EFFECTS OF NITRATE AND CYTOKININ ON NITROGEN METABOLISM AND HEAT STRESS TOLERANCE OF CREEPING BENTGRASS

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ABSTRACT

Creeping bentgrass (*Agrostis stolonifera* L.) is a major low-cut cool-season turfgrass used worldwide. The objectives of this research were to: 1) to gain insight into the diurnal fluctuation of N metabolism and effects of cytokinin (CK) and nitrate; 2) to characterize the impacts of N and CK on creeping bentgrass under heat stress; 3) to investigate the simultaneous effects of CK and N on the antioxidant responses of heat stressed creeping bentgrass; and 4) to examine the expression pattern of the major heat shock proteins (HSPs) in creeping bentgrass during different heat stress periods, and then to study the influence of N on the expression pattern of HSPs.

The transcript abundance of nitrate reductase (NR), nitrite reductase (NIR), plastidic glutamine synthetase (GS2), ferredoxin-dependent glutamate synthase (Fd-GOGAT), and glutamate dehydrogenase (GDH) and N metabolites in shoots were monitored during the day/night cycle (14/8 h). All the measured parameters exhibited clear diurnal changes, except *GS2* expression and total protein. Both *NR* expression and nitrate content in shoots showed a peak after 8.5 h in dark, indicating a coordinated oscillation. Nitrate nutrition increased diurnal variation of nitrate content compared to control and CKHowever, CK shifted the diurnal *in vivo* NR activity pattern during this period.

Grass grown at high N had better turf quality (TQ), higher Fv/Fm, normalized difference vegetation index (NDVI), and chlorophyll concentration at both 15 d and 28 d of heat stress than at low N, except for TQ at 15 d. Shoot NO_3^-, NH_4^+, and amino acids increased due to the high N treatment, but not water soluble proteins. High N also induced maximum shoot nitrate reductase activity (NR_{max}) at 1 d. CK increased NDVI at 15 d and Fv/Fm at 28 d. In addition, grass under 100 μM CK had greatest NR_{max} at both 1 d and 28 d. Under high N with 100 μM CK, root *t*ZR and iPA were 160% and 97% higher than under low N without CK, respectively.

Higher O_2^- production, H_2O_2 concentration, and higher malonydialdehyde (MDA) content in roots were observed in grass grown at high N. The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and guaiacol peroxidase (POD) in roots were enhanced by high N at 19, 22, and 24% levels, respectively, relative to low N. Twenty-eight days of heat stress resulted in either the development of new isoforms or enhanced isoform intensities of SOD, APX, and POD in roots compared to the grass responses prior to heat stress. However, no apparent differences were observed among treatments. No CK effects on these antioxidant parameters were found in this experiment. At week seven, grass at medium N had better TQ, NDVI, and Fv/Fm accompanied by lower shoot electrolyte leakage (ShEL) and higher root viability (RV), suggesting better heat resistance. All the investigated HSPs (HSP101, HSP90, HSP70, and sHSPs) were up-regulated by heat stress. Their expression patterns indicated cooperation between different HSPs and that their roles in creeping bentgrass thermotolerance were affected by N level.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER ONE

LITERATURE REVIEW

OVERVIEW

Temperature is one of the major environmental factors influencing plant distribution and growth. High temperature is a common abiotic stress for higher plants. It is estimated that the annual mean air temperature of about 23% of land on the earth is above 40 ^{o}C (Leone et al., 2003). It could be getting worse due to current trends in global warming. It is anticipated the global temperature will increase another 1.7 to 3.8 ^{o}C by 2100 (Wigley and Raper, 1992). Temperature above the optimum for healthy plant growth is known as heat stress. Heat is understood as the upper temperature range in which active plant life is stressed, but is still possible (normally 10 to 15 ^{o}C above the optimum temperature). Stress intensity increases with increasing magnitude and length of supraoptimal temperature periods (Schulze et al., 2005). Higher temperature affects the balance of plant growth and development by accelerating and redirecting metabolic processes. Under heat stress, plants respond at all levels, from morphological adaptations to physiological changes to molecular regulations. A number of important adaptive changes occur in terms of carbon and nitrogen metabolism, antioxidant responses, hormone homeostasis, and expression of specific stress proteins (e.g. HSPs) (Schulze et al., 2005; Wahid et al., 2007).

In general, cool-season turfgrass species (C3) are adapted to temperate and subarctic climates, and thrive at temperatures from 16 to 24 °C (Turgeon, 2002). The intensity and

duration of heat stress is one of the primary factors limiting environmental adaptation of cool-season turfgrasses. For example, cool-season turfgrasses grow best in the cooler regions of the US. However, cool-season turfgrasses are widely planted in the transition zone (a transitional region between temperate and subtropical climates, e.g. Virginia) and even in some warmer climatic regions, where heat stress is usually expected during summer. Creeping bentgrass (*Agrostis stolonifera* L.), the most widely used cool-season turfgrass species for golf greens over the temperate regions due to its fine texture and excellent tolerance of low mowing heights, is particularly susceptible to heat stress. Additionally, its use is increasing in the transition zone and some sub-tropical regions in the United States because it can offer a year-round high quality playing surface. In turn, high temperature during the summer often becomes a major factor affecting creeping bentgrass performance in these regions (Carrow, 1996). Poor performance is manifested as leaf yellowing, a decline in density, and even loss of grass. For example, in July 2005, many bentgrass putting greens were lost either directly or indirectly due to above-average hot, humid weather in the southeastern US (Bevard, 2006). Managing creeping bentgrass putting greens to provide high quality and consistent putting surfaces in the transition zone and warmer regions during the summer is a big challenge for golf course superintendents.

HEAT STRESS RESPONSES AND TOLERANCE IN COOL-SEASON TURFGRASSES

Heat-Stress Threshold

An upper developmental threshold of temperature is that above which a detectable reduction in physiological parameters and/or growth begins (Schulze et al., 2005). Cool

season and temperate crops often have threshold temperature values below 30 °C, which is variable depending on species and tissues (Table 1-1). Although both high air and soil temperatures limit cool-season grass growth, higher soil temperature was found to be more detrimental for the growth of creeping bentgrass (Wang et al., 2003; Xu and Huang, 2001; Xu et al., 2003). In a heat stress experiment with creeping bentgrasses, Pote et al. (2006) reported that all measured parameters decreased in all nine cultivars as root-zone temperature increased from 20 to 35 ^{o}C, but the time and temperature at which the decline occurred varied among parameters measured. For example, the temperature thresholds for decrease of canopy net photosynthetic rate, cytokinin content, root number, and turf quality after 28 days of exposure were at 23, 27, 27, and 35 ^{o}C, respectively. Furthermore, at 31 ^{o}C, decline in root number, cytokinin content, and turf quality occurred at 19, 37, and 47 days, respectively. However, when creeping bentgrass was subjected to a soil temperature of 35 ^{o}C, root death occurred at 5 days of heat stress, cytokinin decline occurred at 5 to 10 days of stress, followed by water deficit, major nutrient content and grass quality decline at 15, 15 to 20, and 20 days, respectively (Huang et al., 2005) (Table 1-2). Temperature optima for cool-season grass root growth has been proposed to be from 15 to18 ^{o}C (Beard, 1973). The above studies suggest that a root-zone temperature of 23 ^{o}C was the threshold temperature for proper root function. Temperatures at or above this level are detrimental to overall turf growth, and higher temperature would have faster detrimental effects. Moreover, an array of other changes is occurring at the cellular and molecular levels prior to these changes. For example, cell membrane stability is affected shortly after heat exposure, antioxidant response and heat shock proteins can be induced in hours (Cui et al., 2006; Luthe et al., 2000).

Table 1-1. Sequence of changes in different physiological parameters of creeping bentgrass in response to high temperature (all at 35 ^{o}C)

Parameter	Days of heat stress	Note
Cell membrane leakage	hours	Luthe et al., 2000
Heat shock protein up-regulation	hours	Luthe et al., 2000
Canopy photosynthetic rate	1	Xu et al., 2002
Root health decline	5	Huang et al., 2005
Photochemical efficiency	7	Xu et al., 2002
Cytokinin decline	5 to 10	Huang et al., 2005
Water deficit	15	Huang et al., 2005
N, P, K content decline	15 to 20	Huang et al., 2005
Turf quality decline	20	Huang et al., 2005

Table 1-2.Threshold high temperatures for some crop plants (adapted from Wahid, et al., 2007 with modifications)

Crop plants	Threshold temperature (°C)	Growth stage	References
Wheat	28	Post-germination	Monje et al., 2007
Brassica	29	Flowering	Morrison and Stewart, 2002
Cool season pulses	25	Flowering	Siddique et al., 1999
Cool season turfgrass	18	Vegetative, root	Beard, 1973
Cool season turfgrass	24	Vegetative, shoot	Beard, 1973
Creeping bentgrass	23	Vegetative, root	Pote, et al., 2006
Perennial ryegrass	28	Vegetative	Kauffman et al., 2007

Researchers in the past decades have advanced our understanding of the mechanisms of how cool-season turfgrasses respond and adapt to heat stress. Among various mechanisms, carbohydrate metabolism, antioxidant responses, induction of stress proteins, particularly heat shock proteins, and cytokinin synthesis are involved in protecting cool-season turfgrasses from heat stress injuries (Huang, 2004). In one of the early turfgrass studies of abiotic stress responses, Schmidt and Blaser (1967) reported the effects of temperature and other environmental factors on creeping bentgrass. Informed by research advances in other model species and crops, research on creeping bentgrass abiotic stress responses has progressed. In the following literature review, it is not my intention to enunciate the complexity of heat stress in plants, but to highlight some of the most current information as regards cool-season turfgrass heat stress responses. This information will then provide the basis of my research objectives.

Cell Membrane Stability

Like other eukaryotes, plant cells are characterized by their biological membrane system, including cell membranes, plastid membranes and others. All these bio-membranes consist of two basic components (lipids and proteins) and other components, such as carbohydrates (Taiz and Zeiger, 2002). The integrity and functions of biological membranes are sensitive to high temperature. Under high temperature, the lipid bilayer becomes less viscous and more fluid. Moreover, high temperature alters the advanced conformation of membrane-binding proteins. All these changes eventually result in enhanced membrane permeability indicated by higher loss of electrolytes, accompanied by cell and organelle malfunction (Schulze et al., 2005; Senthil-Kumar et al., 2007).

Increased solute leakage, as an indication of cell membrane injury, has long been used as an indirect measure of stress tolerance in various plant species and different abiotic stresses, including heat stress (Wahid et al., 2007). Heat stress decreased cell membrane thermostability in both tall fescue (*Festuca arundinacea* Schreb.) cultivars, but to a greater degree in TF66, the sensitive one (Cui et al., 2006). Recently, a study reported less electrolyte leakage in a hybrid bluegrass (Kentucky bluegrass (*Poa pratensis* L.) × Texas bluegrass (*Poa arachnifera* Torr.) compared with Kentucky bluegrass and tall fescue when exposed to supraoptimal temperature (35/25 °C, d/n) for 48 d, indicating greater heat resistance (Su et al., 2007). Similar results were found between creeping bentgrass and *Agrostis scabra* (Willd.), a naturally thermotolerant bentgrass species (Rachmilevitch et al., 2006). In creeping bentgrass, increased leaf electrolyte leakage has been repeatedly observed under stress. Lower leaf electrolyte leakage of heat-acclimated plants, compared to non-

acclimated plants, indicated an increase of plant tolerance to subsequent heat stress (40 ^{o}C) (He et al., 2005; Huang et al., 2001).

Plants are able to adapt the fluidity of their membranes to temperature change to a certain degree by changing lipids composition, with more saturated fatty acids being incorporated into the membranes upon increased temperature (Schulze et al., 2005). For example, in *Arabidopsis* grown under high temperature, the ratio of unsaturated to saturated fatty acids decreased to one-third of the levels at normal temperatures (Somerville and Browse, 1991). Moreover, changes in membrane fatty acid composition and saturation levels (decrease in double bond index) in both leaves and roots of creeping bentgrass were observed even though only roots were exposed to high temperature (Liu and Huang, 2004). Another study on creeping bentgrass found that the heat tolerant cultivar, 'L-93' had a higher proportion of saturated lipids prior to heat stress in both leaves and roots compared to the sensitive cultivar, 'Penncross' (Larkindale and Huang, 2004a). These adaptive changes can be related to a cultivar's ability to maintain optimal viscosity for membrane function (Schulze et al., 2005). However, it has also been suggested that elevated melting temperature of plasma membranes by an increase in saturated fatty acids in mature leaves reduces heat tolerance of the plant (Wahid et al., 2007). Moreover, heat tolerance does not correlate with the degree of lipid saturation in some species, and complexity of the heat stress response in plants is not a surprise (Kotak et al., 2007). It should be noted that factors other than membrane stability might also play a role in growth limitations at high temperatures.

Photosynthesis and Carbon Metabolism

Sustaining functional cellular membranes under stress is critical for many physiological and biochemical processes, particularly those membrane-related metabolic processes such as photosynthesis and respiration (Blum, 1988). Photochemical reactions in thylakoid lamellae and carbon metabolism in chloroplast stromas have been suggested as the primary sites of injury at high temperatures (Wise et al., 2004). Sensitivity of photosynthesis to heat stress is thought to be due to the effects on PSII (Bukhov et al., 1999; Camejo et al., 2005), with heat stress leading to either dissociation or inhibition of the oxygen evolving complexes (OEC), causing a loss of oxygen evolution capacity. Subsequent restricted electron transport results in an imbalance between electron flow from OEC toward the acceptor side of PSII in the direction of the PSI reaction center (Wahid et al., 2007). Heat-altered PSII function is documented by monitoring chlorophyll fluorescence, the ratio of variable fluorescence to maximum fluorescence (F_v/F_m). It is a widely used physiological parameter to screen for thermotolerance (Maxwell and Johnson, 2000; Schreiber and Berry, 1977). It has also been shown to correlate positively with heat tolerance in 23 tropical crops and one temperate fruit crop (Yamada et al., 1996).

High temperature stress decreased maximum photochemical efficiency of PS II in dark adapted leaves in two heat-shocked tall fescue cultivars. In addition, heat stress led to reductions in chlorophyll content, chlorophyll/carotenoid ratio and an increase in the chlorophyll a/b ratio (Cui et al., 2006). Similar results were also reported in tomato (*Solanum lycopersicum* L.), and these changes were suggested to be related to different thermotolerance of plants (Wahid and Ghazanfar, 2006). In creeping bentgrass under high temperatures, reductions in F_v/F_m and canopy photosynthetic rate have also been reported (Fu

and Huang, 2003b; Liu et al., 2002; Xu et al., 2002). Furthermore, photosynthetic rate decreases while the respiration rate increases with increased temperature in general, resulting in a carbon imbalance. Thus, limited carbohydrate availability has been proposed as one of the major factors that cause decline of plant growth and quality in creeping bentgrass under heat stress (Fu and Huang, 2003b; Xu et al., 2003).

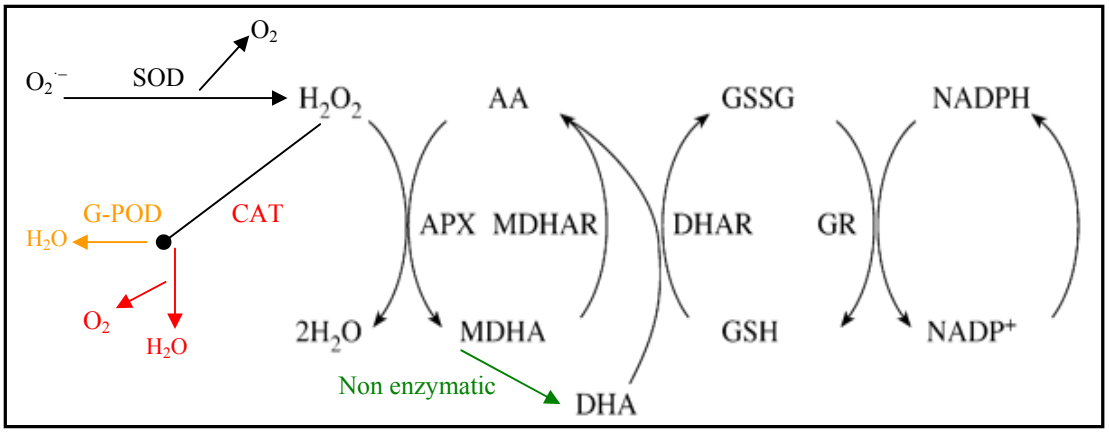
However, the magnitude of such alterations in response to heat stress differs with species and genotypes (McDonald and Paulsen, 1997). Lyons et al. (2007) reported that the thermotolerant cool-season grass, *Agrostis scabra* (Willd.) was able to maintain a better carbon balance by down-regulating its respiration rate faster than the heat-sensitive species, creeping bentgrass. Other studies from the same research group found that the down-regulated respiration rate was due to higher carbon partitioning to the alternative respiratory pathway and an improved capacity to increase respiratory efficiency (Rachmilevitch et al., 2006; Rachmilevitch et al., 2007).

Oxidative Stress and Antioxidants

Although reactive oxygen species (ROS) are normally generated in chloroplasts, mitochondria and cell membranes, heat-stress inhibited photosynthesis in chloroplasts results in an imbalance of the electron-transfer chain and promotes production of ROS, including singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Smirnoff, 1993; Veljovic-Jovanovic et al., 2006). Excess ROS are detrimental due to their high reactiveness with proteins, lipids, nucleic acids, and other important cell components. Further, OH^- formed by the combination of O_2^- and H_2O_2 is very toxic, causing the autocatalytic peroxidation of membrane lipids and pigments and leading to a loss of

membrane semi-permeability and modifying its functions (Senthil-Kumar et al., 2006; Wahid et al., 2007).

Fig. 1-1. The ROS scavenging systems in higher plants. Including Ascorbate-glutathione cycle (Halliwell–Asada pathway). APX, ascorbate-peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase. SOD, Superoxide dismutase, CAT, Catalase, G-POD, Guaiacol type peroxidase. Modified from May et al. (1998).



Levels of ROS are regulated by their rate of generation and degradation as influenced by the scavenging capacity of antioxidant enzymes and antioxidants (Fig. 1-1) (Asada, 1999; Blokhina et al., 2003). Superoxide dismutase (SOD) constitutes the first line of defense against ROS by dismutating the superoxide anion to H_2O_2. Next, H_2O_2 is finely regulated by catalase (CAT). Some H_2O_2 also enters the ascorbate-glutathione cycle and is catalyzed by ascorbate peroxidase (APX) with reduced ascorbate as the electron acceptor (Asada, 1999; Blokhina et al., 2003). Increasing evidence suggests that tolerance of adverse environments is correlated with an increased capacity to scavenge or detoxify ROS. Thus, protection against oxidative stress is an important component in determining the survival of a plant under heat stress (Smirnoff, 1993; Maestri et al., 2002). Higher levels of ROS resulting from a decrease

in antioxidant activity in stressed tissues may contribute to injury (Fadzillah et al., 1996). For example, damage of the photosynthetic apparatus was widely suggested to be associated with the production of ROS (Guo et al., 2006). Liu and Huang (2000) also suggest that greater generation of ROS indicates cellular injury in creeping bentgrass due to high temperature. Studies on heat-acclimated versus non-acclimated cool season turfgrass species, perennial ryegrass (*Lolium perenne* L.) and tall fescue, indicated that the former had lower production of ROS as a result of enhanced synthesis of two important antioxidants, ascorbate and glutathione (Xu et al., 2006). Heat stress caused decline in antioxidant enzyme activities in both creeping bentgrass cultivars ('L-93' and 'Penncross'), such as SOD and CAT (Liu and Huang, 2000), and more specifically, high soil temperature causes more severe oxidative damage to leaves than high air temperature as evidenced by limiting antioxidant activities and increasing lipid peroxidation, manifested by higher malonydialdehyde (MDA) content (Huang et al., 2001).

Cultivar variations in antioxidant enzyme activities are associated with their differences in heat tolerance (Liu and Huang, 2000). Wang et al., (2003) reported that a higher temperature threshold was needed for the tolerant cultivar to reach a similar level of decline of antioxidant enzyme activities compared to the sensitive one. Similar results were also reported in two tall fescue cultivars under heat stress (Cui et al., 2006), further indicating a possible contribution of antioxidant activities to heat tolerance. Furthermore, enhanced heat tolerance in two other cool-season turfgrass species (tall fescue and Kentucky bluegrass) by exogenous Ca^{2+} application was suggested due to the ability to maintain antioxidant activities and lower membrane lipid peroxidation (Jiang and Huang, 2001). Certainly further

research is necessary to identify the signaling molecules, which could stimulate the production of antioxidants in cells exposed to heat stress.

Heat Shock Proteins

Reactive oxygen species (ROS) as a secondary signaling molecule to induce a series of subsequent growth and metabolic responses to the stress could be involved in the stimulation of heat shock response (Kotak et al., 2007; Sun et al., 2002). For example, Banzet et al. (1998) observed the accumulation of small heat shock proteins (sHSP) in tomato cells as an adaptive response induced by oxidative stress. Changes in gene expression at the transcriptional and/or translation level are thought to be a fundamental mechanism in plant response to environmental stresses (Senthil-Kumar et al., 2007). Heat stress redirects protein synthesis in higher plants and results in a nearly exclusive synthesis of stress proteins, particularly heat shock proteins (HSPs) (Schulze et al., 2005). HSPs have been assigned to 11 multiprotein families across different organisms based on a new eukaryotic/*Escherichia coli* nomenclature system, including HSP100/ClpB, HSP90/HtpG, HSP70/DnaK, HSP60/GroEL, HSP40/CbpA and small HSP proteins (Bharti and Nover, 2002; Kotak et al., 2007). HSPs are thought to be important in thermotolerance due to the following observations: (1) coincident and universal induction in response to temperature stress in all investigated organisms, and (2) fast and intensive biosynthesis (Wahid, et al., 2007).

The role of HSPs to counteract negative effects of heat stress in plants was first hypothesized based on correlative evidence (Vierling, 1991). Recently, some specific HSPs have been shown to be causally involved in the capacity to acquire thermotolerance in many studies (Maestri et al., 2002). For example, Ristic et al. (1991) reported that a 45-kDa HSP

was observed in a heat-resistant maize line, but not in a heat-sensitive line, and they later confirmed that the expression of the 45 kDa HSP was correlated with thermotolerance in maize (Ristic et al., 1998). In addition, a thermosensitive *Arabidopsis* mutant was found to lack a 27-kDa HSP (Burke et al., 2000).

Three HSP functions have been proposed: to act as molecular chaperones, to conserve housekeeping mRNA in heat shock granules for future use during recovery from heat stress, and to catalyze proteolysis of irreversibly denatured proteins (Schulze et al., 2005). HSPs function mainly as molecular chaperones that help other proteins to maintain their native conformation and improve protein thermostability. Members of different HSP families usually form multi-subunit chaperone complexes in nearly all compartments of cells with protein synthesis or processing, such as chloroplasts and endoplasmic reticulum (ER) (Bharti and Nover, 2002). Most specific information on how HSP70, HSP90 and HSP60 help survival of heat stress in plants is attributed to their molecular chaperone activity. For example, the system of HSP70 chaperones consists of at least three HSP members, HSP70, HSP40, and HSP23. The HSP70 family of proteins is one of the most well understood systems of chaperone operation. This family, with an energy consumption of about 100 ATP per mid-size protein, works together to interact with partially unfolded proteins, and then helps them to fold back into their native conformation via binding and disassociation (Schulze et al., 2005).

Next we mainly refer to studies on the function of HSP100 family and sHSPs during heat stress since they are the most extensively studied family in higher plants. HSP100 proteins are ATP-binding with chaperone activity to re-solubilize protein aggregates (Bösl et al., 2006). HSP101 has been reported to play a major role in acquiring thermotolerance in

Arabidopsis (Hong and Vierling, 2000; 2001). Follow-up research by the same group indicated that HSP101 interacts with the sHSP chaperone system to re-solubilize protein aggregates after heat stress, a process that requires complex interactions of HSP101 protein domains. In addition to cytosolic HSP101, plants also have nuclear-encoded, chloroplast- and mitochondrion-localized HSP100, but they seem not to be essential to thermotolerance in *Arabidopsis* (Lee et al., 2005; 2007). However, defects in thermotolerance of transgenic lines expressing an antisense construct of tomato chloroplast-localized HSP100 have been reported (Yang et al., 2006). In wheat (*Triticum aestivum* L.), three members of HSP101 have been cloned, but their detailed role in heat tolerance has not been confirmed (Maestri, 2002). HSP101 has been identified in maize (*Zea mays* L.) as a nucleus-localized protein, and levels of HSP101 increased in response to heat shock, with abundance depending on different tissues/organs (Young et al., 2001). In rice, five HSP100 genes have been reported (including predicted cytoplasmic and chloroplast localization), indicating the possible complex of HSP100 family in plants (Batra et al., 2007). Although molecular diversity (i.e., mosaic regulatory elements in the promoter regions) within a family exists, like other high-molecular-weight HSPs, HSP100 are characterized to be highly homologous in higher plants (Vierling, 1991). However, similar HSPs have not been reported in cool season turfgrasses. The role of HSP100 in plant heat stress tolerance, particularly in turfgrasses, warrants further investigation.

sHSPs with a molecular mass of 15 to 42 kDa on denaturing polyacrylamide gel electrophoresis (PAGE) are the most dominant proteins produced in higher plants upon heat stress (Sun et al., 2002). According to their cellular locations, sHSP are classified into 5 classes: cytosolic (class I and II), chloroplastic, mitochondrial, and endoplasmic reticulum

related sHSPs (Lenne et al., 1995; Leone et al., 2003; Vierling, 1991). After separation by native gel electrophoresis, sHSPs are found as high molecular weight oligomers ranging in size from 150 to 800 kDa composed of 9 to 32 subunits (Kim et al., 1998; Lee et al., 1995). However, in some cases they form only dimers or tetramers (Kappé et al., 2002). The sHSP oligomers appear to be homo-oligomers in human HSP27 and murine HSP25. In higher plants, *in vitro* and *in vivo* data indicated class I cytoplasmic, chloroplast and mitochondrial sHSPs also form homo-oligomers of 200-300 kDa, but not hetero-oligomers (Helm et al., 1997; Waters et al., 1996). According to the currently accepted model of sHSP function, sHSP oligomers dissociate to dimers and bind substrates through both their non-conserved amino-terminal domain and the conserved α-crystallin domain. Denatured proteins in the presence of sHSPs can be refolded and reactivated by HSP70 with the participation, in some cases, of HSP100 (Kotak et al., 2007; Nover and Scharf, 1997). Mammalian sHSPs are phosphorylated through a MAP kinase cascade; phosphorylation results in oligomer size reduction accompanied by change in functions (Freshney et al., 1994; Knauf et al., 1994). Whether the phosphorylation is involved in thermotolerance is not clear. Some scientists suggested phosphorylation represented another difference in sHSPs between animals and plants (Leone et al., 2003). It was reported that the phosphorylation of sHSPs did not occur in tomato cells (Nover and Scharf, 1984) and pea (*Pisum sativum* L) (Suzuki et al., 1998). However, Lund et al. (2001) reported the phosphorylation of a maize mitochondrial small heat shock protein, HSP22, which is the first demonstration of sHSP phosphorylation in plants. More studies are needed to investigate sHSP phosphorylation in different plant species and the role of phosphorylation in plant thermotolerance.

The majority of sHSPs are induced by heat stress, demonstrating their crucial role in heat shock response. This is supported by findings related to their accumulation during heat stress response. Indeed, sHSPs accumulation during heat stress is proportional to temperature; furthermore this response is very rapid and related to stress duration. Many data suggest that the maximum synthesis of sHSP is induced by temperature just below lethal temperatures. The most abundant sHSPs induced are class I cytoplasm sHSPs that can account for over 1% total leaf or root water soluble protein (Hsieh et al., 1992). Other sHSPs, such as the chloroplast-located sHSPs can amount to only 0.02% of total leaf protein (Chen et al., 1990). In addition to chaperone function, sHSPs are also proposed to modulate membrane fluidity and composition (Balogi et al., 2005). sHSPs are by far the most complex group of HSPs that may protect practically all cellular compartments in plants under stress due to their unusual abundance and diversity (Klaus-Dieter et al., 2001). Immuno-localization studies have determined that some sHSPs associate with particular cellular structures, such as chloroplasts and mitochondria (Nieto-Sotelo et al., 2002; Yang et al., 2006), suggesting that sHSPs play a role in protecting photosynthetic electron transport and mitochondrial electron transport during heat stress. Protection of electron transport chains during heat stress has been shown by adding low-molecular-weight (LMW) HSP *in vitro* during heat stress (Downs and Heckathorn, 1998; Heckathorn et al., 1998). Over expression of a mitochondrial sHSP enhanced thermotolerance in tobacco (*Nicotiana tabacum* L.) (Sanmiya et al., 2004). In response to heat shock, maize seedlings have four more mitochondrial LMW-HSPs expressed compared to seedlings of wheat and rye, which was suggested to be the possible reason for higher heat tolerance in maize than in wheat and rye (Korotaeva et al., 2001).

Moreover, immunomodulation of cytosolic sHSPs led to plant heat sensitivity (Miroshnichenko et al., 2005).

Heat Shock Proteins in Cool-Season Turfgrasses

Among all cool-season turfgrass species, most heat stress related research has been done on creeping bentgrass. In this species, a LMW-HSP (HSP25) was reported to be genetically involved in heat tolerance (Luthe et al., 2000; Park et al., 1996). Another study showed that heat sensitivity was associated with reduced capacity of bentgrass variants to accumulate chloroplastic HSPs (Wang and Luthe, 2003). Luthe et al., (2000) reported the synthesis of several high-molecular-weight (HMW)-HSPs (97, 83, 70 kD) in creeping bentgrass under heat, but failed to determine the difference between two accessions with different heat tolerance and whether HSP101 was involved. In a later study on creeping bentgrass, new HSPs (54, 57 kD) were synthesized during heat acclimation compared to sudden heat stress, which was suggested to be related to improved heat tolerance. However, no direct correlation has been proved so far (He et al., 2005). In Kentucky bluegrass, He and Huang (2007) suggested that better heat tolerance was associated with induction of HSPs during the early phase of heat stress, but if they are the cause of better heat tolerance needs further work. Similarly, increased HSP70 expression was reported in ABA-treated or drought stressed tall fescue, but if the role of HSP70 was related to heat tolerance in tall fescue was also not clear (Jiang and Huang, 2002).

Thermo Tolerance and Plant Hormone/Growth Regulators

Plant hormones have an important role in regulating the growth, development, and environmental stress tolerance of plants (Delaney, 2004). Cross-talk in hormone signaling affects the ability of plants to respond appropriately to adverse environmental conditions. Under heat stress hormone homeostasis is altered, including hormone stability, content, biosynthesis, and compartmentalization (Maestri et al., 2002). Although the involvement of hormones in plant thermotolerance is complex and the exact signal pathway of hormones under heat is yet mostly unclear, many studies have shown that optimizing status of certain hormones can enhance plant thermotolerance (Kotak et al., 2007; Wahid et al., 2007).

Abscisic Acid (ABA)

ABA is known as an important stress-induced plant hormone with ABA levels increasing in response to different environmental stresses (e.g. osmosis related stresses), including high temperature (Arteca, 1996). A transient peak of ABA levels was observed in response to heat shock in pea plants (Liu et al., 2006). However, in creeping bentgrass ABA level rose during recovery from heat stress, but not during heat stress (Larkindale and Huang, 2004b; 2005). Trewavas and Jones (1991) suggested increased ABA synthesis was regulated at the transcriptional level. Moreover, it was documented that exogenous applications of ABA could increase cold hardiness of plants (Guy, 1990). Similarly, exogenously applied ABA was reported to increase thermotolerance of photosystem II by mimicking water stress in maize (Gong et al., 1998). ABA level resulted in a modification of gene expression in stressed plants. Later studies proved that the action of ABA in response to stress involves modulating the up- or down-regulation of expression of numerous genes (Swamy and Smith,

1999; Xiong et al., 2002). Although several HSPs are ABA-inducible, and it was suggested to be one mechanism that confers thermotolerance (Pareek et al., 1998; Rojas et al., 1999), the ABA pathway involved in the plant response to heat stress is thought to be different from HSP pathway (Senthil-Kumar et al., 2007). For example, a study on two *Arabidopsis* ABA signaling mutants, *abi1*and *abi2*, showed that the accumulation of HSPs was not affected, even though decreased survival after heat stress was observed in these mutants (Larkindale et al., 2005).

