WATER RELATIONS OF BEDDING PLANTS:

WATER USE, DROUGHT PHYSIOLOGY, AND GENE EXPRESSION

by

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(Under the Direction of Marc W. van Iersel)

ABSTRACT

Water scarcity has brought about the need for more efficient water use in agriculture. To better understand the water use and drought responses of bedding plants, I conducted studies on modeling daily water use of petunia based on plant and environmental factors, the drought physiology of vinca in response to different rates of drought stress development, and gene expression and physiological changes of petunia at specific substrate water contents (θ). Daily water use of two petunia cultivars (*Petunia* × *hybrida* 'Single Dreams Pink' and 'Prostrate Easy Wave Pink') was explained with a regression model based on plant age and environmental factors, such as daily light integral, vapor pressure deficit, and temperature (R^2=0.93 and 0.91 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively). Plant age was the most important factor affecting daily water use due to increasing plant size, while the daily light integral was the important environmental factor. A better understanding of plant responses to drought is needed to predict plant responses to deficit irrigation or drought. Reductions in photosynthesis, respiration, and transpiration of vinca (*Catharanthus roseus*) in response to low θ were less severe in plants subjected to slow drying than in plants subjected to fast drying,

suggesting that the rate at which drought stress develops has important implications for the level of acclimation that occurs. Physiology and gene expression of petunia (*Petunia* × *hybrida* 'Apple Blossom') displayed θ specific responses, showing acclimation of stomatal conductance and photosynthesis under mild drought (θ of 0.20 and 0.30 $m^3 \cdot m^{-3}$), but less acclimation and high abscisic acid concentrations in the leaves under severe drought (θ=0.10 $m^3 \cdot m^{-3}$). Regardless of θ, stomatal conductance of petunia was highly correlated with leaf ABA concentration. Although putative ABA biosynthesis genes (*NCED* and *AAO3*) did not significantly respond to drought, the ABA catabolic gene *CYP707A* and ABA response gene *PLDα* responded to the drought, showing significant correlation with ABA level, suggesting that these two genes may be involved in regulating ABA levels and drought signaling. To better understand water relations of bedding plants, precise descriptions of the drought imposition and severity and integrated studies combining gene expression with physiological measurement will be needed for a more comprehensive view of plant responses.

INDEX WORDS: efficient irrigation, evapotranspiration, datalogger, soil moisture sensor, automated irrigation, modeling, petunia, vinca, drying rate, daily light integral, vapor pressure deficit, abscisic acid, whole plant physiology, photosynthesis, respiration, acclimation, gene expression

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DEDICATION

I dedicate my dissertation to my soon-to-be wife (Hyo Jin Nam) for unfailing support, love, and patience that brought me so far, and to my parents for their unconditional love, and always being my heroes whole through my life.

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CHAPTER 1

INTRODUCTION

Purpose of the Study

Efficient water use has become a critical agricultural issue as water scarcity increases. Climate change-related alterations in precipitation patterns and increased urban water use related to population growth are likely to increase water scarcity (IPCC, 2007). In particular, agricultural water use is unsustainable in many areas around the world for reasons including soil salinization, ground water overdraft, and the over allocation of available surface water supplies (Jury and Vaux, 2005). Many states in the U.S. and countries in Europe are addressing these water problems by adopting restrictive laws to limit water wastage and pollution. These laws will force growers to seriously consider their irrigation control systems and strategy, and efficient irrigation will become more essential (Majsztrik et al., 2011).

For horticulture, the most critical issue is product quality, which is closely related to water use. To ensure high quality plants (and high profits), most growers apply water at rates that exceed plant needs. However, growers also need to consider other factors such as irrigation cost, water runoff, and fertilizer for best management practices and regulations. Therefore, more efficient irrigation practices are needed to increase profits and reduce waste of water and fertilizer.

Effective irrigation systems or methods that provide the appropriate amount of water to crops have been suggested by many researchers. Efficient irrigation scheduling and irrigation systems save a significant amount of water and increase plant yield and quality (Beeson, 2005; Blonquist et al., 2006; Pereira et al., 2002). Although several models have been developed for efficient water use, most previous models were developed for field crops, which are not applicable for greenhouse ornamental production. Therefore, better models that can predict water use of ornamental plants in greenhouses are needed for efficient water use.

Although efficient water use is critical, plants still need sufficient water to support their growth and development. Soil water deficit, drought, is common and considered to be the most limiting environmental factor for plant growth (Boyer, 1982). Over the last few decades, many studies have reported plant responses to drought in physiology, gene expression, and biochemistry (Chaves et al., 2003). Although these studies have investigated plant responses to drought, many of them provided imprecise descriptions of the drought treatments (Jones, 2007). Plant responses to drought are usually studied by withholding irrigation until plants are wilted or reached a certain pre-determined soil moisture level. However, observed responses can be confounded by other factors, such as other environmental and temporal variations. Plant responses to water deficit likely depend on the severity of drought stress, the process of drought development, and the duration of drought stress (Bray, 1997; Chaves et al., 2003; Flexas et al., 2006). Imprecise descriptions of how drought treatments are imposed and their effects on plant responses complicate the interpretation of many previous studies (Pinheiro and Chaves, 2011). Therefore, more precisely described drought conditions and its results would be beneficial to better understand plant responses to drought. Further, gene expression

experiments need to be integrated with whole plant responses to explicitly explain physiological responses to drought (Bray, 1997; Jones, 2007).

Recent developments in soil moisture sensor technology have made it easier to quantify the water status of substrates and control the irrigation based on these measurements (Nemali and van Iersel, 2006). Automated measurement and control of substrate/soil water content through soil moisture content (Nemali and van Iersel, 2006) or pot weight (Earl, 2003) also allows for control of both the severity of the drought stress and the rate of drought stress development. Thus, such systems allow for precise control and description of the drought conditions and aid in studying plants responses to specific drought conditions.

Hence, I conducted experiments with soil moisture sensor- and load cell-based automated irrigation systems to improve our understanding of plant water use, particularly for efficient water use of bedding plants in greenhouse production. From these experiments, I hope that greenhouse growers can irrigate their crops more efficiently, and manage their irrigation practices better, improving plant quality and increasing their profits through savings in water, labor, and other costs.

Research Objectives

Based on the perspectives above, my research had three objectives. My first objective was to develop an easy-to-use model that describes the daily water requirement of petunia based on easily acquired environmental parameters, including daily light integral, vapor pressure deficit, temperature, and days after planting. Such model-based quantitative

information regarding plant water use may assist greenhouse growers in making better irrigation decisions.

My second objective was to investigate the effect of different drying rates on whole-plant responses to drought. An integrated system with soil moisture sensors, load cells, and a whole-plant gas exchange system could improve our understanding of drought responses of the annual plant, vinca (*Catharanthus roseus*). Specifically, I investigated how the rate of drought stress imposition affects plant growth, whole-plant CO_2 exchange, evapotranspiration, and water use efficiency. This may provide a better understanding of how different rates of drought stress imposition affect whole-plant physiology and acclimation to drought.

Finally, my third objective was to grow plants under precisely controlled substrate water contents to study their physiological responses to specific drought levels as well as their gene expression, in particular on changes in stomatal regulation through ABA biosynthesis, catabolism, and signaling. This integrated study with physiology and gene expression can provide a better understanding on how genes and physiology were regulated by a certain drought stress levels.

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CHAPTER 2

LITERATURE REVIEW

Water Use of Ornamental Plants

A critical issue in ornamental plant production is plant quality, which is closely related to irrigation practices. Ornamental plant growers commonly apply excess water to reduce the risk of drought stress and ensure high quality plants (and high profits). However, excessive irrigation can increase the plants' vulnerability to diseases such as root rot, which can lead to plant death or decreased quality (Nelson, 1998). Excessive irrigation also leaches fertilizer, which constitutes an economic loss and can lead to environmental pollution. Good water management practices can improve nutrient management (Bilderback, 2002). Proper irrigation management in container plant production is more critical than for field-grown plants due to the limited substrate volume, which restricts water and nutrient availability. Greenhouse growers should consider irrigation and fertilizer costs, as well as the potential for leaching and runoff, when selecting best management practices, and efficient irrigation can reduce costs and increase profits (Bilderback, 2002; Majsztrik et al., 2011).

Appropriate irrigation systems and scheduling saves a significant amount of water, while maintaining or increasing yield and quality in horticultural production (Bacci et al., 2008; Beeson Jr. and Brooks, 2008; Blonquist et al., 2006; Fereres et al., 2003). Although efficient irrigation systems, such as micro-irrigation or sub-irrigation, have been employed by many

growers, overhead irrigation still prevails in ornamental production (Hodges et al., 2008). Soil moisture sensor-based automated irrigation allows for precise irrigation (Jones, 2004; Nemali and van Iersel, 2006), but few greenhouse growers have adapted this approach so far. Although sensors can provide valuable information to help make irrigation decisions, malfunctioning of sensors or lack of uniformity may occur during their use. Sensor based irrigation, together with estimation of needed irrigation amounts, may be beneficial to correctly manage the irrigation of ornamental plants in containers (Bacci et al., 2008). By integrating precise irrigation systems with accurate irrigation scheduling, efficient irrigation management can be achieved, saving water, labor, electricity, and fertilizer.

Water Use Modeling in Greenhouse Production

Many researchers have developed models predicting irrigation needs by estimating evapotranspiration (ET) using environmental factors (Jones and Tardieu, 1998). The most common method of calculating ET is the Penman-Monteith equation, recommended by the United Nations Food and Agriculture Organization (Allen et al., 1998). The Penman-Monteith equation is an energy balance-based method, and requires good estimates of an empirical crop coefficient (Kc) that incorporates specific features of a crop (Allen et al., 1998). This equation was developed for field crops with large, uniform canopies and many field crops have relatively well established Kc values. However, Kc values for ornamental plants have high variability, and require a large amount of empiricism with recalibration (Baille et al., 1994), and the adoption of this approach for ornamental plant production has been questioned (Bacci et al., 2008; Schuch and Burger, 1997).

Estimates of the required irrigation amount also can be based on plant and environmental factors. Plant size affects water use because it affects the evapotranspirational surface area of plants (Ray and Sinclair, 1998). Environmental factors, such as light, air humidity, air temperature, wind, and soil water availability, also affect water use (Allen et al., 1998; Jones and Tardieu, 1998). Light is an important environmental factor affecting plant water use due its effects on evaporation and stomatal opening (Pieruschka et al., 2010). Vapor pressure deficit (VPD), the gradient of water vapor concentration from the leaf to the air, is the driving force for transpiration and also affects stomatal regulation (Bunce, 2006; Taiz and Zeiger, 2006). Temperature can affect evapotranspiration and plant metabolic activity (Allen et al., 1998; van Iersel, 2003). Wind speed and soil water content also affect plant water use (Andersson, 2011; van Iersel et al., 2010). However, air flow in greenhouses is highly variable (Fernandez and Bailey, 1994), making it difficult to quantify.

For estimation of water use of ornamentals in greenhouses, researchers have developed models by modifying the Penman-Monteith equation (Bacci et al., 2008; Baille et al., 1994; Beeson, 2005; Krügera et al., 1999; Rouphael et al., 2008). Baille et al. (1994) derived models for *Begonia* × *hiemalis*, *Cyclamen persicum*, *Gardenia jasminoides*, *Sinningia speciosa*, *Hibiscus rosa-sinensis*, *Impatiens* × *novae-guinea*, *Pelargonium hortorum*, *Euporbia pulcherrima*, and *Shefflera arboricola* based on a simplified Penman-Monteith equation, with radiation and vapor pressure deficit as the two main environmental factors and leaf area index (LAI) as the plant factor for determining hourly evapotranspiration. Their model predicted the hourly evapotranspiration rate of these nine ornamental plants during the daytime with an R^2 ranging

from 0.87 to 0.97. Although LAI is a good indication of plant size, it can be difficult and laborious to measure, especially for commercial growers (Rouphael and Colla, 2004).

Drought Stress Physiology

Drought is the most common stress that limits yield in agricultural production, since water plays pivotal roles in plants, providing turgor for cell elongation and functioning as a metabolic reactant (Boyer, 1982; Hsiao, 1973). Understanding plant responses to drought has become even more of a priority, now that climate change is expected to reduce water availability in many areas (IPCC, 2007; Jury and Vaux, 2005). Because of the ecological and agricultural importance of drought, many researchers have investigated and made progress in understanding plant responses to drought (Chaves et al., 2003; Jones, 2007; Pinheiro and Chaves, 2011).

In general, drought stress inhibits cell elongation due to turgor pressure loss, and the resulting shoot growth inhibition is the earliest drought response (Boyer, 1970; Hsiao, 1973; van Volkenburgh, 1999). Plants under drought stress commonly close stomata by regulating guard cell turgor to minimize water loss. The change in stomatal conductance (g_s) not only decreases efflux of water vapor, but also decreases the influx of CO_2 into the leaves, thus reducing photosynthetic carbon assimilation in the leaves. Critical damage to the biochemical capacity of the photosynthetic apparatus generally occurs only under severe drought stress (Flexas et al., 2006; Lawlor and Cornic, 2002). Therefore, stomatal closure is regarded as the most limiting factor in photosynthesis of plants under drought stress (Flexas and Medrano, 2002; Lawlor and Cornic, 2002; Pinheiro and Chaves, 2011).

However, these drought stress responses depend on the species and genotype, the duration and severity of the stress, the age and stage of plant development, and the stress history of the plants (Bray, 1997; Hsiao, 1973; McDonald and Davies, 1996). Plants reduce drought sensitivity or adjust biochemical processes in order to enhance stress tolerance (Flexas et al., 2006; Lambers et al., 2008; Yordanov et al., 2000). Plants can adjust their water relations and photosynthesis by modifying their physiology, morphology, and gene expression, leading to acclimation to unfavorable conditions (Flexas et al., 2006). For instance, mild drought stress increases the WUE of plants and increases drought tolerance through osmotic adjustment or by altering the root-shoot allocation (Davies et al., 2002; Earl, 2002; Kozlowski and Pallardy, 2002; Lawlor and Cornic, 2002; López et al., 2009; Yordanov et al., 2000). It has been suggested that exposing plants to a slow drying rate lessens the resulting physiological impairment, because of better acclimation (Bray, 1997; Flexas et al., 2006).

A rapid development of drought is especially likely when plants are grown in containers, since the limited soil/substrate volume limits the amount of available water. Growing plants in containers is common in the greenhouse and nursery industry, as well as in many research applications. In addition, soilless substrates are commonly used for container-grown plants, and the moisture retention curves of soilless substrates differ from those of mineral soils. Much of the water in soilless substrates is held at a low tension, but water becomes rapidly less available as θ approaches a substrate-specific threshold (Wallach, 2008). However, despite its potential importance, there is little quantitative information on the effect of the drying imposition rate on physiological responses and drought acclimation.

A previous study looking at different rates of drought stress development found that rapid drying, imposed by removing plants from the soil, provides insufficient time for resurrection plants (*Craterostigma wilmsii*, *Xerophyta humilis*, and *Myrothamnus flabellifolius*) to activate protective mechanisms (Farrant et al., 1999). These resurrection plants failed to survive rapid drying, while all species survived when exposed to slow drying (Farrant et al., 1999). However, ressurection plants have unique drought tolerance mechanisms and may have different physiological responses than other plants. Recently, López et al. (2009) reported that slow drought imposition enhanced osmotic adjustment of seedlings of *Pinus canariensis*. In this experiment, researchers added polyethylene glycol to hydroponic solutions to control drying rates by controlling the osmotic potential of the nutrient solution. However, plants may have different physiological responses to drought stress in soil or substrate compared to osmotic stress in hydroponics.

Gene Expression Related to ABA Biosynthesis and Catabolism

Stomatal control by guard cells is regulated by environmental factors such as light, CO_2, and the water status of the plants (Roelfsema and Hedrich, 2005). Stomatal closure under drought commonly occurs either through a chemical signal (abscisic acid, ABA), a hydraulic signal from roots sensing low soil water potential, or both (Christmann et al., 2007; Schachtman and Goodger, 2008). ABA is the primary chemical signal for drought, increasing in concentration under drought stress and inducing stomatal closure and expression of stress-related genes.

Due to the critical role of ABA in plant responses to various environmental stresses, ABA has been studied for decades (Jiang and Hartung, 2008; Schachtman and Goodger, 2008). ABA

biosynthesis and catabolism related genes have been identified in many species (Seki et al., 2007; Shinozaki and Yamaguchi-Shinozaki, 2007). In the ABA biosynthetic pathway, 9-*cis*-epoxycarotenoid dioxygenase (NCED) cleaves off epoxycarotenoids in plastids to form xanthoxin, a precursor of ABA, and this step is regarded as the regulatory step of ABA biosynthesis (Nambara and Marion-Poll, 2005). The *NCED* genes belong to a multigene family, and have been identified in many species, including *LeNCED1* and *LeNCED2* in tomato, which is in the same family as petunia (Thompson et al., 2000). As the final step of ABA biosynthesis, abscisic aldehyde oxidase converts abscisic aldehyde to ABA in the cytosol, and *AAO3* was identified as the key gene in encoding the enzyme in arabidopsis (Nambara and Marion-Poll, 2005; Seo et al., 2000). The endogenous ABA level is determined not only by ABA biosynthesis but also by ABA catabolism, which contributes to ABA homeostasis in plants (Seiler et al., 2011). In ABA catabolism, there are two types of reactions, hydroxylation and conjugation. ABA 8'-hydroxylases predominantly catalyze the ABA catabolic pathway by isomerizing ABA to phaseic acid. The Cytochrome P450 (CYP707A) family plays an important role in encoding ABA 8'-hydroxylases (Umezawa et al., 2006), and overexpression of *SlCYP707A3* in tomato decreased the ABA concentration in the ovary (Nitsch et al., 2009). ABA can also be stored in the inactive, conjugated ABA glucosyl ester (ABA-GE) form, which can be reactivated by specific *β*-glucosidases, releasing free ABA from ABA-GE (Lee et al., 2006).

Other than ABA biosynthesis and catabolism, ABA signaling in plants also can mediate stomatal opening and closing (Kim et al., 2010). Phospholipase D (PLD) has been suggested as an enzyme that plays a role in ABA signaling by mediating ABA effects on stomata. PLDα1 activity results in the synthesis of phosphatidic acid from phospholipids, which has dual roles in

promoting stomatal closure and inhibiting stomatal opening (Mishra et al., 2006; Zhang et al., 2004). Under environmental stress, various transcription factors are involved in triggering plant responses and adaptation to the environment (Yamaguchi-Shinozaki and Shinozaki, 2006). Zinc finger proteins are one of the transcription factor families, responsive to a very wide variety of abiotic stresses (Yamaguchi-Shinozaki and Shinozaki, 2006). *ZPT2-3* is a transcription factor that encodes a Cys2/His2-type zinc finger protein found in petunia, and is up-regulated in response to wounding, low temperature, drought, and heavy metal treatments (Sugano et al., 2003). Overexpression of *ZPT2-3* in petunia increased drought tolerance and thus survival under drought (Sugano et al., 2003). Similar studies in arabidopsis showed that *ZPT* homologues were up-regulated under drought stress and increased drought tolerance (Sakamoto et al., 2004; Shu-Jing et al., 2010).

Datalogger Controlled Irrigation System

A datalogger is a device that reads various types of electronic signals and stores the data in internal memory for later download to a computer. The advantage of dataloggers is the ability to automatically collect data as frequently as needed. Recently, a capacitance soil moisture sensor (ECH_2O, Decagon Devices, Pullman, WA) was calibrated and examined for measuring substrate water contents in greenhouse production (Nemali et al., 2007). A load cell can measure plant weight and can be used to calculate whole-plant conductance and transpiration rate from the change in weight (Earl, 2003).

Automated irrigation systems using these sensors, connected to a datalogger and relay driver to control solenoid valves, can constantly monitor and control the substrate water

content and/or pot weight as programmed by the user. This approach has been successful in controlling substrate water contents in a variety of studies (Burnett and van Iersel, 2008; Earl, 2003; Kim and van Iersel, 2009; Nemali and van Iersel, 2008; van Iersel et al., 2010). Further, this automated measurement and control of substrate water content or pot weight with a datalogger allows for control of both the severity of the drought stress and the rate of drought stress development. With this system, a well-described and precise substrate water status can be achieved.

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CHAPTER 3

ESTIMATING DAILY WATER USE OF TWO PETUNIA CULTIVARS BASED ON PLANT AND ENVIRONMENTAL FACTORS [1]

[1] Kim, J., S. Burnett, and M.W. van Iersel. To be submitted to *HortScience*.

Abstract

Many ornamental plant growers use excessive irrigation water to reduce the risk of unwanted drought stress. Part of the difficulty of scheduling irrigation in greenhouses is that there is little quantitative information about plant water requirements, and how they depend on environmental conditions. Models to estimate the daily water use (DWU) of greenhouse crops may provide a useful tool to reduce excessive irrigation. Our objective was to develop a model to predict DWU based on plant age and easily acquirable environmental data. Two petunia (*Petunia* x*hybrida*) cultivars, 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', were grown in different size containers (diameter=10, 12.5, and 15 cm) to quantify their DWU for 6 weeks. The substrate water content (θ, v/v) was maintained at 0.40 $m^3 \cdot m^{-3}$ using an automated irrigation system with capacitance soil moisture sensors. Every irrigation event was recorded by the datalogger, and used to calculate the DWU of the plants. On overcast days early in the experiment, plants used only 4.8 to 13.8 mL/d. The maximum DWU of 'Single Dreams Pink' was 63, 96, and 109 mL/d in 10, 12.5, and 15 cm containers, respectively. Later in the experiment, 'Prostrate Easy Wave Pink' used more water than 'Single Dreams Pink' because of their more vigorous growth habit. DWU was modeled as a function of days after planting (DAP), daily light integral (DLI), vapor pressure deficit (VPD), temperature, container size, and interactions between these factors and DAP (R^2=0.93 and 0.91 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively). DAP and container size were the most important factors affecting DWU, and are indicative of plant size. DLI was the most important environmental factor affecting DWU. These models, describing the DWU as a function the DAP and environmental

conditions, may be used as guidelines for water requirements of petunias in greenhouse production, and may improve irrigation scheduling in greenhouses.

Additional index words: capacitance sensor, greenhouse irrigation, efficient irrigation, container size, daily light integral, vapor pressure deficit, multiple regression, modeling

Introduction

Efficient water use has become a critical agricultural issue as water scarcity increases. Climate change-related alterations in precipitation patterns and increased urban water use related to population growth are likely to increase water scarcity (IPCC, 2007). In much of the world, existing water supplies are insufficient to meet all of the urban, industrial, agricultural, and environmental demands. In particular, agricultural water use is unsustainable in many areas around the world for reasons including soil salinization, ground water overdraft, and the over allocation of available surface water supplies (IPCC, 2007; Jury and Vaux, 2005). Many states in the U.S. and countries in Europe are addressing these water problems by adopting restrictive laws to limit water wastage and pollution. These laws will force growers to seriously consider their irrigation control systems and strategy, and efficient irrigation will become more essential (Majsztrik et al., 2011).

A critical issue in ornamental plant production is plant quality, which is closely related to irrigation practices. Ornamental plant growers commonly apply excess water to reduce the risk of drought stress and ensure high quality plants (and high profits). However, excessive irrigation can increase the plants' vulnerability to diseases such as root rot, which can lead to plant death

or decreased quality (Nelson, 1998). Excessive irrigation also leaches fertilizer, which constitutes an economic loss and can lead to environmental pollution. Previous research indicated that good water management practices can improve nutrient management (Bilderback, 2002). Proper irrigation management in container plant production is more critical than for field-grown plants due to the limited substrate volume, which restricts water and nutrient availability. Greenhouse growers should consider irrigation and fertilizer costs, as well as the potential for leaching and runoff, when selecting best management practices, and efficient irrigation can reduce costs and increase profits (Bilderback, 2002; Majsztrik et al., 2011).

Previous studies reported that the use of appropriate irrigation systems and scheduling saves a significant amount of water, while maintaining or increasing yield and quality in horticultural production (Bacci et al., 2008; Beeson Jr. and Brooks, 2008; Blonquist et al., 2006; Fereres et al., 2003). Although efficient irrigation systems such as micro-irrigation or sub-irrigation have been employed by many growers, overhead irrigation still prevails in ornamental production (Hodges et al., 2008). Soil moisture sensor-based automated irrigation allows for precise irrigation (Jones, 2004), but few greenhouse growers have adapted this approach so far. Although sensors can provide valuable information to help make irrigation decisions, malfunctioning of sensors or lack of representativity may occur during their use; thus careful monitoring of the sensor system is needed. Sensor based irrigation, together with estimation of needed irrigation amounts, may be beneficial to correctly manage the irrigation of ornamental plants in containers (Bacci et al., 2008). By integrating precise irrigation systems with proper

irrigation scheduling, efficient irrigation management can be achieved, saving water, labor, electricity, and fertilizer.

Estimates of the required irrigation amount can be based on plant and environmental factors. Plant size affects water use because it affects the transpirational surface area of plants (Ray and Sinclair, 1998). Environmental factors, such as light, air humidity, air temperature, wind, and soil water availability, also affect water use (Allen et al., 1998; Jones and Tardieu, 1998). Light is an important environmental factor affecting plant water use due its effects on evaporation and stomatal opening (Pieruschka et al., 2010). Vapor pressure deficit (VPD), the gradient of water vapor concentration from the leaf to the air, is the driving force for transpiration and also affects stomatal regulation (Bunce, 2006; Taiz and Zeiger, 2006). Temperature can affect evapotranspiration and plant metabolic activity (Allen et al., 1998; van Iersel, 2003). Wind speed and soil water content also affect plant water use (Andersson, 2011; van Iersel et al., 2010). However, air flow in greenhouses is highly variable (Fernandez and Bailey, 1994), making it difficult to quantify. Providing sufficient water can prevent water availability from becoming limiting.

Many researchers have developed models predicting irrigation needs by estimating evapotranspiration (ET) using environmental factors (Jones and Tardieu, 1998). The most common method of calculating ET is the Penman-Monteith equation, recommended by the United Nations Food and Agriculture Organization (Allen et al., 1998). The Penman-Monteith equation is an energy balance-based method, and requires good estimates of an empirical crop coefficient (Kc) that incorporates specific features of a crop (Allen et al., 1998). This equation was developed for field crops with large, uniform canopies and many field crops have relatively

well established Kc values. However, Kc values for ornamental plants have high variability, and require a large amount of empiricism with recalibration (Baille et al., 1994), and the adoption of this approach for ornamental plant production has been questioned (Bacci et al., 2008; Schuch and Burger, 1997).

For estimation of water use of ornamentals in greenhouses, researchers have developed models by modifying the Penman-Monteith equation (Bacci et al., 2008; Baille et al., 1994; Beeson Jr. and Brooks, 2008; Krügera et al., 1999; Rouphael et al., 2008). Baille et al. (1994) derived models for *Begonia* × *hiemalis*, *Cyclamen persicum*, *Gardenia jasminoides*, *Sinningia speciosa*, *Hibiscus rosa-sinensis*, *Impatiens* × *novae-guinea*, *Pelargonium hortorum*, *Euporbia pulcherrima*, and *Shefflera arboricola* based on a simplified Penman-Monteith equation, with radiation and vapor pressure deficit as the two main environmental factors and leaf area index (LAI) as the plant factor for determining hourly evapotranspiration. Their model predicted the hourly evapotranspiration rate of these nine ornamental species during the daytime with an R^2 ranging from 0.87 to 0.97. Although LAI is a good indication of plant size, it can be difficult and laborious to measure, especially for commercial growers (Rouphael and Colla, 2004).

Previously, measurements of plant water use were collected by measuring the change in container weight during a certain time period. In this study, we used a soil moisture sensor based automatic irrigation system (Nemali and van Iersel, 2006) to quantify the irrigation amount. This automated irrigation system maintained θ at a stable level regardless of plant water use, while quantifying the amount of water needed to maintain θ. The objective of this study was to develop an easy-to-use model that describes the daily water requirement of petunia based on easily acquirable parameters (DLI, VPD, temperature, and DAP). Such model-

based quantitative information regarding plant water use may assist greenhouse growers in making better irrigation decisions.

