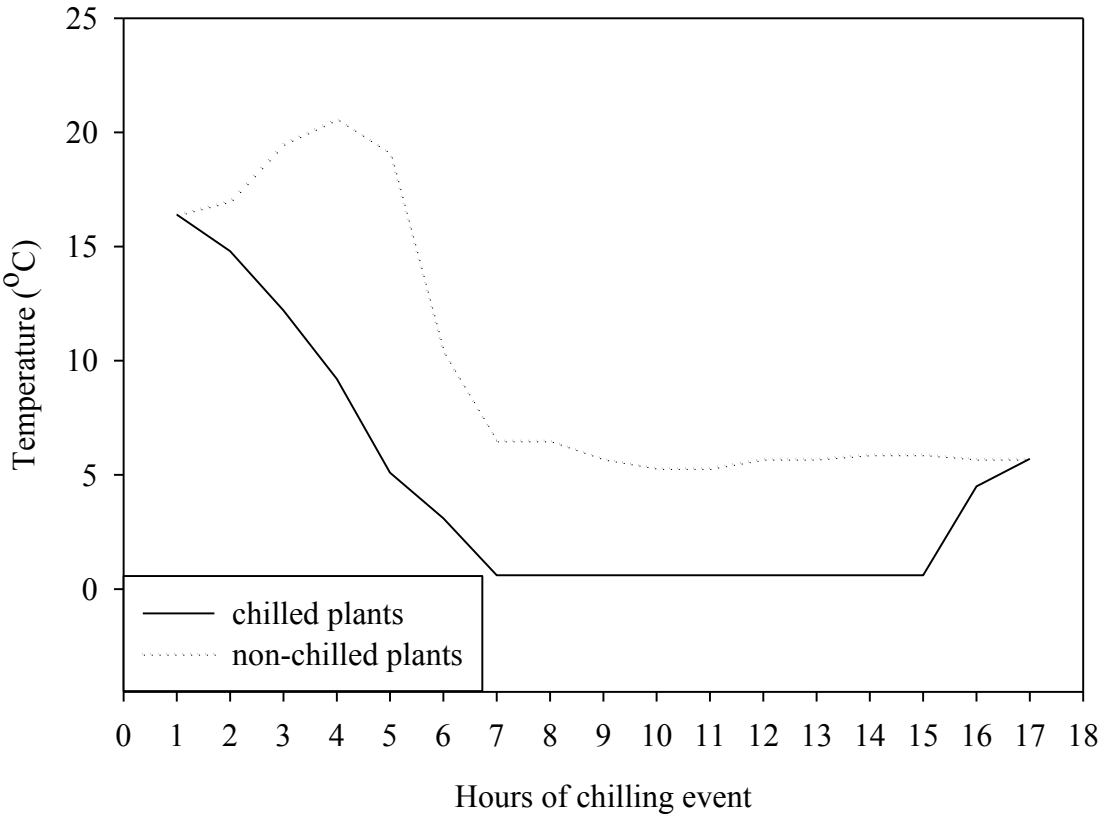

Figure 7.2 On 5 March 2011, air temperature was recorded from 1700 HR (1 h) to 0900HR the following morning (17 h) monitoring *Fragaria* x *ananassa* 'Camarosa' strawberry plants; chilled plants went through a 15 h chilling event in a growth chamber (no light) where temperature was controlled at 0 °C for 8 h. Non-chilled plants remained in a high tunnel overnight, typical of those used for Southeast strawberry production (Expt. 1).