Ethylene (C_2H_4)

Similar to ABA, ethylene is termed a stress hormone since it can be promoted by a number of stresses. Ethylene is involved in the regulation of numerous physiological processes by acting as a secondary signal molecule (Abeles et al., 1992). It is also linked with the signaling pathway in different plant species in response to heat stress (Kotak et al., 2007). Changes in ethylene production under heat stress vary among species. Tan et al. (1988) reported different patterns of ethylene production between soybean hypocotyls and wheat leaves under high temperature. In soybean, ethylene production in hypocotyls was promoted by increasing temperature up to 40 ^{o}C and decreased at 45 ^{o}C. Meanwhile, ethylene production in wheat leaves decreased slightly at 35 ^{o}C and severely at 40 ^{o}C. The difference could be related to different plant tolerance to heat stress. In pepper (*Piper nigrum* L.) the level of 1-aminocyclopropane-1-carboxylic acid (ACC), the main precursor of ethylene, was reported to increase with temperature increase (Huberman et al., 1997). More recently, a study on creeping bentgrass showed that treatment with exogenous ACC prior to heat stress (35 ^{o}C) increased thermotolerance via maintaining longer acceptable grass visual quality,

sustaining greater photosynthesis, and less lipid peroxidation compared to the control. Enhanced activities of antioxidant enzymes were also thought to play an important role (Larkindale and Huang, 2004b). A later study from the same authors observed ethylene production in creeping bentgrass during recovery from heat stress, but not when under stress, which implied that production during recovery could be important (Larkindale and Huang, 2005). However, a commercial ethylene inhibitor (Retain) was reported to increase the number of harvestable buds during a hot fall, but it might not have been due to reduced heat stress (Schrader, 2005). More recently, Xu and Huang (2009) reported another ethylene synthesis inhibitor, aminoethoxyvinylglycine, suppressed leaf senescence and improved heat tolerance of creeping bentgrass. The possible mechanism could be because of their effects on endogenous production of ethylene and on antioxidant metabolism. Although some studies indicate a possible linkage between ethylene signaling and HSPs induction (Suzuki et al., 2005), it is unclear if ethylene signaling is involved in HSPs induction (Larkindale et al., 2005). Ethephon (2-chloroethyl phosphonic acid), a synthetic form of ethylene, has been reported to improve germination of sunflower seed at 25 ^{o}C by breaking thermodormancy (Corbineau et al., 1989). However, its use on turfgrass is mainly as a growth retardant to reduce mowing requirements (chemical mowing) and to suppress seedheads (Ervin and Zhang, 2007a). The effects on heat tolerance of turfgrass, particularly under field conditions could be variable and need further investigation.

Salicylic acid (SA)

SA is important for pathogen defense by participating in systemic acquired resistance (SAR) in plants (Davies, 2004), with recent studies suggesting it is also widely involved in

different abiotic stresses, such as heat stress. One reported function of SA under moderate stress is primarily to promote antioxidative capacity (Dat et al., 2000; Horvath et al., 2007). Dat et al. (1998b) showed that thermotolerance of mustard (*Sinapis alba* L.) seedlings could be obtained by both SA treatment and heat acclimation. Their later observations suggested that SA could be involved in heat acclimation and that oxidative stress may be linked to the action of SA (Dat et al., 1998a; 2000). The effect of SA on thermotolerance may be mediated by HSPs since SA can induce transcription of HSPs, such as HSP70 (Cronje and Bornman, 1999). Maestri et al. (2002) proposed that SA could promote expression of heat shock related genes by stabilizing the trimers of heat shock transcription factors. He et al. (2000) reported the potential use of SA on cool-season turfgrass to heat. Later Ervin et al. (2005) found pre-harvest foliar SA application could improve transplant success of supraoptimally heated cool-season turfgrass sod. In creeping bentgrass, SA-pretreated plants have less oxidative damage and reduced membrane leakage, but higher antioxidant enzyme activity, and increased photosynthetic rate (Larkindale and Huang, 2004; 2005). Similar results have also been found in Kentucky bluegrass treated with SA (He et al., 2005).

Gibberellins (GA)

Gibberellins are known to regulate many phases of plant development, including stem growth. There are about 136 known GAs so far (Sponsel and Hedden, 2004). As opposed to that of ABA, the effects of gibberellins on thermotolerance are adverse (Maestri et al., 2002). More than twenty years ago, Lee et al. (1985) reported the protective property of paclobutrazol (a triazole derivate), a GA biosynthesis inhibitor, against heat damage to snapbean (*Phaseolus vulgaris* L.). GA application resulted in heat-sensitivity of an inherently

heat-resistant dwarf mutant of barley (*Hordeum vulgare* L.), which was identified as impaired biosynthesis of gibberellins, while the application of the triazole paclobutrazol restored heat tolerance (Vettakkorumakankav et al., 1999). In addition, paclobutrazol-treated wheat seedlings have shown increased heat tolerance, which is partly due to enhanced antioxidant enzyme activities and increased transpiration (Kraus and Fletcher, 1994). Zhang et al. (2003) reported paclobutrazol-treated tall fescue sod had less visual injury and greater root strength when stored at 40 ^{o}C for 72 h compared to the non-treated. In addition, several studies have shown two other triazole derivatives (similar to paclobutrazol, but used as fungicides), to have plant growth regulation properties, which increased superoxide dismutase activity in creeping bentgrass (Zhang and Schmidt, 2000) and delayed leaf senescence in Kentucky bluegrass (Goatley and Schmidt, 1990). Their similarity to paclobutrazol, plus those reported observations suggest they could be potentially used to enhance heat tolerance in turfgrass.

Trinexapac-ethyl (TE) is another GA antagonist that is widely studied on turfgrass for different functions, such as a growth retardant and stress tolerance elicitor (Ervin and Zhang, 2007b). Ervin and Zhang (2007a) suggested TE may increase turfgrass heat tolerance since it increased cytokinin level and antioxidant activity. However, Heckman, et al. (2001; 2002) found LT_{50} (lethal temperature 50, the highest temperature that 50% of the population can be killed) of TE-treated Kentucky bluegrass and control was 35.5 ^{o}C and 36.1 ^{o}C respectively, with TE-treated Kentucky bluegrass having less cell-membrane thermostability than the control. The reason for the adverse effects of TE on heat tolerance of Kentucky bluegrass was attributed to the possible block of lipid synthesis by inhibiting acetyl coenzyme-A activity. Those findings warrant further investigation of effects of TE on turfgrass to heat.

Brassinosteroids (BR)

In addition to an essential role in plant development, brassinosteroids appear to have the ability to protect plants against various environmental stresses, such as heat stress (Confraria et al., 2007; Kagale et al., 2007). BR was shown to increase basic thermotolerance to *Brassica napus* (L.) and tomato seedlings (*Lycopersicon esculentum* L.) by accumulating higher HSPs, at least partly (Dhaubhadel et al., 1999). The same research group further reported BR functioned to protect the translational machinery and heat-shock protein synthesis following heat stress (Gampala et al., 2007). Similarly, the positive effect on thermotolerance in bromegrass (*Bromus inermis* Leyss.) cell-cultures was reported (Wilen et al., 1995). However, a study on moth bean (*Vigna aconitifolia* Jacq.) found no enhancement of heat stress tolerance or antioxidant activity by BR. Compared to other hormones, such as ABA or SA, less studies have been done on the role of BR against environmental stresses. More investigation on other plant species would be needed, such as on cool-season turfgrasses.

Cytokinins (CKs)

Contrary to ABA and ethylene as stress hormones that promote leaf senescence under environmental stress, CKs are known to delay it, which is more desirable in turfgrass management. CKs are a group of plant hormones that play an important role in many growth and developmental processes, including promoting cell division and differentiation, enhancing chloroplast development, and counteraction of senescence (Mok and Mok, 2001). The biosynthesis of CKs in plants occurs mainly in root tips with transport to shoots via the xylem (Arteca, 1996). There are large varieties of natural and synthetic compounds

belonging to the CK group. Previous studies have proposed three major CK groups: active forms, translocation forms, and storage forms (Sakakibara, 2006). Natural plant cytokinins are adenines and adenine derivatives which have been substituted at the N^6 terminal either with an aromatic side chain (aromatic cytokinins, such as benzyladenine and its derivatives) or an isoprene-derived side chain (isoprenoid cytokinins, such as zeatin and its derivatives). Free-base cytokinins have been suggested to be active, such as isopentenyladenine (iP), and *trans*-zeatin (*t*Z), whereas cytokinin nucleosides show low activity, such as *trans*-zeatin riboside (*t*ZR) (Delaney, 2004). Zeatin and its derivatives are considered to be the most prevalent base in plants, especially those with a trans-hydroxylated N^6-side chain.

Heat stress affects CK synthesis adversely, with the reduction of CKs being reported in different species under stress, such as *Nicotiana rustica* (L.), and *Phaseolus vulgaris* (L.) (Itai et al., 1973), winter rape (*Brassica napus* L.) (Zhou and Leul, 1999), and creeping bentgrass (Liu and Huang, 2005). In creeping bentgrass, reduction of CK levels is as early as five days of heat stress in roots and is correlated with decreased root biomass. Moreover, application of exogenous CKs has been shown to have potential in alleviating heat injury (Skogqvist, 1974). In creeping bentgrass, retarded leaf senescence and reduced cell membrane lipid peroxidation was observed via exogenous zeatin riboside application (Liu and Huang, 2002). In addition, the beneficial effects of extended day-length on creeping bentgrass under heat were partly attributed to higher endogenous cytokinins content in leaf tissues (Wang et al., 2004). Enhanced antioxidant response was suggested as a possible mechanism for the observed reductions in heat injury due to exogenous CK application (Liu et al., 2002; Wang et al., 2003). However, the exact mechanism of how CKs improve creeping bentgrass performance

under heat stress is far from conclusive. Other mechanisms, such as nitrogen metabolism or HSPs may also be involved.

In a summary, many heat-induced processes at different levels can be mediated by hormones themselves or an altered hormonal status/balance by heat stress (Hoffmann and Parsons, 1991). Better thermotolerance could be achieved if hormonal control of plant processes in the thermotolerant state is optimized (Maestri et al., 2002). Although extensive research has been conducted on heat stress in plants, the understanding of plant hormones and/or plant growth regulator involvement in thermotolerance is still far from completion, especially in turfgrasses.

Nitrogen Metabolism and Heat Stress

Nitrogen is an essential component in many important organic compounds, such as amino and nucleic acids. As a result, plants require greater amounts of nitrogen than of any other mineral nutrient (Epstein and Bloom, 2005). Nitrate (NO_3^-) normally is the major nitrogen form existing in well-aerated soil, although ammonium (NH_4^+) is the preferred inorganic nitrogen source (Bloom, 1997). Moreover, once nitrate is absorbed from soil by roots, it can be translocated and stored at higher levels than ammonium, with ultimate assimilation into amino acids. The first step of nitrate assimilation is the reduction of nitrate to nitrite (NO_2^-) in the cytoplasm by nitrate reductase (NR) (Kaiser et al., 1999). The reaction is as follows: $NO_3^{--} + NAD(P)H + H^+ + 2\ e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O$. Given nitrite's potential toxicity, it is immediately transported into leaf chloroplasts or root plastids. Next it is reduced into ammonium by nitrite reductase and rapidly enters into the ammonium assimilation pathway to amino acids (Epstein and Bloom, 2005). Glutamine synthetase (GS)

and glutamate synthetase (GOGAT) are the two key enzymes involved in the ammonium assimilation pathway (Ruiz et al., 1999).

The step from nitrate to nitrite is rate limiting in the overall nitrate assimilation pathway regulated by nitrate reductase (Botrel and Kaiser, 1997). Nitrate reductases in higher plants are homodimers composed of two identical subunits (Campbell, 1996), and they are tightly regulated by many factors (Kaiser et al., 1999). The activity of nitrate reductase increases with light, carbohydrate, nitrate concentration and cytokinin, with enhanced activity thought to be due to denovo synthesis of enzyme and protein dephosphorylation (Kaiser et al., 1999).

Temperature has been reported to affect nitrate reductase level in creeping bentgrass. The NR activity, not synthesis of the enzyme, was reduced when grown at 35 ^{o}C (Kaufmann et al., 1971). Cui et al. (2006) observed high temperature decreased the activities of NR, GS, and GOGAT in two tall fescue cultivars. Similar results were also reported by Xu and Zhou (2006) in a perennial grass (*Leymus chinensis* Trin.). As mentioned previously, biological membrane integrity begins to unravel with increasing temperature, which increases the leakage of inorganic nutrients. Moreover, nutrient influx can not keep up with nutrient efflux when temperature is above a certain level. In creeping bentgrass, root N, P, and K content declines after exposure to 35 ^{o}C for 15 to 20 days (Huang and Xu, 2000; Liu and Huang, 2005). Cultivars with better heat tolerance tended to maintain a higher nutrient element status. More recently, Rachmilevitch et al. (2006) reported NO_3^- assimilation rate decreased with increasing soil temperatures in both *Agrostis scabra* (Willd.) and *A. stolonifera* (L.), accompanied by decreased root viability, indicating a relation between heat tolerance and carbon and nitrogen allocation pattern. Another study (Fu and Huang, 2003a) demonstrated that foliar nutrient application, including inorganic nitrogen, improved heat tolerance of

creeping bentgrass manifested by slowed leaf senescence and maintenance of photosynthetic activities.

Interaction of Nitrogen and Cytokinins

Inorganic nitrogen, especially nitrate, is not only required for nitrogen assimilation, but also serves as a signal to regulate many metabolic processes, including CK biosynthesis (Sakakibara et al., 2006). CK levels increase when plants are supplied with nitrogen and decrease inversely (Schachtman and Shin, 2007). The induction of CKs by NO_3^- supplementation has been shown in barley (Samuelson and Larsson, 1993), maize (Sakakibara et al., 1998; Takei et al., 2001) and *Arabidopsis* (Takei et al., 2002). In *Arabidopsis* seedlings grown under nitrogen limited conditions, the expression of *AtIPT3,* a gene encoding adenosine phosphatesisopentenyltransferase which has been identified in CK biosynthesis, was rapidly induced by NO_3^- accompanying the accumulation of CKs (Takei et al., 2004), indicating a *de novo* synthesis of cytokinin.

There is a functional balance between shoot and root growth determined by resource levels and plant hormone levels (CKs and auxin). In general, as nutrient levels in plants increase, CK level increases, but auxin levels decrease, which result in stimulated shoot growth, but decreased root growth (Epstein and Bloom, 2005). For example, the root-to-shoot allocation of CKs is influenced by nitrogen (Takei et al., 2002; Sakakibara, 2006), which is well correlated with observed changes in biomass allocation between roots and shoots (Sakakibara, 2006). In addition, Kuiper et al (1989) observed the sum of CKs in BA (benzyladenine)-treated *Plantago major* (L.) plants at low nutrient level was similar to that in plants grown at the high nutrient level. These results suggest a role of CKs in regulating

growth responses to changes in mineral nutrition. In reverse, exogenous CK application has been shown to affect nitrogen partitioning and maintain a relatively higher leaf nitrogen content in wheat (Simpson et al., 1982), *Urtica dioica* (L.) (Beck, 1996), and rice (*Oryza sativa* L.) (Ookawa et al., 2004). A higher leaf nitrogen level was suggested through the accumulation of nitrogenous compounds (such as amino acids) by increasing partitioning to leaves (Jordi et al., 2000; Ookawa et al., 2004). And the higher nitrogen content is well correlated to higher leaf Rubisco (ribulose-1,5-bisphosphate carboxylase oxygenase) content during leaf senescence indicating a higher photosynthesis potential (Ookawa, 2004).

As one of the key enzymes to regulate overall nitrogen assimilation, nitrate reductase (NR) activity can be induced by nitrate and CK alone or in combination. Although CK and nitrate are reported to induce the activity of NR additively by stimulating *de novo* synthesis of NR, enhanced NR activity by CK is suggested to be a direct effect, instead of via nitrate (Henalatha, 2002; Kende et al., 1971; Zielke and Filner, 1971). In addition, the response of NR to CK can be repeated and is independent of light (Henalatha, 2002; Kende et al., 1971), which resulted in the shift of the circadian pattern of NR activity (Steer, 1976).

In summary, although heat stress has been extensively studied in higher plants, the exact physiological responses and mechanisms are complex and still being resolved, especially in cool-season turfgrasses. There are various methods to achieve the goal of improved heat tolerance of cool-season turfgrasses, such as newly developed cultivars by traditional breeding, modern genetic transformation, heat acclimation, and exogenous application of plant growth regulators. Among all the available methods, exogenous application of plant growth regulators is more practical on golf greens since this approach is easily incorporated into standard management practices. Moreover, CKs and natural products

containing CKs, have been documented to have positive impacts on creeping bentgrass under heat stress, with their role in enhancing antioxidative response being emphasized (Liu and Huang, 2002; Wang et al., 2003; Zhang and Ervin, 2008). However, other mechanisms may be important for improved heat stress response, such as induction of HSPs (Heckathorn et al., 1999) and nitrogen metabolism. For example, proper nitrogen application has been shown to alleviate heat injury and/or improve heat tolerance in creeping bentgrass, and there is accumulating evidence of CK and nitrogen interactions, particularly their additive effects on NR, but little information about how CKs affect creeping bentgrass grown at variant nitrogen levels under heat is available. Thus we hypothesize that foliar application of CKs will improve creeping bentgrass performance under heat, with positive effects varying to some extent with nitrogen availability. Further, we hypothesize that positive heat tolerance effects will be associated with induction of specific HSPs and improved nitrogen metabolism, particularly nitrogen use efficiency, characterized by higher activities of nitrate reductase and GOGAT. Therefore, the overall objective of this study is to characterize the function and action mechanisms of CKs in creeping bentgrass grown at different nitrogen levels under heat. The specific research objectives will be:

1) to determine cytokinin status (concentration, formation and distribution) and other physiological responses in creeping bentgrass under heat stress;

2) to explore effects of cytokinin and nitrogen on antioxidant metabolism in shoots and roots of heat stressed creeping bentgrass.

3) to investigate the activity and expression of nitrate reductase, GOGAT, and other nitrogen metabolic enzymes in creeping bentgrass treated with cytokinin and nitrate;

4) to examine HSP expression patterns in cytokinin and nitrogen treated creeping bentgrass under heat stress;

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CHAPTER TWO

CIRCADIAN PATTERNS OF THE MAJOR NITROGEN METABOLISM-- RELATED ENZYMES AND METABOLITES IN CREEPING BENTGRASS AND THE INFLUENCE OF CYTOKININ AND NITRATE

ABSTRACT

Creeping bentgrass (*Agrostis Stolonifera* L.) is a C3 perennial grass species widely used as a major low-cut turfgrass species worldwide. Little information is available for the diurnal variation in nitrogen metabolism of creeping bentgrass, particularly the impact of cytokinin (CK) and nitrate. To gain an insight into the diurnal fluctuation of nitrogen metabolism and effect of CK and nitrate the transcript abundance of the major nitrogen metabolism enzymes in shoots were studied during the day/night cycle (14/8 h), including nitrate reductase (NR), nitrite reductase (NIR), plastidic glutamine synthetase (GS2), ferredoxin-dependent glutamate synthase (Fd-GOGAT), and glutamate dehydrogenase (GDH). In the meanwhile, changes in the nitrogen metabolites (total protein, total free amino acids, nitrate, and ammonium) were monitored. All the measured parameters exhibited clear diurnal changes with either similar or even higher levels between certain phase of light and dark period, except *GS2* expression and total protein. Both *NR* expression and nitrate content in shoots showed a peak at 4:30 (8.5 h in dark), indicating a close relationship between diurnal changes of these two. Nitrate nutrition increased diurnal variation of nitrate content, showing an over 2-fold diurnal difference instead of a 63% and 81% difference for control and grass treated

with CK, respectively. Simultaneously, CK shifted the diurnal *in vivo* NR activity pattern. These results together indicate that creeping bentgrass appears to be able to maintain nitrogen assimilation at night comparable to that during the day, suggesting a nighttime nitrogen use strategy in creeping bentgrass.

Keywords: nitrate, zeatin riboside, nitrogen metabolites, diurnal change, creeping bentgrass

INTRODUCTION

Nitrogen is the most required inorganic nutrient and it is an essential component in many important organic compounds in plants, such as amino and nucleic acids, proteins, and plant hormones (Pessarakli, 2002). Nitrate (NO_3^-) in the soil is normally the main nitrogen source for most higher plants, especially in well-aerated soil (Bloom, 1997). Once nitrate is absorbed from soil by roots, it can be translocated and stored at higher levels than ammonium, with ultimate assimilation into amino acids (Buchanan et al., 2000). The first step of nitrate assimilation is the reduction of nitrate to nitrite (NO_2^-) in the cytoplasm by nitrate reductase (NR) (Kaiser et al., 1999). The reaction is as follows: $NO_3^{--} + NAD(P)H + H^+ + 2\ e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O$. Given nitrite's potential toxicity, it is immediately transported into leaf chloroplasts or root plastids, and then it is reduced into ammonium by nitrite reductase (NIR). Next ammonium rapidly enters into the ammonium pathway to be assimilated into the glutamine amide group, which is then transferred to 2-oxoglutarate, producing two molecules of glutamate by the concerted reaction of glutamine synthetase (GS) and glutamate synthase (GOGAT). Following the formation of glutamate, the α-amino group can be transferred to a wide variety of 2-oxo acid acceptors to form other amino acids in plants (Epstein and Bloom, 2005; Igarashi et al., 2009; Ruiz et al., 1999). Glutamate dehydrogenase (GDH) is another important enzyme in plant nitrogen metabolism, which can either synthesize or catalyze glutamate (Melo-Oliveira et al., 1996). Under normal conditions GDH is responsible for the deamination of glutamate (Masclaux-Daubresse et al., 2006a; Purnell and Botella, 2007; Skopelitis et al., 2007). GDH might be also responsible for incorporating ammonium generated in senescing leaves into glutamate, and subsequently into glutamine by GS (Lam et al., 1996; Miflin and Habash, 2002).

Daily oscillation of nitrogen metabolism-related enzymes and metabolites is common but important for the normal function of plants, such as carbon/nitrogen balance (Huppe and Turpin, 1994; Masclaux-Daubresse et al., 2002; Stitt et al., 2002). NR regulates the rate limiting step in the overall nitrate assimilation pathway (Kaiser et al., 1999). In higher plants, NR is tightly regulated by many factors, including nitrate concentration, cytokinin, and light (Rouzé and Caboche, 1992). Diurnal variation of NR activity (NRA) and/or mRNA level were reported in many plant species, such as tobacco (*Nicotiana tabacum* L.) (Scheible et al., 1997), tomato (*Lycopersicon esculentum* L.) (Tucker et al., 2004), *Arabidopsis* (Schaffer et al., 2001), maize (*Zea mays* L.) (Huber et al., 1994), barley (*Hordeum vulgare* L.) (Lillo, 1984), and kentucky bluegrass (*Poa pratensis* L.) (Jiang and Hull, 2000). In general, the activity of leaf NR increases early in the day and decreases during the later part of the day and the night. Nitrate reductase mRNA has a peak at the beginning of the photoperiod as well (Jiang and Hull, 2000; Rouzé and Caboche, 1992; Stitt et al., 2002). Similarly, diurnal changes of activity, mRNA level, and /or protein level of NIR, GS, GOGAT, and GDH have also been reported in different plant species (Debouba et al., 2006; Lillo, 1984; Masclaux-Daubresse et al., 2002; Rouzé and Caboche, 1992; Stohr and Mack, 2001). However, the diurnal patterns of the enzymes that catalyze the major steps of inorganic nitrogen assimilation appear to depend on plant species, growth stage, and other factors. For example, the expression of chloroplastic glutamine synthetase (GS2) was found to be light-induced and show diurnal rhythmicity in tomato, but not in tobacco (Becker et al., 1992). Seedlings of colonial bentgrass (*Agrostis capillaries* L.) showed no diurnal changes of NR activity in leaves, but 2-week-old plants exhibited an activity peak of NR after 3 h into the photoperiod (Harris and Whittington, 1983).

Cytokinin (CK) or natural products containing CK have received increased attention in the agriculture and turfgrass industries (Schmidt et al., 2003). Cytokinin is a plant hormone regulating the proliferation and differentiation of plant cells, delaying leaf senescence, and functions as a signal to affect nitrogen metabolism in plants (Sakakibara et al., 2006). For instance, other than nitrate, CK can also induce NRA. Enhanced NRA by CK can be repeated and is independent of light (Henalatha, 2002; Kende et al., 1971), and has been shown to result in shifts of the circadian pattern of NRA (Steer, 1976). In addition, exogenous CK application has been shown to affect nitrogen partitioning and function to maintain a relatively higher leaf nitrogen content compared with the control in several plant species, such as wheat (*Triticum aestivum* L.) (Simpson et al., 1982), and rice (*Oryza sativa* L.) (Ookawa et al., 2004). A higher leaf nitrogen level was suggested through the accumulation of nitrogenous compounds (such as amino acids) by increasing partitioning to leaves (Jordi et al., 2000; Ookawa et al., 2004).

Creeping bentgrass is an important perennial grass species used for forage and low-cut turfgrass in golf. Harris and Whittington (1983) reported effect of light duration on NRA in creeping bentgrass. Other than that, little is known about the diurnal regulation of these major nitrogen metabolism enzymes in shoots of creeping bentgrass, particularly the impact of CK and nitrate. Thus the objectives of this study were to determine the diurnal gene expression patterns of the major nitrogen metabolism enzymes in shoots of creeping bentgrass, and to investigate the influence of CK and nitrate on the diurnal expression patterns. The study would provide further information for understanding how CK and nitrate regulate nitrogen metabolism in creeping bentgrass.

MATERIALS AND METHODS

Plant Materials and Treatments

Mature, healthy 'A-4'creeping bentgrass sod plugs (10-cm diam. by 8 cm deep) were collected from a golf green with a root zone that meets USGA (United State Golf Association) sand mix (30 cm deep) specifications at the Turf Research Center at Virginia Tech, Blacksburg, VA. The plugs were trimmed to 2.5-cm thick and then grown in plastic pots (14-cm diameter, 14.5-cm depth) filled with fine to medium particle size diameter sand in the greenhouse for 60 d before being moved into a growth chamber. The grass was fertilized with Bulldog fertilizer (28-8-18, SQM North America, Atlanta, GA) at 5 kg N ha^{-1} every week over the whole experimental period. Growth chamber conditions were a 14 h photoperiod (6:00 to 20:00), 450 μmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR), and 20°C /15°C (day/night) for 7 d before treatments were applied. Grass was hand-clipped at 25 mm using an electric shear (twice a week) throughout the project, but was withheld after being placed in the growth chamber so as to have enough shoot material for diurnal sampling. Grasses in pots were sprayed with 0.05% Tween 20 solution on the foliage or CK (*trans*-zeatin riboside, *t*ZR) at 50 μM in 0.05% Tween 20 solution (500 μL per pot) combined with fertilization without or with nitrogen (nitrate) nutrient at 7.5 kg N ha^{-1} (60 mL Hoagland's solution per pot) (Epstein and Bloom, 2005) at day 0. Both KNO_3 and $Ca(NO_3)_2$ were used as the nitrate sources in the solution. Extra KCl and $CaCl_2$ were used to equalize K and Ca in the nutrient solution for no nitrogen treatments. Irrigation was applied as necessary in the morning to prevent water deficit. The three treatments with 4 replications are listed as below:

1) Control: No nitrate nitrogen, no CK
2) CK: No nitrate nitrogen, CK at 50 μM

3) Nitrate: Nitrate nitrogen at 7.5 kg N ha^{-1} , no CK

Sampling and Measurements

Shoots were harvested five times at 9:30, 13:30, 17:30, 22:30 and 4:30 during the first photoperiod, and at 9:30 in the second photoperiod. All samples were immediately frozen with liquid nitrogen and stored at -80 ^{o}C until analysis, except the portion used for *in vivo* NRA assay.

Shoot NO_3^-, NH_4^+, amino acids and total soluble protein

For analysis of shoot NO_3^-, NH_4^+, amino acids and total soluble protein, one gram of liquid nitrogen powdered shoot tissue was extracted two times for 30 min at 4 ^{o}C in 5 mL of ice-cold deionized distilled (dd) water. After centrifugation at 4000 *g* for 10 min, supernatants were collected and pooled. The pooled supernatants were then filtered through Whatman #42 filter papers before further analysis. Nitrate was estimated using a Lachat QuickChem 8000 Flow-Injection Auto-Analyzer (Lachat Instruments, Milwaukee, WI) with a Cd-reduction column to reduce NO_3^- to NO_2^-. The same analyzer was used for NH_4^+ analysis. Amino acids in the supernatant were determined using ninhydrin with glycine as a standard (Rosen, 1957). In detail, a 200 µL aliquot from the extraction above was used, and the final volume in assay tubes was adjusted to 750 µL with dd H_2O. Then 0.5 mL of 0.2 mM cyanide solution (in 0.2 M acetate buffer, pH 5.3) and 0.5 mL of 3 % (w/v) ninhydrin solution (in ethylene glycol monomethyl ether) were added into the tubes. Tubes were then covered with marbles and boiled for 20 minutes. Tubes were next removed from the water bath and a 5 mL mixture of isopropyl alcohol and water (1:1, v:v) was added. Tubes were

vortexed and allowed to cool to room temperature before being read at 570 nm. All the samples and standards were run in duplicate. For total soluble protein content, a 25 µL aliquot of extract was assayed using BCA reagent (Sigma, USA). The reaction mixture was read at 562 nm after 30 min incubation at 37 °C with bovine serum albumin (BSA) as the standard (Smith et al., 1985).

In vivo nitrate reductase activity

The *in vivo* activity of leaf NR was estimated by using the method of Chanda (2003) with some modifications. Briefly, about 150 mg fresh shoot tissue was cut into 0.5-cm lengths. Then the shoot sections were immersed in 15 mL of 0.05 M potassium phosphate buffer (pH 7.5) with 1% *n*-propanol and 50 mM KNO_3. The samples were vacuum-infiltrated for 4 min to insure infiltration of incubation buffer and then incubated in the dark for 4 h at 30 °C. At the end of the incubation, one milliliter solution was transferred to 10 mL glass culture tubes. The nitrite formed was estimated colorimetrically by adding 750 µL of 1% sulfanilamid in 3 M HCl, and 750 µL of 0.02% N-naphthyl-ethylenediamine hydrochloride. Absorption was determined at 540 nm. For each run, blanks and four nitrite standards (1, 5, 10 and 25 µM KNO_2) were included.

Gene expression analysis

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from the shoots using a commercial RNA isolation reagent (TRI REAGENT® RT, Molecular Research Center Inc., USA). Briefly, about 100 mg liquid nitrogen powdered shoot tissue were mixed with 1 mL TRI Reagent. After being briefly shocked for 2 min, the

homogenate was stored for 5 min at room temperature. Next, 0.2 mL chloroform was supplemented, and the mixture was shaken vigorously for 30 seconds. The resulting mixture was stored at room temperature for 10 min and centrifuged at 12,000 g for 15 minutes at 4 ^{o}C. Then about 500 µL upper aqueous phase was transferred to a new 1.5 mL microtube. RNA from the aqueous phase was precipitated by mixing with 0.5 mL isopropanol. The mixture was stored at 4 ^{o}C for 10 min and centrifuged at 12,000 g for 10 minutes at 4 ^{o}C. The supernatant was removed and the RNA pellet was washed with 1 mL 75% (v) ethanol and subsequent centrifugation at 10,000 g for 5 min at 4 ^{o}C. After removing the ethanol wash, the RNA pellet was briefly air-dried for 3 to 5 min and dissolved into 75 µL RNase-free double deionized water (treated with diethyl pyrocarbonate). The isolated RNA was quantified with a NanoDrop 8000 Spectrophotometer and then used for RT-PCR. First-strand cDNA synthesis was conducted with RT random primers and MultiScribe reverse transcriptase (Applied Biosystems, USA). PCR reactions were performed with a Bio-rad thermocycler (Bio-rad, USA). The primers used for RT-PCR were as follows (in parentheses): *NR* (5′-TACACSATGAAGGGATACGC-3′ and 5-′ ACCAGAAGCACCAGCACC -3′), *NIR* (5′-CTCAAGTGGCTYGGCCTYT-3′ and 5′-CMTTGTCCATGCCRCTCTG-3′), *GS2* (5′-ATGCCTGGKCAGTGGGAGTWCCA-3′ and 5′-CTTGGTGCTGTARTTKGTGTG -3′), *Fd-GOGAT*-(5′-CCTGGAGGTGAATATCATKS-3′ and 5′-GAAGTGTTGRA GAATAMCCATC-3′), *GDH* (5′-AACATTCARGGGTTYATGTGGGA-3′ and 5′-ACT CCCAGGGTGAAKGCBCCCAT-3′), and *18s* (5′- GTGACGGGTGACGGAGAATT-3′ and 5′- GACACTAATGCGCCCGGTAT-3′). *18s* was used as an inner standard of gene expression semi-quantified with RT-PCR. Aliquots of individual PCR products were

resolved by agarose gel electrophoresis. Optical density of bands was determined with the Bio-rad Quantity One image system (Bio-rad, USA).

