Differential Responses of Warm-season and Cool-season Turfgrass Species to Heat Stress Associated with Antioxidant Enzyme Activity

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ABSTRACT. Heat stress may limit the growth of turfgrasses through the induction of oxidative stress, causing cellular and physiological damage. The objective of the study was to examine the association of heat and oxidative stresses between warm-season (C₄) and cool-season (C₃) turfgrasses. Plants of zoysiagrass (Zoysia matrella L. Merr. cv. Manila) (C₄) and tall fescue (Festuca arundinacea Shreber cv. Barlexus) (C₃) were exposed to optimal temperature conditions (24 °C for tall fescue and 34 °C for zoysiagrass) or heat stress (10 °C above the respective optimal temperature for each species) in growth chambers. Zoysiagrass exhibited less severe decline in turf quality and photochemical efficiency and less severe oxidative damage in cellular membranes as demonstrated by lower membrane electrolyte leakage and lipid peroxidation compared with tall fescue when both were exposed to heat stress. The activities of superoxide dismutase (SOD) and peroxidase (POD) declined with heat stress for both species, but to a lesser extent in zoysiagrass than in tall fescue, whereas catalase activity did not change significantly under heat stress and did not exhibit species variation. Our results demonstrate that the superior heat tolerance in zoysiagrass in comparison with tall fescue was associated with greater oxidative scavenging capacity as a result of the maintenance of higher SOD and POD activities.

Cool-season grasses grow most actively within the temperature range of 16 to 24 °C, whereas warm-season grasses have a temperature optimum of 27 to 35 °C (DiPaola and Beard, 1992). Supraoptimal temperatures limit the growth of both cool-season and warm-season grass species, particularly for cool-season grasses. Heat stress injury is associated with photosynthesis inhibition and various other physiological changes such as limited water and nutrient uptake and hormone synthesis (DiPaola and Beard, 1992; Fry and Huang, 2004). Inhibition of photosynthetic activity under heat stress induces oxidative stress by enhancing the production of reactive oxygen species [ROS (such as O₂⁻, ¹O₂, H₂O₂, OH⁻)] because of the imbalance between light capture and its use in carbon fixation (Asada and Takahashi, 1987; Bowler et al., 1992; Dat et al., 2000; Smirnoff, 1993). Reactive oxygen species can cause lipid peroxidation and in turn damage cell membranes and the photosynthetic apparatus, leading to degradation of chlorophyll (Foyer et al., 1994; Smirnoff, 1993). Plants use ROS scavenging mechanisms to protect cells from oxidative injury, including the activation of antioxidant enzymes: superoxide dismutase (SOD), which convert the superoxide radical to H₂O₂; peroxidase (POD); and catalase (CAT) that break down H₂O₂ to water (Smirnoff, 1993). Superior heat tolerance in warm-season species has been attributed to more efficient C₄ photosynthesis metabolism relative to cool-season species using C₃ photosynthetic metabolism, which is more sensitive to heat stress (Furbank and Taylor, 1995). The levels of oxidative damage and antioxidant scavenging capacity may differ between warm-season and cool-season grass species as a result of their differences in photosynthesis sensitivity to stress (Asada and Takahashi, 1987; Wahid et al., 2007).

Oxidative scavenging capacity varies among plant species and cultivars under different environmental stress conditions (Dat et al., 2000; Zhang and Kirkham, 1996). The antioxidant capacity is generally positively related to stress tolerance in various plant species, including turfgrasses. For example, Zhang et al. (2006) reported a correlation between cold tolerance and antioxidant enzyme activities in a cold-tolerant cultivar of bermudagrass [Cynodon dactylon (L.) Pers. var. dactylon]. A positive relationship between antioxidant activities and drought tolerance was also reported in tall fescue, creeping bentgrass (Agrostis stolonifera L.), and kentucky bluegrass (Poa pratensis L.) (Zheng and Schmidt, 1999, 2000). Jiang and Huang (2001a) concluded that turf quality decline and leaf senescence induced by drought, heat, or the combined stresses in tall fescue and kentucky bluegrass were associated with a decrease in antioxidant enzyme activities and an increase in membrane lipid peroxidation. Improvement in heat tolerance in tall fescue treated with exogenous applications of Ca²⁺ was related to the maintenance or increases in antioxidant activities and a decrease in membrane lipid peroxidation (Jiang and Huang, 2001b).

