PHYSIOLOGICAL AND BIOCHEMICAL FACTORS ASSOCIATED WITH DROUGHT TOLERANCE OF *AGROSTIS STOLONIFERA*

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ABSTRACT OF THE DISSERTATION

Physiological and Biochemical Factors Associated with Drought Tolerance of *Agrostis Stolonifera*

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Drought stress is a primary factor limiting the growth and productivity of many plant species and is caused by lack of rainfall and declining availability of fresh water for irrigation. Creeping bentgrass (*Agrostis stolonifera* L.) is a cool-season grass species commonly maintained as high-value turfgrass on golf courses across the world. As global climate change progresses, creeping bentgrass stands may be exposed to increased frequency of drought episodes or longer durations of drought stress. Therefore, a better understanding of the physiological and biochemical factors contributing to drought tolerance in creeping bentgrass may aid turfgrass managers in maintaining high-quality playing conditions when water for irrigation is limited. The goals of this research were to explore which physiological and biochemical factors are associated with drought tolerance of creeping bentgrass and how elevated carbon dioxide (CO_2) may mitigate drought damages in this widely-utilized turfgrass species. This was accomplished in several studies which 1) investigated whether sequential exogenous applications of plant

growth regulators (PGRs) and osmoregulants promote drought tolerance of creeping bentgrass under field conditions, 2) elucidated on the link between fatty acid metabolism in creeping bentgrass leaves and roots and the observed level of drought tolerance, and 3) explored the interactive effects of elevated CO_2 and drought stress on growth, morphology, physiology, and biochemical properties such as protein, carbohydrate, and hormone metabolism of creeping bentgrass turfgrass. The potential implications of the research may help to improve efficacy for various aspects of turfgrass management such as efficient utilization of irrigation water and decreased need for supplementary chemicals including fertility and pesticides. The lessons derived from the research may have implications across many different turfgrass management aspects and provide turfgrass managers new techniques to maintain high-quality playing conditions at a lower economic cost and less environmental impact.

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LITERATURE REVIEW

MECHANISMS OF HORMONE REGULATION FOR DROUGHT TOLERANCE IN PLANTS

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ABSTRACT

Drought stress limits the growth and productivity of plants through significant changes at the physiological, cellular, biochemical, and molecular levels. Considerable progress has been made elucidating on how plant hormones contribute to or influence whole-plant drought responses. Advancements in transcriptomics coinciding with mutant analysis have suggested that specific hormones regulate processes such as leaf senescence, antioxidant metabolism, carbon balance, and gas exchange during periods of drought stress. Hormones including abscisic acid, auxins, cytokinins, gibberellins, ethylene, salicylates, and jasmonates may independently regulate these plant responses through regulation of transcription factors and subsequent downstream induction or repression of stress-responsive genes. Alternatively, hormone-to-hormone or hormone-to-sugar crosstalk may facilitate the drought responses. This chapter provides an overview of the major physiological processes regulated by plant hormones and the roles that different hormones serve during physiological responses to drought stress. Current knowledge of hormone-to-hormone or hormone-to-sugar crosstalk regulating physiological responses to drought stress is also discussed. Suggestions for ongoing and future research are provided, such as which molecules perceive a specific hormone to then initiate signal transduction cascades during drought stress. Crosstalk signals derived from hormone-to-hormone or hormone-to-sugar or secondary-messengers (i.e., calcium or ROS) are not yet clear and also need to be investigated further. Research addressing the unanswered questions of hormone-signaling perception and crosstalk among hormones and other metabolites will provide further insights into molecular factors controlling hormone regulation of plant tolerance to drought stress.

ABBREVIATIONS

ABA: Abscisic acid; APX: Ascorbate peroxidase; ARF: Auxin response factor; ASA: Ascorbic acid; CAT: Catalase; CK: Cytokinin; ERF: Ethylene response factor; GA: Gibberellic acid; GR: Glutathione reductase; GSH: Glutathione; IAA: Indole-3-acetic acid; JA: Jasmonic acid; MDA: Malondialdehyde; MeJA: Methyl jasmonate; POD: Peroxidase; ROS: Reactive oxygen species; SA: Salicylic acid; SOD: Superoxide dismutase

INTRODUCTION

Drought stress caused by lack of rainfall or declining fresh water supplies for irrigation imposes significant limitations to growth and productivity of many plant species across different climatic areas. Predictive models of global climate change have shown that frequency of precipitation events and net volumes have changed drastically during the past one hundred years, suggesting that certain regions may experience drought episodes more frequently and of longer durations in the future (Solomon et al. 2007).

The far-reaching effects of climate change in conjunction with an increasing global population will likely contribute to greater instability in food security and underscores the need for greater knowledge of the specific mechanisms underlying drought tolerance across major and novel plant species. The physiological effects of long- or short-term drought stress have been well characterized for the major grain crops such as maize (*Zea mays*), wheat (*Triticum spp*.), rice (*Oryza sativa*), and barley (*Hordeum vulgare*) and have also been investigated to a lesser extent in certain novel or underutilized crop species (Graham and Vance 2003; Hanjra and Qureshi 2010; Kang et al. 2009; Ruiz et al. 2014; Zwart and Bastiaanssen 2004). Drought stress can also impose functional limitations upon non-crop species such as trees, shrubs, and perennial grasses (Abrams 1994; Bréda et al. 2006; Condit et al. 1995; Hacke et al. 2000; Tester and Bacic 2005).

The extent of damage sustained during drought stress depends on factors including plant species or variety, developmental stage, rate of soil water decline, frequency of drought events, and duration of water deficit (Mahajan and Tuteja 2005;

Reddy et al. 2004). These interacting factors cause significant changes at the physiological, cellular, biochemical, and molecular levels preempting the observed decline in plant performance or net yield (Huang 2003; Huang et al. 2014).

Substantial progress has been made to better understand plant drought tolerance mechanisms through research on physiological processes (water relations, carbon metabolism), protein metabolism, and genomic factors (Atkinson and Urwin 2012; Cattivelli et al. 2008). More recently, there have been significant advancements in the ability to accurately detect and quantify low-concentration plant hormones to elucidate on how hormone metabolism may regulate whole-plant stress responses (Peleg and Blumwald 2011; Robert-Seilaniantz et al. 2011; Vanstraelen and Benková 2012). Investigating the earliest mechanisms through which the drought-response cascade is initiated in plants may aide in breeding and selecting for drought-tolerant lines and in developing new management techniques to minimize plant damages when water for irrigation is limited.

This chapter focuses on the recent advancements in plant hormone metabolism in relation to drought tolerance with the following aims: (1) to provide an overview of several major physiological drought responses that are highly regulated by plant hormones, including leaf senescence and antioxidant metabolism, carbon metabolism, and stomatal movement; (2) to discuss the roles of different hormones including abscisic acid, auxins, cytokinins, gibberellins, jasmonates, salicylates, and ethylene regulating these physiological responses during plant drought responses; (3) to describe the current knowledge of interactions or crosstalk between various hormones or between hormones and other plant metabolites regulating physiological responses to drought stress; and (4)

to summarize and propose future research perspectives for enhancing our understanding of hormone regulatory mechanisms conferring plant drought tolerance.

MAJOR PHYSIOLOGICAL RESPONSES TO DROUGHT STRESS

Leaf Senescence and Antioxidant Metabolism

Leaf senescence is a key developmental process which occurs naturally during plant maturation and is also a common result of prolonged abiotic stress. The coordinated breakdown and translocation of leaf cellular constituents increases the likelihood for plant survival during short-term stress periods and leaf senescence may be reversed if stress conditions are relieved within a certain time period (Buchanan-Wollaston 1997). Chlorophyll degradation is preempted by protein and RNA degradation mobilizing amino acids and nutrients towards other actively growing tissues or storage organs, thereby enhancing likelihood for drought survival (Buchanan- Wollaston et al. 2003; Hörtensteiner and Feller 2002).

In addition, there are extensive reviews detailing the relationship between oxidative stress agents and the antioxidative mechanisms which mitigate cellular damage to chlorophyll (Apel and Hirt 2004; Blokhina et al. 2003; Mittler 2002). The balance between reactive oxygen species (superoxide radical, hydrogen peroxide, hydroxyl radical) and enzymatic (CAT, POD, SOD, APX, and GR) or nonenzymatic (GSH and ASA) antioxidants, as well as carotenoids and tocopherol, determine the extensiveness of lipid peroxidation leading to chlorophyll membrane failure and eventual leaf senescence (Apel and Hirt 2004; Prochazkova et al. 2001). As opposed to other abiotic stresses such as salinity or UV-B radiation, oxidative stress during drought periods may increase tocopherol, carotenoid, and glutathione, while ascorbate pools tend to decrease (Munné-Bosch and Alegre 2000; Smirnoff 1993). Additionally, plants under drought stress may suppress production of reactive oxygen species and mitigate leaf senescence by

decreasing cytochrome respiration and utilizing alternative respiratory pathways, though the influence of plant hormones on distinct respiratory pathways is not well known (Bartoli et al. 2005; Vanlerberghe 2013). Recent studies which use the systems biology approach have begun to shed light on how specific hormones influence the balance between oxidative stressors and antioxidant agents which mitigate their damaging effects on leaf senescence and are discussed below (Jibran et al. 2013).

Carbon Metabolism

Carbon metabolism or carbohydrate production during photosynthesis supplies the substrates needed to drive an array of growth, energy, and signaling processes in plants. The extent to which drought stress impairs carbon metabolic processes depends on the intensity, duration, and onset rate of the stress and varies based on plant species, maturation, and specific tissue type (Jaleel et al. 2009). It is well known that prolonged drought stress impairs photosynthesis either by decreasing stomatal aperture size limiting CO_2 diffusion into the mesophyll cells or by indirectly inhibiting associated biochemical and photochemical processes (i.e., RuBisCO deactivation or slowed RuBP regeneration) (Bota et al. 2004; Chaves et al. 2002, 2009; Flexas and Medrano 2002; Lawlor 2002). However, despite these limitations, carbon-rich molecules such as soluble carbohydrates (hexose, sucrose, trehalose, mannitol), amino acids (proline), organic acids (malate, fumarate, citrate), and structural compounds (cellulose and lignin) typically increase within plant tissues during drought stress (Muller et al. 2011).

Additionally, many of these compounds act as compatible solutes within cells and protect subcellular structures against damaging effects of water deficit, a topic which has

been reviewed in detail (Chaves et al. 2002; Farooq et al. 2009; Yordanov et al. 2000). As soil water deficit is prolonged and level of drought stress becomes more severe, plant growth rates will decrease which lessens net carbon demand while net photosynthetic rates temporarily remain less affected which maintains net carbon gain within the plant system (Poorter and Nagel 2000). Coinciding with the significant reductions in stomatal aperture size and cellular water content, many plants seek to sustain photosynthesis by altering metabolic aspects such as Rubisco activity or activity of sugar-cleaving enzymes, among other enzymes (Muller et al. 2011). The underlying mechanisms by which carbon-containing molecules interact with hormonal stress-signaling pathways to initiate specific growth processes during drought stress have been of particular interest to researchers over the past several decades and current knowledge is discussed below.

Stomatal Movement

Stomatal closure is the primary line of defense by which plants decrease transpirational water loss to maintain cellular water content during drought stress and is induced by either hydropassive or hydroactive mechanisms (Murata and Mori 2014). Hydropassive stomatal closure occurs most often in environments of low humidity and/or high air currents and is characterized by guard cells quickly losing turgor due to rapid evaporative water loss without timely replenishment of water from adjacent epidermal cells (Wang et al. 2001). Alternatively, hydroactive stomatal closure is a more complex process, occurring as a result of whole-plant (root and shoot) water deficit and involves solute expulsion from guard cells increasing their osmotic potential and causing them to become flaccid and close (Kaiser and Legner 2007). The factors which contribute to hydroactive

stomatal movement (opening or closing) under a variety of abiotic stresses have been of particular interest to researchers within the context of global climate change and limited water supplies for irrigation.

Hormone profiling techniques coupled to genetic approaches such as transcript profiling and plant mutants have offered valuable insight into the signal transduction pathways preempting initiation of stomatal closure during drought stress (Daszkowska-Golec and Szarejko 2013; Dodd 2003). Specifically, ABA-mediated stomatal closure through signal transduction pathways and downstream effects on cellular ion content during drought stress has been investigated over the past several decades, though important factors including mechanisms of drought stress perception activating abscisic acid (ABA) as well as upstream genes in the ABA-signaling pathway remain to be discovered (see ABA section below). Furthermore, recent advances in transcriptomics and next-generation sequencing techniques have suggested critical functions of other hormones and metabolites interacting with ABA and further contributing to stomatal closure during drought stress.

ROLES OF HORMONES REGULATING PHYSIOLOGICAL RESPONSES TO DROUGHT STRESS

Abscisic Acid

Among all classes of drought-responsive endogenous hormones currently known to exist within the plant system, ABA has been implicated as the primary chemical signal initiating stomatal responses to drought stress (Wilkinson and Davies 2002). Provided the abundant current knowledge, emphasis of ABA (and subsequent hormones) research within this chapter will be limited to research conducted during the past fifteen years. It is a well-known fact that ABA concentrations increase in response to drought imposition and the series of chemical reactions involving carotenoids for ABA biosynthesis and catabolism have been previously elucidated through a serious of experiments (Ikegami et al. 2009; Schwartz et al. 2003; Schroeder and Nambara 2006; Sridha and Wu 2006).

The mechanisms or intermediate signaling components by which ABA induces stomatal closure have also been characterized, though little was previously known regarding the earliest events in ABA perception initiating specific signal transduction pathways (Hirayama and Shinozaki 2007; Zhang et al. 2006). A recently discovered family of proteins known as PYRABACTIN (4-bromo-N-[pyridin-2-yl methyl] naphthalene-1-sulfonamide) RESISTANCE (PYR)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) has opened additional avenues for ABA-signaling research and continues to prompt new questions about how the ABA-signaling network operates (Cutler et al. 2010). PYR/RCARs are ABA-binding proteins which interact with two other protein classes, Protein Phosphatase 2Cs (PP2Cs) and SNF1-related protein kinase 2s (SnRK2s), to initiate ABA recognition and signaling cascades. Specifically,

PYR/RCARs are ABA receptors while PP2Cs and SnRK2s are negative and positive regulators of the signaling pathway, respectively (Hubbard et al. 2010). When ABA is present and associates with PYR/ RCARs, PP2Cs are inhibited which subsequently allow SnRK2s to become active and phosphorylate downstream transcriptional factors such as ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2 (ABF2) and ABI5-regulating downstream effects on target proteins gene expression, secondary messenger productions, and ion transport (Fujii et al. 2009; Ren et al. 2010). Alternatively, when ABA is not present, PP2Cs are active and inhibit SnRK2s activity thereby preventing downstream ABA responses. For in-depth descriptions of the PYR/RCAR–PP2C– SnRK2-signaling module and associated questions about the hormonal response pathways, see reviews by Hubbard et al. (2010), Joshi-Saha et al. (2011), Melcher et al. (2010), and Raghavendra et al. (2010).

Technological advancements in next-generation sequencing during recent years have produced extensive transcriptome data sets from plants under a variety of abiotic stresses and with or without interacting hormone factors (exogenous or endogenous) (Cramer et al 2011; Huang et al. 2008; van der Graaff et al. 2006; Zawaski and Busov 2014). Comparatively to other classes of plant hormones, the ABA-regulated genomic changes are two to six times greater and can be in excess of 10 % of the genome in Arabidopsis seedlings (Cutler et al. 2010). ABA-induced genes influence a variety of stress-promoting factors including enzymes to detoxify reactive oxygen species, enzymes for compatible solute metabolism maintaining cell turgidity, protein transporters, transcription factors, and enzymes contributing to phospholipid signaling (Cutler et al. 2010; Nakashima et al. 2009; Nemhauser et al. 2006; Zhu 2002). From the transcriptomic

data, there has been an increased focus on ABA-induced changes to WRKY transcription factors, one of the largest families of transcriptional regulators spanning across many different plant processes (Chen et al 2010). For example, rice lines overexpressing OsWRKY11 were more drought tolerant due to enhanced accumulation of compatible solutes such as raffinose (Wu et al. 2009). It was suggested that ABO3/AtWRKY63 functions in ABA-mediated drought stress response pathways since the abo3 mutation impairs ABA-induced stomatal closure (Ren et al. 2010). Overexpression of the ABA-inducible OsWRKY45 gene in Arabidopsis conferred enhanced tolerance to salt and drought stress possibly due to the plants having a higher proportion of closed stomates (Jiang et al. 2012; Qiu and Yu 2009). While the direct link between WRKY transcription factors and stomatal movement remains to be proven, it is well known that stomatal closure upon drought onset is initiated by ABA-mediated calcium increases in the cytosol which stimulate potassium efflux and increased water potential causing guard cell flaccidity (Himmelbach et al. 2003; Sridha and Wu 2006). Identifying additional ion channels or transporters which contribute to polarization state of plasma membranes for potassium movement has been a recent research focus and new evidence is beginning to suggest that inhibitors of protein kinases and protein phosphatases may further affect transporter capabilities (see review by Sirichandra et al. 2009).

The drought-induced increase in endogenous ABA has also been correlated to leaf senescence and carbon remobilization in wheat (Yang et al. 2001b, 2003). The increase in leaf senescence due to ABA has been associated with induction of lipid peroxidation caused mainly by increased hydrogen peroxide content within leaf cells of rice (Hung and Kao 2003). However, low to moderate ABA concentrations may mitigate downstream

oxidative damages preempting leaf senescence by increasing SOD, CAT, APX, and GR activity as well as carotenoid and tocopherol contents in wheat, though these beneficial effects were no longer evident at excessively high exogenous ABA concentrations (Jiang and Zhang 2001). Jiang and Zhang (2002) also suggested an interrelationship between drought stress-induced ABA production and ROS production stimulating an up-regulation of antioxidant defense system. While it may be inferred that ABA-induced oxidative stress and antioxidant metabolism governing leaf senescence as well as stomatal conductance governing carbon influx for photosynthesis will likely have downstream effects on plant growth, ABA has recently been implicated for direct effects on various aspects of plant growth during drought stress. The historical view of ABA affecting plant growth implied that higher concentrations within the plant system would inhibit shoot growth due to stomatal regulation of water status during prolonged drought stress (Trewavas and Jones 1991). However, systems biology research has utilized ABA-deficient mutants of maize, tomato, and Arabidopsis to suggest that ABA sustains growth of plant organs, namely roots, through antagonism with drought stress-induced ethylene production (Sharp 2002). The potential implications of this hormone to hormone crosstalk for enhancing plant drought tolerance are discussed below.

Auxins

The naturally occurring auxin indole-3-acetic acid (IAA) is synthesized within the rapidly dividing tissues of root and shoot apical meristems and young leaves across virtually all plant species (Ljung et al. 2001, 2005). Historically, the biological connection between auxin and stress-induced leaf senescence has been variable depending on plant species or

tissue maturity, and the specific leaf responses may be dependent not only upon auxin concentration but also cellular responsiveness or sensitivity (Schippers et al. 2007). Some of the earliest studies showed that leaf senescence progresses as IAA concentrations decline towards similar levels between stems and leaves in beans, whereas applying IAA exogenously to the distal or proximal end of abscission zone will delay or promote abscission, respectively (Addicott and Lynch 1951; Shoji et al. 1951). The gradient-dependent manner by which auxin delays leaf senescence has been partly explained by studying AUXIN RESPONSE FACTOR1 (ARF1) and ARF2 genes using Arabidopsis arf1 and arf2 mutants (Ellis et al. 2005; Lim et al. 2010). ARF1 and ARF2 mutants, as well as NPH4/ARF7 and ARF19 mutants, all displayed some degree of delayed leaf senescence, suggesting that the respective ARF transcription factors repress auxin signaling and are positive regulators of leaf senescence, or, auxin is involved in the negative regulation of leaf senescence. Lim et al. (2010) further demonstrated that arf2 was more tolerant to oxidative stress since the mutants maintained chlorophyll content and PSII activity compared to wild-type plants. The presence of oxidizing ROS agents can induce auxin degradation, alter auxin transport and distribution, relocate PIN proteins for auxin transport, and induce auxin conjugation (Tognetti et al. 2012). Concurrently, gene expression associated with auxin response factors, transporters, and biosynthetic enzymes has been shown to be stimulated by ethylene, which itself displays antagonism with auxin and is discussed further below (Peleg and Blumwald 2011).

A link between auxin content and antioxidant capacity was hypothesized when various grass species displayed increased abiotic stress tolerance following exogenous treatment with humic acids possessing auxin-like activity (Zhang and Schmidt 1999,

2000; Zhang et al. 2003, 2007). More recently, a mutation of Arabidopsis CATALASE2 resulted in crosstalk between hydrogen peroxide and auxin signaling as mediated by changes in glutathione redox status resulting in a hyponastic phenotype (Gao et al. 2014). Csiszár et al. (2004) showed that auxin autotrophic tobacco callus may resist oxidative damages (less cellular hydrogen peroxide and malondialdehyde) by increasing GPX, GST, and GSH-PX activities, while heterotrophic tobacco callus did so similarly via increases to SOD and CAT activity. Finally, transgenic Arabidopsis with higher endogenous IAA content or wild-type plants treated with exogenous IAA were more drought tolerant due to enhanced SOD, CAT, POD, and GR activity for accelerated ROS mitigation (Shi et al. 2014). These transgenic lines also displayed improved root growth or lateral rooting for water uptake, maintained metabolic homeostasis, and positively modulated specific stress-related genes (RAB18, RD22, RD29A, RD29B, DREB2A, and DREB2B) during drought stress.

Auxin has also been implicated in altering hydrogen peroxide dynamics with downstream signaling effects on stomatal movement and root morphology, both of which are important contributors to whole-plant drought tolerance. It was suggested that hydrogen peroxide contributes to the auxin-dependent responses of plasma membrane H+-ATPase and cytoplasmic pH controlling inward and outward potassium movement to guard cells (Song et al. 2006). Similarly, there is increasing evidence that auxins influence nitric oxide levels within guard cells possibly stimulating ion movement via these cross-membrane channels (Xiao-Ping and Xi-Gui 2006). More specifically, low auxin concentrations induced potassium influx and guard cell opening whereas increasing auxin concentrations induced potassium efflux and closing of guard cells (Acharya and

Assmann 2009; Daszkowska-Golec and Szarejko 2013). However, this can be interpreted as contradictory to the observed effects of exogenous auxins which counteract ABA-induced stomatal closure, possibly through interactions with ethylene in Arabidopsis (Tanaka et al. 2006). Throughout the literature, there exist contradictory reports regarding stomatal responses to auxins stemming from organic versus synthetic forms applied, concentration dependencies, species or organ-specific responses, and potential crosstalk or interactions with other plant hormones (Daszkowska-Golec and Szarejko 2013; Pospíšilová 2003).

Joo et al. 2001 suggested a novel role for auxin-induced ROS in root gravitropism by which unilateral application of auxin caused transient increases in ROS to mediate directional root growth and may also be interdependent with calcium signaling. Alternatively, ROS-mitigating GSH may be closely linked to auxin transport since Arabidopsis treated with the GSH inhibitor, buthionine sulphoximine (BSO), displayed a loss of PIN1, PIN2, and PIN7 auxin carriers (Koprivova et al. 2010). Auxin has been implicated across a wide array of plant developmental processes including organogenesis and up-regulating the AVP1 H + -pyrophosphatase accelerates auxin fluxes and pyrophosphate-driven cation transport into root vacuoles which increases root biomass for enhanced drought tolerance (Li et al. 2005; Park et al. 2005). Similarly, moderate water stress may stimulate auxin transport into root tips and increase plasma membrane H+-ATPase activity for enhanced proton secretion driving root elongation and root hair development (Xu et al. 2013). Along with drought perception, a myriad of environmental response pathways converge on auxin signal transduction with downstream effects for each stage of lateral root development including cell initiation, emergence of the lateral

root primordial, and lateral root growth and orientation (Casimiro et al. 2003; Malamy 2005). Maintaining auxin homeostasis is an essential component of lateral root growth during drought stress and there is increasing evidence that crosstalk interactions between auxin and other hormones, such as ABA, may inhibit auxin-stimulated root growth, as discussed further below.

Cytokinins

Cytokinins are well known to influence many biological functions throughout the plant system, which one of the most well-known positive regulators of senescence, as demonstrated by exogenous applications or endogenous manipulations suppressing the rate of natural or stress-induced leaf senescence (Lim et al. 2003; Taiz and Zeiger 2010). As CK levels decrease during stress-induced leaf senescence, genes for CK synthase and adenosine phosphate isopentenyl-transferase (IPT) are downregulated, genes for CK oxidase are upregulated, and until recently, little was known about which gene(s) directly influence leaf senescence by means of CK signaling (Lim et al. 2007). Unlike other hormone-signaling pathways (except ethylene), CK signaling comprises a histidyl-aspartyl (His-Asp) phosphorelay system by which histidine kinases (HKs) serve as cytokinin receptors which transfer a phosphoryl group to nuclear type-B response regulators (RRs) activating the type-A RR primary response genes (Imamura et al. 1998; To and Kieber 2008). While six distinct HKs have been identified in Arabidopsis, AHK2-4 (A for Arabidopsis) are localized on the endoplasmic reticulum, serve CK receptor functions, and have distinct roles in various aspects of plant growth and development (see comprehensive review by Ha et al. 2012).

The process of stress-induced leaf senescence can be divided into three distinct phases; the initiation phase which involves stress perception and signal transductions, the reorganization phase during which changes in gene expression inducing a wide range of biochemical and metabolic changes including hormonal changes, and the terminal phase during which permanent or nonreversible cell death occurs (Munné- Bosch and Alegre 2004). Drought-induced leaf senescence typically coincides with decreasing endogenous CK concentrations, though the hypothesis that low CK content directly triggers leaf senescence may not be accurate since CK-deficient mutants typically display delayed chlorophyll degradation compared to wild-types suggesting that other factors such as altered source–sink responses or antioxidant profiles may be responsible for the observed senescence during drought stress (Werner et al. 2003). For example, transgenic tobacco plants expressing the IPT gene driven by a maturation- and stress-induced senescence-associated receptor protein kinase (SARK) promoter maintained higher photosynthetic rates and delayed leaf senescence during drought stress and the improved drought tolerance was associated with larger pools of ascorbate and glutathione accounting for the lower levels of hydrogen peroxide (Rivero et al. 2007). Similarly, transgenic creeping bentgrass containing the IPT gene driven by an auto-regulated senescence-activated (SAG12) promoter displayed less lipid peroxidation maintaining cellular integrity and photochemical efficiency during drought stress, possibly associated with maintenance of SOD, POD, and CAT activities (Merewitz et al. 2011). Despite these examples and numerous other reports correlating CK (exogenous or endogenous) to enhanced antioxidant capacity during drought stress, the stress-induced changes to signaling

pathways linking cytokinins (CKs) to antioxidant metabolism, either directly or indirectly, are largely undefined and deserve further investigation.

Historically, CKs were generally regarded as antagonists to ABA throughout the plant system, though the literature provides contradictory reports by which CKs enhance, mitigate, or have neutral effects on stomatal apertures depending upon plant species, CK type, and concentration dependencies (see reviews by Acharya and Assmann (2009) and Pospíšilová (2003)). In certain cases, increasing concentrations of CKs in xylem sap may reduce stomatal sensitivity to ABA and promote stomatal opening or delay the drought-induced decrease of stomatal aperture (Havlova et al. 2008; Wilkinson and Davies 2002). Alternatively, a reduction in endogenous CK content may not imply enhanced sensitivity to ABA as demonstrated by CK-deficient Arabidopsis lines which regulated the endogenous ABA:CK ratio and maintained stomatal aperture for photosynthetic carbon dioxide uptake (Nishiyama et al. 2011). From this, it was suggested that there may exist mutual regulatory mechanisms between CKs and other plant hormones which collectively mediate stomatal responses upon onset of adverse environmental conditions (discussed further below). Environmental cues such as drought stress may induce synergism between CKs and ABA to collectively exert antagonism upon auxin resulting in suppression of lateral root formation and promotion of primary root growth into deeper soil profiles in search of water supplies (Ha et al. 2012). Such effects may have been evident for SAG12-ipt and HSP18.2-ipt transgenic creeping bentgrass plants with greater root:shoot and CK:ABA ratios upon drought stress, though direct correlations between the two parameters were not clear (Merewitz et al. 2010). There are apparently discrepancies on CK effects on root growth between dicots, such as Arabidopsis and

tobacco, and inhibitory effects on monocots, such as creeping bentgrass; however, the mechanisms underlying the differential responses of roots to CKs between different types of plant species are unknown, which is likely influenced by a variety of plant growth factors and associated signaling pathways influencing hormonal, nutritional, and/or source–sink relationships throughout different plant organs, and different sensitivity of plants to endogenous levels of CKs (Werner et al. 2010).

Gibberellins

Gibberellins (GAs) are a large class of diterpenoid plant hormones with over one hundred different chemical structures currently known to exist, though only a select few are biologically active and influence a variety of plant growth and developmental processes including seed germination, stem and root elongation, leaf expansion, transition from juvenile to adult phases, sex determination, and floral initiation (Sun and Gubler 2004; Taiz and Zeiger 2010; Yamaguchi 2008). In comparison to other plant hormones, there is far less information regarding contribution of GAs to drought tolerance and how GA content changes upon increasing level of drought stress (Pospíšilová 2003). Despite GA typically classified as antagonistic to ABA, the few studies investigating GA contribution to stomatal function suggested that GA may not contribute to stomatal movement since exogenous GA had little or no effect on stomatal closure in Arabidopsis and GA-deficient mutants had similar transpiration rates compared to wild-type plants during drought stress (Acharya and Assmann 2009). The direct effect of GA on stomatal movement by means of exogenous applications or endogenous manipulation is a particular research area which deserves further attention. Alternatively, Saibo et al. (2003) demonstrated that GA is the

main hormonal signal inducing stomata formation on Arabidopsis hypocotyls and the GA-induced developmental signal is further regulated through interactions with auxin and ethylene. It would seem inherent that leaf stomatal density strictly regulated by GA would be a major determinant of transpiration rates and leaf water status during drought stress, though this remains to be empirically proven. There is also considerable evidence suggesting that stomatal aperture responding to drought onset is regulated through multiple signaling pathways or crosstalk among various plant hormones, including GA, as discussed in subsequent sections.

Along with the previously mentioned contribution of CKs to leaf senescence, there is increasing evidence suggesting that GAs also serve important regulatory functions during natural or stress-induced leaf senescence. For example, Rosenvasser et al. (2006) summarized that GA1, GA4, and GA9 content all decreased in *Alstromeria* and lettuce leaves upon dark-induced leaf senescence, while exogenous GA delayed leaf senescence in *Pelargonium*, *Taraxacum*, *Rumex*, *Nasturtium*, and *Alstromeria*. Similarly, leaf senescence was mitigated by exogenous applications of GA4 and GA7 in *Lilium* plants following low-temperature storage in darkness and the beneficial effects were associated with increased CAT activity and decreased lipid peroxidation and proteolysis (Ranwala and Miller 2000). GA3 applied as a foliar spray or soil drench enhanced the antioxidant potential of *Catharanthus roseus* by stimulating production of the indole alkaloid ajmalicine and also alleviated the toxic effects of cadmium in a separate study with the same plant species (Pandey et al. 2007; Jaleel et al. 2007). GA3 applications mitigated oxidative stress and slowed the rate of *Paris polyphylla* leaf senescence by increasing endogenous GA4 and GA7 with potential downstream effects on lipid

peroxidation, hydrogen peroxide content, activities of SOD, POD, and APX, and while a potential antagonistic interaction with ABA was suggested, the actual mechanisms underlying these changes remain unclear (Yu et al. 2009). The inhibitory effects of drought stress on various morphological aspects of maize growth were reduced for plants treated with GA3 during the vegetative growth stage, though underlying mechanisms were not explored during the study (Akter et al. 2014). Finally, the beneficial senescence-mitigating effects of GA3 were associated with a significant enhancement of SOD activity in marigold (*Calendula officinalis*) during drought stress (Sedghi et al. 2012). While we can only speculate on the direct link between antioxidant metabolism and GA for delaying leaf senescence during drought periods, it is possible that WRKY transcription factors associated with the gibberellin-signaling pathway are involved, which may exert downstream effects on ROS signaling and hydrogen peroxide accumulation for antioxidant response, though much more work is needed to verify the actual signaling process (Jo and Hyun 2011).

The growth-promoting effects of GA by means of downstream effects on cellular elongation rates have been recognized for many years, though only until recently have advancements in molecular biology techniques been able to shed light on signaling pathways preempting the GA-induced cellular expansion process (Fleet and Sun 2005; Olszewski et al. 2002; Richards et al. 2001; Schwechheimer and Willige 2009). Moreover, despite the well-known adverse effects of drought stress on root and shoot growth rates, the direct influence of GA on the mechanisms underlying cellular expansion under drought stress are not well known. One particular mechanism by which GA may influence cellular expansion under drought stress involves GA3 up-regulating

expansin genes EXPA4 and EXPB4 and xyloglucan endotransglycosylase (XET) genes XET1 and XET1 to maintain leaf elongation rates as demonstrated in tall fescue (*Festuca arundinacea*) under chemically induced drought stress (Xu et al. 2016). A GA-responsive transcription factor, OsPIL1, was repressed by drought stress and was associated with downregulation of expansin in the internode of rice plants (Todaka et al. 2012). A comprehensive study by Ribeiro et al. (2012) investigated changes in Arabidopsis translatome and metabolome as triggered by GA and suggested that there exists a close interaction between energy metabolic processes and GA-mediated growth with downstream effects on cell wall extension, secondary metabolism, and lipid metabolism. Specifically, GA and paclobutrazol (PAC; GA-inhibitor) had opposing effects on the expression of genes encoding expansins and xyloglucan endotransglucosylase/endohydrolases (XTHs), products of primary metabolism including nitrates, total amino acids, and protein, as well as carbon allocation governing growth rates. Based on these studies which demonstrate that potential interactions between GA and carbon metabolism and growth may likely exist, it would be useful for future research to begin identifying which genes are responsible and identify new markers for growth potential (Ribeiro et al. 2012). Additionally, similar studies should be conducted investigating translatomic and metabolomic changes conferred by GA induction or suppression and which changes may contribute to enhanced drought tolerance in Arabidopsis or other model plant species.

Ethylene

The gaseous plant hormone ethylene induces a triple response on plant development encompassing radial swelling, inhibited elongation of the epicotyl, and horizontal growth of the epicotyl, and is also implicated in downstream effects on various aspects of plant stress responses (Chaves et al. 2003; Sharp and LeNoble 2002; Taiz and Zeiger 2010). The current model of ethylene signaling suggests that ethylene molecules are sensed by a family of receptors acting as negative regulators of the ethylene-responsive pathway (Guo and Ecker 2004). More specifically, ethylene binds to the receptors and inactivates the receptor-CTR1 complex which allows EIN3 and EIN3-like transcription factors to accumulate in the nucleus and express transcription factor genes ERF1-4 which initiate activation or repression of hundreds of downstream genes, though until recently, little was known about how these signaling networks may contribute to ethylene responses during drought stress (Stepanova and Alonso 2009). Additionally, signaling pathways mediated by ethylene may also involve crosstalk between calcium-dependent protein kinases (CDPKs) and mitogen- activated protein kinases (MAPKs) preempting the downstream activation of stress-response genes (Fujita et al. 2006; Ludwig et al. 2005; Nakagami et al. 2005).

Recent studies have suggested that expression of specific ethylene response factors (ERFs) exert downstream effects in various plant species responding to osmotic stresses, including drought or salinity, and the potential mechanisms by which ERFs promote stress tolerance have been suggested in several cases. Transgenic sugarcane (*Saccharum officinarum*) plants overexpressing SodERF3 displayed improved drought tolerance as noted by enhanced plant height, leaf weight, and flower production following

20-day water withholding as compared to wild-type plants, though the underlying mechanisms facilitating the enhanced growth were not investigated (Trujillo et al. 2008). Alternatively, OsDERF1 is induced by drought stress and while transgenic rice plants overexpressing OsDERF1 were more drought-susceptible at seedling stage, OsDERF1 knockdown lines had enhanced drought tolerance at seedling and tillering stages associated with lower MDA accumulation and higher accumulation of sugars and proline suggesting specificity of ERF regulation in drought response (Wan et al. 2011). Several studies which regulated ERF expression levels to induce downstream changes in stress tolerance have shown that enhanced drought tolerance may be attributed to ERFs activating transcription of specific stress-responsive genes regulating the observed physiological changes during water withholding. Rice plants overexpressing JERF3 displayed improved drought tolerance similarly associated with proline and sugar accumulation and the increased proline content was most likely due to up-regulation of OsP5CSs encoding two key enzymes in proline synthesis (Zhang et al. 2010a, b). In the same study, JERF3 also upregulated three stress-inducible genes, WCOR413-like, OsEnol, and OsSPDS2, which were attributed to maintaining membrane stability under drought stress. In addition to up-regulating osmotic stress genes, up-regulation of the ethylene-responsive JERF3 has also been shown to reduce ROS by enhancing expression of antioxidative SOD genes and further contributed to drought tolerance by activating photosynthetic carbon assimilation/metabolism genes (Wu et al. 2008). ROS-responsive genes may contain ethylene-responsive cis elements, as was shown for Zat7, Zat12, WRKY25, and Apx1 in Arabidopsis (Miller et al. 2010). Overexpressing OsWR1, a homolog of the wax/cutin synthesis regulatory gene WIN1/SHN1, regulated the

expression of wax-related genes OsLACS2 and OsFAE1'-L, as well as genes related to oxidative stress response and membrane integrity, all of which likely contributed to improved drought tolerance in transgenic rice plants (Wang et al. 2012). Ethylene contributes to long-distance stress signaling upon perception of soil drying by means of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) transported from roots to shoots, though the literature provides inconsistent results as to when and under which specific conditions ACC transport and ethylene production occurs (see review by Wilkinson and Davies 2010). Wilkinson and Davies (2010) also summarized that plants respond to ethylene biphasically with low or high ethylene concentrations increasing or decreasing plant growth, respectively, though there is still no clear role of ethylene in maintaining shoot growth under drought stress conditions. The well-defined effects of ethylene on leaf senescence and growth inhibition under drought stress has retracted from research interests of potential contributions of ethylene to drought tolerance, even though in the absence of detrimental growth effects under induced by high ethylene concentrations, lower concentrations of ethylene may maintain stomatal apertures for leaf cooling and carbon uptake during mild to moderate drought stress (Wilkinson et al. 2012). ERFs such as ETR1 (ethylene response 1) may facilitate signaling functions for stomatal movement, glucose-sensing, and hydrogen peroxide biosynthesis suggesting a potential link between ROS, sugars, and hormone pathways (Pinheiro and Chaves 2011). Furthermore, the ethylene-mediated reductions in shoot growth as well as stomatal responses under drought stress are highly dependent upon ABA accumulation in shoots, since ABA and ethylene exert antagonism upon each other as discussed below (Chaves et al. 2003).

Salicylates

Salicylic acid (SA) is an endogenous phenolic plant hormone which serves diverse regulatory roles in plant metabolism and has been implicated in modulating specific plant responses to oxidative stress, such as the signaling cascades and regulation of chloroplast biogenesis with subsequent promotive effects on photosynthesis (Hayat et al. 2010). SA contributes to the initial development of stress responses and higher concentrations of SA within the plant system tend to induce the beneficial responses promoting tolerance to osmotic stresses such as salinity or drought (Horváth et al. 2007). Exogenous SA applications enhanced the drought tolerance of tomato (*Lycopersicon esculentum*) by increasing photosynthetic parameters, membrane integrity, leaf water potential, chlorophyll content, and activity of nitrate reductase carbonic anhydrase (Hayat et al. 2008). Similarly, higher water content, dry mass accumulation, and chlorophyll content associated with maintenance of carboxylase activity and SOD activity were observed in drought-stressed wheat seedlings following SA application (Singh and Usha 2003). Higher antioxidant enzyme activities limited hydrogen peroxide accumulation and lipid peroxidation in droughted wheat leaves previously sprayed with SA (Agarwal et al. 2005). The stimulative effect of exogenous SA on plant antioxidant components is likely concentration-dependent as SA-deficient mutants lack the ability to mitigate ROS, low concentrations (0.01–0.05 mM) of SA induce slight stimulation of AOX and HSPs, while optimum concentrations (0.1–0.5 mM) of SA initially increase ROS which themselves act as secondary messengers to dramatically enhance CAT, APX, SOD, GR, AOX, and HSP activities conferring the observed drought tolerance (Yuan and Lin 2008). A similar dose-dependent effect of SA was observed in tomato and bean (*Phaseolus vulgaris*)

plants which displayed enhanced drought tolerance for plants grown from seed imbibed in low SA concentrations but not for plants grown from seed imbibed in high SA concentrations (Senaratna et al. 2000).

Interactions between SA and hydrogen peroxide affect the rate of ROS accumulation within plant tissues which stimulates oxidative stress-induced PR gene expression and downstream systemic acquired response (SAR) responses (Horváth et al. 2007; Lee et al. 2006; Mateo et al. 2006). SA-mediated ROS accumulation may also influence stomatal aperture during drought responses as demonstrated by siz1 Arabidopsis mutants lacking SIZ1-mediated endogenous SA accumulation, though this is one particular area which deserves further investigation (Miura et al. 2013). Similarly, increasing SA concentrations by means of endogenous manipulation or exogenous applications stimulates nitric oxide (NO) synthesis, another key component in stress-responsive signaling cascades (Zottini et al. 2007). MAPKs have also been shown to be stimulated by SA and initiate various downstream defense responses including expression of key enzymes for defense signaling and initiation of abiotic and biotic stress responses (Bowler and Fluhr 2000; Yang et al. 2001a; Zhang and Liu 2001). Despite considerable work investigating SA contribution to other oxidative stresses including salinity, ozone, and UV-B radiation, there has been far less investigation of which SA-responsive genes contribute to drought tolerance in plants. Exogenous SA application promoted drought tolerance in wheat seedlings by enhancing the transcription of GST1, GST2, GR, and MDHAR which facilitate the detoxification of ROS, though the focus of this study was narrowed towards genes involved in the ASA-GSH cycle (Kang et al. 2013). The interaction between GSH and SA regulating ROS production under stress conditions may

also regulate a variety of other plant processes due to the effects on cell redox states (Horváth et al. 2007). Tobacco stress-induced gene1 (Tsi1) was shown to be induced by exogenous SA application and subsequently increased expression of drought stress-responsive target genes PR1, PR2, PR3, osmotin, and SAR8.2 (Park et al. 2001). Transcriptional profiling of the WRKY gene family showed that genes encoding certain WRKY transcription factors are upregulated by SA application while others are upregulated by drought stress and that a specific WRKY gene, 12g02400, was upregulated by both SA and drought stress (Ramamoorthy et al. 2008). However, it remains to be determined as to which downstream plant responses are regulated by SA- or drought-induced WRKY gene regulation. It would be interesting to know whether there exists a link between SA-induced WRKY gene expression and downstream proteomic changes, as SA-induced growth and drought tolerance of wheat was associated with altered expression patterns of proteins facilitating signal transduction, stress defense, photosynthesis, carbohydrate metabolism, protein metabolism, and energy production during drought stress (Kang et al. 2012).