Experimental Design and Statistical Analysis

The experiment was a randomized complete block design with four replicates. All measurements were analyzed using the samples collected at the sampling time mentioned above. Data were analyzed using PROC GLM (SAS Institute, Version 9.1, Cary, NC). Treatment means were separated using Fisher's–protected Least Significant Difference (LSD) test at a 0.05 significance level, except as otherwise stated herein.

RESULTS AND DISCUSSION

There was interaction between sampling time of the day and treatment for shoot nitrate content (p=0.025) and NRA (p=0.096), indicating a difference in the diurnal pattern of shoot nitrate content and NRA among treatments. In addition, grass treated with nitrate maintained higher nitrate content and NRA than the control at all sampling times, except at 9:30 in the second photoperiod, but only higher NRA at 17:30 for grass treated with CK was observed when compared to control (Fig.2-1, 2). For the grass treated with nitrate, the nitrate content continued to increase from 9:30 of the first photoperiod (3.5 h in light) at 12.3μg g^{-1} fresh weight (FW) to a peak of 39.1 μg g^{-1} FW at 4:30 (8.5 h in dark) in the morning. Similar peaks at 4:30 were also observed for both grasses under CK and control, but to a much lower extent. However, unlike the grass treated with either nitrate or CK, shoot nitrate content in control plants at 4:30 was not significantly different from other sampling times. Meanwhile, there was a peak of *NR* gene expression at 4:30 regardless of treatment (Fig.2-4), with the

grasses treated with nitrate or CK having relatively higher expression levels at certain sampling times (9:30, 13:30, and 4:30).

Plants cannot use nitrate directly in metabolism; it must first be reduced to NH_4^+ (Schulze et al., 2005). Nitrate reductase catalyzes the first step of nitrate reduction to nitrite in the cytosol, with the nitrite being further reduced to NH_4^+ in chloroplasts by NIR. There are two major forms of NR in higher plants, NADH-NR and NAD(P)H-NR. NADH-NR is found in both leaves and roots. It is the most common form of the enzyme, and constitutes the majority of the total NRA, while NAD(P)H-NR is mainly found in non-green tissues, such as roots (Pessarakli, 2002 ; Taiz and Zeiger, 2002). Most higher plants have been shown to have several *NR* genes. For example, in both Arabidopsis and diploid wheat (*Triticum monococcum* L.), there are at least two *NR* genes encoding NADH-NR (Wilkinson and Crawford, 1993; Zhou et al., 1994). The primers we designed here would not distinguish all these different NR genes. NRA and/or *NR* expression and nitrate content change diurnally (Jiang and Hull, 2000; Stitt et al., 2002; Stohr and Mack, 2001). The peak of *NR* gene expression at 4:30 (8.5 h in dark) was in accordance with the peak of nitrate content at the same time. Nitrate is one of the most critical factors required for the expression of *NR* genes in plants. Nitrate content tends to vary accordingly with *NR* gene expression (Stitt et al., 2002). Similarly, the maximum NO_3^- level observed in the sap has been reported to be correlated with a peak of NRA in the decapitated leaves of *Capsium annuum* (L.) (Steer, 1976). It should be noted that the diurnal oscillation of *NR* expression was not totally coincident with the fluctuation of NRA, indicating activation processes in addition to the regulation at mRNA level, such as post-translation. Indeed, post- translation regulation of NR is well known (Campbell, 1999; Kaiser and Huber, 2001; Kaiser et al., 1999; Lillo et al.,

2004). Nitrate induces the activity of NR and mRNA encoding NR in a variety of plant species and tissues (Kaiser et al., 1999; Rouzé and Caboche, 1992). Our findings showed the same regulation mechanism of NR by nitrate in creeping bentgrass (Fig.2-1, 4). Similar to nitrate, CK showed its induction effect on NRA and *NR* mRNA, but to a lesser extent (Fig. 2-1, 4). This is consistent with many previous studies (GaudinovÁ, 1990; Kende et al., 1971; Lu et al., 1992). In addition, grass treated with CK showed a slightly different diurnal NRA pattern compared to the grasses under other two treatments with an earlier peak (17:30). Steer (1976) reported CK application resulted in the shift of the NRA peaks during light/dark cycle. A possible explanation could be that CK can induce NRA independent of light (Henalatha, 2002; Kende et al., 1971).

In addition to nitrate, the contents of ammonium and free amino acids in shoots showed a slight diurnal fluctuation. Data were presented as averages across treatments because there were no interactions between sampling time and treatment (p=0.135 for ammonium and 0.602 for amino acids). For ammonium, the content increased during the light period and showed a trend of slightly decreasing in the dark phase. Photorespiration represents a major source of ammonium in leaves of C3 plants (Ogren, 1984), and the accumulation of ammonium during the light period might be due to photorespiration (Matt et al., 2001b). Amino acids stayed at a higher level in the light period than in the dark phase (Fig.2-3). Diurnal change of ammonium content was reported in tobacco (Lea et al., 2006; Scheible et al., 1997) and other plant species (Ourry et al., 1996), but little time-wise variation was found in ammonium concentration in Kentucky bluegrass (Jiang and Hull, 2000). Similarly, contradictory results about diurnal fluctuation of amino acids were also reported (Ferrario-Méry et al., 2002; Matt et al., 2001b). An explanation for this apparent difference could be

due to either species, or tissue sampled. Consistent with previous studies (Ferrario-Méry et al., 2002; Stohr and Mack, 2001; Viswanathan et al., 1962), there was no clear diurnal fluctuation for total protein content.

Nitrite reductase in chloroplasts or root plastids reduces nitrite to ammonium (Taiz and Zeiger, 2002). The expression of *NIR* exhibited a diurnal fluctuation, and the overall pattern during the first photoperiod was similar to the *NR* expression pattern, but at a smaller scale (Fig.2-5), indicating a co-regulation of *NIR* and *NR* expression in creeping bentgrass. *NR* and *NIR* genes are co-regulated in plants, which has been considered as a mechanism to prevent the accumulation of nitrite, a toxic intermediate (Faure et al., 1991, Klepper, 1975). Interestingly, there was a sharp increase of *NIR* mRNA at the beginning of the second light period (Fig.2-5). It is possible that light was the cause since *NIR* is light inducible (Schaffer et al., 2001; Vincentz et al., 1993), but there potentially are other unknown factors involved, so we do not know exactly why there was no such a peak at the early stage of the first light phase.

The GS/GOGAT cycle is the principle route for NH_4^+ assimilation in plants, whether it results from NO_3^- reduction by NR and NIR, photorespiration or amino acid deamination. GS catalyzes the ATP-dependent assimilation of ammonium into glutamine with glutamate as a substrate. GS functions in a cycle with GOGAT, which catalyzes the reductive transfer of the amide group from glutamine to α-ketoglutarate, forming two molecules of glutamate (Buchanan et al., 2000; Pessarakli, 2002). There are two major isoenzymes of GS that are located in different subcellular compartments and display non-overlapping roles. GS1, the cytosolic isoform, is the predominant enzyme in roots and non-photosynthetic tissues and is much less abundant in green tissues than GS2, the plastid-located isoform (Ireland and Lea,

1999; Taiz and Zeiger, 2002). GS2 is dominant in the leaves of many angiosperms, and it is responsible for the assimilation of ammonium derived from nitrate reduction and photorespiration (Ireland and Lea, 1999; Lam et al., 1996). GOGAT is present in two distinct forms, one that uses reduced ferredoxin (Fd-GOGAT) as the electron donor and one that uses NADH (NADH-GOGAT). The Fd-dependent enzyme is the predominant GOGAT isoenzyme in leaves and can account for over 90% of total leaf GOGAT activity, as determined in *Arabidopsis* and barley (Buchanan et al., 2000; Lam et al., 1996). The NADH-dependent enzyme, which is present in various plastids, is located predominantly in non-photosynthesizing cells, where reductant is supplied by the pentose phosphate pathway (Bowsher et al., 2007; Igarashi et al., 2009). Such organ-specific distribution suggests a major role for Fd-GOGAT in primary nitrogen assimilation and photorespiration in leaves (Lea and Miflin, 2003). Here we chose to investigate the expression of *GS2* and *Fd-GOGAT* considering GS2 and Fd-GOGAT are predominant isoforms for GS and GOGAT in leaves respectively, and the GS2/Fd-GOGAT cycle is the major pathway for ammonium assimilation in higher plants (Ferrario-Mery et al., 2002).

The expression level of *GS2* showed no clear diurnal changes for grasses under all three treatments (Fig.2-6). Different results concerning the day-night variation of *GS2* expression have been reported in the literature. Becker et al. (1992) reported that the *GS2* mRNA pools in tobacco leaves were light regulated, but exhibited no diurnal fluctuation. Ferrario-Mery et al. (2002) also reported no diurnal changes in amount of *GS2* mRNA in tobacco. Masclaux-Daubresse et al. (2002) used *GS2* mRNA as a negative control when investigating the day-night fluctuation of *GDH* expression. However, Matt et al. (2001a, b) observed that the transcript for plastid glutamine synthetase (GS2) is low at the end of the night and increases

during the day. The discrepancy regarding diurnal variation of *GS2* expression in the literature could be due to different factors, such as species difference, or different growth conditions. *GS2* expression does not change in response to CK addition in wheat (Caputo et al., 2009), which is consistent with our finding herein. Nitrate was reported to up-regulate *GS2* in several studies (Scheible et al., 2004; Wang et al., 2003; Wang et al., 2004). However, other studies reported *GS2* expression was independent of nitrate (Chen and Silflow, 1996; Mattana et al., 1994). In the study herein, there was no apparent difference in *GS2* expression level at any sampling time between grasses treated with nitrate and not (Fig.2-6). The relatively unchanged *GS2* expression level among treatments is likely due to the requirement for a stable and ready NH_4^+ assimilation into non-toxic intermediate products, such as amino acids (Redinbaugh and Campbell, 1993), because NH_4^+ is toxic to plants at a relative high concentration (Temple et al., 1998).

Day-night fluctuation of Fd-GOGAT transcripts and/or activity in plants has been reported in some studies (Debouba et al., 2006; Ferrario-Mery et al., 2002; Suzuki et al., 2001; Valadier et al., 2008). For example, Suzuki et al. (2001) observed a higher mRNA level reached about 4 h after the onset of light, and then decreased to a minimal level at the middle of the day. Then, the mRNA steadily increased in the night. In the study herein, the grasses under nitrate or control showed similar overall diurnal change patterns of Fd-GOGAT. In general, the Fd-GOGAT level increased at the beginning of the light period, and peaked at late afternoon (17:30). Then it started to decrease until 22:30 at night, and increased again after (Fig.2-7). The recovery of Fd-GOGAT expression level during the late stage of dark period, combined with relatively stable *GS2* level during day-night cycle, indicated continuous and comparable NH_4^+ assimilation during the night period with that

during day time (Ferrario-Mery et al., 2002; Suzuki and Knaff, 2005). The decreased or unchanged levels of amino acid and protein in grass shoots during the same period suggested some of the assimilated nitrogen during dark period might not be primarily used for amino acid and protein synthesis but for other organic nitrogen compounds, perhaps, nucleic acid synthesis (Stohr and Mack, 2001). There was no clear diurnal fluctuation of Fd-GOGAT observed for grass treated with CK, although a similar light/dark trend was followed. Many reports have suggested that cytokinins mediate nitrogen metabolism (Sakakibara, 2003; Sakakibara et al., 2006), but it was not clear why the diminished diurnal fluctuation of Fd-GOGAT in grass treated with CK happened. There was no obvious difference in Fd-GOGAT expression level at any sampling time among treatments, except at 9:30 during the first photoperiod, when the grass treated with either nitrate or CK showed a relatively higher level of Fd-GOGAT than the control grass. Nitrate induces Fd-GOGAT mRNA level, particularly when in conjunction with illumination (Lea and Miflin, 2003; Pajuelo et al., 1997; Redinbaugh and Campbell, 1993; Sakakibara et al., 1997). To the best of our knowledge, there is little information about regulation of Fd-GOGAT by CK in plants. One of the primary functions of Fd-GOGAT is to re-assimilate NH_4^+ produced in photorespiration (Lea and Miflin, 2003). We assumed the higher Fd-GOGAT level could be due to the possible cytokinin-mediated occurrence of photorespiration in plants. Rivero et al. (2009) reported higher photorespiration in transgenic tobacco plants with a CK biosynthesis gene when compared with wild type plants, especially under water deficit condition.

GDH can catalyze both the synthesis and catabolism of glutamate. Plants have two types of GDH isoenzymes: one is NAD(P)H-dependent and is found in the chloroplast, and the other one is NADH--dependent localized to the mitochondria (Buchanan et al., 2000).

GDH may assimilate nitrogen when ammonium is plentiful, but it plays primarily a catabolic role, the deamination of glutamate under normal conditions to generate 2-ketoglutarate (Masclaux-Daubresse et al., 2006b; Melo-Oliveira et al., 1996; Miyashita and Good, 2008; Skopelitis et al., 2006).

Masclaux-Daubresse et al. (2002) reported diurnal fluctuation of *GDH* expression in tobacco leaves, increasing between 7:00 and 15:00 to 18:00, and then decreasing steadily from 18:00 to the end of the dark period (dark period: 22:00 to 6:00). Another study on maize reported that *GDH* mRNAs started to accumulate on illumination, reaching a maximum level at 12:00, and a second peak occurred at 0:00 (dark phase: 22:00–6:00) (Valadier et al., 2008). In the study herein, *GDH* expression exhibited diurnal fluctuation with the first peak level at 13:30 and a lower peak at 4:30 (dark period: 20:00 to 6:00) (Fig.2-8). The pattern of *GDH* during light period observed in our study is similar to the findings from both studies (Masclaux-Daubresse et al., 2002; Valadier et al., 2008). The second peak during dark period was not found in tobacco (Masclaux-Daubresse et al., 2002), and this different pattern during dark phase could be just due to species difference. Other factors, such as growth stage, can also affect diurnal pattern of *GDH* expression. For example, mature leaves showed diurnal variation of *GDH* expression, whereas young leaves did not (Masclaux-Daubresse et al., 2002). *GDH* expression is known to be able to be induced by carbon limitation and darkness (Buchanan et al., 2000; Miyashita and Good, 2008), and these could be the reasons for the second peak observed during night. Two-phase specific promoters and/or mRNA stability could be another explanation for two peaks of *GDH* (McClung et al., 2002; Valadier et al., 2008). There was no difference in the diurnal pattern of *GDH* among treatments. However, the grass treated with CK had generally higher expression level of *GDH* than the control, but

not the grass treated with nitrate (Fig.2-8). So far there is no report indicating that nitrate induces *GDH* expression in plants (Sakakibara et al., 2006). CK was found to stimulate GDH activity (Garg and Srivastava, 1992; Helena et al., 2004), which could be due to induced *GDH* expression. A later study in Arabidopsis reported CK can up-regulate *GDH1* and *GDH2* gene expression (Brenner et al., 2005). More recently, CK was suggested to directly regulate *GDH3* in Arabidopsis (Igarashi et al., 2009). The primers we used here would not distinguish different *GDH* genes. If the relatively higher *GDH* expression level in creeping bentgrass treated with CK is *GDH1, 2* or *3* is not clear.

In conclusion, our results show that the major nitrogen metabolism-related enzymes and metabolites exhibit daily oscillation, with some of the parameters being affected by either nitrate or CK treatment. In addition, creeping bentgrass appears to be able to maintain nitrogen assimilation at night comparable to that during the day, indicating a nighttime nitrogen use strategy could be important for the normal growth and development of creeping bentgrass.

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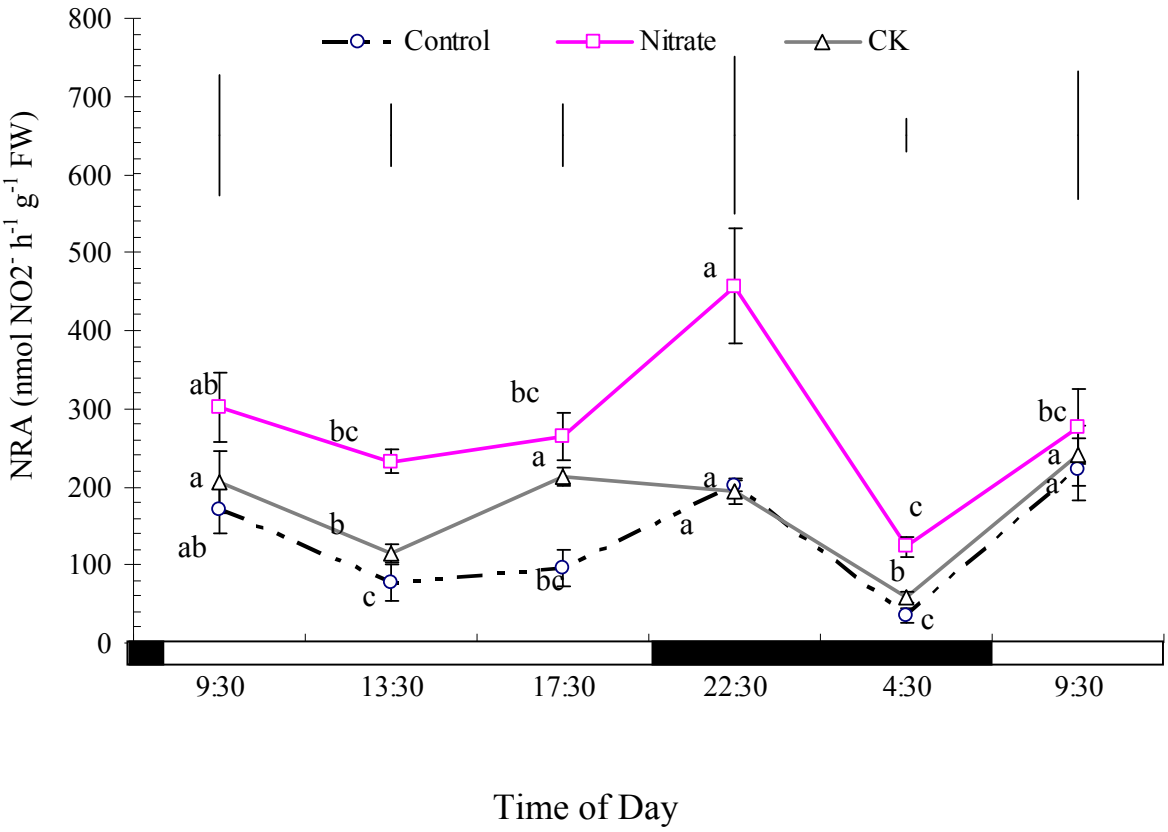


Fig.2-1. Daily fluctuation of shoot *in vivo* nitrate reductase activity (NRA) of creeping bentgrass treated with either 0.05% Tween 20 solution (Control), cytokinin (*trans*-zeatin riboside) at 50 μM (CK), or nitrate at 7.5 kg N ha^{-1} (Nitrate). Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively. Vertical bars indicate LSDs ($p \leq 0.05$) for treatment comparison at a given sampling time. Different letters indicate a significant difference ($p \leq 0.05$) between sampling times for each treatment. Error bars indicate standard error (n=4).

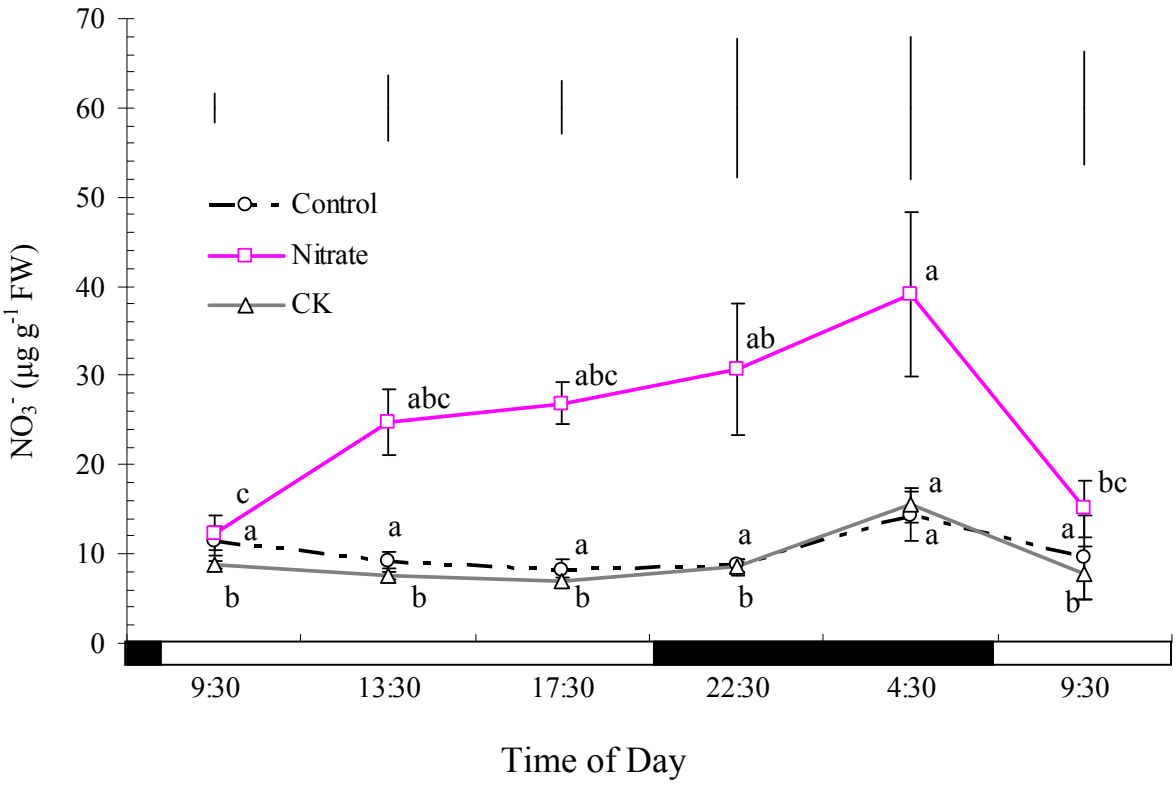


Fig.2-2. Daily fluctuation of shoot nitrate content of creeping bentgrass treated with either 0.05% Tween 20 solution (Control), cytokinin (*trans*-zeatin riboside) at 50 μM (CK), or nitrate at 7.5 kg N ha^{-1} (Nitrate). Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively. Vertical bars indicate LSDs ($p \leq 0.05$) for treatment comparison at a given sampling time. Different letters indicate a significant difference ($p \leq 0.05$) between sampling times for each treatment. Error bars indicate standard error (n=4).

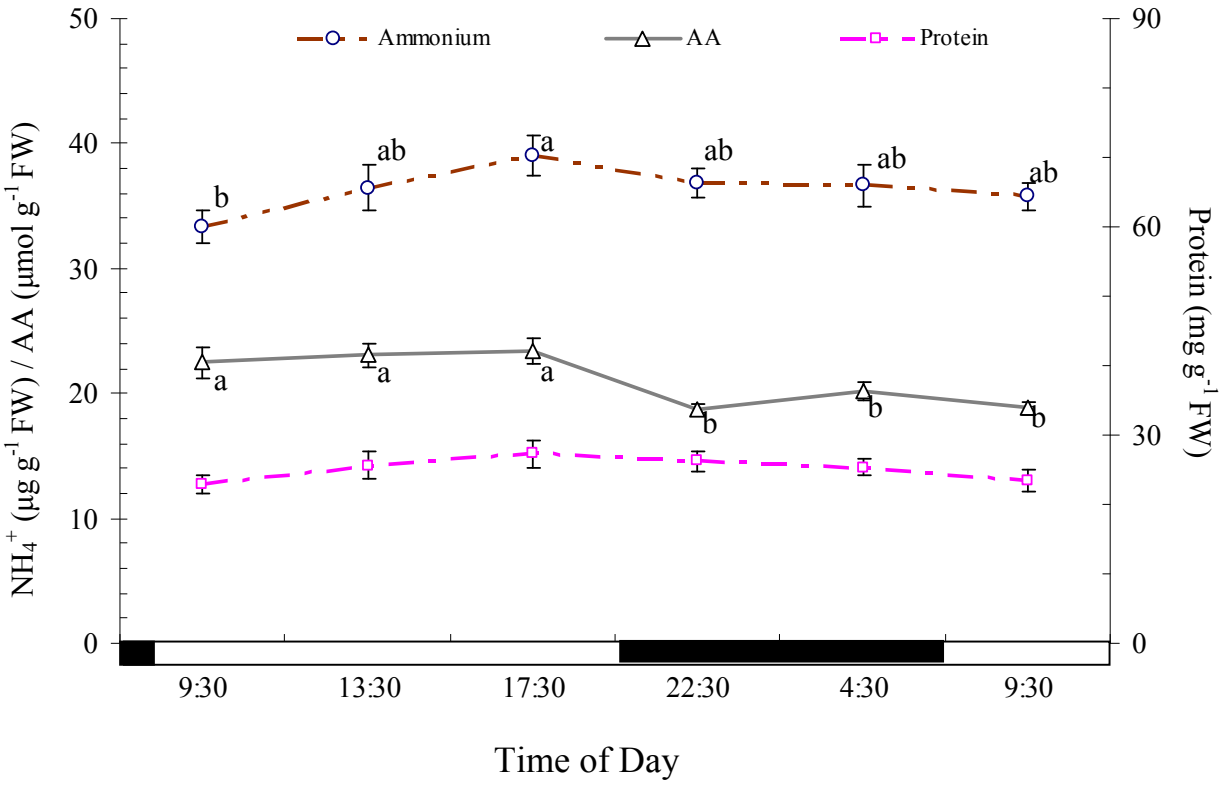
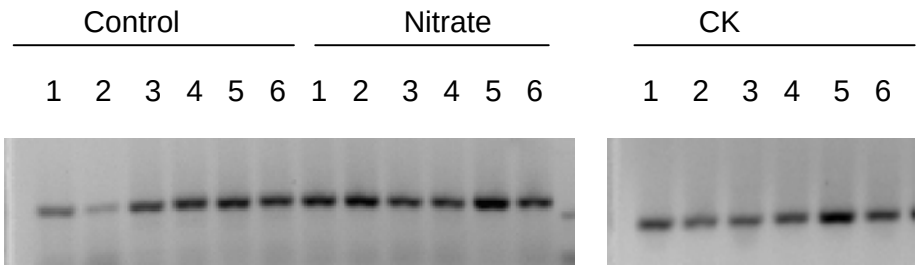


Fig.2-3. Daily fluctuation of shoot NH_4^+, amino acids and total soluble protein of creeping bentgrass. Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively. Different letters indicate a significant difference ($p \leq 0.05$) between sampling times for each measured parameter. Error bars indicate standard error (n=12).

A.



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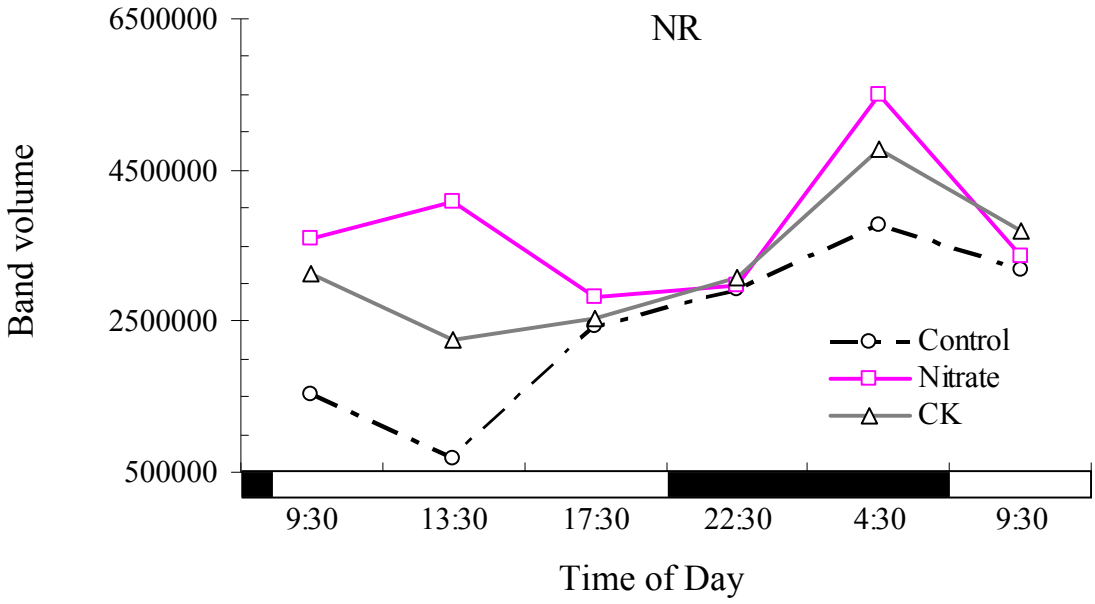
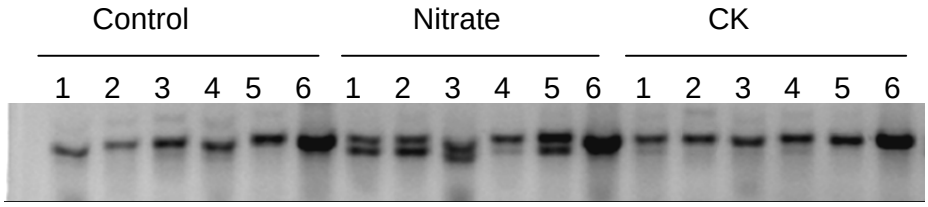


Fig.2-4. Daily fluctuation of *NR* (nitrate reductase) expression (transcripts) in shoots of creeping bentgrass treated with either 0.05% Tween 20 solution (Control), cytokinin (*trans*-zeatin riboside) at 50 μM (CK), or nitrate at 7.5 kg N ha^{-1} (Nitrate). A: the mRNAs were estimated by RT-PCR using an equal amount of total RNA from each sample; B: Corresponding band volume (band intensity×area, arbitrary unit) of *NR* using Bio-rad Quantity One software. Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively.

A.