Materials and Methods

Plant material

Two petunia cultivars (*Petunia* ×*hybrida* 'Single Dreams Pink' and 'Prostrate Easy Wave Pink') were used to quantify the water use of two cultivars with different growth habits (upright, 'Single Dreams Pink' and spreading 'Prostrate Easy Wave Pink', Griesbach, 2006). Seedlings in 288-plug trays were obtained from a commercial greenhouse (C. Raker and sons, Litchfield, MI), and transplanted into 10, 12.5, and 15 cm diameter, round plastic containers (volume of 420, 767, and 1320 mL, respectively). Containers were filled with soilless substrate (Fafard 2P; 60% peat and 40% perlite, Fafard, Anderson, SC) with controlled-release fertilizer (14.0N-6.2P-11.6K, Osmocote 14-14-14; Scotts, Marysville, OH) incorporated at a rate of 7.7 $g \cdot L^{-1}$. Three different container sizes were used to quantify the effect of container size on water use. Plants were grown in a greenhouse at the University of Georgia for 45 d (Oct. 10 to Nov. 24, 2008) with a soil moisture sensor-controlled irrigation system. To allow the seedlings to get established, the plants were hand-irrigated for 5 d after transplanting, after which the sensor-controlled irrigation system was used.

Each experimental unit had 12 plants in 3 rows of 4 pots, and only the two pots in the center of this group were used for data collection to avoid edge effects. During the growing period, average temperature and relative humidity were 18.3 ± 1.6°C and 59 ± 11%, and average daily light integral in the greenhouse was 12.5 ± 5.7 $mol \cdot m^{-2} \cdot d^{-1}$ (average ± sd).

Automatic irrigation system

A previously described automated irrigation system (Nemali and van Iersel, 2006) was modified for this study (Fig. 1). Two multiplexers (AM 16/32; Campbell Scientific, Logan, UT) were connected to a datalogger (CR10; Campbell Scientific), and 32 EC-5 capacitance soil moisture sensors (EC-5; Decagon Devices, Pullman, WA) were connected to each multiplexer. The two center pots in each of the 32 experimental units had one soil moisture sensor each, which measured θ every 10 minutes. Also connected to the datalogger where two relay drivers (SDM-CD16 AC/DC controller; Campbell Scientific), which could control power to 16 solenoid valves (X-13551-72; Dayton Electric Company, Niles, IL) each. When the average θ reading of the two moisture sensors in an experimental unit dropped below 0.40 $m^3 \cdot m^{-3}$, the datalogger sent a signal to the relay driver to power the solenoid valve controlling the irrigation of that particular experimental unit for 4 s. Each container received water from a drip stake with a pressure-compensated emitter (Netafim 8 L/H 4-way Multi-Outlet-Dripper assembly; Netafim USA, Fresno, CA). Each irrigation application was recorded by the datalogger and used to calculate the daily amount of water applied to the plants. We had determined previously that a θ of 0.40 $m^3 \cdot m^{-3}$ resulted in good growth of bedding plants with minimal or no leaching (Burnett and van Iersel, 2008; Kim and van Iersel, 2009; Nemali and van Iersel, 2008; van Iersel et al., 2010).

Four days after starting the automated irrigation, θ reached 0.40 $m^3 \cdot m^{-3}$ in all experimental units and data collection occurred for the next 35 d. The cumulative irrigation amount was calculated based on the last 35 d of the experiment.

To control irrigation precisely and prevent leaching, solenoid valves were opened for 4 s (2.4 mL/plant/application), as needed. This provided enough water to maintain stable θs throughout most of the study. However, late in the growing cycle, plant water use during the daytime exceeded the capacity of the irrigation system to supply water with a 4 s irrigation duration. Thus, at 38 DAP (Nov. 17) the irrigation duration was increased to 6 s (3.6 mL/plant/application) to assure that stable θs could be maintained. When more than 14 solenoid valves were opened at the same time, irrigation amount was decreased due to a lack of water pressure (Fig. 3.2), and the irrigation amount was adjusted for the number of opened valves.

Measurements

Temperature and relative humidity (Vaisala HMP50; Vaisala Inc., Woburn, MA) were measured every 10 minutes and PPF (QSO-Sun; Apogee Instruments, Logan, UT) was measured every 4 s. The datalogger also recorded daily average, maximum, and minimum temperature, relative humidity, and light intensity. Vapor pressure deficit was calculated based on the average temperature and relative humidity in a day. The daily light integral (DLI, $mol \cdot m^{-2} \cdot d^{-1}$) was calculated by integrating all PPF data in a day. Environmental conditions are summarized in Fig. 3. 5.

The datalogger recorded average θ every 2 hours and irrigation events every 10 minutes, and the daily irrigation volume for each experimental unit was calculated from these data.

At harvest, the two plants with soil moisture sensors in each experimental unit were harvested, and growth parameters (shoot fresh and dry weight), leaf size of the uppermost fully expanded leaves, and total leaf area (LI-3100; Li-Cor, Lincoln, NE) were measured.

Statistical analysis

The experimental design was a randomized complete block with a split plot. We had 4 blocks with three container sizes, and two cultivars as a split plot. Linear regression was used to determine the irrigation amount based on the number of opened solenoid valves (SigmaPlot; Systat, San Jose, CA). The effects of container size and cultivar on growth parameters were analyzed by three-way analysis of variance (ANOVA) (proc GLM, SAS, SAS Institute, Cary, NC). When ANOVA indicated significance, means were separated using pair-wise comparisons at α=0.05. The effects of container size and cultivar on the cumulative irrigation amount and the maximum and minimum DWU were also analyzed following the same procedure. The relationship between cumulative irrigation amount and shoot dry weight was analyzed using linear regression.

Multiple regression (α=0.05, proc REG, SAS) was used to describe the effects of DAP, container size, and environmental conditions on DWU. Days after planting was used as a proxy for plant size, and DLI, and daily averages of VPD and temperature were the environmental variables used. We developed separate models for each cultivar to account for differences in DWU between two cultivars. The relationship between DWU and other variables (DAP, DLI, temperature, VPD, and container size), and their interaction terms was analyzed with Pearson's correlation (proc CORR, SAS). To account for the effect of container size on DWU, we tested

correlations for container diameter, surface area, and volume. Container diameter had the strongest correlation with DWU and was used for further model development. Two different models were developed for each cultivar. The simpler model included DAP, container size, DLI, VPD, and temperature, while the other model included the same variables as well as DAP interactions with container size, DLI, VPD, and temperature. DWU regression models for each cultivar were determined by backward selection using proc REG in SAS. Partial R^2 were used to quantify the contribution of each variable to the model.

Results and Discussion

Irrigation system performance

The irrigation system constantly maintained θ close to 0.40 $m^3 \cdot m^{-3}$ (Fig. 3.3). Fluctuations in θ were small, due to the low irrigation volume per application. Jones (2004) indicated that frequent irrigation with small amounts can provide precise irrigation, and θ was maintained at 0.401 ± 0.003 (mean ± sd) $m^3 \cdot m^{-3}$ for 'Single Dreams Pink' without any leaching. However, starting on day 26, the θ of 'Prostrate Easy Wave Pink' dropped below 0.40 $m^3 \cdot m^{-3}$ (to 0.36 $m^3 \cdot m^{-3}$) on sunny days (Fig. 3.3B), when the evapotranspiration during the daytime exceeded the ability of the irrigation system to replenish the θ. To increase the amount of water that could be applied, the irrigation time was increased to 6 s at 38 DAP, but θ still dropped below 0.40 $m^3 \cdot m^{-3}$. The θ increased back to 0.40 $m^3 \cdot m^{-3}$ at night, and this decrease in θ during the day therefore did not affect the measured DWU of the plants, nor was the decrease in θ likely large enough to significantly affect plant growth.

Plant growth and water use

At harvest, shoot fresh and dry weights of the plants in 15-cm containers were greater than those in smaller containers (Table 3.1). Although shoot fresh weight was not significantly different between the two cultivars, shoot dry weight of 'Prostrate Easy Wave Pink' was greater than that of 'Single Dreams Pink'. Leaf size and total leaf area of both cultivars were larger in bigger containers. Similar to shoot dry weight, total leaf area was larger in 'Prostrate Easy Wave Pink' than 'Single Dreams Pink'. Less shoot growth (dry mass and leaf area) in smaller containers was likely due to root growth restriction (Latimer, 1991; Ray and Sinclair, 1998; van Iersel, 1997). Because θ was maintained close to 0.40 $m^3 \cdot m^{-3}$ throughout the growing period (*i.e.* no drought stress), the smaller leaf size in smaller containers was also likely due to the greater effect of root restriction.

Over the 46 d growing period, the minimum DWU was similar among container sizes and cultivars (Table 3.1). All plants used the least water (from 4.8 to 13.8 mL/plant) at 7 DAP, which was during the early growth stage and on a day with a low DLI (2.81 $mol \cdot m^{-2} \cdot d^{-1}$). In contrast, the day of maximum DWU differed among the experimental units. However, maximum DWU in all cases occurred during the later part of the study (DAP > 35), on days with high DLI (> 14 $mol \cdot m^{-2} \cdot d^{-1}$), indicating that water use depends on plant size and light level. The maximum DWU amount ranged from 67.9 mL ('Single Dreams Pink' in 10-cm container) to 218 mL ('Prostrate Easy Wave Pink' in 15-cm container). The maximum DWU was greater in larger containers and in 'Prostrate Easy Wave Pink' compared to 'Single Dreams Pink' (Table 3.1). Maximum daily water use was positively correlated with leaf area ($r = 0.9$, $P < 0.0001$).

Due to their size, plants in larger containers required more irrigation, and 'Prostrate Easy Wave Pink' used more water than 'Single Dreams Pink' (Table 3.1). Differences in cumulative irrigation amount were similar to those in shoot dry weight and total leaf area. 'Single Dreams Pink' and 'Prostrate Easy Wave Pink' in 15-cm containers used 60% and 78% more water than plants in 10-cm containers, respectively. In both cultivars, shoot dry weight was positively correlated with cumulative irrigation amount, regardless of container size (Fig. 3.4, $P < 0.001$). The slope of the regression line (2.46 $g \cdot L^{-1}$) is a measure of water use efficiency (*i.e.*, increase in shoot dry weight per liter of water), accounting for both evaporation from the substrate and transpiration from the plants. These results indicate that container size and cultivar had little or no effect on water use efficiency. This water use efficiency is similar to that of *P.* ×*hybrida* 'Velvet Carpet' (2.5 $g \cdot L^{-1}$, van Iersel et al., 2010) and *Pelargonium* ×*hortorum* (2.2 $g \cdot L^{-1}$, Rouphael et al., 2008).

Models with main effects

Models using only main effects (DAP, container size, DLI, VPD, and temperature) explained 89 and 83% of the variation in DWU of 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively (Table 3.3). Temperature was not significant in the 'Prostrate Easy Wave Pink' model. For both cultivars, DAP had the highest partial R^2, followed by container size (Table 3.3), indicating the importance of plant size (sum of partial R^2 of DAP and container size = 0.60 and 0.74 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively). DAP was positively correlated with DWU (Table 3.2, $r = 0.619$ and 0.758 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively), likely because the increasing plant size over time increased DWU.

Container size also was positively correlated with DWU (r = 0.472 and 0.401 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively), because smaller containers limited plant growth. Previous research reported that transpiration decreased in *Zea mays*, *Glycine max* (Ray and Sinclair, 1998) and *Tagetes erecta* seedling (Latimer, 1991) as container size decreased. Baille et al. (1994) used leaf area index (LAI) to account for plant size in their water use models of nine ornamental plants, but frequent measurements of LAI are time consuming and not practical in production greenhouses (Rouphael and Colla, 2004). Digital imaging can be used to monitor plant size and growth (Klassen et al., 2003), but is not yet commonly used in greenhouses. In our study, DAP and container size were correlated with DWU and appeared to be a good proxy for plant size of petunia.

It was previously reported that DLI is the most important environmental factor affecting evapotranspiration (Kim and van Iersel, 2009; Löfkvist et al., 2009; van Iersel et al., 2010). However, DLI had a weak or no correlation with DWU in this study (Table 3.2, r = 0.201 and -0.001, P = 0.028 and 0.989 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively). This low correlation was mainly due to the negative correlation between DLI and DAP (r = -0.36, P < 0.001). Since this study was conducted in fall, day length and DLI generally decreased throughout the experiment (Fig. 3.5). Furthermore, there were only a few overcast days, resulting in relatively little variation in DLI. Despite the lack of a strong correlation between DLI and DWU, DLI was the most important environmental factor in the model, with a partial R^2 of 0.20 and 0.08 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively.

VPD was positively correlated with DWU, but had a partial R^2 of only 0.07 and 0.01 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively. This low partial R^2 is

surprising, since VPD is the main driving force for the diffusion of water from leaves to the air (Taiz and Zeiger, 2006). Previous transpiration models used VPD to explain convective water loss from leaves in greenhouse crops (Baille et al., 1994; Bakker, 1986; Lorenzo, 1998), and VPD plays an important role in the Penman-Monteith equation as well (Allen et al., 1998). Although high VPD can decrease stomatal conductance under drought (Bunce, 2006), transpiration of *Pelargonium zonale* (Montero et al., 2001) and *Cucumis sativus* (Medrano et al., 2005) and VPD were correlated, even when VPD > 3 kPa. In our study, θ was sufficient throughout the experiment and average daily VPD ranged from 0.2 to 1.2 kPa, and was unlikely to restrict stomatal opening.

Temperature was negatively correlated with DWU (Table 3.2), but had little effect on DWU, with a partial R^2 of only 0.01 for 'Single Dreams Pink', and was not significant for 'Prostrate Easy Wave Pink' (Table 3.3). Similar to the decrease in DLI during the experiment, temperature gradually decreased due to a seasonal weather change, thus resulting in lower temperatures during the period that plants had the highest DWU (Fig. 3.5). Further, relatively stable temperatures in the greenhouse may have made it difficult to detect a temperature effect on transpiration.

Although the coefficients of determination of the model were 0.89 and 0.83 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively, the models for both cultivars overestimated DWU when DWU was low and underestimated DWU when it was high (Fig. 3.6A, B).

Model selection with DAP interactions

Including interactions between DAP and other variables improved the model for both cultivars. The DWU model for 'Single Dreams Pink' included the main effects of DAP, DLI, and container size, as well as the DAP interactions with DLI, VPD, container size, and temperature, and explained 93% of the variation in DWU (Fig. 3.7A). For 'Prostrate Easy Wave Pink', DAP, DLI, VPD and DAP interactions with VPD and container size were significant in its DWU model, and explained 91% of the variation (Fig. 3.7B).

Similar to the models with main effects only, DAP and container size, had the largest contribution to the model (Table 3.4, combined partial R^2 for DAP, container size, and DAP × container size = 0.62 and 0.80 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively). DAP interaction with container size was significant in both cultivars, indicating that the effect of container size became more important over time, as root restriction of growth becomes more important. Container size affected growth of *Salvia splendens* as early as 18 DAP and those effects increased over time (van Iersel, 1997).

For both cultivars, DLI was the most important environmental variable in the DWU model (Table 3.4, partial R^2 = 0.20 and 0.08 for 'Single Dreams Pink' and 'Prostrate Easy Wave Pink', respectively), and for 'Single Dreams Pink' the effect of DLI on DWU depended on DAP (DAP × DLI interaction, partial R^2 = 0.05). The main effect of VPD was statistically significant for 'Prostrate Easy Wave Pink', but had little practical importance (partial R^2 = 0.01). There also was a DAP × VPD interaction, indicating that the VPD effect increased later in the study, but this effect was small (partial R^2 = 0.03 for both cultivars). The interactions between DAP and DLI and VPD indicate that these two environmental factors became more important over time, due to

increasing plant size. Water use is likely to depend on the amount of light reaching the plant, and thus on both DLI and leaf surface area. DLI and VPD were positively correlated (r = 0.528, P < 0.001), which caused difficulties in partitioning the effects of these two environmental factors. Modification of individual environmental factors [*e.g.* DLI by shading or VPD using (de)humidification] may be beneficial to minimize multicollinearity among the environmental variables to more precisely determine their individual contributions to DWU.

Temperature effects were not significant for 'Prostrate Easy Wave Pink', while there was a significant DAP × temperature interaction for 'Single Dreams Pink'. As DAP increased, the effect of temperature on DWU of 'Single Dreams Pink' increased, but the overall effect was small (partial R^2 = 0.02). As mentioned above, the effect of temperature may have been small due to the relatively stable greenhouse temperatures. Previous research reported that temperature and VPD were the most important environmental factors affecting plant water use (Mankin et al., 1998; Treder et al., 1997), but recent research suggested that DLI is more important in greenhouse production (Kim and van Iersel, 2009; Löfkvist et al., 2009; van Iersel et al., 2010). Our results support that DLI is most important, but temperature might have played a bigger role if it had varied more.

The models with DAP interaction terms fitted better than the models with main effects only. The models with DAP interaction terms still tended to overestimate DWU when DWU was low and underestimate DWU when it was high (Fig. 3.6C, D), like the models with simple terms. However, the slope of the regression line between the measured and modeled DWU was closer to 1, when interaction terms were included. These models suggest that DAP, container size and

their interaction may be a good proxy for plant size, while DLI and VPD and their interactions with DAP can account for most of the environment-induced variation in DWU of petunia.

Conclusions

We developed a model of petunia DWU, which can be used to quantify how environmental conditions affect DWU. DAP and container size were the most important factors affecting DWU, indicating the importance of plant size. DLI was the most important environmental factor and a regression model including main effects of DAP, container size, environmental conditions, and interactions between DAP and the other variables explained 93 and 91% of the DWU fluctuations. This model may help greenhouse growers get a better idea of the water requirements of their crop and thus to irrigate more efficiently.

Acknowledgements

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Figure 3.1. Schematic diagram of the capacitance soil moisture sensor-controlled automated irrigation system. 1. Substrate, 2. EC-5 soil moisture sensor, 3. Multiplexer, 4. Datalogger, 5. Relay driver, 6. Temperature/relative humidity sensor, 7. Quantum sensor, 8. Solenoid valve, 9. Pressure-compensated emitter, 10. Irrigation stake. Only one container is shown in detail, although 32 experimental units of 12 plants each were used. Two pots per experimental unit had a soil moisture sensor.

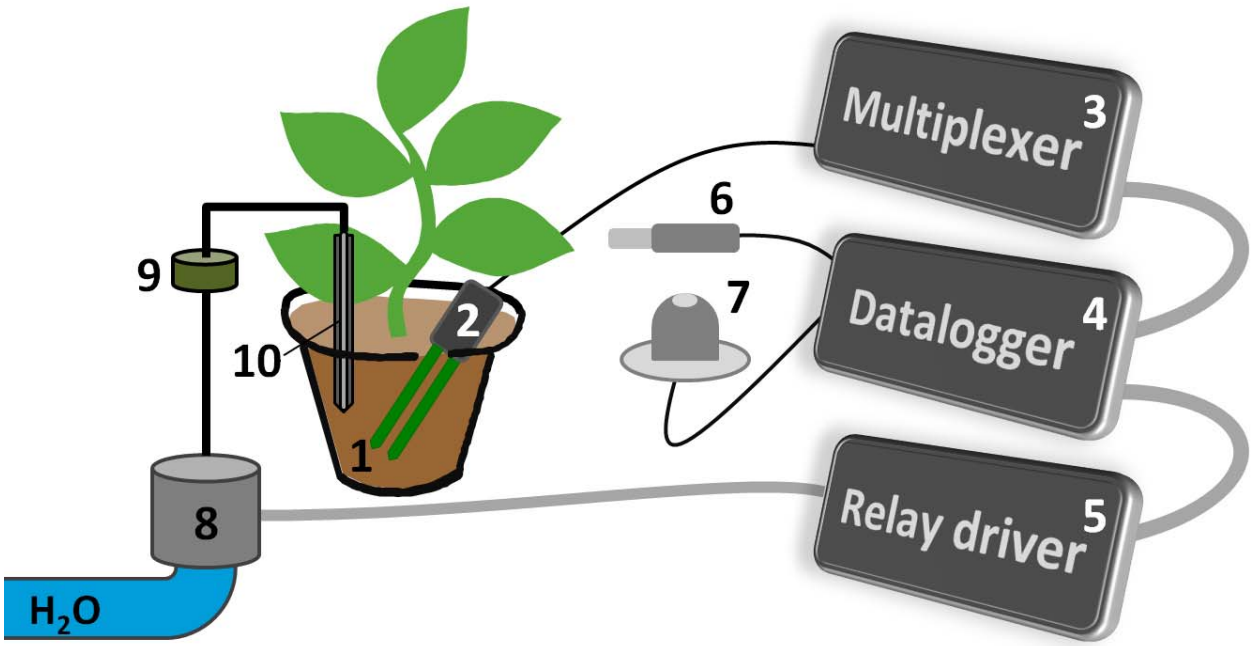
Multiplexer 3
Datalogger 4
Relay driver 5
6
7
9
10
2
1
8
H_2O

Figure 3.2. The Irrigation amount per application (6 sec) decreased as the number of opened solenoid valves exceeded 14, due to a lack of water pressure. To adjust for this decrease, irrigation volume was calculated based on the number of opened solenoid valves.

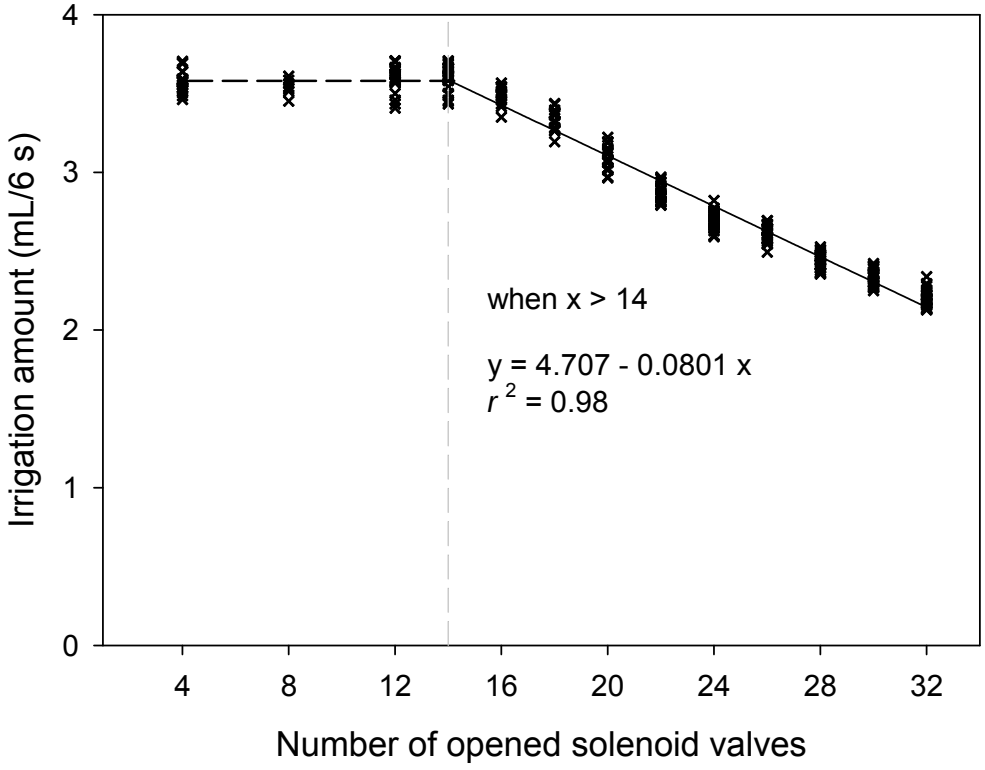
Irrigation amount (mL/6 s)
0
1
2
3
4
4
8
12
16
20
24
28
32
Number of opened solenoid valves
when x > 14
y = 4.707 - 0.0801 x
r 2 = 0.98

Figure 3.3. Average substrate water content (θ, n=4) of two petunia cultivars in 10-, 12.5-, and 15-cm containers, as maintained by a soil moisture sensor-controlled automated irrigation system. Plants were irrigated when θ dropped below 0.40 $m^3 \cdot m^{-3}$. Selected error bars (every five days for data collected at noon) indicate the SE. Error bars that are not visible indicate small SEs.

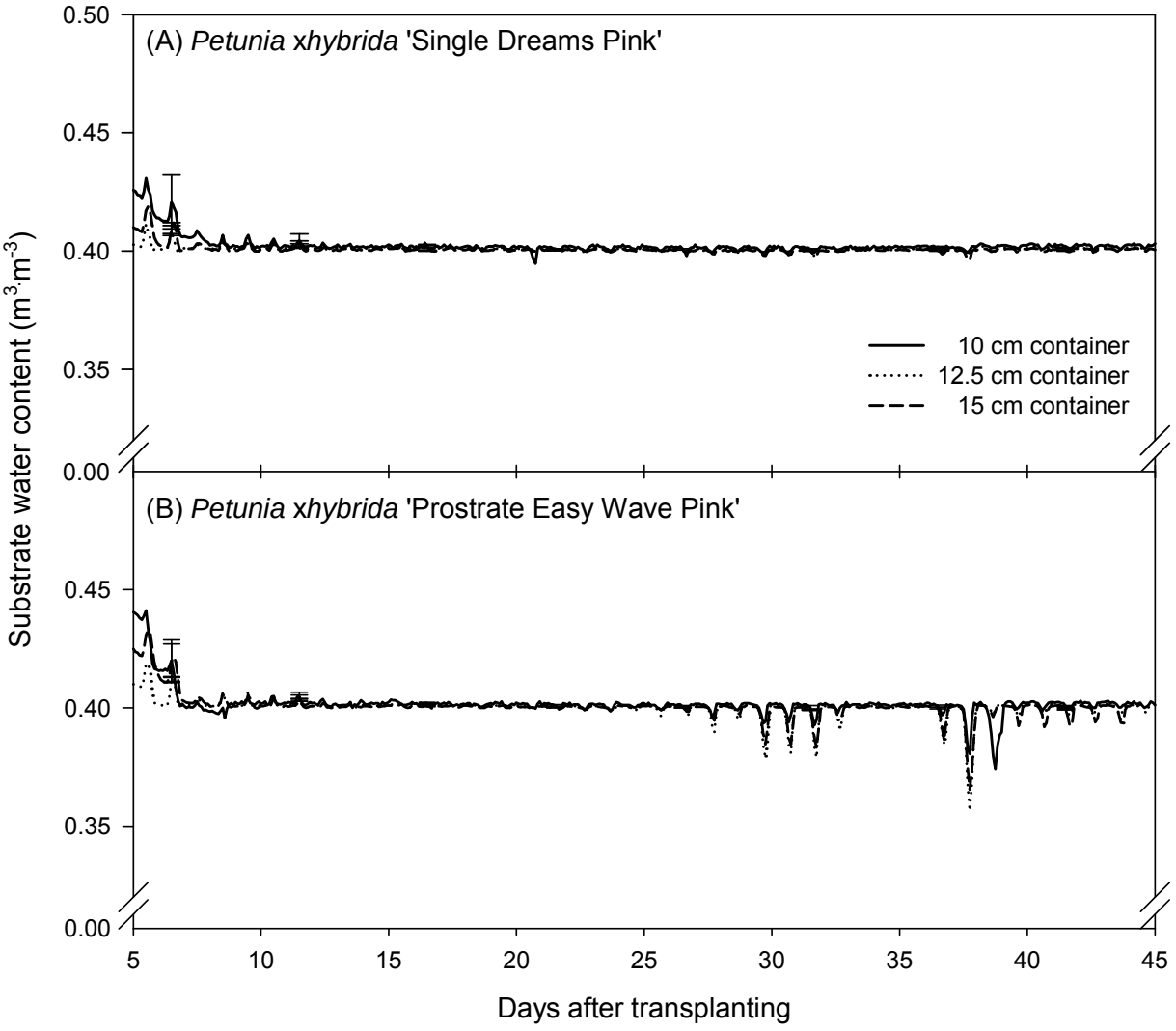
(A) *Petunia* x*hybrida* 'Single Dreams Pink'
(B) *Petunia* x*hybrida* 'Prostrate Easy Wave Pink'
10 cm container
12.5 cm container
15 cm container
Substrate water content ($m^3.m^{-3}$)
Days after transplanting
0.50
0.45
0.40
0.35
0.00
5
10
15
20
25
30
35
40
45

Figure 3.4. Shoot dry weight of two petunia cultivars as a function of cumulative irrigation amount. Solid circles represent *Petunia* x *hybrida* 'Single Dreams Pink' and open circles represent *Petunia* x *hybrida* 'Prostrate Easy Wave Pink'. The regression line was fitted through the data for both cultivars ($P < 0.001$); the slope of the regression line is a measure of water use efficiency.