Jasmonates

Jasmonic acid (JA) and methyl jasmonate (MeJA) are biologically active lipid derivatives formed by fatty acid oxidation and contribute to the regulation of various stress responses in plants including leaf senescence, ROS and NO signaling, antioxidant metabolism, and stomatal movement (Balbi and Devoto 2008; Murata and Mori 2014; Taiz and Zeiger 2010; Wasternack 2007). Similar to the current state of SA research, jasmonates have been implicated in promoting tolerance to abiotic stresses including salinity, ozone, or

UV-B irradiance through downstream effects on antioxidant metabolism, whereas less research has been conducted regarding the contribution of jasmonates to antioxidant-facilitated drought tolerance (Kumari et al. 2006). Nevertheless, several studies which have been conducted suggest jasmonates mitigate the drought-induced oxidative burst in a similar manner as for other oxidative stresses, by means of increased antioxidant enzyme activities. For example, soybean plants treated with 50 μM MeJA had decreased lipid peroxidation associated with increased activities of SOD, POD, and CAT while the observed increase in proline concentration may have further facilitated the higher leaf water content for MeJA-treated plants during irrigation withholding (Anjum et al. 2011). Cellular water retention is also enhanced following JA application by increasing betaine aldehyde dehydrogenase (BADH) for enhanced betaine accumulation and subsequent osmotic adjustment in pear (*Pyrus bretschneideri*) leaf cells (Gao et al. 2004). The increased activities of SOD, POD, CAT, APX, and GR collectively detoxified hydrogen peroxide and, in conjunction with higher proline and soluble sugar content, enhanced the drought persistence of cauliflower (*Brassica oleracea*) seedlings following MeJA or coronatine (COR; a phytotoxin that mimics some biological activities of MeJA) application (Wu et al. 2012). A microarray analysis of over two thousand selected Arabidopsis genes showed that the abundance of 221 mRNAs was highly upregulated following MeJA application and the upregulated mRNAs served putative functions spanning oxidative stress responses, cellular maintenance, as well as low and high molecular weight defense signaling (Schenk et al. 2000). More specifically, the reduction in transcript levels of l-galactono-1,4- lactone dehydrogenase (GalLDH), APX, GR, dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR)

during drought stress was mitigated in crested wheatgrass (*Agropyron cristatum*) leaves following JA application, reinforcing the notion that JA also serves critical roles in regulating ascorbate and glutathione metabolism during drought periods (Brossa et al. 2011; Shan and Liang 2010). Alternatively, the increase in jasmonate content upon drought stress may induce specific NAC transcription factor gene (i.e., ANAC019 and ANAC055) expression with potential downstream effects on abiotic-stress cellular networks, though the ways in which JA-induced NACs may be utilized to promote abiotic stress tolerance remain largely unknown (Bu et al. 2008; Puranik et al. 2012).

Given that jasmonate concentrations increase in a similar manner as ABA during drought onset, it was hypothesized that the biologically active jasmonate derivatives may positively regulate stomatal closure as drought stress severity increases (Acharya and Assmann 2009). Furthermore, whether or not jasmonates regulate similar mechanisms as ABA to influence stomatal movement was of particular interest. Studies which utilized jar1 (MeJA-insensitive) mutants have shown that MeJA-mediated stomatal closure involves guard cell alkalization, ROS and NO production, potassium efflux, and slowed anion channels, all of which are similarly associated with ABA-induced stomatal closure (Evans 2003; Munemasa et al. 2007; Suhita et al. 2004). Further research investigating how MeJA and fluridon (ABA-inhibitor) influence stomatal movement in ABA-deficient mutants suggested that endogenous ABA is required to activate calcium signaling during MeJA-induced stomatal closure (Hossain et al. 2011). Additionally, stomatal closure will not occur for Arabidopsis mutants lacking the coronatine-insensitive1 (COI1) gene likely due to little change in ROS and NO production or anion efflux following MeJA application whereas coi1 mutants will close stomates following ABA application,

indicating that COI1 is upstream of ROS and NO in MeJA signaling (Munemasa et al. 2007). Synergism exists between JA and NO in stimulating stomatal closure in broad bean (*Vicia faba*) leaves such that JA enhances NO synthesis in guard cells and both JA and NO induce stomatal closure in a dose-responsive manner (Liu et al. 2005). A review by Hadiarto and Tran (2011) suggested that JA may serve important regulatory roles during the ABA-dependent drought responses in plants since JAZ (Jasmonate ZIM-domain), ABA-dependent, and drought-inducible AtMYC2 transcription factors all regulate gene expression in jasmonate pathway.

In comparison to the extensive research regarding jasmonate-stimulated antioxidant metabolism or stomatal movement, there is less known regarding how jasmonates directly influence growth and photosynthetic processes during drought stress. One research area which may be of particular interest is in how jasmonates influence homeostasis of various energy-consuming processes during drought stress, as hormone balance likely controls metabolic and physiological stabilization during periods of abiotic stress (Harb et al. 2010). For example, MeJA pretreatment has been shown to have reversible effects on nitrogen uptake inhibition and remobilization of RuBisCO subunits in field-grown oilseed rape (*Brassica napus*), though whether JA-induced changes in these parameters confers drought acclimation remains unknown (Rossato et al. 2001). Furthermore, despite abundant knowledge detailing jasmonate contributions to many different plant physiological processes (i.e., floral development, senescence induction, growth inhibition, root morphogenesis) under non-stress conditions, little is known as to how these parameters may be individually affected by JA under short- or long-term drought stress treatment (Santino et al. 2013).

INTERACTIONS BETWEEN HORMONES AND PLANT METABOLITES DURING DROUGHT STRESS

Hormone to Hormone Interactions

As discussed above, multiple hormones may be involved in regulating a particular growth trait or physiological responses to drought stress through synergistic or antagonistic interactions, although each hormone play unique roles. The analysis of Arabidopsis mutant phenotypes in conjunction with transcriptomic profiling studies has provided convincing evidence supporting the theory that crosstalk between plant hormones results in antagonistic or synergistic effects on various phenotypic responses to abiotic stress (Depuydt and Hardtke 2011). Crosstalk signals derived from hormone to hormone or hormone to secondary-messenger (i.e., calcium or ROS) interactions may converge upon or be transduced by MAPK modules to regulate gene expression by means of transcription factor modulation (Fujita et al. 2006; Smékalová et al. 2014). For example, two specific MAPKs, OsMPK5 and OsEIN2, have been shown to facilitate antagonism between ABA and ethylene in that RNAi suppression of OsMPK5 reduces rice sensitivity to ABA, increases endogenous ethylene, and reduces drought tolerance, while suppression of OsEIN2 reduces sensitivity to ethylene, increases hypersensitivity to ABA, and enhances drought tolerance (Sharma et al. 2013). Ethylene has also been shown to regulate many auxin-related genes including ARFs, transporters, and genes encoding biosynthetic enzymes, while genes encoding rate-limiting enzymes in ethylene biosynthesis are conversely regulated by auxin (Peleg and Blumwald 2011). Given the well-known contribution of ABA to stomatal closure during plant drought response, many studies have investigated how other hormones influence ABA-mediated stomatal

closure upon water deficit. Thus far, it is generally accepted that auxins, CKs, and ethylene are antagonistic with ABA and counteract stomatal closure while SA and jasmonates are in synergism with ABA and positively regulate stomatal closure during drought, though all of these hormones may differentially modulate the downstream expression of stress-related genes (Acharya and Assmann 2009; Nilson and Assmann 2007). For example, one of the ABA-regulated bZIP transcription factors (ABI5-Like1) is typically induced by drought or salinity but may also be regulated by auxin to then activate a variety of stress response genes including ABRE-containing genes related to auxin metabolism (Yang et al. 2011). Wang et al. (2011) also suggested that crosstalk between ABA and MeJA occurs at the transcript level and is consistent with the downstream effects of crosstalk at the physiological level both in guard cells and other tissues. Synergism may exist between CKs and auxins, specifically IAA, in that CKs are positive regulators of auxin biosynthesis and the two hormones may establish a homeostatic feedback regulatory loop to maintain proper proportions in developing root tissues (Jones et al. 2010). Alternatively, the well-known antagonism between ABA and CKs contributing to drought-induced stress responses may be in part facilitated by CK-receptor histidine kinases (AHK2, AHK3, and CRE1) acting as negative regulators of ABA and osmotic stress signaling, whereas another non-ethylene histidine kinase (AHK1) is a positive regulator of these same processes (Tran et al. 2007). It was also proposed that nitrate transporters facilitating nitrate uptake may serve in hormone crosstalk since NRT2.6 is regulated by auxin, CKs, and ABA, whether be individually or interactively (Krouk et al. 2011). GA may be synergistic with SA as exogenous GA applications increased expression levels for two genes encoding SA-synthesis genes, plus

transgenic Arabidopsis plants overexpressing a GA-responsiveness gene had higher endogenous SA content and were more tolerant to oxidative stress (Alonso-Ramírez et al. 2009a). Crosstalk between GA and SA by which GA induces both SA production and action may also contribute to changes in source–sink relationships during drought stress, most notably through the effects on photosynthesis, mobilizing resources, and sink strength (Alonso-Ramírez et al. 2009b).

The recent discovery of the JAZ (JASMONATE-ZIM DOMAIN) family proteins, acting as JA co-receptors and transcriptional repressors in JA signaling, has suggested that JAZ proteins facilitate JA-mediated crosstalk with auxins, ethylene, SA, and interestingly may be antagonistic or synergistic with GA depending on which plant function is of focus (Kazan and Manners 2012). For example, GA and JA are antagonistic with respect to plant growth and defense but synergistic in that both are required for jasmonate- and GA-mediated stamen development and male fertility (Cheng et al. 2009; Pauwels et al. 2009; Navarro et al. 2008). JAZ proteins facilitate synergism between JA and ethylene supporting plant defense functions and antagonism between jasmonates and SA or auxins by which SA- or auxin-mediated signaling is regulated by jasmonates (Broekaert et al. 2006; Leon-Reyes et al. 2010; Sun et al. 2009). Observations of similar developmental changes responding to distinct abiotic stress signals suggests that redundant signaling intermediates, such as DELLA proteins (negative regulators of GA signaling), facilitate crosstalk between different phytohormones (Kohli et al. 2013). For example, JA was shown to interfere with DELLA–PIF3/4 interactions and inhibit GA-mediated hypocotyl elongation (Lyons et al. 2013). DELLAs have also been implicated in orchestrating GA and ABA signaling crosstalk controlling Arabidopsis seed

germination and seedling development under oxidative stress conditions (Yuan et al. 2011). During drought stress, increased ABA and ethylene concentrations exert antagonism on GA signaling and GA-mediated growth and this crosstalk occurs by means of DELLA proteins, though GA interacting with other plant hormones such as SA also contributes to changes in growth under drought stress (Kohli et al. 2013; Wolters and Jürgens 2009). As described previously, ethylene exerts strict control upon drought-induced leaf senescence by controlling gene expression of EIN transcription factors and, more specifically, the EIN2 transcription factor has been shown to be similarly regulated by ABA and MeJA, suggesting a means for crosstalk between the three hormones controlling downstream expression of stress- responsive genes (Kim et al. 2011). Elucidating on how specific points in ethylene pathway interact with other plant hormones and whether similar mechanisms are involved across different interactions to confer the plant drought responses at the physiological level, such as stomatal movement and growth processes, continues to be a primary focus of researchers (Vandenbussche and Van Der Straeten 2007).

Hormone to Sugar Interactions

Hormone regulation of plant growth and responses to drought stress not only involve interactions among hormones, but also interaction with other metabolites, such as sugars, as found in recent research. The ongoing and extensive research into crosstalk between multiple hormone classes also suggests that sugars may exert influence upon biosynthesis or response pathways of other plant hormones, such as those associated with auxin or CK signaling. Sugars have been recognized to serve integral signaling functions modulating a

range of growth processes throughout the plant life cycle and, in an attempt to understand why various genes respond to specific sugars or sugar-phosphorylations, it was noted that Arabidopsis mutants with altered sugar responses displayed phenotypes similar to plant-hormone biosynthesis or signaling mutants suggesting the existence of links between sugar- and hormone-signaling pathways (Gibson 2005; Hanson and Smeekens 2009; León and Sheen 2003; Pinheiro and Chaves 2011). The initial comparable mutant screens coinciding with subsequent genetic and functional analyses suggested that there is extensive overlap between sugar, ABA, and ethylene signaling preempting various downstream plant processes such as root development (Eveland and Jackson 2012). For example, mutants lacking genes encoding for ABA biosynthesis or sensitivity (aba or abi, respectively) are similarly insensitive to high concentrations of glucose and the potential link facilitating sugars and ABA-perception crosstalk might be ABI4, which encodes an AP2 transcription factor required for normal sugar response (Arenas-Huertero et al. 2000). Co-expression of a sucrose synthase gene and ABI3 occurred under stress conditions, as did ABI1 with one neutral invertase, two sucrose synthases, and one β-amylase, all of which may serve to amplify the signaling capacity and phenotypic responses (i.e., stomatal closure) under drought stress (Pinheiro and Chaves 2011). Alternatively, mutants lacking genes encoding for ethylene perception (etr1, ein2, ein3) are hypersensitive to glucose while a mutant with negative regulation of ethylene signaling (*ctr1*) is insensitive to glucose and the antagonistic relationship between ethylene and glucose may similarly be mediated through repression of ABA biosynthetic genes (Ghassemian et al. 2000; Yanagisawa et al. 2003). Studies conducted on ABA and ethylene mutants, Arabidopsis mutants (hxk) unable to catalyze glucose phosphorylation

were resistant to exogenous auxin, insensitive to high glucose concentrations, and the hxk-based signaling negatively interacted with CKs (Moore et al. 2003). Tobacco transgenic lines with reduced levels of ASR (ABA-stress-ripening) protein displayed limited glucose metabolism and altered ABA and GA levels with downstream effects on leaf senescence, suggesting that *Asr* may be a central signaling component between glucose, ABA, and GA (Dominguez et al. 2013). Despite the identification of several novel genes possibly serving as links between sugars and auxin, the overall complexity of crosstalk between hormones, sugars, and interacting secondary metabolites establishes the need for more in-depth genomic studies to show how gene expression levels change across thousands of genes, of which distinct changes can be associated with sugar or hormone signaling under different abiotic stress conditions (Eveland and Jackson 2012; Kissoudis et al. 2014). Mishra et al. (2009) performed genome-wide expression profiling of Arabidopsis seedlings which showed that over two-thirds of genes affected by auxin were regulated by glucose and that glucose and auxin establish either antagonistic or synergistic mechanisms to regulate transcription. Furthermore, the auxin-deficient mutants receiving exogenous glucose displayed phenotypes indicative of various defects in root development, suggesting that glucose contributes to proper root development by means of auxin-based signaling functions.

CONCLUDING REMARKS

There has been increasing evidence supporting the critical roles of various plant hormones involved in regulating plant growth and physiological responses to drought stress in the last decade, although this has been a research area that have been studied for many decades. Among various drought responses, leaf senescence, antioxidant metabolism, carbon metabolism, and stomatal movements are directly impacted or indirectly mediated by a particular or multiple hormones. Physiological and metabolic regulation of drought responses by hormones are well known as demonstrated by extensive research in related areas discussed throughout the chapter. Various transcription factors and downstream genes controlling hormone synthesis, degradation, and responses or sensitivities have been identified through transcriptomic analysis and confirmation through genetic transformation of overexpressing and silencing or mutating specific genes. Through the analysis of transcription factors, recent research is beginning to unravel signaling pathways of a single or multiple hormones and interactions among hormones and between hormones and other metabolites such as sugars, which coordinately mediate drought responses. However, the events or molecules in the perception of a specific hormone initiating specific signal transduction pathways are not completely understood. Furthermore, crosstalk signals derived from hormone to hormone or hormone to sugars or secondary-messenger (i.e., calcium or ROS) are not yet clear. Further research addressing those critical questions regarding hormone-signaling perception and crosstalk among hormones and other metabolites will provide further insights into molecular factors controlling hormone regulation of plant tolerance to drought stress.

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CHAPTER ONE

EFFECTS OF SEQUENTIAL APPLICATION OF PLANT GROWTH REGULATORS AND OSMOREGULANTS ON DROUGHT TOLERANCE OF CREEPING BENTGRASS (AGROSTIS STOLONIFERA)

Burgess, P. and B. Huang. 2014. Effects of sequential application of plant growth regulators and osmoregulants on drought tolerance of creeping bentgrass (Agrostis stolonifera). Crop Sci. 54: 837–844. doi: 10.2135/cropsci2013.03.0200

ABSTRACT

Plant growth regulators and osmoregulants may be involved in protection against drought stress, but their additive effects are not well documented in turfgrass. The objective of this study was to determine physiological effects of trinexapac-ethyl (TE) and glycine betaine on drought tolerance in creeping bentgrass (*Agrostis stolonifera* L.) under field conditions. The experiment was conducted in 2010 and 2011 on mature field plots of creeping bentgrass cultivar 007 planted on a Nixon sandy loam soil. The application of TE before drought in conjunction with glycine betaine at drought onset and during water withholding significantly improved turf performance evaluated as turf quality (TQ) and normalized difference vegetation index (NDVI). Enhanced turf performance was associated with greater osmotic adjustment promoting water retention in leaves and improved cellular membrane stability, indicating less membrane damage during drought stress. Furthermore, the combined TE plus glycine betaine treatment was more effective in maintaining high TQ during soil-water deficit than when either TE or glycine betaine was applied alone. The results suggested that the sequential application of TE before drought onset and glycine betaine during water withholding effectively promoted creeping bentgrass tolerance to prolonged periods of drought stress. Cultural methods to maintain acceptable TQ with limited water resources are of major concern in the turfgrass industry, and this study provided promising results as to the effects of TE and glycine betaine for promoting creeping bentgrass drought tolerance.

INTRODUCTION

The impacts of drought stress on turfgrass utility and aesthetic quality is of major concern in many areas of the world. In cases of prolonged drought, irrigation may be altogether forbidden and managers may have no choice but to let their stands go dormant, characterized by desiccation and browning of the canopy. Exogenous application of certain plant growth regulators (PGRs) and osmoregulants has been found to promote plant survival during drought stress in various agronomic crops (Bochicchio et al., 1991; Ervin et. al., 2009; Kirkham, 1983; Xing and Rajashekar, 1999). However, little is known as to how PGRs and osmoregulants affect drought tolerance of turfgrass species. Enhancing turfgrass survival during drought stress is essential for maintaining functional aesthetics with limited water resources. Plant adaptation to drought stress is associated with various mechanisms, such as slow shoot growth with low water-use rate and high capacity of water retention in leaves through osmotic adjustment during drought stress (Nilsen and Orcutt, 1996). Previous studies have shown that plant drought tolerance can be measured through leaf relative water content (RWC) and leaf electrolyte leakage (EL), which indicate leaf water status and membrane stability, respectively (Fu et al., 2004).

Trinexapac-ethyl (TE) [4-(cyclopropyl-á-hydroxy-methylene)- 3,5-dioxocyclohexanecarboxylic acid ethyl ester] is one of the most widely used PGRs in turfgrass management. Trinexapac-ethyl inhibits late-stage gibberellic acid synthesis (Adams et al., 1992) to suppress vertical shoot growth and reduce clipping accumulation (Lickfeldt et al., 2001) and also reduces evapotranspirational water loss (Ervin and Koski, 2001a). Exogenous application of TE before plant exposure to stress has been shown to improve turf quality and leaf water content under combined drought and heat stress in

turfgrass species (McCann and Huang, 2007). Trinexapac-ethyl has also been shown to improve membrane stability through reduced membrane leakage in Kentucky bluegrass (*Poa pratensis* L.) (Xu and Huang, 2012). Accumulation of amino acids, reducing sugars, and other solutes contribute to osmotic adjustment for cellular water retention (Handa et al., 1983; Nilsen and Orcutt, 1996). Glycine betaine, a quaternary ammonium compound, is a major form of osmoregulant that is positively associated with drought tolerance through osmotic adjustment in various plant species (Khan et al., 2009). Exogenous application of glycine betaine has been proven effective in improving drought tolerance of agronomic crop species such as beans (*Phaseolus vulgaris* L.) (Xing and Rajashekar, 1999) and rice (*Oryza sativa* L.) (Farooq et al., 2008). Although TE and glycine betaine effects were individually examined in different species, their combined effects on plant drought tolerance when applied as a pretreatment or during drought stress are not well documented.

The main objective of this study was to evaluate sequential applications of TE before drought stress and glycine betaine at the onset and during drought stress for effectively promoting turfgrass performance and physiological adjustments during water withholding.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Irrigation Treatments

The experiment was performed May through August in 2010 and 2011. Individual plots (1.52 by 1.83 m) of mature (4 year old) creeping bentgrass (cultivar 007) (Seed Research of Oregon, Corvallis, OR) were mowed weekly at 10-mm height with clippings removed and watered three times per week to maintain soil water content at field capacity (30%) before the drought treatment. The soil type was a Nixon sandy loam (fine-loamy, mixed, semiactive, mesic Typic Hapludult). Emerald fungicide (a.i. boscalid [3-pyridinecarboxamide,2-chloro- N-(4'-chloro(1,1'-biphenyl)-2-yl)]) (BASF Corporation) was applied at manufacturer-recommended rate (548.9 $g \cdot ha^{-1}$) in late April to preventatively control dollar spot disease (*Sclerotinia homoeocarpa* F.T. Benn.) before TE application. Urea (46–0–0) was also applied late April at 15 g $N \cdot m^{-1}$ to promote spring green-up. No fungicides or fertility were applied during the experimental period of 2010 or 2011 as to avoid confounding effects. After the study was terminated in 2010, the field was again treated to control dollar spot and a spoon-feeding fertility regimen implemented to deliver 5 g $N \cdot m^{-1}$ every 2 weeks until the growing season ended. In 2010, irrigation was withheld for 31 d and then rewatered thereafter. In 2011, irrigation was withheld for 48 d and plants were rewatered thereafter. The duration of drought differed between years due to weather conditions. The 2010 trial had very hot and windy weather during the drought period, causing rapid drought onset and prompt initiation of site rewatering. Weather during the 2011 trial was more conducive for gradual drought onset and a steady rate of site dry-down. Drought symptoms took longer to appear in 2011 and site rewatering was prolonged until after 48 d of drought stress, allowing for greater

number of chemical applications during water withholding. Nevertheless, similar trends were observed between years and are discussed below.

Chemical Treatments

Each main plot of drought was divided into replicated subplots which were treated with TE, glycine betaine, or both TE plus glycine betaine. In 2010 and 2011, TE was applied twice, biweekly during the month of May (17 and 31 May 2010; 16 and 30 May 2011) per manufacturer recommended rates for creeping bentgrass turfgrass at 0.8 $L \cdot ha^{-1}$ TE (Primo Maxx, Syngenta Professional Products, Greensboro, NC) (1.95 $mL \cdot L^{-1}$ [v/v]; a.i. TE = 11.3%). Glycine betaine (200 mM) was applied weekly four times over the 31-d dry-down period in 2010 (1, 8, 15, 22 June) and seven times over the 48-d dry-down period in 2011 (31 May; 7, 14, 21, 28 June; 5, 12 July). All chemicals were applied separately to avoid possible tank-mix incompatibility, and carrier volume was 815 $L \cdot ha^{-1}$ applied with a pressurized (276 KPa) backpack sprayer. The concentration of glycine betaine utilized was chosen based on a preliminary test that showed positive effects on creeping bentgrass growth at the 200 mM concentration.

Soil Water Status and Physiological Analysis

Soil volumetric water content was monitored using the time domain reflectometry method (Topp et al., 1980) (Trase Soil Moisture Equipment, Santa Barbara, CA). Two waveguide probes, each measuring 15 cm in length, were inserted into the root zone and soil water content measured for drought-stressed and well-watered conditions. Twenty measurements were collected within each irrigation treatment and averaged together for

the soil volumetric water content value reported. Soil volumetric water content was measured at 1, 8, 13, 16, 20, 23, and 30 d after drought stress initiation in 2010 and at 1, 6, 12, 19, 26, 32, 40, and 48 d after drought stress initiation in 2011.

Visual evaluation of turf quality was performed weekly at 1, 8, 16, 23, and 30 d after initiation of drought stress in 2010 and 1, 7, 14, 20, 26, 33, 41, and 48 d after initiation of drought stress in 2011. Quality was rated on a scale of 1 to 9, with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being green and dense turf. Ratings were based on parameters such as uniformity, visual attractiveness, leaf color, and canopy density (Beard, 1973).

Relative water content of leaves was measured at 1, 8, 16, 23, and 30 d after drought stress initiation in 2010 and at 7, 14, 30, 40, and 48 d after drought stress initiation in 2011 to indicate leaf hydration status. Approximately 1.0 g of fresh leaf blades measuring 1 cm were harvested from within individual plots and divided into three groups for RWC, EL, and osmotic adjustment. Relative water content was calculated based on fresh weight (FW), turgid weight (TW), and dry weight (DW) of leaves using the formula (%) = ([FW - DW]/[TW - DW]) × 100 (Barrs and Weatherley, 1962). Leaf fresh weight was measured with a mass balance immediately after harvesting. Samples were then wrapped in tissue paper and submerged in deionized water for 12 h at 4°C. Leaf tissue was removed from water, blotted dry, and weighed for turgid weight. Following a drying period of 3 d at 80°C, samples were weighed a final time for dry weight.

Electrolyte leakage was measured at 1, 8, 16, 23, and 30 d after drought stress initiation in 2010 and at 7, 14, 30, 40, and 48 d after drought stress initiation in 2011 to

evaluate cellular membrane stability. The sampling procedure was the same as previously stated for RWC measurement. Approximately 0.2 g of fresh leaf tissue was collected, rinsed with deionized water to remove external solutes, and placed in a test tube containing 30 mL of deionized water. Tubes were agitated on a conical flask shaker for 12 h and the initial conductance (C_i) measured using a conductivity meter (YSI Model 32, Yellow Springs, OH). Leaf samples were then autoclaved at 140°C for 20 min and again shaken for 12 h. The maximal conductance of autoclaved tissue (C_{max}) was then measured. Electrolyte leakage was calculated using the formula (%) = (C_i/C_{max}) × 100 (Blum and Ebercon, 1981).

Osmotic adjustment was measured at 1, 8, 16, 23, and 30 d after drought stress initiation in 2010 and at 7, 14, 30, 40, and 48 d after drought stress initiation in 2011 and determined by measuring the osmotic potential of leaf sap at full turgor. The sampling procedure was the same as previously stated for RWC measurement. Fresh leaf tissue was collected, immediately submerged in deionized water, and placed in 4°C for 12 h to fully hydrate leaves. Samples were blotted dry, transferred to 2-mL microcentrifuge tubes, frozen in liquid nitrogen, and stored at -20°C for further analysis. Following thawing, leaves were ground with a micropestle to express leaf sap from which a 10-mL sample was inserted into an osmometer (Wescor, Inc., Logan, UT) to determine osmolality ($mmol \bullet kg^{-1}$). Osmolality was converted to osmotic potential using the formula osmotic potential = -([osmolality] [0.001][2.58]). Osmotic adjustment was then calculated as the difference in osmotic potential between drought-stressed leaves and well-watered control leaves (Blum, 1989).

Objective canopy measurements were determined using a handheld multispectral radiometer (MSR model CT100; Crop Scan Inc., Fargo, ND) at 1, 8, 16, 23, and 30 d after drought stress initiation in 2010 and 1, 7, 12, 20, 34, 41, and 48 d after drought stress initiation in 2011. Measurements were performed on sunny days between 1100 and 1400 h. The MSR quantifies canopy reflectance characteristics to determine normalized difference vegetation index (NDVI), computed as near-infrared (NIR) minus visible reflectance (R) divided by NIR plus R (NDVI = [R935 - R660]/[R935 + R660]) according to Trenholm et al. (1999).

For both years, site rewatering coincided with a large rain event (Fig. 1A and 1B) and resulted in rapid increase of soil water content to the field capacity and daily irrigation maintained soil water content at this level thereafter. Compared to the 30-yr average, May through August 2010 was 3.32°C warmer and experienced 6.35 cm less rain and May through August 2011 was 2.6°C warmer and experienced 3.81 cm less rain. Temperature normals and precipitation normals for the past 30 years were obtained from the New Jersey Weather and Climate Network and the National Weather Service, respectively. Environmental conditions were continuously monitored via the Campbell Scientific weather station operating on Mesonet network (40.4727°, -74.4225°).

Experimental Design and Statistical Analysis

The experimental design was a split-plot design with irrigation as main plot and TE, glycine betaine, or TE plus glycine betaine as subplots. All treatments were replicated in four plots. Treatment effects were determined by analysis of variance according to the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC).

Differences between means were separated by Fisher's protected least significance difference test at the 0.05 probability level.

RESULTS

Volumetric Soil Water Content during Drought Stress and Re-watering

Soil water content was maintained at field capacity (roughly 30%) under well-watered control conditions in both 2010 and 2011. Upon termination of irrigation, soil water content began to decline steadily in 2010 and 2011 (Fig. 2A and 2B, respectively), declining to 9 to 10% in 2010. During 2011, rainfall events at 23 d into dry-down period caused a partial rewetting of the soil with an increased soil water content to 21% in the upper 15-cm soil layer followed by a rapid decline of soil water content to the lowest level at 12 to 14% by 40 and 48 d of drought stress.

Turf Quality and Canopy Characteristics as Affected by TE and Glycine Betaine during Drought Stress

There were no significant differences in TQ among TE-only, glycine betaine–only, and TE plus glycine betaine treatments during the early phases of drought stress at 1 and 8 d in 2010 (Fig. 3A) and 1 and 7 d in 2011 (Fig. 3B). Differences became significant at 16 d in 2010 and 14 d in 2011, with TQ of TE plus glycine betaine plots being significantly higher than the drought-stressed control plots. In 2011, these differences diminished with rainfall occurring at 23 d but returned thereafter. At 23 d of drought stress in 2010 and 33 d of drought stress in 2011, plots treated with TE plus glycine betaine maintained significantly higher TQ than the drought-stressed controls. During 2011, drought-stressed turf treated with TE plus glycine betaine maintained similar TQ as the well-watered control at 41 and 48 d of drought stress. Turf treated with TE alone or glycine betaine alone also had significantly higher TQ than the drought-stressed control treatment at 41

and 48 d in 2011, but neither was similar to the well-watered control. In 2010, plots treated with TE plus glycine betaine or TE-only also had significantly higher TQ than the drought-stressed controls, whereas the glycine betaine applied alone did not. Furthermore, the TE plus glycine betaine plots had significantly higher TQ at 23 d of drought stress in 2010 compared to all other drought-stressed treatments.

The effects of TE and glycine betaine on turfgrass canopy characteristics were objectively evaluated as NDVI. All plots except the TE plus glycine betaine treatment subjected to drought stress in 2011 had significantly lower NDVI compared to the well-watered controls after 20 d of drought stress (Fig. 4B). Separation between treatments was more pronounced by 34 d of drought stress in 2011. Plots treated with TE alone or TE plus glycine betaine had significantly higher NDVI compared to the untreated drought-stressed controls from 34 to 48 d of drought stress, whereas glycine betaine applied singly did not cause NDVI to differ from untreated drought-stressed controls. Similar trends were noted during the 2010 drought stress period, but differences diminished before site rewatering due to excessively hot and windy weather during the drought period. At 16 d of drought stress in 2010, plots receiving TE alone, glycine betaine alone, or TE plus glycine betaine all had NDVI significantly higher than the untreated drought-stressed controls (Fig. 4A). As drought stress progressed by 23 d, there was no difference in NDVI between glycine betaine–only plots and drought-stressed controls, whereas turf treated with TE alone or TE plus glycine betaine remained significantly higher than untreated drought-stressed controls.

Water Relations and Membrane Stability as Affected by TE and Glycine Betaine during Drought Stress

A similar trend was noted for RWC during 2010 and 2011 trial. By 16 d of drought stress in 2010, RWC of TE-only plots dropped to significantly lower levels than the untreated drought-stressed controls, whereas glycine betaine–only and TE plus glycine betaine treatments maintained RWC significantly higher than drought-stressed controls (Fig. 5A). By 23 d of drought stress in 2010, plots treated with TE plus glycine betaine maintained significantly higher RWC than drought-stressed controls, whereas glycine betaine–only plots did not and TE-only plots remained significantly lower than drought-stressed control plots.

At 7 and 14 d of drought stress in 2011, plots treated with TE only, TE plus glycine betaine, or glycine betaine only had significantly higher leaf RWC than the untreated drought-stressed control (Fig. 5B). Rainfall caused RWC to increase for all treatments by 30 d of drought stress, but TE-only, TE plus glycine betaine, or glycine betaine–only plots still had significantly higher RWC than the untreated drought-stressed controls and the TE-only treatment was similar to well-watered controls. At 40 d of drought stress in 2011, all three chemical treatments continued to maintain higher RWC than untreated drought-stressed controls, but TE plus glycine betaine was significantly higher than TE-only or glycine betaine–only plots. Before site rewatering in 2011 at 48 d of drought stress, there was no difference in RWC between TE-only plots and untreated drought-stressed controls. Among the three chemical treatments, TE plus glycine betaine had the highest RWC at 48 d of drought stress followed thereafter by glycine betaine only and both were significantly higher than the drought-stressed controls. None of the

chemical treatments maintained RWC similar to well-watered controls at 40 and 48 d of drought stress in 2011.

For osmotic adjustment, during 2010 trial the TE plus glycine betaine treatment resulted in significantly higher osmotic adjustment than untreated drought-stressed controls for the duration of drought stress (Fig. 5C). The TE-only and glycine betaine–only treatments did not result in significant differences to osmotic adjustment during the trial. Bentgrass treated with glycine betaine only or TE plus glycine betaine maintained significantly higher levels of osmotic adjustment compared to the drought-stressed control treatment across all sampling days in 2011 (Fig. 5D). The TE-only treatment had little effect on osmotic adjustment during mild to moderate level of drought stress at 7, 14, and 30 d in 2011, but did significantly increase osmotic adjustment with prolonged drought at 40 and 48 d compared to untreated drought-stressed controls. The TE plus glycine betaine treatment resulted in significantly higher osmotic adjustment among all treatments at 40 d of drought stress in 2011 and on severe drought stress at 48 d there was no significant difference between TE-only, glycine betaine–only, and TE plus glycine betaine treatments, though all three treatments maintained significantly higher osmotic adjustment than untreated drought-stressed controls.

Similar trends were noted in EL during 2010 and 2011 trial, but the treatment effects on EL were generally more pronounced in 2011 than in 2010. In 2010, the TE-only, glycine betaine–only, or TE plus glycine betaine treatments all maintained EL significantly lower than drought controls by 23 d of drought stress (Fig. 5E). Furthermore, among these three chemical treatments, the TE plus glycine betaine treatment resulted in significantly lower EL compared to TE-only or glycine betaine–only

plots. During 2011 trial, turfgrass treated with TE only, glycine betaine only, or TE plus glycine betaine maintained significantly lower EL across all sampling days compared to untreated drought-stressed controls (Fig. 5F). At 30 d of drought stress in 2011, TE plus glycine betaine plots had significantly lower EL compared to TE-only or glycine betaine–only plots. By 40 d of drought stress in 2011, there was no significant difference in EL between glycine betaine–only and TE plus glycine betaine treatments, whereas

EL of TE-only plants was significantly higher than other chemical treatments and this trend continued to 48 d of drought stress. At no point during the 2011 trial did any of the chemical treatments produce EL levels similar to well-watered controls, which remained at 20% compared with 23 to 50% for all other treatments.

DISCUSSION

Enhancement of Drought Performance in Creeping Bentgrass through Sequential Application of TE and Glycine Betaine

Previous studies have investigated the effects of foliar TE or glycine betaine on stress tolerance in several turfgrass species (Ervin and Koski, 2001b; Ervin et al., 2009; McCann and Huang, 2007) and in agronomic crops (Kirkham, 1983; Bochicchio et al., 1991; Xing and Rajashekar, 1999), but the additive effects of TE plus glycine betaine on drought tolerance, particularly in turfgrass species, have not been reported. It is especially important to investigate cultural practices promoting drought tolerance of turfgrass given the wide usage of turfgrass throughout many different climates. During both years of this study, creeping bentgrass performance measured as visual TQ during drought stress was significantly improved by the application of TE before drought stress and glycine betaine during drought stress. The effects of combining a PGR plus osmoregulant were more pronounced than when either chemical was applied singly, suggesting that sequential application was more effective or the two applications had additive effects in maintaining higher quality turf under prolonged drought stress.

The enhanced TQ of creeping bentgrass during prolonged periods of drought stress was associated with higher NDVI in TE-treated plots compared to untreated drought-stressed controls and suggests changes to plant morphology by TE, such as increased canopy density. Increased NDVI could be related to the suppression of vertical shoot growth, which may allocate carbohydrates or energy towards tiller production as previously reported for other turfgrass species (Ervin and Koski, 1998). Turf treated with TE only also had higher leaf RWC and lower EL during early drought stress compared

with untreated drought-stressed controls, but differences became less pronounced on severe drought stress in 2010 and 2011. Previous studies have shown that foliar application of TE delayed leaf dehydration in creeping bentgrass but had limited or no effects on osmotic adjustment under combined drought and heat stress (McCann and Huang, 2007, 2008). In the current study, TE did not have significant effects on osmotic adjustment during the early phases of drought stress. Similarly, in other studies with perennial ryegrass (*Lolium perenne* L.) and creeping bentgrass, TE application did not significantly alter osmotic adjustment during drought stress (Jiang and Fry, 1998; McCann and Huang, 2007). The consistent results across studies suggest that the positive effects of TE on drought tolerance could be due to an increased canopy density and is not related to altered water status of individual leaves.

Glycine betaine applied during drought stress did not have significant effects on NDVI but did lead to a significantly higher TQ, RWC, and osmotic adjustment, as well as lower EL in bentgrass leaves. Improved turf performance during drought stress due to glycine betaine could be mainly due to the maintenance of cell membrane stability and water retention in individual leaves through osmotic adjustment, which protects leaves from dehydration. Previous studies that investigated stress-mitigating effects of glycine betaine following exogenous application to agronomic crops also reported that foliar application of glycine betaine improved cellular water retention, minimized leaf dehydration, and resulted in increased biomass production (Agboma et al., 1997; Ma et. al., 2007). Farooq et al. (2008) reported that exogenous glycine betaine caused decreased permeability of cellular membranes in rice. Plants of winter wheat (*Triticum aestivum* L.) with increased accumulation of glycine betaine exhibited better tolerance to combined

heat and drought through enhanced protection of photosynthetic machinery (Wang et al., 2010a). A review that described such mechanisms involved with photosynthetic protection concluded that glycine betaine sustained the activity of oxygen-evolving photosystem II by stabilizing extrinsic proteins and aiding in Mn-cluster coordination (Papageorgiou and Murata, 1995). The accumulation of glycine betaine may also enhance antioxidant activity and is associated with greater cellular water status under abiotic stress (Wang et al., 2010b). Whether these specific mechanisms are present in turfgrass species treated with exogenous glycine betaine deserves further investigation.

The additive effects of sequential TE application before drought stress followed by glycine betaine application during drought stress in creeping bentgrass manifested as enhanced TQ and NDVI as well as physiological adjustment of individual leaves promoting water status. Overall, TE plus glycine betaine increased TQ and NDVI and maintained RWC, osmotic adjustment, and cell membrane stability on increasing drought stress severity by 48 d of water withholding in 2011. Similar effects were present during the prior 2010 trial at 23 d of drought stress, but severe weather conditions caused differences to diminish before site rewatering. Combining TE for improved NDVI and glycine betaine for leaf water retention ultimately contributed to the maintenance of TQ and may be a valuable tool for turfgrass managers to combine with current management tactics. New formulations of foliar turfgrass products containing glycine betaine may also be used in a programmatic approach with TE to delay visual appearance of drought stress and minimize the severity of drought stress. Incorporating PGRs and osmoregulants into turfgrass management protocols could be very beneficial for managing cool-season

turfgrass species and for maintaining acceptable quality in climatic areas with limited rainfall or during periods of irrigation water-use restriction.

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FIGURES

Figure 1. Daily maximum temperature and daily rainfall amounts during the experimental period of (A) 2010 trial and (B) 2011 trial.

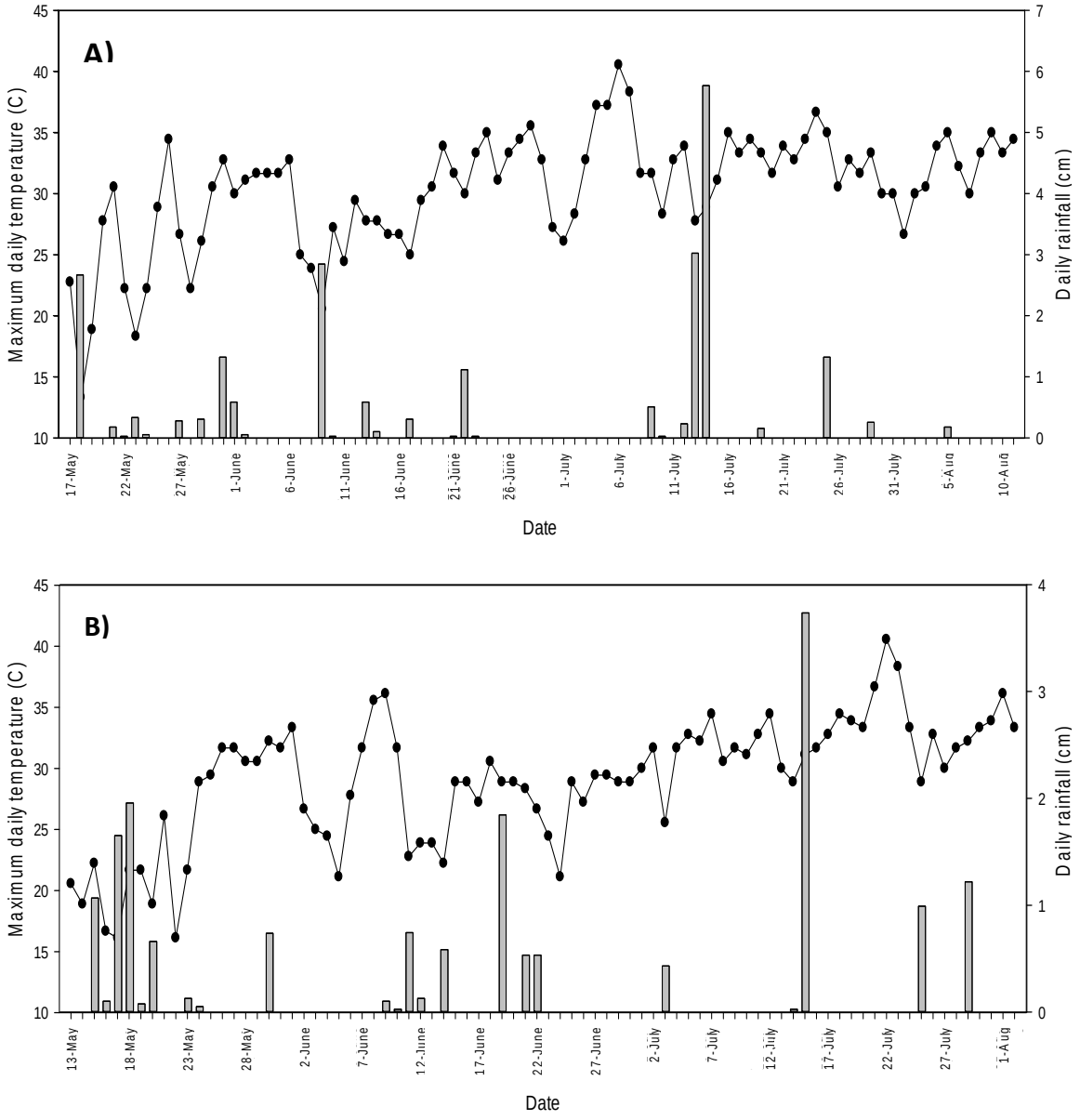


Figure 2. Volumetric soil water content (SWC) in well-watered control and drought treatment during water withholding in (A) 2010 and (B) 2011 in creeping bentgrass. Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.