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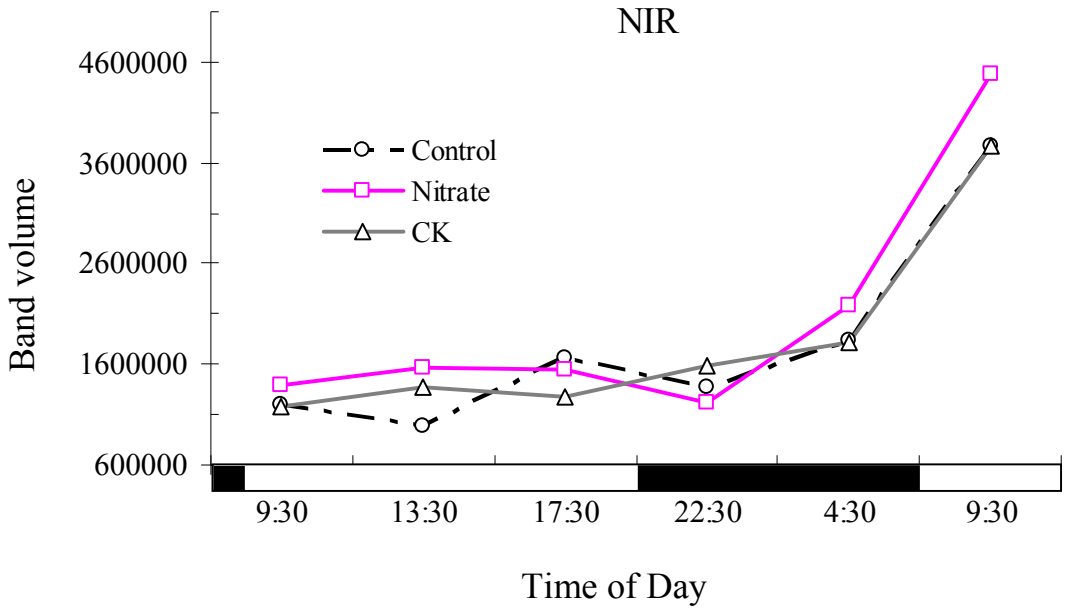
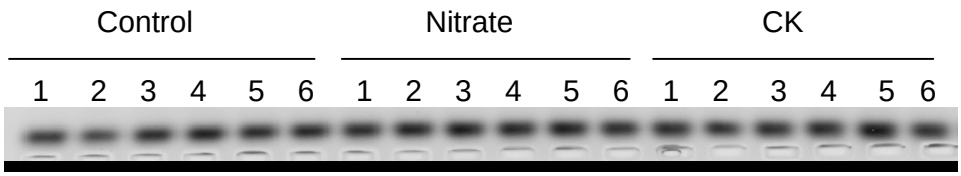


Fig.2-5. Daily fluctuation of *NIR* (nitrite reductase) expression (transcripts) in shoot of creeping bentgrass treated with either 0.05% Tween 20 solution (Control), cytokinin (*trans*-zeatin riboside) at 50 μM (CK), or nitrate at 7.5 kg N ha^{-1} (Nitrate). A: the mRNAs were estimated by RT-PCR using an equal amount of total RNA from each sample; B: Corresponding band volume (band intensity×area, arbitrary unit) of *NR* using Bio-rad Quantity One software. Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively.

A.



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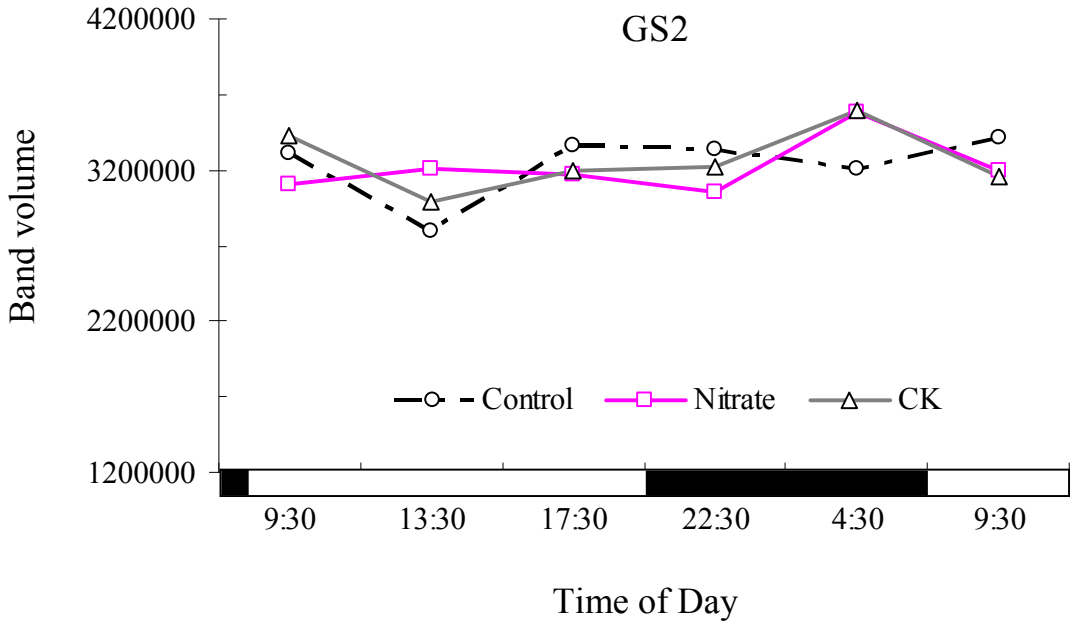
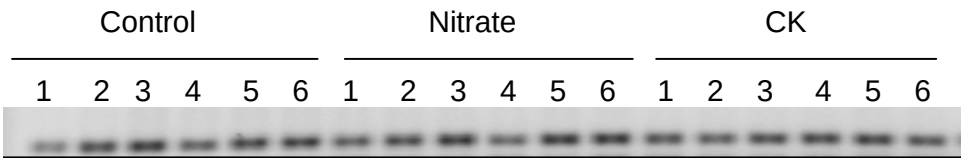


Fig.2-6. Daily fluctuation of *GS2* (plastid glutamine synthetase) expression (transcripts) in shoot of creeping bentgrass treated with either 0.05% Tween 20 solution (Control), cytokinin (*trans*-zeatin riboside) at 50 μM (CK), or nitrate at 7.5 kg N ha^{-1} (Nitrate). A: the mRNAs were estimated by RT-PCR using an equal amount of total RNA from each sample; B: Corresponding band volume (band intensity×area, arbitrary unit) of *NR* using Bio-rad Quantity One software. Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively.

A.



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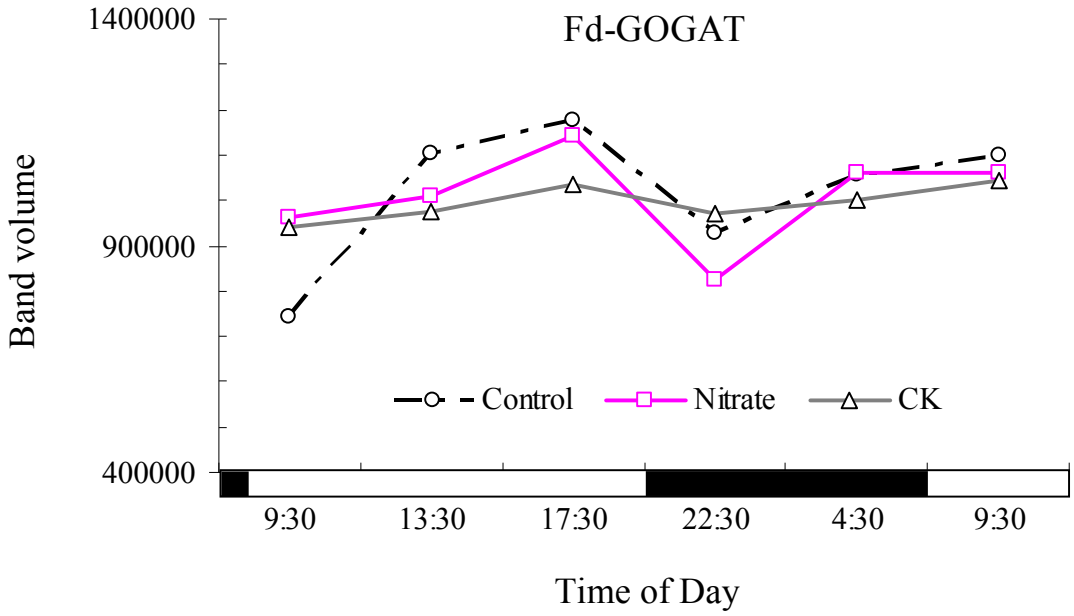
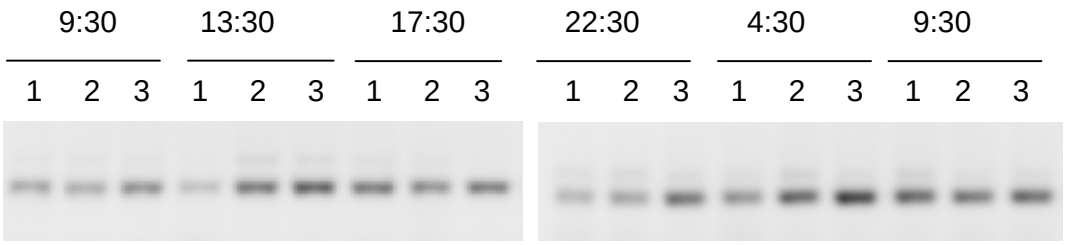


Fig.2-7. Daily fluctuation of *Fd-GOGAT* (Fd-glutamate synthase) expression (transcripts) in shoot of creeping bentgrass treated with either 0.05% Tween 20 solution (Control), cytokinin (*trans*-zeatin riboside) at 50 μM (CK), or nitrate at 7.5 kg N ha^{-1} (Nitrate). A: the mRNAs were estimated by RT-PCR using an equal amount of total RNA from each sample; B: Corresponding band volume (band intensity×area, arbitrary unit) of *NR* using Bio-rad Quantity One software. Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively.

A.



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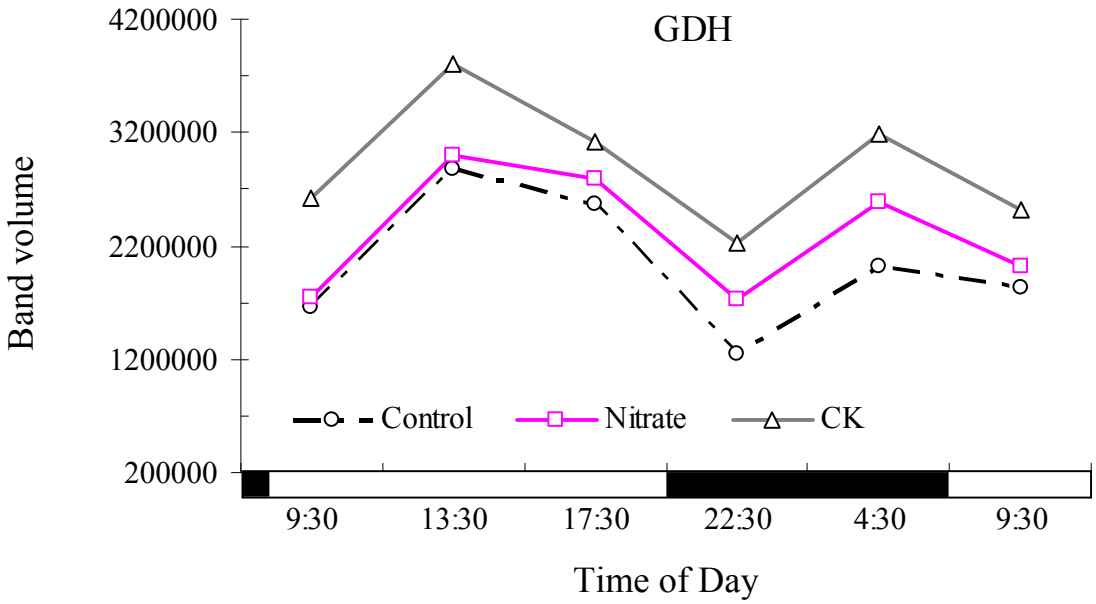

Fig.2-8. Daily fluctuation of *GDH* (glutamate dehydrogenase) expression (transcripts) in shoot of creeping bentgrass treated with either 0.05% Tween 20 solution (Control), cytokinin (*trans*-zeatin riboside) at 50 μM (CK), or nitrate at 7.5 kg N ha^{-1} (Nitrate). A: the mRNAs were estimated by RT-PCR using an equal amount of total RNA from each sample; B: Corresponding band volume (band intensity×area, arbitrary unit) of *NR* using Bio-rad Quantity One software. Photoperiod: 14 h (6:00 to 20:00). Filled and open bars indicate dark and light period, respectively.

CHAPTER THREE

EFFECTS OF NITRATE AND CYTOKININ ON CREEPING BENTGRASS UNDER SUPRAOPTIMAL TEMPERATURES

ABSTRACT

Heat stress is a major problem affecting creeping bentgrass (*Agrostis stolonifera* L.) putting green performance in temperate to sub-tropical climates. The overall objective of this research was to characterize the effects of nitrogen (N) and cytokinin (CK) on creeping bentgrass under heat stress. 'L-93' creeping bentgrass, in a 38 ^{o}C /28 ^{o}C (day/night) growth chamber, was treated with two nitrogen rates (2.5 and 7.5 kg N ha^{-1}, low and high N) and three rates of CK (*trans-zeatin* riboside, *t*ZR, 0, 10 and 100 μM, CK0, 10, and 100) biweekly in a complete factorial arrangement. Grass was harvested at d 1, 15, and 28. Grass grown at high N (averaged across CK rates) had better turf quality (TQ), higher canopy photochemical efficiency of photosystem II (Fv/Fm), normalized difference vegetation index (NDVI), and chlorophyll concentration at both d 15 and 28 than at low N, except for TQ at d 15. Shoot NO_3^-, NH_4^+, and amino acids increased due to the high N treatment, but not water soluble proteins. High N also induced maximum shoot nitrate reductase activity (NR_{max}) at d 1, but not on other sample dates. CK increased NDVI at d 15, with Fv/Fm of the CK100 treatment

(averaged across N levels) being 18% higher than that of CK0 at d 28. In addition, CK100 had greatest NR_{max} at both d 1 and 28. Either N or CK increased root *t*ZR and isopentenyl adenosine (iPA) contents. Under high N with 100 μM CK, root *t*ZR and iPA were 160% and 97% higher than under low N without CK, respectively. These results demonstrate positive impacts of N and CK on creeping bentgrass under heat, with N playing a dominant role. These results can improve the understanding of turfgrass manager's in developing N plus CK programs that enhance bentgrass performance under high temperature stress.

Keywords: nitrate, zeatin riboside, nitrogen metabolites, creeping bentgrass, heat stress

INTRODUCTION

Temperature is one of the primary environmental factors influencing plant distribution and growth. Temperature above the optimum for healthy plant growth is known as heat stress. Supraoptimal temperature affects plant growth and development by accelerating and redirecting metabolic processes. Under heat stress, plants respond at all levels, from morphological adaptations and physiological changes to molecular regulations (Wahid et al., 2007).

Plants require greater amounts of nitrogen (N) than of any other mineral nutrient because N is an essential component in many important organic compounds, such as amino and nucleic acids (Epstein and Bloom, 2005). Nitrate (NO_3^-) normally is the major N form existing in well-aerated soil (Bloom, 1997). With increasing temperature, biological membrane integrity begins to be compromised, increasing leakage of inorganic nutrients (Wahid et al., 2007). Moreover, nutrient influx cannot keep up with nutrient efflux under supraoptimal temperatures. In creeping bentgrass, root N, P, and K content declines after exposure to 35 ^oC for 15 to 20 days (Huang and Xu, 2000; Liu and Huang, 2005). In these studies, cultivars with greater heat tolerance tended to maintain a higher nutrient element status relative to those with less heat tolerance. More recently, Rachmilevitch et al. (2006) reported NO_3^- assimilation rate decreased with increasing soil temperatures in both *Agrostis scabra* (Willd.) (rough bentgrass) and *A. stolonifera* (L.), accompanied by decreased root viability, indicating a relation between heat tolerance and carbon and N metabolism. Fu and

Huang (2003) demonstrated that foliar nutrient application, including inorganic N, improved heat tolerance manifested by slowed leaf senescence and maintenance of photosynthetic activities.

Cytokinin (CK) is known to delay leaf senescence under environmental stresses. The biosynthesis of CKs in plants occurs mainly in root tips with transport to shoots via the xylem (Arteca, 1996). Zeatin and its derivatives are considered to be the most prevalent base in higher plants (Sakakibara, 2006). Heat stress affects CK synthesis adversely, and the reduction of CK has been reported in different species under stress, such as aztec tobacco (*Nicotiana rustica* L.), kidney bean (*Phaseolus vulgaris* L.) (Itai et al., 1973), and creeping bentgrass (Liu and Huang, 2005). In creeping bentgrass, retarded leaf senescence and reduced cell membrane lipid peroxidation were observed via exogenous zeatin riboside application (Liu and Huang, 2002; Liu et al., 2002). In addition, the beneficial effects of extended day-length on creeping bentgrass under heat were partly attributed to higher endogenous CK content in leaf tissues (Wang et al., 2004).

Inorganic N, especially nitrate, is not only required for N assimilation, but serves as a signal to regulate many metabolic processes, including CK biosynthesis (Sakakibara et al., 2006). Cytokinin levels increase when plants are supplied with nitrogen and decrease inversely (Schachtman and Shin, 2007). The induction of CK biosynthesis by NO_3^- supplementation has been shown in barley (*Hordeum vulgare* L.) (Samuelson and Larsson, 1993), maize (*Zea mays* L.) (Takei et al., 2001) and *Arabidopsis* (Takei et al., 2002).

Conversely, exogenous CK application has been shown to affect N partitioning and maintain a relatively higher leaf nitrogen content in other species, such as wheat (*Triticum aestivum* L.) (Simpson et al., 1982), and rice (*Oryza sativa* L.) (Ookawa et al., 2004).

Creeping bentgrass is one of the most widely used cool-season turfgrass species for golf greens, fairways, and tees over temperate and subtropical regions, and supraoptimal summer temperatures often become a major factor in bentgrass decline in these regions (Carrow, 1996). Although heat stress has been extensively studied in higher plants, the exact responses and mechanisms are complex and still being resolved, particularly in creeping bentgrass (Huang, 2003). Moreover, how N and CK improve creeping bentgrass performance under heat stress is far from conclusively known, and their interactive effects on creeping bentgrass are relatively unreported. Herein, we hypothesized that grass treated with low N plus CK would have comparable turf quality and root CK content to that treated with high N alone under high temperature. The objectives of this study were to investigate the effects of NO_3^- and CK on creeping bentgrass under supraoptimal temperatures and to provide useful information for developing improved application programs for enhancing summer creeping bentgrass performance.

MATERIALS AND METHODS

Plant Materials and Treatments

'L-93' creeping bentgrass was planted in late July, 2007 at 49 kg PLS (pure live seed) ha^{-1} in plastic pots (14-cm diameter, 14.5-cm depth) filled with calcined clay (heat-treated montmorillonite clay mineral, 0.015% N, Profile LLC, Buffalo Grove, IL) and grown on a greenhouse bench with a mist system (25±3 /15± 2 ^{o}C, day/night). The grass was fertilized with Bulldog fertilizer (28-8-18, 1% ammoniac N, 4.8% nitrate N, and 22.2% urea N; SQM North America, Atlanta, GA) at 5 kg N ha^{-1} every week over the first two months, then reduced to 2.5 kg N ha^{-1} biweekly. Three months after planting, the grass was transferred into a growth chamber at 38/28°C (day/night), relative humidity 70%/85% (day/night), photosynthetically active radiation at 450 μmol s^{-1} m^{-2} and a 14-h photoperiod. Grass was clipped at 12 mm, three times a week throughout the project, but clipping was periodically stopped in order to collect enough tissue mass for laboratory analyses.

Treatments

At two-week intervals foliar spray treatments of CK (*trans*-zeatin riboside, *t*ZR) at 0, 10 and 100 μM (CK0, 10, and 100) in 0.05% Tween 20 solution (500 μL per pot) combined with sub-irrigation (an 18 cm pan was placed under each pot in which water or nutrient solution was applied) to supply nitrate treatments (60 mL Hoagland's solution per pot) (Epstein and Bloom, 2005) at 2.5 (low N) and 7.5 (high N) kg N ha^{-1} were applied. Both KNO_3 and

$Ca(NO_3)_2$ were used as the nitrate sources in the solution. Potassium and calcium levels were equalized across treatments by adding KCl and $CaCl_2$ into the low N treatment solution, accordingly. All the nutrient levels were the same across treatments, except a higher Cl^- concentration in the high N treatment solution. Pots were sub-irrigated daily to prevent drought stress.

Sampling and Measurements

Shoots were harvested at d 1 (1 day after initial treatment application plus heat stress), d 15, and d 28 in the morning from 08:00 to 09:30. Roots were washed free of soil after the final harvest (d 28). All samples were immediately frozen with liquid nitrogen and stored at -80 ^oC until analysis.

Turf quality (TQ) was rated weekly based on a visual scale of 1 to 9, with 1 indicating yellow, dead leaves, 9 the best possible quality, and 6 minimum acceptable quality according to Wang and Jiang (2007). Normalized difference vegetation index (NDVI = $(Infrared_{850}-Red_{660}) / (Infrared_{850}+Red_{660})$) and canopy photochemical efficiency of photosystem II (PSII) ($Fv/Fm = (Fm_{690}-F0_{690})/Fm_{690}$) were recorded after each TQ reading by using a turf color meter (Fieldscout TCM500, Spectrum technologies, IL) and dual wavelength chlorophyll fluorometer (OS-50II, Opti-sciences, Tynsboro, MA), respectively. Leaf chlorophyll (Chl) concentration was measured according to the procedures described by Lichtenthaler et al. (1987) with some modifications. Leaf Chl was extracted by soaking 20 to

30 mg samples (cut into 0.5-cm fragments) in 10 mL acetone (100%) in the dark at 4 °C for 72 h. Absorbance was read at 662 and 645 nm.

For analysis of shoot NO_3^-, NH_4^+, amino acids and total soluble protein, one gram of liquid nitrogen powdered shoot tissue was extracted two times for 30 min at 4 ^oC in 5 mL of ice-cold deionized distilled (dd) water. After centrifugation at 4000 *g* for 10 min, supernatants were collected and pooled. The pooled supernatants were then filtered through Whatman #42 filter papers before further analysis. Nitrate was estimated using a Lachat QuickChem 8000 Flow-Injection Auto-Analyzer (Lachat Instruments, Milwaukee, WI) with a Cd-reduction column to reduce NO_3^- to NO_2^-. The same analyzer was used for NH_4^+ analysis. Amino acids in the supernatant were determined using ninhydrin with glycine as a standard (Rosen, 1957). In detail, a 200 µL aliquot from the extraction above was used, and the final volume in assay tubes was adjusted to 750 µL with dd H_2O. Then 0.5 mL of 0.2 mM cyanide solution (in 0.2 M acetate buffer, pH 5.3) and 0.5 mL of 3 % (w/v) ninhydrin solution (in ethylene glycol monomethyl ether) were added into the tubes. Tubes were then covered with marbles and boiled for 20 minutes. Tubes were next removed from the water bath and a 5 mL mixture of isopropyl alcohol and water (1:1, v:v) was added. Tubes were vortexed and allowed to cool to room temperature before being read at 570 nm. All the samples and standards were run in duplicate. For total soluble protein content, a 25 µL aliquot of extract was assayed using BCA reagent (Sigma, USA). The reaction mixture was read at 562 nm after 30 min incubation at 37 °C with bovine serum albumin (BSA) as the standard (Smith et al., 1985).

The activity of leaf nitrate reductase (NR) was estimated by using the method of Baki et al. (2000) with some modifications. Briefly, 300 mg liquid nitrogen powdered shoot tissue was mixed in a microtube with 1.5 mL pre-cooled extraction buffer (100 mM HEPES-KOH pH 7.6, 10 μM flavin adenine dinucleotide (FAD), 5 mM DL-dithiothreitol (DTT), 0.05% (w/v) cysteine, 2 mM EDTA, and 1% (w/v) polyvinylpyrrolidone (PVP), plus the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mM). The mixture stayed on the ice for 5 min before centrifugation. The extracts were centrifuged for 15 min at 14,400 *g* at 4°C, the supernatant was transferred to new microtubes and kept on ice. For the maximum and actual NR activity assay (NR_{max}, the maximum NR capacity of the dephospho-form and NR_{act}, the actual dephospho-form), the reaction mixture (1 mL) contained 50 mM HEPES-KOH pH 7.6, 10 μM FAD, 1 mM DTT, 20 mM KNO_3, 0.2 mM NADH plus either 5 mM EDTA or 15 mM Mg^{2+}. The reaction was initiated by adding 200 μL extract. The reaction mixture was then incubated at 30 °C for 20 min before termination by addition of 100 μL zinc acetate solution (1 M). After 10 min in the dark, the mixture was centrifuged for 5 min (4 °C, 8000 *g*), and 900 μL of supernatant was transferred to 10 mL glass cultural tubes. The nitrite formed was estimated colorimetrically by adding 750 μL of 1% sulfanilamid in 3 M HCl and 750 μL of 0.02% N-naphthyl-ethylenediamine hydrochloride. Absorption was determined at 540 nm. For each run, blanks and four nitrite standards (1, 5, 10 and 25 μM KNO_2) were included. An extract from frozen spinach (*Spinacia oleracea* L.) leaf was used as a positive check each time.

Liquid nitrogen powdered root tissues (0.5 g) were used for *trans*-zeatin riboside (*t*ZR) and isopentenyl adenosine (iPA) assay. Cytokinins were extracted and purified following the methods of Zhang and Ervin (2004), with minor modifications. A recovery rate greater than 90% was obtained based on the internal standards. Both *t*ZR and iPA were analyzed using indirect ELISA as described by Zhang and Ervin (2008). Briefly, wells of a 96-unit micro-plate were coated with 100 µL per well of *t*ZR conjugated to bovine serum albumin (BSA) (1:2000 dilution) or iPA-BSA conjugate (1:10 000 dilution), incubated overnight at 5°C, emptied, and then washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBS-T). The reaction was blocked with 200 µL of 1% BSA in PBS for 30 min at 37°C to prevent nonspecific protein adsorption. After the plate was washed twice with PBS-T, 50 µL of the CK extracts or standards and 50 µL of the anti-*t*ZR3 antibody (1:200 dilution) or -iPA3 antibody (1:200 dilution) were added to the wells and the plates were incubated at 37°C for 60 min, emptied, and then washed three times with PBS-T. Both the *t*ZR and iPA antibodies were kindly provided by Dr. G. Banowetz from USDA-ARS at Corvallis, OR, USA. To each well, 100 µL of a 1:5000 dilution of alkaline phosphatase–labeled goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) was added and the plates were incubated at 37°C for 60 min. After three washes with PBS-T, 100 µL of substrate solution (3 mg mL^{-1} of *p*-nitrophenyl phosphate in 10% (w/v) diethanolamine buffer, 0.5 mM $MgCl_2$, pH 9.8,) was added to each well and the plates were incubated at 37°C for 20 min. The color reactions in each well were determined by measuring absorbance at 405 nm

by an enzyme immunoassay microplate reader (Opsys MR, Thermo Labsystems, Chantilly, VA). The concentrations of *t*ZR and iPA were calculated based on the standard curve. Each standard or sample was repeated two times and the averages were used for data analysis.

Experimental Design and Statistical Analysis

The experiment was a randomized complete block design with five replicates. There were two factors (CK and N) with N at two levels, and CK at three. All measurements were analyzed using the samples collected at the sampling days mentioned above. Data were analyzed using PROC GLM (SAS Institute, Version 9.1, Cary, NC). Treatment means were separated using Fisher's–protected Least Significant Difference (LSD) test at a 0.05 significance level, except as otherwise stated herein.

RESULTS AND DISCUSSION

Turf Quality, Photochemical Efficiency, NDVI and Leaf Chlorophyll Concentration

Turf quality (TQ) is an indication of overall turfgrass performance. TQ decreased with the stress regardless of the nitrogen level (Figure3-1A). Decline in TQ is common for grasses under various environmental stresses, including high temperature (Liu et al., 2002; Wang and Jiang, 2007). Fu and Huang (2003) reported foliar application of ammonium nitrate improved TQ and alleviated heat injury. This is consistent with our findings of TQ at d 28. When averaged across all three CK levels, grass treated with high N showed 15% higher quality

than at low N at the last sampling day, d 28, (Figure3-1A). Totten et al. (2007) reported that moderate nitrogen fertilization (7.5 to 11.25 kg N ha^{-1} biweekly) maintained better TQ of creeping bentgrass under summer heat-stressed environment than fertilization at either 3.75 or 22.5 kg N ha^{-1} biweekly. Our findings were similar with regard to N rate effects on TQ.

Canopy normalized differential vegetative index measured as NDVI is one of the most commonly used indices to evaluate the status of plant health in crop management (Tucker, 1979). Huang et al. (1998) reported that a more drought-tolerant tall fescue (*Festuca arundinacea* Schreb.) cultivar (Kentucky-31) had greater NDVI than a drought-sensitive one (MIC-18) under water stress. Higher NDVI readings have been consistently shown to be positively correlated with increased visual TQ, shoot density, and turf color (Fitz-Rodriguez and Choi, 2002). Grass under high N showed consistently higher NDVI readings (9% and 12% higher at d 15 and d 28, respectively), except at d 1 (Figure 3-1C). Meanwhile, a significant effect of CK was found at d 15, with CK100 having the greatest NDVI (Table 3-1). Relatively greater NDVI values at high N and CK100 may have been due to higher density and/or greener leaves, which would indicate beneficial effects of both N and CK on turf under stress conditions.

Both Fv/Fm and Chl concentration decreased steadily with time under low N. Unlike TQ and NDVI, they slightly increased at d 15 under high N (Figures 3-1B, D). This could simply be a response to the high N because plants require nitrogen for the synthesis of chlorophyll and for proteinaceous stroma and grana of the chloroplasts. Photosystem II is

known to be sensitive to heat stress (Critchley and Chopra, 1988), with Fv/Fm decline being reported repeatedly in various plants under heat stress (Liu et al., 2002; Zhao et al., 2008). Heat stress promotes leaf senescence and degradation of Chl (Thomas and Stoddart, 1980). Decreased Chl in heat-stressed turfgrasses has also been reported previously (Liu and Huang, 2002; Zhang and Ervin, 2008; Zhao et al., 2008). Higher Fv/Fm and more Chl under high N at d 15 and 28 were observed, relative to that at low N. Zhao et al. (2008) reported that foliar nitrogen fertilization improved Fv/Fm and Chl concentration of two tall fescue cultivars under heat. Fu and Huang (2003) reported higher Fv/Fm and Chl concentration in heat stressed creeping bentgrass at d 16 and 29 after foliar nitrogen treatment, respectively. A study with corn (*Zea mays* L.) indicated that greater Fv/Fm in plants treated with high N was related to greater chloroplastic and mitochondrial heat shock protein (HSP) production relative to low N plants (Heckathorn et al., 1996). In addition, Fv/Fm in the bentgrass treated with 100 μM *t*ZR at d 28 was 18% higher than that without *t*ZR treatment when averaged across N levels (Table 3-1). In another heat-stressed creeping bentgrass study, Liu et al. (2002) reported that zeatin riboside delayed the decline of Fv/Fm relative to the control. More recently, Zhang and Ervin (2008) found both seaweed extract-based CKs and synthetic *t*ZR alleviated the decline of Fv/Fm of creeping bentgrass under heat stress, which was correlated with increased plant endogenous CK level. Exogenous application of CKs has been reported to retard leaf senescence and decline in Chl (Badenoch-Jones et al., 1996; Liu and Huang, 2002). Surprisingly, in our study no difference in Chl concentration among CK levels was

found. Possible reasons for discrepancies across studies may be differences in stress intensity (temperature setting, 28/38 ^{o}C (night/day) here vs. 30/35 or 25/35 ^{o}C in other studies) and duration (4 weeks here vs. 6 or 8 weeks in others) or the amount of CK applied (5 to 50 nmol per pot here vs. 5 to 500 nmol per pot in others). In addition, the N effect could have functioned to mask CK impacts since its effects seemed to be dominant in this study.