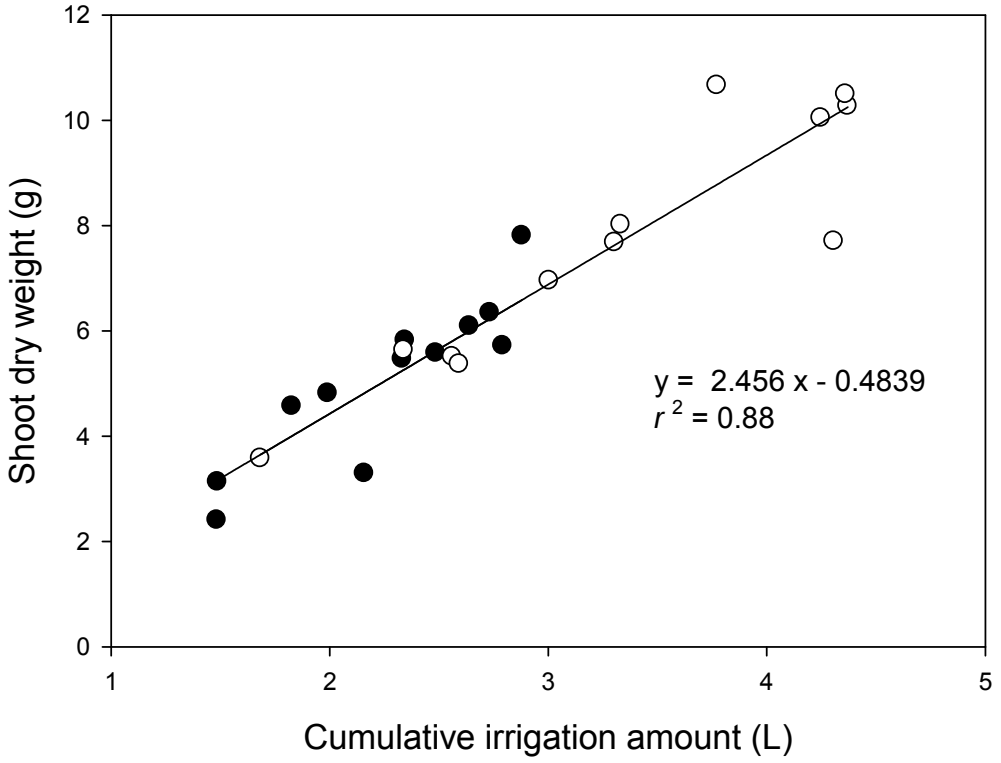
Shoot dry weight (g)
Cumulative irrigation amount (L)
y = 2.456 x - 0.4839
r 2 = 0.88
0
2
4
6
8
10
12
1
2
3
4
5

Figure 3.5. Environmental conditions (DLI, daily light integral; VPD, vapor pressure deficit; and temperature) in the greenhouse during the experiment. VPD and temperature were averaged over the entire day.

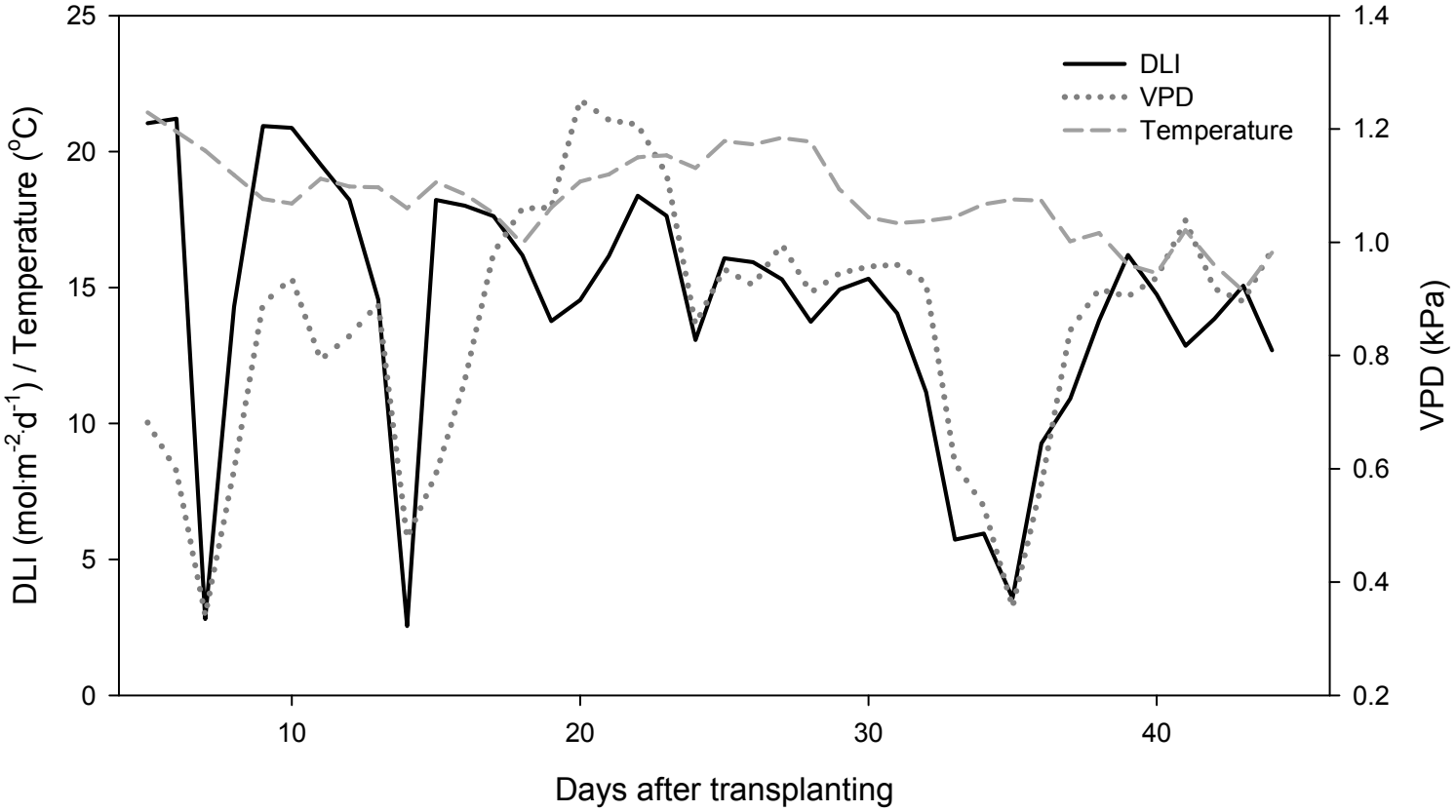
DLI
VPD
Temperature
DLI (mol·m-2·d-1) / Temperature (oC)
VPD (kPa)
Days after transplanting
0
5
10
15
20
25
0.2
0.4
0.6
0.8
1.0
1.2
1.4
10
20
30
40

Figure 3.6. Measured and modeled daily water use (DWU) of two petunia cultivars (*Petunia* x *hybrida* 'Single Dreams Pink' and 'Prostrate Easy Wave Pink') grown in three container sizes (●; 10 cm, ○; 12.5 cm, and ▼; 15 cm container diameter, respectively). Models were developed using multiple regression with the main effects of days after planting (DAP), daily light integral (DLI), vapor pressure deficit (VPD), container size, and temperature (A, B) or with these terms and interactions of the environmental factors and container size with DAP (C, D). See tables 3.3 and 3.4 for details of the models. The solid line indicates the regression line fitted through the data (n=120).

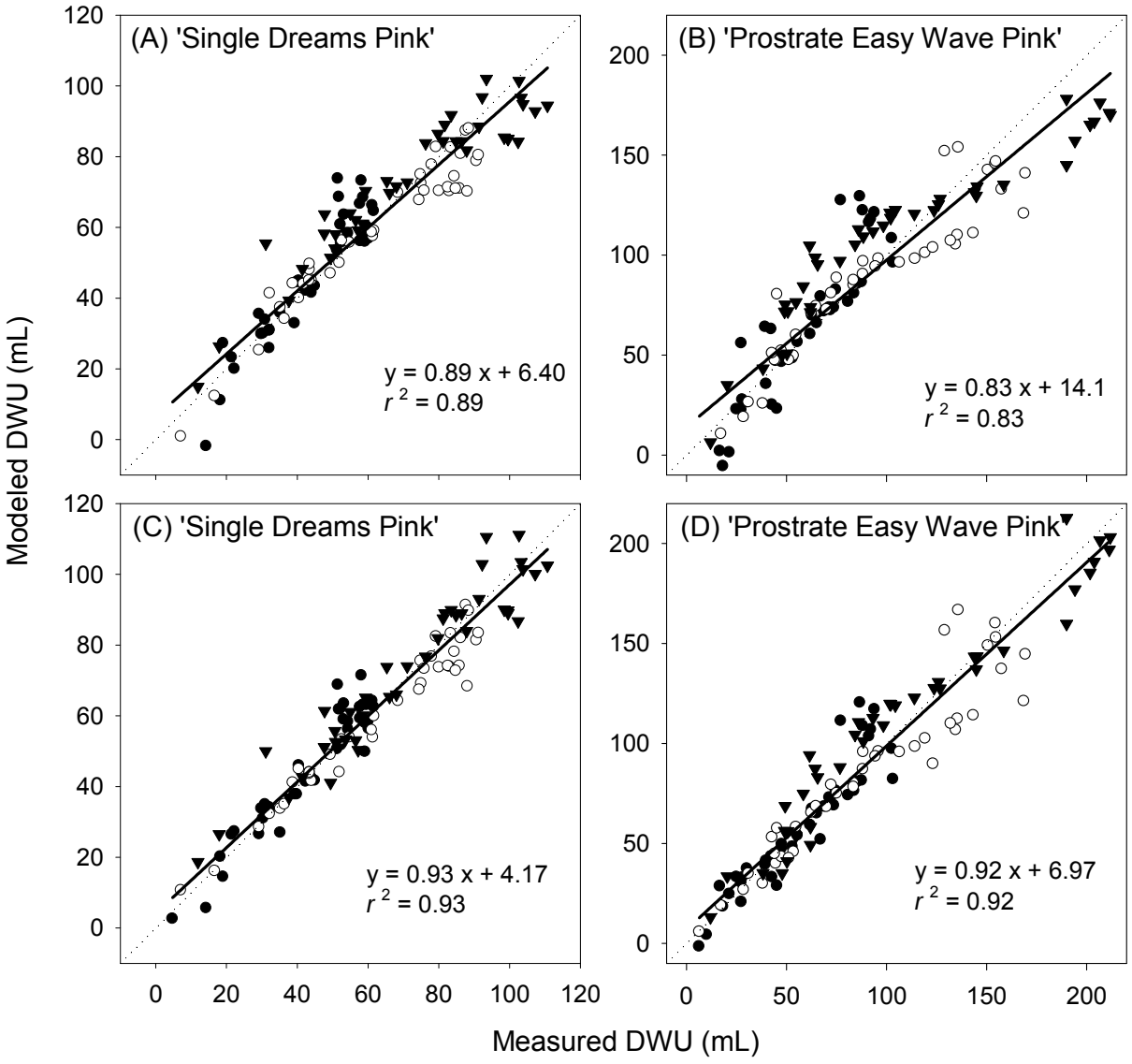
(A) 'Single Dreams Pink'
y = 0.89 x + 6.40
r 2 = 0.89
(B) 'Prostrate Easy Wave Pink'
y = 0.83 x + 14.1
r 2 = 0.83
(C) 'Single Dreams Pink'
y = 0.93 x + 4.17
r 2 = 0.93
(D) 'Prostrate Easy Wave Pink'
y = 0.92 x + 6.97
r 2 = 0.92
Modeled DWU (mL)
Measured DWU (mL)

Figure 3.7. Measured daily water use (DWU) (symbols) and modeled DWU (lines) of two petunia cultivars (*Petunia* x *hybrida* 'Single Dreams Pink' and 'Prostrate Easy Wave Pink') grown in three container sizes. The models were based on the effects of plant age, container size, environmental conditions, and interactions between plant age and environmental conditions. See Table 3.4 for details of the models.

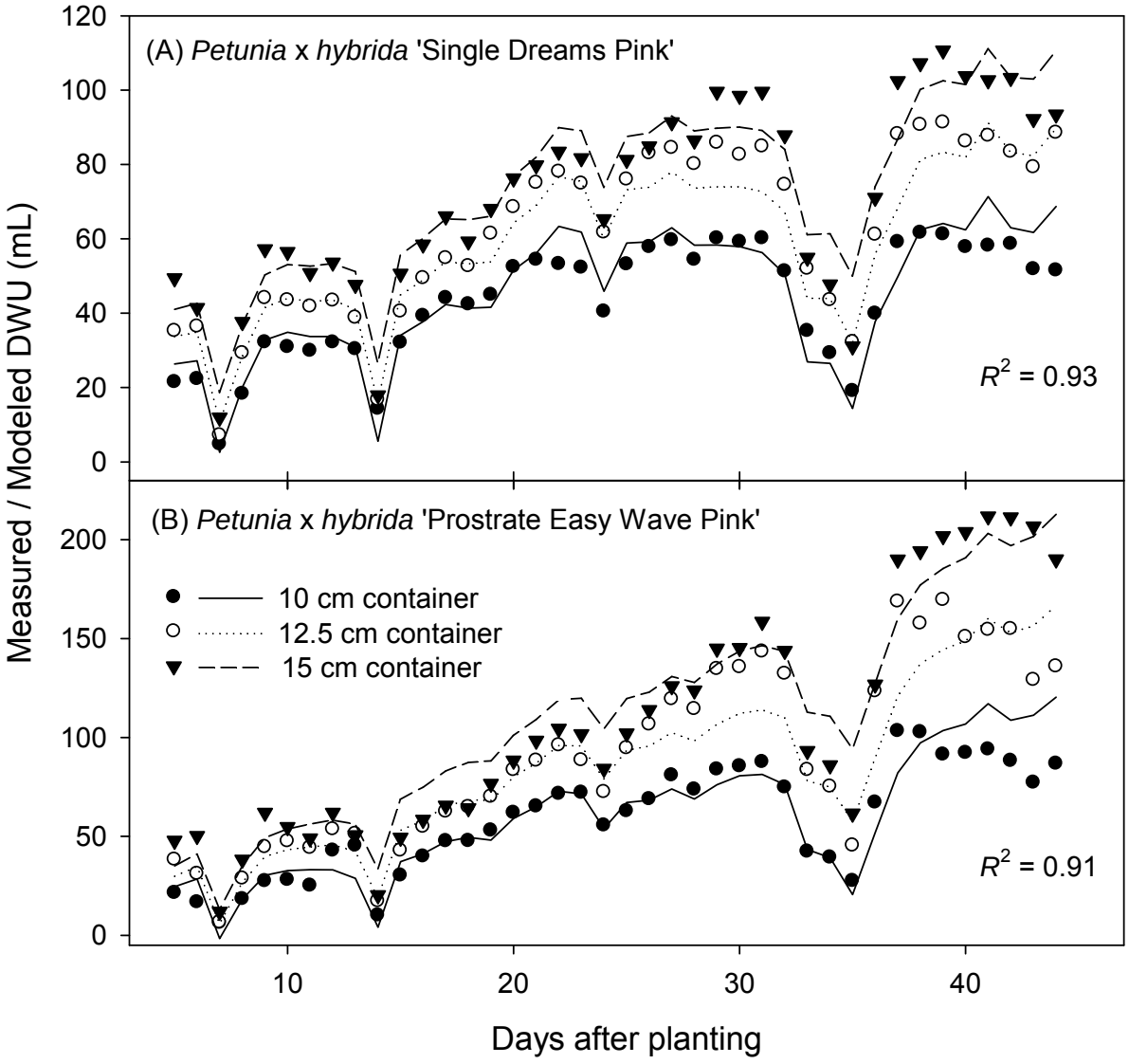
(A) *Petunia* x *hybrida* 'Single Dreams Pink'
R^2 = 0.93
(B) *Petunia* x *hybrida* 'Prostrate Easy Wave Pink'
10 cm container
12.5 cm container
15 cm container
R^2 = 0.91
Measured / Modeled DWU (mL)
Days after planting
0
20
40
60
80
100
120
0
50
100
150
200
10
20
30
40

Table 3.1. Growth parameters and daily water use (DWU) of two *Petunia* × *hybrida* cultivars at harvest. Means followed by the same letter are not significantly different ($P = 0.05$). *** and ** indicate significance at $P < 0.001$ and $P < 0.01$, respectively, and NS represents no significance.

Cultivar and container size	Shoot fresh weight (g)	Shoot dry weight (g)	Leaf size (cm^2)	Total leaf area (cm^2)	Minimum DWU (mL)	Maximum DWU (mL)	Cumulative water use (L)
'Single Dreams Pink'							
10 cm	60 c	3.73 c	11.8 bc	727 d	4.8 b	68 c	1.70 c
12.5 cm	88 b	5.17 bc	14.4 b	1003 cd	7.2 ab	99 c	2.37 bc
15 cm	115 a	6.36 b	17.6 ab	1291 bc	13.1 ab	111 c	2.72 b
'Prostrate Easy Wave Pink'							
10 cm	54 c	5.02 bc	9.8 c	1000 cd	6.6 ab	111 c	2.29 bc
12.5 cm	97 b	8.25 a	14.9 b	1583 b	6.6 ab	165 b	3.60 a
15 cm	127 a	9.71 a	19.7 a	2160 a	13.7 a	218 a	4.08 a
ANOVA							
Container size	***	**	**	***	**	**	***
Cultivar	NS	***	NS	***	NS	***	***
Cultivar × Container size	NS	NS	NS	NS	NS	NS	NS

Table 3.2. Pearson's correlation coefficients for daily water use of two *Petunia* × *hybrida* cultivars (DWU ^{SD}; Daily water use of 'Single Dreams Pink', DWU ^{EW}; Daily water use of 'Prostrate Easy Wave Pink'), days after planting (DAP), container diameter, and environmental variables (DLI, daily light integral; VPD, vapor pressure deficit; temperature). *** and * indicate significance at $P < 0.001$ and $P < 0.05$, respectively, and NS represents no significance, na: not applicable, since container size did not change over the course of the experiment.

Variables	Range	DWU ^{SD}	DWU ^{EW}	Single Variables			
				DAP	DLI	VPD	Temperature
DAP	5 – 44 d	0.619 ***	0.758 ***	-			
DLI	2.6 – 21.2 mol·m^{-2}·d^{-1}	0.201 *	-0.001 NS	-0.357 ***	-		
VPD	0.34 – 1.25 kPa	0.609 ***	0.421 ***	0.193 *	0.528 ***	-	
Temperature	14.9 – 21.4 °C	-0.326 ***	-0.489 ***	-0.642 ***	0.207 *	-0.121 NS	-
Container diameter	10 – 15 cm	0.472 ***	0.401 ***	na	na	na	na

Table 3.3. Regression coefficients (β) and partial R^2 values for the variables in daily water use regression models of *Petunia* × *hybrida* 'Single Dreams Pink' and 'Prostrate Easy Wave Pink' based on the main effects of plant age, container size, and environmental conditions.

Variable (unit)	'Single Dreams Pink'			'Prostrate Easy Wave Pink'		
	β	*P*-value	Partial R^2	β	*P*-value	Partial R^2
Days after planting (d)	1.52	< 0.001	0.38	3.44	< 0.001	0.58
Daily light integral ($mol \cdot m^{-2} \cdot d^{-1}$)	1.25	< 0.001	0.20	2.09	< 0.001	0.08
Vapor pressure deficit (kPa)	40.40	< 0.001	0.07	37.19	0.002	0.01
Temperature (°C)	2.08	0.002	0.01	-	-	-
Container diameter (cm)	5.66	< 0.001	0.22	9.78	< 0.001	0.16
Intercept	-139.60	< 0.001		-183.04	< 0.001	-
Total R^2		0.89			0.83	

Table 3.4. Regression coefficients (β) and partial R^2 values for the variables in daily water use regression models of *Petunia* × *hybrida* 'Single Dreams Pink' and 'Prostrate Easy Wave Pink' based on the main effects of plant age (DAP), container size, and environmental conditions (DLI, daily light integral; VPD, vapor pressure deficit; temperature), as well as their interactions with plant age.

Variable (unit)	'Single Dreams Pink'			'Prostrate Easy Wave Pink'		
	β	*P-value*	Partial R^2	*β*	*P-value*	Partial R^2
Main effects						
DAP (d)	-3.48	< 0.001	0.38	-4.75	< 0.001	0.58
DLI ($mol \cdot m^{-2} \cdot d^{-1}$)	1.03	< 0.001	0.20	1.94	< 0.001	0.08
VPD (kPa)	-	-	-	-41.24	0.008	0.01
Temperature (°C)	-	-	-	-	-	-
Container size (cm)	2.24	0.002	0.22	-	-	-
DAP Interactions						
DAP × DLI	0.03	0.041	0.05	-	-	-
DAP × VPD	1.31	< 0.001	0.03	3.65	< 0.001	0.03
DAP × Temperature	0.11	< 0.001	0.02	-	-	-
DAP × Container size	0.14	< 0.001	0.02	0.42	< 0.001	0.22
Intercept	-26.77	0.008	-	2.16	0.860	-
Total R^2		0.93			0.91	

CHAPTER 4

SLOWLY IMPOSED DROUGHT STRESS INCREASES PHOTOSYNTHETIC ACCLIMATION OF VINCA [1]

[1] Kim, J. and M.W. van Iersel. Submitted to *Physiologia plantarum*.

Abstract

Our understanding of plant responses to drought has improved over the decades. However, the importance of the rate of drought imposition on the response is still poorly understood. To test the importance of the rate at which drought stress develops, whole-plant photosynthesis (P_{net}), respiration (R_{dark}), daily carbon gain (DCG), daily evapotranspiration (DET), and water use efficiency (WUE) of vinca (*Catharanthus roseus*), subjected to different drought imposition rates, were investigated. We controlled the rate at which the substrate dried out with an automated irrigation system that allowed pot weight to decrease gradually throughout the drying period. Fast, intermediate, and slow drying treatments reached their final pot weight [500 g, substrate water content (θ) ≈ 0.10 $m^3 \cdot m^{-3}$] after 3.1, 6.6, and 10 days, respectively. Although all drying treatments decreased P_{net} and R_{dark}, slow drying reduced P_{net} and R_{dark} less than fast drying. At a θ < 0.10 $m^3 \cdot m^{-3}$, DCG and DET in the slow drying treatment were reduced by ≈ 50%, whereas those in the fast drying treatment were reduced by 85% and 70% at a θ of 0.16 $m^3 \cdot m^{-3}$, respectively. Plants exposed to slow drought imposition maintained a high WUE, even at θ < 0.10 $m^3 \cdot m^{-3}$. Overall, physiological responses to low θ were less severe in plants subjected to slow drying than in plants subjected to fast drying, even though the final θ was lower for plants exposed to slow drying. This suggests that the rate at which drought stress develops has important implications for the level of acclimation that occurs.

Index words: drying rate, substrate water content, respiration, transpiration, water use efficiency

Abbreviations – ABA, abscisic acid; ATP, adenosine triphosphate; DCG, daily carbon gain; DET, daily evapotranspiration; P_{net}, net photosynthesis; PSII, photosystem II; R_{dark}, dark respiration; RuBP, Ribulose-1, 5-biphosphate; WUE, water use efficiency; θ, volumetric substrate water content; Ψ_{leaf}, leaf water potential; Ψ_{π}, leaf osmotic potential; Ψ_{P}, leaf turgor potential

Introduction

Drought is the most common stress that limits yield in agricultural production, since water plays pivotal roles in plants, providing turgor for cell elongation and functioning as a metabolic reactant (Boyer 1982, Hsiao 1973). Understanding plant responses to drought has become even more of a priority, now that climate change is expected to reduce water availability in many areas (IPCC 2007, Jury and Vaux 2005). Because of the ecological and agricultural importance of drought, many researchers have investigated and made progress in understanding plant responses to drought (*e.g.*, Chaves et al. 2003, Jones 2007).

Although many studies have investigated plant responses to drought, the severity of these stresses is not often accurately quantified or controlled (Jones 2007). Many previous studies imposed drought stress by withholding irrigation for a certain amount of time or applying osmotica, such as polyethylene glycol, to simulate drought stress. However, simply withholding irrigation may result in an artificially fast development of drought, especially for potted plants with a small root zone. Unfortunately, the rate of drought stress development is rarely controlled, although plant responses to drought may depend on the drought imposition process. Previous research indicated that slowly imposed drought stress may induce better acclimation than rapidly imposed stress (Bray 1997, Flexas et al. 2006, Jones 2007, Kozlowski and Pallardy 2002, McDonald and Davies 1996), but the importance of the rate of drought stress development on the actual physiological acclimation response has not been quantified.

In general, drought stress inhibits cell elongation due to turgor pressure loss, and the resulting shoot growth inhibition is the earliest drought response (Boyer 1970, Hsiao 1973, van Volkenburgh 1999). Stomatal closure, minimizing transpirational water loss, is also a common

response of plants under drought. Stomatal regulation is either through a chemical signal (abscisic acid), a hydraulic signal from roots sensing low soil water potential, or both (Christmann et al. 2007, Schachtman and Goodger 2008). As a consequence of stomatal closure, reduced transpiration alleviates plant water loss, but also decreases the conductance for CO_2 diffusion from the air into the leaf, reducing photosynthetic carbon assimilation. Decreases in stomatal conductance and mesophyll conductance are regarded as the most limiting factor in carbon assimilation under drought (Flexas et al. 2006, Lawlor and Cornic 2002). Critical damage to the biochemical capacity of the photosynthetic apparatus generally occurs only under severe drought stress, (Flexas et al. 2006, Lawlor and Cornic 2002).

However, these drought stress responses depend on the species and genotype, the duration and severity of the stress, the age and stage of plant development, and the stress history of the plants (Bray 1997, Hsiao 1973, McDonald and Davies 1996). Plants reduce drought sensitivity or adjust biochemical processes in order to enhance stress tolerance (Lambers et al. 2008, Yordanov et al. 2000). Plants can adjust their water relations and photosynthesis by modifying their physiology, morphology, and gene expression, leading to acclimation to unfavorable conditions (Flexas et al. 2006). For instance, mild drought stress increases the WUE of plants and increases drought tolerance through osmotic adjustment or by altering the root-shoot allocation (Earl 2002, Kozlowski and Pallardy 2002, Lawlor and Cornic 2002, López et al. 2009, Yordanov et al. 2000). It has been suggested that exposing plants to a slow drying rate lessens the resulting physiological impairment, because of better acclimation (Bray et al. 1997, Flexas et al. 2006). A rapid development of drought is especially likely when plants are grown in containers, since the limited soil/substrate volume limits the amount of

available water. Growing plants in containers is common in the greenhouse and nursery industry, as well as in many research applications. In addition, soilless substrates are commonly used for container-grown plants, and the moisture retention curves of soilless substrates differ from those of mineral soils. Much of the water in soilless substrates is held at a low tension, but water becomes rapidly less available as θ approaches a substrate-specific threshold (Wallach 2008). However, despite its potential importance, there is little quantitative information on the effect of the drying imposition rate on physiological responses and drought acclimation.

A previous study looking at different rates of drought stress development found that rapid drying, imposed by removing plants from the soil, provides insufficient time for resurrection plants (*Craterostigma wilmsii*, *Xerophyta humilis*, and *Myrothamnus flabellifolius*) to activate protective mechanisms. These resurrection plants failed to survive rapid drying, while all species survived when exposed to slow drying (Farrant et al. 1999). However, ressurection plants have unique drought tolerance mechanisms and may have different physiological responses than other plants. Recently, López et al. (2009) reported that slow drought imposition enhanced osmotic adjustment of seedlings of *Pinus canariensis*. In this experiment, researchers added polyethylene glycol to hydroponic solutions to control drying rates by controlling the osmotic potential of the nutrient solution. However, plants may have different physiological responses to drought stress in soil or substrate compared to osmotic stress in hydroponics.

Measuring water potential of plants or substrate/soil water content can aid in quantifying the severity of drought (Jones 2007, Nemali and van Iersel 2008). Automated measurement and control of substrate/soil water content allows for control of both the

severity of the drought stress and the rate of drought stress development. Such control can be achieved based on pot weight (Earl 2003), or soil moisture sensors, which measure θ (Nemali and van Iersel 2006).

The objective of this study was to investigate the effect of different drying rates on whole-plant responses to drought. We combined a load cell system and a whole-plant gas exchange system to improve our understanding of drought responses of the annual plant, vinca (*Catharanthus roseus*). Specifically, we investigated how the rate of drought stress imposition affects plant growth, whole-plant CO_2 exchange, DET, and WUE. This may provide a better understanding of how different rates of drought stress imposition affect whole-plant physiology and acclimation to drought.