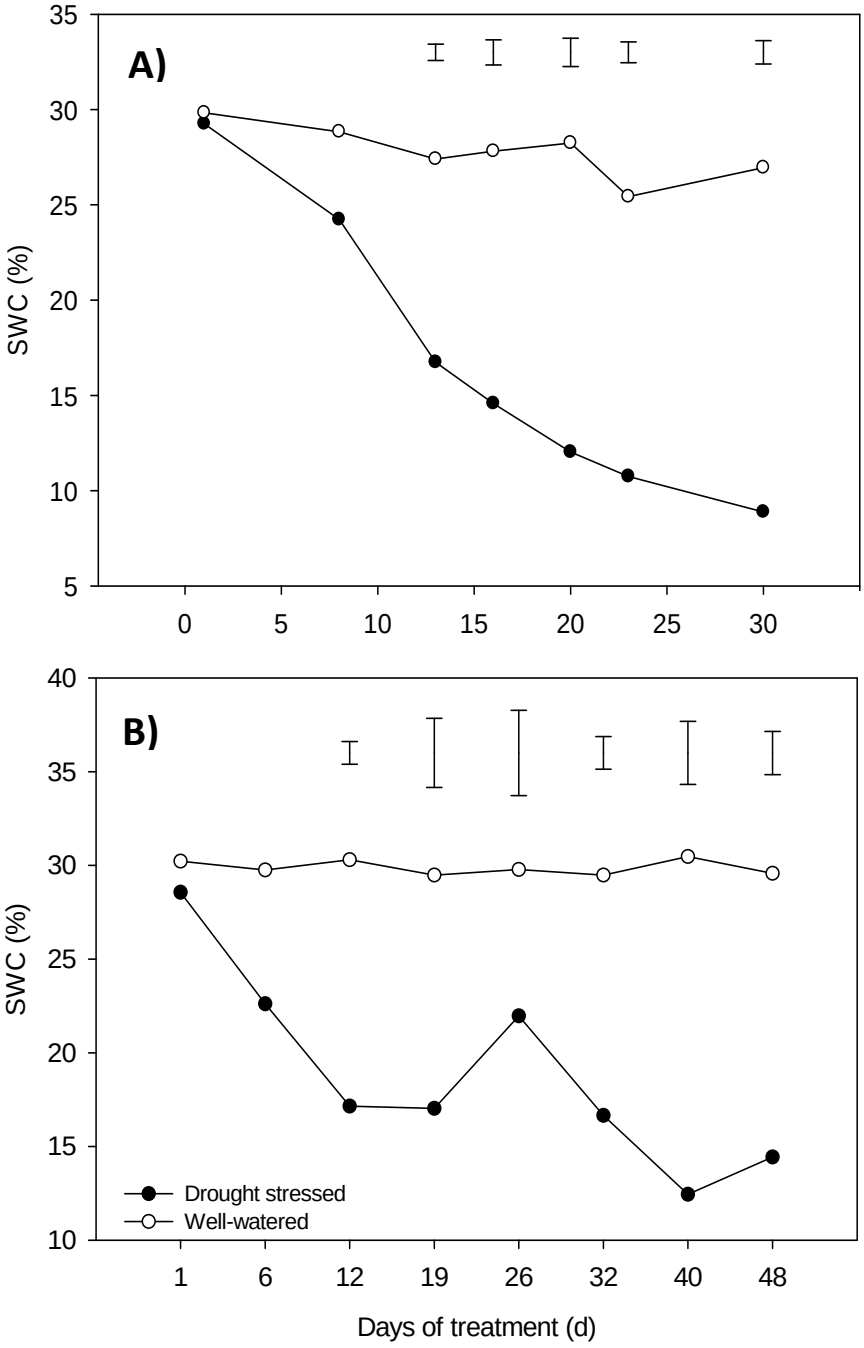


Figure 3. Turf quality (TQ) in well-watered control and drought treatment during water withholding in (A) 2010 and (B) 2011 for creeping bentgrass plants treated with trinexapac-ethyl (TE-only + drought), glycine betaine (GB only + drought), and trinexapac-ethyl plus glycine betaine (TE + GB + drought). Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.

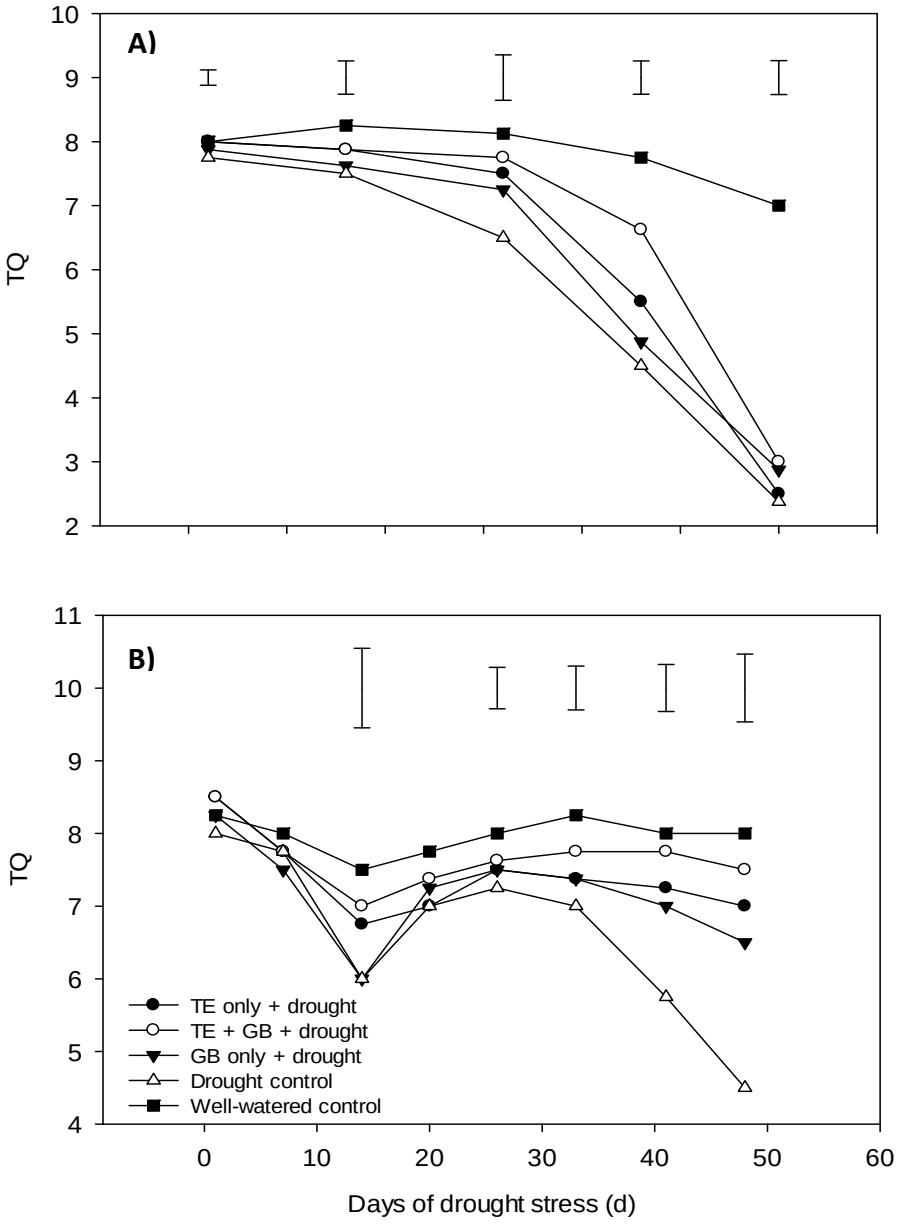


Figure 4. Normalized difference vegetation index (NDVI) in well-watered control and drought treatment during water withholding in (A) 2010 and (B) 2011 for creeping bentgrass plants treated with trinexapac-ethyl (TE-only + drought), glycine betaine (GB only + drought), and trinexapac-ethyl plus glycine betaine (TE + GB + drought). Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.

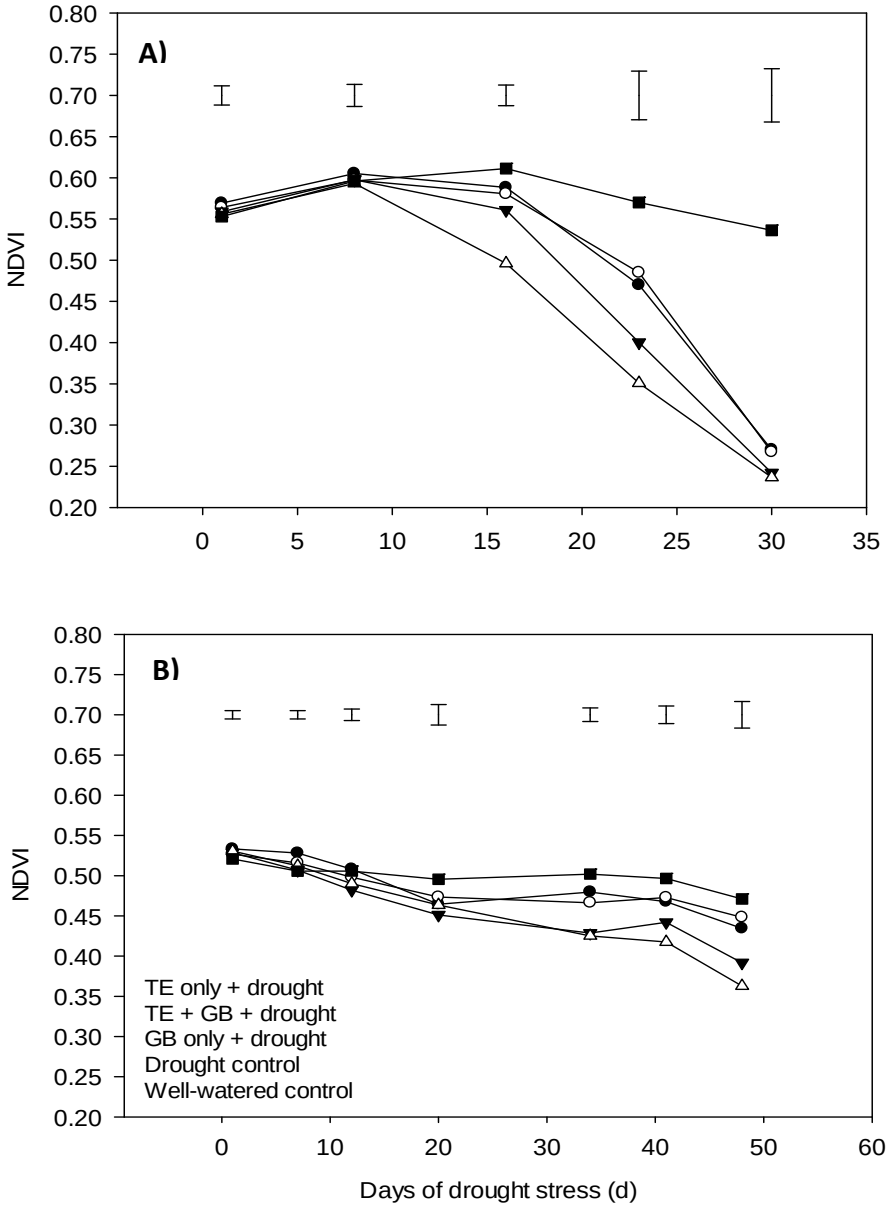
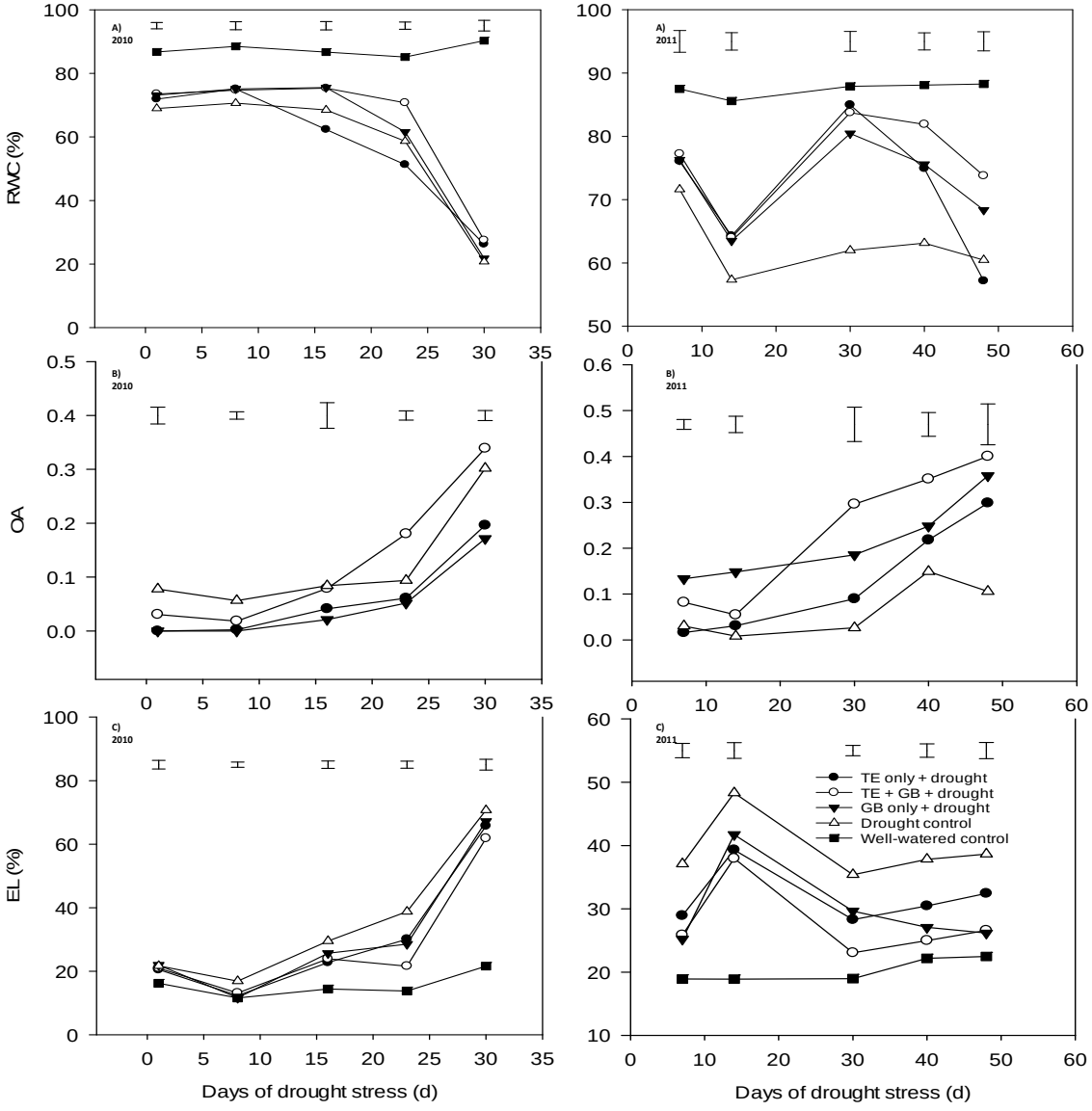


Figure 5. Leaf relative water content (RWC), osmotic adjustment (OA), and electrolyte leakage (EL) in well-watered control and drought treatment during water withholding in (A–C) 2010 and (D–F) 2011 for creeping bentgrass plants treated with trinexapac-ethyl (TE-only + drought), glycine betaine (GB only + drought), and trinexapac-ethyl plus glycine betaine (TE + GB + drought). Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.



CHAPTER TWO

FATTY ACID COMPOSITION AND SATURATION IN LEAVES AND ROOTS ASSOCIATED WITH IMPROVED DROUGHT TOLERANCE IN AGROSTIS STOLONIFERA EXPRESSING SAG12-IPT GENE CONTROLLING CYTOKININ SYNTHESIS

Burgess, P., Gianfagna, T., and B. Huang. 2013. Fatty acid metabolism in leaves and roots associated with improved drought tolerance in Agrostis stolonifera expressing SAG12-ipt gene controlling cytokinin synthesis. Int. Turfgrass Soc. Res. J. 12:497-502.

ABSTRACT

Creeping bentgrass over-expressing the *SAG12-ipt* gene encoding adenine isopentenyl phosphotransferase controlling cytokinin synthesis exhibited improved drought tolerance. This study was designed to elucidate whether changes in membrane fatty acid composition and saturation levels in leaves and roots are associated with improved growth of creeping bentgrass (*Agrostis stolonifera*) under drought stress. *SAG12-ipt* transgenic and wild-type (WT) plants were exposed to well-watered conditions or drought stress for 15 d. Leaves and roots were sampled at 8 d and 15 d of drought and the following morphological and metabolic parameters were compared between the transgenic and WT plants, including visual evaluation of turf quality (TQ), relative water content of leaves (RWC), photochemical efficiency of leaves (F_v/F_m), leaf and root membrane stability via electrolyte leakage (EL), leaf and root lipid peroxidation by quantifying malondialdehyde production (MDA), leaf and root membrane fatty acid content and saturation level. Compared to WT plants, transgenic plants maintained significantly higher TQ, higher leaf RWC and F_v/F_m, as well as lower EL and MDA in leaves and roots under drought stress. Transgenic plants maintained higher levels of long-chain unsaturated fatty acids such as linoleic (C18:2) and linolenic (C18:3) acid during drought compared to WT plants. Increased unsaturated fatty acids could help maintain membrane fluidity, contributing to continued leaf and root growth during drought stress in the transgenic plants as demonstrated by enhanced turf quality and physiological activities.

INTRODUCTION

Drought stress is a major limiting factor for turfgrass growth. Understanding drought tolerance mechanisms is critically important for developing stress-tolerant germplasm for areas with limited rainfall and water availability for irrigation. Plant perception of drought first occurs via membranes which initiate signal transduction and gene expression, subsequently inducing physiological responses (Shinozaki, 1997; Los and Murata, 2004). Membranes are mainly composed of lipids with saturated or unsaturated fatty acids. Maintaining proper proportions of saturated and unsaturated fatty acids is of great importance in plant adaptation to unfavorable growing conditions, as previous demonstrated in the model plant *Arabidopsis thaliana* (Gigon et al., 2004) and agronomic crops such as *Triticum aestivum* (An et al., 2000) and *Avena sativa* (Berglund et al., 2004). Plant adaptation to abiotic stress includes various physiological changes as well as alteration of membrane fatty acid composition. For instance, the amount of unsaturated fatty acids such as linolenic (C18:3) and linoleic (C18:2) decreases as shown in drought-sensitive *Brassica napus* (Dakhma et al., 1995). Plant species which are more tolerant to drought stress maintain higher levels of these unsaturated fatty acids to maintain membrane fluidity and proper cellular functions (Toumi et al., 2008).

One approach to enhancing drought tolerance is by introducing genes underlying stress tolerance. Previous studies have created transgenic creeping bentgrass lines with a gene encoding adenine isopentenyl phosphotransferase (*ipt*) controlling cytokinin synthesis (Xing et al., 2008) and reported improved drought tolerance of the transgenic creeping bentgrass (Merewitz et al., 2010, 2011a,b, 2012). These studies found the improvement in drought tolerance in *ipt* transgenic plants was associated with higher

photosynthetic activity and root viability as well as the accumulation of antioxidant enzyme proteins, carbohydrates, organic acids, and amino acids. However, how changes in membrane fatty acid composition and saturation level may be associated with improved physiological activities and drought tolerance is not well documented in turfgrass species. The transgenic creeping bentgrass with improved growth and physiological activities under drought stress is an excellent germplasm to investigate membrane fatty acids related to drought tolerance in creeping bentgrass.

The objective of the current study was to determine if whole-plant drought tolerance previously seen in transgenic creeping bentgrass expressing the *SAG12-ipt* gene for cytokinin synthesis is associated with changes in membrane fatty acid composition and saturation level when compared to wild-type plants. Given the wide usage of creeping bentgrass throughout the turfgrass industry, elucidating on the factors governing drought response is of high importance.

MATERIALS AND METHODS

Plant materials and Growth Conditions

Tillers of transgenic (*SAG12-ipt*; S32 line) creeping bentgrass and the wild type (WT) (cv. Penncross) were propagated in polyvinyl chloride pots filled with fritted clay medium and allowed to establish in a greenhouse for eight weeks. Growing conditions during establishment were 22.0/17.0 °C (day/night), 50% relative humidity, approximately 11 h sunlight at photosynthetically active radiation (PAR) of 500 μmol•m^{-1}•s^{-1}. Plants were trimmed at 2 cm canopy height and fertilized weekly with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Plants were then transferred to a controlled-climate walk-in growth chamber set to 22.0/17.0 °C (day/night), 50% relative humidity, 12 h photoperiod at 700 μmol•m^{-1}•s^{-1} PAR, and allowed to acclimate for one week before drought stress treatment.

Treatments and Experimental Design

Non-stressed control plants were irrigated every day to maintain soil water content (SWC) at field capacity (28%). Drought was imposed by withholding irrigation for 15 d at which time soil volumetric water content (SWC) of drought stressed plants was 9%. SWC was monitored via the time domain reflectometry method (Trase; Soil Moisture Equipment, Santa Barbara, CA) (Topp, 1980). Each treatment per plant line had four replicated pots of plants. Treatments and plant lines were arranged in a randomized complete block design.

Physiological analysis

Turf quality (TQ) was evaluated based on a scale of 1 to 9, with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being green and dense turf. Ratings were based on parameters such as uniformity, visual attractiveness, leaf color, and canopy density (Beard, 1973).

Relative water content (RWC) of leaves was measured to indicate leaf hydration status and was calculated based on three weights of leaf tissue collected: fresh weight (FW), turgid weight (TW), and dry weight (DW) using the formula (%) = [(FW – DW)/ (TW – DW)] x100. Leaf FW was determined with a mass balance immediately after harvesting. Samples were then wrapped in tissue paper and submerged in deionized water for 12 h at 4°C. Leaf tissue was removed from the water, blotted dry, and again weighed for TW. Following a drying period of three days at 80°C, samples were weighed a final time for DW (Barrs and Weatherley, 1962).

Electrolyte leakage (EL) was measured to indicate cellular membrane stability. Approximately 0.1 g (FW) leaf or root tissue was collected, rinsed with deionized water, and placed in a conical test tube containing 30 mL deionized water. Tubes were placed on a conical flask shaker for 12 h and the initial conductance (C_i) was measured using a conductivity meter (YSI Model 32 , Yellow Spring, OH). Tissue samples were killed by autoclaving at 140 °C for 20 minutes and again shaken for 12 h. The maximal conductance of killed tissue (C_{max}) was then measured. Leaf or root EL was calculated using the formula (%) = (C_i/C_{max}) x100 (Blum and Ebercon, 1981).

Leaf photochemical efficiency (F_v/F_m) was expressed as chlorophyll fluorescence and calculated as the ratio of variable fluorescence (F_v) to maximal fluorescence (F_m)

using a chlorophyll fluorescence meter (Fim 1500; Dynamax, Houston). Leaf-clips were used to dark-adapt leaves for 30 min prior to F_v/F_m measurement and two subsample measurements were taken per plant.

Lipid peroxidation of leaves and roots was determined by measuring malondialdehyde (MDA) content using the method set forth by Dhindsa et al. (1981). 0.2 g tissue was ground in 4 ml solution containing 50 mM phosphate buffer (pH 7.0), 1% (w/v) polyvinylpolypyrrolidone, and 0.2 mM ascorbic acid. The homogenate was centrifuged at 15 000 g for 30 min and supernatant collected. 2 mL supernatant was added to 1 mL 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. The mixture was heated in a water bath at 95°C for 30 min, quickly cooled to room temperature, and centrifuged at 10,000 g for 10 min. Supernatant absorbance was measured at 532 nm using a spectrophotometer (Genesys 2; Spectronic Instruments, Rochester, NY) and the nonspecific absorbance at 600 nm. MDA content was then calculated using the extinction coefficient of 155 $mM^{-1} \bullet cm^{-1}$ (Heath and Packer, 1968).

Analysis of Membrane Fatty Acids

Fatty acid composition and content in leaves and roots were determined using the method described in Browse et al. (1986). Fresh leaf tissues (0.2 g) were acidified with 1 N H2SO4 and fatty acids were methylated by heating at 80 °C for 90 min in solution containing heptadecanoic acid (17:0) as internal standard. After a rapid cooling, 0.9% NaCl solution and hexane were added, samples vortexed, and then centrifuged for 5 min at 250 g. A 1 μL extract was taken from the hexane layer and subjected to gas chromatography and mass spectrometry analysis. Fatty acids were separated and

identified using a HP GC-MS (HP 6890 GC and HP 5973 MS) (Hewlett Packard Co., Palo Alto, CA, USA) equipped with a 60-mHP-5MS capillary column with an inner diameter of 0.25 mm. The GC was programmed to begin at 170 °C for 10 min, followed by a 10 min ramp to 220 °C at a flow rate of 1 ml per minute. Individual fatty acids were identified based on relative peak time. Saturation levels, expressed as a proportion of the total fatty acids present, were determined based on percent recovery against the heptadecanoic acid (17:0) internal standard. Relative unsaturation level of fatty acids was estimated using the double-bond index (DBI) calculated with the flowing equation: DBI = [16:1 + 18:1] + [2(16:2 + 18:2)] + [3(18:3)] (Cyril et al., 2002). Values used are the proportion of each fatty acid expressed as a percent.

Statistical Analysis

Treatment effects and variations between two plant lines (transgenic vs. WT) were determined by analysis of variance (ANOVA) according to the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC). Differences between means were separated by Fisher's protected least significance difference (LSD) test at $p \leq 0.05$ level.

RESULTS AND DISCUSSION

Differential Physiological Responses between Transgenic Plants and Wild-type Plants to Drought Stress and Re-watering

Visual evaluation of turf quality (TQ) showed notable differences between treatments across all three sampling days. The TQ of transgenic plants exposed to 8 d drought stress did not differ from that of well-watered plants, whereas WT plants exposed to drought had significantly lower TQ than the control plants (Table 1). By 15 d of drought stress, TQ of both WT and transgenic plants declined significantly below that of the respective well-watered control, but transgenic plants had significantly higher TQ than WT plants. Leaf water content (RWC) of transgenic plants exposed to drought was 68.2% and 51.7% at 8 and 15 d drought, respectively, and was significantly higher than WT plants exposed to drought (56.2% and 36.2%, respectively) (Table 1). Leaf photochemical efficiency (F_v/F_m) represents chlorophyll fluorescence of photosystem two (PSII) and showed significant differences between the two plant lines at 15 d of drought stress (Table 1). Transgenic plants had significantly lower F_v/F_m compared to WT plants at 15 d drought stress but not at 8 d drought stress.

Membrane stability indicated by percent electrolyte leakage (EL) from leaves also showed distinct response patterns between the two plant lines during the experimental period. Leaves of transgenic plants exposed to 8 and 15 d of drought had significantly lower EL (38.7% and 51.5%, respectively) compared to WT plants (45.5% and 66.4%, respectively) (Table 1). Membrane lipid peroxidation measured as MDA content increased to significantly higher than the well-watered control plants at 15 d of drought

stress in leaves of both transgenic and WT plants but leaves of droughted transgenic plants had significantly lower MDA content than those of WT plants (Table 1).

Root characteristics also showed differential response patterns between the two plant lines in response to drought. Differences in root membrane stability were most notable at 15 d of drought, as transgenic plants had a significantly lower EL (31.6%) compared to WT (41.8%) (Table 2). Root MDA content increased to significantly higher levels than the well-watered control plants at 15 d of drought stress in both transgenic and WT plants, but transgenic plants had significantly lower MDA content than WT plants (Table 2).

These results indicated that transgenic creeping bentgrass plants expressing the *SAG12-ipt* gene displayed improved physiological activities and growth of leaves and roots compared to non-transgenic WT plants under drought stress, confirming the results in previous studies that *SAG12- ipt* transgenic plants exhibited improved TQ, leaf RWC, F_v/F_m, and photosynthetic activities (Merewitz et al. 2010a, 2011a). Previous studies have reported increased levels of certain proteins and metabolites such as carbohydrates, organic acids and amino acids associated with improved drought tolerance in the transgenic creeping bentgrass expressing the ipt gene (Merewitz, 2011b, 2012). The current study found that improved drought tolerance in creeping bentgrass could also be related to changes in fatty acids in leaves and roots, as discussed below.

Differences in Fatty Acid Content and Saturation in Leaves and Roots between Transgenic Plants and Wild-type Plants under Drought and Re-watering

Five fatty acids [palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), linoleic acid (C18:2), and linolenic acid (C18:3)] were detected in leaves and roots of both transgenic and WT plants under well-watered and drought stress conditions. These five fatty acids are found primarily in the plasma membranes, thylakoid, and mitochondria membranes (Millar et al., 2000). Under well-watered conditions, the majority of fatty acids in leaves were linolenic (58-60%), followed by linoleic and palmitic acid (both 17-20%) (Table 3). Lipid composition in root tissue was slightly different under non-stress conditions, with the majority being linoleic acid (39- 41%), followed by palmitic (25-27%) and linolenic acid (16- 17%) (Table 4). Leaves and roots both had similar amounts of stearic acid (>2.0%) while small amounts of palmitoleic acid were detected only in leaves under well-irrigated conditions. These proportions are in accordance with previous research which investigated fatty acid composition and saturation level in *A. stolonifera* during heat stress (Liu and Huang, 2004). The two plant lines did not exhibit differences in the content of palmitic acid (C16:0) and palmitoleic acid (C16:1) in either leaves or roots under well-watered and drought conditions (data not shown).

The content of stearic acid (C18:0) of both plant lines increased at 15 d of drought stress to a significantly higher level than that of well-watered plants in leaves (Table 3). Linoleic acid (C18:2) in leaves increased to a significantly higher level than the well watered plants at 15 d of drought in transgenic plants but did not show differences between the two treatments for the WT plants. The content of linolenic acid (C18:3) of

drought-stressed leaves were significantly lower at both 8 and 15 d of drought stress than that under well-watered conditions in the WT-plants, but was only lower at 8 d of drought in the transgenic plants (Table 3). Leaves of transgenic plants had significantly lower content of stearic acid (C18:0) but significantly higher content of linoleic acid (C18:2) and linolenic acid (C18:3) during drought stress. The double bond index (DBI) in leaves of transgenic plants was significantly greater in transgenic plants than the WT plants under drought stress (Table 3).

Root stearic acid (18:0) content decreased to a significantly lower level in both transgenic and WT-plants at 15 d of drought stress, but transgenic plants exhibited significantly lower amount of stearic acid (Table 4). Root linoleic acid (18:2) content at 15 d of drought stress was significantly greater than that under well-watered conditions in both transgenic and non-transgenic plants and was significantly higher in transgenic plants than that in WT plants (Table 4). Linolenic acid (18:3) content was significantly higher in roots of transgenic plants compared to that in WT plants under drought stress. The DBI in roots were significantly higher in roots of transgenic plants than those in the WT plants at 8 and 15 d of drought.

It is well-accepted that cellular membranes are the primary site for stress recognition and integral to cellular functions (Tuteja and Sopory, 2008) and that there exists a close relationship between fatty acid saturation level and stress sensitivity (Mikimi and Murata, 2003). Upon soil water deficit, cellular membranes of drought-sensitive agronomic plant species undergo a reduction in unsaturated lipids and a concomitant increase in saturated lipids such as demonstrated in *Phaseolus vulgaris* (Junior et al., 2008) and *Carthamus tinctorius* (Hamrouni et al. 2001) whereas drought-

tolerant plant lines tend to maintain better membrane fluidity through higher levels of unsaturated lipids (Guerfel et al., 2008). Membranes composed primarily of unsaturated fatty acids can therefore maintain photosynthetic activities and aide in whole-plant acclimation to abiotic stress (Falcone el al., 2004). The maintenance of higher unsaturation level of fatty acids due to higher content of unsaturated fatty acids in both leaves and roots of transgenic plants relative to the WT plants could at least partially contribute to the improved drought tolerance. Previous research using Kentucky bluegrass (*Poa pratensis* L.) showed similar accumulation of unsaturated fatty acids in drought-tolerant plants and it was suggested that improved performance under water deficit could be due to sustained activity of lipid desaturases (Xu et al., 2010). Drought-tolerant plants maintain higher levels of omega-3 fatty acid desaturase gene expression in chloroplasts which corresponds to successive desaturations of non-branched fatty acids (Torres-Franklin et al., 2009). This ensures adequate membrane stability through proper functioning of integral membrane proteins (Los and Murrata, 2004; Upchurch, 2008). Plants which are particularly sensitive to drought have limited chloroplast function due to lipid peroxidation as demonstrated by Ferraro-Iliou et al. (1994) who used reactive oxygen species to induce peroxidation for three plant lines differing in drought tolerance. Similar results became evident in the present study by quantifying MDA of leaves and roots. Transgenic plants had significantly lower MDA content in roots and leaves compared to WT plants, suggesting less oxidative damages in cellular membranes.

In summary, results from the present study suggest that improved turf quality and physiological activities of transgenic creeping bentgrass during drought stress could be associated with the increased production of long-chain unsaturated fatty acids, which

could help maintain membrane stability and fluidity under drought stress, contributing to improved leaf and root growth. Further research may be conducted to understand the biochemical and molecular factors causing the increased production of unsaturated fatty acids in plants with improved drought tolerance, providing further insights into drought tolerance mechanisms in turfgrasses.

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TABLES

Table 1. Visual rating of turf quality (TQ), leaf relative water content (RWC), leaf electrolyte leakage (EL), and leaf photochemical efficiency (F_v/F_m) of transgenic *SAG12-ipt* creeping bentgrass (line S32) and wild-type 'Penncross' (WT) exposed to 8 and 15 d of drought stress or well-watered conditions (control). Values followed by the same letter are not significantly different based on Fisher's protected LSD test ($P \leq 0.05$) for treatment comparisons at a given day of treatment.

Days	Treatment	Plant line	TQ 1-9 scale	SWC %	RWC %	EL %	F_v/F_m ratio	MDA content nmol • g^{-1} FW
8 d	Drought	WT	7.75c	9.74b	56.18c	45.50a	0.798a	146.58a
	Control	WT	8.75ab	25.32a	79.26a	38.73b	0.787b	60.56c
	Drought	S32	8.63b	10.36b	68.24b	31.76c	0.791ab	93.85b
	Control	S32	9.00a	25.36a	78.14a	33.68c	0.793ab	57.35c
		LSD	0.37	0.76	2.7	2.94	0.0075	13.159
15 d	Drought	WT	5.13c	8.79b	39.20c	66.48a	0.728c	
	Control	WT	8.63a	28.83a	79.10a	51.56b	0.787a	
	Drought	S32	7.12b	9.36b	54.69b	32.16c	0.774b	
	Control	S32	8.88a	28.35a	79.06a	32.47c	0.789a	
		LSD	0.63	0.92	2.52	3.17	0.0104	

Table 2. Root electrolyte leakage (EL) and root malondialdehyde production (MDA) of transgenic *SAG12-ipt* creeping bentgrass (line S32) and wild-type 'Penncross' (WT) exposed to 8 and 15 d of drought stress or well-watered conditions (control). Values followed by the same letter are not significantly different based on Fisher's protected LSD test ($P \leq 0.05$) for treatment comparisons at a given day of treatment.

Days	Treatment	Plant line	EL %	MDA content nmol • g^{-1} FW
8 d	Drought	WT	18.02b	51.98a
	Control	WT	17.01b	25.48c
	Drought	S32	20.25a	38.98b
	Control	S32	13.84c	20.73d
		LSD	1.27	2.19
15 d	Drought	WT	41.80a	
	Control	WT	19.25c	
	Drought	S32	31.63b	
	Control	S32	15.80d	
		LSD	2.34	

Table 3. Content of stearic acid (C18:0), linoleic acid (C18:2), linolenic acid (C18:3), and double-bond index (DBI) in leaves of transgenic *SAG12-ipt* creeping bentgrass (line S32) and wild-type 'Penncross' (WT) exposed to 8 and 15 d drought stress or well-watered conditions (control). Values followed by the same letter are not significantly different based on Fisher's protected LSD test ($P \leq 0.05$) for treatment comparisons at a given day of treatment.

Days	Treatment	Plant Line	Stearic acid	Linoleic acid	Linolenic acid	DBI
			%	%	%	
8 d	Drought	WT	1.56b	17.08a	51.84c	205.0c
	Control	WT	1.67b	18.01a	58.03a	208.3bc
	Drought	S32	1.19c	17.40a	55.41b	217.5a
	Control	S32	1.82a	17.97a	58.62a	211.6b
		LSD	0.127	0.946	1.898	3.643
15 d	Drought	WT	1.78a	18.34b	53.81c	204.27c
	Control	WT	1.29c	18.06b	60.38a	211.75b
	Drought	S32	1.48b	19.44a	59.17b	219.84a
	Control	S32	1.27c	17.46c	60.18ab	215.12b
		LSD	0.069	0.578	1.104	4.18

Table 4. Content of stearic acid (C18:0), linoleic acid (C18:2), linolenic acid (C18:3), and double-bond index (DBI) in roots of transgenic *SAG12-ipt* creeping bentgrass (line S32) and wild-type 'Penncross' (WT) exposed to 8 and 15 d of drought stress or well-watered conditions (control). Values followed by the same letter are not significantly different based on Fisher's protected LSD test ($P \leq 0.05$) for treatment comparisons at a given day of treatment.

Days	Treatment	Plant line	Stearic acid %	Linoleic acid %	Linolenic acid %	DBI
8 d	Drought	WT	0.994a	39.29b	16.31b	129.3b
	Control	WT	0.828c	38.97b	16.92ab	129.7b
	Drought	S32	0.879b	42.56a	17.57a	136.9a
	Control	S32	0.885b	40.28ab	16.27b	129.6b
		LSD	0.0299	2.469	0.977	1.859
15 d	Drought	WT	0.727c	41.69b	17.11a	134.7b
	Control	WT	0.842b	40.38c	17.15a	132.2c
	Drought	S32	0.588d	43.78a	17.83a	141.1a
	Control	S32	0.901a	39.92c	17.16a	130.5c
		LSD	0.039	1.194	0.758	2.967

CHAPTER THREE

GROWTH AND PHYSIOLOGICAL RESPONSES OF CREEPING BENTGRASS (AGROSTIS STOLONIFERA) TO ELEVATED CARBON DIOXIDE CONCENTRATIONS

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ABSTRACT

The atmospheric carbon dioxide level has increased and is predicted to continue increasing, which may affect various aspects of plant growth. The objective of this study was to investigate the effects of doubling the carbon dioxide level on the growth and physiological activities of a widely utilized cool-season turfgrass species, creeping bentgrass (*Agrostis stolonifera* L. 'Penncross'). 'Penncross' plants were established in fritted clay medium and maintained under well-irrigated and well-fertilized conditions in growth chambers. The plants were exposed to either ambient carbon dioxide concentrations (400 ±10 mmol • L^{-1}) or elevated carbon dioxide concentrations (800 ±10 mmol • L^{-1}) for 12 weeks. Plants grown under elevated carbon dioxide displayed a significantly faster growth rate of their lateral stems (stolons) and increased shoot and root dry weight but a reduced specific leaf area compared to those plants at ambient carbon dioxide levels. Fast stolon growth is a highly desirable trait for turfgrass establishment and recovery from physical damage. The root length and surface area were also increased due to the elevated CO_2, which may facilitate water uptake and serve critical drought-avoidance roles when irrigation water is limited. Elevated carbon dioxide caused an increase in the leaf net photosynthetic rate but a reduction in the stomatal conductance and transpiration rate, contributing to improved water use efficiency in creeping bentgrass. Efficient water use is especially important for turfgrass plant survival when irrigation water is limited. Our results suggested that cool-season turfgrass species may greatly benefit from increasingly elevated carbon dioxide concentrations via growth promotion and increasing water use efficiency.

INTRODUCTION

Atmospheric carbon dioxide (CO_2) levels have risen by 69 mmol • L^{-1} from 1958 to 2008 (Dlugokencky, 2009) and the rate of increase is predicted to hasten during the next century (Houghton et al., 2001). Elevated CO_2 concentrations have a significant impact on plant growth, productivity and species composition in agricultural and natural ecosystems (Kirkham, 2011). Meta-analysis of wild grass species has shown that C3 grasses have increased rates of lateral tillering when exposed to elevated CO_2, while C4 grasses display a greater increase in leaf area (Wand et al., 1999). The positive effects of elevated CO_2 on annual crops are linked to increased photosynthesis and water use efficiency (Lee et al., 2011; Morison, 1998; Reddy et al., 2010) as well as root formation and root elongation (Kirkham, 2011; Pritchard et al., 2010) associated with enhanced cell wall extensibility and carbon supply under elevated CO_2 (Taylor et al., 1994). A majority of the research regarding plant response to elevated CO_2 has been focused on agronomic crop species, which themselves vary in growth, productivity and response to interacting environmental stresses (Kirkham, 2011). Few studies have investigated the effects of elevated CO_2 on perennial grasses utilized as fine turfgrass (Yu et al., 2012a; 2012b; 2014). There are over 35 000 km^2 of managed turfgrass within the United States (Milesi et al., 2005) where it serves many important environmental functions, such as erosion control, surface water detoxification and the control of allergens and diseases (Beard and Green, 1994). Hence, changes in turfgrass growth, physiology and stress-response due to rising CO_2 levels are of great importance for many aspects of environmental stewardship and turfgrass management.

Unlike other agronomic or horticultural crops that are grown for grain or fruit yield production, the goal of turfgrass management is to obtain high quality plants as defined by leaf color, canopy density, aesthetics and playability, and the consistency of these traits over time (Stier et al, 2015). To achieve high quality, turfgrasses are managed to maintain active yet slow vegetative growth rates and high water use efficiency, which becomes especially important when water for irrigation is limited. As human populations grow and climate patterns (notably atmospheric CO_2 levels) continue to change, there is an ever-increasing demand for the efficient allocation of water resources, justified through the research of turfgrass water use patterns responding to environmental conditions (Vickers, 2001). Understanding how elevated CO_2 may affect the major traits of turfgrass growth and water use efficiency provides guidelines for how to develop efficient management tactics, such as irrigation, to maintain quality turfgrass in the scenario of increasing CO_2 concentration in the future.

The objective for this research was to examine the effects of a doubled CO_2 concentration on growth, morphological, and physiological processes for cool-season creeping bentgrass maintained under well-watered and well-fertilized conditions.

MATERIALS AND METHODS

Plant materials and Growth Conditions

Individual tillers (40 per pot) of creeping bentgrass (*Agrostis stolonifera* L. 'Penncross') were propagated in plastic pots (10 cm diameter x 40 cm depth) filled with fritted clay medium. The plants were maintained in a greenhouse with an average temperature of 22/17 °C (day/night), 700 mmol • m^{-2} • s^{-1} photosynthetic active radiation from natural sunlight, and 65% relative humidity for 56 days to establish canopy and root systems. During establishment, the plants were irrigated to water-holding capacity daily, fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1950) twice per week and had their leaves trimmed once per week to maintain a 5-cm canopy height. The plants were then trimmed to a 2-cm canopy height and moved to controlled-climate growth chambers (Environmental Growth Chamber, Chargrin Falls, OH, USA) set to 21/18 °C (day/night) temperature, 60% relative humidity, 650 mmol • m^{-2} • s^{-1} photosynthetic active radiation and a 14-h photoperiod for 1 week prior to CO_2 treatment. During the 12-week CO_2 treatment, the plants were maintained under well-watered conditions with daily irrigation and fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1950) twice per week and their leaves were not trimmed.