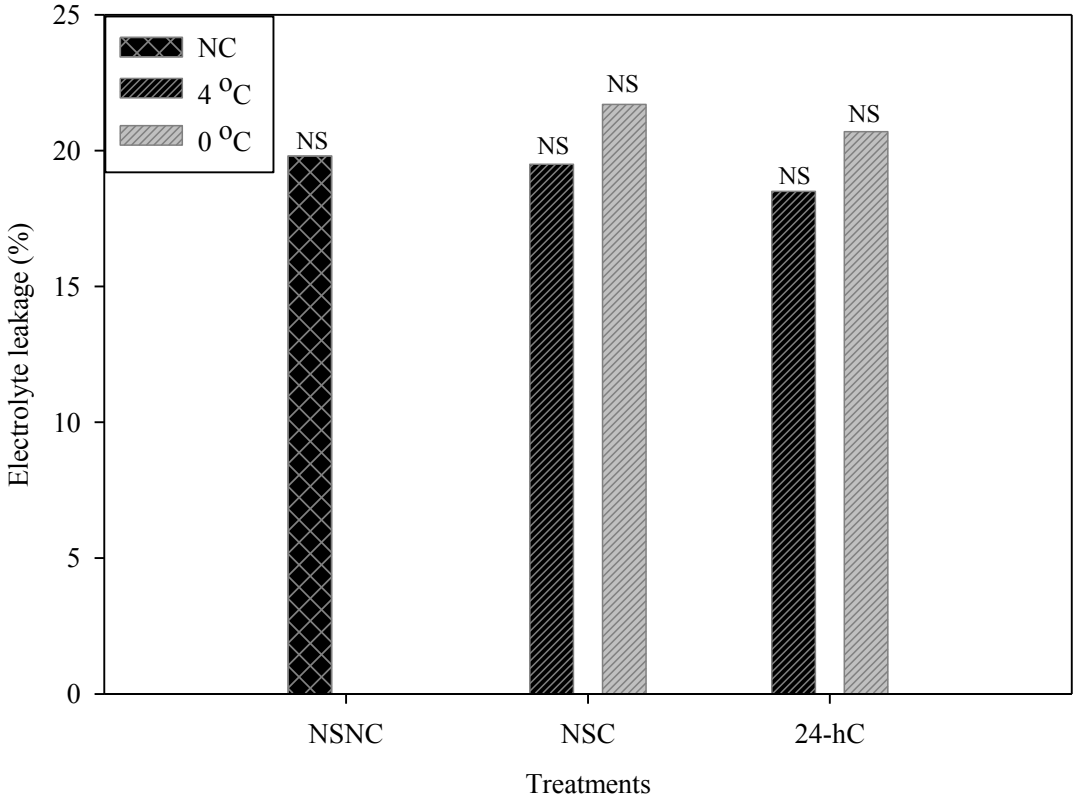

Figure 7.3 Electrolyte leakage (EL) of *Fragaria* ×*ananassa* 'Camarosa' strawberry plants exposed to 4 °C and 0 °C compared to no chilling (NC), following application of Regalia (1× = 10 mL·L^{-1}). Treatments were no spray no chilling (NSNC), no spray chilling (NSC), and Regalia applied at 1× 24 h before chilling (24-hC). EL was determined by placing two flower buds cut in half into 20 mL of distilled water and shaken for 12 h before taking the first electrical conductivity reading (EC_1) then autoclaved at 120 °C and shaken an additional 12 h before taking the second reading (EC_2) [EL = ($EC_1 \div EC_2$) ×100]. ^{NS}, indicates nonsignificant difference $P \leq 0.05$ (Expt. 1).