Materials and Methods

Plant material

Seedlings of vinca (*Catharanthus roseus* L. 'Sun Devil Extreme Purple') in 288-cell plug trays were transplanted into 15 cm, round, plastic containers (1.68 L) filled with a soilless substrate (Fafard 2P; 60% peat and 40% perlite; Fafard, Anderson, SC, USA). Controlled-release fertilizer (14.0N-6.2P-11.6K, Osmocote 14-14-14; The Scotts Co., Marysville, OH, USA) was incorporated at a rate of 7.7 $g \cdot L^{-1}$ (12.9 g per pot). Plants were grown in a glass-covered greenhouse at the University of Georgia for a month (June 22 to July 21, 2009), and were irrigated daily with tap water using a subirrigation system. At the start of the gas exchange measurements, eight plants of similar size were selected. Each plant was placed in a separate gas exchange chamber (Fig. 4.1), and four gas exchange chambers were placed together inside

a growth chamber. Growth chamber conditions were a photosynthetic photon flux of 480 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the canopy level, a 14-h photoperiod, and a constant temperature of 25°C was maintained inside the gas exchange chambers using resistance heaters. Air inside each gas exchange chamber was mixed using fans to assure uniformity within the chambers.

Load cell-based irrigation system

Each pot was placed on a load cell (LSP-2; Transducer Techniques, Temecula, CA, USA) to measure the pot weight inside each gas exchange chamber (Fig. 4.1). A soil moisture sensor (10HS; Decagon Devices, Pullman, WA, USA) was inserted into the substrate to measure θ. The load cells and soil moisture sensors were connected to a datalogger (CR10; Campbell Scientific, Logan, UT, USA) via a multiplexer (AM16/32; Campbell Scientific). Each load cell was powered using the regulated 5V output from the datalogger and calibrated individually by weighing six known weights ranging from 0 to 1.3 kg, and fitting a regression line (r^2 = 1.00 for all load cells). Load cells measured the weight of the eight pots and soil moisture sensors measured θ every 30 seconds. Pot weight and θ data were averaged and recorded in the datalogger every 10 minutes. The soil moisture sensors were calibrated specifically for the substrate [θ ($m^3 \cdot m^{-3}$) = 0.959 × sensor output (V) - 0.3336, r^2 = 0.99]. When the weight of a particular pot dropped below a specific set point, the datalogger used a relay driver (SDM-CD16 AC/DC controller; Campbell Scientific) to open a solenoid valve (57101; Orbit, Bountiful, UT, USA) controlling the irrigation of that pot. Solenoid valves were powered by a 24 VAC transformer (ACME TA-2-81141; ACME electric, Lumberton, NC, USA), whenever the relay driver activated the relay controlling a specific valve. Each pot received water for 10 s from a circular drip tube (dribble

ring, Dramm, Manitowoc, WI, USA) connected to a pressure-compensated emitter (8 L/h, Netafim USA, Fresno, CA, USA) as needed. Each irrigation cycle applied approximately 22 mL of water. All eight plants were maintained at a pot weight of 950 g ($\theta \approx 0.40 – 0.45$ $m^3 \cdot m^{-3}$) throughout a three week period during which the plants acclimated to the growth chamber conditions.

Drought imposition rate

After the acclimation period, plants were exposed to three different drying rates, with drying periods of 10, 7, and 3 d (slow, intermediate, and fast drying), during which pot weight decreased from 950 to 500 g, corresponding to a θ of approximately 0.10 $m^3 \cdot m^{-3}$. When the pot weight reached 500 g, plants were watered to maintain this weight. This weight was chosen because it resulted in a severe, but sub-lethal drought stress. To control the slow and intermediate drying rate, the datalogger was programmed to gradually decrease the weight at which the pots were irrigated (0.318 and 0.477 g every 10 min for the slow and intermediate drying treatments, respectively), resulting in a controlled rate of drought stress development. The plants in the fast drying treatment were no longer irrigated after the initiation of the drying treatment until the pot weight reached 500 g. The pot weight of control plants was maintained at 950 g throughout the experiment.

Whole-Plant Gas Exchange Measurements

Throughout the drying period, each plant was kept inside an acrylic chamber to continuously measure whole-plant carbon exchange. The gas exchange system (van Iersel and

Bugbee 2000) consisted of ten chambers (0.32 × 0.5 × 0.6 m^3), eight of which were placed in one of two growth chambers (E-15, PGR15; Conviron, Winnipeg, Canada). CO_2 exchange of eight plants within each acrylic chamber was measured continuously and recorded using a datalogger (CR10T; Campbell Scientific). Air flow through each gas exchange chamber (≈ 17 $mmol \cdot s^{-1}$) was measured with mass flow meters (HFM200; Teledyne Hasting Inst., Hampton, VA, USA) and the difference in CO_2 concentration between the air entering and exiting the gas exchange chambers was measured with an infrared gas analyzer in differential mode (LI-6262; Li-Cor, Lincoln, NE, USA). Whole plant CO_2 exchange rates ($\mu mol \cdot s^{-1}$) were calculated as the product of mass flow ($mol \cdot s^{-1}$) and the difference between the CO_2 concentration of the air entering and exiting chamber ($\mu mol \cdot mol^{-1}$). Two empty gas exchange chambers were placed outside of the growth chambers and were measured to determine the zero drift of the differential CO_2 analyzer. Gas exchange data were corrected for this zero drift by subtracting the CO_2 exchange rate of the empty chambers from that of the chambers with plants in them. Each chamber was measured for 30 s, once every 10 min. To allow plants to adjust to light or darkness, P_{net} and R_{dark} data from the first 40 min of each light or dark period were not used for data analysis.

Daily Carbon Gain, Daily Evapotranspiration, and Daily Water Use Efficiency

Daily carbon gain (DCG, an indicator of growth rate) and daily evapotranspiration (DET) were calculated by integrating the CO_2 exchange rate and evapotranspiration over 24 h periods. Evapotranspiration was calculated from the pot weight change over 24 hours after correcting for the pot weight increase by irrigation events. WUE was calculated for each day as DCG/DET.

Normalized values of DCG, DET, and WUE were calculated using the data from the day before the start of the slow drying treatment as 100%. This normalization helped to account for differences in plant size.

Harvest measurements

When all drying treatments reached the target final pot weight of 500 g, physiological parameters of the plants were measured. Leaf water potential (Ψ_{leaf}) was measured using leaf cutter thermocouple psychrometers (Model 76; J.R.D. Merrill Specialty Equipment, Logan, UT, USA). Leaf discs were sampled at 15 h into the light period, after which the psychrometers were equilibrated in a water bath at 25.0°C for 4 h, and then Ψ_{leaf} was measured using a datalogger (CR7X; Campbell Scientific). After Ψ_{leaf} was measured, the psychrometers were placed in a freezer overnight to disrupt the cell membranes, and osmotic potential (Ψ_{π}) was measured using the same procedure as for Ψ_{leaf}. Turgor potential (Ψ_P) was calculated as $\Psi_{leaf} - \Psi_{\pi}$. The maximum quantum yield of PSII (F_v/F_m) was measured using a chlorophyll fluorometer (Mini-PAM; Heinz Walz GmbH, Effeltrich, Germany) after 30 min dark adaptation. Total leaf area and the size of ten fully expanded leaves from the upper part of the canopy of each plant were measured using a leaf area meter (LI-3100; Li-Cor) and shoot fresh and dry weights were measured. Shoot water content was calculated as (shoot fresh weight – shoot dry weight) / shoot fresh weight.

Experimental design and analysis

The experimental design was a randomized complete block with four treatments and two blocks. Each growth chamber was a block, and each growth chamber had one of each treatment. The effect of treatments on growth parameters were analyzed by ANOVA followed by Fisher's least significant difference (LSD, $P = 0.05$) procedure using SAS (SAS Institute, Cary, NC, USA). P_{net}, R_{dark}, DCG, DET, and WUE were normalized so that the regression curves reached 100% at a θ of 0.35 $m^3 \cdot m^{-3}$. The responses of P_{net}, R_{dark}, DCG, DET, and WUE to decreasing θ during the drying period were analyzed by fitting quadratic curves to the data and then testing for homogeneity of the slopes of the regression lines. Differences in slopes between treatments indicate that plants in those treatments responded differently to changes in θ. For the analysis of P_{net} and R_{dark}, we used the data from the time that the drying treatment of that particular treatment was started, while all data from the start of the slow drying treatment were used for analysis of DCG, DET, and WUE. This was necessary to assure that we had enough data in the fast drying treatment to fit quadratic curves.

Results and Discussion

Pot weight and θ *changes*

The load cell-based irrigation system successfully controlled pot weights within a narrow range (± 2 g) and was able to impose the different drying rates (Fig. 4.2A). However, there were differences in θ among pots of the same weight (Fig. 4.2B). Shoot fresh weight in the slow drying treatment was greater (≈ 40 g) than that in the fast drying treatment (Fig. 4.3A), and this greater shoot fresh weight accounted for more of the total pot weight (container + plant +

substrate + substrate water) than in the other treatments. Since we controlled total pot weight, rather than θ, the θ in the drought stress treatments at harvest was negatively correlated with shoot fresh weight (r = -0.87, P = 0.026). At the final pot weight of 500 g, the slow drying treatment had a θ of 0.08 $m^3 \cdot m^{-3}$, while the fast drying treatment had a θ of 0.14 $m^3 \cdot m^{-3}$ (Fig. 4.3F). The effect of shoot fresh weight can also be seen in the control treatment. Despite the constant pot weight of 950 g, the θ of control pots decreased from 0.43 to 0.36 $m^3 \cdot m^{-3}$ throughout the experiment, due to a gradual increase in plant weight. The effect of plant fresh weight can be avoided by controlling θ rather than weight. However, soil moisture sensors do not measure all the substrate in the pot, and θ measurements are thus sensitive to sensor placement. Controlling weight has the advantage that it intergrates the entire substrate volume.

Physiological changes at harvest

Despite lower shoot fresh weight in the intermediate and fast drying treatments, shoot dry weight did not differ significantly among the treatments (Fig. 4.3B). The short-term drought stress also did not significantly affect the leaf size of vinca (Fig. 4.3C), although reduction in leaf elongation is a common response to drought (Boyer 1970). However, total leaf area in the intermediate and fast drying treatments was smaller than that of the control (P < 0.05, Fig. 4.3D). In contrast, total leaf area in the slow drying treatment was similar to that of the control. This indicates that slowly imposed drought allows plants to maintain leaf area development.

Although shoot water content was decreased in all drought stress treatments (Fig. 4.3E), only the fast drying treatment significantly reduced Ψ_{leaf} and Ψ_{π} compared to the control; Ψ_{π} in the fast drying treatment also was lower than that in the slow drying treatment (Fig. 4.4). The

slow drying treatment tended to have a higher Ψ_{leaf} than the fast drying treatment (-1.06 MPa vs. -1.64 MPa, $P = 0.067$), despite lower θ (0.08 $m^3 \cdot m^{-3}$ vs. 0.14 $m^3 \cdot m^{-3}$). Nemali and van Iersel (2008) reported that Ψ_{leaf} was lower at a θ of 0.09 $m^3 \cdot m^{-3}$ than at a θ of 0.15 $m^3 \cdot m^{-3}$ or higher, but we only saw a significant decrease in Ψ_{leaf} in the fast drying treatment, despite the fact that θ was higher than in the slow and intermediate drying cycles. This suggests that slowly imposed drought alleviates the large reduction in Ψ_{leaf} compared to rapid drought imposition. Although visual observations (wilting) suggested that Ψ_P was lowest in the fast drying treatment, there was no significant difference in Ψ_P among the treatments (Fig. 4.4).

At harvest, the maximum quantum yield of PSII (F_v/F_m) averaged 0.79 and was similar among treatments. Similarly, previous studies showed that drought stress had little effect on F_v/F_m of other herbaceous ornamental plants (*Lantana camara* L., *Lobelia cardinalis* L., *Salvia farinacea* Benth., *Scaevola aemula* R. Br., and *Pelargonium* × *hortorum* L.) (Bukhov and Carpentier 2004, Sanchez-Blanco et al. 2009, Starman and Lombardini 2006). Although a θ around 0.1 $m^3 \cdot m^{-3}$ can be regarded as severe drought, the F_v/F_m data do not show any damage to PSII, regardless of the rate of drying imposition. This lack of a response of F_v/F_m is consistent with the suggestion that chlorophyll fluorescence is not as good an indicator of drought stress, as it is of other environmental stresses (Fracheboud and Leipner 2003).

Whole-plant CO_2 exchange rate during the drying period

Similar to previous research that showed that plants, including vinca, decrease photosynthesis under drought (Chaves et al. 2003, Nemali and van Iersel 2008, Niu et al. 2006), P_{net} decreased in all drying treatments throughout the drying period. As hypothesized, the rate

of reduction in P_{net} depended on the drying rate (Fig. 4.5). Plants in the slow and intermediate drying treatments had a 20% reduction in P_{net} at seven and five days after drying initiation, respectively, whereas the fast drying reduced P_{net} by 20% within a day. At harvest, P_{net} of control plants was 24% higher than at the start of the study, likely due to increased plant size. Slow, intermediate, and fast drying treatments reduced P_{net} at harvest by 42, 58, 61%, respectively. It is noteworthy that the reduction in P_{net} in the fast drying treatment was greatest, even though this treatment had the highest θ at the end of the study. The slow drying treatment had the least reduction in P_{net}, despite having the lowest θ (Fig. 4.6A).

The analysis of the relationship between θ and P_{net} showed that the reduction in P_{net} differed among the three treatments ($P < 0.0001$); a fast drying rate resulted in a much steeper decrease in P_{net} as θ decreased (Fig. 4.6A). For example, the fast drying treatment resulted in a 47% reduction in P_{net} at θ of 0.20 $m^3 \cdot m^{-3}$ and by 69% at the final θ of 0.15 $m^3 \cdot m^{-3}$, whereas slow drying reduced P_{net} by only 12% and 19% when θ reached 0.20 and 0.15 $m^3 \cdot m^{-3}$. Even when the slow drying treatment reached a final θ of 0.09 $m^3 \cdot m^{-3}$, the plants still had a photosynthetic rate of 69% of the pre-drought rate (Fig. 4.6A). This reduction in photosynthesis under drought results from a low CO_2 concentration inside the chloroplast due to decreased stomatal conductance and/or mesophyll conductance, while severe drought also induces metabolic inhibition of photosynthesis by decreasing ATP and RuBP synthesis and Rubisco activity (Flexas et al. 2006, Galmés et al. 2011, Lawlor and Cornic 2002). Previous research suggested that the reduction in photosynthesis may be alleviated by gradual development of drought stress, allowing for acclimation (Bray 1997, Flexas et al. 2006, Jones 2007). Our results support the

hypothesis that slow imposition of drought stress allows for more photosynthetic acclimation than rapidly developing drought.

Whole-plant R_{dark} of vinca under drought also decreased as θ decreased (*i.e.* respiration rates closer to zero; Figs. 4.5 and 4.6B), but the reduction was less severe than that in P_{net}. The reduction in R_{dark} in response to decreasing θ depended on the drying rate, with significant differences among all three treatments ($P < 0.0001$). When θ reached 0.16 $m^3 \cdot m^{-3}$, fast drying had reduced R_{dark} by 20%, compared to 12% in the slow and intermediate drying treatments (Fig. 4.6B). Root and whole-plant respiration generally decrease under severe water stress, but results of drought on leaf respiration are not consistent among previous studies (see review by Atkin and Macherel 2009). This lack of consistentency may depend on the duration, severity, and the rate of drought imposition. In our study, R_{dark} decreased in all treatments as θ decreased, but the reduction in R_{dark} was alleviated by slowly imposed drought as compared to rapidly imposed drought, similar to our findings for P_{net}.

DCG, DET, WUE

Throughout the experiment, DCG and DET of control plants gradually increased (Figs. 4.7A and 7B). In all drying treatments, DCG and DET decreased after drying was initiated; DCG and DET decreased at a more rapid rate in the fast drying treatment compared to the slow and intermediate drying treatments. DCG and DET of plants in the slow drying treatment began to decrease seven days after the initiation of drought (Fig. 4.7, $\theta \approx 0.18$ $m^3 \cdot m^{-3}$). In contrast, DCG and DET decreased within two days after the start of the fast drying treatment. This rapid decrease in DCG and DET may be due to rapid stomatal closure, which would reduce CO_2

diffusion into and transpiration from the leaves (Kim and van Iersel 2011, Niu et al. 2006). The more gradual decrease of DCG and DET in the slow and intermediate drying treatments suggests a more gradual decrease in stomatal conductance (Fig. 4.7).

Both DCG and DET in all drying treatments decreased as θ decreased (Figs. 4.8A and 4.8B). DCG is the integration of P_{net} and R_{dark} over a day and is an indicator of plant growth rate (van Iersel and Bugbee 2000). Therefore, this reduction in DCG indicates that all drying treatments reduced plant growth rate. As with P_{net} and R_{dark}, the effect of θ on DCG depended on the rate of drought imposition ($P < 0.0001$). Slowly imposed drought decreased DCG more gradually than rapidly imposed drought. At a θ of 0.18 $m^3 \cdot m^{-3}$, the relative DCG in the fast drying treatment decreased to 30%, compared to 78% in the slow drying treatment. The slow drying treatment reduced DCG by only 46%, even when θ reached 0.09 $m^3 \cdot m^{-3}$. In contrast, the DCG in the fast drying treatment was reduced by 50%, even though θ was still 0.21 $m^3 \cdot m^{-3}$ (Fig. 4.8A).

Similar to the DCG response, the decrease in DET with decreasing θ depended on the drying rate ($P < 0.0001$, Fig. 4.8B). The fast drying treatment decreased DET by 50% at θ of 0.20 $m^3 \cdot m^{-3}$, whereas the slow and intermediate drying treatments decreased DET by 50% at θ of 0.09 and 0.14 $m^3 \cdot m^{-3}$. When θ reached 0.15 $m^3 \cdot m^{-3}$, the relative DET in the fast drying treatment was 24% compared to 69% in the slow drying treatment. Stomatal closure, decreasing transpiration and water loss, is a well known drought response (Hsiao 1973). Although all drying treatments decreased DET, the slowly imposed drought likely alleviated stomatal closure at low θ, thus resulting in a higher transpiration rate than the rapid drying treatment.

As expected, WUE of control plants did not change much during the experiment (Fig. 4.7C). Changes in WUE also depended on the rate of drought imposition ($P < 0.0001$, Fig. 4.8C). The WUE in the intermediate and fast drying treatments decreased by more than 20% at a θ of 0.18 and 0.23 $m^3 \cdot m^{-3}$, respectively. The reduction in WUE in response to decreasing θ was similar in the fast and intermediate drying treatments ($P = 0.09$). Conversely, WUE of vinca exposed to slow drying was not significantly affected by θ ($P = 0.78$), showing that vinca has the ability to acclimate to severe drought, if it does not develop too quickly. Previous studies reported that mild drought stress increases WUE by reducing stomatal conductance, and the WUE increase is due to the nonlinear relationship between stomatal conductance and photosynthesis (Davies et al. 2002, Liu et al. 2005). Liu et al. (2005) reported that WUE of soybean (*Glycine max*) increased under mild drought stress, but decreased when plants experienced severe drought stress. Our results showed no increase in WUE in drought stressed plants, but a decrease in WUE in the intermediate and fast drying treatments at low θ. This decreased WUE under severe drought indicates that the reduction in photosynthesis was larger than the reduction in transpiration, suggesting that non-stomatal limitations to photosynthesis were at least partially responsible. Since we found no differences in F_v/F_m among the treatments, there apparently was no drought-related damage to photsystem II. Other non-stomatal limitations to photosynthesis, such as decreased mesophyll conductance, photoinhibition, and reduced production of ATP and synthesis of RuBP, may be responsible for part of the reduction in photosynthesis, especially under more severe drought (Flexas et al. 2006, Flexas et al. 2008, Lawlor 2002, Lawlor and Cornic 2002, Misson et al. 2010, Pastenes et al. 2005).

Overall, the results from P_{net}, R_{dark}, DCG, DET, and WUE measurements showed that a slowly imposed drought alleviated vinca's physiological responses to drought compared to rapidly imposed drought. Although it has long been suggested that the rate of drought imposition affects plant responses (Bray 1997, Chaves et al. 2003, Hsiao 1973, Jones 2007, Kozlowski and Pallardy 2002, McDonald and Davies 1996), there has been little quantitative, physiological information. Our data confirm that a slow rate of drought imposition facilitates acclimation and limits the resulting reduction in carbon assimilation and growth. Jones (2007) stated that, as methods for monitoring and controlling plant and soil water status improve, precisely-quantified drought conditions are necessary to achieve a better understanding of plant responses to drought. Our findings confirm this, and also suggest that the rate at which the drought stress develops needs to be quantified and, when possible, controlled. Whether plants grown in mineral soil and with an unrestricted root volume would react similarly remains to be seen.

Conclusions

A load cell-based irrigation system was able to control pot weight precisely (± 2 g) and could quantify evapotranspiration. Maintaining a steady pot weight actually resulted in a gradual decrease in θ, due to the increasing weight of the growing plant. Drought stress decreased P_{net}, R_{dark}, DCG, and DET as θ decreased, but the severity of these decreases depended on the rate at which the drought stress was imposed. Plants that were gradually exposed to drought had less severe physiological responses and were able to maintain their WUE even under severe drought ($\theta < 0.10\ m^3 \cdot m^{-3}$), while fast or intermediate drying decreased

WUE. Previous drought stress studies seldom consider the rate of drought stress imposition, and the process by which drought stress is imposed is often neglected. However, our results show that plants respond very differently to drought stress, depending on how quickly it is imposed. Therefore, detailed descriptions of the drought process are needed in future studies, to acquire a better understanding of drought responses of plants.

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Figure 4.1. Diagram of the load cell-based irrigation system inside the whole plant CO_2 gas exchange chamber. 1) Acrylic plate, 2) load cell, 3) load cell support, 4) soil moisture sensor, 5) multiplexer, 6) datalogger, 7) relay driver 8), water source, 9) solenoid valve, 10) pressure-compensated emitter, 11) circular drip tube, and 12) whole-plant gas exchange chamber.

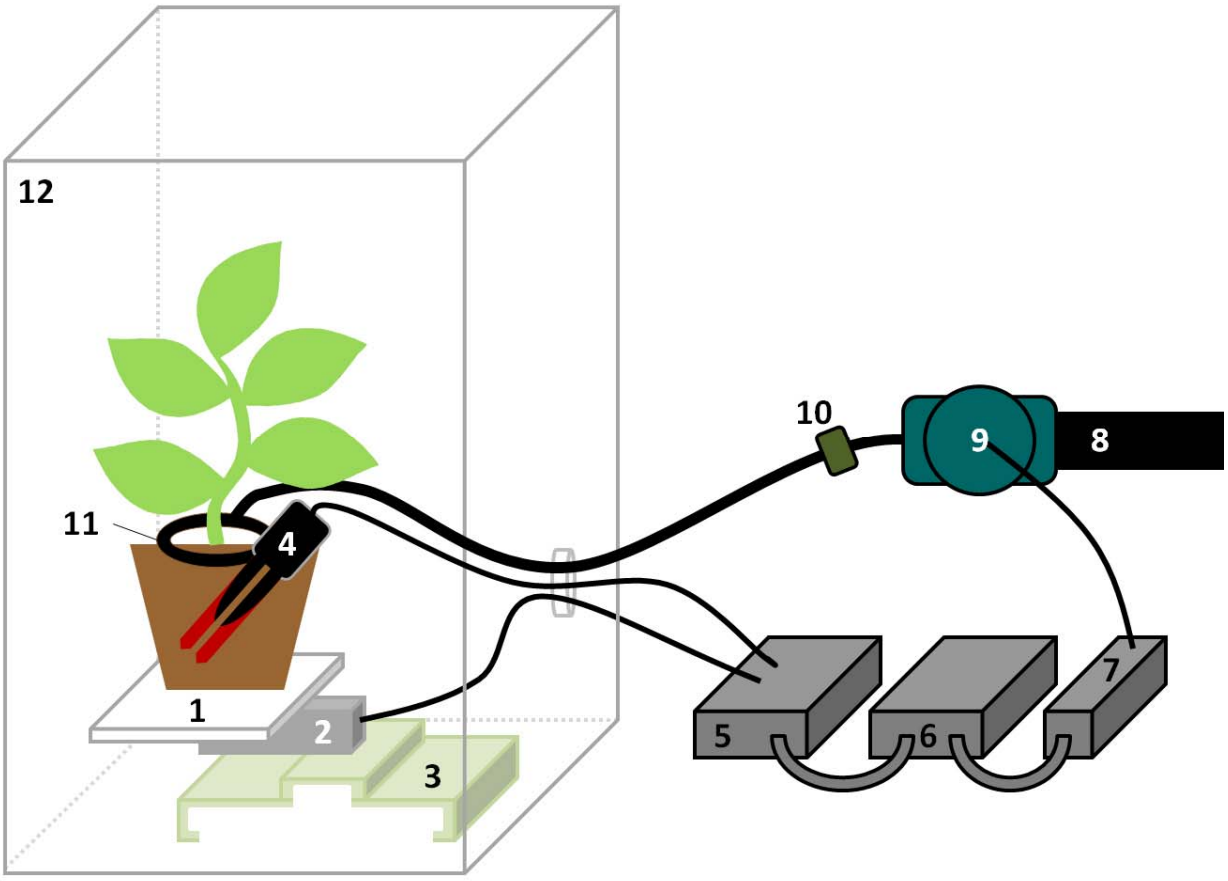

12
11
4
10
9
8
1
2
3
5
6
7

Figure 4.2. (A) Pot weight and (B) substrate volumetric water content changes over time with different drought imposition rates. Drying was controlled by gradually decreasing the pot weight at which the pots were irrigated. Arrows and dashed vertical lines indicate the start of the different drying treatments, and Δ indicates the time when each treatment reached the threshold pot weight (500 g). Weight of the control pots was maintained at 950 g. Error bars indicate the standard error (n=2).

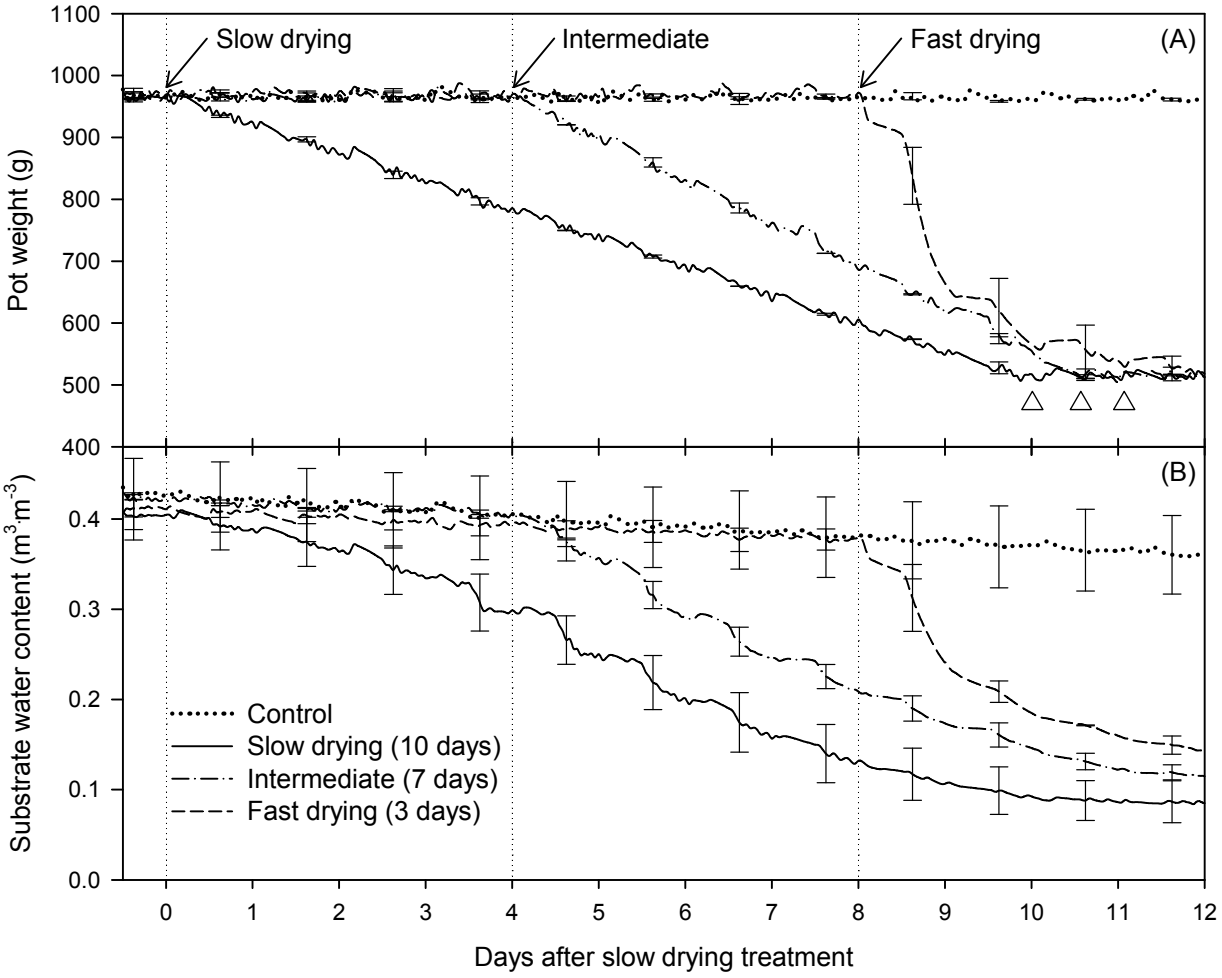

Slow drying
Intermediate
Fast drying
(A)
Pot weight (g)
1100
1000
900
800
700
600
500
400
(B)
Substrate water content (m3·m-3)
0.4
0.3
0.2
0.1
0.0
Control
Slow drying (10 days)
Intermediate (7 days)
Fast drying (3 days)
0
1
2
3
4
5
6
7
8
9
10
11
12
Days after slow drying treatment

Figure 4.3. General growth parameters of *Catharanthus roseus* after drought stress with different drought imposition rates. All drying treatments had the same pot weight (500 g) at the time of measurement, and drying took 10, 6.6, and 3.1 days for the slow, intermediate, and fast drying treatments, respectively. Leaf size was measured on uppermost fully expanded leaves. Mean separation followed ANOVA with Fisher's least significant difference (LSD) at α=0.05. Error bars indicate the standard error (n=2).

