Sitosterol-mediated Antioxidant Regulation to Enhance Heat Tolerance in Creeping Bentgrass

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ABSTRACT. Heat stress symptoms in cool-season plants are characterized by loss of chlorophyll (Chl) and membrane stability, as well as oxidative damage. The objectives of this study were to determine whether foliar application of β-sitosterol, a naturally occurring plant metabolite, may promote heat tolerance by suppressing heat-induced leaf senescence as indicated by the maintenance of healthy turf quality (TQ), and Chl and membrane stability; and to determine its roles in regulating antioxidant metabolism in creeping bentgrass (*Agrostis stolonifera*). 'Penncross' plants were exposed to heat stress (35/30 °C day/night) optimal temperature conditions (nonstressed control, 22/17 °C day/night) for a duration of 28 days in environment-controlled growth chambers. Plants were foliar-treated with β-sitosterol (400 μM) or water only (untreated control) before heat stress, and at 7-day intervals through 28 days of heat stress. Plants treated with β-sitosterol had significantly greater TQ and Chl content, and significantly less electrolyte leakage (EL) than untreated controls at 21 and 28 days of heat stress. Application of β-sitosterol reduced malondialdehyde (MDA) content significantly at 21 and 28 days of heat stress, and promoted the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) from 14 through 28 days of heat stress. β-Sitosterol effectively improved heat tolerance through suppression of leaf senescence in creeping bentgrass exposed to heat stress in association with the alleviation of membrane lipid peroxidation and activation of the enzymatic antioxidant system.

Heat stress is detrimental for cool-season turfgrasses and is characterized symptomatically by a marked decrease in TQ in cool-season turfgrass species as a result of heat-induced leaf senescence. Heat-induced leaf senescence is associated with loss of Chl and disruption of cellular membranes, leading electrolytes and ions to leak freely out of cells (Hu et al., 2016; Liu and Huang, 2000; Wang et al., 2009; Wassie et al., 2019; Wilson et al., 2014). Membrane damage induced by heat stress and other abiotic stresses are associated with the production of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical, that cause lipid peroxidation in plasma, mitochondria, peroxisome, and chloroplast membranes and are toxic to various macromolecules, such as lipids, proteins, and DNA (Hopkins, 1999; Jimenez et al., 1997; Roy and Basu, 2009). Lipid peroxidation occurs, producing MDA as ROS attack the conjugated double bonds of the unsaturated fatty acids comprising the liquid-disordered membranes (Berlett and Stadtman, 1997; Dean et al., 1997; Halliwell and Gutteridge, 1989). Antioxidant enzymes, including SOD, POD, CAT, and APX, play crucial roles in suppressing the production and accumulation of ROS and mitigate oxidative damage (Asada, 1999); however, severe heat stress can also damage antioxidant enzymes, restricting ROS scavenging ability (De la Haba et al., 2014). Because ROS exacerbate leaf senescence under stress, it is necessary to identify the mechanisms or natural metabolites that may be effective in mitigating heat-induced oxidative damage.

Sterols are important components in membranes and play roles in maintaining membrane fluidity and stability (Dufourc, 2008; Hartmann, 1998). Phytosterols are present in high quantities in plant tissues, with β-sitosterol alone accounting for ≈50% of the total sterol content in leaves of various plant species (Nomura et al., 1999), including cool-season grass species such as hard fescue [*Festuca trachyphylla* (Wang et al., 2017)]. Seed priming or foliar spraying sterols have been found to have positive effects on improving plant tolerance to abiotic stresses, such as heat stress in tomato [*Solanum lycopersicum* (Gamel et al., 2017)], salt stress in pepper [*Capsicum annuum* (Abu-Muriefah, 2015)] and sunflower [*Helianthus annuus* (Fawzia et al., 2016)], and drought stress in wheat [*Triticum aestivum* (Elkeilsh et al., 2019)] and white clover [*Trifolium repens* (Li et al., 2019)]. Sitosterol reinforces the stabilization of liquid-disordered membranes in plants exposed to heat stress (Dufourc, 2008). Sitosterol content correlated positively to heat tolerance of hard fescue, as demonstrated by its increased content in a heat-tolerant cultivar compared with a sensitive cultivar exposed to heat stress (Wang et al., 2017). Improvement of drought tolerance by exogenous application of β-sitosterol has been associated with increased total antioxidant capacity in white clover (Li et al., 2019) and antioxidant enzyme activities in wheat (Elkeilsh et al., 2019). β-Sitosterol, as a naturally occurring sterol molecule in plants, has been found to exhibit antioxidant potential for decreasing the level of ROS in animal cells by increasing the antioxidant activity of CAT, SOD, glutathione peroxidase, glutathione reductase, and glutathione S-transferase in rats [*Rattus norvegicus* (Baskar et al., 2012)]. The antioxidant functions of β-sitosterol against abiotic stress, including heat stress-induced oxidative damages in plants, are not well-understood. In