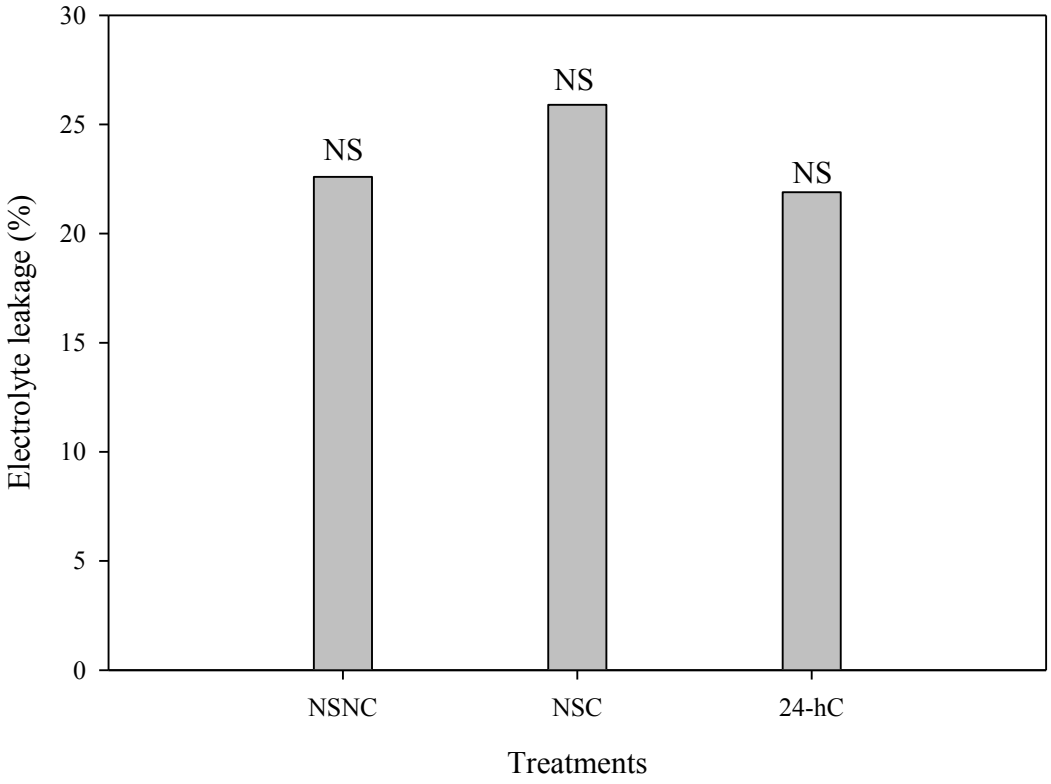

Figure 7.4 Electrolyte leakage (%) of *Fragaria ×ananassa* 'Camarosa' 24 h after exposure to 0 °C compared to no chilling, following application of Regalia (1× = 10 mL·L^{-1}). Treatments were no spray no chilling (NSNC), no spray chilling (NSC), and Regalia applied at 1× 24-h before chilling (24-hC). EL was determined by placing two flower buds cut in half into 20 mL of distilled water and shaken for 12 h before taking the first electrical conductivity reading (EC_1) then autoclaved at 120 °C and shaken an additional 12 h before taking the second reading (EC_2) [$EL = (EC_1 \div EC_2) \times 100$]. ^{NS}, indicates nonsignificant difference $P \leq 0.05$ (Expt. 1).

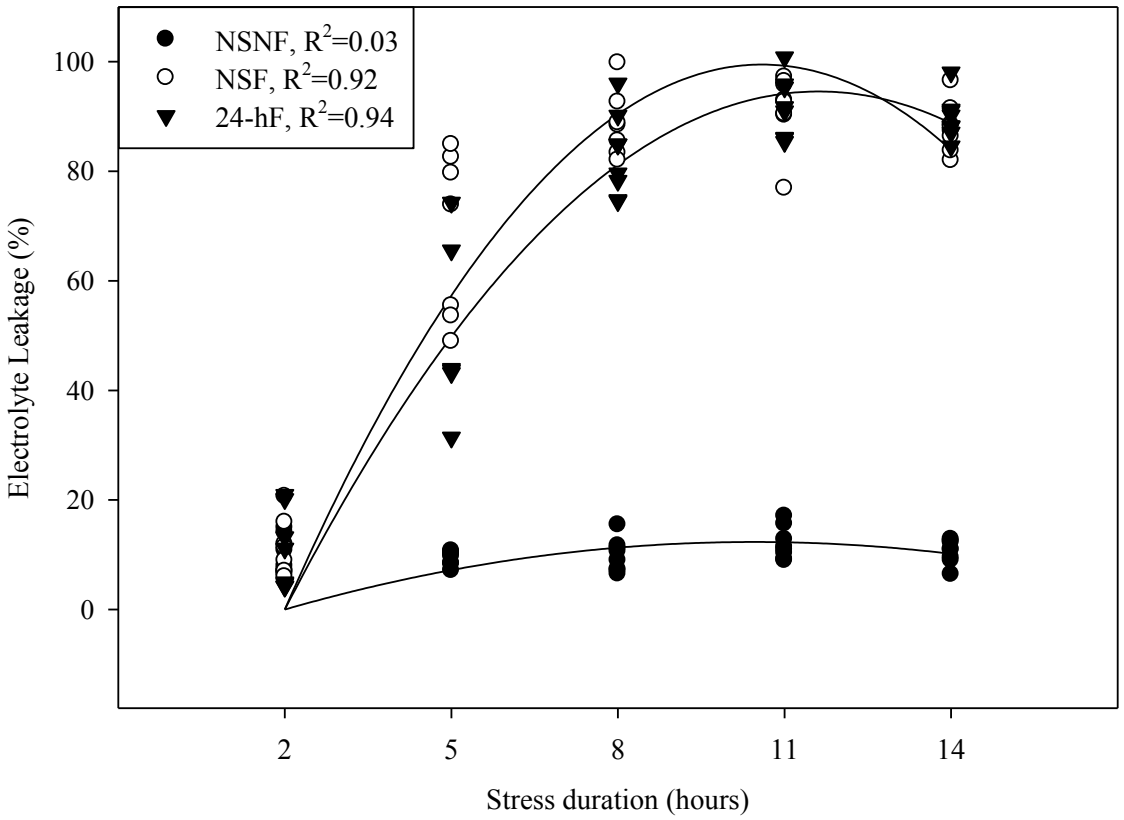

Figure 7.5 Electrolyte leakage (EL) of *Citrus unshui* 'Owari' satsuma leaves: no spray no freeze event [NSNF (leaves were sampled and kept at 15°C], no spray freeze event (NSF) and Regalia applied 24 h 1× = 10 mL·L^{-1}) before freeze event (24-hF). Freeze event was conducted in a programmable freezer lowered at 2 °C·h^{-1} and held for 1 h before leaves were sampled at 2 (4°C), 5 (0°C), 8 (-4°C), 11 (-8°C), and 14 (-12°C). EL at each temperature and fit to a regression model yielding the following equations: NSNF, y = 9.78 + 0.23temp + 0.02temp^2; NSF, = -14.0 + 57.3*temp - 9.89*temp^2; 24-hF = 9.9+ 50.7temp - 7.70temp^2 (Expt. 2).