Most previous studies examined antioxidative responses of either C₃ or C₄ plant species to a specific environmental stress...
separately (Jiang and Huang, 2001a, 2001b; Zhang et al., 2006; Zhang and Schmidt, 1999, 2000). Limited studies compared the differential antioxidant responses of C₄ and C₃ plant species to the same stress such as drought (Nayyar and Gupta, 2006; Zhang and Kirkham, 1996) and salinity (Stepien and Klobus, 2005). Stepien and Klobus (2005) concluded that maize (Zea mays L.) (C₄) developed more efficient antioxidant systems than wheat (Triticum aestivum L.) (C₃) in response to salinity or drought. Nayyar and Gupta (2006) reported that the better drought and salinity tolerance in maize than in wheat was associated with the higher level of enzymatic and nonenzymatic antioxidants. However, Zhang and Kirkham (1996) found no consistent differences in the level of antioxidant accumulation between sorghum (Sorghum bicolor L.) (C₄) and sunflower (Helianthus annuus L.) (C₃). It appears that stress responses of the antioxidant system differ between C₃ and C₄ plants depending on plant species, the type of stress, and the severity of stress. Under heat stress, warm-season grass species possessing the C₄ photosynthetic pathway may have more active antioxidant scavenging metabolism than cool-season grass species with C₃ photosynthesis metabolism as a result of its more efficient photosynthesis. However, information on the comparative antioxidant responses of C₃ and C₄ turfgrass species to heat stress is lacking. To better understand the mechanism underlying turfgrass heat tolerance associated with oxidative stress and antioxidant metabolism, the study was designed to examine differential photochemical and antioxidant responses to heat stress for warm-season zoysiagrass with C₄ photosynthesis and cool-season tall fescue with C₃ photosynthesis. Zoysiagrass is a commonly used turfgrass in warm climatic regions and tall fescue is widely used in cool climatic regions (Fry and Huang, 2004). Plants were subjected to supraoptimal temperatures (10 °C above the optimal temperatures) for both species. Turf quality was examined for the evaluation of overall turf performance. Leaf photochemical efficiency was measured as an indicator of integrity of the photosynthetic system, whereas cell membrane stability and lipid peroxidation were determined for evaluation of oxidative stress levels. The activities of three major antioxidant enzymes, SOD, CAT, and POD, were quantified for the examination of antioxidant capacity.

Materials and Methods

Mature sods of tall fescue (‘Barlexus’) and ‘Manila’ zoysiagrass were transplanted from field plots to plastic pots (30 cm diameter and 40 cm deep) filled with sandy loam (fine-loamy, mixed mesic Typic Hapludult). Plants were grown in growth chambers (HP1500 GS-B; Wuhan Ruihua Instrument & Equipment, Wuhan, China) for 42 d before treatments were imposed. Turf was cut at 4-cm canopy height every 3 d. Plants were watered every other day until water drained from the bottom of the pot; 2) heat stress: plants were exposed to supraoptimal temperatures of 10 °C above the optimum range for each species (34 °C for tall fescue and 44 °C for zoysiagrass) while watered daily until water drainage from the bottom of the pot; The three temperature regimes (24, 34, or 44 °C maintained constant at day and night) were conducted in three chambers simultaneously, each chamber set at one temperature regime with three pots for each grass species. The 24 °C chamber was used for the control treatment of tall fescue, the 34 °C chamber contained control treatment for zoysiagrass and heat treatment for tall fescue, and the 44 °C chamber was used for heat treatment of zoysiagrass. The three temperature treatments were repeated three times on three different sets of plants. The temperature in each chamber was randomly reassigned such that each temperature treatment for each turfgrass species was repeated in three different growth chambers. All growth chambers had a 14-h photoperiod, photosynthetically active radiation of 600 μmol·m⁻²·s⁻¹, and 70% to 80% relative humidity regulated by a humidifier (YCD-205; Beijing Yadu Indoor Environmental Protection Science and Technology, Beijing, China).