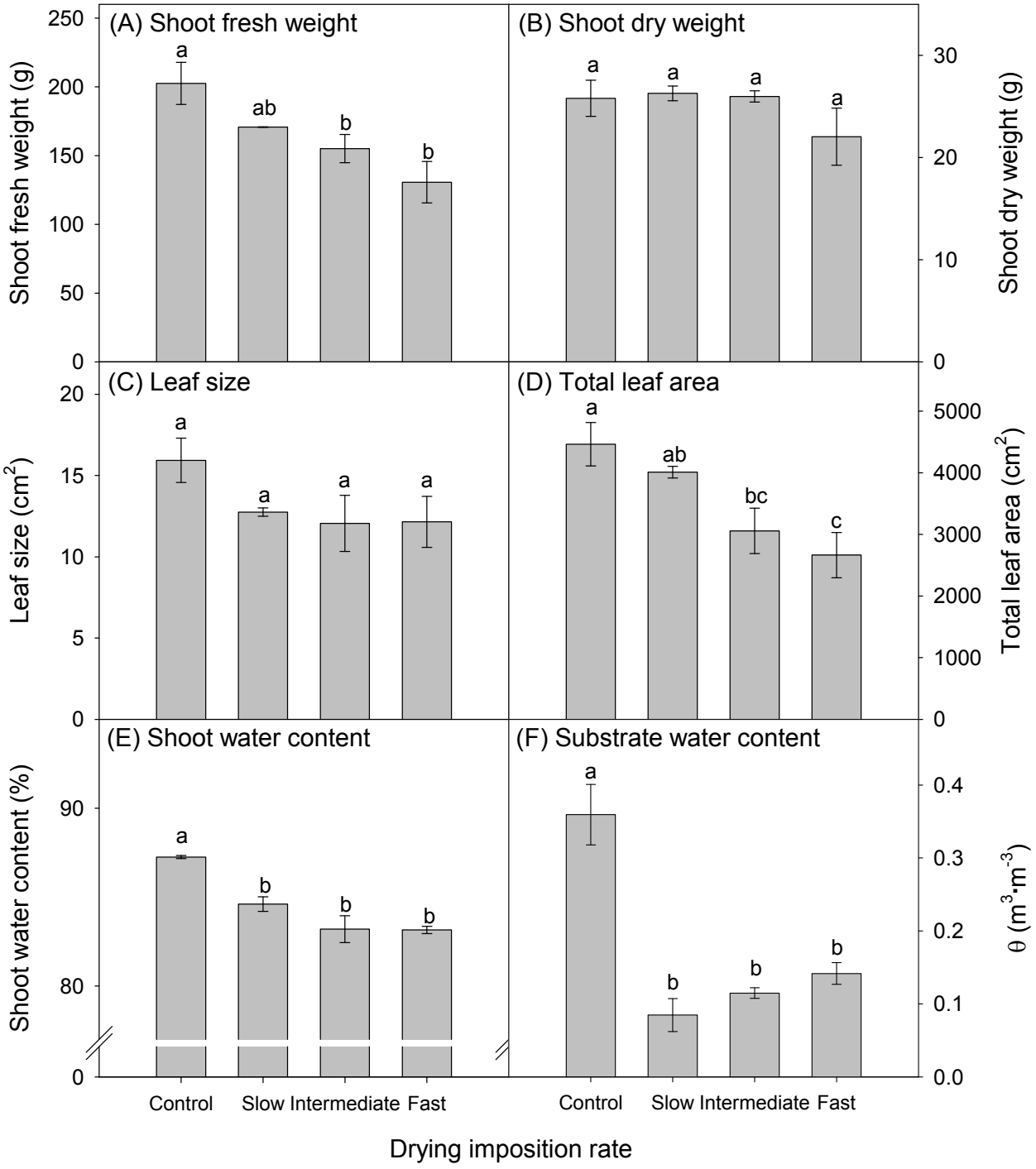

(A) Shoot fresh weight
Shoot fresh weight (g)
250
200
150
100
50
0
a
ab
b
b
(B) Shoot dry weight
Shoot dry weight (g)
30
20
10
0
a
a
a
a
(C) Leaf size
Leaf size (cm2)
20
15
10
5
0
a
a
a
a
(D) Total leaf area
Total leaf area (cm2)
5000
4000
3000
2000
1000
0
a
ab
bc
c
(E) Shoot water content
Shoot water content (%)
90
80
0
a
b
b
b
(F) Substrate water content
θ (m3·m-3)
0.4
0.3
0.2
0.1
0.0
a
b
b
b
Control
Slow
Intermediate
Fast
Control
Slow
Intermediate
Fast
Drying imposition rate

Figure 4.4. Water, osmotic, and turgor potential of *Catharanthus roseus* as affected by different drought imposition rates. All the drought treatments had the same pot weight (500 g) at the time of measurement, and drying took 10, 6.6 and 3.1 days for the slow, intermediate, and fast drying treatments, respectively. Substrate water contents at this stage were 0.08, 0.11, and 0.14 $m^3 \cdot m^{-3}$ for the slow, intermediate, and fast drying treatments, respectively. Mean separation with Fisher's least significant difference (LSD) at α=0.05 followed ANOVA. Error bars indicate the standard error (n=2).

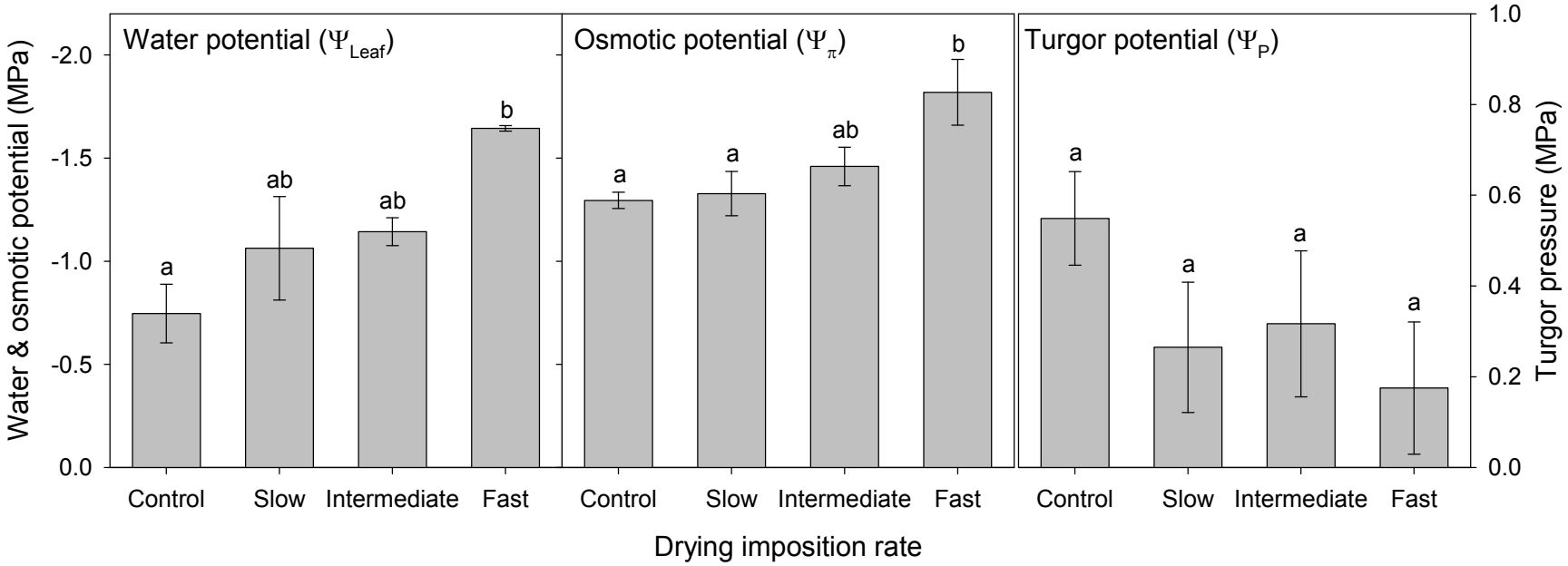

Water potential (Ψ_{Leaf})
Osmotic potential (Ψ_{π})
Turgor potential (Ψ_{P})
Water & osmotic potential (MPa)
Turgor pressure (MPa)
-2.0
-1.5
-1.0
-0.5
0.0
1.0
0.8
0.6
0.4
0.2
0.0
a
ab
ab
b
a
a
ab
b
a
a
a
a
Control
Slow
Intermediate
Fast
Drying imposition rate

Figure 4.5. Normalized whole-plant CO_2 exchange rates of C*atharanthus roseus* when exposed to different drying rates. Arrows and dashed vertical lines indicate the start of the different drying treatments. CO_2 exchange rates were normalized to the average photosynthetic rates of each plant during day 0. Data points above zero represent net photosynthesis, and ones below zero represent dark respiration. Selected error bars indicate standard errors (n=2).

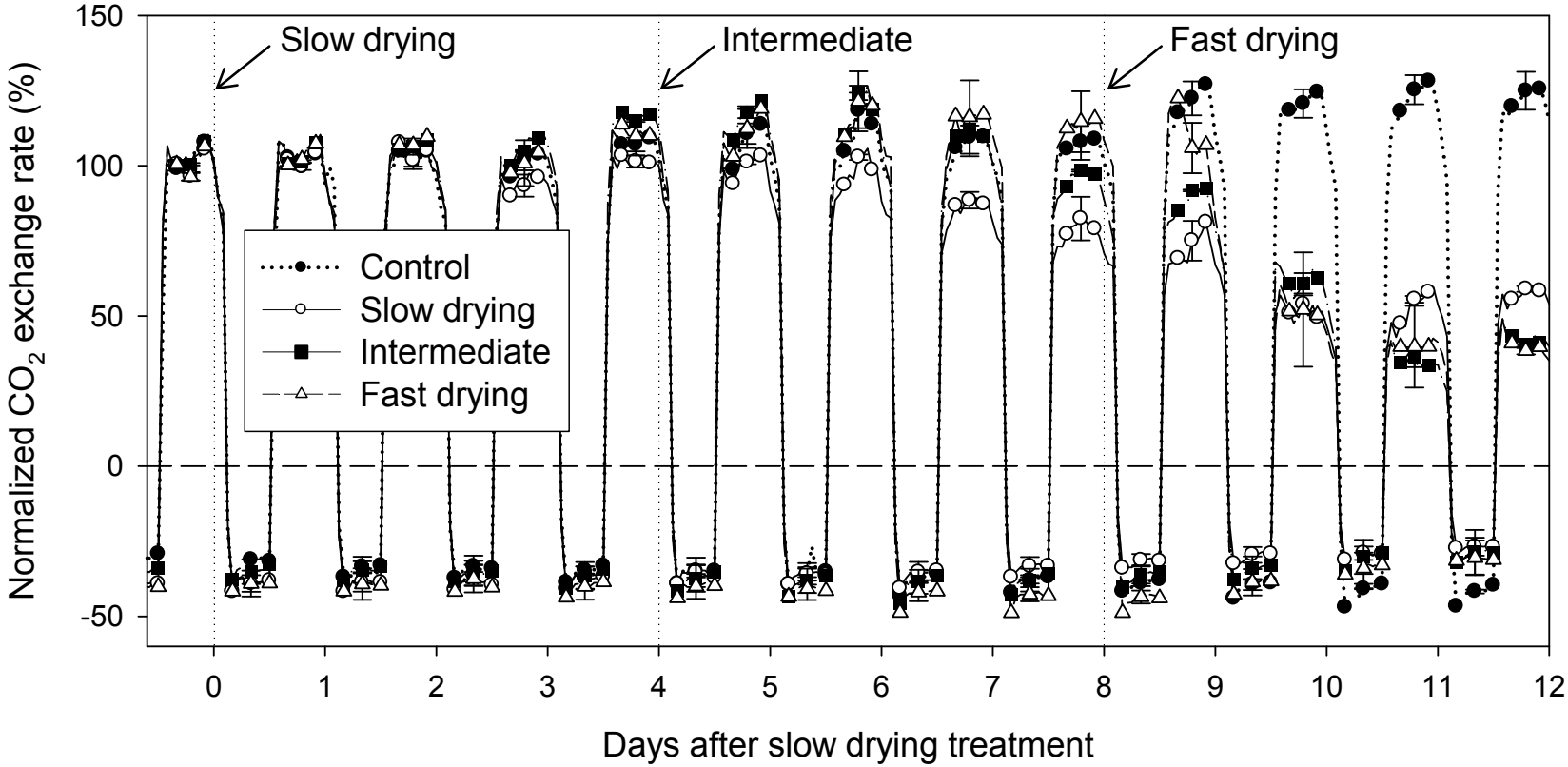

Slow drying
Intermediate
Fast drying
Control
Slow drying
Intermediate
Fast drying
Normalized CO_2 exchange rate (%)
150
100
50
0
-50
0
1
2
3
4
5
6
7
8
9
10
11
12
Days after slow drying treatment

Figure 4.6. (A) Relative net photosynthesis (P_{net}) and (B) relative dark respiration (R_{dark}) of *Catharanthus roseus* as a function of substrate water content during the drying period. Relative values were standardized to be 100% at 0.35 $m^3 \cdot m^{-3}$. Curves represent the quadratic regression lines for each treatment. *P*-values in the graph indicate the significance of homogeneity of slope tests between the treatments.

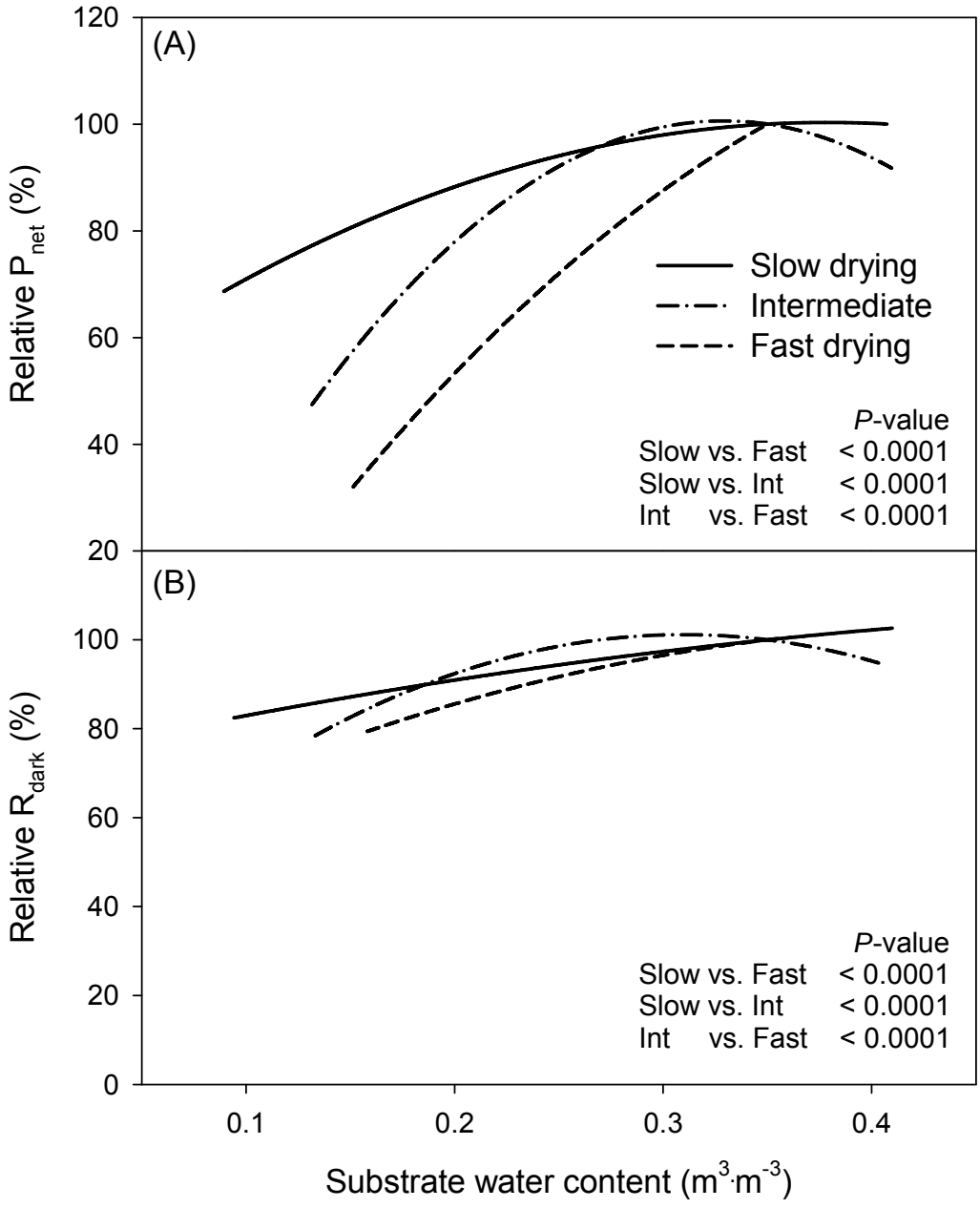

(A)
Relative P_{net} (%)
120
100
80
60
40
20
Slow drying
Intermediate
Fast drying
P-value
Slow vs. Fast < 0.0001
Slow vs. Int < 0.0001
Int vs. Fast < 0.0001
(B)
Relative R_{dark} (%)
100
80
60
40
20
0
P-value
Slow vs. Fast < 0.0001
Slow vs. Int < 0.0001
Int vs. Fast < 0.0001
0.1
0.2
0.3
0.4
Substrate water content ($m^3 \cdot m^{-3}$)

Figure 4.7. Normalized (A) daily carbon gain (DCG), (B) daily evapotranspiration (DET), and (C) water use efficiency (WUE) of *Catharanthus roseus* exposed to different drying rates throughout the experiment period. Values were normalized to the values of the day before the slow drying treatment started. Error bars indicate the standard error (n=2). The $LSD_{0.05}$ bar indicates Fisher's least significant difference at α=0.05. *** and ** indicate significant difference among treatments at $P < 0.001$ and 0.01, respectively.

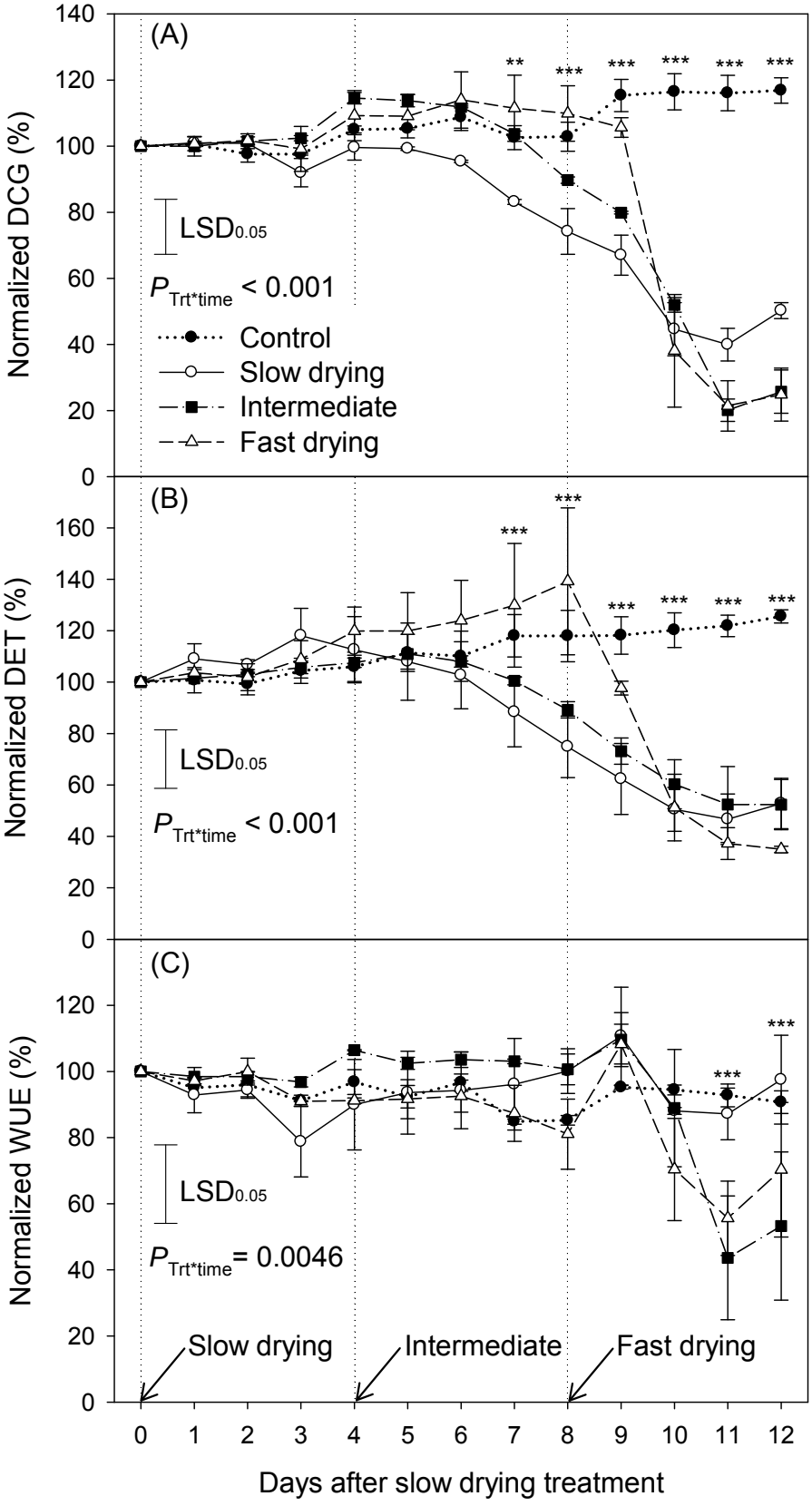
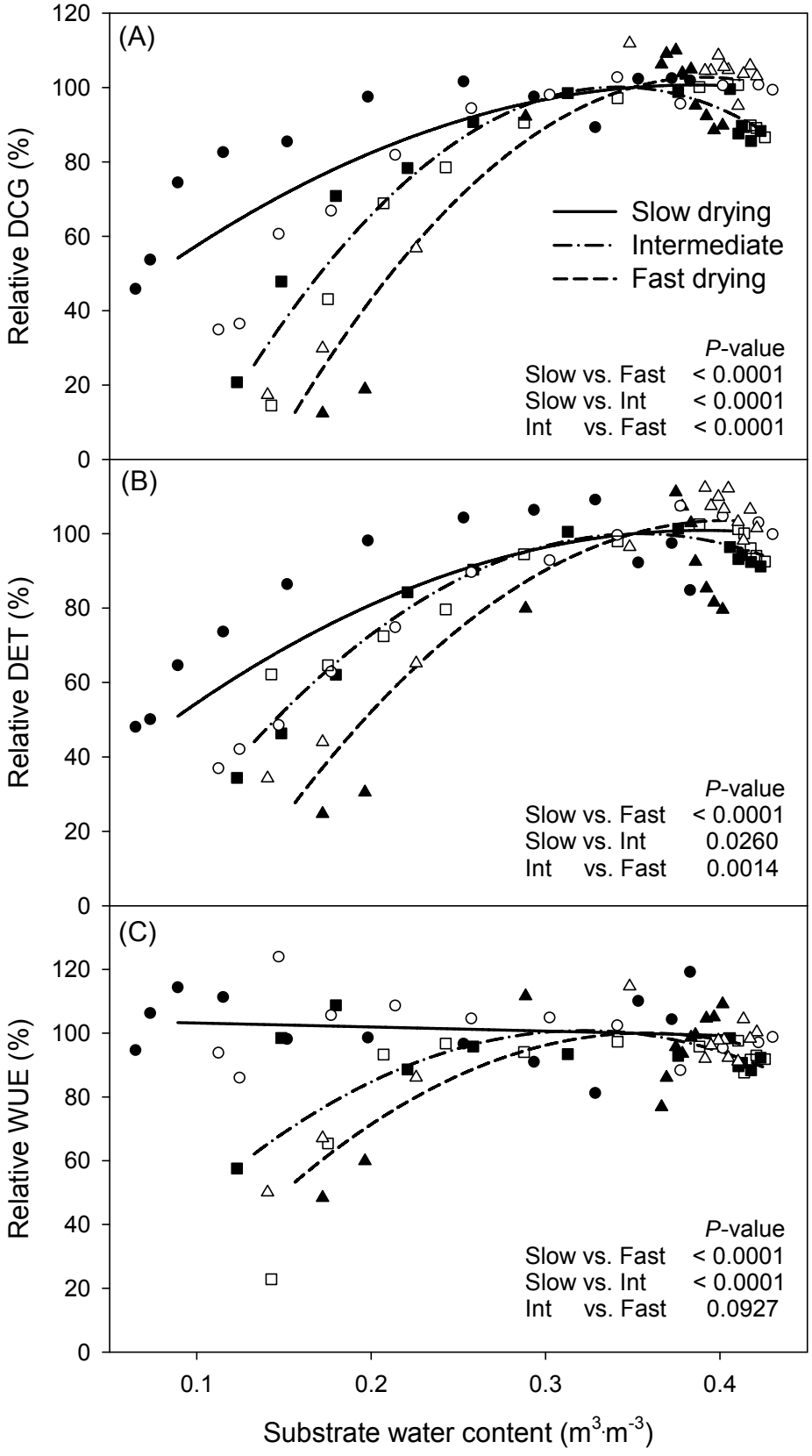

(A)
Normalized DCG (%)
LSD0.05
PTrt*time < 0.001
Control
Slow drying
Intermediate
Fast drying
(B)
Normalized DET (%)
LSD0.05
PTrt*time < 0.001
(C)
Normalized WUE (%)
LSD0.05
PTrt*time= 0.0046
Slow drying
Intermediate
Fast drying
Days after slow drying treatment

Figure 4.8. (A) Relative daily carbon gain (DCG), (B) relative daily evapotranspiration (DET), and (C) relative water use efficiency (WUE) of *Catharanthus roseus* exposed to different drying imposition rates as a function of substrate water content. Relative values were normalized to the value at 0.35 $m^3 \cdot m^{-3}$. Closed and open symbols represent different replications (●, ○; slow drying, ■, □; intermediate drying, ▲, Δ; fast drying). Curves represent the quadratic regression lines for each treatment. *P*-values in the graph indicate the significance of homogeneity of slope tests between the treatments.


(A)
Relative DCG (%)
Slow drying
Intermediate
Fast drying
P-value
Slow vs. Fast < 0.0001
Slow vs. Int < 0.0001
Int vs. Fast < 0.0001
(B)
Relative DET (%)
P-value
Slow vs. Fast < 0.0001
Slow vs. Int 0.0260
Int vs. Fast 0.0014
(C)
Relative WUE (%)
P-value
Slow vs. Fast < 0.0001
Slow vs. Int < 0.0001
Int vs. Fast 0.0927
0
20
40
60
80
100
120
0.1
0.2
0.3
0.4
Substrate water content (m3·m-3)

CHAPTER 5

ABSCISIC ACID RELATED GENE EXPRESSION AND PHYSIOLOGICAL RESPONSES OF PETUNIA AT DIFFERENT SUBSTRATE WATER CONTENTS [1]

[1] Kim, J., A. Malladi, and M.W. van Iersel. To be submitted to *Plant physiology*.