Shoot NO_3^-, NH_4^+, Amino Acids, Total Soluble Protein and Nitrate Reductase Activity

The ability to accumulate and maintain high N reserves has been suggested as critical for heat tolerance (Gerard, 1997). He et al. (2005) suggested that changes in N metabolic parameters, such as amino acid and total protein content, might contribute to poor performance under prolonged heat stress for creeping bentgrass. In the study herein, greater shoot concentrations of all the measured N metabolites were found at high N at d 1 and 15, except total water soluble proteins (Figure 3-2). In particular, four to six-fold amounts of NO_3^- were accumulated in shoots under high N compared with that at low N (Figure 3-2A). Nitrate accumulation in plant tissues is common when its uptake exceeds its reduction and subsequent assimilation. In addition, there was an interaction on shoot NO_3^- at d 1 between the two factors, N and CK. The highest shoot NO_3^- was observed under high N plus high CK (Table 3-2), which could have been due to the induction effects of CK on NO_3^- transporters (Sakakibara et al., 2006). Interestingly, a similar interaction was not observed at d 15 and 28. We conjecture that stress duration may have played a role in this result. Ammonium

accumulation under high N was not as pronounced relative to NO_3^- (Figure 3-2A, B). Ammonium is toxic to plants at a relatively high concentration, requiring fairly rapid assimilation into non-toxic intermediate products, such as amino acids (Temple et al., 1998). A pattern parallel to NH_4^+ was found for shoot amino acids content with more amino acids being measured under high N (Figure 3-2C), indicating more NH_4^+-N was assimilated into amino acids.

The rate limiting step in the overall nitrate assimilation pathway is the reduction of nitrate to nitrite (NO_2^-) in the cytoplasm by nitrate reductase (NR) (Kaiser et al., 1999). The activity of NR measured in reaction buffer containing either 5 mM EDTA or 15 mM Mg^{2+} gives the maximum NR capacity of the dephospho-form (NR_{max}) and the actual NR activity (NR_{act}) equivalent to dephospho-NR, respectively. Both NR_{max} and NR_{act} showed a trend of decline with heat stress regardless nitrogen level, particularly NR_{act} (Figures 3-2E and F). High temperature has been reported to decrease NR activity in several different grass species, including creeping bentgrass grown at 35 ^oC (Kaufmann et al., 1971), tall fescue (Cui et al, 2006) and *Leymus chinensis* (Trin.) (Xu and Zhou, 2006). Nitrate reductases in higher plants are tightly regulated by many factors, such as light, carbohydrate, nitrate concentration and CK (Kaiser et al., 1999). NR activity can be induced by nitrate and cytokinin alone or in combination. Enhanced NR activity by cytokinin is suggested to be a direct effect, instead of via nitrate (Henalatha, 2002). As expected, NR_{max} under high N at d 1 was significantly higher than that under low N (Figure 3-2E). Similarly, CK100 had 14% and 16% higher

NR_{max} than CK0 at d 1 and d 28, respectively. However, no N or CK effects were observed for NR_{act} (Figure 3-2F, Table 1), which indicates the different NR_{max} between treatments could be mainly due to *de novo* synthesis of NR, instead of post-translation modification, such as dephosphorylation. Both cytokinin and nitrate are thought to induce the activity of NR by stimulating *de novo* synthesis of NR (Kende, 1971; Zielke, 1971; Henalatha, 2002). Interestingly, no significant difference in NR_{max} was observed among treatments for N at both d 15 and d 28, and d 15 for CK, although similar patterns among treatments were found (Figure 3-2E, Table 3-1).

Root CK Content

Both N and CK had effects on root *t*ZR and iPA. Under high N, root *t*ZR was 14% higher than under low N. The grass under CK100 (averaged across two N levels) had the highest root *t*ZR content, which was 18% and 57% higher than that for CK10 and CK0, respectively. Similar patterns were found for root iPA content (Table 3-2). These results are consistent with many other studies (Liu and Huang, 2002; Takei et al., 2002; Zhang and Ervin, 2004; 2008). There were interactive effects of N and CK on root iPA and *t*ZR contents. For instance, the grasses under low N without CK had the lowest root iPA content (8.6 ng g^{-1} FW), with those under high N with 100 μM CK having the highest root iPA content (22.5 ng g^{-1} FW) (Table 3-2). Nitrogen, especially NO_3^-, can stimulate CK biosynthesis (Sakakibara et al., 2006). CKs are implicated in N responses as signals communicating N availability

between roots and shoots (Vidal and Gutiérrez, 2008). Nitrate application to *Arabidopsis* roots results in a rapid increase in CK levels and its translocation into xylem vessels (Takei et al., 2002). This effect is probably partly due to upregulation of transcript levels of the IPT3 gene, which encodes a key enzyme for CK biosynthesis by nitrate (Takei et al., 2004). Exogenous CK application has also been reported to increase endogenous CK content in plants. Liu et al. (2002) found CK contents in both shoots and roots of plants treated with 10 μmol zeatin riboside were significantly higher than those of untreated plants, and suggested that exogenous CK application to the root zone stimulated CK synthesis in roots. More recently, Zhang and Ervin (2004; 2008) reported the application of synthetic CK or CK-containing natural products increased shoot CK content under either drought or heat stress. Higher root viability was found by Liu et al. (2002) and Zhang and Ervin (2008). We posit that reported increases in endogenous CK content could be due to higher root viability since roots are the primary organ to synthesize CK, and a higher content of leaf CKs are thought to be mainly due to translocation from roots (Sakakibara, 2006). Cytokinin is thought to protect plants under stress via its antioxidant properties, and possibly also through its role in protecting the photosynthetic apparatus, as demonstrated by higher Fv/Fm, Chl concentration and other parameters (Sabater and Rodriguez, 1978; Liu and Huang, 2002; Liu et al., 2002; Zhang and Ervin, 2008).

In conclusion, both N and CK had beneficial effects on creeping bentgrass under heat stress, with N playing a dominant role as manifested by its effects on most of the measured

parameters. As we described before, our hypothesis was that grass treated with low N plus CK would have comparable turf quality and root CK content to that treated with high N alone under high temperature. Our results partially supported the hypothesis. Interestingly, exogenous cytokinin application did not show effects on some parameters as reported in other studies, such as TQ and Chl concentration. This could have been due to variable treatment application methods (e.g., foliar vs. root), sampling times, stress intensity, or other factors as we described before. This disagreement warrants further study to specify the effects of CKs on turfgrass under heat stress, especially at a wider range of nitrogen levels.

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Table 3-1. Effects of cytokinin on photochemical efficiency (Fv/Fm), NDVI, shoot maximum NR activity (NR_{max}) and actual NR activity (NR_{act}) of creeping bentgrass under heat. (n=10).

CK	Day 0	Day 1	Day 15	Day 28
			Fv/Fm	
0	0.670	0.616	0.568	0.438 b†
10		0.609	0.559	0.485 ab
100		0.625	0.558	0.515 a
			NDVI	
0	0.720	0.644	0.589 b	0.584
10		0.648	0.598 ab	0.574
100		0.682	0.636 a	0.611
		NR_{max}	nmol NO_2^{-1}	g^{-1} FW h^{-1}
0	833.89	878.94 b	837.24	766.56 b
10		868.14 b	855.99	791.27ab
100		997.67 a	902.98	886.29 a
		NR_{act}	nmol NO_2^{-1}	g^{-1} FW h^{-1}
0	708.92	685.21	554.94	550.56
10		746.80	599.49	553.42
100		797.23	615.64	586.22

† Means followed by the same letters within a column are not significantly different according to Fisher's Protected LSD test at p=0.05 level, except NR_{max} (Day 28) at p=0.1 level. Treatments were applied at D 0 and D 14.

Table 2-2. Interactive effects of nitrogen and cytokinin on NO_3^- and root cytokinin content of creeping bentgrass under heat (n=5).

		NO_3^- (μg g^{-1} FW)			
Nitrogen	CK	Day 0	Day 1	Day 15	Day 28
Low	0	7.60	7.64 c†	14.44†	4.54
	10		6.34 c	12.98	4.70
	100		8.96 c	13.50	4.40
High	0		37.16 b	61.84	8.20
	10		43.68 b	53.60	8.66
	100		53.98 a	48.04	6.94

		RtZR (ng g^{-1} FW)	RiPA (ng g^{-1} FW)
Nitrogen	CK	Day 28	Day 28
Low	0	5.71 c	8.59 d
	10	8.79 b	19.01 b
	100	8.86 b	19.06 b
High	0	7.06 bc	14.90 c
	10	8.27 b	19.75 ab
	100	11.23 a	22.51 a

†Means followed by the same letters within a column are not significantly different based on LSD test at p=0.05 level, except RtZR at p=0.1 level. Treatments were applied at D 0 and D 14.

† Means followed without letters within a column indicate there are no interactive effects of nitrogen and cytokinin.

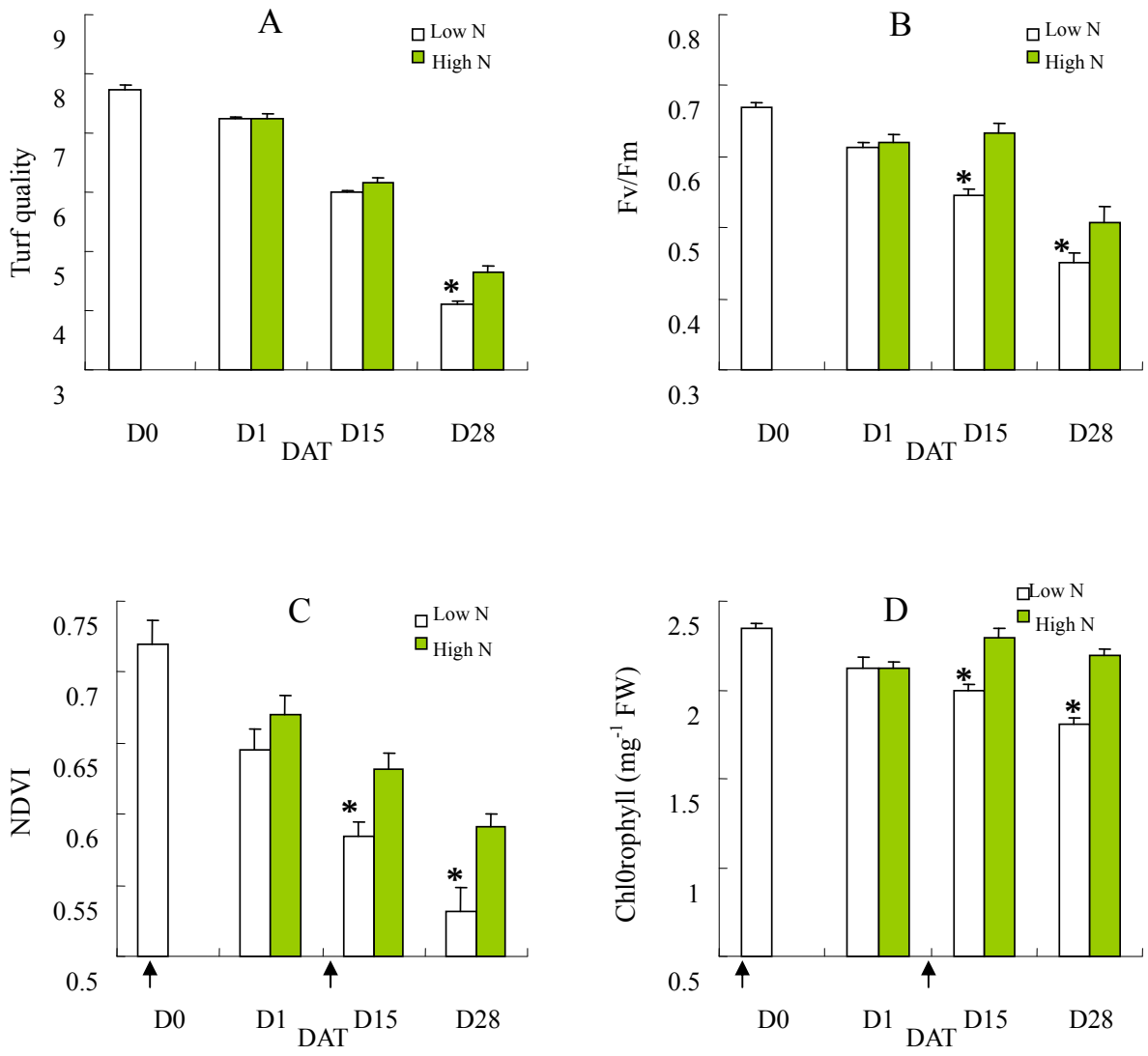

Figure 3-1. Effects of nitrogen on turf quality, photochemical efficiency (Fv/Fm), NDVI, and chlorophyll concentration (Chl) of creeping bentgrass under heat. A: turf quality, B: photochemical efficiency, C: turf color index and D: chlorophyll concentration. An asterisk (*) indicates a significant difference (p<0.05) between treatments on the same day. Arrows indicate the application dates of treatments (D0 and D14). DAT: day after initial treatment application. Error bars indicate standard error (n=15).

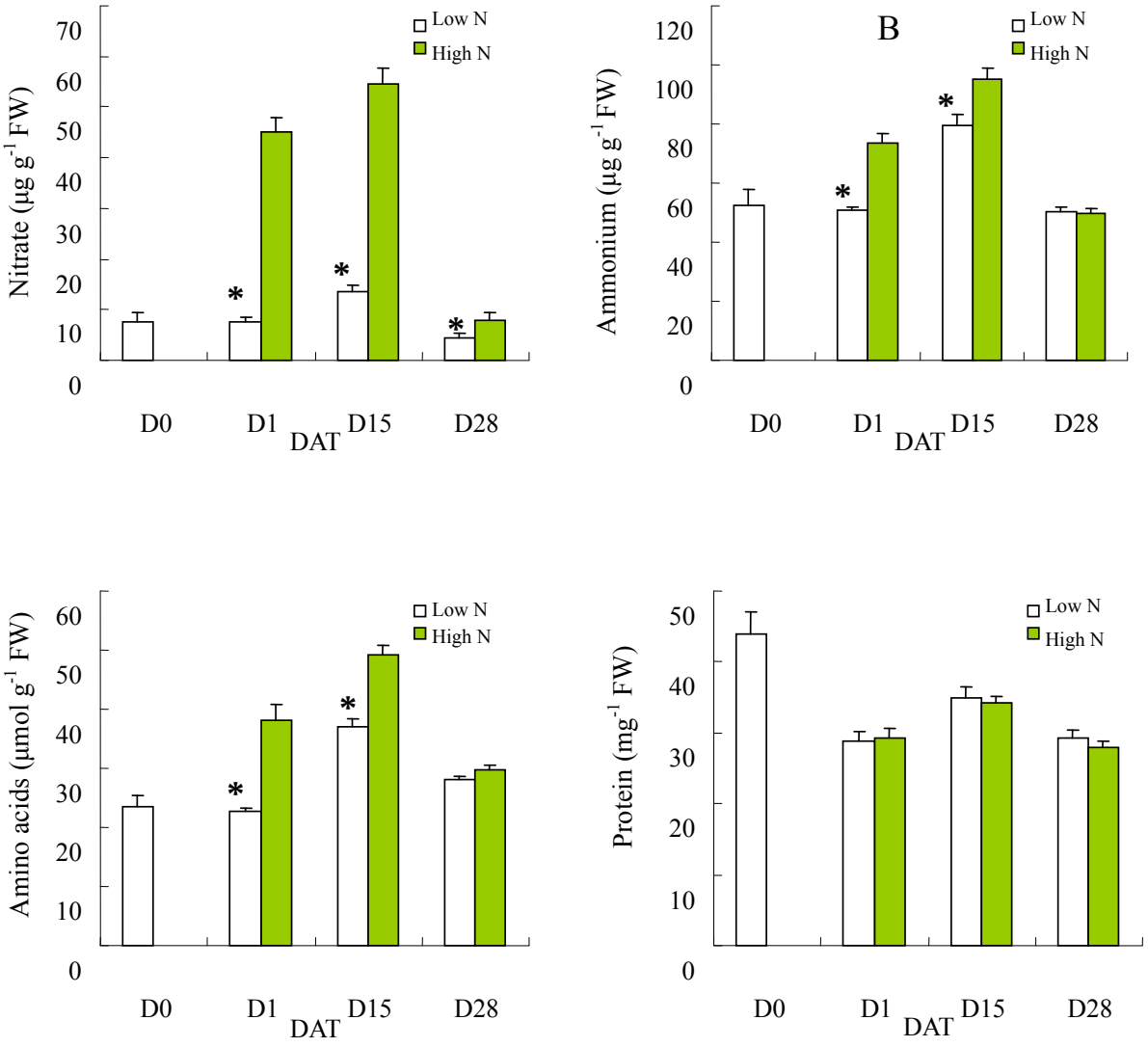

Figure 3-2. Effects of nitrogen on shoot NO_3^-, NH_4^+, amino acids, and total soluble protein of creeping bentgrass under heat. A: NO_3^-, B: $NH4^+$, C: amino acids, D: total soluble protein. An asterisk (*) indicates a significant difference (p<0.05) between treatments on the same day. Arrows indicate the application dates of treatments (D0 and D14). DAT: day after initial treatment application. Error bars indicate standard error (n=15).

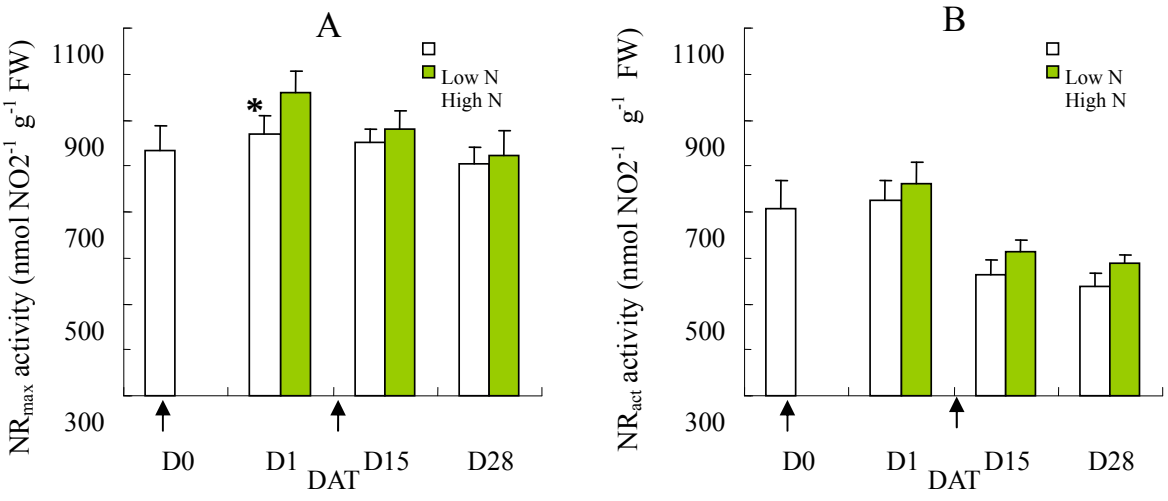

Figure 3-3. Effects of nitrogen on shoot maximum nitrate reductase activity (NR_{max}) and actual nitrate reductase activity (NR_{act}) of creeping bentgrass under heat. A: NR_{max} activity and B: NR_{act} activity. An asterisk (*) indicates a significant difference ($p<0.05$) between treatments on the same day. Arrows indicate the application dates of treatments (D0 and D14). DAT: day after initial treatment application. Error bars indicate standard error (n=15).

CHAPTER FOUR

EFFECTS OF NITROGEN AND CYTOKININ ON ANTIOXIDATIVE RESPONSES IN THE ROOTS AND SHOOTS OF CREEPING BENTGRASS UNDER HIGH TEMPERATURE

ABSTRACT

It has been previously reported that foliar applications of either nitrogen or cytokinins will alleviate heat stress injury on creeping bentgrass, with some studies reporting enhanced antioxidant metabolism being related to stress protection. The objective of this research was to investigate the simultaneous effects of CK and N on the antioxidant enzyme activity and isoforms of heat stressed creeping bentgrass. 'L-93' creeping bentgrass treated with three rates of CK (trans-zeatin riboside, *t*ZR, 0, 10 and 100 μM, designed by CK0, 10, and 100) and two nitrogen rates (2.5 and 7.5 kg N ha^{-1} biweekly, low and high N) in a complete factorial arrangement was maintained in a 38 ^{o}C /28 ^{o}C (day/night) growth chamber for 28 d and then harvested. Grass grown at high N (averaged across CK rates) had higher O_2^- production, H_2O_2 concentration, and higher malonydialdehyde (MDA) content in roots. The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and guaiacol peroxidase (POD) in roots were enhanced by high N at 19%, 22%, and 24%, respectively, relative to low N. Twenty-eight days of heat stress resulted in either the development of new isoforms or enhanced isoform intensities of SOD, APX, and POD in roots compared to the grass responses prior to heat stress. However, no apparent differences were observed among

treatments (N+CK). Both SOD and POD showed different isoform patterns between roots and shoots, suggesting the function of these isoforms could be tissue specific. Interestingly, no CK effects on these antioxidant parameters were found in this experiment. These results demonstrated the impacts of N on antioxidant metabolism of creeping bentgrass under heat stress with some differences between roots and shoots, but no simultaneous impacts of CK and N.

Keywords: Antioxidant metabolism, Cytokinin, Nitrogen, Creeping bentgrass，SOD, ROS, isoform, *trans*-zeatin riboside

INTRODUCTION

Heat stress is usually defined as a period where temperatures are high enough for sufficient time to cause injury to plant function or development. Heat stress inhibition of photosynthesis in chloroplasts results in an imbalance of the electron-transfer chain and promotes production of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide radical (O_2^-), and hydrogen peroxide (H_2O_2) (Smirnoff, 1993). Although ROS can function as signal molecules for plant growth and development, excess ROS are detrimental and can cause the autocatalytic peroxidation of membrane lipids and pigments, leading to the loss of membrane semi-permeability and modifying its functions (Senthil-Kumar et al., 2007; Wahid et al., 2007). Due to this biological paradox, levels of ROS are generally well regulated by their rate of generation and degradation as influenced by the scavenging capacity of two systems, the enzymatic system (antioxidant enzymes) and non-enzymatic system (small molecular antioxidants) (Asada, 1999; Blokhina et al., 2003). Superoxide dismutase (SOD) constitutes the first line of defense against ROS by dismutating the superoxide anion to H_2O_2. Next, H_2O_2 is finely regulated by catalase (CAT) and an array of peroxidases localized in almost all compartments of the plant cell, such as ascorbate peroxidase (APX) and guaiacol peroxidase (POD) (Blokhina et al., 2003). There is increasing evidence suggesting that tolerance of adverse environments is correlated with an increased capacity to scavenge or detoxify ROS, and protection against oxidative stress is thought to be an important component in determining the survival of a plant under heat stress (Maestri et al., 2002; Smirnoff, 1993).

As an important plant hormone, cytokinin was targeted in many plant species to improve their tolerance to different environment stresses (Barna et al., 1996; Havlova et al.,

2008; Huynh et al., 2005; Zhang and Ervin, 2004; Zhang and Ervin, 2008). Exogenous application of cytokinin has been shown to have potential in alleviating heat injury in various higher plants (Liu et al., 2002; Schrader, 2005; Skogqvist, 1974). Nitrogen is the mineral that most often limits plant growth because relatively large quantities of N are required for incorporation into numerous organic compounds that are crucial for plant growth and development, such as proteins, nucleic acids, and some plant hormones (Pessarakli, 2002). A proper level of nitrogen nutrition is essential to maintain adequate plant growth to withstand heat stress. Tawfik et al., (1996) suggested that NDS (N fertilization during heat stress) plants benefitted from greater rhizospheric N levels during heat stress compared to NBS (N fertilization before heat stress) plants, indicated by greater fresh and dry weight, and significantly higher membrane thermostability.

Creeping bentgrass is a widely used cool-season turfgrass on golf greens and fairways in northern regions and transitional zones, where heat stress continues to be the primary factor limiting its summer performance (Zhang and Ervin, 2008). Some studies have reported that high temperature stress affected antioxidant responses in creeping bentgrass and other cool-season turfgrass species (He et al., 2005; Jiang and Huang, 2001; Liu and Huang, 2000; Xu et al., 2006). For instance, decline in antioxidant enzyme activities (SOD and CAT) accompanied by greater generation of ROS and increasing lipid peroxidation were reported in creeping bentgrass under high temperature (Liu and Huang, 2000; Huang et al., 2001). A later study on heat-acclimated versus non-acclimated cool season turfgrass species, perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.), indicated that the ability to protect against oxidative stress is an important component in determining the heat stress tolerance of a plant (Xu et al., 2006). In addition, several studies reported either

the effects of cytokinin or N nutrition on creeping bentgrass, with antioxidant response as one of the mechanisms accounting for less damage. For example, retarded leaf senescence and reduced cell membrane lipid peroxidation in creeping bentgrass was observed via exogenous zeatin riboside application, and enhanced antioxidant response was suggested as a possible mechanism for the observed reductions in heat injury due to exogenous cytokinin application (Liu and Huang, 2002; Liu et al., 2002). Zhang and Ervin (2008) also reported that cytokinin is thought to protect plants under stress via its antioxidant properties. For nitrogen, Fu and Huang (2003) reported foliar application of NH_4NO_3 increased CAT and POD activity and reduced MDA content at during heat stress, but no effects on SOD activity was observed. The maintenance of the scavenging ability of antioxidants and inhibition of lipid peroxidation by NH_4NO_3 was thought to be related to alleviating heat injury. However, there has been no study regarding how CK and N simultaneously affect antioxidant metabolism in roots and shoots of creeping bentgrass under heat stress. In addition, changes in the amount of a particular isoform of an antioxidant enzyme can be more important than alterations in the total activity (Mallineaux and Creissen, 1997). Pinhero et al. (1997) also suggested that synthesis of new antioxidant enzyme isoforms could be more beneficial for antioxidant metabolism than mere enhancement of the activity. Thus, the analysis of isoform changes could provide additional information about antioxidant responses in creeping bentgrass. Moreover, studies on the isoform patterns of antioxidant enzymes in cool-season turfgrasses under heat stress are very limited.

Thus the objective of this study was to investigate the effects of N and CK on the antioxidant response of heat stressed bentgrass, including any changes of antioxidant enzyme isoforms. Knowledge of antioxidant metabolism would provide valuable information for

understanding the mechanisms underlying creeping bentgrass responses to heat stress, perhaps leading to enhanced summer management programs.

MATERIALS AND METHODS

Plant Materials and Treatments

'L-93' creeping bentgrass was planted in late July, 2007 at 49 kg PLS (pure live seed) ha^{-1} in plastic pots (14-cm diameter, 14.5-cm depth) filled with calcined clay (heat-treated montmorillonite clay mineral, 0.015% N, Profile LLC, Buffalo Grove, IL). The grass was fertilized with Bulldog fertilizer (28-8-18, 1% ammoniac N, 4.8% nitrate N, and 22.2% urea N; SQM North America, Atlanta, GA) at 5 kg N ha^{-1} every week over the first two months, then reduced to 2.5 kg N ha^{-1} biweekly. Three months after growing on a greenhouse bench with a mist system (25±3 /15± 2 ^{o}C, day/night), the grass was transferred into a growth chamber with the detailed setting as following: 38/28°C (day/night), relative humidity 70%/85% (day/night), photosynthetically active radiation (PAR) at 450 μmol s^{-1} m^{-2} and a 14-h photoperiod. Grass was cut at 12 mm three times a week with an electric clipper during the project, except the last week in order to collect enough tissue samples for further analysis.

Treatments

Sub-irrigation (a 18-cm pan was placed under each pot in which water or nutrient solution was applied) to supply nitrate treatments (60 mL Hoagland's solution per pot) (Epstein and Bloom, 2005) and foliar spray treatments of CK (*trans*-zeatin riboside, *t*ZR) in 0.05% Tween 20 solution (500 μL per pot) was applied biweekly (0 d and 14 d). Both KNO_3 and Ca $(NO_3)_2$ were used as the nitrate sources in the Hoagland's solution. KCl and $CaCl_2$

was added into low N treatment solution accordingly to equalize the potassium and calcium levels across treatments (All the levels of other nutrients were the same, except higher Cl^- concentration in low N treatment solution than in high N treatment solution). Grass was sub-irrigated daily to prevent drought stress.

The treatments included:

1) Nitrate N at 2.5 (low N) kg N ha^{-1} , no CK (CK0)
2) Nitrate N at 2.5 (low N) kg N ha^{-1} , CK at 10 μM (CK10)
3) Nitrate N at 2.5 (low N) kg N ha^{-1} , CK at 100 μM (CK100)
4) Nitrate N at 7.5 (high N) kg N ha^{-1} , no CK (CK0)
5) Nitrate N at 7.5 (high N) kg N ha^{-1} , CK at 10 μM (CK10)
6) Nitrate N at 7.5 (high N) kg N ha^{-1} , CK at 100 μM (CK100)

Sampling and Measurements

Shoots were harvested at d 28 (28 days after initial treatment application plus heat stress). Roots were washed free of soil at the same time. All samples were immediately frozen with liquid nitrogen and stored at -80 ^{o}C until analysis.

To extract the soluble protein, a frozen sample (0.5 g) from the entire root or shoot tissue was ground into fine powder using liquid nitrogen and 4-mL of extraction buffer (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpyrrolidone (PVP), 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl (PMSF), pH 7.8) was then added. Samples then were centrifuged at 15 000 x g for 15 min at 4°C, and supernatant was collected for enzyme assay. The protein content was determined

using the method of Bradford (1976). All enzyme assays were performed the same day as the Bradford assay using Spectronic Genesys 10 Bio Spectrophotometer (Thermo Electron Corporation, Waltham, MA).

Total SOD activity was measured according to the method of Giannopolitis and Ries (1977). The assay medium contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM p-nitro blue tetrazolium chloride (NBT), 2 µM riboflavin, 0.1 mM EDTA, and 30 to 40 µL of enzyme extract. A reaction mixture was illuminated under 80 to 90 µmol m^{-2} s^{-1} for 10 min. The reaction mixture without illumination served as the control, and the mixture lacking of enzyme developed maximum color as maximum reduction of NBT. One unit of SOD activity was defined as the amount of enzymes that can cause 50% inhibition in the rate of NBT reduction.

The activity of APX was assayed by recording the decrease in absorbance at 290 nm for 1 min. The 1.5-mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, and 0.15 mL of enzyme. The reaction was started with the addition of 0.1 mM H_2O_2 (Nakano and Asada, 1981).

The activity of POD was determined by an increase in absorbance at 470 nm for 1 min. The assay contained 50 µL of 20 mM guaiacol, 2.83 mL of 10 mM phosphate buffer (pH 7.0), and 0.1 mL of enzyme extract. The reaction was initiated by adding H_2O_2 (Kochhar et al., 1979).

The H_2O_2 content was determined using the methods of Bernt and Bergmeyer (1974). Root tissue (1 g) was homogenized in 3 mL of 100 mM sodium phosphate buffer (pH 6.8) and extractions were centrifuged at 18000 g for 5 min at 4°C. Then 0.17 mL of supernatant was added to 0.83 mL peroxidase reagent containing 83 mM sodium phosphate (pH 7.0),

0.005% (w/v) o-dianisiden, and 40 μg peroxidase per mL. The mixture was incubated at 30°C for 10 min, and 0.17 mL of 1 M perchloric acid was added to stop the reaction. The absorbance at 436 nm was read against a blank.

The O_2^- production rate was measured as described by Jiang and Zhang (2002) with modification according to Hakan et al. (2005). A 0.2 g powder of shoot or root tissue was homogenized in 1 mL of 50 mM sodium phosphate buffer (pH 7.5) and centrifuged at 5000 *g* for 5 min at 4 ^oC. The reaction mixture of 1 mL contained 100 μL supernatant and 0.5 mM 3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H- tetrazolium-5-carboxanilide inner salt (XTT sodium salt) and 0.2 mM NADH. The reaction was initiated by adding NADH and the reduction of XTT was recorded for 10 min. Background absorbance was corrected in the presence of 50 units SOD (Sigma, USA). The O_2^- production rate was calculated using an extinction coefficient of 2.16 x 10^4 M^{-1} cm^{-1}.

Lipid peroxidation of the root tissue was measured in terms of MDA content (Dhindsa et al., 1981). A 1-mL aliquot of supernatant was mixed with 4 mL of 20% trichloroacetic acid containing 0.5% thiobarbituric acid. The mixture was heated at 100°C for 30 min, quickly cooled, and then centrifuged at 10000 *g* for 10 min. The absorbance was read at 532 and 600 nm. Concentrations of MDA were calculated using an extinction coefficient of 155 $mm^{-1}cm^{-1}$ (Heath and Packer, 1968).