Measurements. Turf quality was rated based on canopy color, density, and uniformity on the scale of 0 to 9 with 9 being the best (green, dense canopy) and 0 being the worst (dead turf) (Beard, 1973). Turfgrass visual quality, electrolyte leakage, chlorophyll fluorescence, and enzyme activities (SOD, POD, and CAT) were measured the day before treatments (0 d) and every 6 d until 18 d of heat stress when turf quality dropped below 2.0 in tall fescue. Cell membrane stability of leaves was estimated using the measurement of electrolyte leakage (EL) from leaf cells (Blum and Ebercon, 1981). For EL analysis, first and second fully expanded leaves with fresh weight of 0.3 g per pot were excised and cut into 1-cm segments. After being rinsed three times with distilled water, leaf segments were placed into 50-mL vials containing 20 mL distilled water. After shaking for 24 h, initial conductivity (Ci) of the bathing solution was measured with a conductivity meter (DDS-11C; Analytical Equipment, Tianjin, China). Leaves were then killed in an autoclave at 121 °C for 30 min and placed on a shaker for 24 h before final conductivity (Cmax) of the bathing solution was measured. The EL was calculated as (Ci/Cmax) × 100. Leaf photochemical efficiency was determined by measuring chlorophyll fluorescence, the ratio of variable fluorescence to maximal fluorescence (Fv/Fm), with a leaf photochemical efficiency analyzer (OS 1FL; Opti-Sciences, Hudson, NH). Leaves were exposed to darkness for 30 min before readings were taken.

For the analysis of activities of SOD, CAT, and POD and the level of lipid peroxidation, 0.25 g fresh leaf tissues was sampled at 0, 6, 12, and 18 d of treatment, frozen immediately with liquid nitrogen, and then stored at −80 °C until use. Frozen leaves were homogenized with 4 mL cold extraction solution (150 mM phosphate buffer, pH = 7.0). The homogenate was centrifuged at 15,000 g for 20 min at 4 °C. The supernatant was used to measure SOD, CAT, and POD activity and the level of lipid peroxidation.

The activity of SOD was determined according to the method of Giannopolitis and Ries (1977) with modifications. The reaction solution (3 mL) contained 50 mM phosphate buffer (pH 7.8), 60 μM riboflavin (7,8-dimethyl-10-ribitylisoalloxazine), 195 mM methionine [2-amino-4-(methyl-thio)-butyric acid], 3 μM EDTA,
1.125 mM nitro blue tetrazolium \( \text{NBT} \) [2,2'-di-p-nitrophenyl-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride]], and 100 \( \mu \text{L} \) of extracted enzyme solution. A solution containing no enzyme solution was used as the control. Test tubes were irradiated under fluorescent lights at \( \approx \)100 \( \mu \text{mol}-\text{m}^{-2}\cdot\text{s}^{-1} \) for 20 min and then transferred into the dark for 10 min. The absorbance of each solution was measured at 560 nm using a spectrophotometer (Helios Alpha; ThermoSpectronic, Rochester, NY), and one unit of enzyme activity was defined as the amount of enzyme that would inhibit 50% of NBT photoreduction.

Activities of POD and CAT were determined using the method of Chance and Maehly (1955) with modifications. For POD analysis, the 3-mL reaction mixture contained 1.85 mL 0.1 M HAc-NaAc buffer (pH 5.0), 1 mL 50% guaiacol solution, and 50 \( \mu \text{L} \) enzyme extraction. The reaction was started with 100 \( \mu \text{L} \). 0.75% \( \text{H}_2\text{O}_2 \). Reading at 460 nm was recorded every minute within the first 3 min. One-unit POD activity was defined as the absorbance change of one unit per minute. The activity of CAT was determined based on the oxidation of \( \text{H}_2\text{O}_2 \) using the method of Chance and Maehly (1955) with modifications. The reaction solution (3 mL) contained 50 \( \text{mM} \) phosphate buffer (pH 7.0), 45 \( \text{mM} \) \( \text{H}_2\text{O}_2 \), and 100 \( \mu \text{L} \) of extracted solution. The reaction was initiated by adding the enzyme solution. Changes in absorbance at 240 nm were read every 10 s for 60 s using a spectrophotometer. One unit of CAT activity was defined as the absorbance change of 0.01 unit per minute.

The level of lipid peroxidation was determined by measuring malondialdehyde (MDA content), which is a secondary breakdown product of lipid peroxidation (Halliwell and Gutteridge, 1989). The content of MDA was determined according Heath and Packer (1968) with modifications. Briefly, 1 mL of enzyme extraction was added to 2 mL of a reaction solution containing 20% \( \text{v/v} \) trichloroacetic acid and 0.5% \( \text{v/v} \) thiobarbituric acid. The solution was placed in a water bath at 95 \( ^\circ \text{C} \) for 30 min and then quickly cooled in an ice-water bath. After the solution was centrifuged at 10,000 \( \text{g}_\text{n} \) for 10 min, the absorbance of the supernatant was read at 532 and 600 nm. Absorbance at 600 nm was subtracted from that at 532 nm, and MDA content was calculated using this adjusted absorbance and the extinction coefficient of 155 \( \text{mM}^{-1}\cdot\text{cm}^{-1} \) (Heath and Packer, 1968).