Abstract

Drought stress commonly limits plant growth. To improve understanding of plant responses to different severities of drought stress, we investigated the leaf physiology, ABA concentration, and expression of genes associated with ABA metabolism and signaling in *Petunia* × *hybrida*. Petunias were grown at different specific substrate water contents (θ = 0.10, 0.20, 0.30, or 0.40 $m^3 \cdot m^{-3}$), using an automated irrigation system. Stomatal conductance (g_s) and net photosynthesis (A) decreased after drought imposition. g_s and A of plants at θ of 0.20 and 0.30 $m^3 \cdot m^{-3}$ partially recovered after the target θ was reached. In contrast, plants at θ of 0.10 $m^3 \cdot m^{-3}$ did not acclimate and maintained low g_s and A. Drought stress increased leaf ABA concentration, which was highly correlated with g_s (r^2 = 0.85). Despite the increase in leaf ABA concentration, we saw no significant effects on the relative expression of ABA biosynthesis genes (*NCED* and *AAO3*) in response to drought stress. However, the ABA catabolic gene, *CYP707A2* was down-regulated in plants at a θ of 0.10 $m^3 \cdot m^{-3}$, suggesting a decrease in ABA catabolism under severe drought. The relative expression of *PLDα*, involved in regulating stomatal responses to ABA, and *ZPT2-3*, a transcription factor related to drought tolerance, at different θ was related to changes in stomatal sensitivity and drought tolerance in petunia. Our results suggest that combining gene expression with physiological measurements can provide a more integrated view of plant responses to environmental stress then either set of measurements by itself.

Introduction

Drought is common and considered to be the most limiting environmental factor for plant growth (Boyer, 1982). Over the last few decades, many studies have reported physiological, gene expression, and biochemical plant responses to drought (Chaves et al., 2003). Generally, a decrease in turgor, limiting cell elongation and shoot growth, is the earliest response to drought (Hsiao, 1973). Plants under drought stress commonly close stomata by regulating guard cell turgor to minimize water loss. The decrease in stomatal conductance (g_s) not only decreases efflux of water vapor, but also decreases the influx of CO_2 into the leaves, thus reducing photosynthetic carbon assimilation. Therefore, stomatal closure is regarded as the most limiting factor for photosynthesis of plants under drought stress (Flexas and Medrano, 2002; Lawlor and Cornic, 2002).

Stomatal control by guard cells is regulated by environmental factors such as light, CO_2, and the water status of the plants (Roelfsema and Hedrich, 2005). Stomatal closure under drought commonly occurs either through a chemical signal (abscisic acid, ABA), a hydraulic signal from roots sensing low soil water potential, or both (Christmann et al., 2007; Schachtman and Goodger, 2008). ABA is the primary chemical signal for drought, increasing in concentration under drought stress and inducing stomatal closure and expression of stress-related genes. Due to the critical role of ABA in plant responses to various environmental stresses, ABA has been studied for decades (Jiang and Hartung, 2008; Schachtman and Goodger, 2008).

ABA biosynthesis and catabolism related genes have been identified in many species (Seki et al., 2007; Shinozaki and Yamaguchi-Shinozaki, 2007). In the ABA biosynthetic pathway, 9-*cis*-epoxycarotenoid dioxygenase (NCED) cleaves off epoxycarotenoids to form xanthoxin, a

precursor of ABA, and this step is regarded as the regulatory step of ABA biosynthesis (Nambara and Marion-Poll, 2005). The *NCED* genes belong to a multigene family, and have been identified in many species, including *LeNCED1* and *LeNCED2* in tomato (Thompson et al. 2000), which is in the same family as petunia. As the final step of ABA biosynthesis, abscisic aldehyde oxidase converts abscisic aldehyde to ABA and *AAO3* was identified as the key gene in encoding the enzyme in arabidopsis (Seo et al., 2000; Nambara and Marion-Poll, 2005). The endogenous ABA level is determined not only by ABA biosynthesis but also by ABA catabolism, which contributes ABA homeostasis in plant (Seiler et al. 2011). There are two types of ABA catabolism, hydroxylation and conjugation. ABA 8'-hydroxylases predominantly catalyze the ABA catabolic pathway by isomerizing ABA to phaseic acid. The cytochrome P450 CYP707A family plays an important role in catalyzing ABA 8'-hydroxylases (Umezawa et al., 2006), and overexpression of *SlCYP707A3* in tomato resulted in decreased ABA concentrations in the ovary (Nitsch et al., 2009). ABA can also be stored in the inactive, conjugated ABA glucosyl ester (ABA-GE) form, which can be reactivated by specific *β*-glucosidases, releasing free ABA from ABA-GE (Lee et al., 2006). Other than ABA biosynthesis and catabolism, ABA signaling in plants also can mediate stomatal opening and closing (Kim et al., 2010). Phospholipase D (PLD) has been suggested as an enzyme that plays a role in ABA signaling by mediating ABA effects on stomata. PLDα1 produces phosphatidic acid, which has dual roles in promoting stomatal closure and inhibiting stomatal opening (Zhang et al., 2004; Mishra et al., 2006). Under environmental stress, various transcription factors are involved in triggering plant responses and adaptation to the environment. Zinc finger proteins are one of these transcription factor families; they are responsive to a wide variety of abiotic stresses (Yamaguchi-Shinozaki and Shinozaki, 2006).

ZPT2-3 is a transcription factor that encodes a Cys2/His2-type zinc finger protein found in petunia, and it is up-regulated in response to wounding, low temperature, drought, and heavy metal treatments. Overexpression of *ZPT2-3* in petunia increased drought tolerance and survival under drought (Sugano et al., 2003). Similar studies in arabidopsis showed that *ZPT* homologues were up-regulated under drought stress and increased drought tolerance (Sakamoto et al., 2004; Shu-Jing et al., 2010).

Although many studies have looked at plant responses to drought, from the molecular to whole plant level, many of these studies do not give a detailed description of the drought treatments (Jones, 2007). Plant responses to drought are usually studied by withholding irrigation until plants are wilted or reached a certain pre-determined soil moisture level. However, observed responses can be confounded by other factors, such as other environmental and temporal variations. Plant responses to water deficit likely depend on the severity of drought stress, the process of drought development, and the duration of drought stress (Bray, 1997; Chaves et al., 2003). Imprecise descriptions of how drought treatments are imposed complicate the interpretation of many previous studies (Pinheiro and Chaves, 2011). Therefore, more precise descriptions of how drought treatments are imposed should be beneficial to better understand plant responses to drought. Further, gene expression experiments need to be integrated with whole plant responses to explicitly explain physiological responses to drought (Bray, 1997; Jones, 2007).

To investigate plant responses to well-controlled specific drought conditions, we controlled substrate water content (θ) using an automated irrigation system based on soil moisture sensor readings. Comprehensive physiological and molecular studies on the response

of petunia to specific drought conditions have not yet been reported. We analyzed responses of petunia to different severities of drought, from the level of gene expression to whole plant physiology. Particularly, we focused on changes in stomatal regulation through ABA biosynthesis, catabolism and signaling.

Results

Drought Stress Treatments and Plant Growth

To impose specific drought conditions, an automated irrigation system with a datalogger and capacitance soil moisture sensors was used (Fig. 5.1, Nemali and van Iersel, 2006). The automated irrigation system applied a small amount of water when θ dropped below the threshold θ (0.10, 020, 0.30, or 0.40 $m^3 \cdot m^{-3}$) and was able to maintain θ close to the threshold θ (± 0.02 $m^3 \cdot m^{-3}$, Fig. 5.2A). Fluctuation in θ was greater in treatments with lower θ, which is consistent with previous findings (van Iersel et al., 2010), and may be related to a decrease in hydraulic conductivity of peat-based substrates as they dry out (Wallach, 2008). Control plants were maintained at a θ of 0.40 $m^3 \cdot m^{-3}$ throughout the experiment, and the drought treatments reached their threshold θ of 0.30, 0.20, and 0.10 $m^3 \cdot m^{-3}$ after 2.5, 3.7, and 8.8 d, respectively (Fig. 5.2A). At harvest, plants in lower θ treatments had smaller leaves and lower shoot dry weight (Table 5.1).

Leaf Physiological Responses to Specific Substrate Water Contents

Plants in all drought treatments had lower g_s than control plants at 2 d after drought initiation (Fig. 5.2B). Although the decrease in θ reduced g_s to 20% (≈150 $mmol \cdot m^{-2} \cdot s^{-1}$) of that

of control plants at 2 d after drought imposition, the g_s of plants at θ of 0.30 and 0.20 $m^3 \cdot m^{-3}$ partially recovered after θ reached the threshold levels (50-70% and 30-40% of control g_s for 0.30 and 0.20 $m^3 \cdot m^{-3}$, respectively). However, plants at 0.10 $m^3 \cdot m^{-3}$ maintained low g_s (< 50 $mmol \cdot m^{-2} \cdot s^{-1}$), less than 5% of g_s of control plants, from day 4 until day 16. Photosynthetic rate also decreased in drought treatments as θ decreased (Fig. 5.2C). Plants at θ < 0.40 $m^3 \cdot m^{-3}$ decreased A by 50-70% of the control at 2 d after drought initiation. Plants at 0.30 and 0.20 $m^3 \cdot m^{-3}$ maintained A at ≈67 and ≈50% of control plants until the end of the experiment. However, A of plants at θ of 0.10 $m^3 \cdot m^{-3}$ subsequently decreased to 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$, approximately 5% of A of control plants. Chlorophyll fluorescence measurements indicated that only the 0.10 and 0.20 $m^3 \cdot m^{-3}$ treatments significantly decreased the quantum yield of photosystem II (Φ_{PSII}). In contrast to g_s and A results, the 0.30 $m^3 \cdot m^{-3}$ treatment did not show a significant decrease in Φ_{PSII} compared to control plants, and maintained Φ_{PSII} at ≈90% of that of control plants throughout the experiment (Fig. 5.2D). CO_2-saturated assimilation rate (A_{max}) was lower as the threshold θ decreased (Table 5.1).

In contrast to the quick responses of g_s and A, midday Ψ_{leaf} only decreased during later stages of drought imposition (Fig. 5.2E). Plants at θ < 0.40 $m^3 \cdot m^{-3}$ had lower midday Ψ_{leaf} than plants at 0.40 $m^3 \cdot m^{-3}$ a week after the start of the drought treatment, but Ψ_{leaf} of all plants partially recovered after the threshold θ was reached. At harvest, midday Ψ_{leaf} of the plants at θ < 0.40 $m^3 \cdot m^{-3}$ was not different among the drought treatments, but lower than that of plants grown at θ of 0.40 $m^3 \cdot m^{-3}$. Plants at a θ of 0.10 $m^3 \cdot m^{-3}$ had lower leaf relative water contents than other treatments (Table 5.1).

Control plants maintained the leaf ABA concentration at ≈0.15 nM·g^{-1} FW, while all drought treatments resulted in an ≈3 fold increase in leaf ABA concentration after two days of drought (Fig. 5.2F). Plants at a θ of 0.30 m^3·m^{-3} maintained a 2-3 fold higher ABA concentration for a week, but had ABA concentrations similar to that of the 0.40 m^3·m^{-3} treatment from 9 to 16 d after the start of the treatments. Plants in the 0.10 m^3·m^{-3} treatment gradually increased their ABA concentration up to 1.2 nM·g^{-1} FW (9 fold of control) as the substrate dried out, and maintained high leaf ABA concentrations until the end of the experiment.

Expression of ABA Biosynthesis, Catabolism and Signaling Genes

To study the effect of θ on gene expression, drought-related genes were quantified using quantitative RT-PCR. Putative homologues of ABA biosynthesis genes (*NCED1, NCED2, AAO31,* and *AAO32*) and catabolism-related genes (*CYP707A1 and CYP707A2*) in *Petunia* × *hybrida* 'Apple Blossom' were identified. Although leaf ABA concentration was increased in all three drought treatments, our results did not show any effect on the expression of the putative *NCED* and *AAO3* genes in petunia leaves (Figs. 5.3A-D). Although there was an increase in expression of *NCED2* in drought treatments at 2 d after the start of the treatments, control plants also increased *NCED2* expression 4 d after treatment, and the relative expression was not significantly different among different θ treatments. Relative expression of the putative *AAO3* genes also did not differ significantly among treatments throughout the experiment. Expression of the *CYP707A2* gene in the 0.10 m^3·m^{-3} treatment was ≈3 fold lower than in the control and 2 fold lower than in other drying treatments as θ reached 0.10 m^3·m^{-3}, suggesting a decrease in ABA catabolic activity.

To investigate the potential changes in ABA signaling, the putative homologue *PLDα* gene was identified from tomato *LePLDα1* (Bargmann et al., 2009). Relative expression of *PLDα* in petunia leaves increased rapidly after the drought imposition, and subsequently declined after the threshold θ was reached (Fig. 5.3G). All drought treatments displayed a ~2 fold increase in the expression of *PLDα* after 2 d. The relative expression of *PLDα* in plants at a θ of 0.30 $m^3 \cdot m^{-3}$ decreased at 4 d after treatment, when the θ reached the threshold level. A similar pattern was observed with the 0.20 $m^3 \cdot m^{-3}$ treatment. However, plants in the 0.10 $m^3 \cdot m^{-3}$ treatment displayed up to a 2.5 fold higher relative expression of *PLDα* than control plants and maintained higher expression rates than other treatments until θ reached the threshold level.

We also examined the expression of the *ZPT2-3* gene in petunia leaves to see how this drought tolerance-related transcription factor changed. All drought treatments increased the expression of *ZPT2-3* between 2 and 4 d after the start of the treatments, but only plants at a θ of 0.20 $m^3 \cdot m^{-3}$ maintained higher expression levels throughout the study (Fig. 5.3H).

Relationship among ABA Related Genes and Leaf ABA Concentration

We analyzed the relationship between ABA-related gene expression and θ and leaf ABA concentration. Relative expression of ABA biosynthesis genes (*NCED1*, *NCED2*, *AAO31*, and *AAO32*) showed no relationship with either θ or leaf ABA concentration. However, high expression of *CYP707A2* was associated with low ABA concentration (Fig. 5.4B). Expression of *CYP707A2* increased in the 0.20 and 0.30 $m^3 \cdot m^{-3}$ treatments after the θ reached the threshold levels (Fig. 5.4A). In contrast, plants at a θ of 0.1 $m^3 \cdot m^{-3}$ maintained lower expression of

CYP707A2 after θ reached the threshold levels, which suggests lower ABA catabolic activity in the 0.10 $m^3 \cdot m^{-3}$ treatment.

Expression of *PLDα* increased as θ decreased during the drying period, but then decreased after θ reached the threshold levels (Fig. 5.5A). As ABA concentration increased during the drying period, the expression of *PLDα* increased as well, suggesting a positive ABA effect on PLDα metabolism (Fig. 5.5B). However, expression of *PLDα* in all drought treatments decreased after the θ reached the threshold levels, although they generally maintained higher ABA concentrations than the control.

Discussion

Plant responses to drought may vary depending on the severity and duration of drought imposition (Bray, 1997; Jones, 2007; Pinheiro and Chaves, 2011). Control and precise quantification of drought conditions are important in understanding plant responses and for comparing results with other studies. In our study, an automated irrigation system with soil moisture sensors provided the ability to precisely control θ levels. By collecting data over a 16 d period, we were able to determine how the plant responses changed during the drying period and after the threshold θ was reached. This soil moisture sensor-based automated irrigation system has been successful in investigating θ specific physiological and morphological changes in plants under drought conditions (Burnett and van Iersel, 2008; Nemali and van Iersel, 2008), and also for quantifying the water use amount of plants depending on environmental conditions (van Iersel et al., 2010). Our current study shows that this approach is valuable for looking at relationships between gene expression and physiological responses as well.

Gene Expression at Specific θ

NCED is regarded as the key gene encoding the enzyme that cleaves epoxycarotenoid to produce a precursor of ABA, the first step in ABA biosynthesis (Nambara and Marion-Poll, 2005). Previous research reported that *LeNCED1* mRNA increased in both leaves and roots of tomato during drought stress (Thompson et al., 2000), and drought stress induced a 4 fold increase in the expression of *NCED3* in arabidopsis (Harb et al., 2010). Additionally, *AAO3* is considered the key gene in the final step in ABA biosynthesis, the oxidation of abscisic aldehyde to ABA. Seo et al. (2000) reported that an *AAO3* deficient arabidopsis mutant had lower leaf ABA concentrations. However, we did not observe drought-related changes in the expression of putative *NCED* or *AAO3* genes in petunia leaves, although the ABA concentration in leaves was increased 6-9 fold during drought stress. The lack of changes in the expression of these genes may possibly be due to that the putative *NCED* homologue genes identified in petunia might not be the *NCED* genes involved in ABA biosynthesis under drought, and there might be another regulatory gene in ABA biosynthesis. It is likely that additional *NCED* homologues are present in petunia and ABA biosynthesis in leaves under drought might be regulated by those genes. In Arabidopsis, at least three *NCED* genes are present, but *AtNCED3* was suggested as the gene responsive to drought stress (Iuchi et al., 2001). It is possible that *NCED1* and *NCED2* in petunia have roles in maintaining normal ABA levels, but are not responsive to drought. Alternatively, the increase in ABA concentration under drought in petunia leaves may be due to ABA synthesized elsewhere in the plant and subsequently transported to the leaves. Previous research suggested that translocation of root produced ABA is critical for drought sensing and signaling (Wilkinson and Davies, 2002). However, Christmann et al. (2007) suggested *de novo*

ABA biosynthesis in the leaves, regardless of ABA translocation from root to shoot in arabidopsis. Additionally, Harb et al. (2010) showed an increase in *AtNCED3* expression and an increase in ABA concentration in arabidopsis leaves under drought. The relative contribution of *de novo* synthesis of ABA under drought may differ among species, and petunia leaves may depend more on transported ABA than *de novo* synthesis in the leaves under drought stress. Alternatively, the increase in leaf ABA concentration could be due to the release of free ABA from conjugated ABA stored in the leaves (Lee et al., 2006; Jiang and Hartung, 2008). The key gene in releasing free ABA from conjugated ABA was identified as *AtBG1* in Arabidopsis (Lee et al., 2006), but we were unable to identify a homologue of this gene in petunia.

Although we did not see an effect of drought on the expression of putative ABA biosynthetic genes, the relative expression of the ABA catabolic gene, *CYP707A*, was related to the ABA concentration in petunia leaves. ABA concentration in plants is modulated by a balance between biosynthesis and catabolism, and reduced ABA catabolic activity results in higher ABA concentration (Umezawa et al., 2006). Petunia plants at a θ of 0.10 $m^3 \cdot m^{-3}$ had lower expression of *CYP707A2* than those at a higher θ, suggesting that severe drought stress may decrease ABA catabolism. Relative expression of putative *CYP707A2* as a function of θ shows relatively low expression during the drying period, but expression of this gene increased in the 0.20 and 0.30 $m^3 \cdot m^{-3}$ treatments after the threshold θ was reached (Fig. 5.4A). However, plants at a θ of 0.10 $m^3 \cdot m^{-3}$ maintained low expression of *CYP707A2* even after the threshold θ was reached. This low level of *CYP707A2* expression in plants at a θ of 0.10 $m^3 \cdot m^{-3}$ was associated with high levels of ABA (Fig. 5.4B). ABA 8'-hydroxylase, encoded by cytochrome P450 CYP707A families, catalyzes the conversion of ABA into phaseic acid (Nambara and Marion-Poll, 2005). In

arabidopsis, the expression of *CYP707A3* was induced under dehydration and subsequent rehydration conditions, and a *CYP707A3*-deficient mutant had higher ABA concentrations and enhanced drought tolerance (Umezawa et al., 2006). Overexpression of the *SlCYP707A1* gene in tomato decreased ABA concentration in tomato ovaries (Nitsch et al., 2009), suggesting that ABA 8'-hydroxylase played a pivotal role in regulating ABA concentrations. Our results of the expression of the putative *CYP707A2* gene agree with previous findings, and suggest that low ABA catabolic activity may be important in maintaining high ABA concentrations in petunia leaves exposed to severe drought (θ = 0.10 $m^3 \cdot m^{-3}$).

PLDα1 generates phosphatidic acid by degradation of phospholipids, and the phosphatidic acid binds to a negative regulator of ABA responses, ABI1 protein phosphatase 2C (PP2C) , thus promoting ABA-induced stomatal closure (Zhang et al., 2004). Additionally, PLDα1 and phosphatidic acid also interact with the GTP-binding proteins, and can mediate ABA inhibition of stomatal opening (Mishra et al., 2006). In our study, putative *PLDα* was up-regulated in petunia leaves as θ decreased (Fig. 5.5A). Harb et al. (2010) also reported the increase of *PLDα1* as an early response of arabidopsis under moderate drought stress. This suggests that decreasing θ induced higher expression of *PLDα*, thus promoting stomatal closing through phosphatidic acid binding to ABI1. The relative expression of *PLDα* during the drying period was associated with leaf ABA concentration (Fig. 5.5B, $P < 0.001$), suggesting that ABA may promote the expression of *PLDα*, which agrees with previous research (Zhang et al., 2004). This increased expression of *PLDα* during the drying period showed a good correlation with g_s (Fig. 5.5C, $P < 0.001$). However, the expression of putative *PLDα* decreased in all drought treatments after the threshold θ was reached, although leaf ABA concentration in the 0.10

$m^3 \cdot m^{-3}$ treatment remained high (Fig. 5.5A and 5B). This may be due to different mechanisms by which PLDα affects stomatal control. The increased expression of *PLDα* during the drying period was associated with leaf ABA concentration and may be an ABA-dependent response to promote stomatal closure (Fig. 5.5C). However, the decreased expression of *PLDα* after the threshold θ level had been reached may be an ABA-independent response. When θ reached the threshold level, g_s was already low and additional stomatal closure may not be needed. Rather, inhibition of stomatal opening under severe drought may be more important to prevent water loss. Although PLDα can both promote stomatal closure and inhibit stomatal opening, it is possible that inhibition of stomatal opening does not require the same level of *PLDα* expression as the promotion of stomatal closure.

Many transcription factors involved in drought tolerance have been identified in arabidopsis and other plants (Yamaguchi-Shinozaki and Shinozaki, 2006), and *ZPT2-3* was reported as a drought tolerance transcription factor in petunia (Sugano et al., 2003). Sugano et al. (2003) reported that *ZPT2-3* in petunia was up-regulated under cold, drought, and heavy metal stress, and overexpression of *ZPT2-3* increased drought tolerance of petunia. Homologues of this gene family in arabidopsis were also up-regulated under drought and other environmental stress, and overexpression of this transcription factor also increased drought tolerance of arabidopsis (Sakamoto et al., 2004). Our results indicated that *ZPT2-3* in petunia leaves was up-regulated as θ decreased, and had the highest expression level in plants at a θ of 0.20 $m^3 \cdot m^{-3}$ (Fig. 5.3). Plants at θ of 0.30 $m^3 \cdot m^{-3}$ and 0.10 $m^3 \cdot m^{-3}$ also increased expression of *ZPT2-3* as θ decreased, but *ZPT2-3* expression decreased again after the threshold θ had been reached. Possibly, a θ of 0.30 $m^3 \cdot m^{-3}$ might not induce a severe enough stress to induce high

ZPT2-3 expression, while the drought at a θ of 0.10 $m^3 \cdot m^{-3}$ might be too severe to allow acclimation of petunia to drought.

Physiological Responses to Specific θ

The physiological responses of petunia to θ partially agree with the results from previous research (Niu et al., 2006; Nemali and van Iersel, 2008). Nemali and van Iersel (2008) reported that g_s of petunia decreased at θ < 0.22 $m^3 \cdot m^{-3}$. They also reported that A was lowest at a θ of 0.09 $m^3 \cdot m^{-3}$, but did not differ among θ levels of 0.15, 0.22, and 0.32 $m^3 \cdot m^{-3}$. However, their measurements were conducted 20 to 40 d after θ reached threshold levels, thus allowing plants to acclimate low θ. In our study, g_s, A, Φ_{PSII}, and Ψ_{leaf} decreased with decreasing θ during the drying period (Fig. 5.6, $P < 0.001$). In particular, g_s and A drastically decreased as θ decreased, similar to previous research reporting a sharp decrease in g_s and A in petunia with decreasing θ (Niu et al., 2006). However, g_s and A of plants at θs of 0.20 and 0.30 $m^3 \cdot m^{-3}$ partially recovered after θ reached the threshold levels (Figs. 5.6A and 5.6B). Only the g_s and A of plants in the 0.10 $m^3 \cdot m^{-3}$ treatment did not recover after the θ threshold was reached, suggesting less acclimation ability under severe drought stress. Supporting these results, it was suggested that plants under severe drought can experience metabolic impairment of photosynthesis without acclimation, but acclimated plants to water stress can lead homeostatic compensation of their physiological responses, as well as gene expression (Flexas et al., 2006). However, references to 'severe' drought are vague, and our results show that physiological responses of petunia vary based on θ and duration of drought. Therefore, better descriptions of drought conditions, such as substrate and/or plant water status and the duration of drought,

may provide a greater ability to make generalization across experiments with different drought treatments, improving our understanding of plant drought response.

Photosynthetic decreases under drought are largely due to stomatal restrictions, but non-stomatal limitations can contribute under severe drought stress (Flexas and Medrano, 2002; Lawlor and Cornic, 2002; Pinheiro and Chaves, 2011). Plants at a θ of 0.10 $m^3 \cdot m^{-3}$ had greatly reduced Φ_{PSII} (≈50% of control plants), indicating non-stomatal limitations of photosynthesis under severe drought. Although plants at 0.30 and 0.20 $m^3 \cdot m^{-3}$ also decreased Φ_{PSII} as θ decreased, they maintained Φ_{PSII} around 90% and 80% of that of control plants, respectively (Fig. 5.6D). A reduction in Φ_{PSII} at low θ has been reported previously in petunia (Nemali and van Iersel, 2008).

Ψ_{leaf} has been commonly used for determining the severity of drought stress in drought studies (Jones, 2007). Midday Ψ_{leaf} of petunia at a θ of 0.10 $m^3 \cdot m^{-3}$ was the lowest among drought treatments (Fig. 5.6D). However, midday Ψ_{leaf} increased from day 9 to day 12, after θ had been maintained at 0.10 $m^3 \cdot m^{-3}$, and there was no significant difference in Ψ_{leaf} among the three drought treatments at harvest (Fig. 5.2E). Our results also showed that midday Ψ_{leaf} decreased later than other physiological responses (Fig. 5.2).

Leaf ABA Concentration and g_s of Petunia

Previous research suggested that g_s is better correlated with xylem ABA concentration than with leaf ABA concentration (Tardieu and Davies, 1993; Heilmeier et al., 2007; Jiang and Hartung, 2008). However, our results showed a strong correlation between leaf ABA concentration and g_s in petunia, regardless of θ or time, suggesting g_s is closely controlled by

leaf ABA concentration (Fig. 5.7). Tardieu and Davies (1993) also reported that the effect of xylem ABA on g_s depends on Ψ_{leaf}. Our results showed that g_s was affected by both leaf ABA concentration and Ψ_{leaf}, but their interaction was not significant. The partial R^2 of ABA and Ψ_{leaf} in the regression were 0.84 and 0.02, respectively, indicating that most variation in g_s could be explained by changes in leaf ABA concentration, with only minor contribution of Ψ_{leaf}.

Conclusions

By precisely controlling θ and conducting temporal analyses of plant genetic and physiological responses, we were able to demonstrate correlated drought responses from the gene to the whole plant level. Our results showed θ-specific drought responses in petunia. As θ decreased, g_s and A decreased, but g_s and A of plants in the 0.20 and 0.30 $m^3 \cdot m^{-3}$ treatments partially recovered after the threshold θ was reached, whereas no recovery of g_s and A was seen at a θ of 0.10 $m^3 \cdot m^{-3}$. All petunia plants at θ < 0.40 $m^3 \cdot m^{-3}$ increased leaf ABA concentrations after the start of the drought treatment, but we did not see any significant changes in relative expression of ABA biosysnthesis genes. Although we could not find the source of the ABA increase, there was a significant decrease in the expression of *CYP707A2*, a putative ABA catabolic gene, in plants at a θ of 0.10 $m^3 \cdot m^{-3}$. This suggests that maintaining high ABA level in leaves of plants under severe drought is at least partially caused by decreased ABA catabolism. High ABA concentrations were correlated with higher expression of *PLDα* during the drought period, possibly promoting stomatal closure mediated by ABA. Plants in the 0.20 $m^3 \cdot m^{-3}$ treatment showed increased expression of the drought tolerance gene *ZPT2-3*, suggesting that θ of 0.20 $m^3 \cdot m^{-3}$ increased drought tolerance, but this was not the case under severe drought (θ

= 0.10 $m^3 \cdot m^{-3}$). Our results also indicated close correlation between leaf ABA concentration and g_s, regardless of time or θ, suggesting that g_s was regulated by leaf ABA concentration.