Treatments and Experimental Design

Plants were exposed to two CO_2 treatments: ambient concentration (400 ± 10 mmol • L^{-1}) or elevated concentration (800 ± 10 mmol • L^{-1}). Each CO_2 treatment was applied in four different growth chambers and the plants were moved between the chambers every 3 days to eliminate the potential confounding effects of environmental variations between

chambers. The experiment was arranged in a randomized complete block design with four replicates (pots) per treatment.

The ambient and elevated CO_2 concentrations within the chambers were maintained through an automatic CO_2 controlling system connected to the CO_2 source-tank containing 100% research-grade CO_2 using a previously-described method (Yu et al., 2012a). The CO_2 concentrations inside the chambers were continuously monitored using an infrared gas analyzer (Li-820; LICOR, Inc., Lincoln, NB, USA) connected to a computer data logger. The CO_2 concentration was maintained using an automatic controlling system consisting of a programmable logic controller unit, solenoid valves and a laptop computer with software capable of monitoring and maintaining the CO_2 concentration within 10 mmol • L^{-1} of the ambient or elevated target levels.

Morphological Analysis

The lengths of the lateral stems (stolons) were measured at 70 and 84 days of CO_2 treatment from 10 randomly-selected stolons per replicate, and the values were averaged together within each replicate. The specific leaf area was measured at 63, 70, 77 and 84 days of CO_2 treatment. Twenty of the second fully expanded leaves per replicate were excised from atop the stolons and were immediately scanned using a hand-held digital scanner. The leaves were then dried in an oven set to 80 6C for 7 days, and their dry weight was subsequently measured. The leaf area was calculated with Digimizer software (MedCalc Software bvba, Mariakerke, Belgium) using scanned digital images of the fresh leaf samples. The specific leaf area was then expressed as the leaf area per unit dry weight leaf tissue. The number of stomates per unit leaf area was measured at 63, 70, 77

and 84 days of CO_2 treatment. Twenty of the second fully expanded leaves were detached from atop stolons and placed on a glass slide in immersion oil to maintain the original state of the stomates. Images (1344 x 1024 pixels) of the adaxial leaf surface were captured under a light microscope (NIKON Inc., Melville, NY, USA) equipped with a digital camera. The camera was calibrated prior to usage for the accurate conversion of pixel size to actual leaf area. All plants were destructively sampled at 84 days of CO_2 treatment for an analysis of the root and shoot biomass accumulation. The roots were severed from the shoots at the soil line and washed free of fritted clay medium. A subset of the root tissue was divided into four zones (0–10, 10–20, 20–30 and 30–40 cm), stained in 1% crystal violet solution and scanned with a digital scanner to generate the root images. The images were then analyzed with WinRHIZO Basic V.2002 software (Regent Instruments Inc., Quebec City, Que., Canada) for their root length and surface area. All of the tissue was then dried in an oven at 80 6C for 7 days, and the dry weight was subsequently measured, yielding the shoot weight, root weight and root to shoot ratio (R/S).

Physiological Analysis

The leaf net photosynthetic rate (Pn), stomatal conductance (g_s) and transpiration rate (T) were measured at 63, 70, 77 and 84 days of treatment. Six of the second fully expanded leaves from atop stolons were arranged in a 6 cm^2 cuvette chamber attached to a portable infrared gas analyzer (Li- 6400; LICOR, Inc.). The leaf Pn, g_s and T were measured in the leaf chamber using a red and blue light source at 800 mmol photon • m^{-2} • s^{-1} and a flow rate of 500 mmol • s^{-1}. The leaf area was determined using a hand-held digital

scanner immediately following leaf removal from the cuvette. Water use efficiency (WUE) was also measured at 63, 70, 77 and 84 days of CO_2 treatment according to the formula WUE = Pn/T.

Statistical Analysis

The main effects of the CO_2 treatment were determined by an analysis of variance according to the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC, USA). Differences between the treatment mean values were distinguished by Fisher's protected least significance difference (LSD) test at the 0.05 probability level.

RESULTS AND DISCUSSION

Shoot and Root Growth of Creeping Bentgrass as Affected by Elevated Carbon Dioxide Concentrations

Creeping bentgrass plants grown under elevated CO_2 had significantly longer stolons at 70 and 84 days of CO_2 treatment compared to the control plants maintained under ambient CO_2 (Fig. 1A). Elevated CO_2 increased the stolon length by approximately 25% at 70 days of CO_2 treatment. The treatment effects became more pronounced over time; the stolon length increased by 40% due to elevated CO_2 at 84 days of CO_2 treatment. A visual depiction showing the growth differences between the elevated and ambient CO_2 treatments just prior to plant harvest is provided in Figure 1B. Increased rates of lateral spread through stolon growth is a highly desirable turfgrass trait and serves to improve aesthetics, functionality, and recovery from stress damage (Turgeon, 2008). The promotion of lateral spread through elevated CO_2 may offset the need for other supplemental management tactics, including inorganic nutrient fertilization, to promote stand density or recovery from stress, as well as for turfgrass establishment from seeds or sprigs. Plant hormones such as gibberellic acid serve roles in cell and stem elongation (Taiz and Zeiger, 2010) but whether stolon elongation affected by elevated CO_2 is related to the changes in hormone concentration or balance is unknown. The underlying mechanisms of stolon elongation in creeping bentgrass as stimulated by CO_2 deserve further investigation.

The specific leaf area, expressed as the leaf area per unit dry weight, is one of the most widely used traits for describing leaf characteristics (Hoffmann et al, 2005). Specific leaf area in creeping bentgrass was significantly reduced due to the elevated CO_2

from 63 to 84 days of treatment (Fig. 2A). During any of the four sampling days, there was a 22-25% reduction in the specific leaf area for plants grown under elevated CO_2 compared to those grown under ambient CO_2. In addition, leaves developed under elevated CO_2 were shorter than those formed under ambient CO_2 (Fig. 2B). The combination of shorter leaves and a lower specific leaf area indicated that plants developed under elevated CO_2 had a smaller leaf area per unit biomass or the leaves became smaller and thicker. Smaller leaves are highly desirable in turfgrass because they require less clipping accumulation and require a lower mowing frequency (Fry and Huang, 2004). It was shown that poplar (*Populus trichocarpa*) grown in elevated CO_2 had thicker leaves and a greater leaf weight per unit leaf area (Radoglou and Jarvis, 1999). It was suggested that the thicker leaves with increased mesophyll cells of poplar plants may be a reason for the observed increase or maintenance of photosynthetic rates. A review summarized the results for the cellular expansion, division and patterning of plant species grown under elevated CO_2 and showed that increased leaf thickness due to cell expansion is a common CO_2-induced response for many plant types (Pritchard et al., 1999). Leaf thickness has also been shown to have an inverse relationship with the transpiration rate wherein thicker leaves have greater transpiration efficiency (Giuliani et al., 2013). Therefore, smaller and thicker leaves due to elevated CO_2 may promote drought tolerance in cool-season turfgrass by slowing the transpirational water loss and prolonging plant survival when water for irrigation is limited.

The shoot biomass was approximately 35% greater for creeping bentgrass plants grown under elevated CO_2 compared to plants maintained under ambient CO_2, while the root biomass increased by 37% due to elevated CO_2 (Fig. 3A). The increased shoot

biomass in creeping bentgrass was mainly due to enhanced growth of the lateral stems, as discussed above. An early review described the effects of doubling the CO_2 concentration on 37 different plant species, including agricultural crops, herbaceous species and woody ornamentals, and concluded that the doubled CO_2 levels increased the grain and vegetative yield by an average of 33% compared to ambient controls (Kimball, 1983). A more recent review of soybean (*Glycine max*) research showed that the vegetative biomass and harvest yield increased by 37% and 18%, respectively, for plants grown under elevated CO_2 compared to plants under ambient CO_2 conditions (Ainsworth and Long, 1980). It was shown that doubling the CO_2 concentration will increase the shoot dry weight two-fold for colonial bentgrass (*Agrostis capillaris*) after 79 days of treatment, while a similar but slower response was noted after 189 days for sheep fescue (*Festuca vivipara*) (Baxter et al., 1994). A higher shoot biomass or increased number of shoots per plant will promote the canopy density and improve the overall functionality and aesthetics of the turfgrass stand (Beard, 1973).

The root biomass typically increases in C3, C4 and CAM plants growing in elevated CO_2 environments but the degree of change relative to ambient controls is highly dependent upon the plant species and other interacting factors, such as drought stress and nutrient availability (Kirkham, 2011). It was reported that the root biomass of a tallgrass prairie mixture composed of big bluestem (*Andropogon gerardii*), little bluestem (*A. scoparius*), Indian grass (*Sorghastrum nutans*) and Kentucky bluegrass (*Poa pratensis*) increased by 55% from 120 to 270 g • m^{-2} for ambient versus elevated CO_2 conditions (Owensby et al., 1993). However, as these grasses were grown as a mixture, it was impossible to delineate which species (C3 versus C4) contributed most to the

observed increase in roots, a topic that deserves further consideration in future studies. Another study that investigated the effects of elevated CO_2 for two contrasting grassland swards dominated by either sheep fescue (*Festuca ovina*) alone or a combination of matgrass (*Nardus stricta*) and heath rush (*Juncus squarrosus*) reported a 40-50% increase in the root biomass for either system under elevated CO_2 (Fitter et al., 1997). Reports of R/S responding to changes in CO_2 concentrations are conflicting and vary across different plant species with both increases and decreases to R/S previously reported (Farrar and Williams, 1991; Bassiri-Rad., 2001). In this study, the root to shoot ratio (R/S) of creeping bentgrass did not change with CO_2 treatment (Fig. 3B), suggesting that shoot and root growth was maintained in balance even with elevated CO_2 for creeping bentgrass.

The enhanced total root biomass in creeping bentgrass was due to increases in the total root length and surface areas, particularly in upper soil profiles under elevated CO_2. Creeping bentgrass plants grown under elevated CO_2 had significantly greater root length in the upper 0-10 cm and middle 10-20 cm root zones, but there were no differences in root length in the 20-30 or 30-40 cm root zones (Fig. 4A). Similarly, the greatest effects of elevated CO_2 on the creeping bentgrass root surface area were observed in the uppermost 0-10 cm zone, whereas no significant differences occurred in the lower root zones (Fig. 4B). A visual depiction of stained root subsets in the upper 0-10 cm zone between elevated and ambient CO_2 treatment is provided in Figure 4C. A review described the effects of elevated CO_2 on plant root systems and reported that both the root length and root number are significantly increased due to elevated CO_2 across C3 and C4 species (Rogers et al., 1994). In this comprehensive review, it was also reported

that other structural aspects of root growth tend to increase when plants are maintained at elevated CO_2 levels, including the volume, branching and relative growth rate, whereas reports of changes to the root surface area due to CO_2 level are lacking. Root proliferation through increased length or surface area serves critical drought-avoidance functions for water uptake (Baker et al., 1990) and has been implicated in prolonging turfgrass survival during periods of drought stress (Carrow, 1996; Marcum et al., 1995). The extent to which elevated CO_2 may promote the drought tolerance of creeping bentgrass plants through changes to root structure deserves further consideration.

Photosynthesis, Transpiration, Water Use Efficiency, and Stomatal Density of Creeping Bentgrass as Affected by Elevated Carbon Dioxide Concentrations

The net photosynthetic rate of single leaves (Pn) was significantly higher for creeping bentgrass plants grown under elevated CO_2 compared to ambient CO_2 treatment for all sampling days (Fig. 5). Elevated CO_2 resulted in an approximately 21% increase in Pn after 84 days of CO_2 treatment. Previous studies have reported that net photosynthesis almost always increases for plants under elevated CO_2 conditions but the extent and duration of this enhancement varies with plant species and interacting environmental conditions (Sage et al., 1989; Ainsworth et al., 2002). The enhanced photosynthesis of plants under elevated CO_2 is likely due to an abundance or high availability of CO_2 as a substrate for carboxylation but may also be related to the activation state of Rubisco for carbon fixation (Habash et al., 1995). Whether the stimulation of photosynthesis in creeping bentgrass is related to those factors deserves further investigation in future studies.

The water use rate from leaves was evaluated as the stomatal conductance (g_s) and transpiration rate (T). The leaf T and g_s were both reduced by 40% due to the elevated CO_2 treatment (Figs. 6A and 6A, respectively). During all sampling days, the plants under elevated CO_2 maintained more than 30% greater WUE, expressed as the ratio of Pn/T, compared to plants under ambient CO_2 (Fig. 6C). The improved WUE by elevated CO_2 in this study was due to the CO_2 stimulation of Pn and CO_2 inhibition of T. Similar results with regard to all three parameters were reported for the flag leaves of winter wheat (*Triticum aestivum*) plants grown at elevated levels of CO_2 (Tuba et al., 1994). Stomatal conductance was reduced by 60% and 75% for amaranth (*Amaranthus hypochondriacus*) and soybean (*Glycine max*), respectively, when the CO_2 levels were doubled (Bunce, 1993). A literature review showed that the water use efficiency almost always increases for agronomic crop species grown under elevated CO_2 conditions (Allen et al., 1985). Specifically regarding a cool-season grass species, doubling the CO_2 level resulted in a two-fold increase in the water use efficiency of perennial ryegrass (*Lolium perenne*) (Nijs et al., 1989) and corroborates the observed increase in water use efficiency of creeping bentgrass in the current study. As previously mentioned, lower amounts of water loss from leaves may lower the irrigation requirements of turfgrass, which is particularly important in areas with limited water availability for irrigation.

Transpiration through the stomates is controlled by the stomatal density and stomatal conductance (Taiz and Zeiger, 2010). The stomatal density was greatly increased for creeping bentgrass grown under elevated CO_2 conditions from 63 to 84 days of treatment (Fig. 7A). On average, there was a 39% increase in the stomatal density; plants grown in elevated CO_2 had 128 stomates per square cm, while plants

under ambient conditions had 78 stomates per square cm (Fig. 7B). Several studies have also reported small changes to the stomatal density through changes in the total leaf area in response to elevated CO_2 (Radoglou and Jarvis, 1990a; Estiarte et al., 1994). The increase in stomatal density may facilitate the observed promotion of photosynthesis by providing more entry points for CO_2 diffusion within less leaf area. However, there was an inverse relationship between the stomatal density and transpiration rate under elevated CO_2 in this study as the transpiration rate was lowered under elevated CO_2 despite the higher number of stomates. Given that stomatal conductance is highly correlated with the transpiration rate, it can be inferred that CO_2-induced stomatal closure is a controlling factor for the reduced transpiration rate in creeping bentgrass exposed to elevated CO_2 levels.

In summary, creeping bentgrass plants grown under elevated CO_2 conditions displayed changes in their growth rate, leaf and root morphology, and water use that are favorable for turfgrass growth and highly desirable for turfgrass management. Specifically, the CO_2 stimulation of lateral spread and production of smaller and thicker leaves, as well as increased root growth, is critically important for rapid turfgrass establishment from seeds or sprigs. Improved water use efficiency by elevated CO_2 will have a significant impact on water use and may lead to changes in the irrigation management of turfgrass. Future research may address the underlying mechanisms of growth promotion by elevated CO_2, such as changes in hormone metabolism.

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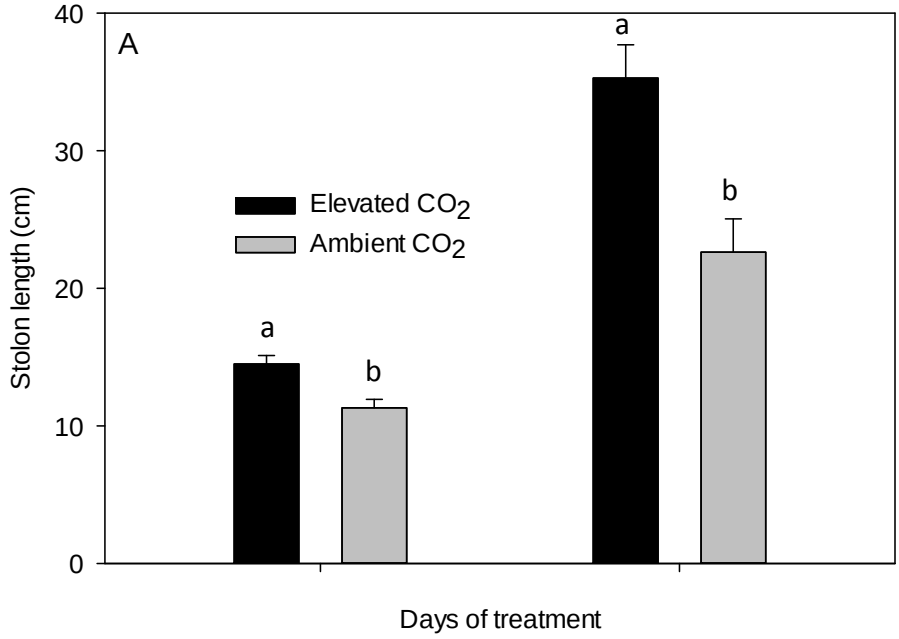
Figure 1. Stolon lengths (A) at 70 and 84 d of elevated carbon dioxide treatment. Different letters atop bars indicate significant differences ($p \leq 0.05$) between treatments on a given sampling day. Photos (B) of creeping bentgrass plants at 84 d of treatment with elevated or ambient CO_2 concentrations.



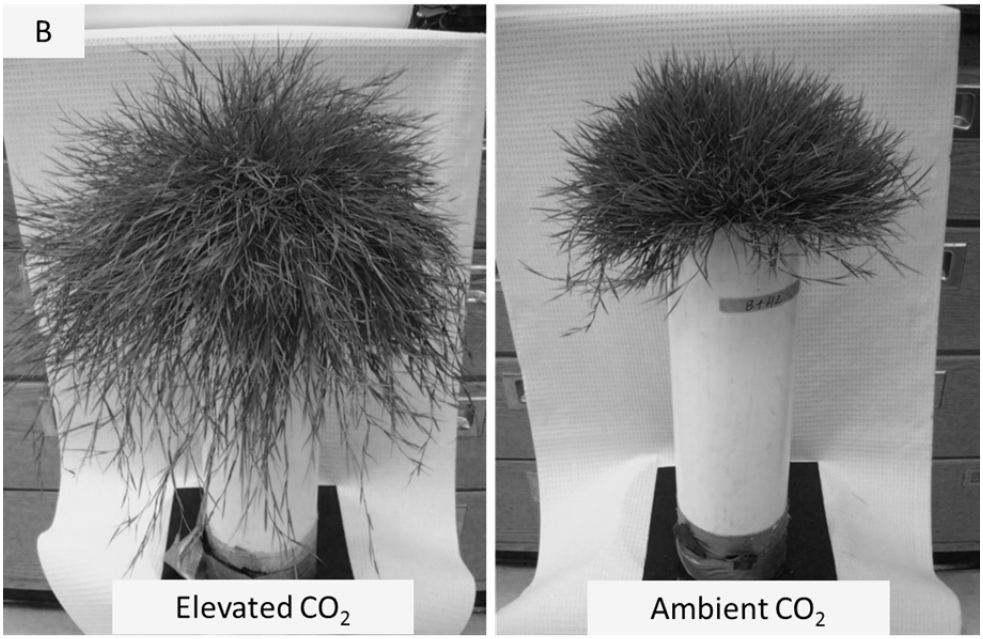
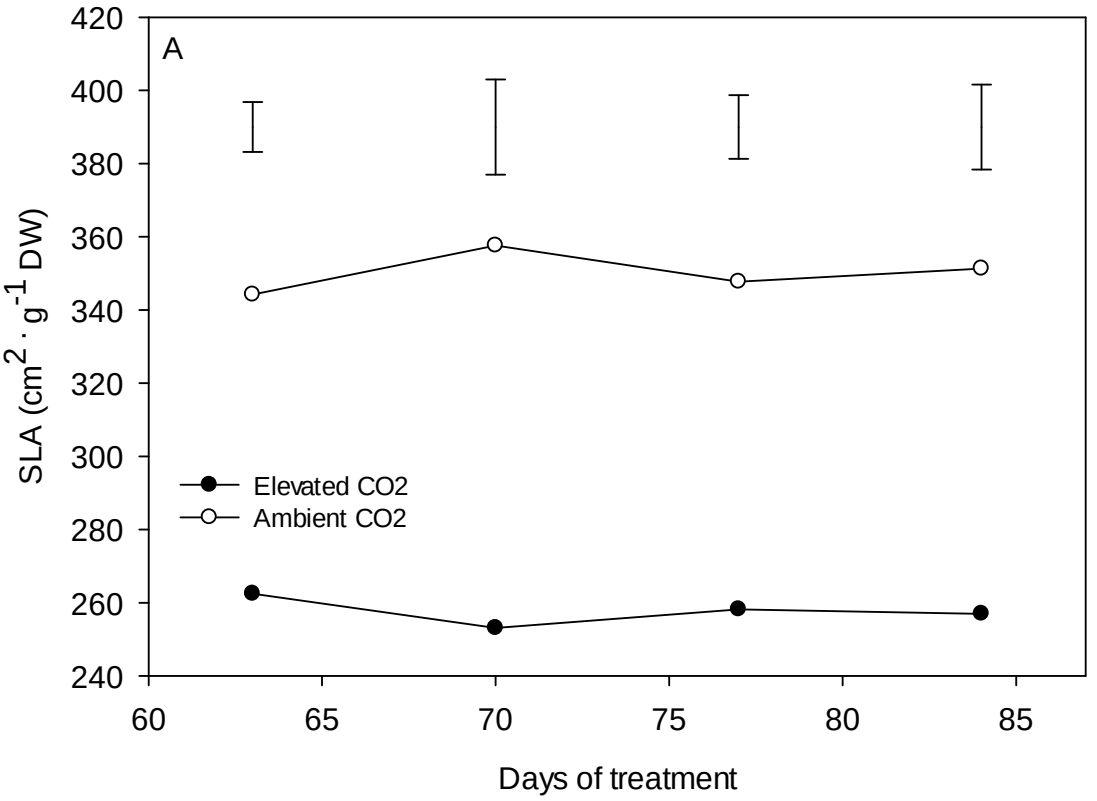


Figure 2. Specific leaf areas (A) at 63, 70, 77, and 84 d of elevated carbon dioxide treatment. Vertical bars indicate LSD values ($p \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected. Photos (B) of creeping bentgrass leaves at 84 d of treatment with elevated or ambient CO_2 concentrations.



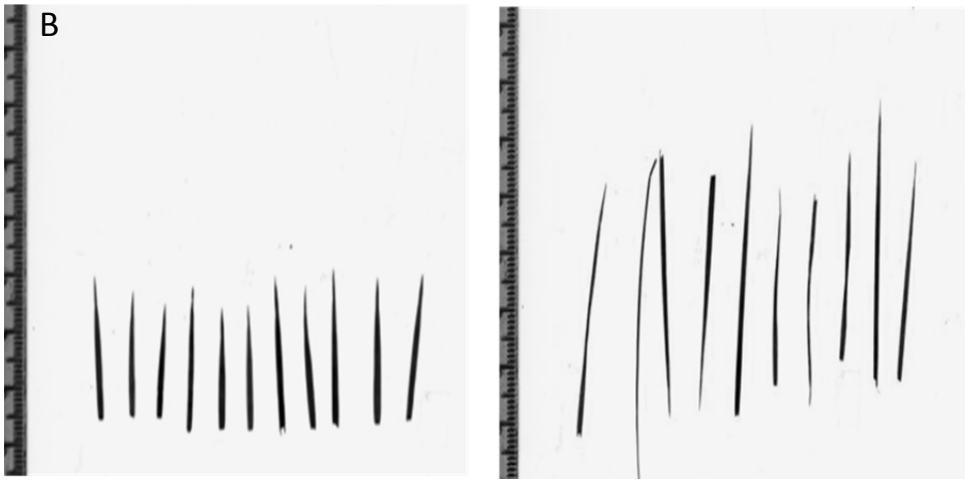


Figure 3. Root and shoot dry weights (A) and root to shoot ratios (B) after 84 d of elevated carbon dioxide treatment. Different letters atop bars indicate significant differences ($p \leq 0.05$) between treatments on a given sampling day.

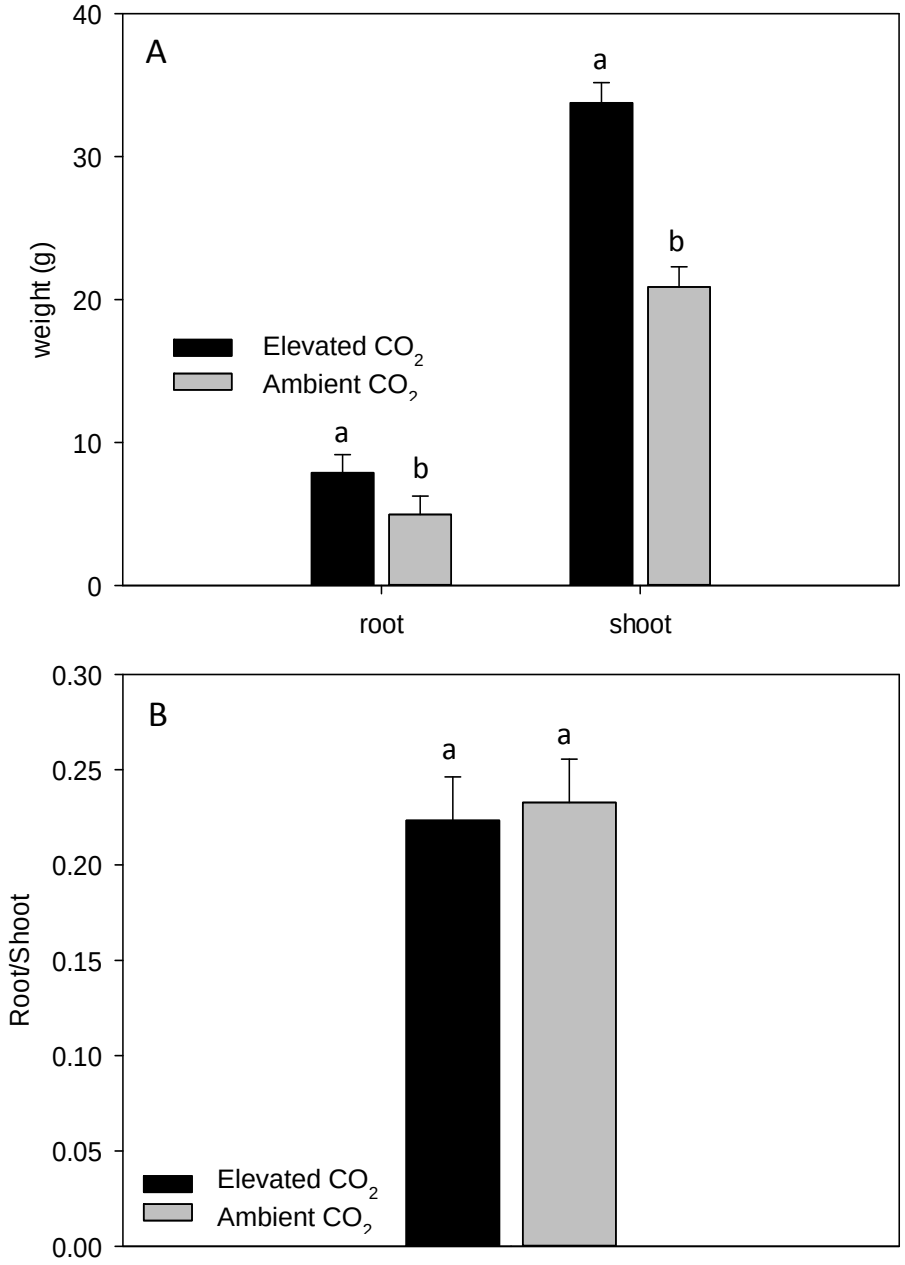


Figure 4. Root length (A) and surface area (B) in the 0-10, 10-20, 20-30, and 30-40 cm root zones for elevated and ambient carbon dioxide treatments. Different letters atop bars indicate significant differences ($p \leq 0.05$) between treatments. Photos (C) of stained creeping bentgrass root subsets in the 0-10 cm zone after 84 d of treatment with elevated or ambient CO_2 concentrations.

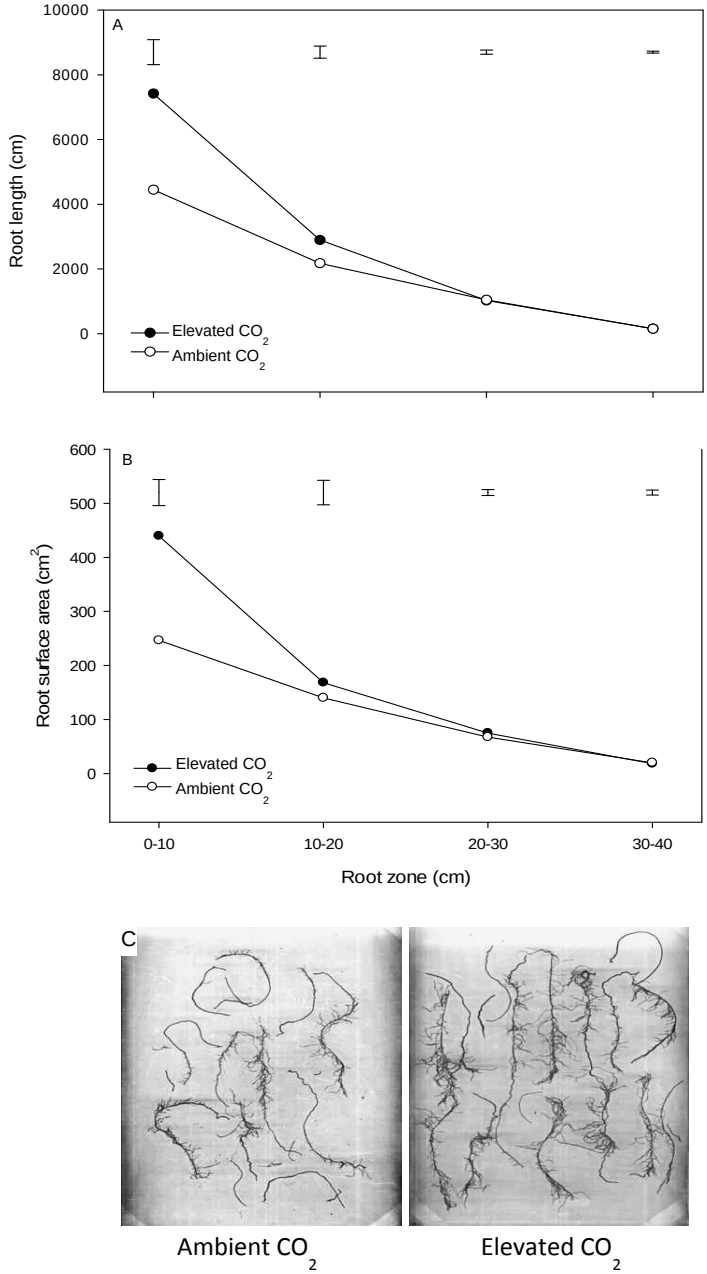


Figure 5. Net photosynthetic rates at 63, 70, 77, and 84 d of elevated carbon dioxide treatment. Vertical bars indicate LSD values ($p \leq 0.05$) for comparison between treatments on a given day where significant differences were detected.

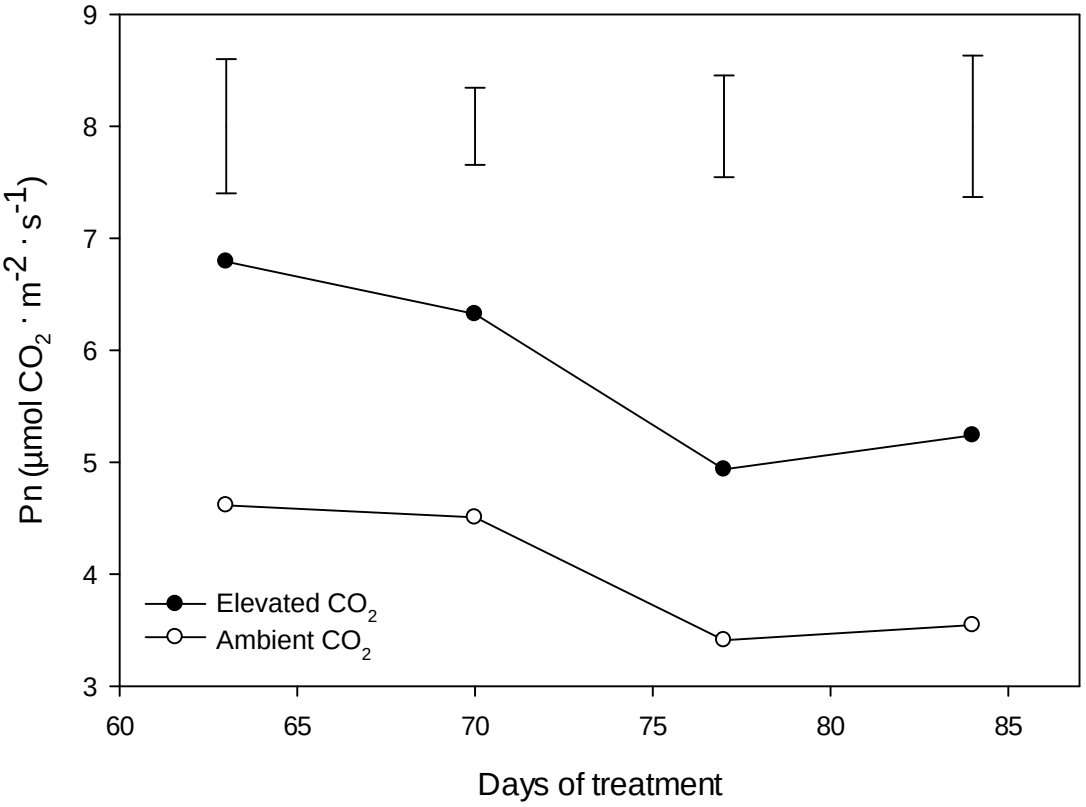


Figure 6. Leaf transpiration rate (A), stomatal conductance (B), and water use efficiency (C) at 63, 70, 77, and 84 d of elevated carbon dioxide treatment. Vertical bars indicate LSD values ($p \leq 0.05$) for comparison between treatments on a given day where significant differences were detected.

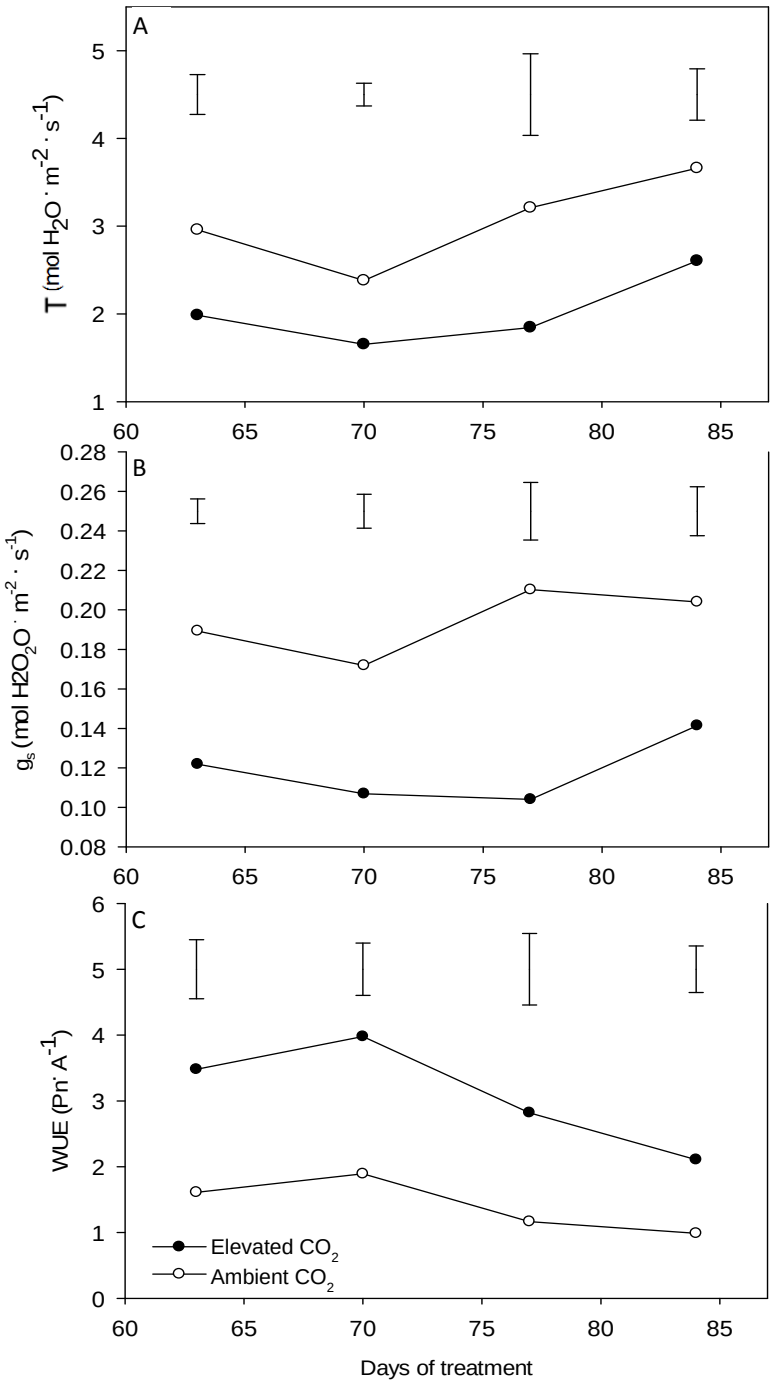
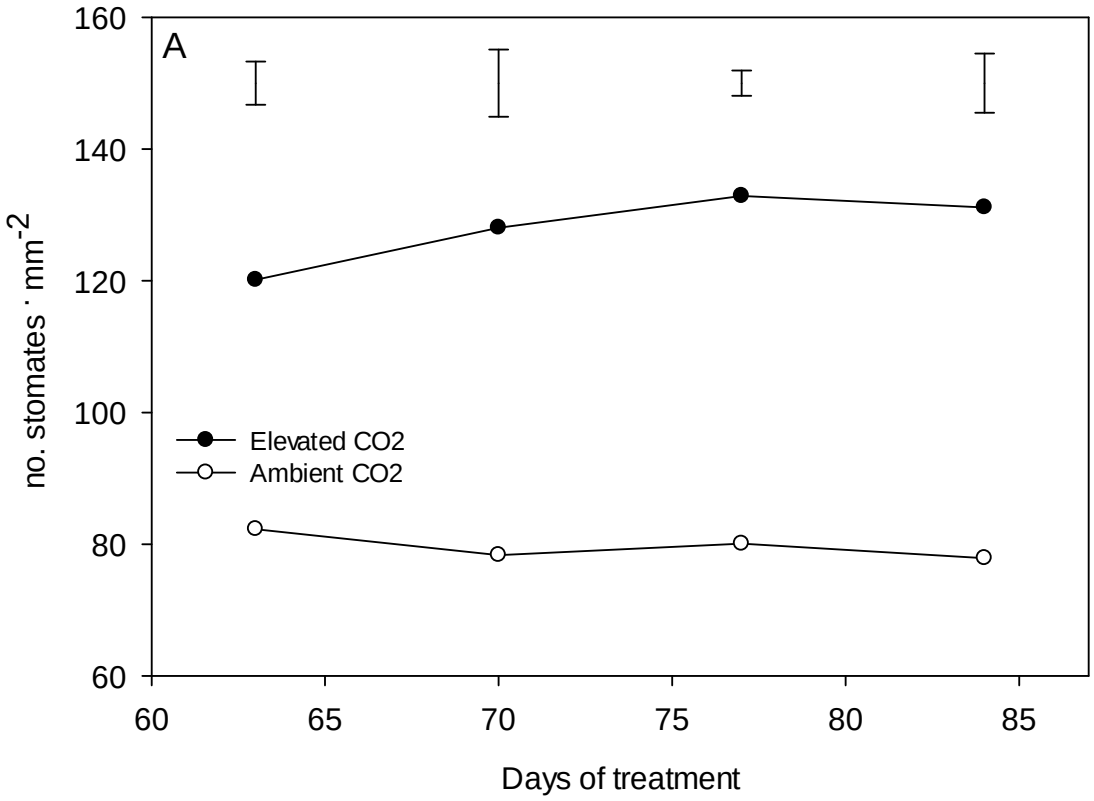
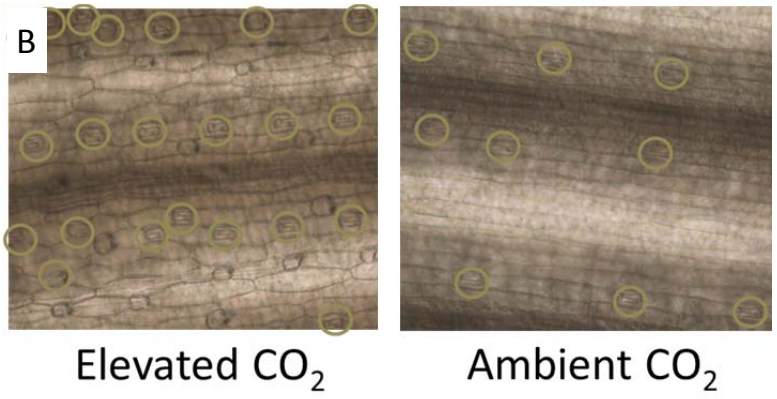


Figure 7. Stomatal density (A) at 63, 70, 77, and 84 d of elevated carbon dioxide treatment. Vertical bars indicate LSD values ($p \leq 0.05$) for comparison between treatments on a given day where significant differences were detected. Photos (B) of creeping bentgrass stomates at 84 d of treatment with elevated or ambient CO_2 concentrations.





CHAPTER FOUR

LEAF PROTEIN ABUNDANCE ASSOCIATED WITH IMPROVED DROUGHT TOLERANCE BY ELEVATED CARBON DIOXIDE IN CREEPING BENTGRASS

Burgess, P. and B. Huang. 2016. Leaf protein metabolism associated with improved drought tolerance by elevated carbon dioxide in creeping bentgrass (Agrostis stolonifera). J. Amer. Soc. Hort. Sci. 141(1): 85-96.

ABSTRACT

Elevated CO_2 may contribute toward plant tolerance to prolonged drought stress. The objective of this study was to investigate changes in protein abundance associated with mitigation of drought stress by elevated CO_2 in leaves of a cool-season grass species used as fine turfgrass. Plants of creeping bentgrass (*Agrostis stolonifera* cv. Penncross) were grown at either ambient CO_2 concentration (400 mL • L^{-1}) or elevated CO_2 concentration (800 mL • L^{-1}) for 35 days under well-irrigated and fertilized conditions and then subjected to drought stress for 21 days by withholding irrigation. Plants exposed to elevated CO_2 concentration maintained higher leaf water content, membrane stability, and visual turf quality (TQ) under drought stress compared with plants grown under ambient CO_2 conditions. The abundance of proteins involved in photosynthetic carbon fixation and assimilation, including chloroplastic glyceraldehyde phosphate dehydrogenase A (GAPDH-A) and ribulose 1,5-bisphosphate carboxylase (RuBisCO) decreased less and the abundance of proteins involved in respiratory metabolism (i.e., cytosolic GAPDH) increased less during drought due to elevated CO_2. The results suggest that elevated CO_2 lessened growth and physiological damages during drought by facilitating ribulose 1,5-bisphosphate regeneration and adenosine triphosphate (ATP) production in photosynthesis and down-regulating factors contributing to respiratory metabolism.