**Experimental Design and Statistical Analysis.** Species and treatments were arranged as a randomized block design. Species comparisons and responses of each species to heat stress were analyzed by the analysis of variance using SAS (Version 8.1; SAS Institute, Cary, NC). Differences among species and treatment means were assessed by Fisher’s protected least significance difference test at \( P = 0.05 \).

**Results and Discussion**

Turf quality was unchanged and no species differences were observed under the optimal temperature conditions during the 18-d treatment period (Fig. 1A). Turf quality declined after 6 d of heat stress for both zoysiagrass and tall fescue (Fig. 1B). The decline in turf quality was more pronounced in tall fescue than in zoysiagrass under heat stress. Zoysiagrass maintained turf quality between 2 and 4, whereas tall fescue quality dropped to below 2 after 12 d of heat stress. These results confirmed that zoysiagrass with a \( \text{C}_4 \) photosynthetic pathway was better able to maintain turf quality than tall fescue with \( \text{C}_3 \) photosynthesis at a temperature of 10 \( ^\circ \text{C} \) above their respective optimum levels. Our previous study suggested that heat stress was more detrimental than drought stress for both species, and tall fescue was more sensitive to both heat and drought stress than zoysiagrass, particularly heat stress (Du et al., 2008).

The ratio of \( \text{Fv/Fm} \) provides an estimate of the quantum yield of PS II photochemistry or photochemical efficiency for dark-adapted leaves, which is used as an indicator of photosynthetic activity with a maximum value of \( \approx \)0.83 in most plant species (Maxwell and Johnson, 2000). Leaf \( \text{Fv/Fm} \) did not change or exhibit species variation during the treatment period under control conditions (Fig. 2A) but decreased significantly after 6 d of heat stress for both species (Fig. 2B), and the decline was more dramatic in tall fescue than in zoysiagrass. After 12 d of heat stress, \( \text{Fv/Fm} \) of zoysiagrass was significantly higher than that of tall fescue (Fig. 2B). These results suggest that an increase in temperature by 10 \( ^\circ \text{C} \) above the optimum level had detrimental damages to photochemical activities in PSII in both tall fescue and zoysiagrass. Lability of PSII is a primary limitation of photosynthesis at high temperatures and direct high temperature injury to PSII may result from the injury to the components at the oxidizing side, perhaps the oxygen-evolving complex (Al-Khatib and Paulsen, 1989). Higher \( \text{Fv/Fm} \) in zoysiagrass suggests that this \( \text{C}_4 \) grass species was able to maintain more active PSII than tall fescue under heat stress.

Cell membrane is one of the first targets of many stresses and it is generally accepted that the maintenance of their integrity
and stability under stress conditions is critical for plant survival (Blum and Ebercon, 1981). Photosynthetic membranes appear to be especially sensitive to high temperatures (Al-Khatib and Paulsen, 1989). Marcum (1998) has shown that cell membrane stability was positively correlated to turf quality and concluded that this parameter can be used to predict whole-plant heat tolerance in kentucky bluegrass cultivars. No significant change in EL was observed with treatment duration for both species and species differences were not detected under control conditions (Fig. 3A). Leaf EL in tall fescue increased with heat stress, beginning at 6 d of treatment; the EL values were 10, 16, and 18 times of the control level at 6, 12, and 18 d, respectively (Fig. 3B). Significant increases in EL did not occur until 12 d of heat stress in zoysiagrass. After 6 d of treatment, tall fescue had significantly higher EL than zoysiagrass. These results suggest that zosygiagrass had more thermostable membranes, which may contribute to the better turf quality and higher photochemical efficiency than tall fescue under heat stress.

Cell membrane damage may also be associated with oxidative stress as a result of lipid peroxidation. Physiological injury resulting from heat stress has been associated with the induction of oxidative stress in different C₃ turfgrass species and cultivars (Fu and Huang, 2001; Jiang and Huang, 2001a, 2001b; Liu and Huang, 2000). The production of MDA is the result of peroxidation of unsaturated fatty acids in phospholipids, which has been associated with cell membrane damage (Halliwell and Gutteridge, 1989). The level of lipid peroxidation, expressed as MDA content, has been used as an indicator of free radical damage to cell membranes. Lipid peroxidation level in leaves of two species measured as the content of MDA did not show significant changes or species difference under control conditions (Fig. 4A). However, significant increases in MDA content were observed after 12 d of heat stress in tall fescue and 18 d of treatment in zoysiagrass (Fig. 4B). These results demonstrated that oxidative stress was an important component of heat stress injury in both zoysiagrass and tall fescue with C₄ or C₃ photosynthesis. In addition, at both 12 and 18 d of heat stress, tall fescue had significantly higher MDA content than zoysiagrass, suggesting that heat stress induced more severe oxidative damage in tall fescue than in zoysiagrass.