Native Polyacrylamide Gel Electrophoresis

The procedure of protein extraction was the same as for soluble protein except for using 0.5 and 1.0 g tissue with 2 mL of extraction buffer for shoot and root, respectively. The root protein fractions were concentrated using Centricon Centrifugal Filter Units (Millipore

Corporation, Billerica, MA, USA) with 10 kDa cutoff before loading into the gels. Native polyacrylamide gel electrophoresis (PAGE) was performed by Bio-rad mini-gel system at 4°C, 120 V for 90 min (Laemmli, 1970), except that SDS was omitted. For SOD, APX, and POD, the enzyme extracts were subjected to native PAGE with 10% resolving gel and 4% stacking gel.

The total activity of SOD was stained using the method of Beauchamp and Fridovich (1971), with some modifications. The gels were incubated in 50 mM potassium phosphate buffer (pH 7.5) containing 2.5 mM NBT in dark 25 min. After briefly being washed twice with the same buffer, the gels were soaked in 50 mM potassium phosphate buffer (pH 7.5) containing 30 μM riboflavin and 0.4% N, N, N', N'-tetramethylethylenediamine (TEMED) in the dark for 40 min. The gels were then illuminated for 10 to 15 min with gentle agitation until an appearance of enzyme bands and were transferred to 1% (v/v) acetic acid to stop the reaction.

The activity of POD was detected using the method of Fielding and Hall (1978). The gels were soaked in a sodium phosphate solution (10-mM sodium phosphate and 150-mM sodium chloride, pH 6.0) for 45 min to lower the pH. After briefly being washed with 100 mM potassium phosphate buffer (pH 6.4), the gels were stained in 100 mM potassium phosphate buffer (pH 6.4) containing 20 mM guaiacol and 5.55 mM H_2O_2 for 5 to 10 min until the bands were clearly visible. The gels were then washed with distilled water to stop the reaction.

The activity of APX was detected using the method of Lopez-Huertas et al. (1999). The gels were preincubated in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate, 2 mM H_2O_2 for 20 min. The gels were then washed briefly and submerged in 50

mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 1.25 mM NBT for 10 min.

Experimental Design and Statistical Analysis

The experiment was a randomized complete block design with five replicates. There were two factors (CK and N) with N at two levels, and CK at three. All measurements were analyzed using the samples collected at the sampling days mentioned above. Data were analyzed using PROC GLM (SAS Institute, Version 9.1, Cary, NC). Treatment means were separated using Fisher's–protected Least Significant Difference (LSD) test at a 0.05 significance level, except as otherwise stated herein.

RESULTS

Analysis of variance indicated that N had effects on all measured parameters in stressed creeping bentgrass roots, but only affected the activities of SOD, POD, and H_2O_2 content in shoots. There were no effects of cytokinin on any measured parameters. No significant N × cytokinin interactions were observed (Table 4-1).

Hydrogen Peroxide, Superoxide and Malondialdehyde Content

High N treated plants had significantly higher H_2O_2, O_2^- and MDA contents in roots (Fig. 4-3, 4-4), which were 34.7%, 98.8%, and 26.4% (averaged across CK levels) higher than those in the low N plants, respectively. However, in shoots, these contents were similar in both high and low N plants, except for H_2O_2, where the higher level was observed in high N plants.

Protein, Antioxidant Enzymes, and Isoforms

High N grass had higher water soluble protein content in comparison with low N grass in roots, but not in shoots (Fig.4-2). The activities of SOD and APX in both shoots and roots increased at the high N level. POD activity in roots of high N plants was 23% higher than that of low N plants, but there was no POD activity difference in shoots (Fig. 4-1, 4-2).

Six isoforms of SOD were revealed via the native PAGE gel in both roots and shoots of stressed creeping bentgrass under all the treatments. Based on their migration distance, here we arbitrarily assigned them as SODr1 to 6, and SODsh1 to 6. There was no apparent SOD isoform pattern or relative stain intensity difference in either roots or shoots between treatments, except that SODsh6 in the grasses treated with low N and 10 or 100 μM CK showed stronger stain intensity than the grasses treated with low N but no CK. In addition, we found roots had relatively higher top 3 isoform activities (SODr1 to 3), and shoots in reverse showed relatively higher activities of the bottom 3 isoforms (Fig. 4-5). Only one isoform of APX was identified in both the roots and shoots, and no obvious difference in the abundance of this band was found among treatments (Fig. 4-6). Five POD isoforms were detected in roots under all treatments with the two top bands strongly exhibited (Fig. 4-7). The POD isoform pattern in shoots was different from the pattern in roots, with two strongly stained bottom bands (total 4 isoforms). From the top to the bottom of the gels, we arbitrarily assigned them as PODr1 to 5, and PODsh1 to 4. No difference in the abundance of these isoform bands was found.

DISCUSSION

Exogenous cytokinin application is known to reduce heat stress injury in different plant species, including creeping bentgrass (Schrader, 2005; Skogqvist, 1974; Zhang and Ervin, 2008). Heat stress affects antioxidant response in creeping bentgrass and other cool-season turfgrass species (Jiang and Huang, 2001; Liu and Huang, 2000). Liu and Huang (2002) reported exogenous cytokinin application by soil injection mitigated heat stress damage to creeping bentgrass and helped to maintain higher antioxidant level and less lipid oxidation. More recently, the same research group reported foliar applications of zeatin riboside suppressed leaf senescence and resulted in elevated antioxidant metabolism in creeping bentgrass (Xu and Huang, 2009). Interestingly, we didn't observe any cytokinin effects on the measured parameters here, such as antioxidant enzyme activities, ROS production, or MDA content. Here we pose that this inconsistency with other studies could be due to different cytokinin application methods, high temperature setting, stress period, or other factors. Here we used foliar application, instead of soil injection used by Liu and Huang (2002). In addition, our high temperature setting was 28/38 ^{o}C (n/d), instead of 25/35 ^{o}C (n/d), or 35 ^{o}C constantly (Xu and Huang, 2009). This harsher temperature stress might have diminished the beneficial effects of exogenous cytokinin. Moreover, the sampling time could also play a role. For example, differences in shoot MDA content between the control and CK treatment was only found at one of the six sampling dates (Xu and Huang, 2009). This inconsistency between studies warrants further research to systematically investigate cytokinin effects on the antioxidant system in heat stressed creeping bentgrass.

Nitrogen treatment had significant effects on SOD activity under heat stress, with a 19% and 13% increase in roots and shoots under high N compared to low N, respectively.

Similar effects of N on APX and POD were observed, except no difference in shoot POD activity between N levels. SOD is the key enzyme to scavenge O_2^- produced in the cells by catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide. The ascorbate-glutathione-cycle is a metabolic pathway that is thought to play a very important role for H_2O_2 detoxification in higher plants. In the first step of this pathway, H_2O_2 is reduced to water by ascorbate peroxidase (APX) using ascorbate as the electron donor. Nevertheless, other peroxidases, such as guaiacol peroxidase, also contribute to H_2O_2 removal in plants (Apel and Hirt, 2004; Blokhina and Fagerstedt, 2010; Mittler, 2002).

In general, the activities of these antioxidant enzymes increase concomitantly with a decrease of ROS production, or vice versa (Blokhina et al., 2003; Miller et al., 2010). For example, Rivero et al., (2004) reported a negative correlation between POD activity and H_2O_2 accumulation in tomato (*Solanum lycopersicum* L.) plants under heat stress. Similarly, Yan et al., (1996) found that declines in SOD activity in corn (*Zea mays* L.) paralleled the enhanced production of O_2^- in corn under flooding stress. Interestingly, in this experiment root O_2^- production and H_2O_2 concentration was greater in heat stressed bentgrass under high N, and higher shoot H_2O_2 concentration was found under low N, although greater antioxidant enzyme activities were observed accordingly. These findings are contradictory to commonly accepted interpretations of plant responses to oxidative stress. However, similar results to ours have been reported. Wang and Jiang (2007) reported that grass with higher APX activity under waterlogging (hypoxic) conditions did not maintain lower H_2O_2 concentration compared to the grass under aerobic conditions. Lily plants (*Lilium longiflorum* Thunb.) showed higher SOD and POD activity accompanied with equal or much greater O_2^- production and higher H_2O_2 concentration after 6 or 8 hour at 47 ^oC, although lower SOD

and POD activity was concurrent with greater O_2^- production and higher H_2O_2 concentration after 10 h at 47 ^oC (Yin et al., 2008). This indicated the stable antioxidant system status could depend on stress duration, intensity, and probably other factors.

As an important and essential nutrient element for plant growth and development, nitrogen is also important for the resistance of turfgrass to different stressful conditions (Liu et al., 2007). Higher N was reported to help to maintain higher photosynthesis and photosynthetic N-use efficiency in plants under heat stress (Wang et al., 2008). Higher growth rate, photosynthesis and metabolic activity were proposed as a mechanism for higher ROS production in three high N treated plant species, maize, barley (*Hordeum vulgare* L.), and *Arabidopsis* (Medici et al., 2004). Another possible explanation for greater ROS accumulation in high N grass is the possible higher ratio of unsaturated to saturated membrane lipids. In general, nitrogen-deficient chloroplasts contained a higher proportion of saturated fatty acids than tissues grown on a complete nutrient solution (Newman, 1966; Newman, 1967). Li et al., (2009) reported that higher N level increased the ratio of unsaturated to saturated fatty acids of the membrane lipids. The membranes with higher proportion of unsaturated fatty acids are more likely to sustain electron leakage and promote superoxide formation (Schönfeld and Wojtczak, 2008). In order to address this question, a further study would be needed to investigate the membrane lipid composition in heat stressed creeping bentgrass at different N levels.

Lipid peroxidation has been often been used to monitor ROS damage. Polyunsaturated fatty acids within the lipids are a preferred target of ROS attack (Apel and Hirt, 2004). As the product of peroxidation of unsaturated fatty acids, malondialdehyde (MDA) content increased in roots of bentgrass at high N, concomitant with elevated O_2^- and H_2O_2 production,

indicating that the increase in lipid peroxidation was probably caused by more ROS production. This result is consistent with many previous plant studies (Xu et al., 2006; Yin et al., 2008). In addition to being potentially toxic molecules, ROS and lipid peroxidation are also thought to participate in the signal pathway to activate stress defense mechanisms (Apel and Hirt, 2004; Kurganova et al., 1999; Schönfeld and Wojtczak, 2008). In response to higher ROS production and/or accumulation and membrane lipid peroxidation under high N, an enhancement of ROS scavenging would be required to prevent potential damage since excessive ROS would be toxic to cells (Medici et al., 2004; Mullineaux and Creissen, 1997). Fu and Huang (2003) also reported a foliar nutrient treatment with NH_4NO_3 improved heat stress tolerance of creeping bentgrass with increased shoot antioxidant enzyme activities, such as SOD and APX. It is worthwhile to mention that our unpublished data showed grass at high N had a lower shoot electrolyte leakage, with higher root viability measured as TTC (2, 3, 5-triphenyl tetrazolium chloride) reduction, suggesting that higher ROS production and lipid peroxidation were more likely due to higher metabolic activity at high N, instead of more stress damage.

Enzyme isoforms could be more sensitive / meaningful compared to total activity changes because different isoforms may function differently due to their distinct properties, such as being stress inducible or not, and different sub-cellular locations (Apel and Hirt, 2004; Blokhina et al., 2003; Shigeoka et al., 2002). For example, there are three different types of SOD isoforms based on their metal cofactors, namely Fe-SOD in chloroplasts, Mn-SOD in mitochondria, and Cu/Zn-SOD in cytosol and chloroplasts (Blokhina et al., 2003). In lily plants, total SOD activity was increased at 42 ^{o}C. Native PAGE further found the increase was due to induced Cu/Zn-SOD isoforms, with the Mn-SOD being inhibited (Xin et al, 2008).

In our study after 28 d of heat stress the roots showed two more SOD isoform bands (SOD r-4 and 5) than in the roots prior to heat stress initiation. A previous report on root SOD isoforms of creeping bentgrass under hypoxia (Wang and Jiang, 2007) suggested these two bands were most likely Cu/Zn-SOD isoforms. Heat stress was reported to increase Cu/Zn-SOD isoform activity in other species, such as lily (Xin et al, 2008) and *Chenopodium murale* (Chopra and Sabarinath, 2004). In our study there was no apparent evidence of a difference in root or shoot SOD isoform profiles among treatments. This result was similar to what other studies reported. Medici et al. (2004) did not observe any SOD isoform regulated by N in plant roots. Other studies reported there were no effects of cytokinn on SOD isoform pattern (Bakardjieva et al., 2001; Szechyńska-Hebda et al., 2007). However, the activity SODsh6 in the grasses treated with low N and 10 or 100 μM CK seemed to be higher than the grasses treated with low N but no CK, although no CK effects on total shoot SOD activity were observed. More quantitative data would be needed before a certain conclusion could be made. In addition, the different SOD isoform activity patterns between roots and shoots could be simply due to the tissue difference.

Like SOD, several distinct isozymes of APX are localized in cytosol and various organelles such as chloroplasts, mitochondria, and peroxisomes (Shigeoka et al., 2002). However, here only one band of APX isoform was observed in both roots and shoots, which is consistent with what Wang and Jiang (2007) reported in creeping bentgrass under hypoxia. In addition, Yahubyan et al. (2005) reported only one APX isoform in fresh leaves of tobacco (*Nicotiana tabacum* L.), but they found two APX isoforms in *Haberlea rhodopensis* (Friv.), suggesting the amount of APX isoform observed is species dependent. Another possible explanation would be that our extraction buffer mainly extracted the water soluble part of

APX, cytosolic APX (Ranieri et al., 2001). Increased activity of APX in response to heat stress has been reported in plants (Sharma and Dubey, 2004). High temperature increased the isoform intensity in roots, which was consistent with the activity assay, indicating that this isomer was at least partially responsible for the observed activity increase under heat. Native gel did not show an obvious effect of CK or N on isoform pattern and stain intensity. There was no difference of APX isoform pattern between shoots and roots of creeping bentgrass.

For POD, high temperature induced stronger intensity for PODr1 and PODr2, which were the most dominant isoforms among all the five isoforms showing on the native gel in roots. In addition, three new isoform bands were observed in the samples after 28 d heat stress, regardless of treatment. Gulen and Eris (2004) reported enhanced POD activity and a newly induced POD isoform in strawberry (*Fragaria x ananassa* Duch.) under high temperature. In contrast, Liu et al. (1995) reported a decline in the intensity of a POD isoform in wheat seedlings in response to heat stress. In addition, Xin et al. (2008) found a POD isoform in lily increased at 37 and 42 ^{o}C, but decreased at 47 ^{o}C. These contradictory results may be due to differences in heat stress tolerance among plants and differences in stress intensity and duration. Native gel did not show obvious difference of POD isoform pattern among treatments in both roots and shoots, suggesting no effect of CK or N on isoform pattern and stain intensity. Shoots have different POD isoform patterns with four bands from roots. PODsh3 and 4, instead of POD r1 and 2 showed stronger intensity, plus shoots did not have the 5th POD band at the bottom. This difference suggested a tissue specific POD isoform distribution pattern, which could be related to their specific function.

In summary, our results found nitrogen had effects on measured parameters in both roots and shoots, suggesting a strong impact on antioxidant response of heat stressed

creeping bentgrass. The up-regulated antioxidant system at high N could be related to better grass performance under heat. Interestingly, CK seemed to play no role in antioxidant response of heat stressed bentgrass, which was unexpected. Both SOD and POD showed different isoform patterns between roots and shoots, indicating some of the responses could be tissue specific.

ACKNOWLEDGEMENT

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Table 4-1. Analysis of variance of the activities of super oxide dismutase (SOD), ascobate peroxidase (APX), and guaiacol peroxidase (POD), malondialdehyde (MDA) content, hydrogen peroxide (H_2O_2) concentration, super oxide (O_2^-) production rate, and water-soluble protein concentration (WSP) in creeping bentgrass after 28 d heat stress.

Plant tissue	Variance	SOD	APX	POD	MDA	H_2O_2	O_2^-	WSP
Root (R)	Nitrogen (N)	**	**	**	**	**	**	*
	Cytokinin (CK)	NS	NS	NS	NS	NS	NS	NS
	N x CK	NS	NS	NS	NS	NS	NS	NS
Shoot (Sh)	Nitrogen (N)	**	**	NS	NS	**	NS	NS
	Cytokinin (CK)	NS	NS	NS	NS	NS	NS	NS
	N x CK	NS	NS	NS	NS	NS	NS	NS

****, and * mean significant level at $p < 0.01$ and 0.05, respectively.**
NS: not significant.

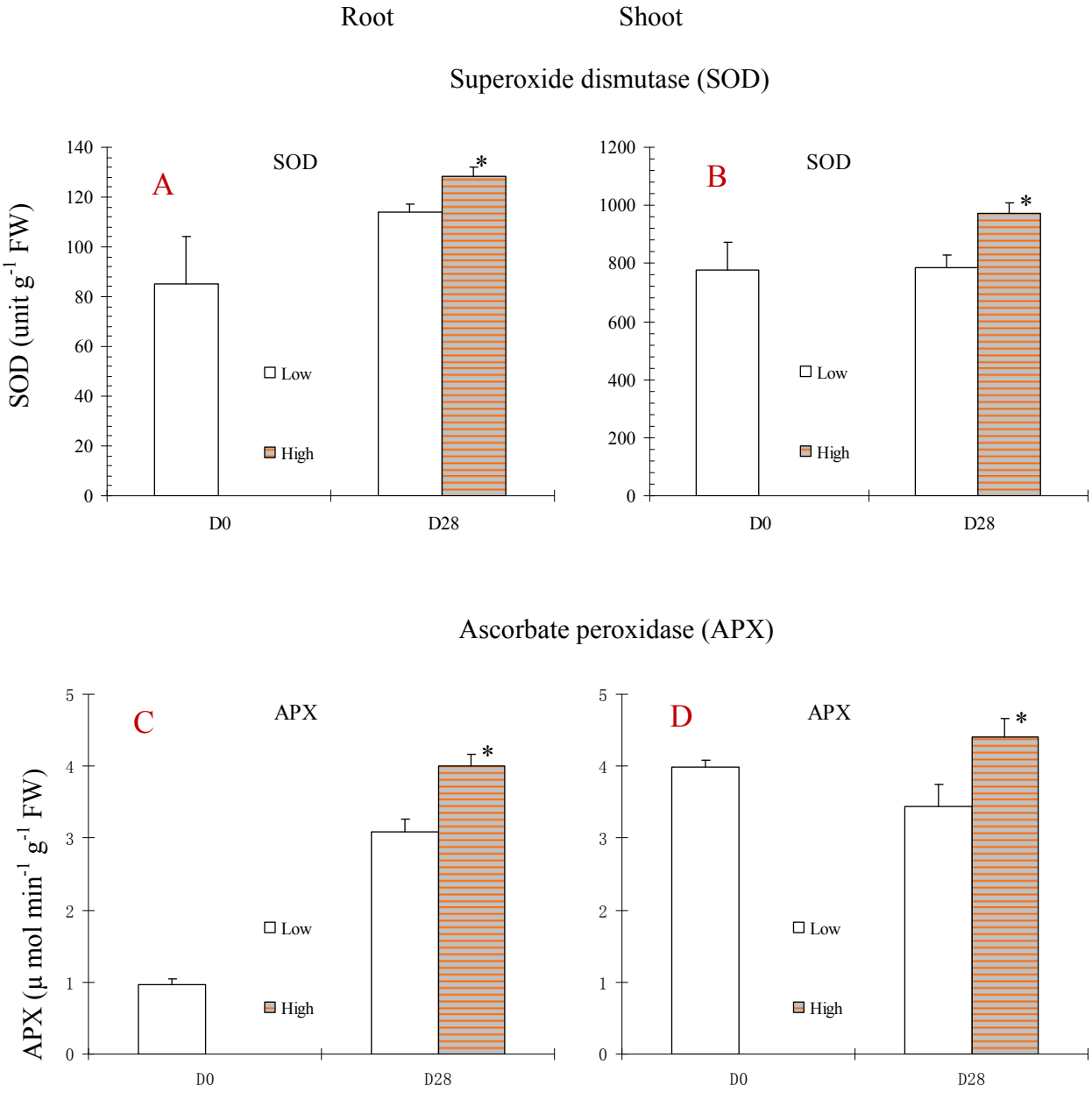

Fig.4-1. Activity of superoxide dismutase (SOD) in roots (A) and shoots (B), ascorbate peroxidase (APX) in roots (C) and shoots (D) of heat stressed creeping bentgrass as affected by two nitrogen levels (Low and High). D0: the day before heat stress was initialed, D28: 28 days after heat stress.

* indicated significantly different at $P < 0.05$. Vertical bars show ± S.E. of mean.

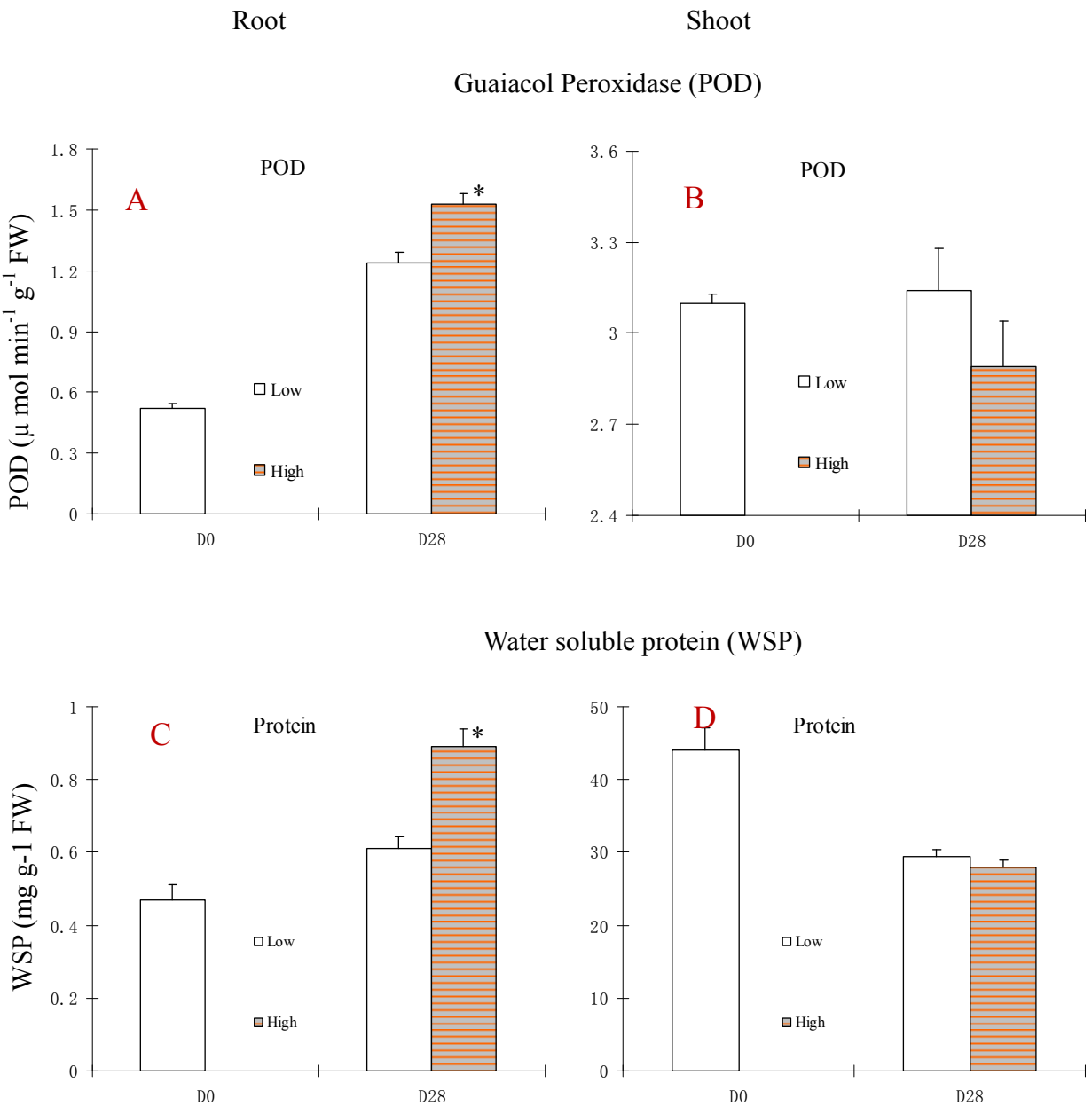

Fig.4-2. Guaiacol peroxidase (POD) activity in roots (A) and shoots (B), water soluble protein content (WSP) in roots (C) and shoots (D) of heat stressed creeping bentgrass as affected by two nitrogen levels (Low and High). D0: the day before heat stress was initialed, D28: 28 days after heat stress.

* indicated significantly different at $P < 0.05$. Vertical bars show ± S.E. of mean.

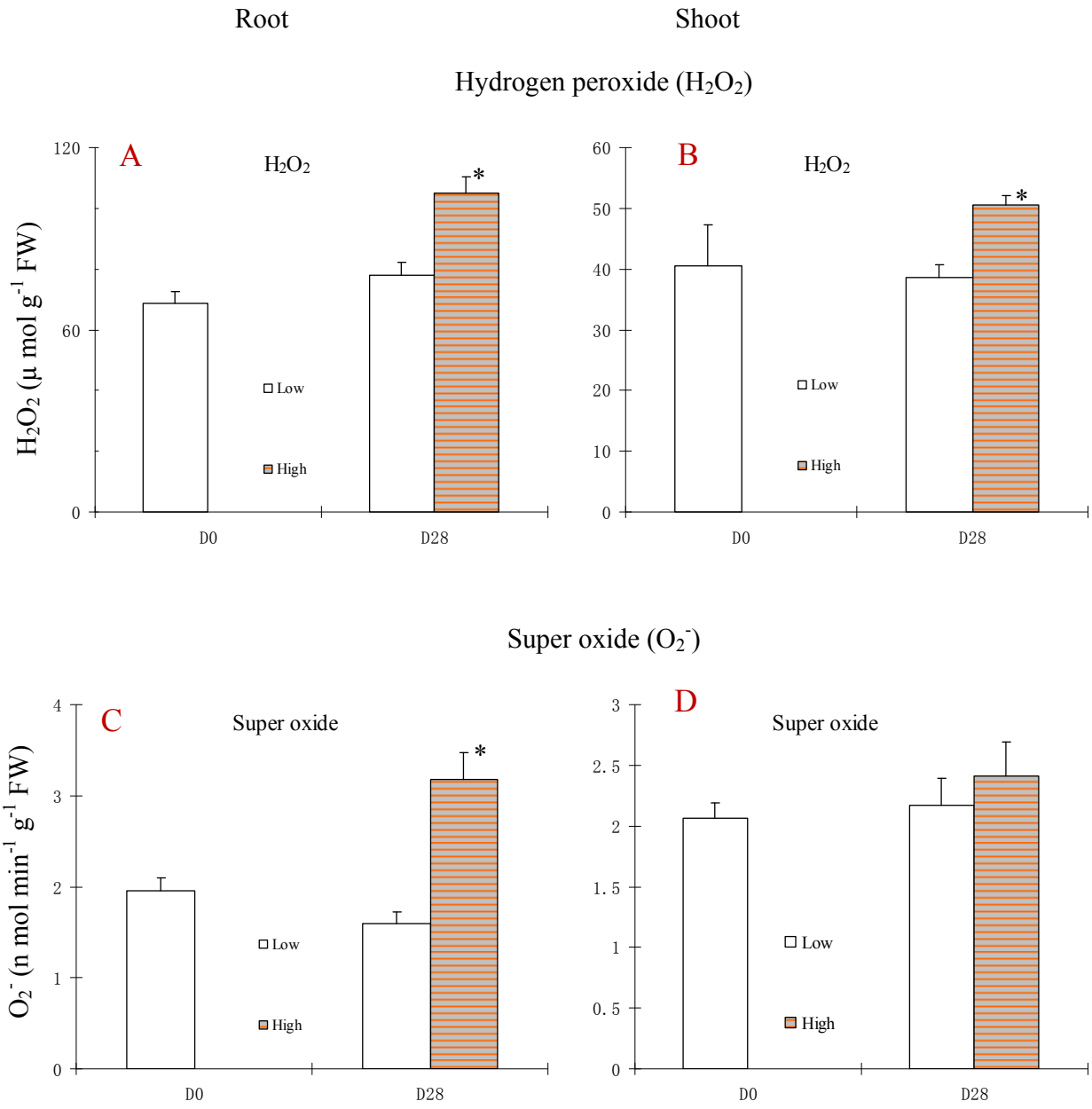

Fig.4-3. Hydrogen peroxide (H_2O_2) concentration in roots (A) and shoots (B), super oxide (O_2^-) production rate in roots (C) and shoots (D) of heat stressed creeping bentgrass as affected by two nitrogen levels (Low and High). D0: the day before heat stress was initialed, D28: 28 days after heat stress.

* indicated significantly different at $P < 0.05$. Vertical bars show ± S.E. of mean.

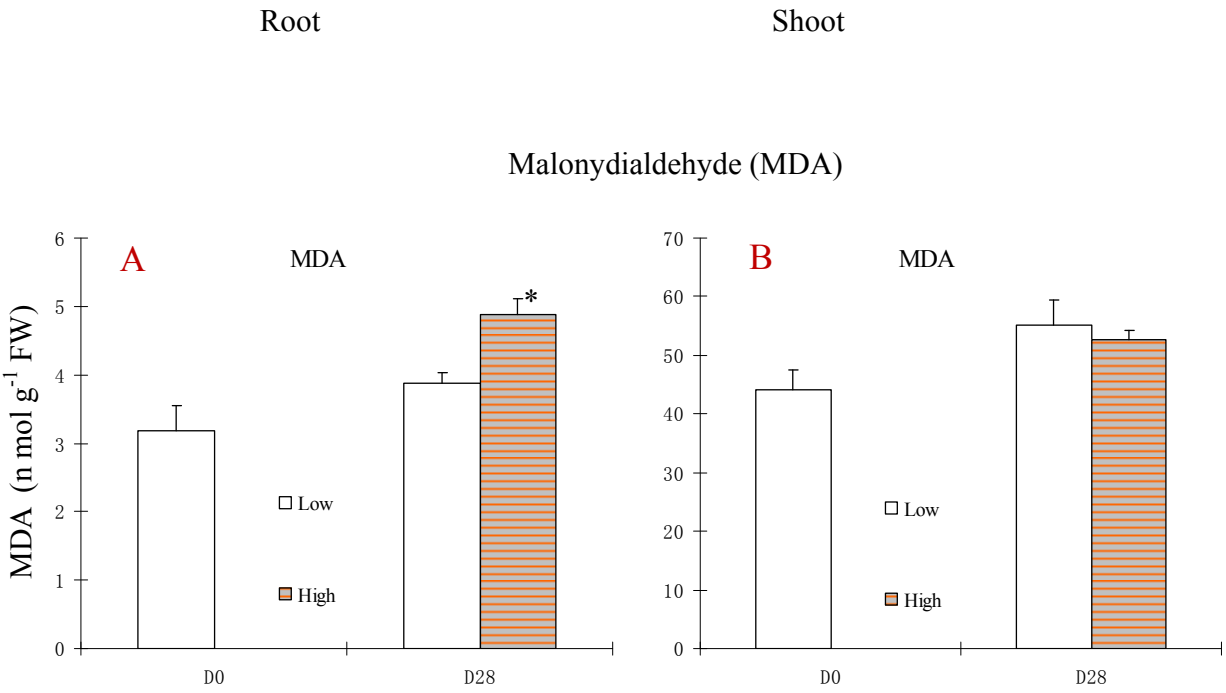

Fig.4-4. Malonydialdehyde (MDA) concentration in roots (A) and shoots (B) of heat stressed creeping bentgrass as affected by two nitrogen levels (Low and High). D0: the day before heat stress was initialed, D28: 28 days after heat stress.

* indicated significantly different at $P < 0.05$. Vertical bars show ± S.E. of mean.