INTRODUCTION

Drought stress due to the lack of rainfall and declining availability of fresh water for irrigation is a primary factor limiting growth and productivity of many plant species. Within the context of global climate change, precipitation amounts and frequencies have changed drastically over the past century and many regions are now experiencing drought episodes more frequently (Solomon et al., 2007). Drought stress imposes many physiological limitations throughout the plant system encompassing damages at the biochemical, metabolic, and cellular levels (Aroca, 2012). Along with an increasing frequency of drought events, anthropogenic CO_2 emissions are driving a steady increase in atmospheric CO_2 concentrations of 2-3 mL • L^{-1} per year and plants may therefore be exposed to prolonged drought stress under elevated CO_2 concentrations in the near future (Solomon et al., 2007). The effects of elevated CO_2 concentration on many aspects of plant development and function under non-stress conditions have been well-documented and generally positive effects of elevated CO_2 concentration on plant growth are reported in various plant species (Ainsworth et al., 2002; Ceulemans and Mousseau, 1994; Huang and Xu, 2015; Kirkham, 2011; Leakey et al., 2009; Peterson et al., 1999). Recent research has also demonstrated that elevated CO_2 may mitigate physiological damages due to abiotic stress, such as drought and heat, in various plant species including perennial grasses such as kentucky bluegrass (*Poa pratensis*) and tall fescue (*Festuca arundinacea*) (Lin and Wang, 2002; Qaderi et al., 2006; Wall et al., 2001; Yu et al., 2012). Despite the abundant knowledge regarding the positive effects of elevated CO_2 on plant growth under non-stress or stress conditions, the underlying mechanisms by which

elevated CO_2 attenuates the damaging effects of prolonged drought stress remain unclear and require further investigation.

Proteomic profiling of stress-responsive proteins by means of two-dimensional polyacrylamide gel electrophoresis separation and mass spectrometry (MS) identification has effectively described changes in proteomic abundance within various tissues of different plant species responding to abiotic stresses (Burgess and Huang, 2014; Ferreira et al., 2006; Huang et al., 2014; Jespersen et al., 2015; Kosova et al., 2011; Merewitz et al., 2011). In response to drought stress alone, the abundance of proteins involved in photosynthesis, membrane synthesis, cell wall loosening, cell turgor maintenance, and antioxidant defense decrease in drought-susceptible grasses and the decrease is less severe in drought-tolerant species or cultivars (Xu and Huang, 2010a, 2012a). Under elevated CO_2 alone, many of the proteins serving integral photosynthetic functions in the light reactions and light-independent reactions have decreased abundance but compensate with significantly higher enzyme activity or activation state (Yu et al., 2014). The majority of proteomic research related to drought stress has identified drought-responsive changes in protein abundance under ambient CO_2 concentration only while changes in protein abundance responding to the combined drought stress and elevated CO_2 are not well documented, although differential abundance of proteins affected by elevated CO_2 under drought stress from those under well-irrigated conditions could serve critical roles for CO_2 mitigation of drought damages. The main focus of the current study was on the analysis of CO_2-responsive proteins under well-irrigated or drought conditions or protein abundance altered by the combined CO_2 and drought stress.

Therefore, the objective of this study was to investigate changes in protein abundance responding to interactive effects of drought and CO_2 in leaves of creeping bentgrass, widely used as fine turfgrass, with a goal to suggest potential metabolic factors regulated by elevated CO_2 contributing to improved drought tolerance.

MATERIALS AND METHODS

Plant materials and Growth Conditions

Thirty uniform-size tillers from creeping bentgrass (cv. Penncross) plants were transplanted from the Rutgers University turfgrass research farm (New Brunswick, NJ) into each pot (10 cm diameter and 40 cm depth) filled with fritted clay medium (Profile Products, Deerfield, IL) on 10 May 2014 and plants were maintained in controlled-environment growth chambers (Environmental Growth Chambers, Chagrin Falls, OH) set to 21/18 °C (day/night), 650 mmol • m^{-2} • s^{-1} photosynthetically active radiation, 60% relative humidity, and 14-h photoperiod for 7 d to allow plant acclimation to growth chamber conditions before exposing plants to CO_2 treatments on 18 May 2014.

Treatments and Experimental Design

Twenty pots (4 treatments x 5 replicates) of plants were established for 35 d (18 May to 21 June 2014) at ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration under well-irrigated conditions with excess water draining from pot bases and fertilized twice per week with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Following establishment under either CO_2 concentration, all plants were irrigated to pot capacity on 22 June 2014 (0-d drought stress) and subjected to drought stress for 21 d (23 June to 13 July 2014) by withholding irrigation until volumetric soil water content (SWC) decreased to 7.0% or irrigated to maintain SWC at the pot capacity (~29%) as the non-stress control. During 21-d drought stress, plants were continually exposed to either ambient or elevated CO_2 concentration.

The ambient and elevated CO_2 concentrations within growth chambers were maintained through an automatic CO_2 controlling system connected to a source tank containing 100% research-grade CO_2 following the method described in Yu et al. (2012). CO_2 concentrations inside the chambers were continuously monitored and recorded using an infrared gas analyzer (LI-820; LI-COR, Lincoln, NE) connected to a computer data logger. The CO_2 concentration was maintained using an automatic controlling system consisting of a programmable logic controller unit, solenoid valves, and a laptop computer with software capable of monitoring and maintaining CO_2 concentration within 10 mL • L^{-1} of the ambient or elevated target levels.

The experiment was arranged in a split-plot design with CO_2 treatment (ambient or elevated) as the main plot and irrigation treatment (well irrigated or drought stress) as the sub-plot. Each CO_2 treatment was performed in four different growth chambers and five replicate pots of well-irrigated or drought treatments were randomly placed inside each growth chamber. All plants were relocated between the four growth chambers every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

Soil Water Status and Physiological Analysis

The SWC was monitored daily using a time reflectometer (Trase System 1; Soilmoisture Equipment Corp., Santa Barbara, CA). Three buriable waveguide probes, each measuring 30 cm in length, were inserted into the root zone and SWC was measured in drought stressed and well-irrigated treatments (Topp et al., 1980).

Leaf relative water content (RWC) was measured to indicate leaf hydration status following 21 d of drought treatment. About 0.2 g leaf tissue of second and third fully expanded leaves was collected and fresh weight (FW) measured using a mass balance immediately after harvesting. Leaves were then wrapped in tissue paper, submerged in deionized water for 12 h at 4 °C, removed from water, blotted dry, and again weighed to measure turgid weight (TW). Leaves were then dried in an oven at 80 °C for 3 d, weighed to determine dry weight (DW) and RWC (%) calculated using the formula [(FW – DW)/(TW – DW)] · 100 (Barrs and Weatherley, 1962).

Leaf membrane stability was estimated by measuring cellular electrolyte leakage (EL) following 21 d of drought treatment. About 0.2 g leaf tissue of second and third fully expanded leaves was collected, rinsed with deionized water to remove external solutes, and placed in a test tube containing 30 mL deionized water. Tubes were agitated on a conical flask shaker for 12 h and the initial conductance (C_i) of incubation solution measured using a conductivity meter (YSI, Yellow Springs, OH). Leaf tissue was then killed by autoclaving at 121 °C for 20 min, cooled to room temperature, agitated for 12 h, and the maximal conductance (C_{max}) of incubation solution was measured. Leaf EL (percent) was calculated using the formula (C_i/C_{max}) x 100 (Blum and Ebercon, 1981).

Visual evaluation of TQ was performed to indicate overall turfgrass performance on a scale of 1 to 9 with 1 being brown and dead turf, 6 being the minimum acceptable quality level, and 9 being green and healthy turf. TQ ratings were based on parameters such as canopy uniformity, density, and color (Beard, 1973).

Protein Extraction, Separation, Quantification, and Identification

Protein extraction and separation were performed using the acetone/trichloroacetic acid extraction and two-dimensional gel electrophoresis method of Xu et al. (2008). Following 21 d of drought stress, second and third fully expanded leaves were collected, immediately frozen in liquid nitrogen, and stored at –80 °C for protein analysis. About 0.4 g leaf tissue was ground to powder in liquid nitrogen and further homogenized in 4 mL ice-cold precipitation solution (10% trichloroacetic acid, 0.07% 2-mercaptoethanol in acetone) for 12 h at -20 °C. Precipitated leaf tissue was centrifuged at 11,600 g for 15 min at 4 °C, the supernatant was removed and the remaining pellet was washed three times with rinse solution (0.07% 2-mercaptoethanol in acetone). The remaining pellet was vacuum dried at room temperature and resuspended in 2 mL resolubilization solution {8 M urea, 2 M thiourea, 1% 3 [(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 1% dithiothreitol, 1% 3/10 biolytes}. Aliquots of the resulting protein solution were then used to determine protein concentration according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as the standard. Immobiline DryStrips (pH 3–10, linear gradient, 13 cm; GE Healthcare, Piscataway, NJ) were rehydrated with 250 mg resolubilized protein and loaded onto an IPGphor apparatus (GE Healthcare) for first dimension separation. The voltage settings for first dimension isoelectric focusing were 50 V for 14 h, 500 V for 1 h, 1000 V for 1 h, 5000 V for 1 h, and 8000 V to a total of 80 kVh. Following first dimension focusing, strips were denatured in 10 mL equilibration buffer [50 mM Tris–Base (pH 8.7), 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 0.002% bromophenol blue, and 1% dithiothreitol] for 20 min and incubated again in the same

buffer with dithiothreitol replaced with 2.5% iodoacetamide. An electrophoresis unit (Hoefer SE 600 Ruby; GE Healthcare) was used to perform second dimension electrophoresis on a 12.5% sodium dodecyl sulfate–polyacrylamide gel {42% monomer solution (30% acrylamide and 0.8% N,N#-Methylenebiscarylamide), 25% resolving gel buffer [1.5 M Tris-Base and 6 N hydrochloric acid (pH 8.8)], 0.01% sodium dodecyl sulfate, 3.4 ppm tetramethylethylenediamine, and 50 ppm ammonium persulfate}. Voltage settings for second dimension electrophoresis were 5 mA per gel for 30 min followed by 20 mA per gel for 6.5 h. Gels were stained with colloidal Coomassie Brilliant Blue G-250 stain (Neuhoff et al., 1988) and scanned on a Typhoon FLA 9500 (GE Healthcare) to generate digital gel images. Gel images were analyzed using SameSpots software (version 4.5; Nonlinear USA, Durham, NC) and protein volumes were normalized as a percentage of total protein volume to correct for variability during staining. Proteins with probability values less than or equal to 0.05 were chosen for further identification by reversed-phase liquid chromatography (RPLC).

Proteins chosen for identification were manually excised from gels and washed with 30% acetonitrile in 50 mM ammonium bicarbonate solution before dithiothreitol reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37 °C overnight. The resulting peptides were extracted with 30 mL 1% trifluoroacetic acid followed by C18 ziptip desalting to simultaneously remove salts and concentrate the peptides. Peptides were further fractionated by RPLC on a LC system (Ultimate 3000; Dionex, Sunnyvale, CA) coupled to a mass spectrometer (Q-Exactive; Thermo Fisher Scientific, Waltham, MA) with a nano-electrospray ionization source (Thermo Fisher Scientific). Source ionization parameters included a 2.2-kV spray voltage, 275 °C

capillary temperature, and 50.0 s-lens. Full-scan MS mode [300–1650 m/z (mass-to-charge ratio)] was operated at a resolution of 70,000, automatic gain control (AGC) target was 1 x 106, and maximum ion transfer time (IT) was 500 ms. MS/MS parameters for selected ions included 17,500 resolution, 5 x 104 AGC, 250 ms IT, 4.0 m/z isolation window, 25.0 normalized collision energy, 5.0% underfill ratio, and a 30 s dynamic exclusion.

Raw files were analyzed using the Proteome Discoverer software platform (version 1.3, Thermo Fisher Scientific) with Mascot (2.4.1) search engine against the Green plant protein sequences (1,474,035 entries) of non-redundant National Center for Biotechnology Information protein database. Mascot parameters included trypsin, two missed cleavages, 10 ppm precursor mass tolerance, 0.1 Da fragment mass tolerance, as well as methionine oxidation and cysteine carbamidomethylation dynamic modifications with decoy search option for Mascot engaged. Proteins with 100% peptide spectral match were considered to be present throughout the majority of analyzed proteins.

Statistical Analysis

The effects of CO_2 level, irrigation regimen, and their interactions on physiological parameters, protein abundance, and relative protein accumulation were determined by analysis of variance according to the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC). Differences between treatment means were separated by Fisher's protected least significance difference test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

A characteristic response of drought-susceptible plants is a steady decline in cellular water content concurrent with dysfunction and eventual failure of cellular membranes, along with numerous other metabolic and biochemical changes (Kopp and Jiang, 2013). Drought stress caused significant reduction in RWC under both ambient and elevated CO_2 concentrations (Fig. 1A) when SWC decreased to ~7.0% following 21 d of drought stress (Fig. 2). Drought-induced reduction in RWC was more pronounced (by 42%) under ambient CO_2 concentration than that under elevated CO_2 concentration (by 28%) following 21 d of drought stress. Elevated CO_2 treatment led to significantly higher (by 19%) RWC compared with the ambient CO_2 treatment following drought stress whereas no significant changes in RWC were observed with elevated CO_2 under well-irrigated conditions (Fig. 1A). Maintaining adequate water content within cells or delaying cellular dehydration during stress periods in CO_2-enriched plants may be due to the effects of elevated CO_2 on the induction of stomatal closure restricting transpirational water loss and enhanced osmotic adjustment due to the accumulation of solutes, such as soluble sugars, as well as enhanced root growth for water uptake (Leakey et al., 2009; Yu et al., 2015). Maintenance of photosynthetic processes has also been associated with improved RWC during drought stress in other cool-season turfgrass species, such as tall fescue (Yu et al., 2012). Moreover, elevated CO_2 has been shown to enhance root growth in creeping bentgrass (Burgess and Huang, 2014), which could access more water from soil and contribute to the improvement of leaf water status under drought stress.

Cellular membrane stability evaluated by quantifying ion leakage is a commonly used indicator for cell integrity and viability in various plant tissues (Jambunathan, 2010).

Leaf EL significantly increased with drought stress under either ambient or elevated CO_2 concentration following 21-d drought treatment (Fig. 1B). Drought-induced increase in EL was greater (by 51%) under ambient CO_2 concentration that under elevated CO_2 concentration (by 43%) following drought stress. Elevated CO_2 treatment led to significantly lower (by 18%) EL compared with the ambient CO_2 treatment following drought stress whereas no significant changes in EL were observed with elevated CO_2 under well-irrigated conditions (Fig. 1B). The results demonstrate that elevated CO_2 treatment effectively mitigated drought-induced cellular membrane deterioration or facilitated maintenance of membrane integrity. Similar results of elevated CO_2 effects on membrane stability of drought-stressed plants have been reported in tall fescue (Yu et al., 2012). Despite elevated CO_2 promoting osmotic adjustment and stomatal constriction with secondary effects on membrane status, the mechanisms by which elevated CO_2 contributes directly to cellular membrane stability remain largely unknown, though it was suggested that elevated CO_2 treatment increases leaf antioxidant content to reduce reactive oxygen species (ROS) content in spring wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) cultivars (Lin and Wang, 2002; Perez-Lopez et al., 2009). Whether elevated CO_2 conditions stimulate cool-season turfgrass species to use similar antioxidant mechanisms mitigating ROS accumulation thereby delaying significant membrane damages during stress periods is not yet known and deserves further investigation.

Visual evaluation of turfgrass quality is a subjective criteria commonly used to evaluate overall turfgrass performance based on visual characteristics including canopy density, leaf color, and uniformity (Beard, 1973). Visual TQ displayed significant decrease with drought stress under either ambient or elevated CO_2 concentration

following 21-d drought treatment (Fig. 1C). Drought-induced reduction in TQ was more pronounced (by 38%) under ambient CO_2 concentration than that under elevated CO_2 concentration (by 28%) following 21 d of drought stress. Elevated CO_2 treatment led to significantly higher (by 13%) TQ compared with the ambient CO_2 treatment following drought stress whereas no significant changes in TQ were observed with elevated CO_2 under well-irrigated conditions (Fig. 1C). The promotive effects of elevated CO_2 on TQ corresponded to enhanced RWC and decreased EL during drought stress, both of which are strongly correlated to TQ for creeping bentgrass during drought stress in turfgrasses (Jespersen et al., 2013; Sun et al., 2013). The improved growth and physiological characteristics favoring plant tolerance to drought stress as affected by elevated CO_2 concentration could be associated with changes in abundance for specific proteins involved in several major metabolic processes, as discussed below.

As discussed in the introduction, most proteomic research related to drought stress has identified drought-responsive changes in protein abundance under ambient CO_2 concentration only. Our study focused on the analysis of CO_2-responsive changes in protein abundance under well-irrigated or drought conditions or protein abundance altered by the combined CO_2 and drought stress. Over 300 proteins were detected on each two-dimensional gel in leaves of creeping bentgrass (Fig. 3) and 37 proteins successfully identified by RPLC-MS exhibited differential abundance (upregulated or downregulated) in response to elevated CO_2 concentration under well-irrigated or drought-stress conditions. Those proteins were categorized into five functional categories according to the criteria set forth by Bevan et al. (1998): energy production, stress defense, metabolism, protein destination and storage, and protein synthesis (Table 1). Among the

37 identified proteins, 67.6%, 8.1%, 16.2%, 5.4%, and 2.7% served functions in energy production, stress defense, metabolism, protein destination and storage, and protein synthesis functions, respectively (Fig. 4). A total of 18 proteins (1–6, 11, 17, 18, 23, 24, 26, 30–33, 36, and 37) were upregulated and 19 proteins (7–10, 12–16, 19–22, 25, 27–29, 34, and 35) were downregulated by elevated CO_2 under well-irrigated conditions (Figs. 5, 6A and B).

Elevated CO_2 upregulated 20 proteins (1, 2, 4–7, 10, 11, 14, 19, 23–25, 30–33, and 35–37) and downregulated 17 proteins (3, 8, 9, 12, 13, 15–18, 20–22, 26–29, and 34) under drought stress conditions while 20 proteins (1, 4–7, 10–12, 14, 23–25, and 30–37) were upregulated and 17 proteins (2, 3, 8, 9, 13, 15–22, and 26–29) were downregulated under ambient CO_2 concentration following drought-stress treatment (Figs. 5, 6A and B). Moreover, the fold change in abundance was significantly different between elevated CO_2 well-irrigated and elevated CO_2 drought-stress treatments for 24 proteins (2, 3, 7, 8, 10, 13–15, 17–26, 29, 30, 32, and 35–37), between ambient CO_2 drought stress and elevated CO_2 drought-stress treatments for 22 proteins (1, 2, 4, 8–13, 15, 16, 19, 21, 27–29, and 32–37), and among all three treatments for 11 proteins (2, 8, 10, 13, 15, 19, 21, 29, 32, 35, and 36) (Figs. 5, 6A and B). The biological functions of those proteins with upregulated or downregulated abundance by elevated CO_2 are discussed, with an emphasis on several notable proteins regulated by elevated CO_2, which may contribute to CO_2 mitigation of drought-stress damages in creeping bentgrass.

The majority of soluble proteins responding to elevated CO_2 with or without drought stress were involved in energy metabolism in creeping bentgrass leaves. Elevated CO_2 increased the abundance of several major proteins involved in the Calvin–Benson

cycle including fructose bisphosphate aldolase precursor (FBA), chloroplastic GAPDH-A, and chloroplastic sedoheptulose bisphosphatase precursor (SBPase) under well-irrigated and drought conditions. The drought-induced decrease in RuBisCO abundance was less severe due to elevated CO_2 compared with ambient CO_2. The drought-induced increase in cytosolic GAPDH abundance contributing to respiratory glycolytic breakdown of glucose was less severe due to elevated CO_2 compared with ambient CO_2. Our results suggest that the enhanced drought tolerance observed in creeping bentgrass under drought stress may be in part due to changes in abundance for those proteins regulating key energy functions within leaf tissues and are discussed in detail below.

FBA catalyzes the sixth reaction of the Calvin–Benson cycle converting fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate yielding dihydroxyacetone phosphate and ATP and has been shown to have direct effects on the regeneration rate ribulose-1,5-bisphosphate (RuBP) for carbon fixation (Iwaki et al., 1991; Taiz and Zeiger, 2010). Research describing changes in FBA abundance responding to abiotic stresses is limited, although Abbasi and Komatsu (2004) reported FBA abundance was upregulated in rice (*Oryza sativa*) leaf sheaths during salinity stress, while gene transcript level analysis revealed differential responses of eight FBA genes in *Arabidopsis thaliana* shoots responding to chilling, heat, or drought stress (Lu et al., 2012). Leaf FBA content at the transcript and protein level significantly increased due to elevated CO_2 treatment for rice under non-stress conditions and tall fescue under heat-stress conditions (Fukayama et al., 2009; Yu et al., 2014). In this study, FBA abundance increased by 1.0- and 1.1-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, but increased by 2.3-fold due to drought stress under ambient CO_2 concentration. The

regulation of FBA abundance under elevated CO_2 suggested that elevated CO_2 could sustain constant ATP production and RuBP regeneration rates supporting plant growth during drought periods.

Chloroplastic GAPDH is composed of A and B subunits, which catalyze the nicotinamide adenine dinucleotide phosphate-consuming reduction of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate during the reduction phase of the Calvin–Benson cycle (Sparla et al., 2005; Taiz and Zeiger, 2010). In a manner similar to FBA limitation, plants with decreased chloroplastic GAPDH abundance may experience reduced CO_2 assimilation due to a reduction in RuBP regeneration, which can then cause subsequent declines in photosynthetic rates and net biomass accumulation (Price et al., 1995). Abiotic stresses such as drought cause significant decreases in chloroplastic GAPDH abundance whereas plants with increased stress tolerance typically exhibit greater abundance of chloroplastic GAPDH compared with stress-sensitive plants (Merewitz et al., 2011; Xu and Huang, 2010a, 2010b). Reports on elevated CO_2 regulation of chloroplastic GAPDH abundance are limited and vary across different plant species and different stress conditions. Yu et al. (2014) reported that the abundance of chloroplastic GADPH decreased due to elevated CO_2 under non-stress conditions but did not change due to elevated CO_2 following heat-stress treatment in tall fescue. Furthermore, chloroplastic GAPDH enzyme activity was either significantly increased or relatively unchanged due to elevated CO_2 under non-stress conditions in other plant species (Haake et al., 1999; Ribeiro et al., 2012; Zhang et al., 2005). In this study, chloroplastic GAPDH abundance increased by 2.2- and 1.7- fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, and was significantly

greater during drought stress under elevated CO_2 compared with ambient CO_2 concentration (Fig. 6A). The observed changes in GAPDH abundance indicated that elevated CO_2 treatment effectively mitigated the decrease in chloroplastic GAPDH abundance upon prolonged drought stress. The ability of elevated CO_2 to alleviate the decline in chloroplastic GAPDH abundance may aide in RuBP regeneration and a continuation of CO_2 assimilation driving photosynthesis during drought stress periods.

RuBisCO is the most abundant soluble protein responsible for catalyzing the first step of the Calvin–Benson cycle, reacting CO_2 and water with RuBP yielding 3-phosphoglycerate (Cleland et al., 1998). Abiotic stresses such as drought impose metabolic limitations on photosynthesis by reducing net abundance of large and small RuBisCO subunits concurrent with inhibition of RuBisCO activation state, among many other biochemical changes (Huang et al., 2014; Shi et al., 2013). Grass species displaying improved tolerance to various abiotic stresses by means of endogenous or exogenous modification typically maintain sufficient RuBisCO abundance or mitigate the extent of decline in RuBisCO abundance (Jespersen et al., 2015; Xu and Huang, 2012b, 2010c; Zhao et al., 2011). It is well accepted that a key component of photosynthetic acclimation to long-term elevated CO_2 treatment is a substantial decrease in RuBisCO both at the transcript and protein level associated with disproportionate resource allocation favoring RuBP regeneration (Bowes, 1991; Feng et al., 2014; Ziska and Teramura, 1992). The stress-mitigating effects of elevated CO_2 facilitated by changes to photosynthetic constituents have been well described for a number of plant species under various abiotic stresses (Alonso et al., 2009; Huang and Xu, 2015). Vu et al. (1998) reported that the decline in RuBisCO abundance and activity was significantly less for drought-stressed

rice plants grown at elevated CO_2 compared with those at ambient CO_2 and similar effects were also reported with regard to RuBisCO enzyme activity in sugarcane (*Saccharum officinarum*) under drought stress (Vu and Allen, 2009). In this study, RuBisCO abundance decreased by 1.1- and 1.5-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, and the decrease was significantly less during drought stress under elevated CO_2 compared with ambient CO_2 concentration (Fig. 6A). Decreased RuBisCO abundance by elevated CO_2 may lead to suppression of photorespiration in cool-season plants (Ehleringer et al., 1991), which could help to avoid inefficient oxygenation of RuBP.

Chloroplastic SBPase is the second bisphosphatase enzyme of the Calvin–Benson cycle, the first being fructose 1,6- bisphosphatase, and is responsible for catalyzing sedoheptulose 1,7-bisphosphate dephosphorylation to sedoheptulose- 7-phosphate during the regeneration phase (Raines et al., 1999). Research using transgenic antisense tobacco (Nicotiana tabacum) lines has suggested a direct link between SBPase content and capacity for RuBP regeneration and subsequent carbon assimilation (Harrison et al., 1998, 2001). Despite the crucial role of SBPase in plant energy metabolism, there exists far less information regarding effects of abiotic stresses on SBPase abundance. In a review by Kosova et al. (2011), it was reported that SBPase abundance was downregulated in rice subjected to ozone stress but upregulated in poplar (*Populus euphratica*) tolerant of heavy metal hyperaccumulation. Transgenic rice overexpressing and accumulating SBPase prevented RuBisCO activase sequestration thereby maintaining sufficient RuBisCO activation for improved tolerance to heat stress (Feng et al., 2007). More specifically, SBPase abundance is significantly downregulated in creeping

bentgrass and kentucky bluegrass upon drought stress but the downregulation was significantly less for drought-tolerant cultivars of each species (Xu and Huang 2010a, 2010c). Similar effects were also noted in creeping bentgrass lines expressing differential tolerance to heat stress (Xu and Huang, 2010b). Given that SBPase is another limiting factor of RuBP regeneration, tobacco plants overexpressing SBPase displayed increased photosynthesis and biomass accumulation when grown at elevated CO_2 compared with plants at ambient CO_2 (Rosenthal et al., 2011). However, the direct effects of elevated CO_2 on SBPase protein abundance and transcript level are variable based on plant species and possible interacting stress effects. SBPase transcript level increased in rice plants exposed to elevated CO_2 across varied nitrogen fertility regimens, but decreased due to high CO_2 when soil temperature was increased above the optimal range (Fukayama et al., 2009, 2011). Protein abundance and transcript levels of SBPase remained unaffected in durum wheat (*Triticum durum*) exposed to elevated CO_2 under non-stress conditions and similar results were also reported for perennial ryegrass (*Lolium perenne*) protein abundance (Aranjuelo et al., 2013; Nie et al., 1995; Rogers et al., 1998). In this study, SBPase protein abundance increased by 1.1- and 1.0-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively (Fig. 6A). The response in SBPase abundance was similar to that of FBA, likely because condensation of SBPase and fructose 1,6- bisphosphatase are both catalyzed by FBA in the Calvin– Benson cycle (Lu et al., 2012). The results suggest that ATP production and RuBP regeneration rates may be supported by increased FBA abundance and further sustain plant growth during drought periods.

Cytosolic GAPDH catalyzes the sixth step of glycolysis by oxidizing aldehyde to carboxylic acid releasing energy to phosphorylate glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate in the presence of nicotinamide adenine dinucleotide and inorganic phosphate (Ramzan et al., 2013; Taiz and Zeiger, 2010). Abiotic stresses including heat, drought, or anoxic conditions have been shown to significantly increase cytosolic GAPDH abundance, which maintains glycolytic breakdown of carbohydrates for energy production and suppresses the production of inhibitory ROS (Chang et al., 2000; Ferreira et al., 2006; Xu and Huang, 2012b; Yang et al., 1993). However, increasing or maintaining the abundance of glycolytic chemical constituents driving respiration can promote whole-plant stress tolerance only when photosynthetic carbon input equals or exceeds respiratory carbon consumption (Song et al., 2014). A review of plant proteomic changes under abiotic stresses showed that excessive abundance of cytosolic GAPDH is typically associated with susceptibility to abiotic stresses such as drought, waterlogging, and hypoxia/anoxia (Kosova et al., 2011). In this study, cytosolic GAPDH protein abundance increased by 3.2- and 3.4-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, and the increase was significantly less under drought due to elevated CO_2 compared with ambient CO_2 concentration (Figs. 5 and 6A). Elevated CO_2 effectively maintained an increased abundance of cytosolic GAPDH content under drought-stress conditions, which may sustain energy production while avoiding a potential excess of cytosolic GAPDH abundance observed during drought stress under ambient CO_2 concentrations.

In summary, this study suggests beneficial effects of elevated CO_2 for lessening the drought damages and improving physiological functions in creeping bentgrass

potentially facilitated by changes in protein abundance supporting energy metabolism of leaves in creeping bentgrass. Elevated CO_2 improved creeping bentgrass growth by maintaining leaf hydration and membrane integrity, which may be in part a result of changes in abundance for proteins of the Calvin–Benson cycle including FBA precursor, chloroplastic GAPDH-A, RuBisCO, and chloroplastic SBPase precursor. Elevated CO_2 also decreased cytosolic GAPDH abundance during drought, which may have downstream effects on certain aspects of plant respiration. Further research is needed to confirm the biological functions and associated molecular factors of CO_2-responsive proteins identified in this study contributing to improved drought tolerance in cool-season grass species.

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TABLES AND FIGURES

Table 1. Creeping bentgrass leaf soluble proteins differentially accumulated following exposure to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. Spot numbers correspond to highlighted proteins in Fig. 3. MW = molecular weight in kilodaltons; PI = isoelectric point; NADP = nicotinamide adenine dinucleotide phosphate; ATP = adenosine triphosphate; HCF = high chlorophyll fluorescence; PSI = photosystem I.

Functional category	Spot no.	Protein name	Abbreviation	Accession	MW/PI[z]
Energy	1	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	GAPDH	120668	33.2/6.20
	2	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	GAPDH-A	115458768	42.7/7.62
	3	Fructose 1,6-bisphosphate aldolase, cytoplasmic isozyme	FBA (cyt)	115468886	37.7/7.57
	4	Fructose 1,6-bisphosphate aldolase precursor	FBA	8272480	41.9/9.01
	5	Triosephosphate isomerase, cytosolic	TIM	2507469	26.79/5.39
	6	Cytoplasmic aldolase	Cyt. ald.	218157	38.7/6.56
	7	Phosphoglycerate mutase	PGAM	32400802	29.6/5.43
	8	Rubisco activase beta form precursor	RCA β	32481063	47.1/7.57
	9	Ribulose 1,5-bisphosphate carboxylase	RuBisCO	1488586	45.3/6.28
	10	Ribulose-5-phosphate 3-epimerase, chloroplast precursor	R5P3E	2833386	30.3/8.23
	11	Sedoheptulose-1,7-bisphosphatase, chloroplast precursor	SBPase	1173347	42/6.04
	12	Ferredoxin-NADP+ reductase, chloroplastic isozyme	FNR	115443657	38.7/7.98
	13	Crystal Structure Complex Between Ferredoxin And Ferredoxin NADP+ Reductase	FDI-FNR	13096165	35.3/7.01
	14	ATP synthase F1 sector beta subunit	ATPF1 β	50401827	53.8/5.17
	15	ATP synthase CF1 alpha subunit	ATPCF1 α	14017569	55.3/6.11
	16	ATP synthase alpha subunit	ATP α	51556908	55.5/6.03
	17	Oxygen-evolving complex protein 1	OEE1	739292	26.5/5.13
	18	Oxygen-evolving enhancer protein 2, chloroplast precursor	OEE2	131394	27.3/8.84
	19	HCF136 photosystem II stability/assembly factor, chloroplast precursor	HCF136	75252730	45.4/9.02
	20	PSI type III chlorophyll a/b-binding protein	PSI a/b	430947	29.1/8.61
	21	Light-harvesting complex I	LHC1	544700	24.2/8.11
	22	Carbonic anhydrase, chloroplast precursor	CAH	729003	35.1/8.93
	23	Malate dehydrogenase	MDH	10798652	35.4/5.91
	24	Dihydrolipoyl dehydrogenase-1, mitochondrial isoform	DLD	115436320	52.6/7.21
	25	Glycerate dehydrogenase HPR, peroxisomal	HPR	115443619	42/6.56
Stress defense	26	Thioredoxin peroxidase	TPX	3328221	28.1/6.34
	27	Catalase-1	CAT	2493543	56.8/6.52
	28	Heat shock protein 70	HSP70	1143427	75.4/5.15
Metabolism	29	Alpha-glucan phosphorylase, H-isozyme	AGP	14916632	93.6/7.27
	30	Glutamate-ammonia ligase, chloroplast precursor	GAL	121340	47.1/5.11
	31	Aspartate carbamoyltransferase	ACT	21535795	42.6/6.06
	32	Phosphoribulokinase	PRK	21839	45/5.84
	33	Aminomethyltransferase, mitochondrial	AMT	115460656	43.9/8.53
	34	Methionine synthase	MS	50897038	84.5/5.68
Protein destination and storage	35	PFTF precursor, FTSH-like	PFTF	52075838	72.5/5.54
	36	Protein disulfide isomerase 2 precursor	PDIM	13925726	56.4/5.03
Protein synthesis	37	Tu translational elongation factor	EF-Tu	17225494	50.4/6.19

Figure 1. Changes in (A) leaf relative water content, (B) leaf electrolyte leakage, and (C) turf quality of creeping bentgrass plants exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. Vertical lines atop bars represent SE of five replicates for each treatment and different letters atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test (α= 0.05).

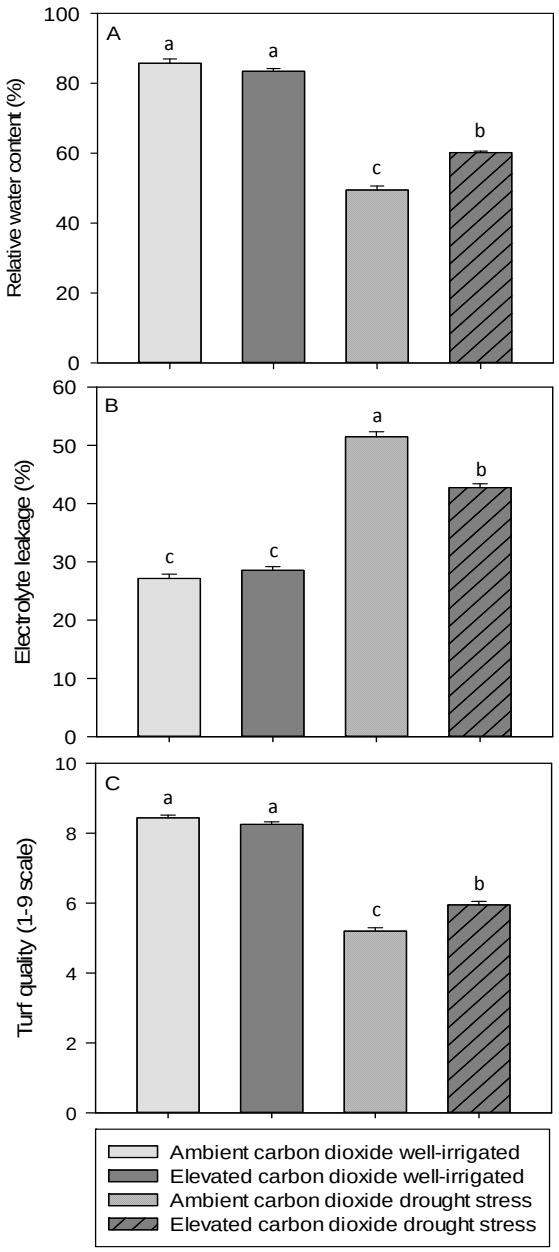


Figure 2. Soil volumetric water content of creeping bentgrass exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. Vertical lines represent least significant difference values where significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

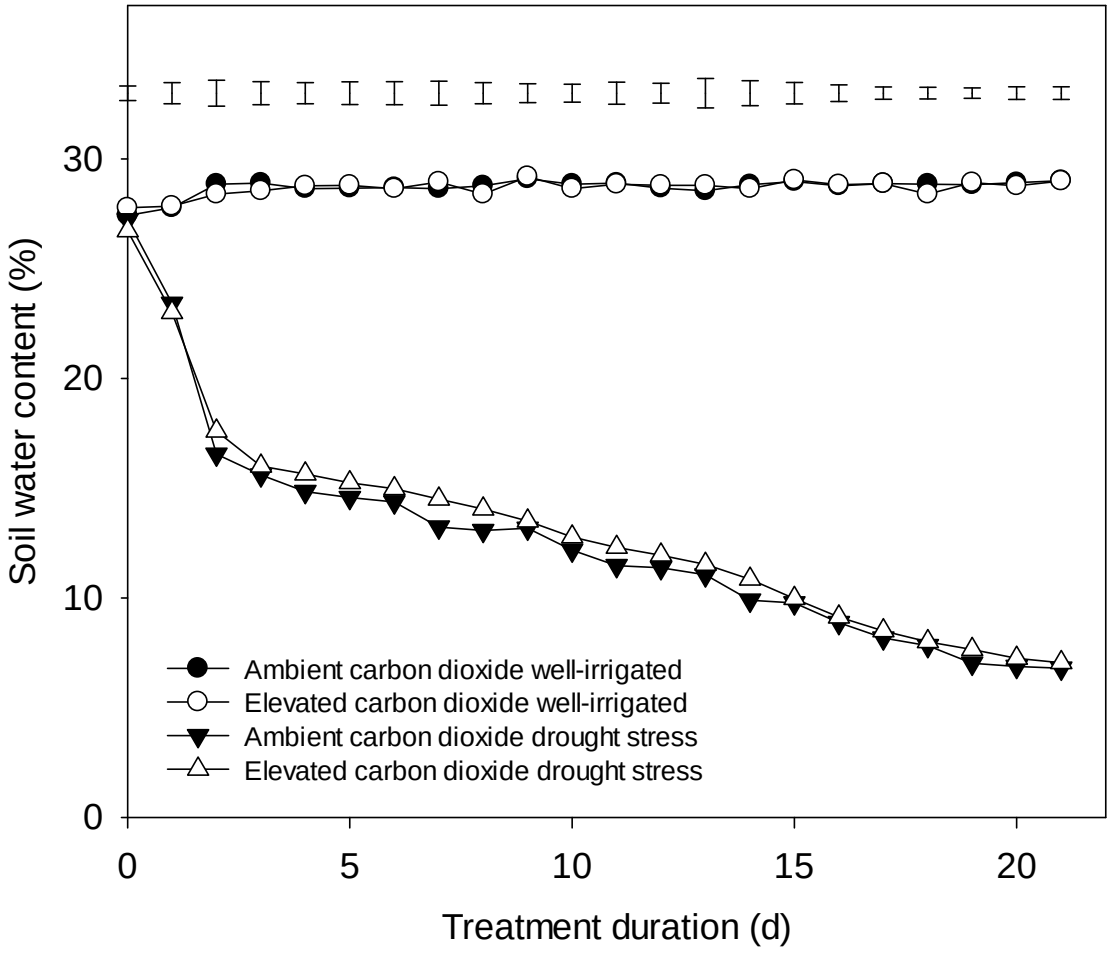


Figure 3. An example representative two-dimensional electrophoresis gel of creeping bentgrass leaf soluble proteins stained with Coomassie Brilliant Blue. Numbers of highlighted proteins correspond with proteins numbers in Table 1 for proteins exhibiting significant changes in abundance following exposure to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d.

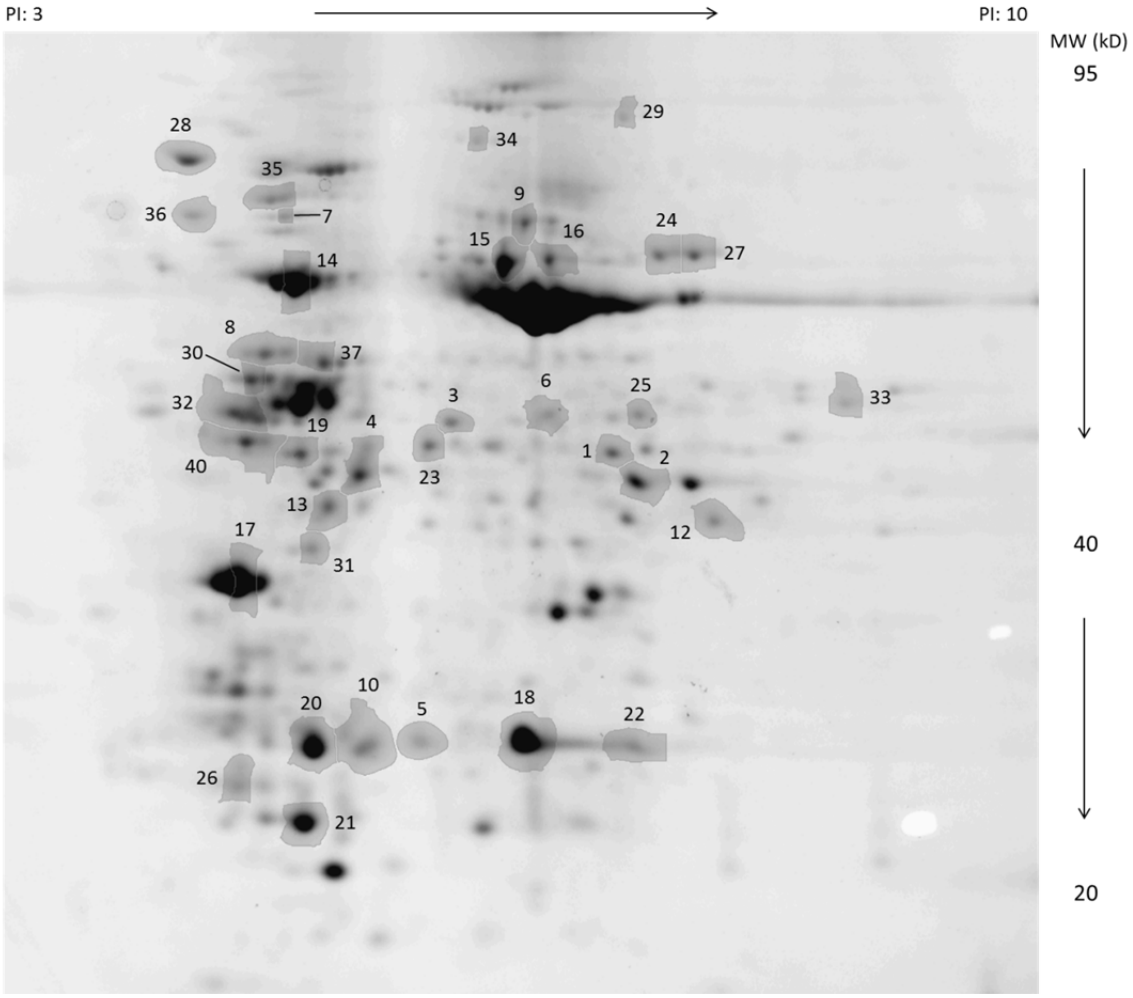


Figure 4. Functional classification and percent of proteins with differential responses in abundance in creeping bentgrass leaves following exposure to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d.