The maintenance of lower lipid peroxidation may be the result of greater antioxidant defense in zoysiagrass than in tall fescue. Several studies suggested that the differences in abiotic stress tolerance may be partially the result of higher constitutive antioxidant enzyme activities in C₄ than in C₃ plants or in tolerant than in intolerant species (Stepien and Klobus, 2005; Türkan et al., 2005). In our study, SOD activity was significantly higher in tall fescue than zoysiagrass under optimal temperature conditions (Fig. 5A), suggesting that tall fescue had constitutively higher SOD activity. In addition, SOD activity also increased with treatment duration in tall fescue
under the optimal temperature conditions, which could be related to antioxidant effects associated with leaf aging or maturation. However, under heat stress, decline in SOD activity was observed beginning 6 d of treatment in both species, to a greater extent in tall fescue (Fig. 5B), indicating that the scavenging ability for superoxide anion was diminished under heat stress. Zoysiagrass maintained significantly higher SOD activity than tall fescue during the entire 18-d period of heat stress. This is in good agreement with results from other studies that heat-tolerant cultivars or species of turfgrasses had superior SOD activity. There are multiple enzymatic forms (isozymes) of SOD (Scandalio, 1993). The identification of individual isozymes responsible for heat tolerance in zoysiagrass might provide further insights into antioxidant protection mechanisms for heat stress. Leaf POD activity did not differ between the two grass species under control conditions (Fig. 6A). Under heat stress, POD activity decreased significantly after 6 d of treatment in both species (Fig. 6B). Compared with tall fescue, zoysiagrass exhibited less pronounced decline in POD activity. At 18 d of heat stress, POD activity in zoysiagrass was 7.0 times higher than that in tall fescue. POD is among the major enzymes that scavenge H₂O₂ produced through dismutation of O₂⁻ catalyzed by SOD (Asada and Takahashi, 1987). The maintenance of higher POD activity may provide further oxidative protection by detoxifying H₂O₂ induced by heat stress through weakening the SOD enzyme system. Catalase is another enzyme for breaking down H₂O₂ (Asada and Takahashi, 1987); however, its activity did not exhibit species variation under either well-watered or heat stress conditions (data not shown). The lack of species difference in CAT activity indicated that CAT may not be involved in antioxidant defense against heat stress in the two grass species examined in this study. Zhang and Kirkham (1996) found no difference in CAT activities between sorghum (C₄) and sunflower (C₃) when plants were exposed to drought stress. The protective action of CAT is limited because it has relatively poor affinity for its substrates and is sensitive to light-induced inactivation compared with other antioxidant enzymes (Asada and Takahashi, 1987; Scandalio, 1993).

Previous studies have reported various physiological and metabolic processes may account for variations in temperature responses between C₃ and C₄ species, particularly photosynthesis (Sage, 2002). The present study revealed that zoysiagrass and tall fescue with different photosynthetic pathways had varying abilities to deal with oxidative stress that may at least partially contribute to their differential tolerance to heat stress. Our results suggest that the combined higher activities of SOD and POD in zoysiagrass may increase protection of cell membranes to oxidation and photochemical reactions in photosynthesis as manifested by the lower EL and higher Fv/Fm under heat stress. Manipulation of endogenous level and activities of antioxidant enzymes through breeding or genetic modification might elevate the defense ability of plant tolerance to heat stress, particularly cool-season grass species.
Fig. 6. Peroxidase (POD) activity in leaves of ‘Manila’ zoysiagrass and ‘Barlexus’ tall fescue under optimal temperature conditions (34 °C for zoysiagrass and 24 °C for tall fescue) (A) and heat stress (44 °C for zoysiagrass and 34 °C for tall fescue) (B). Vertical bars indicate the SE of each mean. Columns marked with lower-case letters indicate significant differences between measurements made at 0, 6, 12, and 18 d of treatment for a given species based on a least significant difference test ($P = 0.05$). Columns marked with an asterisk represent statistical significance for comparison between zoysiagrass and tall fescue at a given day of treatment ($P = 0.05$).

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