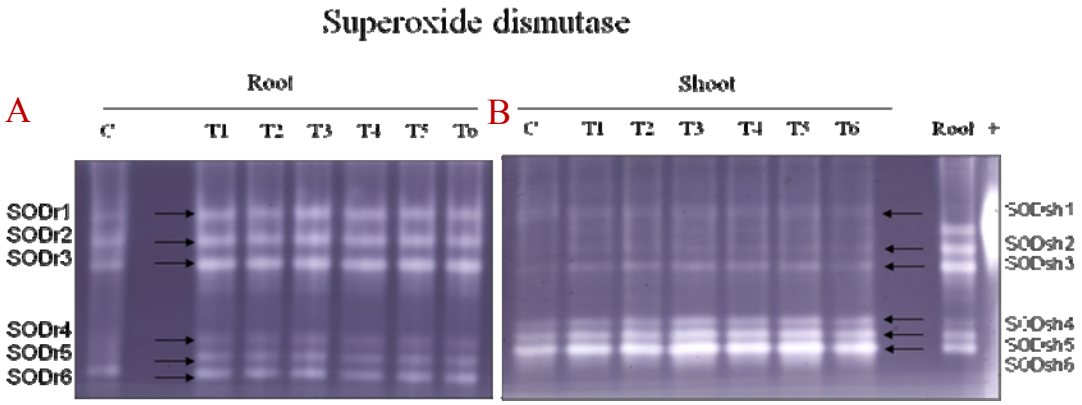

Fig.4-5. Native gel stained for the activity of superoxide dismutase (SOD) isoforms in roots (A) and shoots (B) of heat stressed creeping bentgrass treated with cytokinin (CK) and N nutrition. C: root or shoot samples before heat stress; T1 to T6 represent low N+ no CK, high N+ no CK, low N+ 10 μM CK, high N+ 10 μM CK, low N+100 μM CK, and high N+100 μM CK in roots or shoots of creeping bentgrass 28 d after heat stress, respectively. Root: a root sample at d 28 (for comparing SOD isoform pattern in shoots); +: a positive staining control (Bovine SOD, Sigma). Equal amounts of protein (80 μg) were loaded to each lane. Arrows indicate the isoforms.

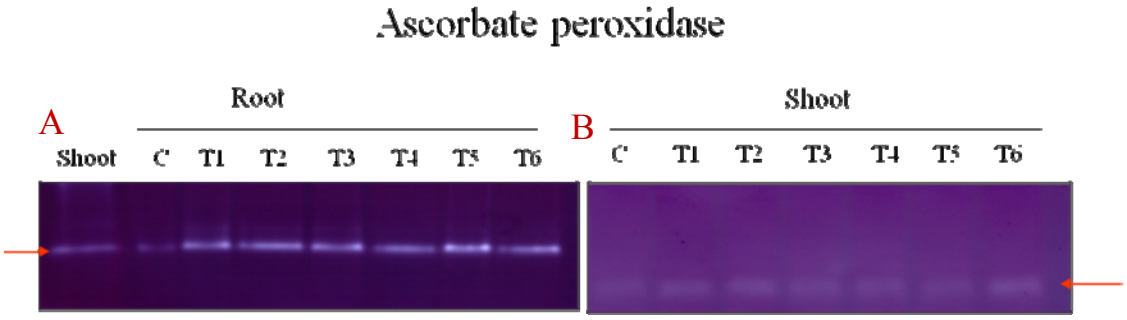

Fig.4-6. Native gel stained for the activity of ascorbate peroxidase (APX) isoforms in roots (A) and shoots (B) of heat stressed creeping bentgrass treated with cytokinin (CK) and N nutrition. C: root or shoot samples before heat stress; T1 to T6 represent low N+ no CK, high N+ no CK, low N+ 10 μM CK, high N+ 10 μM CK, low N+100 μM CK, and high N+100 μM CK in roots or shoots of creeping bentgrass 28 d after heat stress, respectively. Shoot: a shoot sample at d 28 (for comparing APX isoform pattern in roots); Equal amounts of protein (80 μg) were loaded to each lane. Arrows indicate the isoforms.

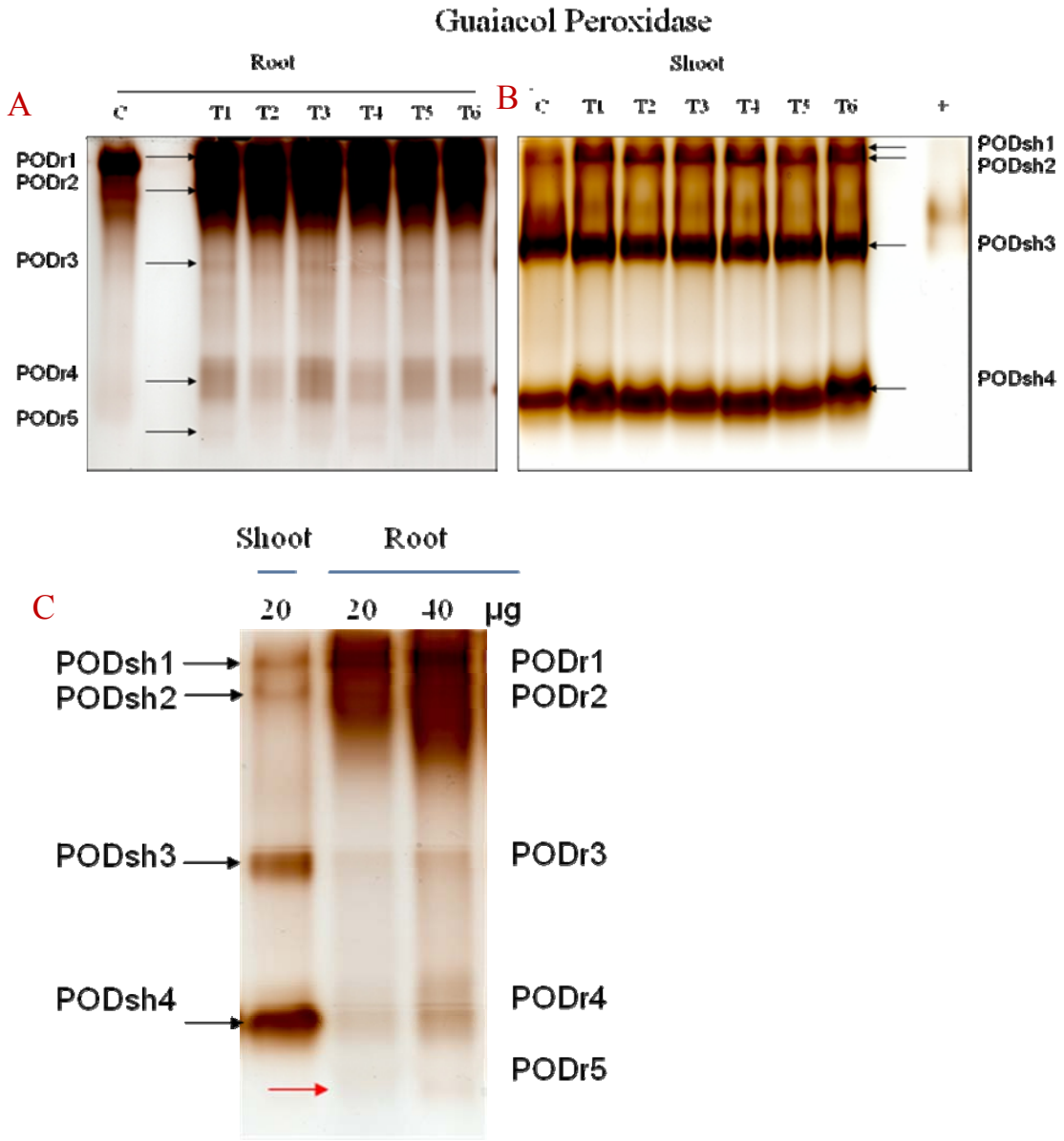

Fig.4-7. Native gel stained for the activity of guaiacol peroxidase (POD) isoforms in roots (A) and shoots (B) of heat stressed creeping bentgrass treated with cytokinin (CK) and N nutrition, and POD isoform pattern comparison (C) between roots and shoots. C: root or shoot samples before heat stress; T1 to T6 represent low N+ no CK, high N+ no CK, low N+ 10 µM CK, high N+ 10 µM CK, low N+100 µM CK, and high N+100 µM CK in roots or shoots of creeping bentgrass 28 d after heat stress, respectively. +: a positive staining control (Horseradish POD, Sigma). Equal amounts of protein (60 µg) were loaded to each lane, except panel C. Arrows indicate the isoforms.

CHAPTER FIVE

HEAT SHOCK PROTEINS IN RELATION TO HEAT STRESS TOLERANCE OF CREEPING BENTGRASS AT DIFFERENT N LEVELS

ABSTRACT

Heat stress is a primary factor causing summer bentgrass decline. Previous studies suggest that nitrogen may improve creeping bentgrass (*Agrostis stolonifera* L.) heat tolerance. Changes in gene expression at the transcriptional and/or translational level are thought to be a fundamental mechanism in plant response to environmental stresses. Heat stress redirects protein synthesis in higher plants and results in a nearly exclusive synthesis of stress proteins, particularly heat shock proteins (HSPs). The goal of this work was to analyze the pattern of expression of the major members of the HSP group in creeping bentgrass during different heat stress periods, and then to study the influence of nitrogen on the expression pattern of HSPs. A growth chamber study on 'Penn-A4' creeping bentgrass subjected to 38/28 ^{o}C day/night for 50 days, was conducted with four nitrate rates (no N-0, low N-2.5, medium N-7.5, and high N-12.5 kg N ha^{-1}) applied biweekly. Visual turf quality (TQ), green color index (NDVI), photochemical efficiency (Fv/m), shoot electrolyte leakage (ShEL) and root viability (RV) were monitored, along with the expression pattern of HSPs as measured by western blot. There was no difference in measured parameters between treatments until week seven, except TQ at week five. At week seven, grass at medium N had better TQ, NDVI, and Fv/Fm accompanied by lower ShEL and higher RV, suggesting better heat tolerance. All the

investigated HSPs (HSP101, HSP90, HSP70, and sHSPs) were up-regulated by heat stress. Their expression patterns indicated cooperation between different HSPs and their roles in creeping bentgrass thermotolerance. In addition, their production seems to be resource dependant. This study could further improve our understanding about how different N levels affect bentgrass thermotolerance and the role of specific HSPs in heat tolerance of creeping bentgrass.

Keywords: Nitrogen, Heat shock protein, heat stress, thermotolerance

INTRODUCTION

Heat stress due to increased temperature is a problem in agriculture worldwide. Heat stress induces a series of growth and metabolic responses in higher plants (Kotak et al., 2007; Sun et al., 2002; Wahid et al., 2007). For example, heat stress redirects protein synthesis in higher plants by decreasing the synthesis of normal proteins accompanied by a dramatic increase in transcription and translation of a new set of proteins: heat shock proteins (HSPs) (Schulze et al., 2005). Based on their approximate molecular weight, the principal heat shock proteins are grouped into five conserved classes: HSP100, HSP90, HSP70, HSP60, and the small heat-shock proteins (a molecular mass of 15 to 42 kDa identified by denaturing polyacrylamide gel electrophoresis) (Bharti and Nover, 2002; Schulze et al., 2005). HSPs function mainly as molecular chaperones that help other proteins to maintain their native conformation, thus improving protein stability under stresses (Wahid et al., 2007). The role of HSPs to counter negative effects of heat stress in plants was first hypothesized based on correlative evidence (Vierling, 1991). There is currently accumulating evidence that HSPs play important roles in thermotolerance, and some specific HSPs are causally involved in the capacity to acquire thermotolerance of plants in many studies, such as HSP101 in maize (*Zea mays* L.) and *Arabidopsis* (Hong and Vierling, 2001; Nieto-Sotelo et al., 2002), HSP90 in *Arabidopsis* (Ludwig-Muller et al., 2000), HSP70 in tobacco (*Nicotiana tabacum* L.) (Cho and Choi, 2009), and small heat-shock proteins (sHSPs) in maize and creeping bentgrass (Heckathorn et al., 1998; Luthe et al., 2000).

Cool-season turfgrasses are often subjected to high temperature stress during summer in warm climatic regions such as the transition zone that often results in turfgrass quality decline. Some studies have reported protein changes in response to heat stress or changes in

specific HSP expression are related to heat stress tolerance in cool-season turfgrass species, including creeping bentgrass. He et al. (2005) found two new proteins (54, 57 kD) were synthesized during heat acclimation compared to sudden heat stress in creeping bentgrass, and suggested that the two HSPs might be related to improved heat tolerance by heat acclimation. More recently, He and Huang (2007) reported the synthesis of several heat-inducible proteins in cytoplasm and membranes of Kentucky bluegrass, and indicated that better heat tolerance in certain cultivars was associated with induction of these proteins during the early phase of heat stress. Park et al. (1996) reported that a small heat shock protein (HSP25) was genetically involved in heat tolerance in creeping bentgrass. A later study from the same research group showed that heat sensitivity was associated with reduced capacity of bentgrass variants to accumulate this chloroplastic sHSP (Wang and Luthe, 2003). However, there are no specific data existing for the role of other major HSPs, such as HSP101, HSP90, and HSP70 in heat tolerance of creeping bentgrass. Moreover, how nitrogen affects the expression pattern of these major HSPs in creeping bentgrass under stress is unclear, although a study found N availability influenced HSP production in maize, demonstrated by high-N plants producing greater amounts of mitochondrial HSP60 and chloroplastic HSP24 per unit protein than their low-N counterparts over a decade ago (Heckathorn et al., 1996).

Nitrogen (N) is the most needed mineral nutrient for plants, and it is also important to maintain good turf quality, including color, density, growth, and resistance to stress conditions (Liu et al., 2007). Plants fertilized with N during heat stress had greater fresh and dry weight, and significantly higher membrane thermostability than those fertilized with N before heat stress. This result was suggested to be due to greater rhizospheric N availability

during heat stress (Tawfik et al., 1996). A more recent study reported that higher N helped to maintain higher photosynthesis and photosynthetic N-use efficiency in maize under heat stress (Wang et al., 2008). In cool-season turfgrasses, additional foliar N supply was found to be beneficial to heat stressed plants (Fu and Huang, 2003; Zhao et al., 2008), and enhanced antioxidative response was suggested as a mechanism accounting for improved heat stress tolerance by N in creeping bentgrass (Fu and Huang, 2003). However, other mechanisms may be important for improved heat stress response by N, such as induction and change of expression pattern of the major HSPs. In addition, although annual N fertilization programs for sand-based creeping bentgrass greens are well developed, recommendations for optimum nitrogen application during summer heat stress periods are not well defined. For instance, Beard (1997; 2002) suggested minimizing N application during summer heat stress. He also indicated a need for N to maintain health turf, but no specific rate was recommended. Duble (2001) also pointed out very little fertilizer should be used in summer on bentgrass greens with possible monthly applications of nitrogen at 12.5 kg N ha^{-1}.

The objectives of this study were to find optimum N fertilization rate ranges for creeping bentgrass under summer heat stress, to analyze the pattern of expression of the major members of the HSPs during different heat stress periods, and then to study the influence of nitrogen on the expression pattern of the HSPs. The N rates chosen in this study were based on a literature search, our previous studies, and the senior author's personal communications with golf course superintendents in Virginia and other states in the transition zone.

MATERIAL AND METHODS

Plant Materials and Treatments

'Penn A4' creeping bentgrass was planted in late April, 2009 at 49 kg PLS (pure live seed) ha^{-1} in 19-cm diameter plastic pots (20-cm depth) filled with gravel 2.0 cm above the bottom and then the remaining pots were filled with a soil mixture of sand and calcined clay (heat-treated montmorillonite clay mineral) at a volume ratio of 80% to 20%. The grass was fertilized with Bulldog fertilizer (28-8-18, 1% ammoniac N, 4.8% nitrate N, and 22.2% urea N; SQM North America, Atlanta, GA) at 5 kg N ha^{-1} every week over the first two months, then reduced to 2.5 kg N ha^{-1} biweekly. Three months after growing on a greenhouse bench with a mist system (20±3 /15± 2 ^{o}C, day/night), the grass was moved into a growth chamber at the Virginia Bioinformatics Institute, Blacksburg, VA. The detailed growth chamber settings were: 38/28°C (day/night), relative humidity 70%/85% (day/night), 450 µmol s^{-1} m^{-2} photosynthetically active radiation (PAR) and a 14-h photoperiod. Grass was hand-clipped at 12 mm using an electric shear (3 times a week) throughout the project, except the weeks when grass tissues were sampled.

Treatments

Foliar spray treatments of N as NO_3^- at 0 (no N), 2.5 (low N), 7.5 (medium N) and 12.5 (high N) kg N ha^{-1} were applied every two weeks (Day 0, 14, 28, and 42) in Hoagland's solution (Epstein and Bloom, 2005) (2.5 mL per pot) with a spray bottle. Leaf burning was observed after the first spray at medium and high N rates, particularly the high N rate. Thus the later N solution application was followed by an immediate leaf rinse with 100 mL potable water per pot, and no fertilization burn was observed later on. Both KNO_3 and $Ca(NO_3)_2$

were used as the nitrate sources in the solution. Potassium and calcium levels were equalized across treatments by adding KCl and $CaCl_2$ into the lower N treatment solution. Thus, all nutrient levels were the same, except higher Cl^- concentration in the high N treatment solution. Grass was sub-irrigated with equivalent volumes each morning to prevent drought stress.

Sampling and Measurements

Shoots were harvested at Day 1 (one day after initial treatment application plus heat stress), 15, 36, and 50 in the morning. Roots were washed free of soil after the final harvest (Day 50). All samples were immediately frozen with liquid nitrogen and stored at -80 ^oC until analysis, except the portion used for shoot electrolyte leakage and root viability assays.

Turf quality (TQ) was visually rated weekly based on a scale of 1 to 9, with 1 indicating poorest or dead turf, and 9 the best possible quality according to Ervin and Zhang (2004). Normalized difference vegetation index or a green color index ($NDVI = (Infrared_{850}-Red_{660}) / (Infrared_{850}+Red_{660})$) and canopy photochemical efficiency of photosystem II (PSII) ($Fv/Fm = (Fm_{690}-F0_{690})/Fm_{690}$) were recorded after each TQ reading by using a turf color meter (Fieldscout TCM500, Spectrum technologies, IL) and a dual wavelength chlorophyll fluorometer (OS-50II, Opti-sciences, Tynsboro, MA), respectively.

Shoot electrolyte leakage (ShEL) and root viability (RV) were measured on samples at the last sampling day (Day 50). ShEL was measured according to the method of Blum and Ebercon (1981) and Marcum (1998), with modifications. The detailed procedure for measuring the electrolyte leakage was described by Wang and Jiang (2007). Fresh shoots (100 mg) were excised and cut into 1-cm segments. After being rinsed twice with double

deionized H_2O, shoot segments were placed in each test tube containing 20 mL of double deionized H_2O. Test tubes were shaken on a shaker for 17 to18 h, and the initial conductivity (C1) was measured (Conductivity Meter, VWR, USA). Shoot samples were then killed by autoclaving at 121°C for 20 min, and the conductivity of solution was re-measured (C2) after the tubes cooled to room temperature. The relative electrolyte leakage was calculated as (C1 / C2)*100.

Root viability was determined on whole roots with intact base and tips by measuring dehydrogenase activity with a modified 2,3,5-triphenyltetrazolium chloride (TTC) reduction method (Knievel, 1973; Zhang and Ervin, 2008). About 300 mg fresh root tissue was cut into 2-cm lengths. Then the root sections were immersed in 15 mL of 0.6% TTC solution (dissolved in 50 mM phosphate buffer plus 0.05% Triton X-100, pH 7.4). The samples were vacuum infiltrated for 5 min to insure infiltration of TTC and then incubated in the dark for 24 h at 30 °C. Then the roots were drained and rinsed with deionized water twice. Formazan in the roots were extracted with 5 mL 95% ethanol at 80 °C twice and combined extracts were brought to 10 mL. The absorbance of the extract solution was measured at 490 nm with a spectrophotometer (Biomate 3, Thermo spectronic). Root viability was expressed as the absorbance per g fresh weight.

Protein isolation, SDS PAGE, and Western blot

About 250 mg of liquid nitrogen powdered shoots and root tissue were carefully mixed in a microtube with either 1.5 (shoots) or 1.0 (roots) mL pre-cooled 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA (ethylenediaminetetraacetic acid), 10% (v/v) glycerin, 1 mM PMSF (phenylmethylsulphonyl fluoride), 1% PVP (polyvinylpyrrolidone) (w/v) and 1

mM DTT (dithiothreitol). The extracts were centrifuged for 20 min at 16,000 g at 4°C, and the supernatant was collected for further analysis. Protein concentration was determined by the method of Bradford (1976). Briefly, 25 µL of protein extract of roots or diluted protein extract of shoots was mixed with 1 mL of Bradford protein regent (Sigma, St. Louis, MO), and the absorbance was measured at 595 nm after 15 min using a spectrophotometer (Biomate 3, Thermo spectronic). Bovine serum albumin was used as a standard (Sigma, St. Louis, MO).

Proteins were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) with some modifications. Protein extract was mixed with same volume of 2 x SDS-PAGE sample buffer containing 125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, and 0.02% bromophenol blue. An equal amount of protein (40 µg for HSP101, HSP90, HSP70 protein and 20 µg for small HSP) was loaded in each lane. A pre-stained protein standard was loaded on each gel for molecular weight identification. A PROTEIN III electrophoresis unit (Bio-Rad, La Jolla, CA) was used to separate the proteins. All the protein extracts were subjected to SDS-PAGE with 5% stacking gel and 10% resolving gel, except small HSP for which a 12% resolving gel was used. Electrophoresis was performed at 160 V for 50 min at room temperature. The separated proteins were transferred for 1 h at constant volts of 100 and blotted onto 0.25-µm nitrocellulose membrane (Bio-Rad Laboratories). After blotting, the membrane was blocked with 5% nonfat milk in TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 2 h at room temperature. After a brief rinse with TBS, the membrane was incubated in TBS with primary antibodies against HSP101 (Abcam plc.), HSP 90 (a kind gift from Dr. Shirasu at University of Tokyo, Japan)

(Takahashi et al., 2003), HSP70 (Stressgen Biotechnologies), and sHSP (a kind gift from Dr. Heckathorn, University of Toledo, Ohio, USA) (Heckathorn et al., 1996) at a dilution of 1:1500, 1:2500, 1:1000, and 1:2000 for 2 h, respectively. After that the membrane was rinsed in TBS containing 0.1% Tween 20 (TBS-T) 5-min four times and then placed for 1.5 h in a solution of either goat anti-rabbit or anti-mouse IgG (secondary antibodies, dilution 1:15,000) conjugated to alkaline phosphatase (Sigma). The membrane was rinsed in TBS-T four times and then developed using a pre-mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Immunoblotting was conducted for three replications and the representative data are presented here.

Experimental Design and Statistical Analysis

The experiment was a randomized complete block design with four replicates. All measurements were analyzed using the samples collected at the sampling days mentioned above. Data were analyzed using PROC GLM (SAS Institute, Version 9.1, Cary, NC). Mean separations were performed using Fisher's–protected Least Significant Difference (LSD) test at a 0.05 significance level, except as otherwise stated herein.

RESULTS

Turf Quality, NDVI, and Photochemical Efficiency

Overall, turf quality (TQ) decreased with the stress regardless of the N level (Figure 1A). No difference in TQ between N treatments was observed until Day 36 ($p<0.1$). At Day 36, grass treated with medium N showed 14% higher TQ than at high N. At Day 50, grass treated with medium N had the highest TQ among the treatments (Figure 1A). Both grass

canopy normalized differential vegetative index (NDVI) and photochemical efficiency (Fv/Fm) followed similar patterns as TQ (Figure 1B, 2A). Significant differences between treatments were found at Day 50 for both NDVI and Fv/Fm, but not at other sampling dates. Grass at high N had lowest NDVI, which was less than half of that at medium N at Day 50. Grass under medium N showed the highest Fv/Fm readings, which was 38%, 35% and over 200% higher than grass without N, under low N, and high N, respectively.

Shoot electrolyte leakage (ShEL) and root viability (RV)

Shoot electrolyte leakage increased after 50 d of heat stress regardless of N treatment, simultaneous with decreased root viability (Figure 2B, C). The grass at medium N had lower ShEL than that at both no N and high N, but not the grass at low N. Similarly, the grass at medium N showed higher root viability than the rest.

Expression of Heat Shock Proteins

Western blot analysis showed that HSP101 was induced under heat stress in both shoots and roots of creeping bentgrass. In shoots, a greater amount of HSP101 was present as stress was prolonged. In addition, the grass with higher N generally had more HSP101in shoots at all sampling dates, except Day 50, when compared to that with lower N. Both roots and shoots of the grass at medium N showed a higher level of HSP101 than others at Day 50.

Levels of HSP90 in both shoots and roots of creeping bentgrass indicated that HSP90 was induced by heat stress. In general, there was a trend that HSP90 at each N level increased with stress until Day 36. In addition, there was a general increase of HSP90 with

increased N level at the earlier sampling days (Day 1, 15, and 36). The grass roots without N had less HSP90 at Day 50 than others.

HSP70 was present in plants in all treatments before and after heat stress. There were more HSP70 in plants after stress than before stress. Similar to HSP101, the levels of HSP70 increased with stress regardless of treatment within the first five weeks. A general trend of greater HSP70 with increased N level at the earlier sampling days was also observed. In addition, both roots and shoots of grass at medium N showed higher level of HSP70 than others at Day 50.

Like other HSPs investigated here, high temperature induced sHSP in both roots and shoots. Unlike the others, the amount of sHSP did not increase with stress during the first five weeks, with a relatively higher level of sHSP accumulation at higher N treatments only being observed at Day 15.

DISCUSSION

Heat stress affects cool-season turfgrasses negatively. Many studies have reported TQ decline, reduced photochemical efficiency, and other changes under heat stress (Liu et al., 2002; Pote et al., 2006; Zhang and Ervin, 2008). As expected, TQ, NDVI and photochemical efficiency all decreased with stress. The grass receiving medium N level demonstrated positive treatment responses at five weeks of heat stress, and showed higher TQ, NDVI and photochemical efficiency than other N treatments at Day 50. Overall, the grass under medium N performed better under stress than at both lower N levels and at the higher N level. Nitrogen is an important nutrient for plant growth and development. Proper N availability is also important for plant resistance to stress conditions (Liu et al., 2007). Fu and Huang

(2003) found better TQ and higher photochemical efficiency in creeping bentgrass with foliar nitrogen treatment than untreated grass four weeks after heat stress. Zhao et al. (2008) also reported that foliar N fertilization improved photochemical efficiency of heat stressed tall fescue (*Festuca arundinacea* Schreb.). Similar beneficial effects of higher N were also reported in a study with corn (*Zea mays* L.) under heat (Wang et al., 2008). It should be noted the lower TQ and NDVI at Day 1 were due to fertilization burn. We started to rinse the canopy right after fertilization treatment in later applications and no further damage was observed.

In order to further evaluate whether grass under medium N was more heat tolerant, we measured ShEL and RV. Both electrolyte leakage and RV have been widely used to evaluate stress resistance/damage in higher plants (Heckman et al., 2002; Huang and Gao, 2000; Rachmilevitch et al., 2007; Su et al., 2007; Wang and Jiang, 2007; Zhang and Ervin, 2008). Lower ShELe was observed in grass at medium N concurrently with higher RV at Day 50. These data further proved better resistance of grass at medium N to long-term heat stress.

However, excess N can reduce grass heat tolerance. In Kentucky bluegrass, plants with high N showed reduced resistance to high temperature (Pellett and Roberts, 1963). In general, before reaching an optimum N status the stress tolerance of turfgrass increases with an increase of N input and carbohydrate reserves. Excessive N makes the turfgrass less stress tolerant possibly due to excess shoot growth with a cost of carbohydrate reserve (Carrow et al., 2001; Fry and Huang, 2004; Liu et al., 2007). Here we did not monitor the carbohydrate status, but we did find grass at high N performed worst. Similarly, Totten et al. (2007) reported in a field study that TQ in summer season peaked at 195 kg N ha^{-1} per year. TQ started to drop at 293 kg N ha^{-1} per year, and decreased further at 390 kg N ha^{-1} per year.

However, their N levels are based on annual rates, and are not specifically for summer heat-stress period, for which their N application rates are not known. Overall, the results indicated the medium N level in this study could be an optimum N rate for creeping bentgrass under heat stress.

Heat shock proteins are widely known to play important roles in heat stress tolerance of higher plants (Wahid, 2007). In order to seek the mechanism for the observed better performance of grass at medium N under long-term heat stress, we investigated the expression of several major HSPs, including HSP101, 90, 70, and sHSP.

HSP100 are a family of ATP-binding proteins with chaperone activity to re-solubilize protein aggregates (Bösl et al., 2006), which then can be refolded with the assistance of the HSP70 system (Glover and Lindquist, 1998; Goloubinoff et al., 1999). HSP101 proteins have been found in many other grass species, such as rice (*Oryza sativa* L.) (Agarwal et al., 2003; Batra et al., 2007), wheat (*Triticum aestivum* L.) (Campbell et al., 2001), maize (Young et al., 2001), and a perennial grass, *Dichanthelium lanuginosum* (Sw.) (Al-Niemi and Stout, 2002). In the study herein we found that HSP101 expression was induced in both roots and shoots of creeping bentgrass under heat stress. In addition, the accumulation of HSP101 protein in shoots seemed to be proportional to stress duration within the first five weeks regardless N treatments. Young et al. (2001) reported that levels of HSP101 in maize increased in response to heat shock, with abundance depending on different tissues/organs. Al-Niemi and Stout (2002) observed HSP101 induction in *Dichanthelium lanuginosum* under both short and long-term heat stress. In maize, HSP101 plays important roles in both induced and basal thermotolerance (Nieto-Sotelo et al., 2002). HSP101 has also been reported to be a major player in acquiring thermotolerance in *Arabidopsis* (Hong and Vierling, 2000; 2001). In the

study herein, grass at medium N continued to maintain high levels of HSP101 in both roots and shoots at Day 50, which coincided with better grass performance. Our results indicate that HSP101 could play a role in enhanced resistant to heat stress.

HSP90 is an essential molecular chaperone in eukaryotic cells, with major roles in managing protein folding, protein degradation, and activation of proteins involved in signal transduction and control of the cell cycle (Krishna and Gloor, 2001; Wang et al., 2004). Rutherford and Lindquist (1998) proposed a dual involvement of HSP90 in signal transduction and cellular responses to stress, including temperature changes. Some members of the HSP90 family are constitutively expressed, and others are stress inducible (Krishna and Gloor, 2001). Similar to HSP101, HSP90 in both roots and shoots of creeping bentgrass showed response to heat stress with increased HSP90 protein level along extended stress periods. An *Arabidopsis* mutant originally identified as deficient in glucosinolate metabolism was found to be thermosensitive due to defective cytosolic HSP90 expression after heat stress. Transient transformation with HSP90 increased its thermostability (Ludwig-Muller et al., 2000). However, another study found an HSP90 inhibitor produced in a fungus enhanced *Arabidopsis* thermotolerance (McLellan et al., 2007). In our study it remains unclear as to whether a relatively high level of HSP90 in shoots at medium N at Day 50 would be related to better overall grass performance. Additionally, the level of HSP90 in roots of grass under high N at Day 50 was still high, although turf quality was low. Considering the possible multiple members of HSP90 existing in a single species (e.g., seven were found in *Arabidopsis,* Krishna and Gloor, 2001), and the fact that HSP90 functions as a capacitor to buffer phenotypic variation in plants (Queitsch et al., 2002), further characterization of the

role of HSP90 in creeping bentgrass is needed to determine its importance for creeping bentgrass heat tolerance.