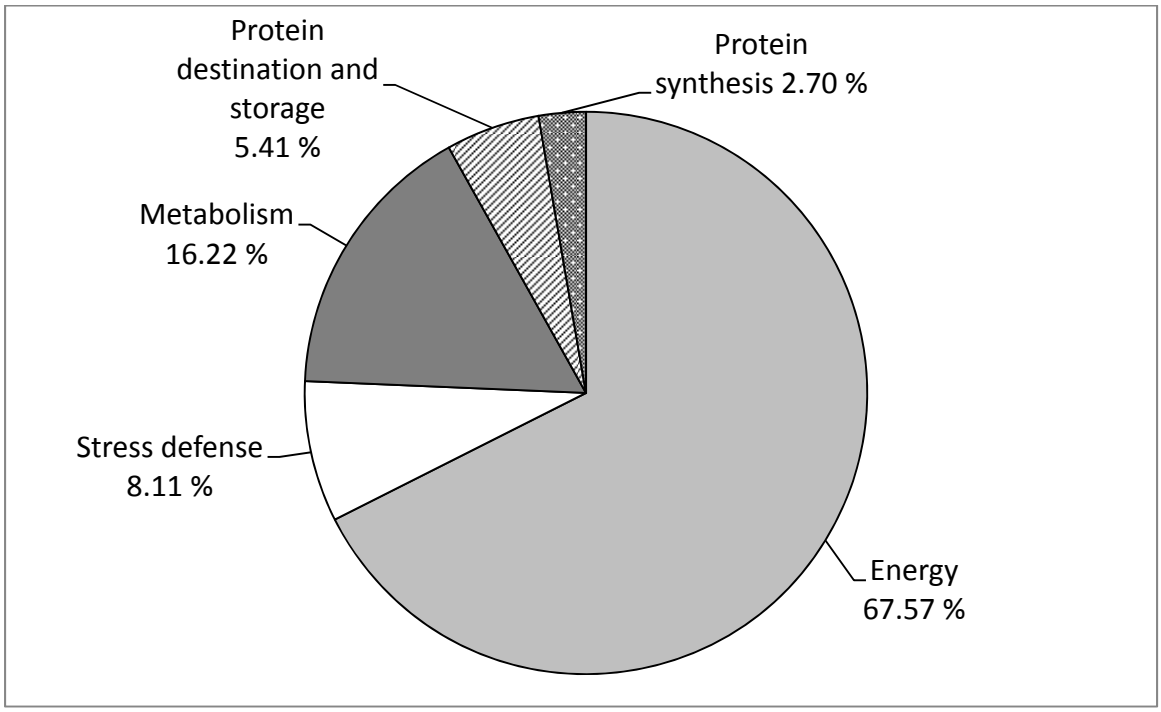


Figure 5. Examples of creeping bentgrass leaf soluble proteins with differential responses in abundance following exposure ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. GAPDH = cytosolic glyceraldehyde-3- phosphate dehydrogenase; LHC1 = light harvesting complex I; MDH = malate dehydrogenase; OEE2 = oxygen-evolving enhancer protein 2, chloroplast precursor.

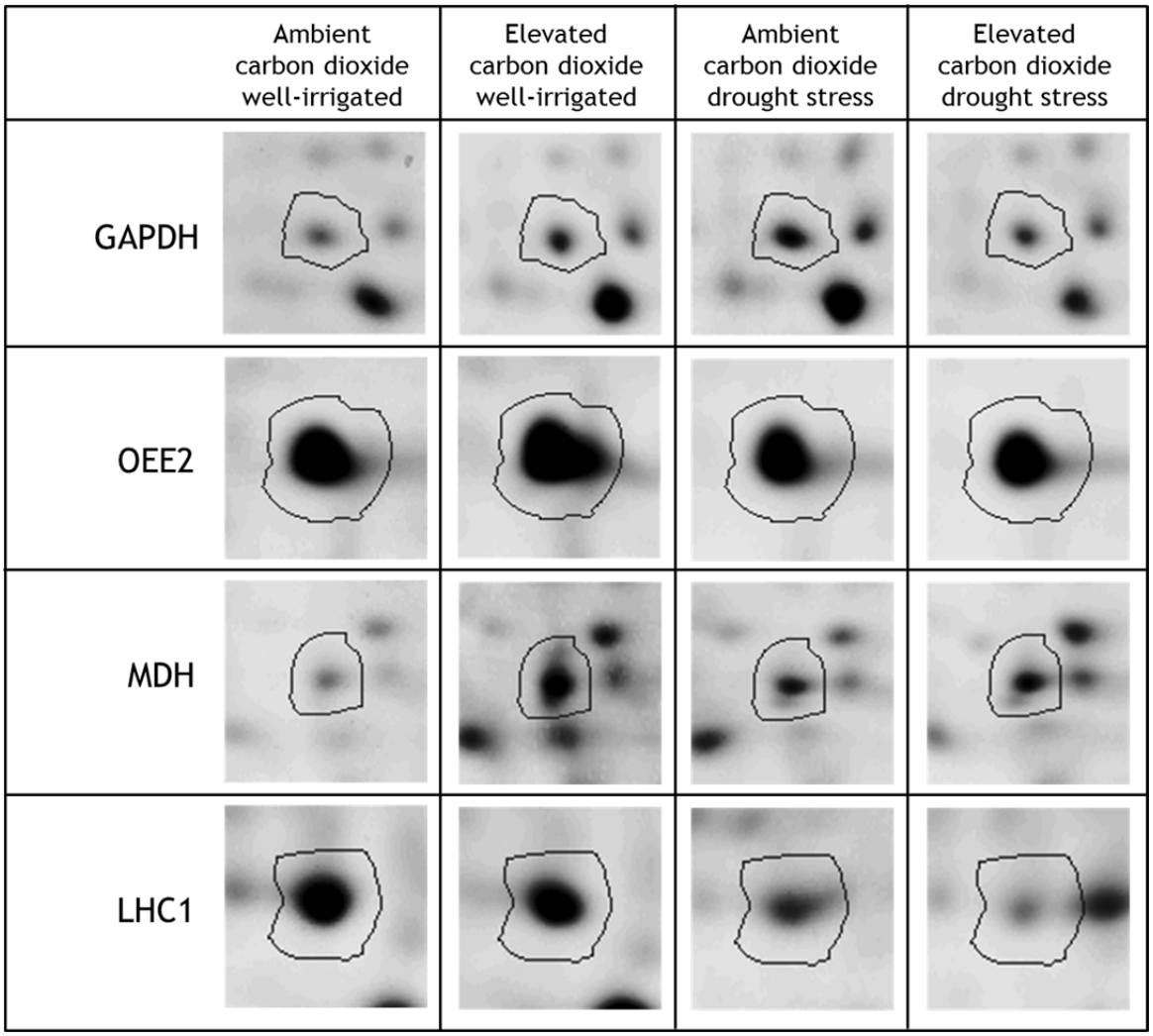


Figure 6A. Fold changes in soluble protein abundance in creeping bentgrass leaves following exposure to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d relative to ambient CO_2 well-irrigated control treatment. Vertical lines atop bars represent SE of five replicates for each treatment and asterisks atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$). Protein abbreviations correspond with respective protein information provided in Table 1.

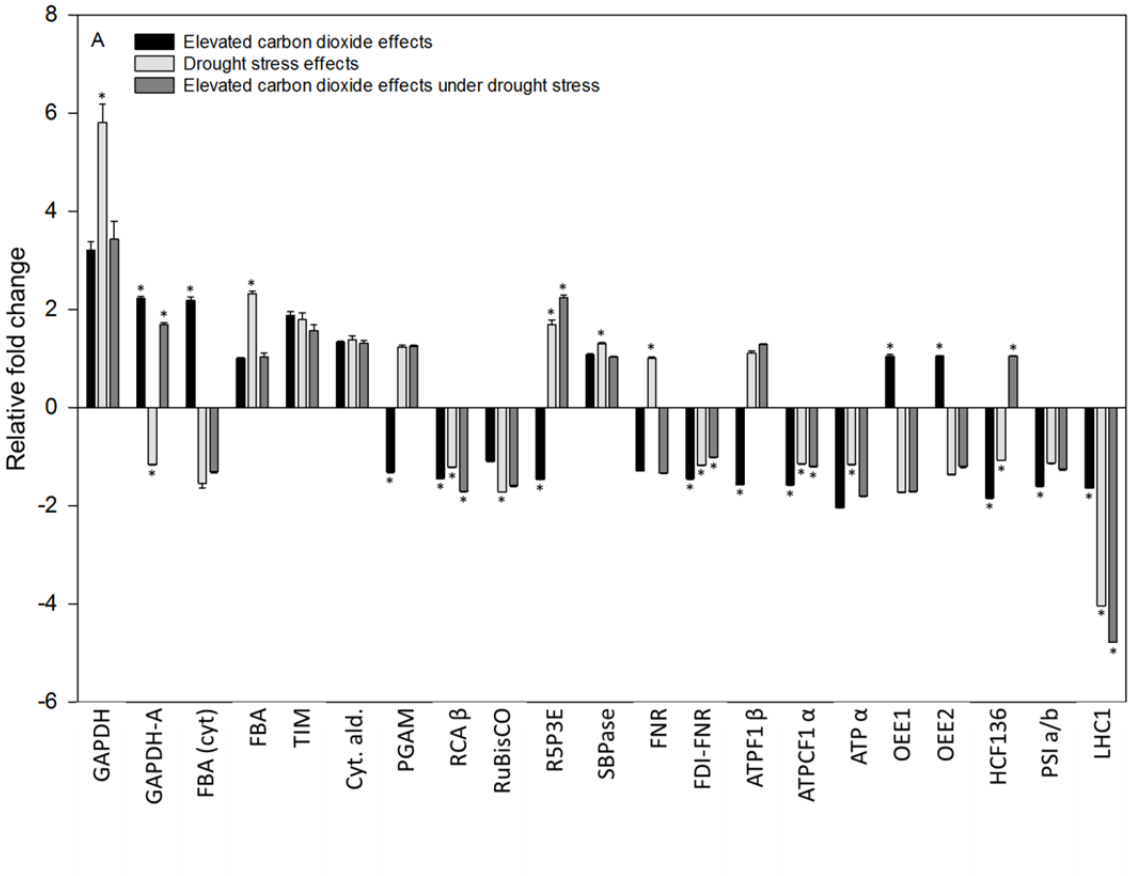
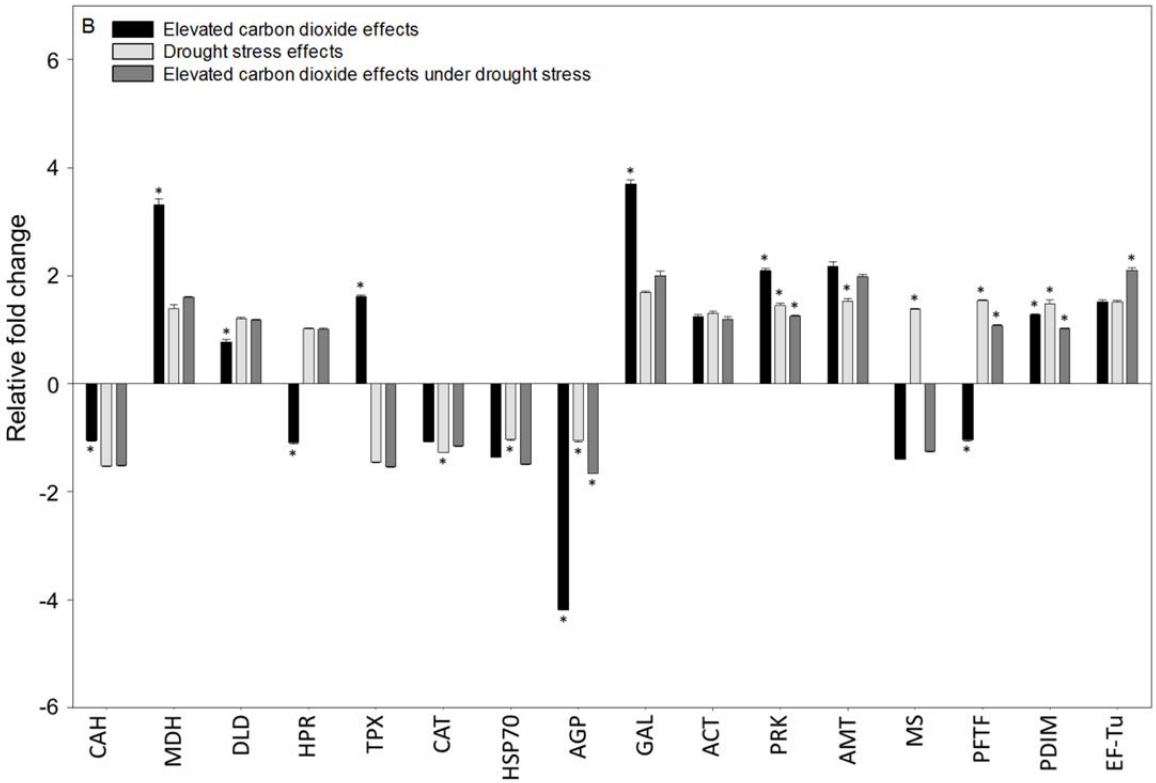


Figure 6B. Fold changes in soluble protein abundance in creeping bentgrass leaves following exposure to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d relative to ambient CO_2 well-irrigated control treatment. Vertical lines atop bars represent SE of five replicates for each treatment and asterisks atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$). Protein abbreviations correspond with respective protein information provided in Table 1.



CHAPTER FIVE

ROOT PROTEIN METABOLISM IN ASSOCIATION WITH IMPROVED ROOT GROWTH AND DROUGHT TOLERANCE BY ELEVATED CARBON DIOXIDE IN CREEPING BENTGRASS

Burgess, P. and B. Huang. 2014. Root protein metabolism in association with improved root growth and drought tolerance by elevated carbon dioxide in creeping bentgrass, Field Crops Res. 165: 80-91. doi: 10.1016/j.fcr.2014.05.003

ABSTRACT

Atmospheric carbon dioxide (CO_2) concentration has been increasing and is predicted to further increase in the future along with the climatic changes which may increase evaporative demand on plants. Elevated CO_2 concentration has a positive effect on plant growth and tolerance to drought stress with regard to above-ground plant organs but limited information is available describing effects of elevated CO_2 concentration on root growth and the subsequent impact on plant responses to drought stress. The specific proteins and metabolic pathways controlling root functions regulated by CO_2 that may contribute to improved root growth and drought stress damages are not well understood. In this study with creeping bentgrass (*Agrostis stolonifera* cv. Penncross), a widely-used perennial grass for forage and turf, elevated CO_2 concentration (800 mL • L^{-1}) promoted root proliferation compared to the ambient CO_2 concentration (400 mL • L^{-1}). Under drought stress, roots developed under elevated CO_2 concentration were able to maintain improved membrane integrity as demonstrated by lower electrolyte leakage. Proteins were extracted from roots of creeping bentgrass exposed to both elevated and ambient CO_2 concentration under well-watered and drought stress conditions. Drought- and CO_2-responsive proteins were separated with two-dimensional electrophoresis and identified using mass spectrometry. Root proteins were mainly classified into the following functional categories: cellular growth, energy, metabolism, and defence. The improved root growth and mitigation of drought stress in creeping bentgrass under elevated CO_2 could be mainly associated with alteration of proteins governing primary metabolism involving nitrogen metabolism (glutamine synthetase), energy metabolism involving respiration (glyceraldehyde-3-phosphate dehydrogenase), and stress defence by

strengthening antioxidant metabolism (ascorbate peroxidase, superoxide dismutase, and catalase) and chaperone protection (HSP81-1).

INTRODUCTION

Atmospheric carbon dioxide (CO_2) levels have risen by 69 mL • L^{-1} between years 1958 and 2008 (Dlugokencky, 2008) and recent environmental trends suggest the rate of increase will continue to hasten over the next century (Houghton et al., 2001). Water availability for irrigation of plants is also becoming limited which may increase frequency and severity of drought stress adversely affecting plant growth and productivity (Cattivelli et al., 2008). Numerous studies have demonstrated that elevated CO_2 may promote plant growth and mitigate damages from abiotic stresses, including drought stress, which has been largely attributed to increases in net photosynthetic rate of leaves and improved water and nutrient use efficiency (Kirkham, 2011). Most of the previous studies focused on responses of the above-ground parts (leaves and shoots) to elevated CO_2, such as increased tillering (Hocking and Meyer, 1991), leaf area, and total shoot biomass (Wand et al., 1999). Limited information is available regarding effects of elevated CO_2 on root growth and subsequent impact on plant adaptation to drought stress, although root systems control water uptake capacity and play key roles in a plant's ability to avoid drought stress. Previous studies found positive effects of elevated CO_2 on root formation and root elongation under non-stress conditions (Taylor et al., 1994; Pritchard et al., 2000; Kirkham, 2011). Here we investigated proteins that are involved in root growth responses and mitigation of drought stress by elevated CO_2.

Proteomic profiling is a powerful tool for the identification and quantification of proteins involved in various metabolic processes controlling plant growth and has been successfully utilized to reveal a wide array of proteins and associated metabolic processes in shoots regulating plant growth and responses to drought stress alone (Xu and Huang,

2010; Merewitz et al., 2011) or elevated CO_2 alone (Bae and Sicher, 2004; Bokhari et al., 2007; Yu et al., 2014). These studies demonstrate that the majority of stress-responsive or CO_2-reponsive proteins in leaves are related to photosynthesis, carbon metabolism, protein synthesis, energy pathways, and stress defence. Drought-responsive proteins in roots are mainly involved in respiration metabolism and energy production functions (Xu and Huang, 2010; Merewitz et al., 2011) though root proteomic responses to elevated CO_2 and interacting CO_2 and drought stress are unknown. Understanding protein responses to elevated CO_2, particularly under drought stress is critically important for unraveling the mechanisms underlying improved root growth and drought tolerance under the scenarios with increasing CO_2 concentration and climate changes. The objective of this project was to identify proteins and metabolic processes involved in root- growth responses to elevated CO_2 concentration under well-watered and drought stress conditions for creeping bentgrass plants.

MATERIALS AND METHODS

Plant materials and Growth Conditions

Individual tillers (20 per pot) (without roots) of creeping bentgrass (*Agrostis stolonifera* cv. Penncross) were collected from a single stock plant and transplanted into pots (10 cm diameter x 40 cm depth) filled with fritted clay medium (Profile Products, Deerfield, IL). Plants were maintained in a controlled-climate growth chambers (Environmental Growth Chamber, Chagrin Falls, Ohio, USA) set to 21/18 °C (day/night), 650 mmol • m^{-2} • s^{-1} photosynthetic active radiation (PAR), 60% relative humidity, and 14 h photoperiod for a week to allow plant acclimation to the growth chamber conditions prior to exposing plants to CO_2 treatments.

Treatments and Experimental Design

Plants were initially established for 35 d (March 1 to April 4, 2013) at ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration under well-watered conditions and fertilized twice per week with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Following 35 d of plant establishment (formation of new roots) under either of the two CO_2 treatments, plants were subjected to drought stress for 20 d (April 5 to April 24, 2013) by withholding irrigation. Soil water content was monitored daily using the time domain reflectometry method (Topp et al., 1980) (Trase Soil Moisture Equipment, Santa Barbara, CA).

The ambient and elevated CO_2 concentrations within chambers were maintained through an automatic CO_2 controlling system connected to the CO_2 source-tank containing 100% research-grade CO_2 following the method described in Yu et al.,

(2012a). CO_2 concentrations inside the chambers were continuously monitored using an infrared gas analyzer (Li-820, LICOR, Inc., Lincoln, NE) connected to a computer data logger. The CO_2 concentration was maintained using an automatic controlling system consisting of a programmable logic controller unit, solenoid valves, and a laptop computer with software capable of monitoring and maintaining CO_2 concentration within 10 mL • L^{-1} of the ambient or elevated target levels.

The experiment was arranged in a split-plot design with CO_2 treatment as the main plot and water treatment as the sub-plot with four replicates for each treatment. The ambient or CO_2 treatments were applied concurrently and each treatment was imposed in four different growth chambers. Plants were relocated between the chambers every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

Shoot Growth and Root Growth Analysis

Plants in four pots (replicates) from ambient or elevated CO_2 treatment which were either well-watered or drought-stressed were destructively sampled for shoot and root analysis parameters at 20 d of drought stress. Shoots were severed from roots, and roots were washed free of fritted clay, stained in 1% crystal violet solution, and scanned with a digital scanner (Epson Expression 1680, U.S. Epson, Inc., Long Beach, CA) to generate root images. Images were then analyzed with WinRHIZO Basic V.2002 software (Regent Instruments Inc., Quebec, QC, Canada) for root length, surface area, and diameter. All tissues (shoots and roots) were dried in an oven at 80 °C for 7 d. Shoot and root dry weight was measured, and root to shoot biomass ratio was calculated.

Root Physiological Analysis

Root electrolyte leakage (EL) was measured on four replicated samples from each treatment following 20 d of drought stress to evaluate root cellular membrane stability (Blum and Ebercon, 1981). Fresh root tissue was collected, rinsed with deionized water to remove external solutes, and placed in a test tube containing 30 mL deionized water. Tubes were agitated in a conical flask shaker for 12 h and the initial conductance (C_i) of immersion liquid measured using a conductivity meter (YSI Model 32, Yellow Springs, OH). Root samples were then autoclaved at 121 °C for 20 min and again shaken for 12 h. The maximal conductance (C_{max}) of autoclaved immersion liquid was then measured and electrolyte leakage calculated ($EL(\%) = (C_i/C_{max}) \times 100$). Roots were then dried in an oven at 80 °C for 7 d and the dry weight was incorporated into growth analysis parameters for each plant.

Root Protein Extraction and Separation

Proteins were extracted from roots in four replicated samples from each treatment collected at 20 d of drought stress using the acetone/trichloroacetic acid (TCA) protein extraction method (Xu et al., 2008) with modifications. Roots were washed free of fritted clay, immediately frozen in liquid nitrogen, and stored at 80 °C for further analysis. Approximately 3 g of root tissues were homogenized and incubated in 35 mL ice-cold precipitation solution (10% TCA, 0.07% 2-mercaptoethanol in acetone) for 12 h at 20 °C. Precipitated proteins were pelleted through centrifugation at 11,600 g at 4 °C and washed three times with rinse solution (0.07% 2-mercaptoethanol in acetone) to remove pigments and lipids yielding colorless supernatant. The pellet was vacuum-dried and suspended

and sonicated in 8 mL resolubilization solution (8 M urea, 2 M thiourea, 1% CHAPS, 1% dithiothreitol, 1% IPG buffer (GE Healthcare), in deionized water) and centrifuged at 11,600 g to separate insoluble tissue from soluble proteins. The supernatant was then used for determination of protein concentration according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as the standard.

Proteins were separated using two-dimensional electrophoresis. Immobiline DryStrips (13 cm, pH 3–10, linear; GE Healthcare, Piscataway, NJ)) were rehydrated with 300 mg of protein sample plus rehydration solution (8 M Urea, 2 M thiourea, 2% CHAPS, 1% IPG buffer (GE Healthcare), 0.002% bromophenol blue, in deionized water). The voltage settings for first-dimension isoelectric focusing were 50 V for 14 h, 500 V for 1 h, 1000 V for 1 h, 5000 V for 1 h, and 8000 V to a total of 80 kV h. DryStrips with protein were then incubated in equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% dithiothreitol, in deionized water) for 20 min and again incubated a second time in the same buffer containing 2.5% iodoacetamide and no dithiothreitol. A Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ) was used to perform second dimension electrophoresis on a 12.5% gel (42% monomer solution (30% acrylamide and 0.8% N,N0-methylenebiscarylamide, in deionized water), 25% resolving gel buffer (1.5 M tris-base and 6 N HCl, pH 8.8, in deionized water), 0.01% sodium dodecyl sulfate, TEMED, and 0.005% ammonium persulfate, in deionized water). Voltage settings for second dimension electrophoresis were 5 mA per gel for 30 min and 20 mA per gel for 6.5 h. Gels were stained with Coomassie Brilliant Blue G-250 (CBB) according to the

procedure set forth by Neuhoff et al. (1988) and scanned with a Typhoon FLA 9500 (GE Healthcare, Piscataway, NJ). Gel images were analyzed using SameSpots software (v4.5, Nonlinear USA Inc., Durham, NC) which utilizes analysis of variance to determine differential treatment effects. Spots with corresponding analysis of variance values less than 0.05 were selected for further characterization. Spot volumes were normalized as a percentage of total spot volume to correct for variability during staining.

Root Protein Identification via Reversed Phase Liquid Chromatography Mass Spectrometry Analysis (RPLC-MS)

Protein spots from CBB-stained 2-D gels were manually excised for protein identification. The gel spots were washed with 30% acetonitrile (ACN) in 50 mM ammonium bicarbonate before dithiothreitol reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37 °C overnight. The resulting peptides were extracted with 30 ml of 1% trifluoroacetic acid followed by C18 ziptip desalting to simultaneously remove salt and concentrate the peptides. Peptides were further fractionated by reversed phase liquid chromatography (RPLC) on an Ultimate 3000 LC system (Dionex, Sunnyvale, CA, USA) coupled with an Q-Exactive mass spectrometer (Thermo Scientific) via a Thermo Scientific nano-electrospray ionization source. The mass spectrometer was operated in a top 15 data dependent mode with automatic switching between MS and MS/MS. Source ionization parameters were as follows: spray voltage: 2.2 kV; capillary temperature: 275 °C; s-lens: 50.0. Full-scan MS mode (300-1650 m/z) was operated at a resolution of 70,000; automatic gain control (AGC) target: 1 x 106; maximum ion transfer time (IT): 500 ms. Ions selected for MS/MS were subjected to the

following parameters: resolution: 17,500; AGC: 5 x 104; IT: 250 ms; isolation window: 4.0 m/z (mass to charge ratio); normalized collision energy: 25.0; underfill ratio: 5.0%; and dynamic exclusion of 30 s.

Each of the raw files was analyzed using the Thermo Proteome Discoverer (V.1.3) platform with Mascot (2.4.1) as search engine against the Green plant protein sequences (1,474,035 entries) of non-redundant NCBI (National Center for Biotechnology Information) protein database (downloaded on July 29, 2013, total entries: 31,242,881). The following Mascot parameters were used: trypsin, two missed cleavages, precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.1 Da; dynamic modifications: methionine oxidation and carbamidomethylation of cysteine. Decoy search option for Mascot was engaged. Proteins identified with highest number of peptide spectral match (PSM) were considered to be present in the majority of respective spots. Proteins with PSM less than 100% were considered hypothetical or predicted, putative, or unknown. Hypothetical and predicted proteins show some level of similarity to known, annotated proteins but lack experimental evidence for definitive identification. Putative proteins show greater similarity to known, characterized proteins compared to hypothetical proteins. Unknown proteins have been experimentally proven to exist but have not been biochemically characterized or cannot be linked to a known gene (Lubec et al., 2005).

Statistical Analysis

Main effects of drought or CO_2 treatment and their interactions were determined by analysis of variance according to the general linear model procedure of SAS (version 9.2;

SAS Institute, Cary, NC). Differences between treatment means were separated by Fisher's protected least significance difference (LSD) test at the 0.05 probability level.

RESULTS

Soil Water Status during Drought Stress under Ambient or Elevated CO_2

Volumetric soil water content (SWC) declined rapidly to only 6.8% at 20 d of drought treatment, indicating plants were subjected to severe drought stress (Fig. 1). There were no significant differences in SWC between the ambient and elevated CO_2 treatments during drought stress at any time, indicating that plants treated with ambient or elevated CO_2 were exposed to the same level of soil water deficit under drought stress.

Root and Shoot Biomass Accumulation and Root to Shoot Ratio of Creeping Bentgrass Plants as Affected by Elevated CO_2 and Drought Stress

Shoot biomass significantly increased by 25% due to elevated CO_2 for plants under well-watered conditions (Fig. 2A). Plants exposed to elevated CO_2 had significantly higher shoot biomass than those at ambient CO_2 under drought stress. There was a 26.4% reduction in shoot biomass due to drought stress for creeping bentgrass under ambient CO_2 conditions. Elevated CO_2 treatment suppressed drought-induced decline in shoot biomass, which showed only a slight decline of 5% following 20 d of drought stress. Root biomass significantly increased by 26% or 18% due to elevated CO_2 for plants under well-watered conditions or drought stress, respectively (Fig. 2B). There was a 19% reduction in root biomass due to drought stress for creeping bentgrass under ambient CO_2 conditions. Elevated CO_2 mitigated the decline in root biomass due to drought stress.

Root to shoot ratio significantly increased by 9% under drought stress for plants exposed to ambient CO_2 conditions (Fig. 2C). There was no difference in root to shoot ratio due to elevated CO_2 for creeping bentgrass plants under well-watered conditions.

Elevated CO_2 resulted in a significantly higher root to shoot ratio than ambient CO_2 treatment under drought stress.

Root Morphology of Creeping Bentgrass Plants as Affected by Elevated CO_2 and Drought Stress

Total root length significantly increased by 26 or 4.7% due to elevated CO_2 for plants under well-watered conditions or drought stress, respectively (Fig. 2D). Drought stress caused significant reduction of 27% in total root length under ambient CO_2 conditions. The elevated CO_2 level suppressed drought-induced decline in total root length for plants subjected to drought stress.

Total root surface area significantly increased by 15% due to elevated CO_2 for plants under well-watered conditions (Fig. 2E). Drought caused significant reduction in total root surface area under ambient or elevated CO_2, but the reduction was only 14% under elevated CO_2 compared to 40% reduction under ambient CO_2. The diameter of individual roots was reduced under drought stress at both ambient and elevated CO_2 (Fig. 2F). The CO_2 treatment had no significant effects on root diameter under either under well-watered or drought conditions.

Root Electrolyte Leakage in Creeping Bentgrass Affected by Elevated CO_2 and Drought Stress

Root EL was significantly increased by 33.6 or 13.7% due to drought stress for plants under ambient CO_2 or elevated CO_2 conditions, respectively (Fig. 3). There were no significant differences in root EL due to CO_2 level for plants under well-watered

conditions. Elevated CO_2 mitigated the large increase in root EL due to drought stress and resulted in significantly lower root EL than plants exposed to ambient CO_2 under drought stress.

Identification and Functional Classification of Root Proteins in Creeping Bentgrass

Approximately 530 root protein spots were successfully detected and separated on two-dimensional polyacrylamide gels. A representative CBB gel showing specific locations of spots is presented in Fig. 4. A total of 55 spots with their abundance significantly altered by drought stress and elevated CO_2 treatments compared to ambient CO_2 well-watered treatment were identified via RPLC–MS analysis. A majority of the selected spots were successfully identified (Table 1). All identified proteins were categorized according to the functional categorization set forth by Bevan et al. (1998). Among the 55 analyzed spots, 5.45% were involved in metabolism functions, 29.09% in energy functions, 5.45% in cell growth and division functions, 1.82% in intracellular traffic functions, 5.45% in transcription functions, 20.0% in stress defence functions, 3.64% in protein destination and storage functions, and 29.09% remain unclassified (Fig. 5A). The proteins which abundances were significantly altered by elevated CO_2, drought stress, or the combined elevated CO_2 and drought stress treatment are discussed below.

Differential Root Proteomic Response of Creeping Bentgrass Plants as Affected by Elevated CO_2 and Drought Stress

Table 1 presents the functional categories, protein names, and changes in protein abundance due to drought stress, elevated CO_2 under well-watered conditions or elevated CO_2 under drought stress conditions. Protein abundance is expressed as fold-change compared to the protein abundance under well-watered and ambient CO_2 conditions. A ratio less than 1.0 indicates reduction or down-regulation of protein abundance while a ratio greater than 1.0 indicates increase or up-regulation of protein abundance by drought, elevated CO_2, or elevated CO_2 and drought stress.

Drought stress led to up-regulation of 32 protein spots (2, 3, 5-9, 11-13, 15, 17-19, 23, 25-31, 33-36, 38, 39, 43, 45, 52, and 55) and down-regulation of 23 protein spots (1, 4, 10, 14, 16, 20-22, 24, 32, 37, 40-42, 44, 46-51, 53, and 54) in creeping bentgrass roots under ambient CO_2 conditions. Among root protein spots which were upregulated by drought stress, 6.25% had putative functions in metabolism, 37.5% to energy metabolism, 6.25% to transcription, 6.25% to protein destination/storage, 3.13% to intracellular traffic, 28.13% to stress defence, and 12.5% had unclear classification (Fig. 5B). Among down-regulated protein spots in roots due to drought stress, 4.35% had putative functions in metabolism, 17.39% in energy metabolism, 13.04% in cell growth and division, 4.35% in transcription, 8.70% in stress defence, and the remaining 52.17% of spots had unclear classification.

Creeping bentgrass roots grown under elevated CO_2 and well-watered conditions showed up-regulation of 25 spots (3, 5-7, 9, 10, 14-18, 20-22, 25-27, 29, 31, 33, 35, 36, 39, 43, and 49) and down-regulation of 30 spots (1, 2, 4, 8, 11-13, 19, 23, 24, 28, 30, 32, 34, 37, 38, 40-42, 44-48, and 50-55). Among root protein spots which were up-regulated by CO_2, 4.17% were related to metabolism, 41.67% to energy metabolism, 8.33% to cell

growth and division, 4.17% to transcription, 8.33% to protein destination/storage, 4.17% to intracellular traffic, 20.83% to stress defence, and 8.33% had unclear classification (Fig. 5C). For the down-regulated root proteins by elevated CO_2, 6.45% of the spots were related to metabolism, 19.35% to energy metabolism, 6.45% to transcription, 3.23% to protein destination/storage, 19.35% to stress defence, and the remaining 45.16% of spots had unclear classification.

Creeping bentgrass roots grown in elevated CO_2 conditions and exposed to drought stress showed up-regulation of 31 spots (2, 3, 5-9, 11-13, 15, 17, 18, 23, 25-31, 33-36, 38, 39, 43, 45, 52, and 55) and down regulation of 24 spots (1, 4, 10, 14, 16, 19-22, 24, 32, 37, 40-42, 44, 46-51, 53, and 54). Among root protein spots which were up-regulated by elevated CO_2 under drought stress, 6.45% were related to metabolism, 35.48% to energy metabolism, 6.45% to transcription, 6.45% to protein destination/storage, 3.23% to intracellular traffic, 29.03% to stress defence, and 12.9% had unclear classification (Fig. 5D). Among the down-regulated proteins by elevated CO_2 under drought stress, 4.17% of the spots had putative functions in metabolism, 20.83% in energy metabolism, 12.5 in cell growth and division, 4.17% in transcription, 8.33% in stress defence, and the remaining 50% of spots had unclear classification.

DISCUSSION

Effects of Elevated CO_2 and Drought Stress on Shoot and Root Growth and Membrane Stability of Creeping Bentgrass

Both shoot and root growth of creeping bentgrass decreased following prolonged water withholding or severe drought stress under ambient CO_2 conditions, but root to shoot biomass ratio was greater in drought-stressed plants than the well-watered plants. Increased root to shoot ratios during drought have also been reported in various agronomic crop species, which is considered an adaptive mechanism in plant responses to drought stress (Sharp and Davies, 1989). Increased amounts of carbon is partitioned to root organs when soil water becomes limited, which may contribute to the increased root to shoot ratio (DaCosta and Huang, 2006). These results indicated that higher root to shoot ratio in drought-stressed creeping bentgrass was merely an adaptive response to drought stress.

In contrast to drought effects, elevated CO_2 enhanced both shoot and root biomass production of creeping bentgrass under well-watered and drought conditions. The growth-promoting effects of elevated CO_2 concentration were greater under drought stress, as manifested by increased root to shoot ratio in plants subjected to elevated CO_2 relative to those under ambient CO_2. The growth-promoting effects of CO_2 on root growth have been associated with increased cellular turgor and carbohydrate accumulation under elevated CO_2 (Taylor et al., 1994) though the underlying mechanisms responsible for promotive effects are not well described. The enhanced root growth and root to shoot ratio could facilitate increased water uptake capacity of the root system, thereby mitigating drought damages by elevated CO_2. The increased root

biomass due to elevated CO_2 in this study could be attributed to the enhanced root proliferation, as discussed below.

Much of the previous work investigating the interactive effects of elevated CO_2 and drought stress on woody plant species suggest that enhanced drought tolerance by elevated CO_2 is associated with both improved water-use efficiency (WUE) and increased proliferation of roots (Wullschleger et al., 2002). In the present study, total length and surface area of creeping bentgrass roots was significantly reduced by drought stress alone but increased with elevated CO_2 treatment under well-watered or drought stress conditions. Root proliferation through increased length and surface area may enhance soil exploration and increase water uptake capacity of the plant in soils with limited water availability. Extensively-proliferated root systems with large surface area serve critical drought avoidance functions for water uptake, and have been implicated in prolonging turfgrass survival of drought stress (Carrow, 1996; Marcum et al., 1995). The increase in total root length due to elevated CO_2 in creeping bentgrass could be due to faster rates of elongation for individual roots or alternatively faster root production increasing the total root number. The stimulation of root elongation by elevated CO_2 has been attributed to increased cell-wall extensibility, carbon supply, and carbon metabolism under elevated CO_2 (Taylor et al., 1994). Rogers et al. (1994) reported that root length and root number were significantly increased due to elevated CO_2 for a majority of the reviewed studies, and several studies also reported increased root diameter. Increased rooting capacity in the current study for plants under elevated CO_2 was mainly due to increases in total root length or more proliferative root systems and not due to increases in root diameter, as this parameter was not affected by elevated CO_2.

Electrolyte leakage indicates cellular membrane stability or integrity for estimating cellular damages of plants responding to stress conditions, such as drought stress (Martin et al., 1987). The positive effects of elevated CO_2 on maintaining cellular membrane stability of leaves has been reported in poplar (*Populus alba* x *tremula*) exposed to cold or chemical stress (Schwanz and Polle, 2001a). Elevated CO_2 has also been shown to maintain better membrane stability and lower electrolyte leakage in leaves of tall fescue (*Festuca arundinacea*) during heat stress (Yu et al., 2012b), drought stress, or the combined drought plus heat stress (Yu et al., 2012a). In this study, root electrolyte leakage increased due to drought stress for creeping bentgrass plants at both CO_2 levels, but electrolyte leakage was significantly lower in plants exposed to elevated CO_2 than those under ambient CO_2 during drought stress. This suggests that elevated CO_2 alleviated membrane damages caused by drought stress, which could help to sustain root function in soils with limited water availability.

Effects of Elevated CO_2 and Drought Stress on Differential Protein Accumulation Regulating Metabolism Functions of Creeping Bentgrass

Among the 55 drought- and CO_2-responsive proteins, three spots were classified as serving important metabolic functions within the plant system. ATP-citrate synthase (spot 1) is a key enzyme in the acetyl coenzyme A (CoA) pathway (Hynes and Murray, 2010) and catalyzes the ATP-dependent reaction of citrate and CoA forming acetyl-CoA and oxaloacetic acid (Fatland et al., 2005). Acetyl-CoA serves as the primary metabolite in the tricarboxylic acid (TCA) cycle of dark respiration (Fatland et al., 2002) and was down-regulated most by drought stress alone in the current study. Elevated CO_2 mitigated

this down-regulation during drought stress which suggests that respiratory metabolism of creeping bentgrass roots may be suppressed through elevated CO_2 growing conditions. Despite lack of previous research regarding root proteomic response to elevated CO_2, leaf respiration was shown to be inhibited by elevated CO_2 in some plant species, including a perennial grass species (Ainsworth and Rogers, 2007; Yu et al., 2012a). The inhibitory effect of elevated CO_2 on leaf respiration was postulated to be related to the direct inhibition of activities for two respiratory enzymes (succinate dehydrogenase and cytochrome c oxidase) (Gonzàlez-Meler et al., 1996). Whether or not these specific respiratory enzymes are inhibited for creeping bentgrass under elevated CO_2 remains to be determined in future studies.

Lichenase (spot 2) is one of the many enzymes of the glycoside hydrolase family responsible for hydrolyzing glycosidic bonds and degrading polysaccharides to remobilize carbohydrates from cell walls of plants (Henrissat et al., 1998). In the present study, lichenase was up-regulated in roots of creeping bentgrass due to drought stress alone and this up-regulation was suppressed by elevated CO_2 during drought. Under well-watered conditions, elevated CO_2 caused down-regulation of the lichenase protein which suggests that elevated CO_2 may inhibit lichenase within the plant system. This may suppress the breakdown of polysaccharides in cell walls to maintain cell wall integrity, which is particularly important for maintaining cellular turgidity under drought stress. Alternatively, suppression of lichenase may suggest less metabolic demand for carbohydrates otherwise needed to sustain plant growth processes during drought stress under elevated CO_2 conditions. This could be investigated in the future.

Glutamine synthetase (GS, spot 3) functions as an assimilatory enzyme which reassimilates ammonia following photorespiration and breakdown of proteins and other nitrogen-containing compounds (Miflin and Habash, 2002). Nitrogen metabolism and reassimilation is greatly affected by plant age, nutritional status, and environmental conditions (Pate, 1973). Drought stress caused an up-regulation of GS under both CO_2 levels, but the up-regulation was greater for creeping bentgrass roots under elevated CO_2 conditions. This may suggest that GS metabolism was increased by drought and further increased by elevated CO_2. Higher amounts of GS may be an important factor sustaining root growth under drought stress through re-assimilation of nitrogen sourced from proteins and other nitrogen-containing compounds. Whether this is a significant contributing factor affecting nutritional status of creeping bentgrass plants under elevated CO_2 during drought remains to be determined.

Effects of Elevated CO_2 and Drought Stress on Differential Protein Accumulation Regulating Energy Metabolism Functions of Creeping Bentgrass

The largest proportion of identified proteins (16 spots) responding to drought stress and CO_2 concentration were related to energy metabolism in roots of creeping bentgrass. Functions of several specific proteins exhibiting differential responses to drought and elevated CO_2 were chosen for further discussion based on their roles in plant respiration, carbohydrate status, and plant nutrition. All of these plant processes serve critical functions greatly affecting plant tolerance to drought stress, as well as other abiotic stresses.

Of the 16 energy-function proteins, four spots (5, 6, 9, and 12) were identified as cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH). There are three active forms of GAPDH within the plant system, two of which are chloroplastic and support photosynthetic roles, and another cytosolic form which supports glycolytic roles in respiration (Taiz and Zeiger, 2010). Drought stress caused up-regulation of all four cytosolic GAPDH proteins in the current study and the up-regulation was enhanced by elevated CO_2. Previous studies on creeping bentgrass have shown that GAPDH protein was initially up-regulated in response to drought or heat stress and eventually declined upon prolonged stress and permanent stress damage (Merewitz et al., 2011; Xu et al., 2010a). The increase in root GAPDH may serve to enhance glycolysis which provides substrates for ATP energy production during drought stress.

Among all the root proteins identified in the current study, fructokinase (spot 7) showed the greatest amount of increase due to drought stress alone. This increase of fructokinase was much less under elevated CO_2 and drought stress, suggesting that elevated CO_2 may suppress fructokinase production under drought stress conditions. Sucrose is the stable form of carbohydrate first metabolized by sucrose synthase and further phosphorylated by fructokinase in the citric acid cycle of plant respiration (Pego and Smeekens, 2000; Odanaka et al., 2002). Suppressed up-regulation of fructokinase may be an important factor contributing to slower or more-efficient conversion of sucrose. Additionally, suppressed fructokinase may benefit plant survival during drought stress through lower respiration rates and carbohydrate conservation or slower carbohydrate metabolism, though this is another aspect of proteomic response to elevated CO_2 and drought stress which deserves further investigation.