Gene expression may be a good indication of plant responses to environmental stresses. However, as our results indicated, gene expression analysis from only one part of the plant may not allow complete comprehension of whole plant responses to the environment. Neither gene expression nor physiology by themselves can provide a complete picture of plant responses to drought. By determining expression of ABA related genes, leaf ABA concentrations, and leaf physiological responses we were able to provide a more integrated of plant responses to drought. Our results suggests that petunia can acclimate to drought, when the drought stress is not too severe (θ 0.20 – 0.30 $m^3 \cdot m^{-3}$), but that severe drought prevents acclimation.

Materials and Methods

Plant Materials and Growth Conditions

Eight *Petunia* x*hybrida* 'Apple Blossom' seedlings were transplanted into sixteen 8-L trays filled with soilless substrate (Fafard 2P; 60% peat and 40% perlite; Fafard) mixed with a controlled-release fertilizer (Osmocote 14-14-14 ; 14.0N-6.2P-11.6K; Scotts) at a rate of 7.7 $kg \cdot m^{-3}$. Plants were grown for three weeks (Mar. 23 to Apr. 14, 2010) in a greenhouse at the University of Georgia using a soil moisture sensor-based, automated irrigation system (Nemali and van Iersel, 2006) which maintained substrate water contents (θ, v/v) at 0.40 $m^3 \cdot m^{-3}$. During the growing period, the average daily temperature and relative humidity in greenhouse were 21.0 ± 1.0°C and 53 ± 10%, and the daily light integral averaged 26.9 ± 12.2 $mol \cdot m^{-2} \cdot day^{-1}$ (mean ± sd).

Drought Treatments

The soil moisture based automated irrigation system was modified from previous research (Nemali and van Iersel, 2006). Two capacitance soil moisture sensors (EC-5; Decagon Devices) were placed in each tray and connected to a datalogger (CR10; Campbell Scientific) via a multiplexer (AM16/32; Campbell Scientific) to monitor and control θ. The soil moisture sensors were calibrated for the specific substrate (θ = 1.7647 × sensor output (V) - 0.4745, r^2 = 0.95). When the average reading of the two sensors dropped below the set moisture level for that tray, a datalogger opened the specific solenoid valve using a relay driver (SDM-CD16 AC/DC controller; Campbell Scientific) to irrigate the tray for 20 seconds (approximately 90 mL per application). Each tray was watered with tap water using a custom grid with two pressure compensated emitters (8L/h; Netafim USA) (Fig. 5.1). θ was measured every 10 minutes, and averages were logged hourly. The different θ thresholds, 0.40, 0.30, 0.20, and 0.10 $m^3 \cdot m^{-3}$, were initiated at midnight Apr. 14, and irrigation was withheld until a tray θ reached its threshold θ.

Leaf Water Potential and Gas Exchange Measurement

Leaf sampling and physiological measurements were conducted every 2 or 3 days from the start of the drying treatment. Leaf samples for RNA extractions and ABA assays were collected by excising the leaf at the petiole using a razor blade and immediately frozen in liquid N_2 at noon and stored at -80°C. Leaf discs for leaf water potential measurement were sampled at noon, and midday leaf water potential (Ψ_w) was measured using leaf cutter thermocouple psychrometers (Model 76; J.R.D. Merrill Specialty Equipment) after equilibration in a water bath at 25^{o}C for 4 hours. Stomatal conductance (g_s), CO_2 exchange rate (A), and quantum yield of PSII

(Φ_{PSII}) were measured with a leaf photosynthesis system (CIRAS-2; PP Systems) equipped with an LED light and chlorophyll fluorescence module. Measurements were taken at a photosynthetic photon flux of 1000 µmol·m^{-2}·s^{-1} and at a CO_2 concentration of 388 µmol·mol^{-1}. Φ_{PSII} was calculated as (F'_m-F_t)/F'_m (Maxwell and Johnson, 2000).

Relative Water Content, A/Ci Curves, Leaf Size, and Shoot Dry Mass at Harvest

All plants were harvested 16 days after initiation of the drought treatments. At harvest, uppermost, fully expanded leaf samples were collected at noon and fresh weight of the leaves was measured immediately after excision. Fully turgid fresh weight of the leaves was obtained after floating the samples on deionized water at 4°C for 6 h, and dry weight was determined after drying the sample at 60°C for a day. Relative water content was calculated as (Fresh weight – Dry weight) / (Turgid weight – Dry weight) × 100%. CO_2 response curves (A/Ci curves) were collected using the CIRAS-2, by changing the CO_2 concentration from 0 to 1200 µmol·mol^{-1} in 200 µmol·mol^{-1} increments. The CO_2 saturated assimilation rate (A_{max}) was calculated using empirical A/Ci curve analysis (Photosyn Assistant; Dundee Scientific). Leaf size was measured on eight uppermost fully expanded leaves per tray using a leaf area meter (LI-3100; Li-Cor) and shoot dry weight was obtained after drying samples in an oven at 70°C for 4 days.

RNA Extraction and cDNA Synthesis

Leaf samples were frozen in liquid nitrogen immediately after excision and stored at -80°C. RNA was extracted from ground, frozen leaf tissue using the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). Approximately 1 g of ground sample was used for RNA extraction in 7mL of extraction buffer (38% acid phenol, 0.8 M guanidine thiocyanate, 0.4 M

ammonium thiocyanate, 0.1 M Sodium acetate, and 5% glycerol). After centrifugation, the supernatant was re-extracted with chloroform:iso-amyl alcohol (24:1 v/v). The aqueous supernatant was precipitated with isopropanol and a salt solution (0.8 M sodium citrate and 1.2 M NaCl). After centrifugation, the RNA was washed with 70% ethanol and dissolved in DEPC (diethylpyrocarbonate) treated water. This mixture was washed with 3 M sodium acetate and chloroform:iso-amyl alcohol (24:1), and the aqueous supernatant was precipitated with 70% ethanol overnight at -20°C. After centrifugation, the RNA was washed with 70% ethanol, dissolved in DEPC-treated water and stored at -80°C. RNA quality was checked by gel electrophoresis and RNA quantification was performed using Nanodrop 8000 (Thermo scientific).

1 µg of RNA was treated with DNase (Promega) to remove genomic DNA contamination, according to the manufacturer's instructions. Reverse transcription was performed using oligo dT (Promega) and ImPromII reverse transcriptase (Promega) according to the manufacturer's instructions. Subsequently, the cDNA was diluted 5 times with autoclaved distilled water and stored at -20°C until further analysis.

Quantitative RT-PCR Primer Design

Petunia expressed sequence tags (ESTs) coding for ABA- and drought-related genes were identified from petunia EST databases (454 Petunia database: http://140.164.45.140/454petuniadb/). ABA biosynthesis and catabolism-related genes (*NCED*, *AAO3*, and *CYP707A*) were selected based on research on ABA metabolism (Seki et al., 2007). *PLDα* was selected to investigate the stomatal sensitivity to ABA (Mishra et al., 2006; Hong et al.,

2010), and *ZPT2-3* TF gene was selected to analyze drought tolerance changes in petunia (Sugano et al., 2003). *Cyclophilin-2* (*CYP*) and *Elongation factor 1α* (*EF1α*) genes were used as reference genes based on previous research on petunia reference genes (Mallona et al., 2010). All genes used in this study, except *ZPT2-3* and reference genes, were identified based on sequence similarity after tblastx analysis with related genes from tomato and potato in the same family (Solanaceae). Candidate genes were further confirmed by sequencing. Primers used for quantitative RT-PCR were designed and primers are presented in supplement II.

Putative homologues *NCED1* and *NCED2* genes were identified based on sequence similarity with the tomato *NCED* gene, *LeNCED1* (Taylor et al., 2000). The putative petunia *NCED1* gene had 93% identity with *LeNCED2* and putative *NCED2* gene had 86% identity with *LeNCED1*, respectively. Putative homologues of *AAO31* and *AAO32* genes were identified based on similarity with *AAO3* from Arabidopsis (Seo et al., 2000). Putative *AAO31* and *AAO32* genes had 80% and 90% identity with potato *AAO* gene (Accession number; DQ206634.1), respectively. Putative homologue *CYP707A1* and *CYP707A2* genes were identified from tomato ABA catabolic genes, *SlCYP707A1* (Nitsch et al., 2009), and had 91% identity with potato *CYP707A1* (DQ206630.1) and 95% identity with potato *CYP707A2* (DQ206631.1). The putative homologue *PLDα* gene was identified from tomato *LePLDα1* gene (Bargmann et al., 2009), and had 79% identity with tomato *PLD* (AF154425.1).

Quantitative RT-PCR

The quantitative RT-PCR analyses were performed on the Staratagene Mx3005P real-time PCR system using 1 µL of cDNA in a 14 µL reaction with 2× SYBR Green Master Mix

(Applied Biosystems). Reaction parameters were: 95°C for 10 min; 95°C for 30 s followed by 60 °C for 1 min (40 cycles); and melting curve analysis. Normalization of gene expression was calculated using the geometric mean of expression of *CYP* and *EF1α*. All analyses were performed using four replicates.

Leaf ABA Content Determination

Leaf tissue was ground in liquid nitrogen and extracted in darkness in an ABA extraction buffer (80% methanol with butylated hydroxytoluene at 100 $mg \cdot L^{-1}$, and citric acid at 500 $mg \cdot L^{-1}$) for 16 h, with constant shaking at 4°C. After overnight extraction, the supernatant was collected after centrifugation and diluted 10 fold with TBS buffer (50 mM Tris, 1 mM $MgCl_2$, 150 mM NaCl, pH 7.8). Subsequently, the ABA concentration was quantified using ELISA with the Phytodetek ABA test kit (Agdia) following the manufacturer's instructions.

Experimental Design and Statistical Analysis of Data

A randomized complete block design with four blocks was used in this experiment. Physiological data and relative gene expression data were analyzed using *proc glm* in SAS 9.2 (SAS Systems) using repeated measures at α=0.05. ABA concentration was analyzed using log transformed [ABA] data. To indicate the physiological and gene expression changes during the drying period, regression analysis was performed in SigmaPlot (Systat). The regression analysis between g_s, ABA concentration, Ψ_{leaf}, and the interaction between ABA concentration and Ψ_{leaf} was performed in SAS 9.2 (SAS Systems) combining data from all sampling times after log transformation of ABA concentration and g_s. Leaf size, leaf relative water content, and shoot

dry weight were analyzed using *proc anova* in SAS (SAS Systems). Mean separation for the harvest data was done using Fisher's protected least significant difference (LSD) procedure.

Supplemental Data

Supplemental Table 5.S1. List of genes and sequence of the identified genes in *Petunia* × *hybrida* 'Apple Blossom'

Supplemental Table 5.S2. Sequence of primers used and annotation of corresponding genes.

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Figure 5.1. Schematic diagram of the soil moisture sensor based irrigation system. 1) capacitance soil moisture sensor, 2) multiplexer, 3) datalogger, 4) relay driver, 5) solenoid valve, 6) pressure compensated emitter. Only one tray is shown in detail although 16 trays were irrigated with the same system.

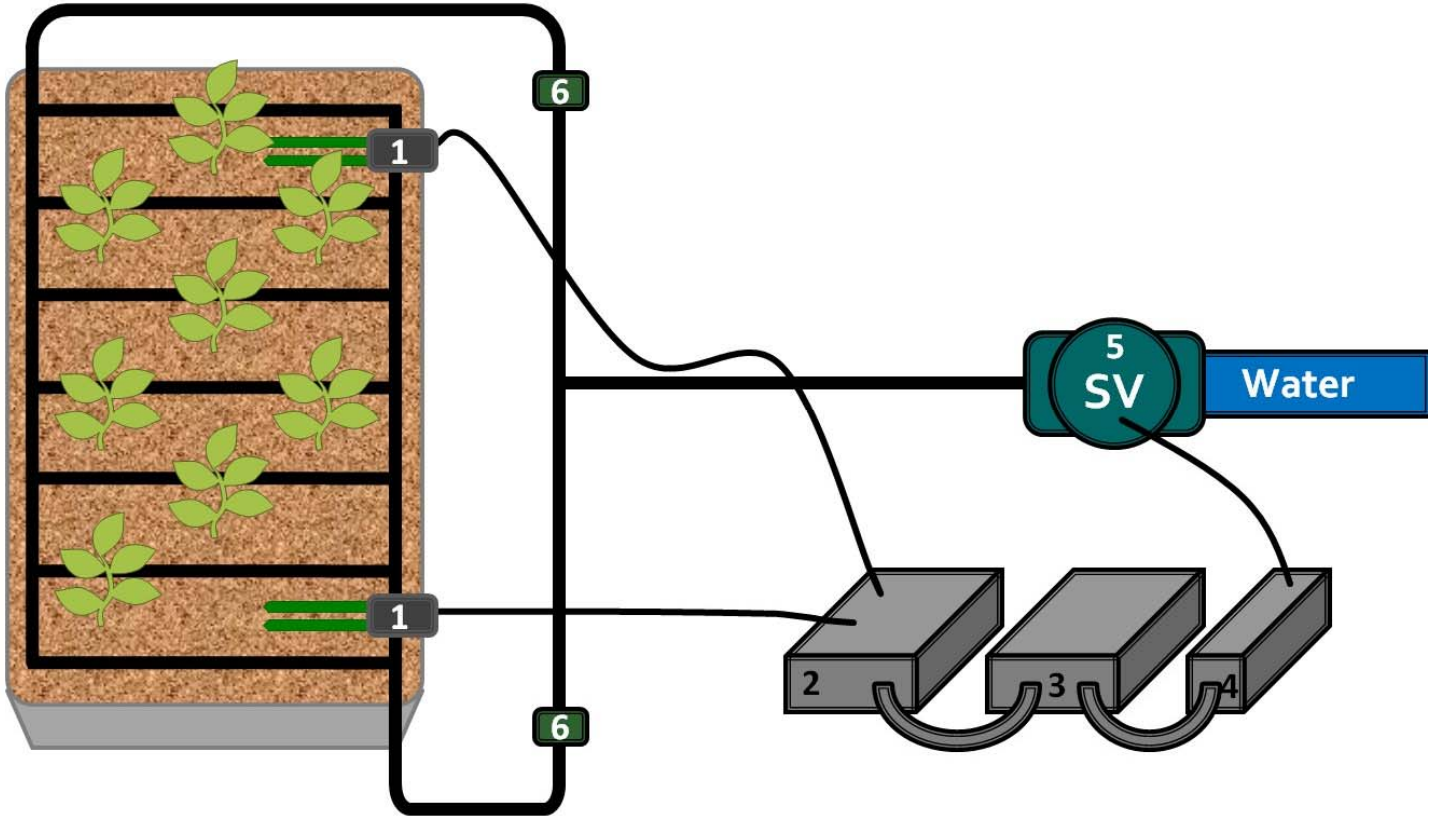

6
1
5
SV
Water
1
2
3
4
6

Figure 5.2. (A) Substrate water contents (θ) and (B-F) leaf physiological responses of *Petunia* × *hybrida* to substrate water content (0.40, 0.30, 0.20, and 0.10 $m^3 \cdot m^{-3}$) over a 16 day period. θ was monitored by soil moisture sensors. Stomatal conductance, photosynthesis, and quantum yield of PSII were measured by leaf photosynthesis measurement system (CIRAS-2; PP Systems), and water potential was measured using thermocouple psychrometers. All the measurements were conducted at noon. The drought treatments reached their threshold θ of 0.30, 0.20, and 0.10 $m^3 \cdot m^{-3}$ after 2.5, 3.7, and 8.8 d, respectively. Error bars indicate the SE.

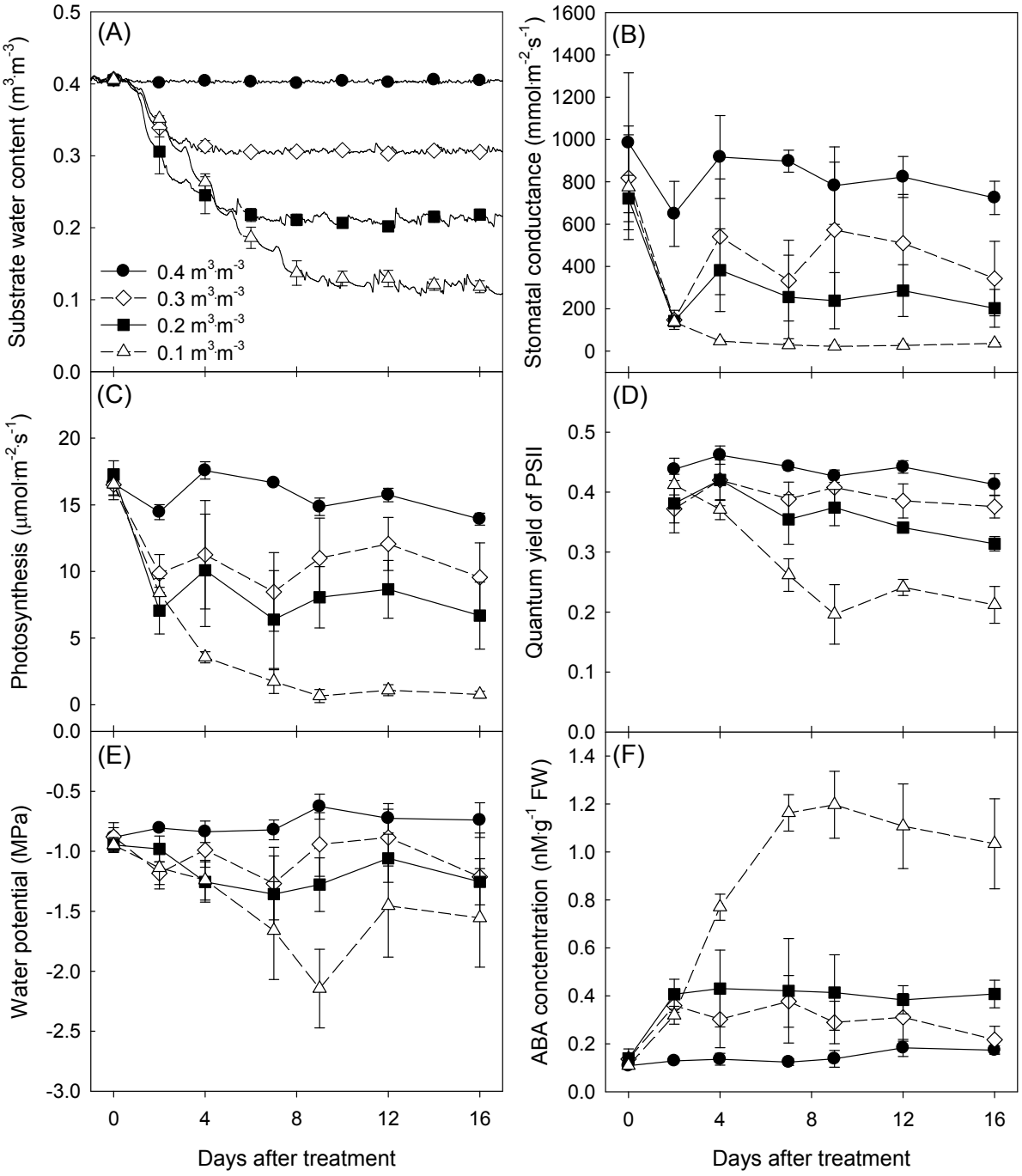

(A)
Substrate water content (m3·m-3)
0.4 m3·m-3
0.3 m3·m-3
0.2 m3·m-3
0.1 m3·m-3
(B)
Stomatal conductance (mmol·m-2·s-1)
(C)
Photosynthesis (μmol·m-2·s-1)
(D)
Quantum yield of PSII
(E)
Water potential (MPa)
(F)
ABA conctentration (nM·g-1 FW)
Days after treatment
Days after treatment

Table 5.1. Morphological and physiological changes of *Petunia* × *hybrida* 'Apple Blossom' in response to various substrate water contents at 16 days after the start of the drying treatment. Leaf relative water content (RWC) was calculated as (fresh weight – dry weight) / (turgid weight – dry weight) × 100%. CO_2-saturated assimilation rates (A_{max}) were calculated using empirical A/Ci curve analysis (Photosyn Assistant; Dundee Scientific). Mean separation was done by Fisher's protected LSD at α=0.05.

Substrate water content ($m^3 \cdot m^{-3}$)	Leaf size (cm^2)	Shoot dry weight (g)	Leaf RWC (%)	A_{max} ($\mu mol \cdot m^{-2} \cdot s^{-1}$)
0.1	6.62 c	2.00 b	72.8 b	31.30 c
0.2	9.31 b	4.06 ab	87.8 a	36.65 bc
0.3	10.71 b	3.82 ab	88.3 a	41.96 ab
0.4	13.35 a	5.58 a	89.3 a	44.66 a

Figure 5.3. Relative expression of drought-related genes in leaves of *Petunia* × *hybrida* in response to various substrate water contents (0.40, 0.30, 0.20, and 0.10 $m^3 \cdot m^{-3}$) during a 16 day period. The drought treatments reached their threshold θ of 0.30, 0.20, and 0.10 $m^3 \cdot m^{-3}$ after 2.5, 3.7, and 8.8 d, respectively. Error bars indicate SE.

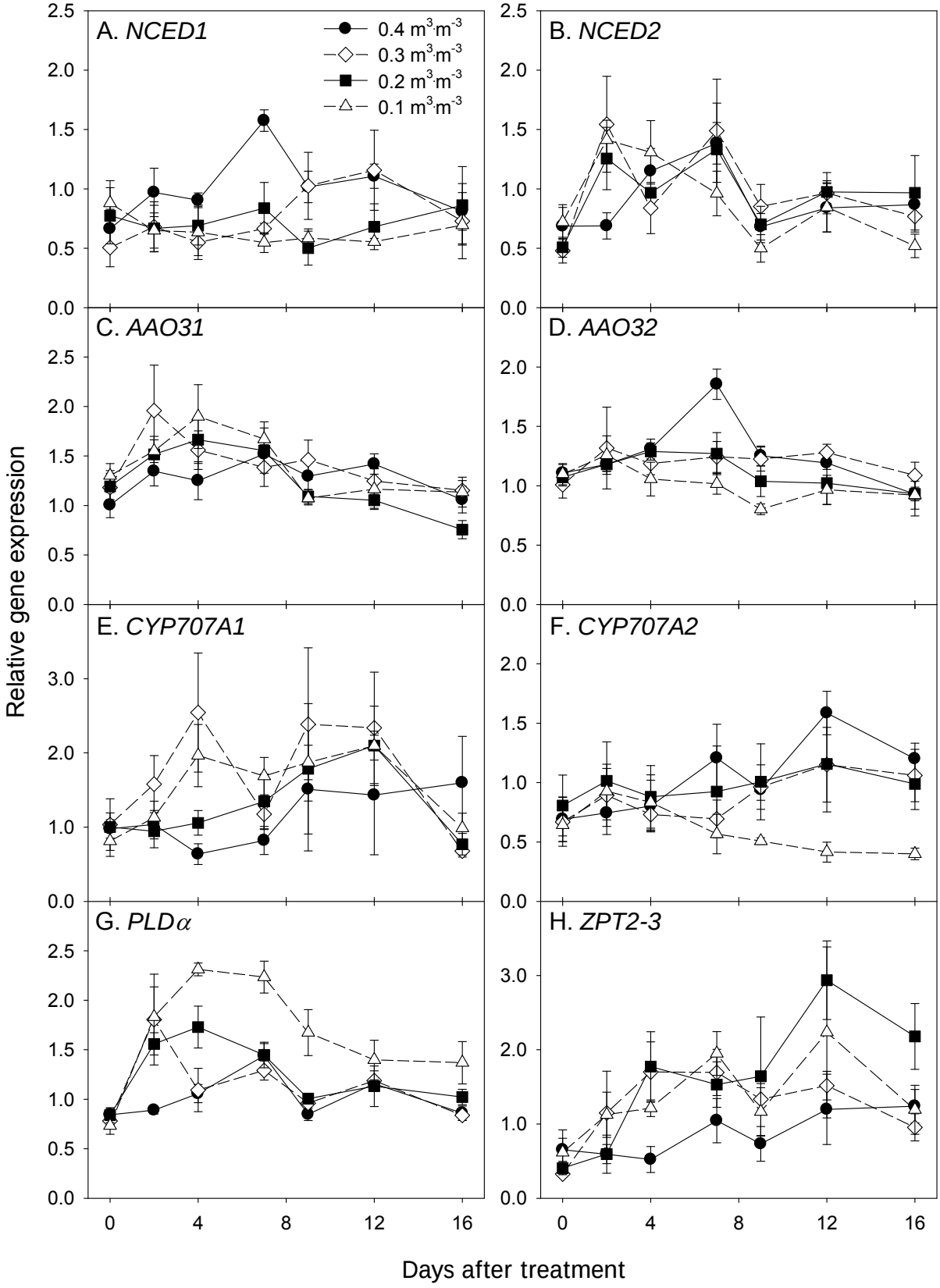

A. NCED1
B. NCED2
C. AAO31
D. AAO32
E. CYP707A1
F. CYP707A2
G. PLDα
H. ZPT2-3
0.4 m3·m-3
0.3 m3·m-3
0.2 m3·m-3
0.1 m3·m-3
Relative gene expression
Days after treatment

Figure 5.4. Relative expression of the putative homologue *CYP707A2* in petunia leaves as a function of substrate water content (A), and ABA concentration (B) (substrate water content treatments: ○ 0.40; ◆, ◇ 0.30; ■, □, 0.20; and ▲, △ 0.10 $m^3 \cdot m^{-3}$). Closed symbols represent data collected during the drying period and open symbols represent data collected after substrate water content thresholds had been reached.

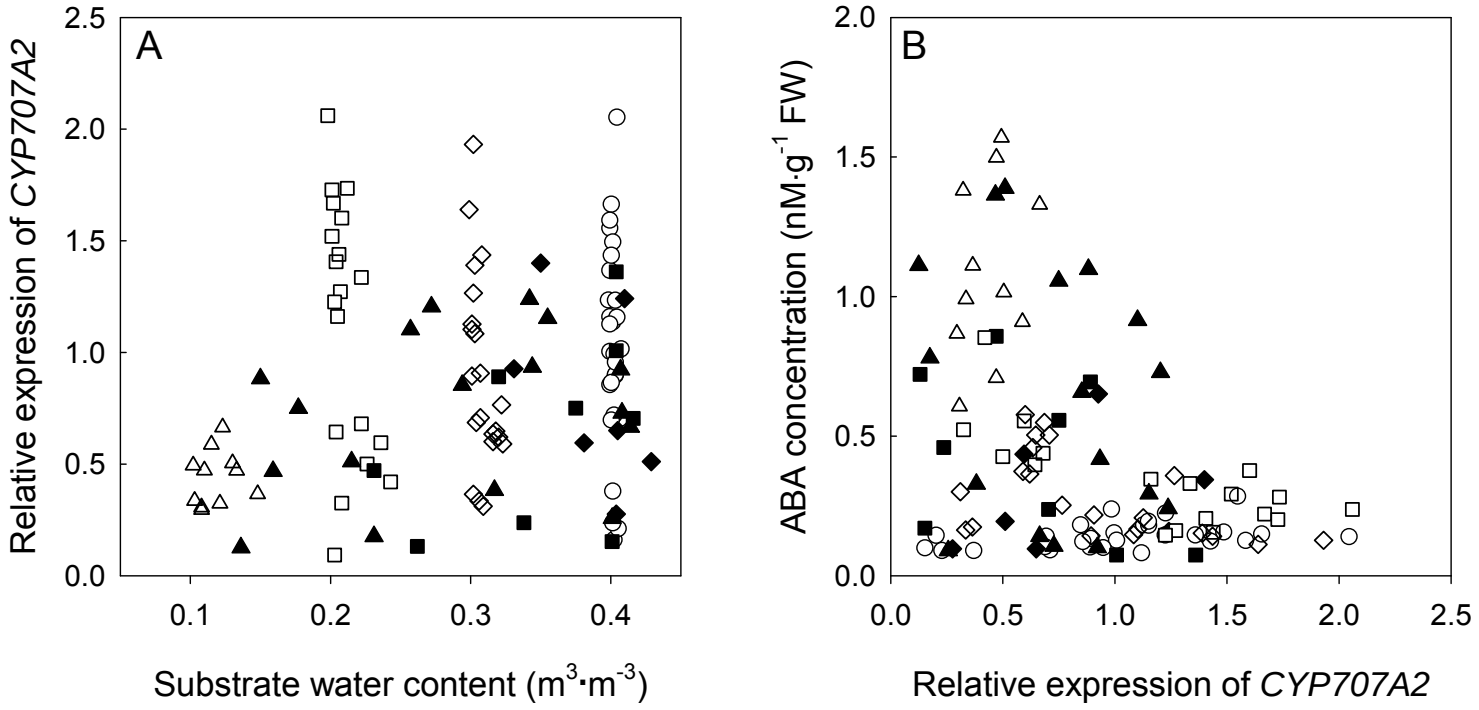

A
Relative expression of CYP707A2
Substrate water content (m3·m-3)
0.0
0.5
1.0
1.5
2.0
2.5
0.1
0.2
0.3
0.4
B
ABA concentration (nM·g-1 FW)
Relative expression of CYP707A2
0.0
0.5
1.0
1.5
2.0
2.5

Figure 5.5. Relative expression of the putative homologue *PLDα* in petunia leaves as a function of (A) substrate water contents and (B) ABA concentration, and (C) its effect on stomatal conductance (substrate water content treatments: ○ 0.40; ◆, ◇ 0.30; ■, □, 0.20; and ▲, △ 0.10 $m^3 \cdot m^{-3}$). Closed symbols represent data collected during the drying period and open symbols represent data collected after substrate water content thresholds had been reached.