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CHAPTER VIII

CONCLUSIONS

Plant health protectants are reported to increase frost tolerance, heat tolerance and drought tolerance in some agronomic crops; however, research with ornamentals is limited or nonexistent.

Impatiens are a common ornamental bedding plant for the southeastern U.S. They bloom from spring into fall and prefer shaded environments which can help ease the stress caused by the high temperatures and minimal rainfall, typically seen in July and August. However, because of the intense heat in the southeast, injury symptoms associated with heat and drought stress are typically seen in impatiens via wilted leaves.

Pageant (pyraclostrobin + boscalid) increased shoot growth in well watered 'Super Elfin XP White' impatiens following 4 weekly applications. However, there was no indication Pageant treated impatiens had enhanced tolerance to water stress since enhanced shoot growth was only seen in impatiens maintained at 85% following weekly applications of Pageant at the 1.0× rate.

Growth differences in 'Super Elfin XP White' impatiens and 'BHN 640' tomato plants following weekly applications of Regalia and MBI-501 were observed. Regalia applied to moderately water stressed impatiens and tomato plants at the 0.5× rate increased leaf chlorophyll content and photosynthetic rate compared to the nontreated.

WUE was improved in impatiens following application of MBI-501 at the 1.5× rate; however, there was a negative effect on Ψ_{stem} following the 1.5× rate of MBI-501. However, the main interests of these experiments were to see if drought tolerance was enhanced following the application of Regalia or MBI-501. While there were indications Regalia or MBI-501 enhanced growth or water relations in impatiens and tomato plants, results tended to be correlated with higher and moderately water stressed conditions and not drought conditions.

Application of Pageant, Regalia, or MBI-501 to impatiens exposed to a three day heat event did not increase tolerance to heat. Wilted leaves and an increase in photosynthetic rate were seen in impatiens exposed to ~ 10°C above growing temperatures. However, the impatiens (with or without application of plant protectant) were able to recover three days after heat event. Therefore, exposing impatiens to 12-h days at 32.2°C (90 °F) and 12-h nights at 28 °C (83 °F) was not a severe heat stress. Furthermore, there was no evidence indicating increased heat tolerance of impatiens after application of Pageant.

GR activity was greater in impatiens exposed to 14-h days at 38°C (100.4°F) and 10-h nights at 32.2°C (90°F) compared to 21.1°C/18.3°C (day/night) temperatures; however, there was no difference between rate of Regalia and exposure to high temperatures. While the heat event did effect metabolic changes, there were no indications Regalia enhanced heat tolerance of impatiens.

Impatiens exposed to 14-h days at 38°C (100.4°F) and 10-h nights at 32.2°C (90°F) and treated with MBI-501 resulted in similar EL values 1 and 9 days after the heat event. Therefore, MBI-501 did not increase heat tolerance to impatiens.

Shoot growth was significantly less in tomato plants exposed to 12-h days at 44 °C (111.2 °F) and 12-h nights at 33 °C (91.4 °F) compared to plants at 24 °C day and 21 °C night temperatures. Additionally, there was a reduction in photosynthesis (Pn) in plants exposed to the higher temperatures; however, by the end of the experiment there were no differences. It was apparent plants were affected by the heat event but there were no indications application of Regalia protected or enhanced heat tolerance in 'BHN 640' tomato plants.

Application of Regalia prior to a chilling event (4°C to 0 °C for 15 h) did not enhance growth or yield in 'Camarosa' strawberry plants; however, there was an increase in antioxidant activity. There was around 40-d from time of chilling event and peak harvest. During this time frame, the flowers that went through the chilling event were spent; however, new uninjured flowers emerged which would explain no differences in any of the parameters measured at the end of the experiment. The development of new uninjured flowers also indicates the chilling event did not damage developing flowers which would have decreased yields.

Application of Regalia prior to a freeze event (temperature set points were 4, 0, -4, -8, and -12 °C for 1 h) did not protect satsuma leaves from EL injury, compared to the no spray freeze treated plants. Low temperatures were the determining factor regarding EL damage.