addition, the manners in which foliar application of β-sitosterol may affect plant tolerance to heat stress by suppressing heat-induced leaf senescence and oxidative damage involving the regulation of enzymatic antioxidant metabolism have not been documented. Understanding how β-sitosterol may affect heat tolerance in cool-season turfgrass species involving the modulation of antioxidant metabolism is important for developing strategies in breeding selection or genetic modification and management practices to improve heat tolerance in cool-season turfgrass species.

Cool-season turfgrass species are widely grown in temperate climate regions, but they are sensitive to heat stress during summer months, and heat-induced leaf senescence and oxidative stress greatly hinder the performance of these species in warm-climate regions (Fry and Huang, 2004; Turgeon, 2002). The objectives of this study were to determine whether foliar application of β-sitosterol could promote heat tolerance by alleviating leaf senescence or maintaining Chl and membrane stability, as well as by enhancing antioxidant metabolism in creeping bentgrass (*Agrostis stolonifera*).

Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. Sods of mature creeping bentgrass (cv. Penncross) (diameter, 10 cm) were collected from field plots at a turfgrass research farm and planted immediately in 16 plastic pots (diameter, 15 cm; height, 24 cm) filled with sterilized sand. Plants were grown in a greenhouse for 28 d until root systems were established under natural sunlight, supplemented with sodium vapor lamps at average temperature conditions of 24/16 °C (day/night). Turf was hand-trimmed twice weekly to maintain a canopy height of 3.8 cm, and was irrigated to pot capacity on alternating days. Plants were fertilized on a biweekly basis with full-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). After the establishment period, plants were moved to environment-controlled growth chambers set to 22/17 °C (day/night), 60% relative humidity, and a 14-h photoperiod with a photosynthetically active radiation value of 700 μmol·m^{-2}·s^{-1}, where they acclimated for 14 d before initiation of the experiment.

EXPERIMENTAL DESIGN AND TREATMENTS. Each plant was treated with either 40 mL of 400 μM β-sitosterol or 40 mL of water (untreated control) by foliar spraying until droplets formed, and then were exposed to optimal temperature (non-stressed control, 22/17 °C day/night) or heat stress (35/30 °C day/night) in growth chambers. This concentration of β-sitosterol was selected from a preliminary concentration screening test that showed it was the most effective in improving TQ under heat stress. β-Sitosterol treatments were applied subsequently every 7 d for the duration of the experiment (28 d). The β-sitosterol or water treatment each consisted of four replicate plants per temperature treatment. Plants were placed in four growth chambers (Environmental Growth Chambers, Chagrin Falls, OH), two controlled at 22/17 °C and the other two at 35/30 °C, and were relocated across growth chambers twice per week to minimize the potentially confounding effects of variable environmental conditions among chambers. The experiment was carried out in a split-plot layout, with temperature (nonstress or heat stress) assigned as the main factor and chemical treatment (β-sitosterol or water) as the subfactor.