Another notable protein involved in energy metabolism in roots of creeping bentgrass was ferredoxin-NADP reductase (spot 19), which was up-regulated by drought stress under ambient CO_2 levels, but down-regulated by elevated CO_2 level. Ferredoxin and ferredoxin-NADP reductase (FNR) are iron-sulfur proteins which have been well-characterized for their roles in photosynthesis (Taiz and Zeiger, 2010), and have also been detected in non-photosynthetic root tissues of spinach (*Spinacia oleracea*) (Morigasaki et al., 1990) and corn (*Zea mays*) (Suzuki et al., 1985). It was suggested that ferredoxin and FNR support nitrogen assimilation processes which occur in amyloplasts and proplastids (Green et al., 1991). Up- regulation of FNR in response to drought stress may indicate increased usage of nitrogen-containing compounds whereas down-regulation of FNR by elevated CO_2 conditions may suggest efficient nitrogen usage for enhanced tolerance to drought stress in creeping bentgrass plants.

Effects of Elevated CO_2 and Drought Stress on Differential Protein Accumulation Regulating Stress Defence Functions of Creeping Bentgrass

The second largest proportion of identified root proteins responding to CO_2 and drought stress in the current study was related to stress defence functions. Among the 10 stress-defence proteins, four spots were identified as superoxide dismutase (SOD), one spot (31) being MnSOD and the other three spots (31, 33, 38) being Cu/ZnSOD subunits. SOD functions to rapidly convert damaging superoxide (O_2^-) radicals to hydrogen peroxide (H_2O_2) in multiple cellular compartments (Noctor and Foyer, 1998). All four SOD proteins were up-regulated in response to drought stress at both CO_2 levels and the increase was greater for creeping bentgrass exposed to elevated CO_2. Another antioxidant

enzyme protein, ascorbate peroxidase (APX), exhibited similar responses to drought and elevated CO_2. APX is responsible for detoxifying hydrogen peroxide both within the vacuole and in the apoplast (Mehlhorn et al., 1996) and may serve important roles in reactive-oxygen species (ROS) signaling functions for stress response (Mittler, 2002). Most previous work on antioxidant protein responses to drought stress or elevated CO_2 focused on leaves and little is known on root antioxidant protein responses to drought and elevated CO_2. It was reported that elevated CO_2 mitigated drought-induced damages in leaves through higher stability of antioxidant enzymes and increased responsiveness of SOD of leaves to drought stress (Schwanz and Polle, 2001b). Another study investigated the effects of elevated CO_2 for promoting drought tolerance of spring wheat (*Triticum aestivum*) and reported that activity of SOD (Cu/ZnSOD, FeSOD, and MnSOD) increased rapidly in leaves upon drought stress and was further enhanced for plants under elevated CO_2 conditions (Lin and Wang, 2002). The up-regulation of SOD and APX proteins by drought stress alone or in combination with elevated CO_2 suggests that drought stress may induce oxidative stress and the enhanced abundance of these proteins under elevated CO_2 may facilitate root antioxidant capacity. Among the four SOD proteins identified, two spots (spot 30 and 38) exhibited down-regulation in response to elevated CO_2 under well-watered conditions in roots of creeping bentgrass plants. Similar results were also reported in leaves in other plant species (Schwanz and Polle, 2001b; Lin and Wang, 2002) though the mechanisms underlying CO_2-induced down-regulation of specific SOD subunits needs to be described further.

Catalase (CAT) is also a critical antioxidant enzyme for oxidative protection and catalyzes the detoxification of hydrogen peroxide in peroxisomes (Taiz and Zeiger,

2010). The CAT protein (spot 37) was down-regulated by drought stress under both CO_2 levels, and elevated CO_2 caused less down-regulation compared to creeping bentgrass plants under ambient CO_2. Changes in CO_2 and O_2 concentration, among other environmental changes, have been shown to influence CAT activity in leaves of barley (*Hordeum vulgare*) (Fair et al., 1973). Leaves of tobacco (*Nicotiana sylvestris*) plants grown under elevated CO_2 showed a rapid reduction in CAT within the first 12 h of treatment followed by a steady decline in CAT thereafter (Havir and McHale, 1989). Imposition of drought stress has been shown to decrease CAT activity in various turfgrass species including tall fescue (*Festuca arundinacea*) and Kentucky bluegrass (*Poa pratensis*) and limits H2O2 detoxification within the plant system (Jiang and Huang, 2001). These results suggested that drought may weaken the antioxidant capacity of CAT while elevated CO_2 helped to sustain the level of CAT detoxification and less oxidative damage to roots during drought stress.

Heat shock protein 81-1 (HSP81-1, spot 28) was up-regulated by drought stress under both CO_2 levels in the current study but the up-regulation was greater for plants under elevated CO_2 compared to ambient CO_2. HSP81-1 is part of the heat-shock protein 90 (HSP90) family which collectively act as ATP-dependent molecular chaperones with unique substrate specificity for proteins involved in transcription and signal transduction pathways (Xu et al., 2011). Changes in HSP abundance has been shown to occur in response to not only heat stress, but also to drought and chilling stress in various plant species (Wang et al., 2004). Higher levels of upregulated HSPs in roots of creeping bentgrass under elevated CO_2 during drought stress may reflect enhanced protection against drought stress. In contrast to CO_2 effects under drought stress, elevated CO_2

under well-watered conditions resulted in a reduction in the abundance of HSP18-1, suggesting that such protective proteins were not required when plants were provided with adequate water, and extra energy could be used to support root growth.

Effects of Elevated CO_2 and Drought Stress on Differential Protein Accumulation Regulating Protein Synthesis Functions of Creeping Bentgrass

Roots of creeping bentgrass also showed differential protein responses to drought and elevated CO_2 for proteins involved in cell growth and division functions. Two protein spots (20 and 21) were identified as elongation factor 2 (EF-2) and showed down-regulation by drought stress and less down-regulation with elevated CO_2 under drought stress. Alternatively to drought stress response, EF-2 proteins were up-regulated in response to elevated CO_2 under well-watered conditions. EF-2 is one of several elongation factors involved in ribosomal protein synthesis of higher plants and was shown to be especially sensitive to carbohydrate depletion and nutritional status (Vogel et al., 1999). Changes to EF-2 have also been shown to cause downstream effects in *Arabidopsis thaliana* responding to low-temperature stress (Guo et al., 2002). The suppression of drought-induced decline in EF-2 abundance and the up-regulation of EF-2 by elevated CO_2 under well-watered conditions may help sustain continued protein synthesis to support root growth, which is particularly important for root survival under drought stress.

SUMMARY

This study provides the first root proteomic analysis describing root responses of a perennial grass species used as forage or turf to elevated CO_2 under well-watered and drought stress conditions. Elevated CO_2 promoted shoot and root growth of creeping bentgrass under non-stress conditions and mitigated the inhibitory effects of drought on shoot and root growth, which was manifested through promotion of total root length, surface area, root and shoot biomass, and root to shoot ratio, as well as maintenance of root membrane integrity during soil drying. Proteins governing primary metabolism involving nitrogen metabolism (glutamine synthetase), energy metabolism involving respiration (glyceraldehyde-3-phosphate dehydrogenase), and stress defence by antioxidant metabolism (ascorbate peroxidase, superoxide dismutase, and catalase) and protective chaperones (HSP81-1) could serve major roles in root responses to elevated CO_2, particularly under drought stress conditions. Additionally, the abundance of proteins imparting other biological functions (protein synthesis, transcription, protein destination/storage, intercellular traffic) were also altered by elevated CO_2. The molecular factors controlling CO_2-repsonsive root proteins involved in the regulation of root growth and drought tolerance deserve further investigation.

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TABLES AND FIGURES

Table 1. Changes in protein abundance in roots of creeping bentgrass in response to drought under ambient CO_2, elevated CO_2 under well-watered conditions, and elevated CO_2 under drought stress compared to well-watered control under ambient CO_2 treatment. Value above 1.0 indicates increase in abundance or up-regulation; value below 1.0 indicates decrease in abundance or down-regulation. SID: spot ID, MW: molecular weight (kDa), score: MO MOWSE score, PM: the number of unique peptides matched, AccN: accession number.* 0.05>P≥0.01; **0.01>P≥0.001, ***0.001>P

SID	Protein name	MW [kDa]	calc. pI	Score	PM	AccN	Ambient CO_2 drought	Elevated CO_2 well-watered	Elevated CO_2 drought	Sig. Level
1	ATP-citrate synthase	65.9	7.28	121.89	3	473926455	0.44	0.82	0.64	***
2	Lichenase-2	25.1	7.03	135.9	1	473971036	2.83	0.77	2.15	***
3	Cytosolic glutamine synthetase	38.9	5.48	249	3	10946357	2.49	1.19	2.93	**
4	Citrate synthase 4,	56.7	8.05	141.8	3	475481084	0.52	0.89	0.48	***
5	Glyceraldehyde-3-phosphate	36.5	7.12	215.59	3	258642943	2.85	1.11	2.93	***
6	GAPDH, cytosolic	33.2	6.2	346	4	120668	2.02	1.17	2.67	***
7	Fructokinase-1	34.7	5.27	172.38	1	158512869	25.56	1.20	8.50	**
8	Phosphoglucoisomerase	60.7	7.66	589.27	7	82754716	1.39	0.97	1.56	**
9	GAPDH, cytosolic	36.5	6.67	473	6	120680	3.95	1.24	4.45	**
10	Class III Alcohol dehydrogenase	40.8	6.78	171	2	1675394	0.83	1.18	0.59	**
11	Nucleoside diphosphate kinase	25.0	8.94	145.32	2	357132312	1.17	0.87	1.19	**
12	Glyceraldehyde 3-phosphate	32.0	7.96	469.95	7	255537011	1.47	0.99	1.67	**
13	Cytosolic 3-phosphoglycerate	31.3	5.05	596	6	28172913	1.02	0.59	1.14	**
14	Fumarate hydratase 2,	53.2	7.61	2121.8	10	475609150	0.69	1.04	0.83	*
15	RuBisCO small subunit	18.7	8.6	379	6	6573206	1.35	1.14	1.76	*
16	Cytoplasmic aldolase	38.7	6.56	311	4	218157	0.83	1.15	0.67	*
17	Nucleoside diphosphate kinase	16.5	6.8	1208.16	5	9652119	1.60	1.07	1.13	*
18	Formate-tetrahydrofolate ligase	68.1	7.01	450.18	2	51536102	1.23	1.10	1.43	*
19	Ferredoxin-NADP reductase,	40.8	8.54	169.39	3	475571381	1.36	0.78	0.83	*
20	Elongation factor 2	93.7	5.93	385	3	6015065	0.45	1.12	0.59	***
21	Elongation factor 2	93.7	5.93	299	4	6015065	0.54	1.45	0.88	***
22	Dynamin-related protein 1C	66.1	7.9	797.84	4	475573181	0.82	1.32	0.97	*
23	Glycine-rich RNA binding	14.2	8.51	66.52	1	6911142	1.41	0.76	1.18	***
24	Polyadenylate-binding protein 2	68.9	8.72	1144.04	1	474111415	0.58	0.82	0.60	**
25	Os07g0642900 transcription	29.6	6.92	331.58	1	115473681	1.38	1.22	1.38	*
26	Protein disulfide isomerase 3	56.6	4.96	203	3	13925728	1.66	1.07	1.33	*
27	Mitochondrial processing	53.3	6.54	414	4	11993905	1.93	1.12	1.10	*
29	ADP-ribosylation factor GTPase-	18.4	6.93	164.59	2	357122721	1.85	1.23	1.87	***
30	Mn-superoxide dismutase	18.5	6.18	920	1	378724804	1.62	0.87	1.66	***
31	Cu-Zn-superoxide dismutase	15.1	6.18	90.42	1	226897529	1.82	1.22	2.26	***
32	L-gulonolactone oxidase-like	63.3	8.12	93.98	1	514794292	0.56	0.80	0.66	**
33	Cu-Zn-superoxide dismutase	15.1	6.18	255.36	1	226897529	1.52	1.16	1.54	**
34	CBS domain protein	15.6	8.15	519.69	1	149392473	1.34	0.94	1.08	**
35	Ascorbate peroxidase	26.6	5.82	1507.89	1	226897533	1.83	1.46	2.36	**
36	Flavoprotein wrbA-like isoform	21.8	6.7	269.87	3	357133098	1.68	1.06	1.41	*
37	Catalase isozyme 1	56.6	7.17	781.14	9	1705612	0.59	0.94	0.67	*
38	Cu-Zn-superoxide dismutase	15.1	6.18	143.85	1	226897529	1.31	0.98	1.39	*
39	Flavoprotein wrbA-like isoform	21.8	6.7	382.85	4	357133098	1.51	1.19	1.50	*
28	Heat shock protein 81-1	80.1	5	718	10	417154	1.56	0.87	1.87	*
40	Uncharacterized protein	84.0	7.78	109.1	3	514744719	0.36	0.74	0.37	***
41	Uncharacterized protein	84.0	7.78	175.24	3	514744719	0.34	0.85	0.40	***
42	Hypothetical protein	19.2	6.68	115.54	1	475554365	0.41	0.54	0.48	***
43	Hypothetical protein OsI_27579	35.4	5.17	190.32	4	125559927	6.06	1.03	10.88	***
44	Hypothetical protein	19.2	6.68	102.88	1	475554365	0.31	0.53	0.39	**
45	Hypothetical protein	18.4	4.78	140.07	2	474060775	1.13	0.75	1.69	**
46	Predicted protein	96.5	6.81	568.42	5	326509431	0.12	0.52	0.21	***
47	Predicted protein	41.2	8.66	1173.45	10	326488467	0.50	0.76	0.52	***
48	Predicted protein [*Hordeum*	60.1	9.35	57.17	1	326522959	0.30	0.88	0.36	***
49	Predicted protein	41.2	8.66	1107.15	9	326488467	0.66	1.07	0.70	**
50	Predicted protein	61.0	5.55	1084.64	1	326491001	0.33	0.52	0.46	**
51	Predicted protein	18.8	6.8	103.11	2	326508766	0.30	0.58	0.39	**
52	Predicted protein	59.3	6.24	4239.45	7	326492854	1.76	0.70	2.32	**
53	Predicted protein	96.5	6.81	246.91	5	326509431	0.28	0.61	0.39	**
54	Predicted protein [*Hordeum*	41.2	8.66	369.33	7	326488467	0.70	0.82	0.66	*
5	Unknown [Zea	8.2	5.8	138	1	2108	1.14	0.70	1.08	*

Figure 1. Soil water content (SWC) during 20 d of drought treatment for well-watered or drought-stressed creeping bentgrass at ambient or elevated CO_2 levels. LSD bars indicate significant differences exist at $P \leq 0.05$.

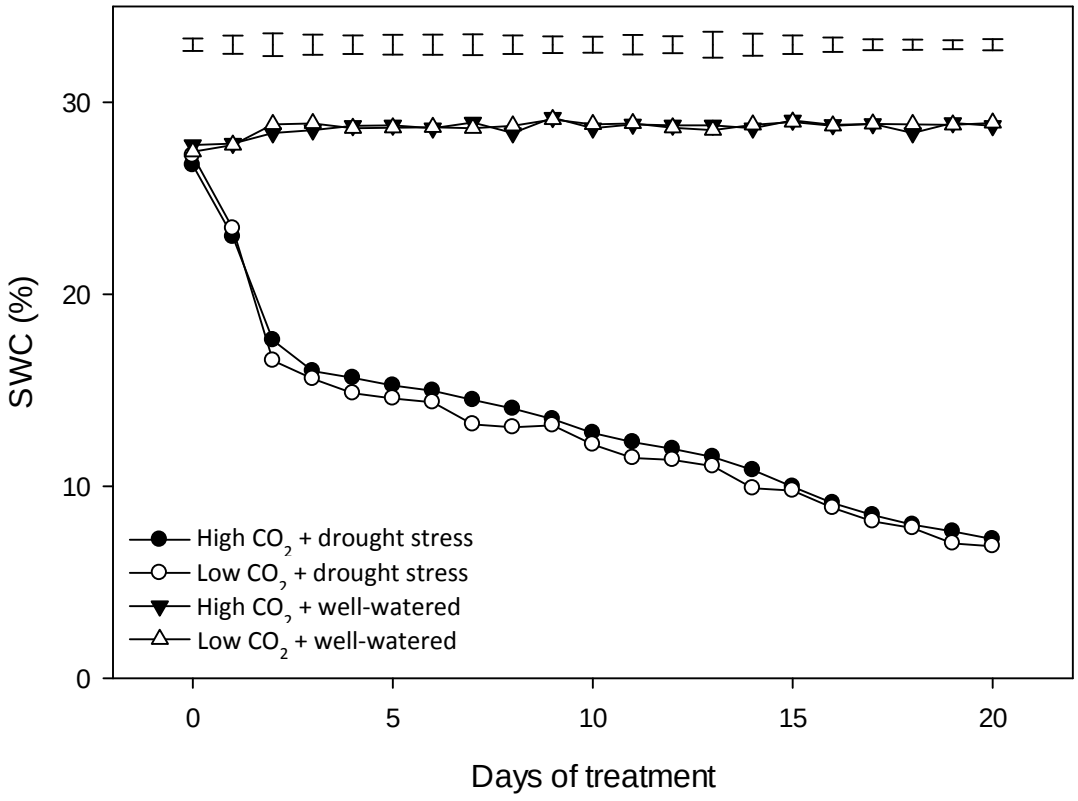


Figure 2. Shoot weight (A), root weight (B), root to shoot ratio (C), root length (D), root surface area (E), and root diameter (F) at 20 d of drought treatment for well-watered or drought-stressed creeping bentgrass at ambient or elevated CO_2 levels. LSD bars and different letters atop bars indicate significant differences exist at $P \leq 0.05$.

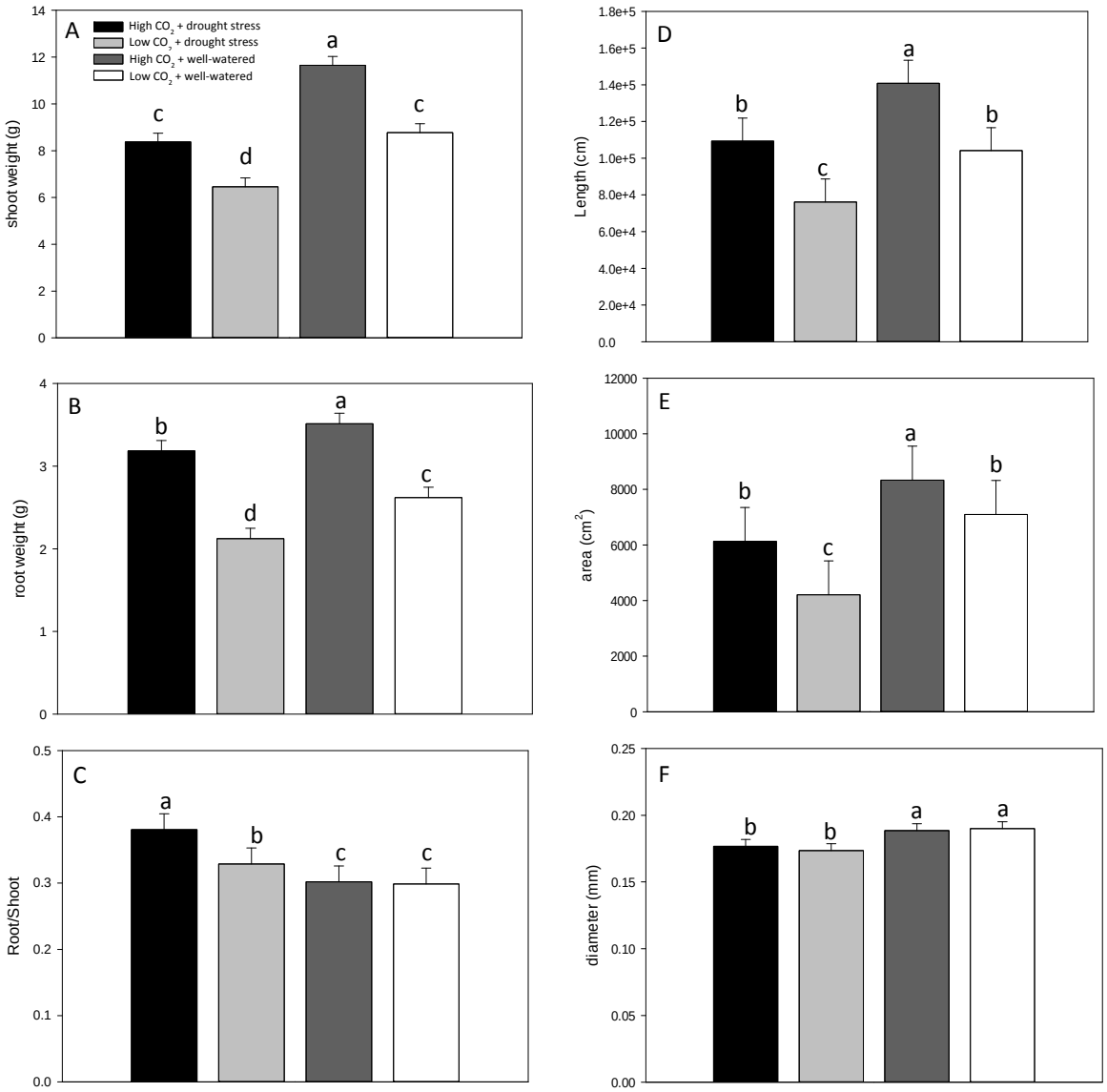


Figure 3. Root electrolyte leakage during 20 d of drought treatment for well-watered or drought-stressed creeping bentgrass at ambient or elevated CO_2 levels. LSD bars and different letters atop bars indicate significant differences exist at $P \leq 0.05$.

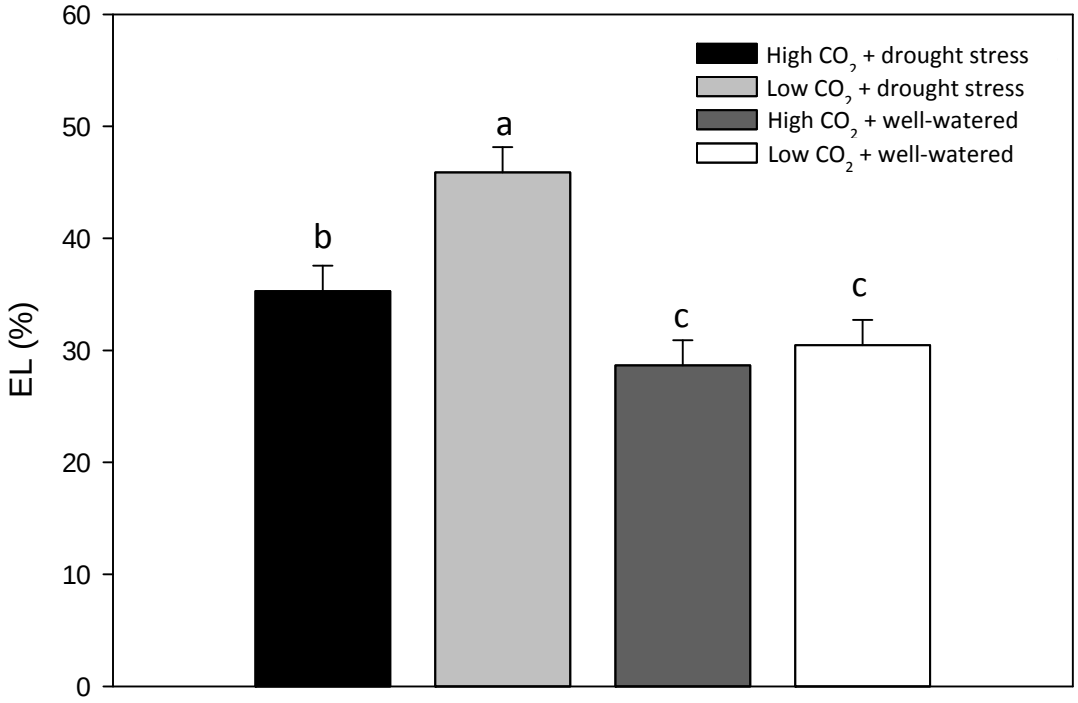


Figure 4. An example representative two-dimensional electrophoresis gel of creeping bentgrass root soluble proteins stained with Coomassie Brilliant Blue. Numbers of highlighted proteins correspond with proteins numbers in Table 1 for proteins exhibiting significant changes in abundance following exposure to ambient or elevated CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d.

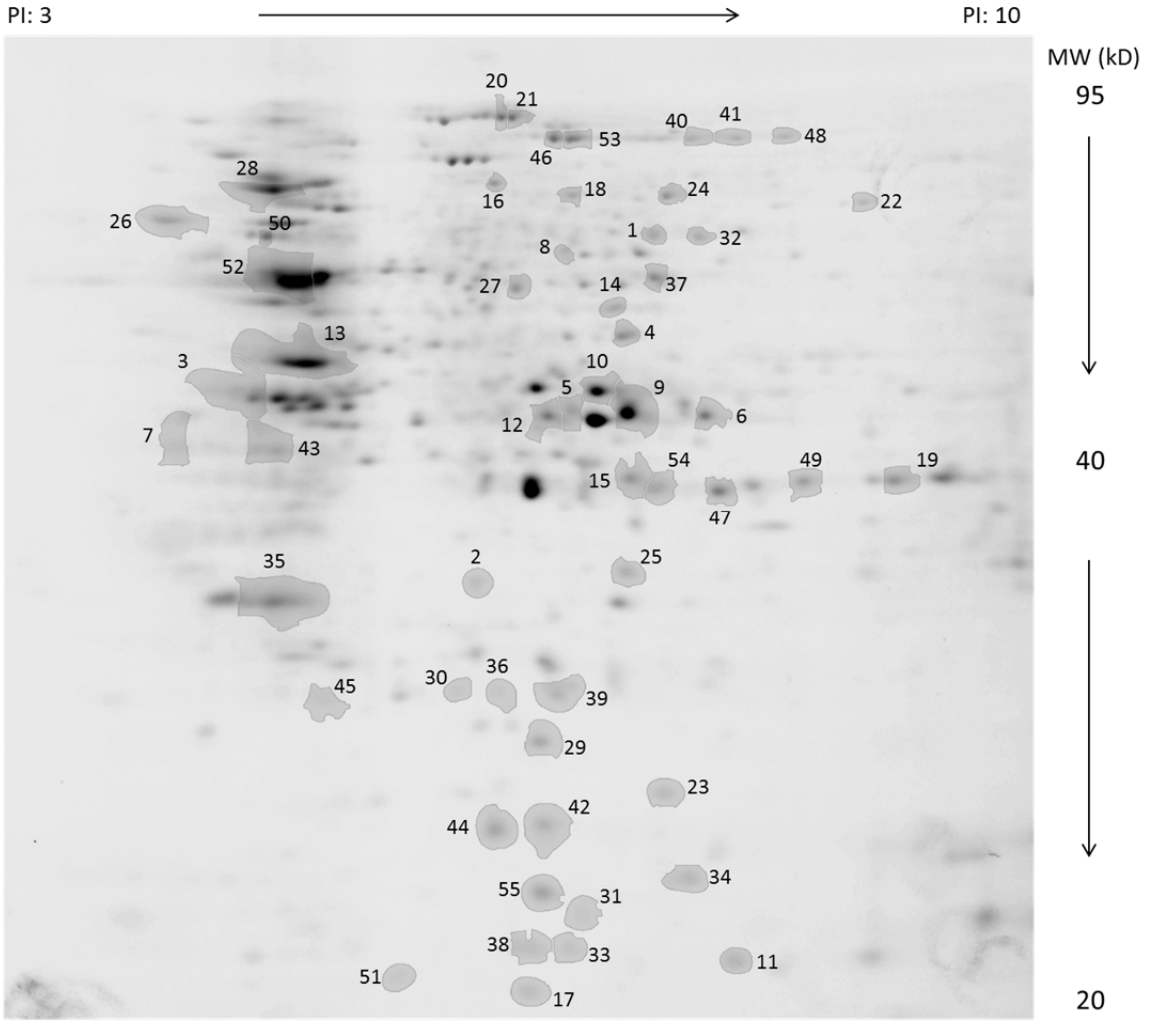
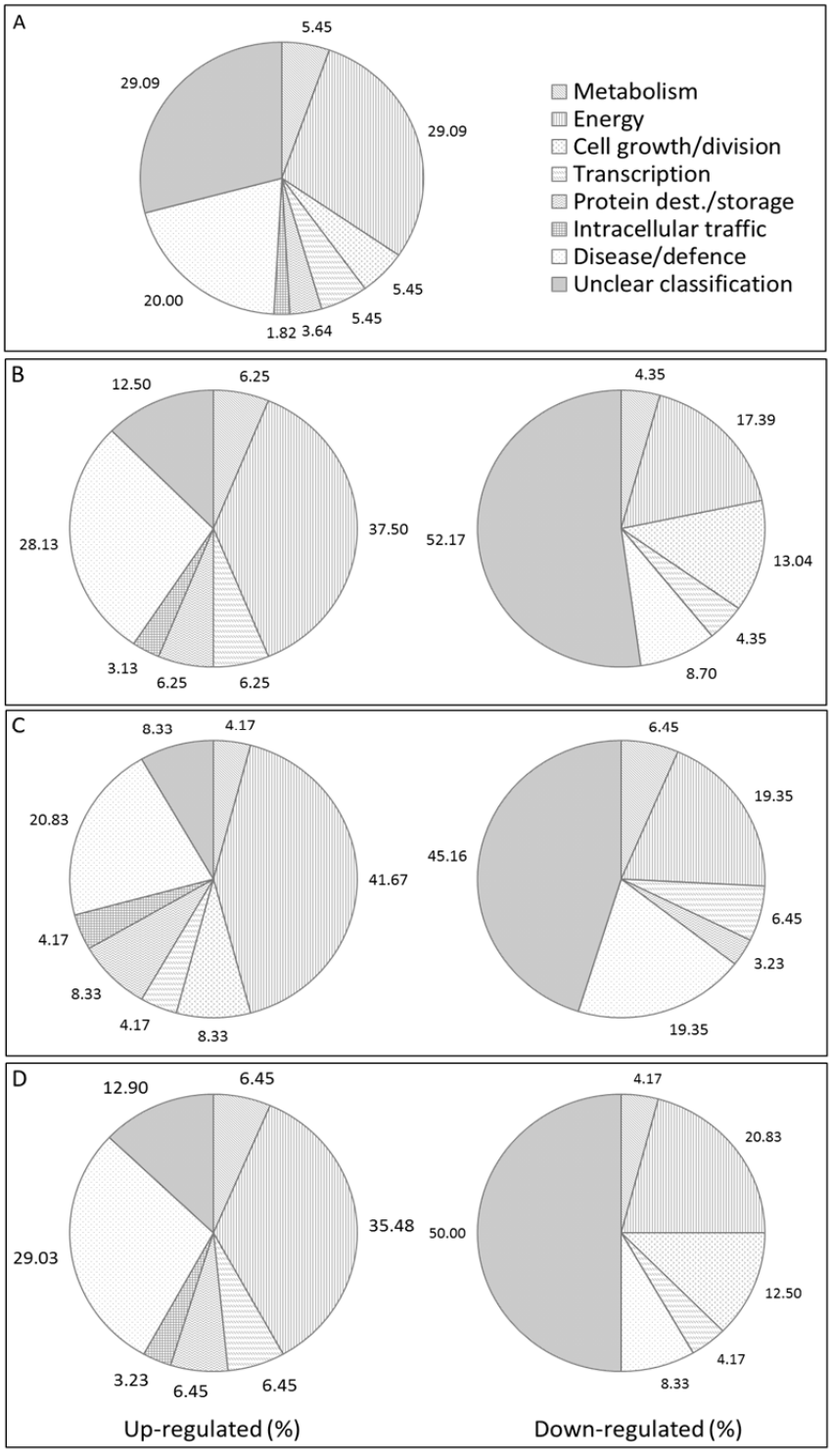


Figure 5. Functional categorization of all identified root proteins (A), protein changes due to drought stress only (B), protein changes due to elevated CO_2 only (C), and protein changes due to elevated CO_2 and drought stress interaction (D).



CHAPTER SIX

ELEVATED CARBON DIOXIDE IMPROVING DROUGHT TOLERANCE ASSOCIATED WITH HORMONAL CHANGES AND ENHANCED TILLER AND STOLON GROWTH IN CREEPING BENTGRASS

Burgess, P., X. Zhang, and B. Huang. 2016. Elevated carbon dioxide improving drought tolerance associated with hormonal changes and enhanced tiller and stolon growth in creeping bentgrass. J. Amer. Soc. Hort. Sci. Expected submission December 2016.

ABSTRACT

Drought stress inhibits shoot growth of cool-season turfgrass species and elevated CO_2 concentration may mitigate the adverse effects of drought through alteration of hormone production. The objective of this study was to determine whether elevated CO_2-enhanced drought tolerance in creeping bentgrass (*Agrostis stolonifera*) was associated with the stimulation of tiller and stolon growth and the alteration of stress-regulating and grow-regulating hormone accumulation. Creeping bentgrass (cv. Penncross) plants were established for 24 d at ambient (400 μL • L^{-1}) or elevated (800 μL • L^{-1}) CO_2 concentration and subsequently exposed to drought stress for 23 d by withholding irrigation. Drought stress caused significant reduction in leaf relative water content and tiller density whereas both parameters, as well as stolon length, were maintained at significantly higher values in CO-treated plants compared to those at ambient CO_2 under drought stress. The positive effects of elevated CO_2 on the maintenance of leaf hydration and the promotion of tiller density and stolon growth in creeping bentgrass exposed to drought stress could be associated with the suppression of drought-induced ABA accumulation and the increase in the endogenous content of isopentenyladenosine (iPA), jasmonic acid (JA), the JA precursor 12-oxo-phytodienoic acid (OPDA), and salicylic acid (SA).

INTRODUCTION

Drought stress is a major abiotic stress which imposes limitations to plant growth, development, and productivity throughout arid and semiarid regions of the world. For perennial grass species, such as those used as turfgrass, extensive tillers and rapid extension of lateral shoots (i.e. stolons) are highly desirable traits for canopy establishment and maintaining high turf quality. However, drought inhibits tiller formation and stolon growth of grass species, which leads to thinning of grass density and slow stand establishment and ultimately low grass quality or productivity (Stier et al., 2013).

Along with the intensified drought stress due to global climate changes, atmospheric CO_2 concentrations have been steadily increasing by 2-3 mL • L^{-1} every year (Solomon et al., 2007). Elevated atmospheric CO_2 independently or interacting with abiotic stress has been shown to influence many aspects of plant development and most studies have reported positive effects for mitigation of abiotic stress damages in various plant species (Ainsworth et al., 2002; Ceulemans and Mousseau, 1994; Huang and Xu, 2015; Kirkham, 2011), including perennial grasses used in turfgrass settings, such as kentucky bluegrass (*Poa pratensis*), tall fescue (*Festuca arundinacea*), and creeping bentgrass (*Agrostis stolonifera*) (Burgess and Huang, 2014b; 2016; Lin and Wang, 2002; Qaderi et al., 2006; Wall et al., 2001; Yu et al., 2012a, 2012b). The positive effects of elevated CO_2 concentrations have been mainly associated with its regulation of leaf water relation, photosynthesis, carbohydrate metabolism, and protein metabolism (Ainsworth et al., 2002; Ceulemans and Mousseau, 1994; Huang and Xu, 2015; Kirkham, 2011; Burgess and Huang, 2014b; Yu et al., 2012a). Several studies have found that elevated

CO_2 promoted tiller formation and growth in grass species under non-stress conditions (Baker et al. 1990, 1992; Nicolas et al., 1993; Ziska et al., 1997); however, the effects of elevated CO_2 on tiller and stolon growth for grass species subjected to drought stress are not well documented.

Plant hormones serve key roles in regulating plant growth and development, such as tiller formation and lateral shoot growth, as well as plant tolerance to drought stress (Fahad et al., 2015; Peleg and Blumwald 2011; Robert-Seilaniantz et al. 2011; Han et al., 2008; Yang et al., 2016). Whether tiller formation and stolon growth negatively affected by drought stress could be mitigated by elevated CO_2 through alteration of hormone metabolism is not well understood. A recent review reported that the transcript levels for genes related to synthesis or transport of several growth-regulating hormones, such as auxins or indole-acetic acid (IAA), cytokinins, and gibberellins either increased or decreased, depending on hormone types, in response to elevated CO_2 treatment in various plant species (Huang and Xu, 2015). However, there has been limited information on the changes in the endogenous content of various hormones due to elevated CO_2 in plants exposed to drought stress. In addition, it is well known that stress-regulating hormones, such as abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) are involved in stress signaling or protection (Hayat et al., 2010; Riemann et al., 2015); however, there has been less focus on the CO_2-induced changes of those hormones related to the mitigation of drought damages in grass species.

Within the context of turfgrass management, several of the most important factors associated with the management of fine turfgrass species is rapid stand establishment, quick recovery from physical damages, and maintenance of turf canopies with high

density and delayed thinning due to abiotic stresses or (Stier et al., 2013). Therefore, the objective of this study was to determine whether elevated CO_2-enhanced drought tolerance in creeping bentgrass (*Agrostis stolonifera*) was associated with the stimulation of tiller and stolon growth and the alteration of stress-regulating and growth-regulating hormone accumulation by quantifying the endogenous content of six major hormones (abscisic acid (ABA), indole-3-acetic acid (IAA), isopentenyladenosine (iPA), jasmonic acid (JA), trans-zeatin riboside (tZR), salicylic acid (SA), as well as the JA precursor 12-oxo-phytodienoic acid (OPDA) in leaves. Within the context of global climate change, the results from this study may aide in the development of new management practices or plant health products able to effectively maintain high-quality turfgrass stands during exposure to elevated CO_2 levels and periods of limited irrigation supply.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Thirty uniform-size tillers from creeping bentgrass (cv. Penncross) plants were harvested from stock plants maintained in a greenhouse at Rutgers University (New Brunswick, NJ) and planted into pots (10 cm diameter, 40 cm depth) filled with fritted clay medium (Profile Products, Deerfield, IL) on 29 December 2014. The plants were grown in a controlled-environment growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) set to 21/18 °C (day/night), 650 μmol • m^{-2} • s^{-1} photosynthetically active radiation, 60% relative humidity, and 14 h photoperiod for 7 d to allow plant acclimation to growth chamber conditions and emergence of root initials prior to exposing plants to CO_2 treatments on 05 January 2015.

Treatments and Experimental Design

Forty pots (4 treatments x 10 replicates) of plants were established for 24 d (05 January to 29 January 2015) at ambient (400 μL • L^{-1}) or elevated (800 μL • L^{-1}) CO_2 concentration. During establishment, the pots were irrigated daily with excess water draining from pot bases, fertilized twice per week with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950), and leaves were not trimmed to allow sufficient tissue growth prior to destructive sampling. Following establishment at either CO_2 concentration, all plants were irrigated to pot capacity on 29 January 2015 (0 d drought stress) and subjected to drought stress for 23 d (30 January to 21 February 2015) by withholding irrigation until volumetric soil water content (SWC) decreased to 9.0% or

irrigated to maintain soil water content at the pot capacity (approximately 25%) as the non-stress control. During 23 d drought stress, plants were continually exposed to either ambient or elevated CO_2 concentration. SWC was monitored daily using a time reflectometer (Trase 1 System; Soil Moisture Equipment Corp., Santa Barbara, CA). Three buriable waveguide probes, each measuring 30 cm in length, were inserted into the root zone and SWC was measured in drought-stressed and well-irrigated treatments (Topp et al., 1980).

The ambient and elevated CO_2 concentrations within growth chambers were maintained through an automatic CO_2 controlling system connected to a source-tank containing 100% research-grade CO_2 following the method described in Yu et al. (2012a). CO_2 concentrations inside the chambers were continuously monitored and recorded using an infrared gas analyzer (LI-820; LI-COR, Lincoln, NE) connected to a computer data logger. The CO_2 concentration was maintained using an automatic controlling system consisting of a programmable logic controller unit, solenoid valves, and a laptop computer with software capable of monitoring and maintaining CO_2 concentration within 10 $\mu L \cdot L^{-1}$ of the ambient or elevated target levels.

The experiment was arranged in a split-plot design with CO_2 treatment (ambient or elevated) as the main plot and irrigation treatment (well-irrigated or drought stress) as the sub-plots. Each CO_2 treatment was performed in four different growth chambers and each watering treatment had 10 replicate pots, which were randomly placed inside each growth chamber. All plants were relocated between the four growth chambers every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

Leaf Water Status and Morphological Analysis

Leaf relative water content (RWC) was measured to indicate leaf hydration status following 23 d of drought treatment. Approximately 0.2 g leaf tissue of second and third fully expanded leaves was collected and fresh weight (FW) measured using a mass balance immediately after harvesting. Leaves were then wrapped in tissue paper, submerged in deionized water for 12 h at 4 °C, removed from water, blotted dry, and again weighed to measure turgid weight (TW). Leaves were then dried in an oven at 80 °C for 3 d, weighed to determine dry weight (DW) and RWC calculated using the formula % = [(FW – DW) / (TW – DW)] x100 (Barrs and Weatherley, 1962).

Plants were destructively sampled at 23 d of drought stress. The length of stolons of plants within each pot was measured using a rule from the base of the plant to the tip of the stolon. The total number of tillers was also counted for all plants within each pot.

Quantification of Endogenous Hormones JA, OPDA, tZR, ABA, SA, and IAA using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS)

Hormone content of JA, JA-Ile, OPDA, tZR, tZ, ABA, SA, IAA, and IAA-Asp was analyzed using LC-MS/MS. Leaf tissues (0.5 g) were lyophilized and ground to powder using a mortar and pestle in liquid N. 900 µL of ice-cold methanol/acetonitrile (MeOH/ACN, 1:1 [v/v]) and 10 µL of a 2.5 µM deuterium-labeled standard ([d5]-trans-zeatin riboside) was added to each sample and homogenized with the TissueLyser II (QIAGEN, Valencia, CA) for 5 min at a frequency of 20 Hz/sec. Then the samples were centrifuged at 16,000 g for 10 min at 4 °C. The supernatant was transferred to a new 2

mL tube and the remaining pellet was re-extracted again. The resulting supernatant from second extraction was added to the first supernatant and evaporated under a vacuum. The evaporated pellet was dissolved in 200 μL of 30% [v/v] methanol, centrifuged to remove any non-dissolved matter, and the supernatant was transferred to LC-MS/MS vials for analysis. The sampled volume injected for each LC-MS/MS analysis was 50 μL.

The LC-MS/MS system used was composed of a Shimadzu LC system (Shimadzu, Kyoto, Japan) with two Shimadzu solvent delivery pumps (LC20AD), an auto-sampler (SIL20AC) with a 100 uL sample loop, and a Valco two-position diverter valve (VICI, Houston, TX). The LC system was interfaced with an AB Sciex 4000 QTRAP mass spectrometer equipped with a TurboIonSpray (TIS) electrospray ion source (SCIEX, Framingham, MA). Source parameters were set as follows: Curtain gas, 20 arbitrary units (a.u.); Source gas 1, 50 a.u.; Source gas 2, 50 a.u.; Collision activated dissociation, high; Interface heater, on; Temperature, 550 °C; Ionspray voltage, +5500. Both quadruples (Q1 and Q3) were set to unit resolution. Analyst software (version 1.5) was used for sample acquisition and data analysis. The 4000 QTRAP mass spectrometer was tuned and calibrated according to the manufacturer's recommendations. The hormone contents were detected using MRM transitions which were previously optimized using a standard and a deuterium-labeled standard. All data were presented as the mean (the average content in g DW) ± SE (the standard error) of ten biological replicates.