Since there are contradicting reports about the use of strobilurins and other plant health protectants in regards to plant health, further research in ornamentals is warranted. Additionally, there was an increase in leaf chlorophyll content, higher photosynthetic rate, increased antioxidant activity and greater soluble protein content following Regalia application. However, these results may not be seen in every plant or every stress condition, therefore, individual assessment on stress tolerance or enhancements under stress should be determined before trying to use these products as a plant health protectant. Research is particularly warranted in controlled environment production since few if any researchers have reported success when conducting studies with these plant protectants on ornamentals in controlled environments.

APPENDIX A

BUFFER PREPARATIONS

A.1. 50 mM sodium phosphate buffer (pH 7.5)

a. Sodium phosphate monobasic stock - 100 mL
   - add 100 mL of distilled water to stock container
   - weigh out 55.2 g of 2 M sodium phosphate monobasic (M.W. 137.99) and add it to the stock container filled with 100 mL of distilled water
   - finish adding the required amount of distilled water to reach 200 mL
   - place a stir bar in the bottle and place on low heat and low stir until dissolved

b. Sodium phosphate dibasic stock - 100 mL
   - add 100 mL of distilled water to stock container
   - weigh out 56.8 g of 2 M sodium phosphate dibasic (anhydrous M.W. 141.96)
   - finish adding the required amount of distilled water to reach 200 mL
   - if needed place on a hot/stir plate to dissolve

A.2. 1 mM penylmethylsulfonyl fluoride (PMSF) stock solution

a. Get a 50 mL plastic vial and cut a piece of parafilm large enough to wrap around vial
b. Fill vial with 20 mL of isopropanol
c. Weigh out 0.87 g of PMSF and add to vial containing 20 mL of isopropanol
d. Add enough isopropanol to bring vial to 50 mL
e. Close lid and wrap with parafilm, store at 4°C (refrigerator)

A.3. Plant Extraction Buffer

a. 100 mL stock solution

   - add a small amount of cold distilled water to stock container
   - pipette 400 µL of sodium phosphate monobasic into container
   - pipette 2100 µL of sodium phosphate dibasic into container
   - weigh out 0.8g of 1 mM polyethyleneglycol (PEG) (MW 8000) and add to container

- weigh out 8 g of 8% polyvinylpyrolydine (M.W. 40000) and add to container
- pipette 100 µL of 0.01% Triton X-100 to container
- bring volume to 100 µL with cold distilled water
- store at 4°C (refrigerator)

b. 200 mL stock solution

- add a small amount of cold distilled water to stock container
- pipette 800 µL of sodium phosphate monobasic into container
- pipette 4200 µL of sodium phosphate dibasic into container
- weigh out 1.6g of 1 mM polyethyleneglycol (PEG) (MW 8000) and add to container
- weigh out 16 g of 8% polyvinylpyrolydine (M.W. 40000) and add to container
- pipette 200 µL of 0.01% Triton X-100 to container
- bring volume to 100 µL with cold distilled water
- store at 4°C (refrigerator)

A.4. 1 M Tris/HCL buffer (pH 7.8) stock solution

a. add 200 mL of distilled water to stock container
b. weigh out 63.04 g of Tris/HCL M.W. 157.56
c. add Tris to distilled water and check pH before adding any more distilled water
d. if pH is not 7.8 then add either HCL (to lower the pH) or NaOH (to raise pH)
e. once pH has reached 7.8 then add remaining volume of distilled water (200 mL) to bring it to desired volume of 400 mL

A.5. 0.5 M EDTA stock solution

a. add 50 mL of distilled water to stock container
b. weight out 18.6 g EDTA M.W. 372.24
c. add EDTA to distilled water and swirl container to mix
d. add the remaining volume of distilled water (50 mL) to bring it to the desired volume of 100 mL (if EDTA will not dissolve place on low heat)

A.6. NADPH stock solution stored in aliquots of 700 and 300 µL, stored at -20°C.

a. NADPH stock solution

- 0.0294 g of NADPH, M.W. 833 added to 4.2 mL distilled water stored at -20°C in aliquots of 700 or 300 µL

A.7. Oxidized Glutathione stock solution stored at -20°C

a. Oxidized Glutathione stock solution

- 0.306 g of Oxidized Glutathione M.W. 612.7 added to 10 mL distilled water stored at -20°C in aliquots of 350 or 225 µL
- 25 tubes have 350 µL and 4 tubes have 225 µL

A.8 2 M potassium phosphate monobasic stock solution

a. 2.72 g of potassium phosphate monobasic (M.W. 136.09) added to 10 mL of distilled water