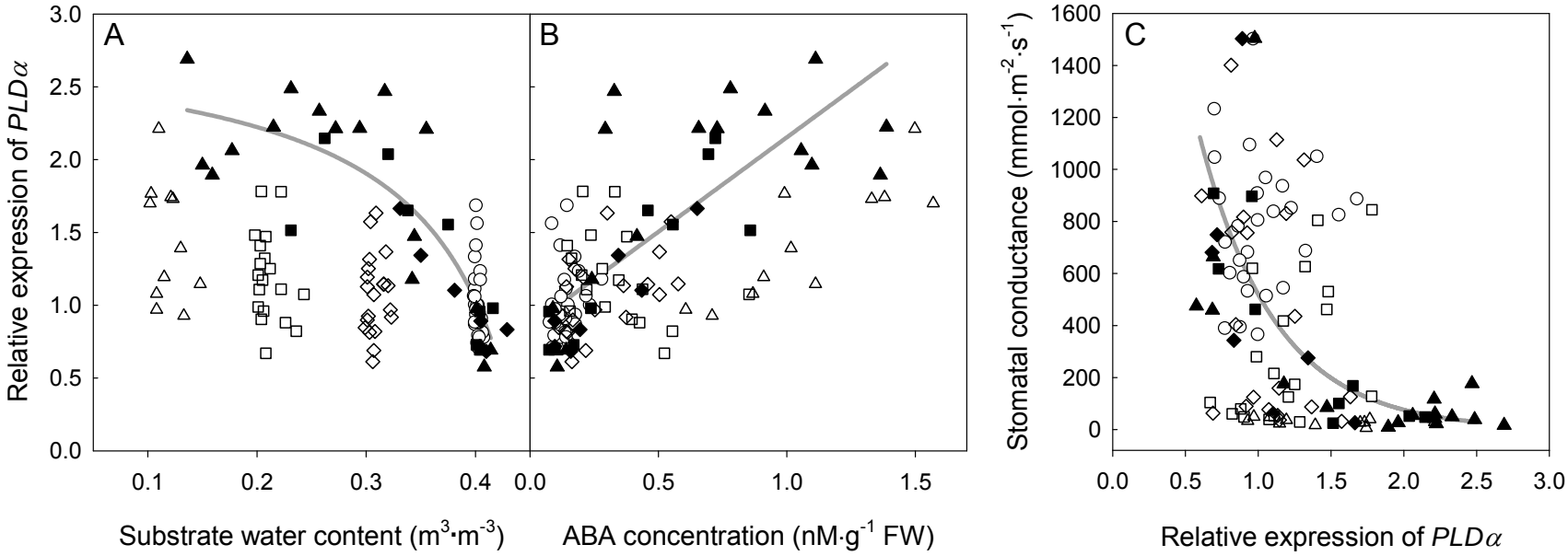

A
B
C
Relative expression of PLDα
Substrate water content (m3·m-3)
ABA concentration (nM·g-1 FW)
Stomatal conductance (mmol·m-2·s-1)
Relative expression of PLDα

Figure 5.6. Leaf physiological responses of petunia as a function of substrate water contents (substrate water content treatments: ○ 0.40; ◆, ◇ 0.30; ■, □, 0.20; and ▲, △ 0.10 $m^3 \cdot m^{-3}$). Closed symbols represent data collected during the drying period and open symbols represent data collected after substrate water content thresholds had been reached.

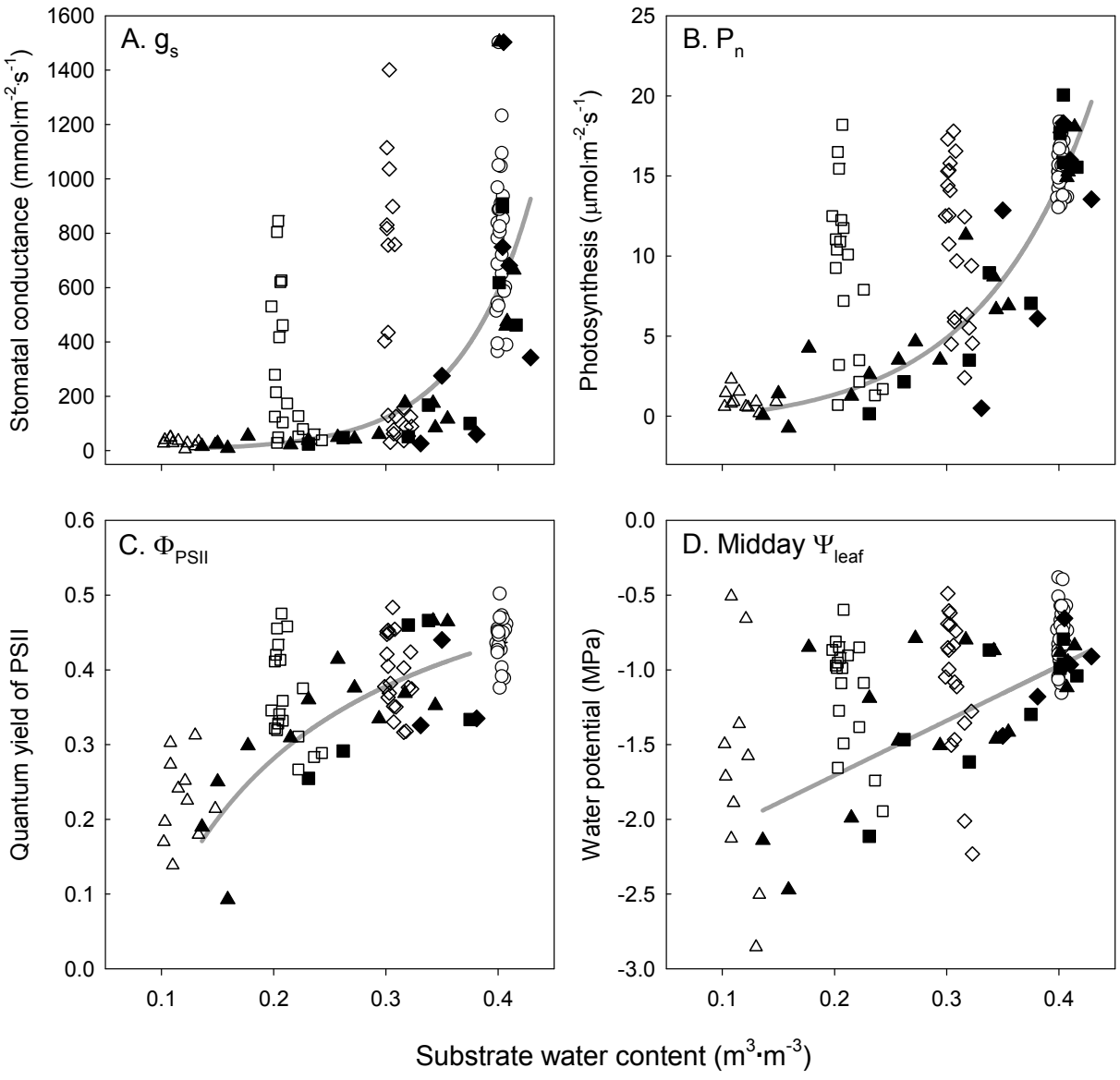
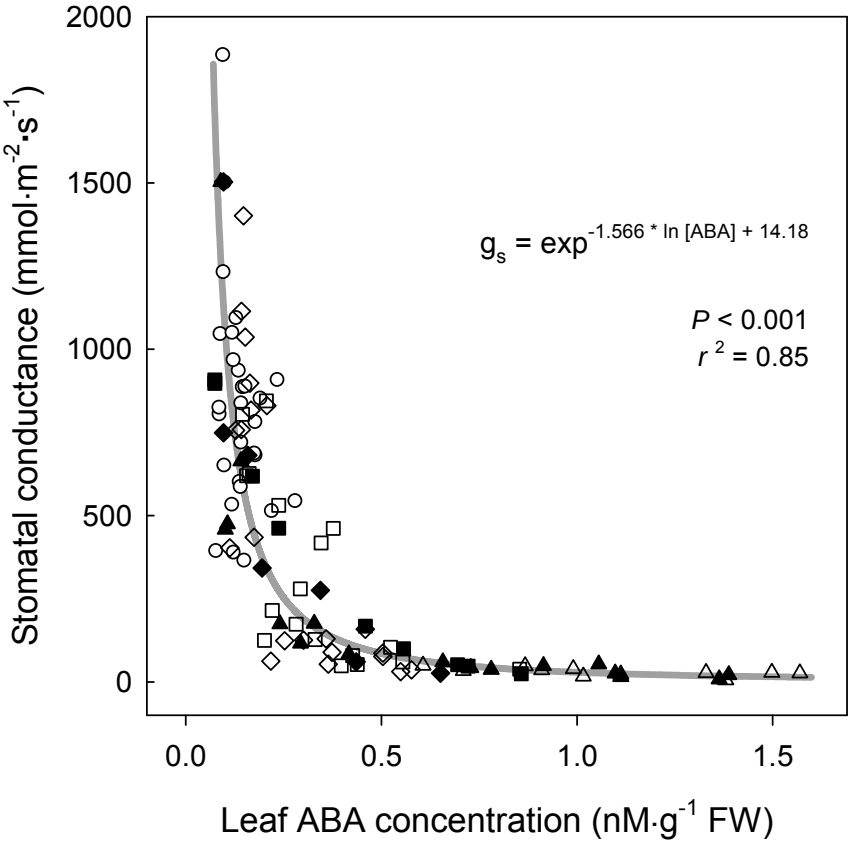

A. g_s
Stomatal conductance (mmol·m^{-2}·s^{-1})
1600
1400
1200
1000
800
600
400
200
0
B. P_n
Photosynthesis (μmol·m^{-2}·s^{-1})
25
20
15
10
5
0
C. Φ_{PSII}
Quantum yield of PSII
0.6
0.5
0.4
0.3
0.2
0.1
0.0
D. Midday Ψ_{leaf}
Water potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
0.1
0.2
0.3
0.4
Substrate water content (m^3·m^{-3})

Figure 5.7. Leaf ABA concentration effects on stomatal conductance. Data from all treatments and the entire study period are combined. Substrate water content treatments: ○ 0.40; ◆, ◇ 0.30; ■, □, 0.20; and ▲, △ 0.10 $m^3 \cdot m^{-3}$. Closed symbols represent data collected during the drying period and open symbols represent data collected after substrate water content thresholds had been reached.


2000
1500
1000
500
0
0.0
0.5
1.0
1.5
Stomatal conductance (mmol·m-2·s-1)
Leaf ABA concentration (nM·g-1 FW)
gs = exp-1.566 * ln [ABA] + 14.18
P < 0.001
r 2 = 0.85

Supplemental Table S5.1. List of genes and sequence of the identified genes in *Petunia* × *hybrida* 'Apple Blossom'

Original Source	Annotation	Expressed Sequence Tag from *Petunia* × *hybrida* 'Apple Blossom'
> Tomato gi|2769641|emb|Z97215.1| *Lycopersicon esculentum* mRNA for nine-cis-epoxycarotenoid dioxygenase	*NCED1*	AGAAGANGATGGATACNTTCTTGCATTTTGCCATGATGAGAAGACATGGAAATCAGAACTTCA AATTGTGAATGCCATGACTCTAGAAT**TGGAGGCTACTGTTGAGCTTCCTT**CAAGAGTTCCATAT GGTTTTCATGGGACTTTCATTACCTCAAAGGACTTGCAAAATCAAGTATAGTAGTAAAAAAAAA TTACNGGTATATAAGTACATAAAAATTAAGGGTGGGATATTATTTATACTTACNAGTGTGTTAT TGGACAAGGGAGATTTTCAA**AAACCAAGCTTGAAGCTTGTAGGT**
	NCED2	TTCTTGCTTTTGTGNNTGATGAAAANNGAATGGAAATCTGAGTTACAAATTGTCAATGCAATGA CCATGAAANNNGA**GGCTTCAGTGAAACTTCCATCAAGAGTCC**CTTATGGTTTTCATGGCACAT TTATAAATGCCAAGGACTTGGTTAATCAGGCCTAATTTGGACTATTACAGAGGAAATTTA**CCAG AGGGATGGTTTAGAAATACGTCCC**CGGAATTTCCTCTATAATAGGGTTCTATAGTTTTTT
>gi|145329960|ref|NM_001084497.1| *Arabidopsis thaliana* AAO3 (Abscisic ALDEHYDE OXIDASE 3)	*AAO31*	TCTGGTGACC**ACCACTACTTCTGGCAGCTTCAGT**CCATTGTGCAACAAAAGCAGCTACTAAAGC AGCAAGGGAACAGCTAAAACATTGGGACAAGCTTGAGGGGNCAGTTTCAGAATTCTATCTGNA TGTCCCTGCCATATTACCTACTG**TGAAGACACTCTGTGGCCTGGATT**ATGTGGAGAAATTCTTG GAAAGTNTACTGGNTCAACAATCTAACTAAATTCCAGAGCACGATGTGGNNGNAGACATTACA TGTGGCCNGNANCACAAGGATATG
	AAO32	GCTNGCAGCTTCNGTCCNTT**GTGCAACAAGAGCTGCGATTAAAGC**AGCAAGAGAACAGCTCA AACTTTGGGGCAAGCTTGAGGGATCTGTTTCAGAATTCTATCTGGATGTTCCTGCCATATTACCT GTTGTCAAGACAC**AGTGTGGCCTGGATTATGTGGAGA**AATTCTTGGAAAGTTTTTTGGAAAGC CACTAGTGGGTAATTGAATTTGGGTGTAATTGAAAGTAATTACAGAATGAAATCTACGCAGTTG GAGACATTTTCTGTGCCCTGGATCAGAAGGATAAGTGCTCATTATGGCTCAATGATTCTGTATA GTTGCTTTACTTTAAACTCTTTTAGGAATTTGAATCGTTCTGTTTGCACAAAGTGTATGATTAAAA GTTAAAAGTTCTTTGTAATCCTGTCTATGGCGATGTCAGTGTAAATTTAGATTGTATAGTAGTAG AAGTGATGTTGTGTATTGAACTAG
> CYP707A1_Tomato gi|160369825|gb|EU183	*CYP707A1*	AAAGTTCTAAATTGGTTAGATACTAAACAGATGCCTATGACTACNAGGGTGATTCAAGAAACTC TTAGAGTTGCTTCAATCTTATCTTTTACTTT**CAGAGAAGCTGTTGAGGATGTTGAGT**TTGATGG

406.1| *Solanum lycopersicum* ABA 8'-hydroxylase (CYP707A1) mRNA, complete cds		ATACTTAATACCTAAAGGATGGAAAGTACTACCACTCTTTAGGAACATTCATCACAGTCCAGAC AACTTTACAGAACCAGAAAAGTTTGATCCTTCAAGATTTGAGGTTNCNCCAAANCCCAATAC**AT TCATGCCATTTGGCAATGGGACC**CACGCATGTCCAGGGAATGAGTTAGCTAAAATGGAAATTT TGATCCTAGTACATCATCTGACTACAAAGTACAGGTGGTCTTTGATGGGCCCACAAAATGGAAT TCAATATGGGCCATTG
	CYP707A2	ACCCAATACATTTATGCCATTTGGCAGTGGAGTACA**TGCTTGTCCAGGAAACGAACTTGC**CAAG CTGGAAATTCTAATTATGACACATCATCTAGTCTCCAAGTTCAGGTGGGAAGTGGTAGGATCTG GTAGTGGGATTCAATATGGACC**ATTTCCAGTTCCAGTGGGTGGACT**ACCAGCAAGATTTTGGA AAGAATCTACTACCTCAACCTAAAGGCACTGAGCAGCAAAAATATGACTCTTCTGATTTGCATAT CTCAAATGATTTCATCAATCTTATCCAAGATTGTTACTCATCCCCTCAAAACATTATAGGGGAGG AAAGAGATGTCCATTTTTCACCCTACAAATTAGTTTTATTCTATATTCCAGAGTTTACTAAAGGT GCCCTAAATTTGAGTACCTTTAAAGCTAGCAACTTGTNCCATTTCCAATTT
>gi|6573118|gb|AF201661.1| *Lycopersicon esculentum* phospholipase D alpha mRNA, partial cds	*PLDα*	GCTNGGGGTCAGGTCCNTGGCTTCCGAATGTCATTATGGTATGAACACTTGGGCATGTTAGAC AACAATTT**CCTTTATCCAGAGAGCTTGGAATGC**ATCCNAAAGGTGAACCAAGTAGGTGAGAAA TATTGGGATTTGTATTCAAGTGAGAGCTTGACTCAAGATTTGCCTGGCCACCTGCTTAGTTACCC TATAGGGGTCACTGAAAATGGACAGGTGAC**TGAACTACCTGGAGCAGAAA**ACTTCCCAGACA CCAAGGCTCCNGTTCTTGGTACCAAATCTGATTTTCTTCCTCCAATTCTCACAACTTAAAACTGGT CAAGTTTTGTTTTTTCTCCTCTCTAGTCATACCATTATATACNATGTTAATAAGGTTTTTAATTAA GGAGAAAAAGGGTATTGCTGGCAATACTATTATAATTTTGTGTGTACCAGCTCTNGTATTTTCT ACATCCNGTT
>gi|92834401|dbj|DD138887.1| Uses of zinc finger transcription factor ZPT2-3 for improving desiccation-resistance of plants	*ZPT2-3*	As in http://www.ncbi.nlm.nih.gov/
> Reference genes	*CYP* *EF1α*	As in SGN-U207468 As in SGN-U207595

Supplemental Table S5.2. Sequence of primers used and annotation of corresponding genes. Primers for real RT-PCR were designed using standard set of reaction conditions and a set of stringent criteria: T_m of 60°C ± 2°C, PCR amplicon length of 60 to 150 bp, primer length of 20 to 30 bp, and a GC content of 35 to 55%.

	Annotation	Forward primers 5' 3'	Reverse primers 5' 3'
> NCED1|Tomato gi|2769641|emb|Z97215.1| *Lycopersicon esculentum* mRNA for nine-*cis*-epoxycarotenoid dioxygenase			
PETIN084121	*NCED1*	TGGAGGCTACTGTTGAGCTTCCTT	ACCTACAAGCTTCAAGCTTGGTTT
PETIN041019	*NCED2*	GGCTTCAGTGAAACTTCCATCAAGAGTCC	GGGACGTATTTCTAAACCATCCCTCTGG
>gi|145329960|ref|NM_001084497.1| *Arabidopsis thaliana* AAO3 (Abscisic ALDEHYDE OXIDASE 3)			
PETAX039740	*AAO31*	ACCACTACTTCTGGCAGCTTCAGT	AATCCAGGCCACAGAGTGTCTTCA
PETIN023342	*AAO32*	GTGCAACAAGAGCTGCGATTAAAGC	TCTCCACATAATCCAGGCCACACT
> CYP707A1_Tomato gi|160369825|gb|EU183406.1| *Solanum lycopersicum* ABA 8'-hydroxylase (CYP707A1) mRNA, complete cds			
PETIN061494	*CYP707A1*	CAGAGAAGCTGTTGAGGATGTTGAGT	GGTCCCATTGCCAAATGGCATGAAT
PETIN048226	*CYP707A2*	TGCTTGTCCAGGAAACGAACTTGC	AGTCCACCCACTGGAACTGGAAAT
>gi|6573118|gb|AF201661.1| *Lycopersicon esculentum* phospholipase D alpha mRNA, partial cds			
PETIN026822	*PLDα*	CCTTTATCCAGAGAGCTTGGAATGC	TTTCTGCTCCAGGTAGTTCAGTCACC
>gi|92834401|dbj|DD138887.1| Uses of zinc finger transcription factor ZPT2-3 for improving desiccation-resistance of plants			
NCBI	*ZPT2-3*	TGTCAGCATGGGAGGAGATGAACA	TACCACCGTCATAGTGGCACCTTT
> Reference genes			
SGN-U207468	*EF1α*	CCTGGTCAAATTGGAAACGG	CAGATCGCCTGTCAATCTTGG
SGN-U207595	*CYP*	AGGCTCATCATTCCACCGTGT	TCATCTGCGAACTTAGCACCG

CHAPTER 6

CONCLUSIONS

The objectives of this research were to improve our understanding of the water relations of bedding plants for efficient irrigation, better water management, and determining the effects of drought stress. Using a datalogger-based, automated irrigation system with soil moisture sensors and load cells allowed for a more descriptive and repeatable approach to plant-water relations research, with precise control of irrigation and/or drought severity. A well-monitored and -controlled substrate water status was also beneficial in acquiring θ data corresponding to the time of plant physiological and gene expression measurements at specific θ.

As a result of estimating the daily water use of petunia, regression models with plant age and easily acquired environmental factors could explain more than 90% of fluctuations in daily water use of two petunia cultivars (*Petunia* × *hybrida* 'Single Dreams Pink' and 'Prostrate Easy Wave Pink'). These models may help greenhouse growers plan their irrigation management and reduce excessive use of water, fertilizer, electricity, and labor. However, these models still need evaluation to test their predictive ability to estimate daily water requirements. To evaluate the model, another independent study should be conducted in a variable environment. As my results showed, environmental factors in greenhouses are not independent from one another (*e.g.*, daily light integral, temperature, and vapor pressure

deficit were correlated with each other). Therefore, modification of individual environmental factors [*e.g.* light intensity by shading or vapor pressure deficit using (de)humidification] may be beneficial to minimize multicollinearity among the environmental variables to more precisely determine their individual contributions to daily water use. Further, larger environmental variation during such studies may provide for broader applications of the model.

Only one substrate and fertilizer rate were used for estimating water use. However, water use of plants might depend on substrate type or fertilizer rate, and be affected by substrate physical properties, such as water holding capacity, or electrical conductivity. Therefore, evaluation of water use with various substrate types or fertilizer rates might be helpful to improve the models.

A sensitivity analysis may improve our understanding of water use of the plants. My plant water use model was developed in the greenhouse, where several environmental factors fluctuated at the same time. This may be acceptable for practical use, but the effect of the different environmental variables could not be evaluated independently. Environmental factors, such as light, temperature, relative humidity not only affect plant physiology, but also plant water use. Therefore, if we vary only one environmental factor at a time, we may be able to determine how much each environmental factor influences plant water use. This experiment needs to be carried out in a well-controlled environment, such as a growth chamber. We can install an automated irrigation system inside the growth chamber, and control the environmental conditions inside the growth chamber. Such a study could be used to quantify how light intensity, temperature, and relative humidity influence plant water use.

The automated irrigation system in my daily water use study replenished substrate water whenever θ dropped below 0.40 $m^3 \cdot m^{-3}$, at 10 min intervals. However, in commercial greenhouses, such frequent irrigation might not be practical, unless the greenhouse uses a similar irrigation system. This irrigation frequency may also affect substrate water status and plant growth. Commercial greenhouse growers generally irrigate plants once, twice, or three times a day, and the effect of this frequency may need to be taken into account. Modification of the automated irrigation system by changing the irrigation interval and duration can simulate the different irrigation frequencies. Thus, this study may be able to identify the effect of the frequency of irrigation on water dynamics in the substrate and plant water use.

As the drying rate and θ-specific drought studies showed, our automated irrigation system provided many benefits in research, by maintaining precise and well-controlled θs. In the drying rate study, the automated irrigation system controlled the different rates of drought imposition as designed. Although the study showed that reductions in photosynthesis and transpiration of vinca were less severe at slower drying rates, this response may differ among species. Therefore, similar studies with different species might be worthwhile to compare their acclimation ability to drought stress.

Imposing mild drought to plants before marketing provides benefits such as longer postproduction life and better landscape performances after transplanting. Therefore, intentionally imposed, mild drought can be used as a production strategy to increase acclimation of plants to drought, but the term 'mild' in mild drought is very ambiguous. My results show that petunias at θ of 0.20-0.30 $m^3 \cdot m^{-3}$ partially recovered g_s and photosynthesis after θ reached the threshold levels, and plants at θ of 0.20 $m^3 \cdot m^{-3}$ showed increased

expression of the drought tolerance gene *ZPT2-3*. Also, the drying rate study showed that slowly-developed drought increased photosynthetic acclimation by providing plants more time to adjust themselves to drought environment. Based on my results, the optimal strategy may be to impose mild drought by irrigating with slightly less water than the amount used by the plants (*e.g.* ≈50% of estimated amount from daily water use study, but maintaining θ at least at 0.20 $m^3 \cdot m^{-3}$) for a week or two before marketing. Such a strategy may produce acclimated (*i.e.* drought tolerant), high quality plants, as well as save irrigation cost. To test this, the study investigating drought tolerance of plants previously exposed to different severities of drought stress might be helpful.

In the gene expression study, I found that the expression of the drought tolerance gene *ZPT2-3* increased under mild drought, but I did not investigate the effects on drought tolerance. Previous research showed that overexpression of *ZPT2-3* in petunia induced higher survival rates than wild type plants after 30 d of drought. However, drought tolerance of plants is not merely about survival, but also about physiological responses and acclimation that may make plants more drought tolerant. Therefore, a study combining gene expression with physiological responses, to identify whether this increased expression of drought tolerance genes actually results in physiological responses and increases drought tolerance may be beneficial to understanding drought tolerance and acclimation.

Although single-leaf physiological measurement can provides a good indication of plant responses to stress, single leaves do not always accurately represent whole plants, making it hard to predict whole plant responses based on single leaf measurements. In the drying rate study, a whole-plant gas exchange system with load cells provided benefits looking at whole-

plant level physiology (whole-plant photosynthesis, respiration, and transpiration rates). Semi-continuous, whole plant measurements showed the physiological responses of plants in response to drought over time. The petunia study with different θ levels indicated that plant physiological responses were different during the drying period as compared to the period that θ was at the threshold level (*i.e.*, acclimation). Since plant responses to environmental stress are not static, but dynamic, temporal measurements of plant responses are important in identifying changes in physiology over time. In the gene expression study, temporal analyses of plant genetic and physiological responses showed changing responses over time, indicating the importance of taking measurements over time.

Although the expression of the ABA catabolic gene *CYP707A2* was associated with ABA levels, ABA biosynthesis genes (*NCED* and *AAO3*) did not show any significant responses to drought. Although ABA is regarded as the primary hormone in signaling drought in plants, its signaling mechanism is still under debate. Much research identified root-synthesized ABA as the main source of ABA, but recent studies showed that *de novo* ABA synthesis in arabidopsis leaves plays an important role in regulating stomata. This might be due to species-specific ABA signaling. To better understand ABA signaling, further studies on identifying the ABA source and transport mechanism should be conducted with different species.

Gene expression may be a good indication of plant responses to environmental stresses. Lack of genetic information complicates gene expression studies of ornamental plants, but this may improve as more genetic information becomes available. However, as my results from the gene expression study indicate, gene expression analysis using only one plant tissue may not allow for complete comprehension of whole plant responses to the environment. Ideally, gene

expression and physiological responses would be monitored in multiple tissues. Neither gene expression nor physiology by themselves can provide a complete picture of plant responses to drought. Therefore, more integrated studies, with collaboration between plant physiologists and plant molecular biologists, might improve our understanding of plant responses. Although many drought responses of plants have been studied, integrated studies on understanding the physiological, biochemical, and genetic responses of plants are still rare. To improve our understanding of plant-water relations, such integrated studies are needed.