Extraction, Purification, and Analysis of Isopentenyl Adenine (iPA) via ELISA

The iPA was extracted from leaves and purified using the procedure as described by Zhang et al. (2013). Briefly, leaf tissues were ground with a mortar and a pestle in liquid N and 50 mg of sample was mixed with 1.8 mL sodium-phosphate buffer (50 mM, pH 7.0) containing 0.02% sodium diethyldithiocarbamate as an antioxidant. The iPA was then extracted by continuous shaking for 1 h at 4 ºC. The pH for each sample was adjusted to approximately 2.6 and each sample was then slurried with Amberlite XAD-7 (150 mg) (Sigma. St. Louis, MO) for 30 min. Following removal of the buffer, the XAD-7 was washed twice with 1 mL 1% acetic acid and again slurried twice more with 1 mL dichloromethane for 30 min each. The combined dichloromethane fractions were reduced to dryness with N gas. The samples were then dissolved in 210 µL methanol and diluted to 700 µL in deionized water with 0.1% formic acid added. Finally, the sample was filtered with a syringe filter (0.2 µm).

The iPA was analyzed using an indirect enzyme linked immunosorbent assay (ELISA) as described by Zhang and Ervin (2004). Briefly, the wells of a 96-unit plate were coated with 100 µL per well of iPA conjugated to bovine serum albumin (BSA) (1:10000 dilution), incubated overnight at 4 ºC, emptied, and washed three times with phosphate buffered saline (PBS; 50 mM, pH 7.2)-Tween-20 (PBS containing 0.05% Tween 20). The reaction was halted with 200 µL of 1% BSA in PBS (37 ºC, 30 min) to prevent nonspecific protein absorption. After the plate was washed twice with PBS-Tween, 50 µL of the iPA extract or iPA standard and 50 µL of the antibody iPA (1:200 dilution) were added to individual wells and the plate was incubated at 37 ºC for 60 min, emptied, and washed three times with PBS-Tween. A series of iPA concentrations (0,

3.13, 6.25, 12.5, 25, and 50 ng•mL^{-1}) were made for a standard curve. 100 uL of alkaline phosphatase-labeled goat anti-mouse IgG (1:1000 dilution; Sigma, St Louis, MO) was added to each well and the plate were incubated at 37 ºC for 60 min. Following three washes with PBS- Tween, 100 μL of substrate solution (3 mg•mL-1 of p-nitrophenyl phosphate in 10% diethanolamine with 0.5 mM MgCl2, pH 9.8) was added to each well and the plate was incubated at 37 ºC for 30 min. The color reaction in each well was determined by measuring absorbance at 405 nm with a microplate reader. iPA concentration was then calculated based on the standard curve after logarithmic conversion of the data.

Statistical Analysis

The effects of CO_2 concentration, watering treatments, and their interactions on morphological parameters and hormone content were determined by analysis of variance according to the general linear model procedure of SAS (version 9.2, SAS Institute, Cary, NC). Differences between treatment means were separated by Fisher's protected least significance difference (LSD) test ($\alpha = 0.05$).

RESULTS

Soil and Leaf Water Status during Drought Stress under Elevated or Ambient CO_2 concentrations

Volumetric SWC was maintained at 24% (pot capacity) under well-watered conditions with ambient or elevated CO_2 treatment (Fig. 1A). SWC decreased rapidly after withholding irrigation, to 9.0% by the end of drought treatment (23 d) (Fig. 1A). No significant differences in SWC between elevated and ambient CO_2 treatments under drought stress, so that plants at either CO_2 treatment were exposed to the same level of soil water deficit.

Leaf RWC was maintained at same level (~92%) for plants grown at elevated or ambient CO_2 treatments under well-irrigated conditions (Fig. 1B). Under drought stress, RWC decreased by 41.0% and 11.8% under ambient and elevated CO_2 concentrations, respectively. Plants at elevated CO_2 concentration maintained significantly higher (82.2%) RWC compared to those plants grown at ambient CO2 concentration (66.2%) at 23 d of drought stress.

Tiller and Stolon Growth as Affected by Elevated CO_2 and Drought Stress

Tiller density was significantly higher (by 16.8%) for plants grown at elevated CO_2 concentration compared to plants grown at ambient CO_2 concentration under well-irrigated conditions (Fig. 2A). Drought stress caused a significant decrease in tiller density regardless of CO_2 concentration, with 49.7% and 33.3% reduction, under ambient

and elevated CO_2 concentrations, respectively. Under drought stress conditions, tiller density was also significantly higher (by 37.2%) for plants grown at elevated CO_2 concentration compared to plants grown at ambient CO_2 concentration.

There were no significant differences in stolon length between plants grown at elevated and ambient CO_2 treatments under well-irrigated conditions (Fig. 2B). Drought had no significant effects on stolon length at ambient CO_2 concentration. Stolon length was significantly higher (by 15.8%) for plants grown at elevated CO_2 concentration compared to plants grown at ambient CO_2 concentration under drought stress.

Leaf Hormone Content as Affected by Elevated CO_2 and Drought Stress

There was a significant increase in leaf ABA content for plants grown at either CO_2 concentration under drought stress, 2.74-fold and 88.9% increases, in plants at ambient and elevated CO_2 concentrations, respectively. There were no significant differences in leaf ABA content between plants grown at ambient CO_2 concentration and plants grown at elevated CO_2 concentration under well-irrigated conditions (Fig. 3A). Under drought stress conditions, leaf ABA content was significantly higher (by 2.03-fold) for plants grown at ambient CO_2 concentration compared to plants grown at elevated CO_2 concentration. Leaf SA content decreased significantly (by 66.0%) in plants grown at ambient CO_2 concentration while it increased by 5.42-fold at elevated CO_2 under drought stress (Fig. 3B), Leaf SA content was significantly higher (by 1.69-fold) for plants grown at elevated CO_2 concentration compared to plants grown at ambient CO_2 concentration under drought stress..

There were no significant differences in leaf JA content between elevated and ambient CO_2 treatments under well-irrigated conditions (Fig. 4A). Under drought stress conditions, leaf JA content was significantly higher (by 49.6%) for plants grown at elevated CO_2 concentration compared to plants grown at ambient CO_2 concentration. Leaf JA content decreased significantly (by 57.8%) due to drought stress at ambient CO_2 concentration, but did not change significantly with drought at elevated CO_2 concentration. There were no significant differences in leaf OPDA content between plants grown at ambient CO_2 concentration and plants grown at elevated CO_2 concentration under well-irrigated conditions (Fig. 4B). However, under drought stress conditions, leaf OPDA content was significantly lower (by 68.7%) for plants grown at ambient CO_2 concentration compared to plants grown at elevated CO_2 concentration. There was a significant decrease in leaf OPDA content due to drought stress for plants grown at either CO_2 concentration, with 84.3 and 48.0% reduction, respectively, under ambient and elevated CO_2 concentrations, respectively.

The content of leaf tZR decreased significantly due to drought stress at ambient or elevated CO_2 concentration, with 64.6 and 53.5% reduction under ambient and elevated CO_2 concentrations, respectively (Fig. 5A). There were no significant differences in leaf tZR content between plants grown at elevated or ambient CO_2 treatment under drought stress.

Leaf iPA content did not change due to drought stress at ambient CO_2 concentration, but increased (by 48.9%) at elevated CO_2 concentration (Fig. 5B). Leaf iPA content was significantly higher (by 68.0%) for plants grown at elevated CO_2

concentration compared to plants grown at ambient CO_2 concentration under drought stress.

There were no significant differences in leaf IAA content between plants grown at ambient CO_2 concentration and plants grown at elevated CO_2 concentration under well-irrigated conditions (Fig. 6). Under drought stress conditions, leaf IAA content was significantly higher (by 53.8%) for plants grown at ambient CO_2 concentration compared to plants grown at elevated CO_2 concentration. There was a significant increase in leaf IAA content due to drought stress at either CO_2 concentration, with 2.37-fold and 98.9% increase, under ambient and elevated CO_2 concentrations, respectively.

DISCUSSION

Maintaining cellular hydration is a critical factor for enhanced tolerance to soil water deficit (Chaves et al., 2009). Drought caused significant decline in leaf RWC of creeping bentgrass plants regardless of CO_2 concentration in the current study. However, the drought-induced water deficit was less severe for plants grown at elevated CO_2 concentration, suggesting that elevated CO_2 facilitated the maintenance of leaf hydration under drought stress. Reduction in water loss by elevated CO_2 concentration has been attributed to the induction of stomatal closure and reduction in transpiration rate (Ainsworth and Rogers, 2007; Huang and Xu, 2015; Leakey et al., 2009; Burgess and Huang, 2014a; 2016; Yu et al., 2012a). The maintenance of higher leaf hydration level also indicated plants exposed to elevated CO_2 could suffer less cellular metabolic injury.

Abscisic acid is known as a stress-induced hormone, with increasing accumulation under drought stress, such as found in this study and numerous previous reports (Wilkinson and Davies, 2002; Hirayama and Shinozaki, 2007; Zhang et al., 2006). In this study, there was lesser extent of increases in the endogenous content of ABA in drought-stressed plants under elevated CO_2 compared to that under ambient CO_2. Wei et al. (2013) reported that there was a significant down-regulation in transcripts for two ABA-associated genes (ABA2 xanthoxin dehydrogenase and ABA-responsive protein-related) in aspen (*Populus tremuloides*) trees under long-term elevated CO_2 enrichment. The question regarding how ABA may interact with CO_2 for plant tolerance to drought at molecular levels deserves further investigation. In addition, JA and SA are known to play positive roles in plant stress tolerance, and the increases in the endogenous content of those hormones may facilitate cellular stress defense (Horvath et al., 2007;

Poltronieri et al., 2014). In this study, the endogenous content of JA and SA was significantly greater in plants grown at elevated CO_2 compared to that at ambient CO_2 when both were exposed to drought stress. These results demonstrated elevated CO_2-mitigation of drought-induced leaf dehydration was associated with stress-related hormonal responses. SA has been suggested as influencing the earliest phases of whole-plant stress response to activate stress defense, including drought (Horváth et al. 2007; Ashraf et al., 2010; Borsani et al., 2001; Janda et al., 2007). In comparison to studies with transgenic plants, Munné-Bosch and Peñuelas (2002) quantified the content of endogenous SA in narrow-leaved mock privet (*Phillyrea angustifolia*) and the results suggested a strong correlation between SA and leaf RWC. Thus far, the effect of elevated CO_2 concentrations on endogenous SA levels under non-stress conditions or with the presence of abiotic stress is not well documented and reports in the literature are contradictory (Kurepin et al., 2013). For example, the review by Kurepin et al. (2013) provides several examples of elevated CO_2 increasing endogenous SA levels in tobacco (*Nicotiana tabacum*), decreasing the precursors to SA in canola (*Brassica napus*), or having no effect on endogenous SA levels in tomato (*Solanum lycopersicum*) (Jwa and Walling, 2001; Matros et al., 2006; Prins et al., 2011). While it remains plausible that the significant increases in endogenous SA were due to elevated CO_2 in leaves of creeping bentgrass under drought stress, further research is needed in order to determine the underlying mechanisms of interactive effects of CO_2 and SA regulating drought tolerance. Jasmonic acid and its associated precursors (OPDA) have been implicated in the regulation of plant stress-responses such as those involved with reactive oxygen species (ROS) and nitric oxide (NO) signaling, and antioxidant metabolism (Poltronieri et

al., 2014). In a recent review by Riemann et al. (2015), there is strong evidence that JA is likely involved in the plant response to drought, but whether or not higher endogenous JA concentrations result in enhanced drought tolerance remains controversial and is likely dependent upon plant species and tissues under analysis, as well as the duration of drought stress. Alternatively, exogenous application of JA have been shown to result in enhanced drought tolerance by means of increased antioxidant capacity across a variety of plant species including corn (*Zea mays*), barley (*Hordeum spontaneum*), and muskmelon (*Cucumis melo*) (Bandurska et al., 2003; Li et al., 1998; Nafie et al., 2011). In comparison to what is currently known about JA responses to drought stress under ambient CO_2, there is far less known regarding JA responses under elevated CO_2 and those studies which have been conducted focused primarily on the CO_2-induced changes to JA-regulated insect herbivory without interacting drought stress (Casteel, 2010; Casteel et al., 2012; Zhang et al., 2015). However, a study which investigated the effect of elevated CO_2 on JA-induced volatiles reported that methyl jasmonate (MeJA) volatility was significantly increased by elevated CO_2 treatment in lima bean (*Phaseolus lunatus*) and may represent an efficient defense mechanism by the inducing the synthesis of proteinase-inhibitors (Ballhorn et al., 2011). Nevertheless, the involvement of JA and SA in CO_2-regulation of drought tolerance deserves further investigation.

As described in the introduction, critical traits associated with high-quality turfgrass are high canopy density and rapid stand establishment, which are determined by tiller density and lateral spread by stolon growth (Stier et al., 2013). In this study, drought stress caused a significant decline in tiller density while elevated CO_2 mitigated drought inhibition of tiller formation, as shown by the increased tiller density. In addition,

elevated CO_2 enhanced stolon growth under drought stress, suggesting that elevated CO_2 stimulated stolon elongation in creeping bentgrass plants under drought stress. In a review of cool and warm-season plant responses to elevated CO_2 conditions, Huang and Xu (2015) summarized that the majority of cool-season plant species exposed to prolonged periods of elevated CO_2 exhibit increases in shoot biomass production; however, there has been limited information on the effect of elevated CO_2 concentration on tillering or stoloniferous spread of turfgrass species such as creeping bentgrass under drought stress conditions. A number of studies have investigated the effect of elevated CO_2 on lateral spread of grass species under well-irrigated conditions and results are inconsistent. For example, plants of colonial bentgrass (*Agrostis capillaris*) and alpine bluegrass (*Poa alpina*) had significantly higher tiller number following extended growth periods (79-105 d) at elevated CO_2 concentration, whereas plants of sheeps fescue (*Festuca vivipara*) did not show increases in tiller number (Baxter et al., 1994). Elevated CO_2 concentration increased tiller production rates in rice (*Oryza sativa*) under non-stress or heat stress conditions (Baker et al. 1990, 1992; Ziska et al., 1997). There is far less known regarding the stoloniferous responses of various plant species, especially grass species, resulting from extended exposure to elevated CO_2 concentrations. While a study by Ryle and Powell (1992) reported that defoliated stolons of white clover (*Trifolium repens*) were longer and regenerated thicker and more-numerous leaves following elevated CO_2 treatment, there has been minimal subsequent research investigating the underlying mechanisms of CO_2-induced stolon growth. To our knowledge, our study is the first to report enhanced stolon growth by elevated CO_2 in perennial grass species exposed to drought stress.

Cytokinins are key hormones regulating cell division and formation of tillers in grass species, and also serve roles in plant tolerance to drought stress (Taiz and Zeiger 2010). The most abundant form of cytokinins in grass species, such as creeping bentgrass, is iPA (Xu et al., 2016). In this study, elevated CO_2 resulted in significant increases in iPA content in plants subjected to drought stress, which could contribute to the increased tiller density. Considering the responses of CK to elevated CO_2 concentrations, Wei et al. (2013) reported that aspen trees exposed to elevated CO_2 concentrations had significant up- or down-regulation of transcripts for specific genes related to the synthesis or transport of CK, including *cytokinin response factor*, *cytokinin oxidase 5 and 7*, and *cytokinin transporter 2 and 3*. More specifically, there was a remarkable increase in endogenous CK (iPA and ZR) in leaves of a tropical epiphytic CAM orchid (*Arachnis* × *Ascocentrum* × *Vanda*) following CO_2 enrichment under non-stress conditions (Li et al., 2002). Li et al. (2007) reported that elevated CO_2 treatments resulted in significant increases in endogenous CK (iPA, DHZR, ZR) in *Ginkgo biloba* leaves compared to those at ambient CO_2 concentrations. Despite the various reports on the enhanced CK production with elevated CO_2, the mechanisms underlying the interactive effects of CK- and CO_2-regulating tiller formation in grass species are not well understood, and our study provides the foundation for further evaluation in this aspect.

The naturally-occurring indole-3-acetic acid (IAA) is synthesized within rapidly-dividing tissues of root and shoot meristems, as well as in young leaves of plants (Ljung et al. 2001, 2005). However, the relationship between IAA and stress-induced plant responses varies, depending on plant species or tissue age, and the plant responses during

stress are likely dependent upon both the IAA concentration as well as cellular responsiveness or sensitivity (Schippers et al. 2007). In the current study, there was a significant increase in leaf IAA due to drought stress for creeping bentgrass plants regardless of CO2 concentration, while plants at elevated CO2 had lower IAA content than that at ambient CO2. There is little current knowledge pertaining to how endogenous IAA responds to elevated CO2 under drought stress. In a study investigating CO2 effects on IAA content in *Gingko biloba* plants exposed to ozone stress, leaf IAA content was significantly increased due to elevated CO2 but this increase was far less for plants under the combined CO2 and ozone treatment (Li et al., 2011). In a similar study investigating the response of IAA in Chinese pine (*Pinus tabulaeformis*) following elevated CO2 treatment with or without ozone enrichment, Li et al. (2007) reported that trees pre-exposed to elevated CO2 and subsequently exposed to ozone stress had lower IAA content compared to those at ambient CO2 conditions. How the lower level of IAA or lesser degrees of increased in IAA content in response to drought stress under elevated CO2 in plants could be related to the morphological changes and whole-plant tolerance to drought stress in grass species and requires further examination.

SUMMARY

Elevated CO2-enhanced leaf hydration, tiller formation, and stolon growth in creeping bentgrass exposed to drought stress. The differential changes in the endogenous content of stress-regulating hormones (ABA, SA, and JA) and growth-regulating hormones (tZR, iPA, and IAA) in response to elevated CO2 under drought stress could contribute, at least in part, to the positive effects of elevated CO2 on maintaining leaf hydration, tiller, and stolon growth during drought stress. The interactive mechanisms of elevated CO2 and hormonal regulation of drought tolerance and growth traits deserves further investigation.

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FIGURES

Figure 1. Soil volumetric water content (A) and leaf relative water content (B) of creeping bentgrass plants exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration and well-irrigated or drought stress conditions. Vertical lines represent least significant difference values (A) or ±SE (B) and different letters (a, CO_2 effects; A, drought effects) atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

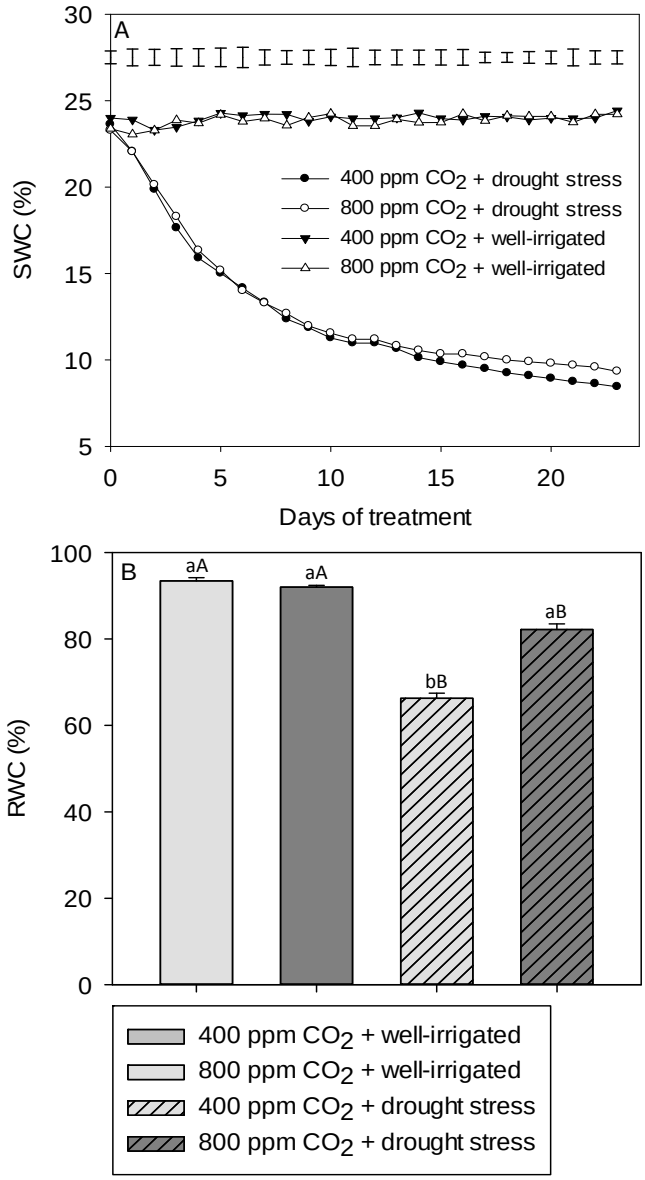


Figure 2. Tiller count (A) and average stolon length (B) of creeping bentgrass plants exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration and well-irrigated or drought stress conditions. Vertical lines represent ±SE of ten replicates and different letters (a, CO_2 effects; A, drought effects) atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

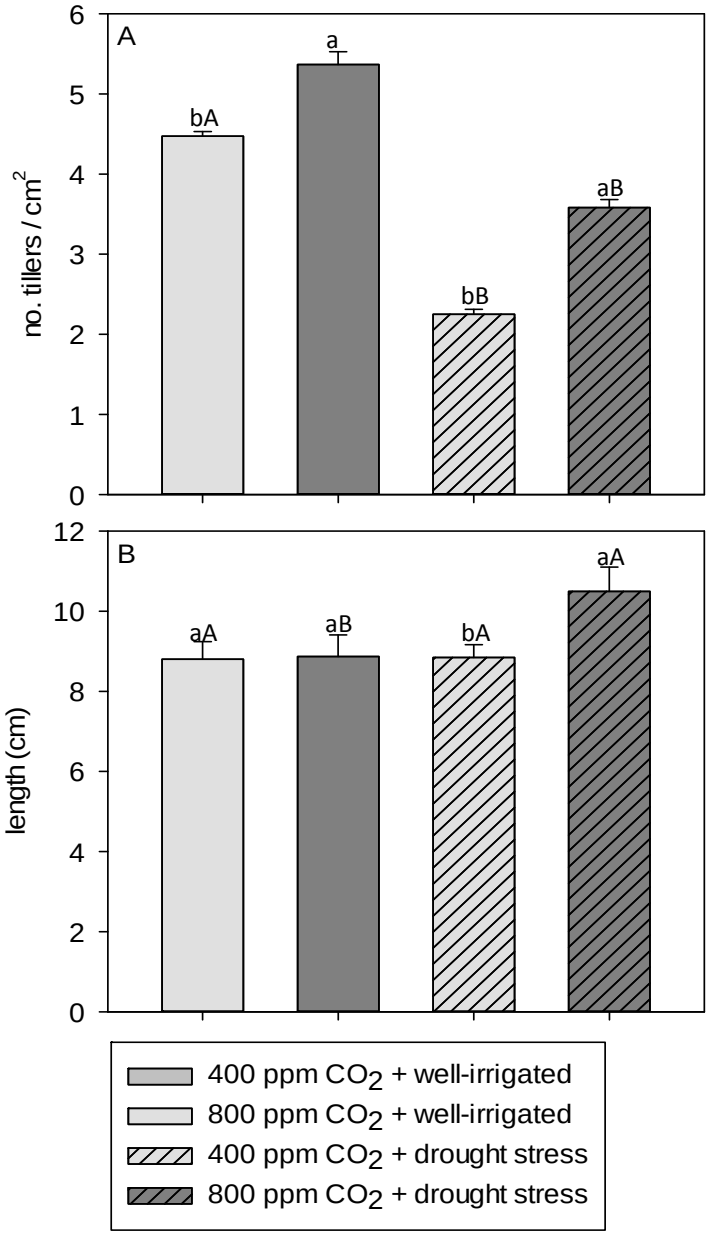


Figure 3. Abscisic acid (A) and salicylic acid (B) content of creeping bentgrass leaves exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration and well-irrigated or drought stress conditions. Vertical lines represent ±SE of ten replicates and different letters (a, CO_2 effects; A, drought effects) atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

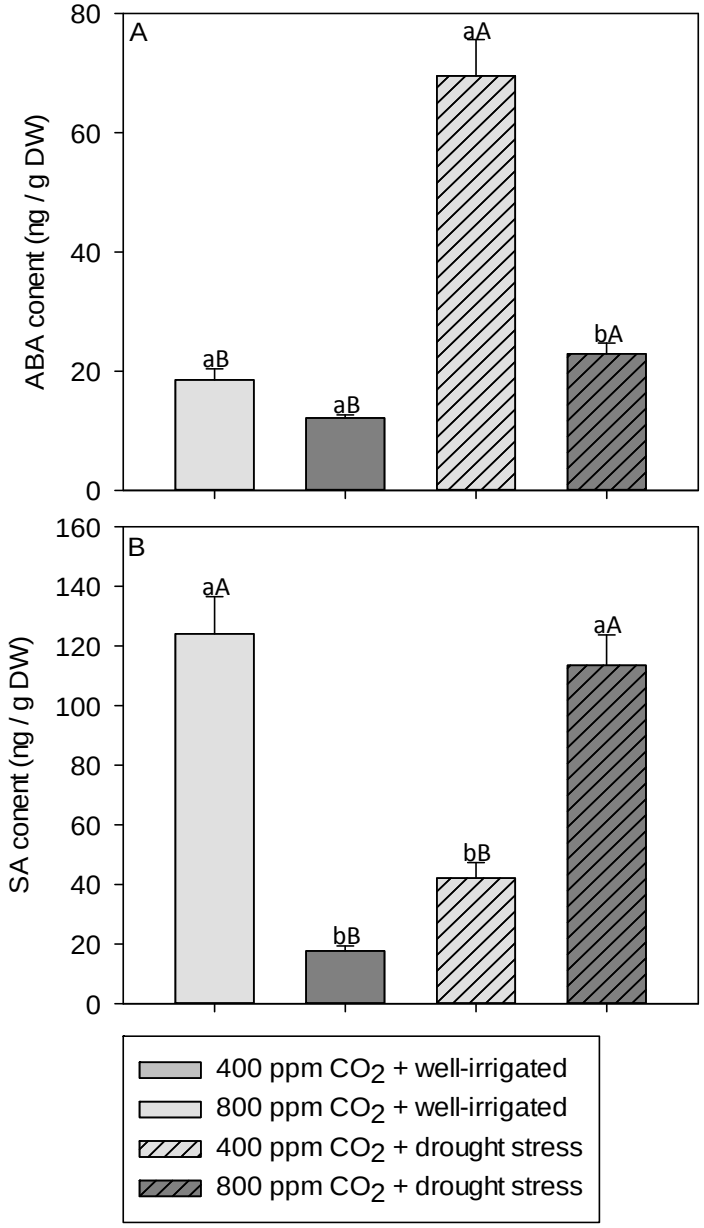


Figure 4. Jasmonic acid (A) and 12-oxo-phytodienoic acid (B) content of creeping bentgrass leaves exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration and well-irrigated or drought stress conditions. Vertical lines represent ±SE of ten replicates and different letters (a, CO_2 effects; A, drought effects) atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

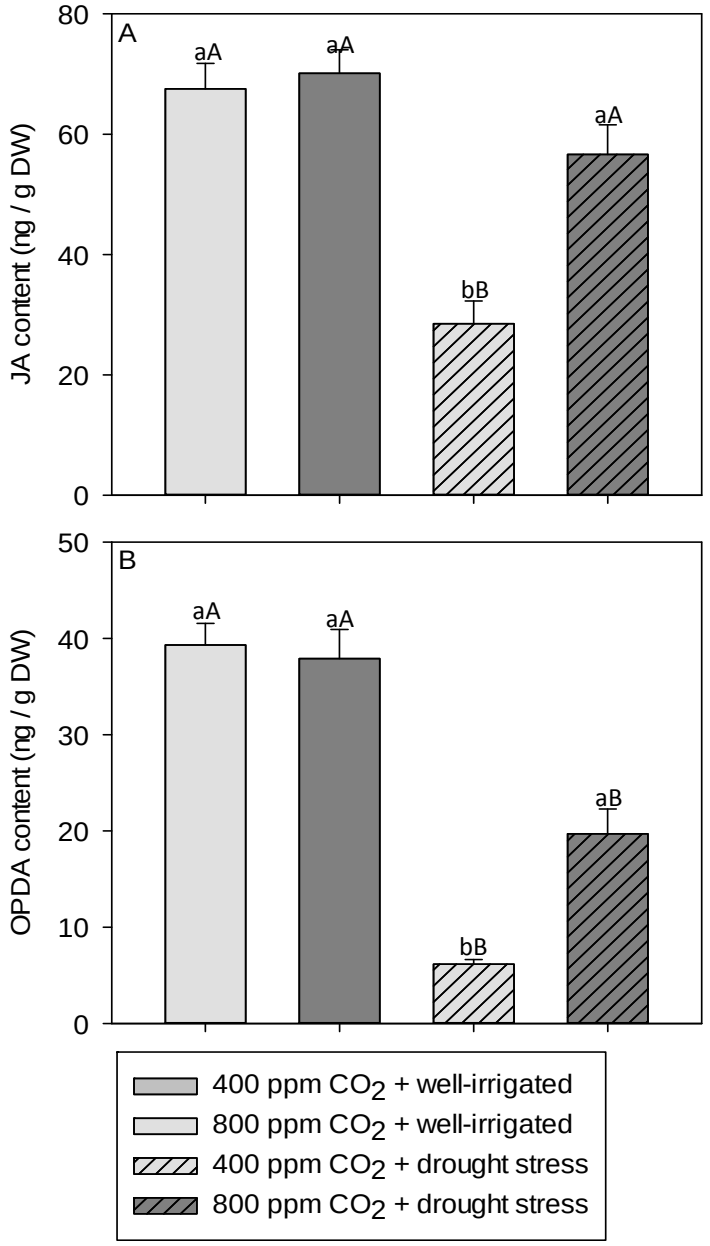


Figure 5. Trans-zeatin riboside (A) and isopentenyladenosine (B) content of creeping bentgrass leaves exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration and well-irrigated or drought stress conditions. Vertical lines represent ±SE of ten replicates and different letters (a, CO_2 effects; A, drought effects) atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

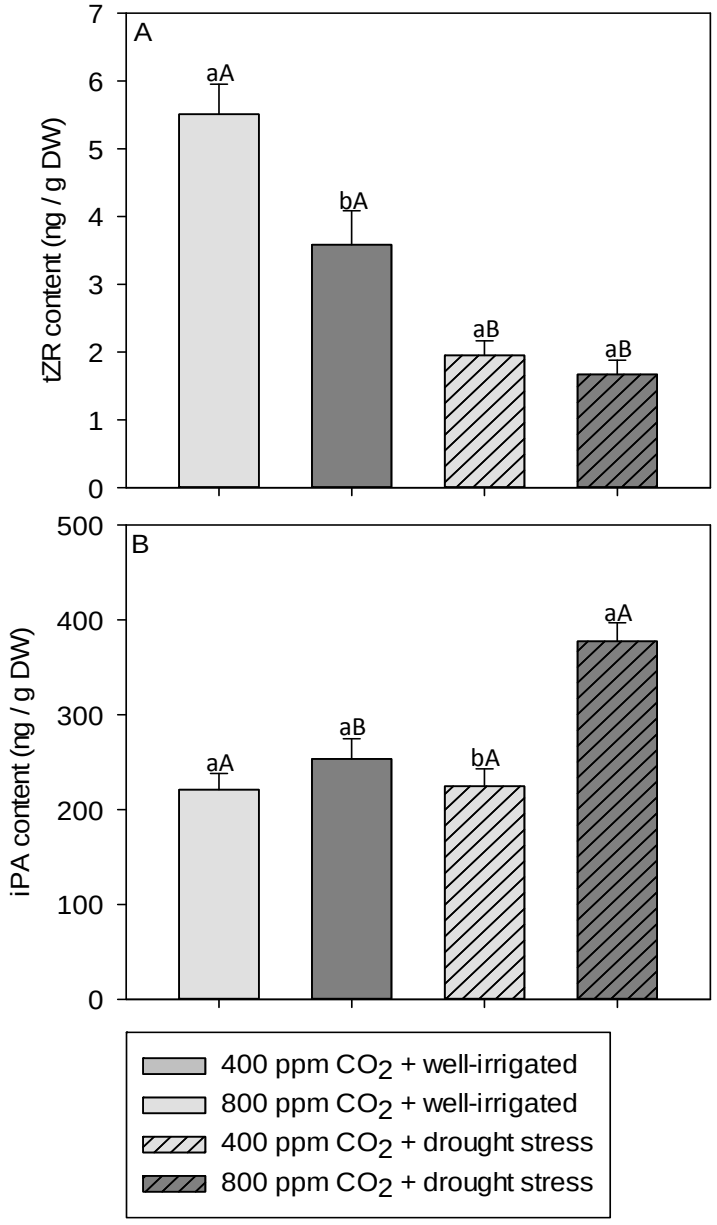
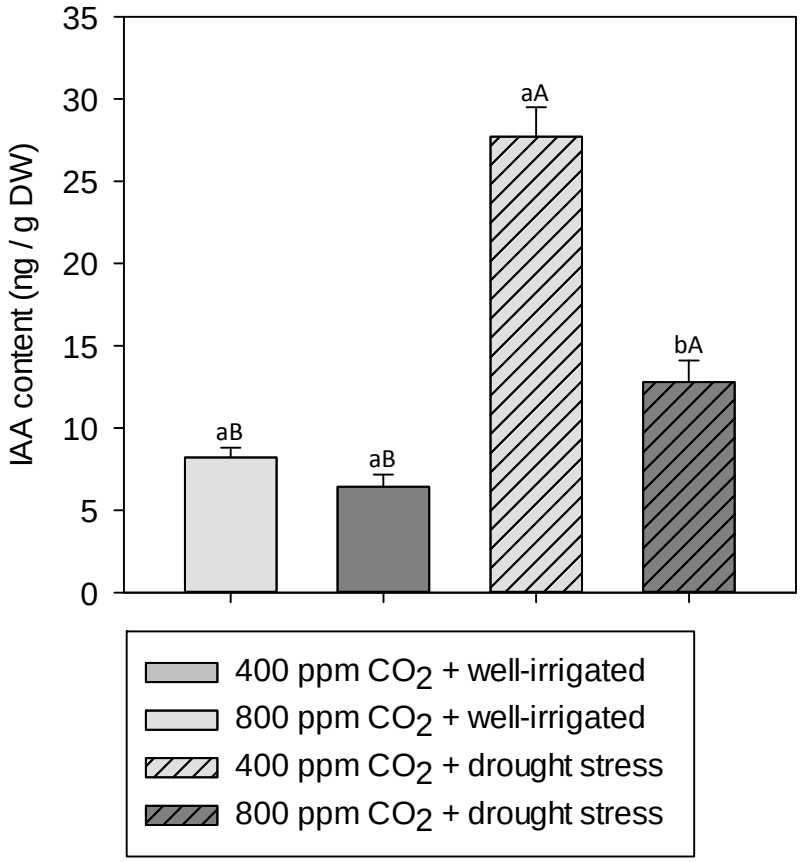


Figure 6. Indole-3-acetic acid content of creeping bentgrass leaves exposed to ambient (400 mL • L^{-1}) or elevated (800 mL • L^{-1}) CO_2 concentration and well-irrigated or drought stress conditions. Vertical lines represent ±SE of ten replicates and different letters (a, CO_2 effects; A, drought effects) atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).



CONCLUSIONS

The goal of this dissertation research was to explore which physiological and biochemical factors are associated with drought tolerance of creeping bentgrass and how elevated carbon dioxide (CO_2) may mitigate drought damages in this widely-utilized turfgrass species. In order to accomplish this, multiple studies were performed which 1) investigated whether sequential exogenous applications of plant growth regulators (PGRs) and osmoregulants promote drought tolerance of creeping bentgrass under field conditions, 2) elucidated on the link between fatty acid metabolism in creeping bentgrass leaves and roots and the observed level of drought tolerance, and 3) explored the interactive effects of elevated CO_2 and drought stress on growth, morphology, physiology, and biochemical properties such as protein, carbohydrate, and hormone metabolism of creeping bentgrass turfgrass.

The initial study showed that plant growth regulators (i.e. TE) and osmoregulants (i.e. GB) have additive effects on promoting drought tolerance when TE is applied multiple times before drought stress and GB is applied during drought stress in creeping bentgrass. The enhanced performance was manifested as enhanced visual quality and canopy health, as well as physiological adjustment of individual leaves promoting water status. Moreover, the TE plus GB increased promoted osmotic adjustment and cell membrane stability during prolonged periods of soil water deficit. Incorporating PGRs and osmoregulants into turfgrass management protocols could be very beneficial for managing cool-season turfgrass species in climatic areas with limited rainfall or during periods of irrigation water-use restriction. The second study took a different approach to explore drought tolerance and utilized transgenic creeping bentgrass with improved

growth and physiological activities under drought stress as a germplasm to investigate membrane fatty acids related to drought tolerance in creeping bentgrass. The objective was to determine if whole-plant drought tolerance previously seen in transgenic creeping bentgrass expressing the *SAG12-ipt* gene for cytokinin synthesis is associated with changes in membrane fatty acid composition and saturation level when compared to wild-type plants. Results from the study showed that improved turf quality and physiological activities of transgenic creeping bentgrass during drought stress could be associated with the increased production of long-chain unsaturated fatty acids, which could help maintain membrane stability and fluidity under drought stress, contributing to improved leaf and root growth. Given the wide usage of creeping bentgrass throughout the turfgrass industry, elucidating on the factors governing drought response is of high importance and fatty acid analysis may be useful for future selections of germplasm for breeding new varieties with enhanced drought tolerance.

From this point onward, the subsequent research studies were focused towards elucidating on various aspects of creeping bentgrass growth, physiological, and biochemical changes associated with elevated CO_2 effects under drought stress conditions. The first study within this focus examined the effects of a doubled CO_2 concentration on growth, morphological, and physiological processes for cool-season creeping bentgrass maintained under well-watered and well-fertilized conditions. Creeping bentgrass plants grown at the elevated CO_2 concentration displayed beneficial changes in their growth rate, leaf and root morphology, and water use which would be considered highly desirable for turfgrass management. Specifically, the CO_2-stimulation of lateral spread and production of smaller and thicker leaves, as well as increased root

growth, would be critically important for rapid turfgrass establishment from seeds or sprigs, as well as reestablishment following physical damages. Improved water use efficiency by elevated CO_2 would also have a significant impact on water use and may lead to changes in the irrigation management of turfgrass.

Proteomic profiling of stress-responsive proteins by means of two-dimensional polyacrylamide gel electrophoresis separation and mass spectrometry (MS) identification was then utilized to describe changes in protein abundance within creeping bentgrass roots and leaves following drought stress treatment at elevated CO_2 concentration. Specifically, the objective of these two studies were to investigate changes in protein abundance responding to interactive effects of drought and CO_2 in leaves or roots of creeping bentgrass with a goal to suggest potential metabolic factors regulated by elevated CO_2 contributing to improved drought tolerance. Within creeping bentgrass leaf tissue, elevated CO_2 improved growth by maintaining leaf hydration and membrane integrity, which may be in part a result of changes in abundance for proteins of the Calvin–Benson cycle including FBA precursor, chloroplastic GAPDH-A, RuBisCO, and chloroplastic SBPase precursor. Elevated CO_2 also decreased cytosolic GAPDH abundance during drought, which may have downstream effects on certain aspects of plant respiration. Within creeping bentgrass root tissue, elevated CO_2 promoted growth of creeping bentgrass under non-stress conditions and mitigated the inhibitory effects of drought on root growth, which was manifested through promotion of total root length, surface area, root biomass, as well as maintenance of root membrane integrity during soil drying. Proteins governing primary metabolism involving nitrogen metabolism (glutamine synthetase), energy metabolism involving respiration (glyceraldehyde-3-

phosphate dehydrogenase), and stress defence by antioxidant metabolism (ascorbate peroxidase, superoxide dismutase, and catalase) and protective chaperones (HSP81-1) could have facilitated the enhanced growth by serving major roles in root responses to elevated CO_2, particularly under drought stress conditions. Additionally, the abundance of proteins imparting other biological functions (protein synthesis, transcription, protein destination/storage, intercellular traffic) were also altered by elevated CO_2.

Lastly, the final study of the dissertation aimed to determine whether elevated CO_2-enhanced drought tolerance in creeping bentgrass was associated with the stimulation of tiller and stolon growth and the alteration of stress-regulating and growth-regulating hormone accumulation by quantifying the endogenous content of six major hormones (abscisic acid (ABA), indole-3-acetic acid (IAA), isopentenyladenosine (iPA), jasmonic acid (JA), trans-zeatin riboside (tZR), salicylic acid (SA), as well as the JA precursor 12-oxo-phytodienoic acid (OPDA) in leaves. Plant hormones serve key roles in regulating plant growth and development, such as tiller formation and lateral shoot growth, as well as plant tolerance to drought stress and there was little information available describing whether tiller formation and stolon growth negatively affected by drought stress could be mitigated by elevated CO_2 through alteration of hormone metabolism. Overall, elevated CO_2-enhanced leaf hydration, tiller formation, and stolon growth in creeping bentgrass exposed to drought stress. The differential changes in the endogenous content of stress-regulating hormones (ABA, SA, and JA) and growth-regulating hormones (tZR, iPA, and IAA) in response to elevated CO_2 under drought stress could have contributed to the positive effects of elevated CO_2 on maintaining leaf hydration, tiller, and stolon growth during drought stress.

In conclusion, this research provides valuable information for advancing our understanding of creeping bentgrass drought tolerance and also has potential implications to aide in improving efficacy for various aspects of turfgrass management such as efficient utilization of irrigation water and decreased need for supplementary chemicals including fertility and pesticides. The lessons derived from the research will likely have implications across many different turfgrass management aspects and provide turfgrass managers new techniques to maintain high-quality playing conditions at a lower economic cost and less environmental impact.

BIBLIOGRAPHY

Patrick Burgess, a lifelong resident of New Jersey, spent nearly a decade employed by Secor Farms, Inc., one of the most prominent and successful greenhouse establishments in north-NJ, specializing in high-quality ornamental bedding plants and agronomic crop products. The extensive training which he received during his time at Secor's encompassed all aspects of greenhouse and farm management and preempted his enrollment into Rutgers University within the Plant Science, Horticulture and Turfgrass Industry major. Burgess was then accepted into the Plant Biology graduate program and served three consecutive years as a teaching assistant of plant pathology, physiology, and propagation. Since then, he has been a Lab Researcher IV in the Department of Plant Biology and Pathology at Rutgers University managing the lab of Dr. Bingru Huang, herself one of the most prominent and acclaimed scientists of plant stress physiology. During his time at Rutgers, Burgess has developed excellence across many areas of plant-environment interactions with special emphasis on turfgrass stress physiology and plant health products. Not only does he have extensive experience in the development and execution of basic and applied research projects both in the field and laboratory, but he also serves as a valued instructor in the Rutgers Professional Golf Turf Management School specializing in turfgrass growth regulators and stress physiology. During his time at Rutgers, Patrick has authored and co-authored twelve refereed journal articles, several trade articles, and a book chapter focused on hormone regulation of drought stress responses in plants. His professional dedication has been recognized at the local, state, and international levels through numerous research awards and scholarships.