A.9 2 M potassium phosphate dibasic stock solution

a. 3.48 g of potassium phosphate dibasic (M.W. 174.18) added to 10 mL of distilled water

A.10 3.6 mM reduced glutathione stock solution (M.W. 307.3)

a. 1.106g reduced glutathione + 10 mL distilled water and aliquoted into 600 µL and stored at -20°C

A.11 1 mM 1-chlor-2,4-dinitrobenzene (M.W. 202.55)

a. mixed as a 100 mM stock consisting of 0.405g CDNB + 20 mL ethanol, stored at -20°C

APPENDIX B

LABORATORY PROCEDURES

B. 1. Plant Extraction Protocol - (Venisse et al., 2001 with modifications)

1. Check to see if a 50 mM sodium phosphate buffer (pH 7.5) is prepared. If not, see Appendix A.1 for instructions on how to prepare stock solution.

2. Check to see if a 1 mM penylmethylsulfonyl fluoride (PMSF) stock solution is prepared. If not, see Appendix A.2 for instructions on how to prepare stock solution.

3. Determine how much extraction buffer will be needed for the day (Table B.1).

4. Prepare plant samples for extraction.

    a. fill a styrofoam cooler with liquid nitrogen and place a metal tray directly on the liquid nitrogen
    b. prepare previously frozen plant samples on tray to allow samples to stay cold
    c. weigh out 0.2 g of plant sample into microcentrifuge tubes
    d. add 1 mL of plant extraction buffer to each tube
    e. centrifuge at 14,000 rpm for 20 min at 4°C. Note: when placing the tubes into the centrifuge rack place where the hinge is pointed towards the top.
    f. after the samples have been taken out of the centrifuge, place on ice and transfer the crude extract (pipette the liquid from the plant tissue) into a new microcentrifuge tube
    g. pipette out enough crude extract from each sample for Bradford protein assay, then place in freezer
    h. remaining crude extract will be used for enzyme analysis

5. Determine which enzyme will be analyzed and follow protocol:

    a. Glutathione Reductase - GSH
    b. Glutathione-S-transferase - GS

B.2. Protein Extraction (Quick Start Bradford Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA)

1. Materials needed

    a. 96 well microplates
    b. Vial of BSA that came with kit from Bio-Rad
    c. Bottle of 1x dye reagent that came with kit from Bio-Rad
    d. Package of 2 ml eppendorf tubes

2. Microplate standard assay

    a. Take vial of BSA and bottle of dye reagent out of fridge and let warm to room temperature
    b. Take eight 2 ml eppendorf tubes and place in a rack
    c. Then follow the protocol from Quick Start Bradford Protein Assay Instruction Manual, Bio-Rad
    d. Preparing standards (Table B.2)
    e. Preparing samples (Table B.3)
    f. Transfer standards and samples to microplate (Table B.4)

Table B.1 Required amounts of stock plant extraction buffer and PMSF needed to mix daily extraction buffer.

Bottle/Vial Volume (needed for analysis)	Protein Extraction Buffer (Stock Orange Lid)	PMSF
200 mL	200 mL = 200,000 µl	2,000 µl
100 mL	100 mL = 100,000 µl	1,000 µl
50 mL	50 mL = 50,000 µl	500 µl
25 mL	25 mL = 25,000 µl	250 µl
12.5 mL	12.5 mL = 12,500 µl	125 µl

*100 units of Extraction Buffer to 1 unit of PMSF

*Ex. 1,000 µl extraction buffer requires 10 µl of PMSF

Table B.2 Bradford Protein Assay - Standard curve preparation.

Tube #	Standard Volume (µl)(vial from kit)	Source of Standard	Diluent Volume (our extraction buffer)	Final [Protein] (µg/ml)
1	40	2 mg/ml stock	0	2000
2	60	2 mg/ml stock	20	1500
3	40	2 mg/ml stock	40	1000
4	40	Tube 2	40	750
5	40	Tube 3	40	500
6	40	Tube 5	40	250
7	40	Tube 6	40	125
8 (blank)	-	-	40	0

*Tubes 1 - 3 are filled with BSA Stock + extraction buffer(small vial from kit).

*To fill 4 - 7:

tube 4 - take 40 µl from tube 2 and place in 4

tube 5 - take 40 µl from tube 3 and place in 5

tube 6 - take 40 µl from tube 5 and place in 6

tube 7 - take 40 µl from tube 6 and place in 7

Table B.3 Preparation of plant samples.

Dilutions	Crude Extract
100%	- + 150 µl sample
75%	37.5 µl EB + 112.5 µl sample
50%	75 µl EB + 75 µl sample
25%	112.5 µl EB + 37.5 µl sample

Table B.4. Plate set up for Protein Determination.