HSP70 proteins are central components of the cellular network of molecular chaperones and are essential for normal cell function (Frydman, 2001; Hartl, 1996). There was basal expression of HSP70 in both roots and shoots of creeping bentgrass before heat stress. After heat stress, an increase over basal levels of HSP70 was observed. Similar results were also reported in a perennial grass, *Dichanthelium lanuginosum* (Sw.), under heat stress (Al-Niemi and Stout, 2002). Jiang and Huang (2002) observed HSP70 expression under both drought and well-water conditions, with higher levels under drought in tall fescue. Some members of the HSP70 family are induced by environmental stresses, such as heat or cold. These members are suggested to be involved in refolding and proteolytic degradation of non-native proteins; others are constitutively expressed and referred to as heat-shock cognates (Karlin and Brocchieri, 1998). The constitutively expressed form of HSP70 could account for the basal level of HSP70 detected in the grass under normal temperature. In yeast, a study has shown that HSP70 is required for survival at moderately high temperatures, but not for surviving extreme temperatures (Deloche et al., 1997). Lee and Schöffl (1996) reported that acquisition of thermotolerance was negatively affected in HSP70 antisense *Arabidopsis* plants, accompanied by significantly reduced levels of HSP70/HSC70 proteins. *Arabidopsis* plants overexpressing HSP70 were more tolerant to heat shock (Sung and Guy, 2003). A more recent study found that a nuclear-localized HSP70 confers thermoprotective activity in tobacco plants (Cho and Choi, 2009). Like HSP101, the higher level of HSP70 in both shoots and roots at the last sampling day in our study could be important for creeping bentgrass survival of long-term heat stress.

Low molecular weight (LMW) heat shock proteins or small HSPs are the most dominant proteins produced in higher plants upon heat stress (Sun et al., 2002). On the basis of their cellular locations, sHSP are classified into 5 classes: cytosolic (class I and II), chloroplastic, mitochondrial, and endoplasmic reticulum related sHSPs (Lenne et al., 1995; Leone et al., 2003; Vierling, 1991). sHSPs play a role as molecular chaperones that bind to partially folded or denatured substrate proteins and thereby prevent irreversible aggregation or promote correct substrate folding to protect cells from stress damage (Sun et al., 2002). However, there is no evidence that they are required for normal cellular function (Zhang et al., 2005). In our study, no sHSP expression was observed in either roots or shoots before heat stress. However, similar to other HSPs analyzed here, they were expressed quickly in response to heat stress. Zhang et al. (2005) confirmed there was no expression of sHSP genes during non-stressed condition, buta strong activation of this gene in both genotypes of tall fescue under heat stress.

There is accumulated evidence showing that sHSPs are important in plant theromotolerance (Harndahl et al., 1999; Heckathorn et al., 1999; Malik et al., 1999). For instance, Malik et al. 1999 reported significantly higher thermotolerance in carrots (*Daucus carota* L.) overexpressing HSP18.1, and less tolerance to heat shock in plants overexpressing antisense HSP18.1. In creeping bentgrass, a small HSP was reported to be genetically involved in heat tolerance (Luthe et al., 2000; Park et al., 1996). Heat sensitivity of creeping bentgrass variants was associated with reduced capacity to accumulate chloroplastic small HSPs (Wang and Luthe, 2003). Like other HSPs analyzed here, the level of sHSP in grass shoots was relatively higher under medium N at Day 50, which may confer, at least partially, better grass performance. Heckathorn et al. (1996) found a correlation between chloroplast-

sHSP production and PSII efficiency, as measured by Fv/Fm. Their later study further indicated that this chloroplast-sHSP plays a direct role in the protection of PSII (Heckathorn et al., 1998).

As we reported above, all the investigated HSPs were up-regulated under heat stress. In addition, at the last sampling day, higher levels of all of them were observed in shoots or /and roots. All these data indicate a possible coordination and cooperation between these HSPs. The different classes of HSPs/chaperones cooperate and play complementary and overlapping roles in protein protection (Huang and Xu, 2008). For example, *in vitro*, sHSPs bind to partially unfolded proteins in an ATP-independent manner, preventing their irreversible aggregation. Substrates that are denatured in the presence of sHSPs can be refolded and reactivated by HSP70/DnaK (the prokaryotic analogue of eukaryotic Hsp70) with the participation, in some cases, of HSP100/ClpB (caseinolytic peptidase B) and HSP60/GroEL (the prokaryotic analogue of eukaryotic Hsp60) (Friedrich et al., 2004, Huang and Xu, 2008). Some HSPs/chaperones (HSP70 and HSP90) involved in signal transduction and transcript activation may lead to the synthesis of other HSPs/chaperones (Wang et al. 2004).

Nitrogen is required in a relatively large amount for biosynthesis of many crucial organic compounds, such as nucleic acids, amino acids, and proteins (Pessarakli, 2002). Heat stress stimulates a dramatic synthesis of HSPs. Upon heat stress, the fraction of HSPs increases from 1-2% to 4–6% of total cellular proteins (Crevel et al., 2001). In plants, just class I sHSPs can account for up to 1% of the total protein in cells (Derocher et al., 1991; Hsieh et al., 1992). Thus production of HSPs involves significant nitrogen and other resource costs (Ackerly et al., 2000). All the investigated HSPs in this study showed an increased

accumulation pattern with increased N levels at certain stages during the lengthy heat stress period, such as the levels of shoot HSP70 at both Day 1 and 15, which indicated the synthesis of HSPs could be resource dependant. Heckathorn et al. (1996) found that high-N plants produced greater amounts of mitochondrial HSP60 and chloroplastic sHSP than their low-N counterparts, suggesting that HSP production confers significant N costs and that N availability influences HSP production in higher plants. However, excessive N stimulates excess shoot growth with a cost of carbohydrate reserve (Carrow et al., 2001; Fry and Huang, 2004; Liu et al., 2007), which could account for the lower accumulation of HSPs in the grass under high N at Day 50.

In summary, medium N helped the grass to better survive long-term heat stress. In addition, the expression patterns of the major HSPs suggested they played a role in the improved heat resistance of grass at medium N and that their production was resource dependant. Caution should be taken when making field turfgrass management recommendations based on the data from growth chamber experiments. However, our growth chamber research does suggest that a good starting point for future field research would be to apply more than 2.5, but less than 12.5 kg N ha^{-1} every two weeks, when day time high temperatures are between 30 and 40 ^{o}C.

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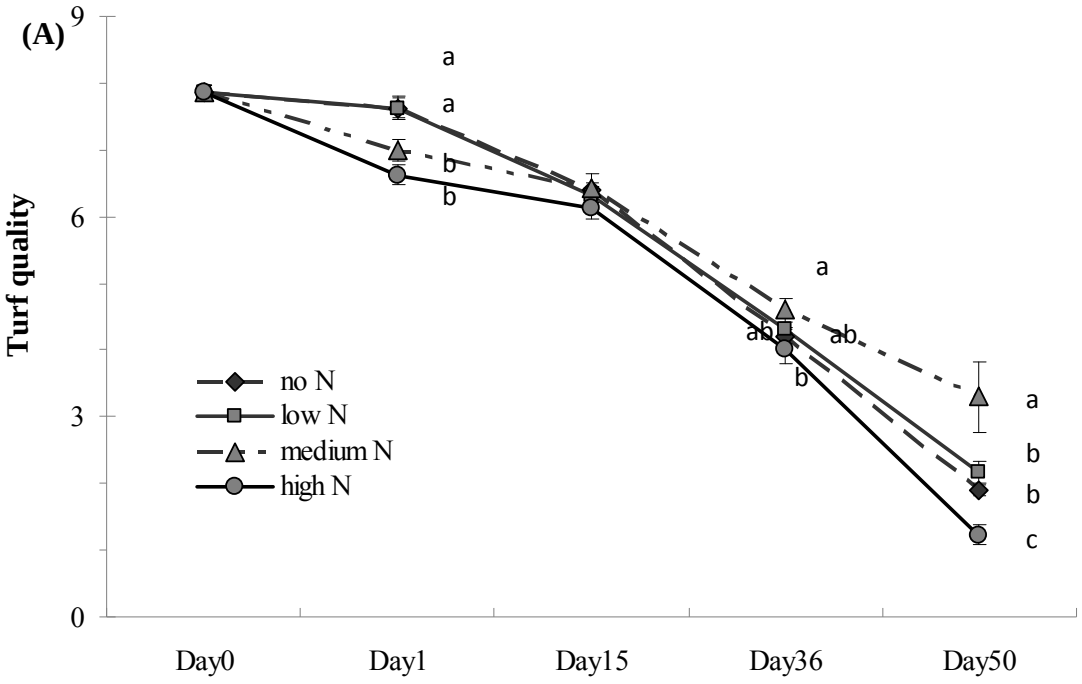
(A)
Turf quality
9
6
3
0
a
a
b
b
a
ab
ab
b
a
b
b
c
no N
low N
medium N
high N
Day0
Day1
Day15
Day36
Day50

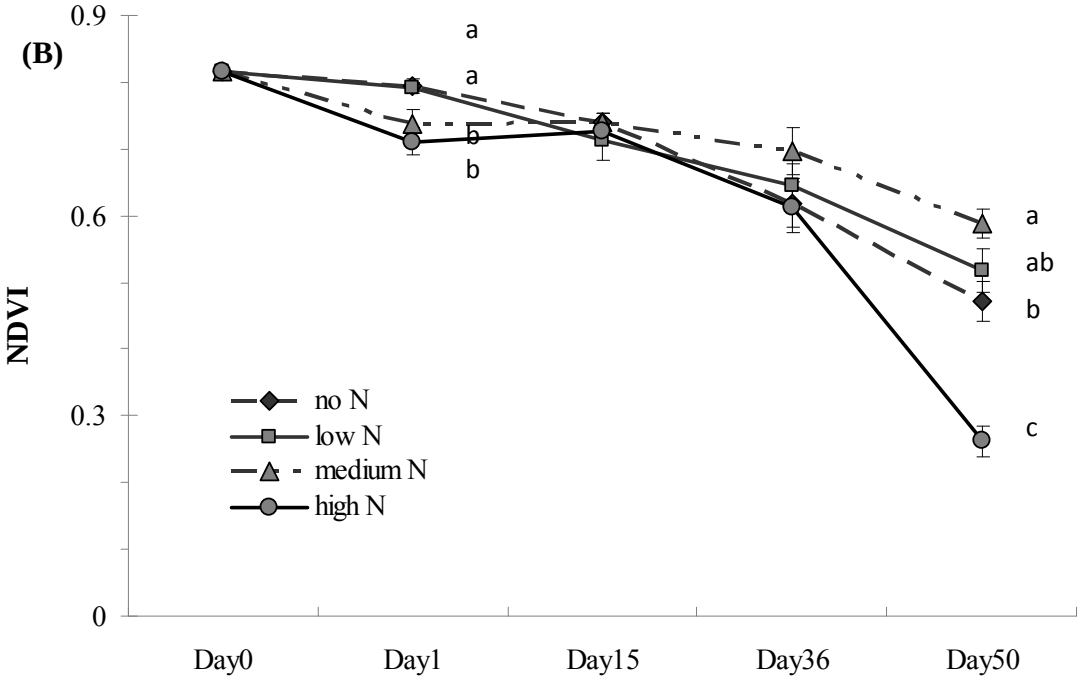
(B)
NDVI
0.9
0.6
0.3
0
a
a
b
b
a
ab
b
c
no N
low N
medium N
high N
Day0
Day1
Day15
Day36
Day50

Fig.5-1. Effects of different N levels on turf quality (TQ) (A) and normalized difference vegetation index (NDVI) (B) of creeping bentgrass under heat stress. Means followed by the same letters at each sampling day are not significantly different based on LSD test at p=0.05 level, except TQ (Day 36) at p=0.1 level. Day50: Fifty days after heat stress.

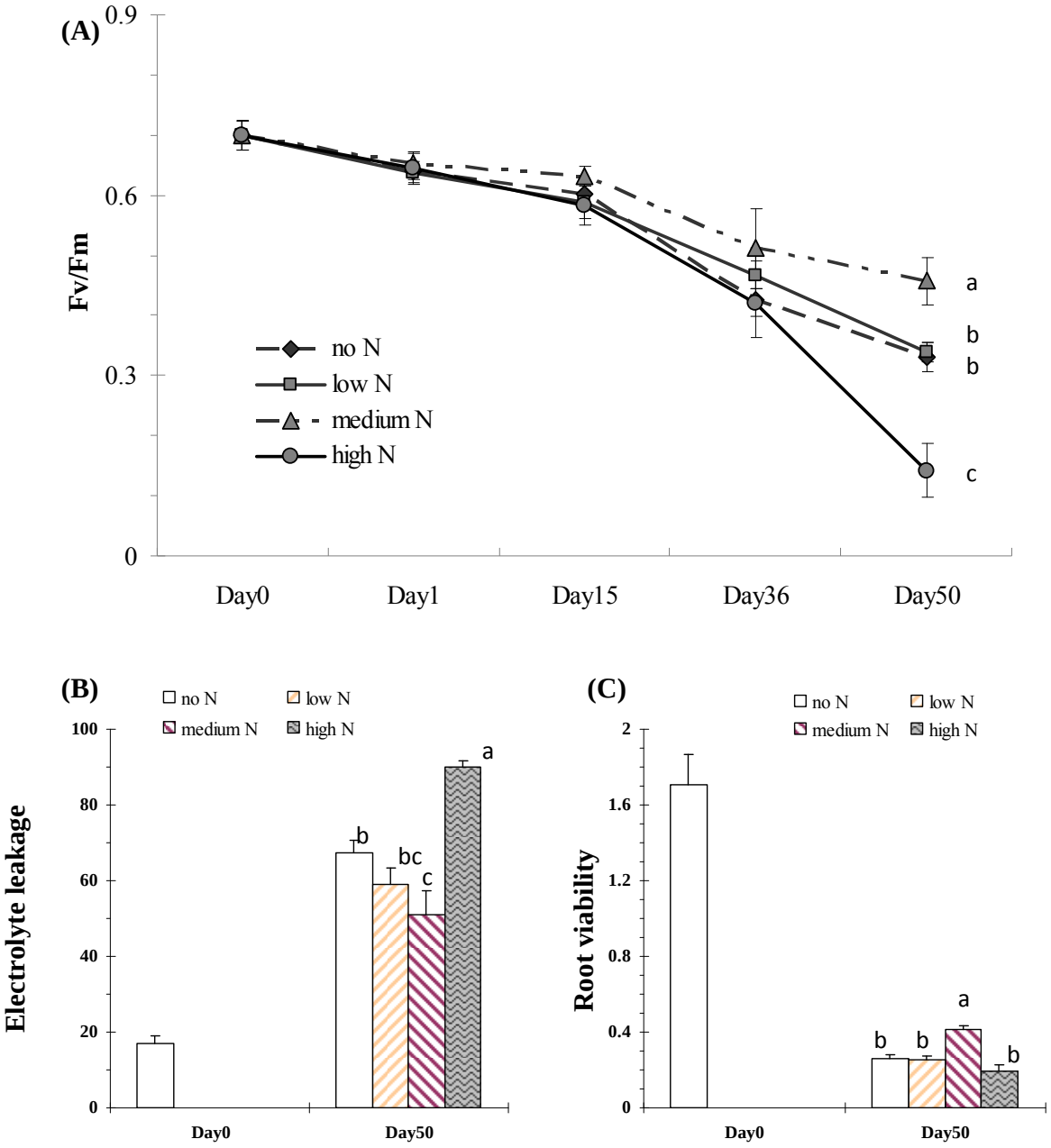

Fig.5-2. Effects of different N levels on photochemical efficiency (Fv/Fm) (A), shoot electrolyte leakage (ShEL) (B) and root viability (RV) (C) of creeping bentgrass under heat stress. Means followed by the same letters at each sampling day are not significantly different based on LSD test at p=0.05 level. Day50: Fifty days after heat stress.

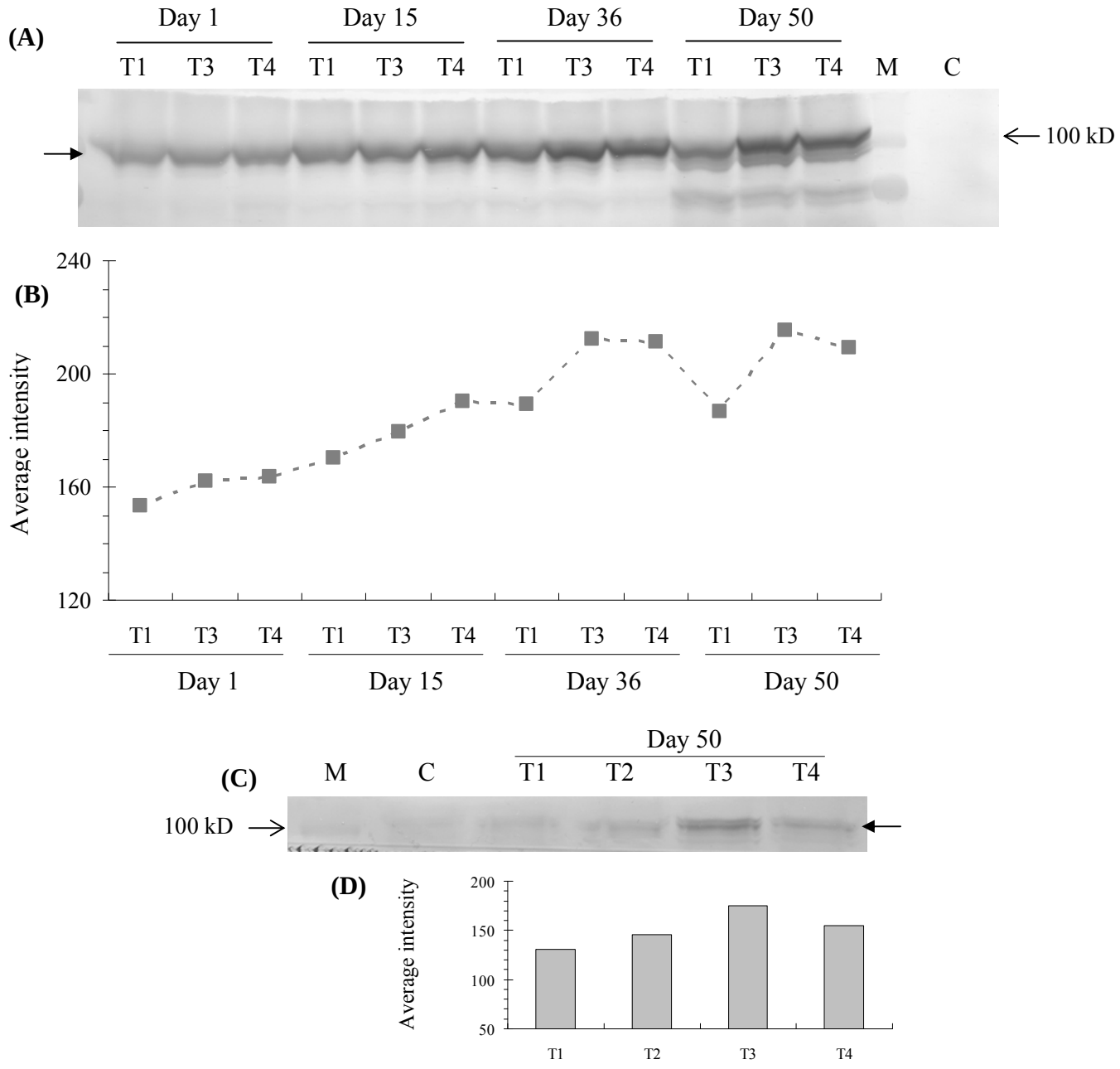

Fig.5-3. Expression of HSP101 in shoots (A) and roots (C) of heat stressed creeping bentgrass under different N levels using immunoblot and correponding band intensity of HSP101 in shoots (B) and roots (D) using Bio-rad Quantity One software. T1, T2, T3, and T4 represents the treatments of no N, low N, medium N, and high N, respectively. M: protein standard for molecular weight; C: sample before heat stress. Equal amounts of protein (40 µg)

were loaded to each lane. Solid arrow indicates the HSP, and the open arrow(s) indicate protein standard.

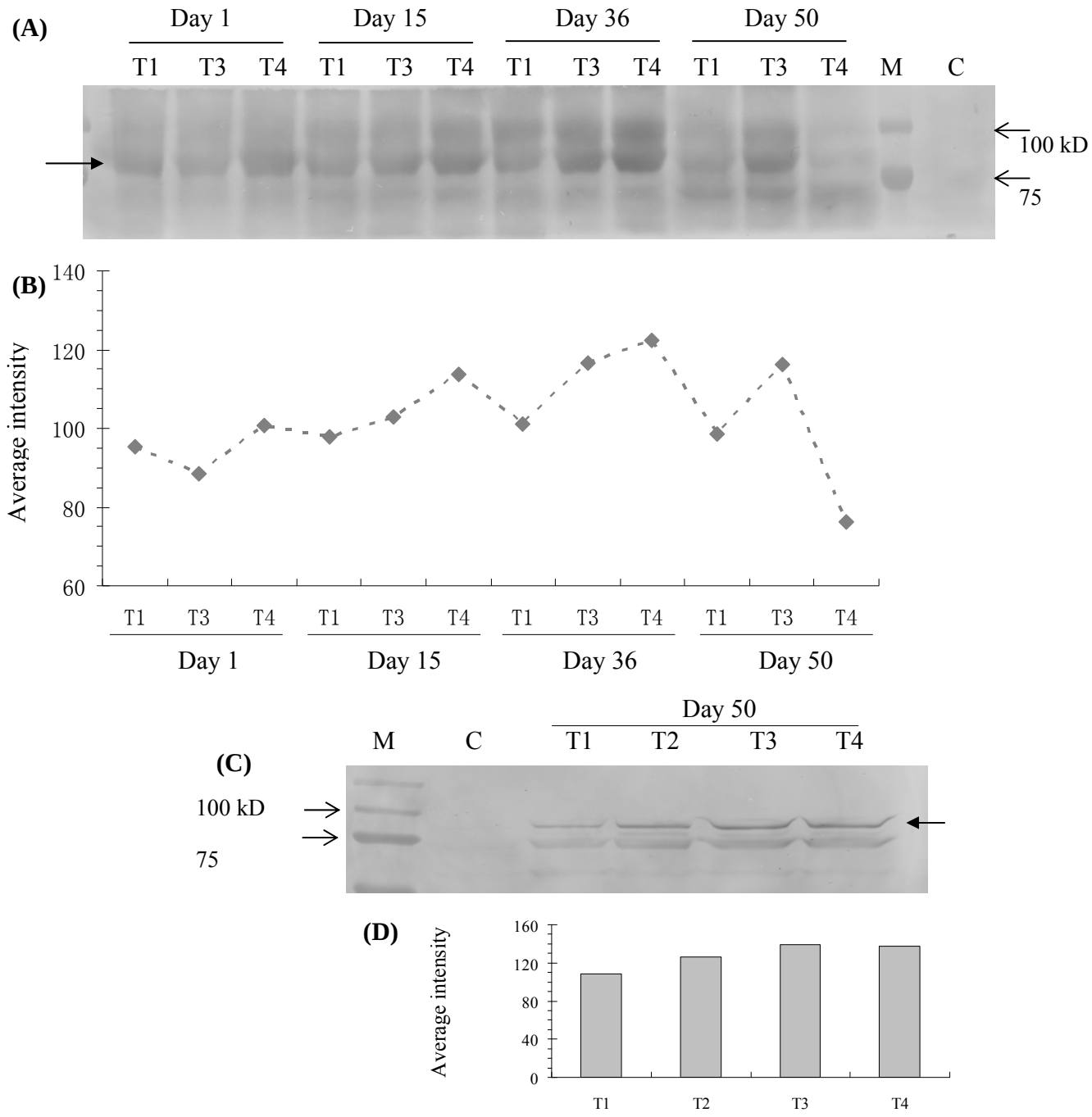

Fig.5-4. Expression of HSP90 in shoots (A) and roots (C) of heat stressed creeping bentgrass under different N levels using immunoblot and correponding band intensity of HSP90 in shoots (B) and roots (D) using Bio-rad Quantity One software. T1, T2, T3, and T4 represents the treatments of no N, low N, medium N, and high N, respectively. M: protein standard for

molecular weight; C: sample before heat stress. Equal amounts of protein (40 μg) were loaded to each lane. Solid arrow indicates the HSP, and the open arrow(s) indicate protein standard.

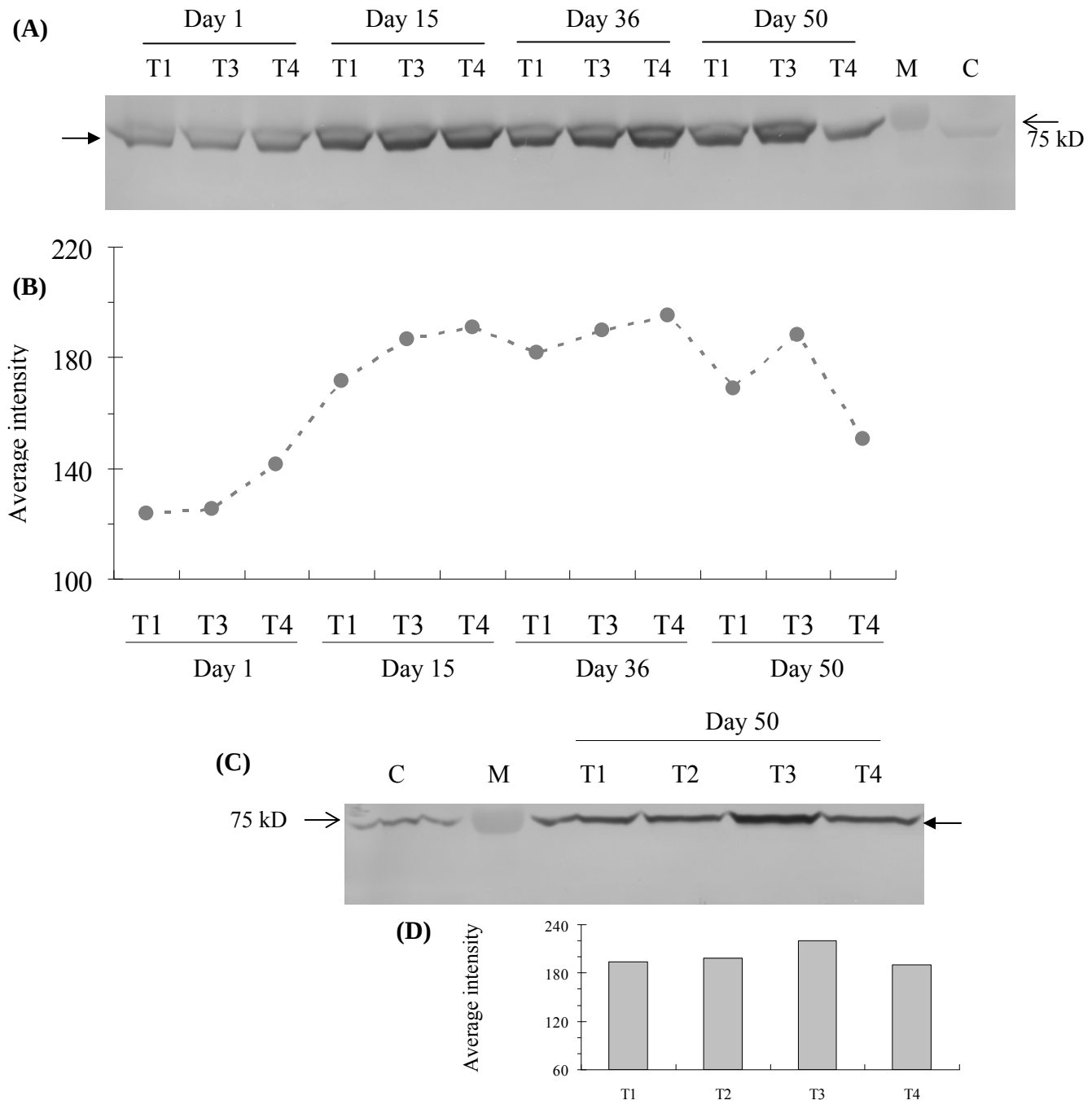

Fig.5-5. Expression of HSP70 in shoots (A) and roots (C) of heat stressed creeping bentgrass under different N levels using immunoblot and correponding band intensity of HSP70 in shoots (B) and roots (D) using Bio-rad Quantity One software. T1, T2, T3, and T4 represent the treatments of no N, low N, medium N, and high N, respectively. M: protein standard for

molecular weight; C: sample before heat stress. Equal amounts of protein (40 μg) were loaded to each lane. Solid arrow indicates the HSP, and the open arrow(s) indicate protein standard.

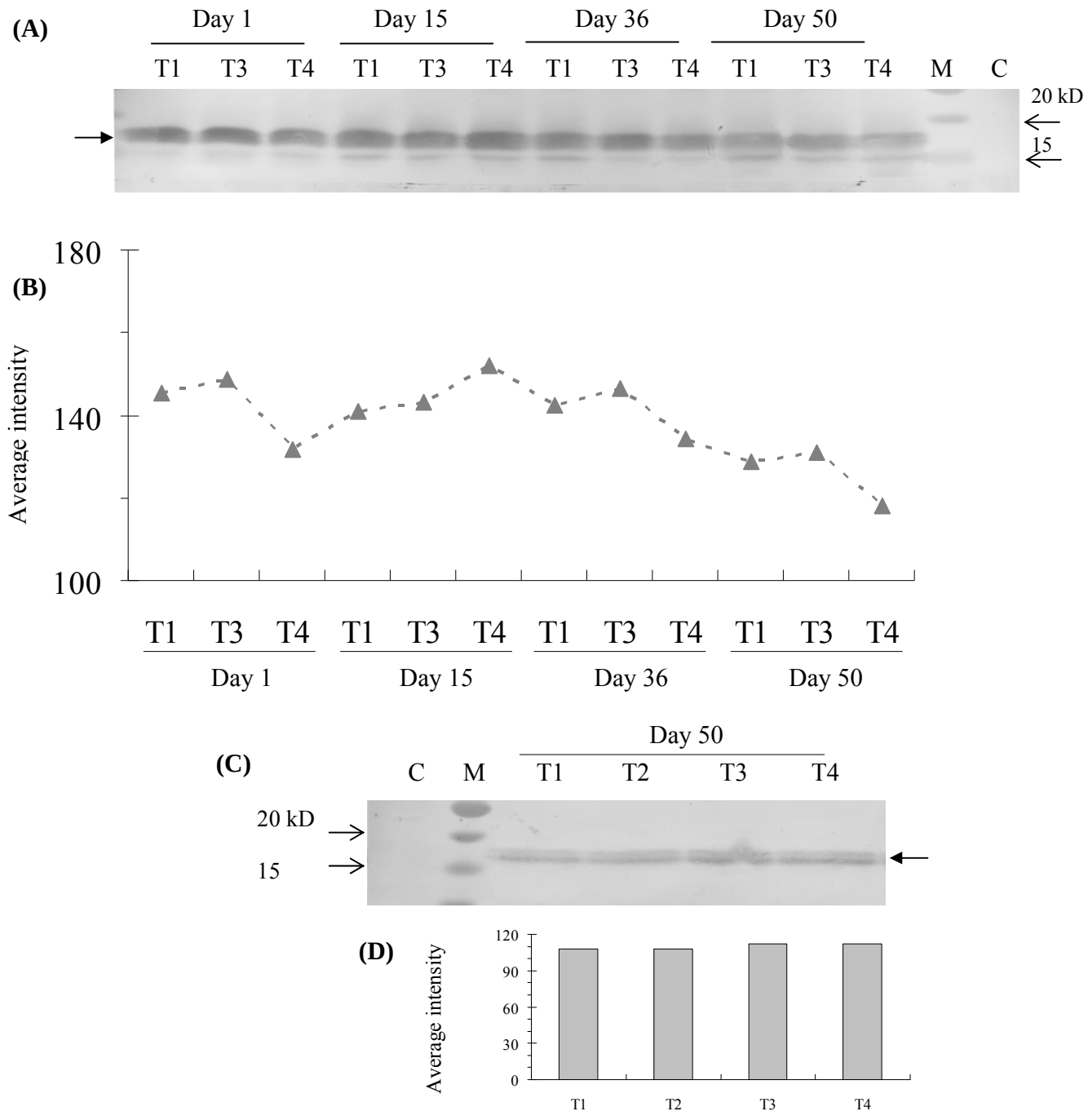

Fig.5-6. Expression of sHSP in shoots (A) and roots (C) of heat stressed creeping bentgrass under different N levels using immunoblot and correponding band intensity of sHSP in shoots (B) and roots (D) using Bio-rad Quantity One software. T1, T2, T3, and T4 represents the treatments of no N, low N, medium N, and high N, respectively. M: protein standard for

molecular weight; C: sample before heat stress. Equal amounts of protein (30 μg) were loaded to each lane. Solid arrow indicate the HSP, and the open arrow(s) indicate protein standard.