Assay	Volume of standard	Volume of sample	Volume of 1x dye reagent
microplate	5 µl + 15 µl water	20 µl	250 µl

B.3 Glutathione Reductase Protocol [GSH (Esterbauer and Grill, Plant Physiology)]

1. Check to see if there is enough plant extraction buffer.

2. Check to see if there is a 1M Tris/HCL buffer (pH 7.8) prepared. If not, see appendices A.4.

3. Check to see if a stock solution of NADPH is prepared in aliquots of 700 and 300 µL. (stored in the freezer at -20°C) If not, see appendix A.6.

   a. NADPH stock solution
      - NAD 700 or NAD 300
   b. Decide how much will be needed based on how many samples will be run (Table B.5)
   c. Place tubes on ice

4. Check to see if a stock solution of Oxidized Glutathione is prepared in aliquots of 350 and 225 µL, stored at -20°C. If not, see appendix A.7.

   a. Oxidized Glutathione stock solution - blue labeled microcentrifuge tubes (OG 350 or OG 225)
      - 25 tubes have 350 µL and 4 tubes have 225 µL
   b. Decide how much will be needed based on how many samples will be run (25µL per well)
   c. Place tubes on ice

5. Prepare stock solution of Glutathione reductase fresh (enzyme standard).

   a. Glutathione reductase (GSH) is stored in refrigerator
   b. Pipette 1.32 µL of GSH into 1 mL of our plant extraction buffer (bottle with orange top + PMSF)
   c. Place tube on ice

6. Standard Curve set up.

    a. 8 tubes labeled S1 - S8 (Table B.6)

7. Get a UV microplate and determine the plate set up (Table B.7).

8. Mix reaction buffer containing BSA, Tris, and EDTA (leave enough room for NADPH and leave on ice).

9. Have ready enzyme standard, plant samples, reaction mix (Tris, EDTA and BSA), NADPH, and OG to and set up computer and Spectrophotometer for 340nm.

10. After set up of equipment, follow Table B.8.

    a. Add standards, then plant samples to microplate and keep plate on ice
    b. Add NADPH to the reaction mix, then add reaction mix to microplate

11. As the last step, just before reading the plate, add Oxidized Glutathione (OG stock).

    a. 25 µL per well

12. Read plate absorbance at 340 nm.

13. Save readings and write down name of file and directory saved.

    a. File_____

Table B.5 Glutathione reductase reaction mixture.

Volume Needed	0.2 M TRIS/HCL	1% EDTA	8.4 mM NADPH	BSA (stock)
4	400 µL	0.01 g	60 µL	10 µL
8	800 µL	0.02 g	120 µL	20 µL
12	1200 µL	0.03 g	180 µL	30 µL
16	1600 µL	0.04 g	240 µL	40 µL
20	2000 µL	0.05 g	300 µL	50 µL
24	2400 µL	0.06 g	360 µL	60 µL
28	2800 µL	0.07 g	420 µL	70 µL
32	3200 µL	0.08 g	480 µL	80 µL
36	3600 µL	0.09 g	540 µL	90 µL
40	4000 µL	0.10 g	600 µL	100 µL
80	8000 µL	0.20 g	1200 µL	200 µL

Table B.6 Glutathione Reductase Standard Curve - 1:2 serial dilution

Tube	Standard (Pure GSH)	Extraction Buffer	Protein u/mL
1	100	-	0.6
2	100	100 µL	0.3
3	100 µL from tube 2	100 µL	0.15
4	100 µL from tube 3	100 µL	0.075
5	100 µL from tube 4	100 µL	0.0375
6	100 µL from tube 5	100 µL	0.01875
7	100 µL from tube 6	100 µL	0.009375
8	-	100 µL	0

Table B.7. Plate set up.

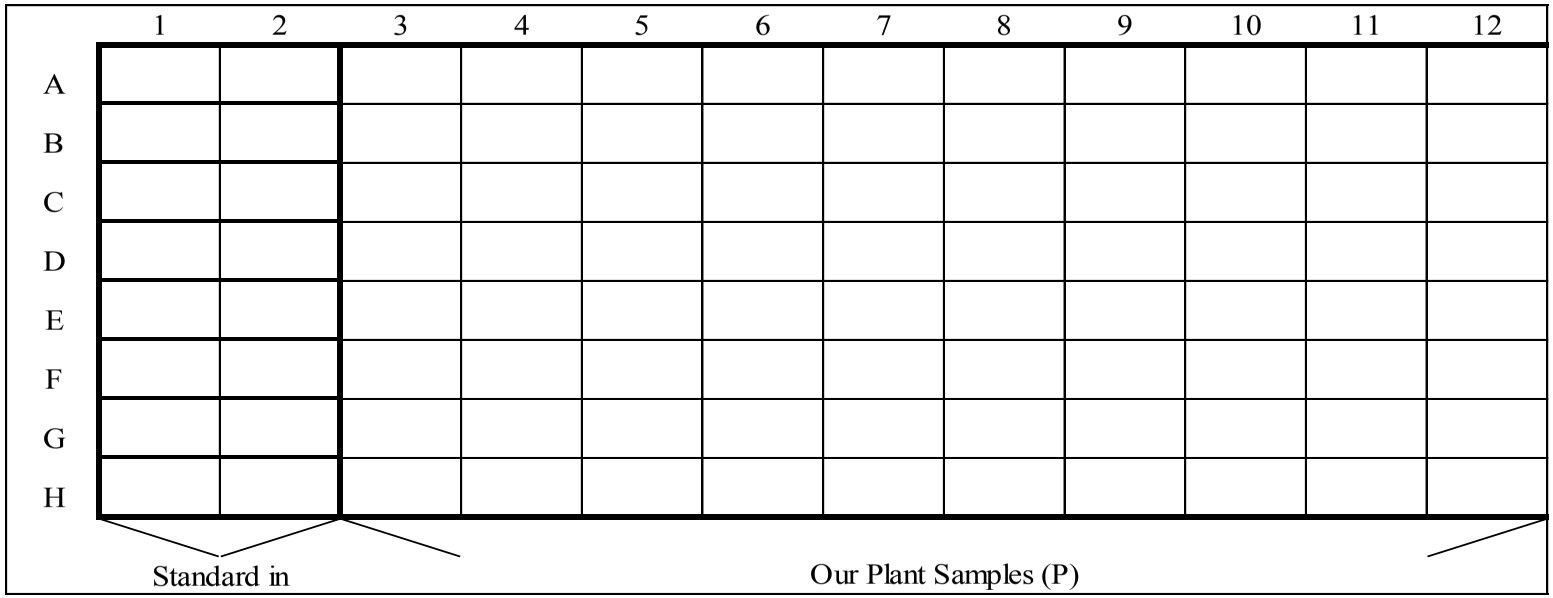

Table B.8 Glutathione reductase plate preparation.

Assay	Standards	Plant Samples	Reaction Buffer
microplate	15 µL	15 µL	200 µL

B. 4. Glutathione-S-transferase (GST)

1. Make sure there is a 2 M potassium phosphate monobasic stock solution (M.W. 136.09) and a 2 M potassium phosphate dibasic stock solution (M.W. 174.18), if not see appendix A.8 and A.9.

2. Determine how many samples will be analyzed and mix GST reaction buffer.

    a. Check to see if a 3.6 mM reduced glutathione stock solution is prepared, if not see appendix A.10.
    b. Check to see if a 100 mM CDNB + EtOH stock solution is prepared, if not see appendix A.11.
    c. Then mix according to Table B.9.

3. Prepare standard curve.

    a. Eight tubes (Table B.10).

4. Transfer standards and samples to microplate (Table B.11).

    a. cover microplate with parafilm for 10 minutes prior to first reading, to prevent oxidation

    b. read plate every 5 minutes for 20 minutes to determine end point

5. Read plate absorbance at 340 nm.

6. Save readings and write down name of file and directory saved.

    a. File_____

Table B.9 Glutathione-*S*-transferase reaction buffer.

Amount Needed (mL)	Potassium Phosphate Monobasic	Potassium Phosphate Dibasic	Glutathione Reductase	CDNB
12.5	838 µL	412 µL	125 µL	125 µL
25	1675 µL	824 µL	250 µL	250 µL
50	3350 µL	1648 µL	500 µL	500 µL
100	6700 µL	3295 µL	1000 µL	1000 µL
400	26800 µL	13180 µL	2000 µL	2000 µL

Table B.10 Glutathione-*S*-transerase standard curve

Tube	Standard (Pure GST)	Enzyme Extraction Buffer
1	50 µL	-
2	100 µL	100 µL
3	50 µl from tube 2	150 µL
4	50 µl from tube 3	150 µL
5	50 µl from tube 4	150 µL
6	50 µl from tube 5	150 µL
7	50 µl from tube 6	150 µL
8	-	150 µL

Table B.11 Glutathione-*S*-transferase plate preparation.

Assay	Standards	Plant Samples	Reaction Buffer
microplate	15 µL + 5 µL	20 µL	230 µL