PHYSIOLOGICAL MEASUREMENTS. Several commonly used parameters, including TQ, leaf Chl content, and membrane stability, were evaluated to determine the effects of β-sitosterol on heat tolerance of plants or the extent of leaf senescence. TQ was rated visually every 7 d by assigning a value from 1 through 9 points depending on turf canopy color, density, and uniformity (Beard, 1972). On this scale, a rating of 9 points was designated to turf that was uniformly green and dense, whereas a rating of 1 point indicated turf that was completely brown, desiccated, and dead.

Using the methods of Hiscox and Israelstam (1979) with slight modifications, Chl content was analyzed every 7 d by immersing 0.1 g of fresh leaf tissue in 10 mL dimethyl sulfoxide and leaving the Chl to extract from the tissue in total darkness for 72 h. After the extraction period, absorbance of the Chl solution was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at wavelengths of 663 and 645 nm, and leaves were dried in a gravity convection oven (Cole-Parmer, Vernon Hills, IL) at 80 °C for a minimum of 72 h, after which dry weights were obtained. Chlorophyll content was calculated using the formulas provided by Arnon (1949).

To measure the stability of cell membranes, leaf EL was measured on a weekly basis by excising ≈0.1 g of mature leaves, cutting them into 1-cm sections, and submerging them in a conical centrifuge tube containing 30 mL deionized water. Samples were shaken on an orbital shaker for 12 h, and initial conductance was measured using a conductance meter (model 32; YSI, Yellow Springs, OH). Leaves were killed by autoclaving at 121 °C for 20 min and shaken again for a period of 12 h, after which maximum conductivity was measured. EL was calculated as a ratio of initial conductance to maximum conductivity and was expressed as a percentage, according to the equations provided by Blum and Ebercon (1981).

EVALUATION OF LIPID PEROXIDATION AND ANTIOXIDANT ENZYME ACTIVITY. Fresh leaf tissue (0.25 g) collected at 7-d intervals was flash-frozen in liquid nitrogen, ground mechanically into a fine powder using a mortar and pestle, and stored immediately at –80 °C in an ultra-low temperature freezer (Thermo Fisher Scientific) to be used in the lipid peroxidation and antioxidant enzyme activity reactions. A chilled extraction solution (1 mL) containing 50 mM phosphate buffer, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 1.0% polyvinylpolypyrrolidone was added to the frozen leaf tissue and ground over ice for ≈5 min, after which 2 mL of extraction solution was added to the slurry. After centrifuging the homogenate at 15,000 g_n at 4 °C for 20 min, the supernatant was decanted and saved to be used to measure MDA content and antioxidant enzyme activities.

The activity of SOD was determined using the methods of Giannopolitis and Ries (1977), with the modifications specified by Du et al. (2009). SOD-mediated inhibition of the photochemical reduction of Nitro blue tetrazolium chloride (NBT) was initiated by reacting the enzyme extract of each sample with a solution containing 50 mM phosphate buffer (pH, 7.8), 195 mM methionine, 60 μM riboflavin, 3 μM EDTA, and 1.125 mM NBT under fluorescent lighting for 20 min. To halt the reaction, the solution was incubated in complete darkness for 10 min, and SOD activity was measured immediately at 560 nm via spectrophotometer. One enzyme activity unit was expressed as the quantity of enzyme necessary for 50% inhibition of NBT photochemical reduction. The enzymatic activities of POD and CAT were measured using the procedure outlined in Chance and

Maehly (1955), using the modifications detailed by Du et al. (2009). To analyze POD, crude enzyme extract from each sample was added to a solution comprised of 0.1 M acetic acid–sodium acetate buffer (pH, 5.0) and 50% guaiacol, followed by the addition of 0.75% H_2O_2 to initiate the reaction. To quantify the oxidation of guaiacol by H_2O_2, absorbance of the mixture was read via spectrophotometer at a wavelength of 460 nm every 10 s for 1 min. A single unit of POD activity was represented by a decrease in absorbance by 1 $U \cdot min^{-1}$. CAT activity was analyzed by combining crude enzyme extract with 50 mM phosphate buffer (pH, 7.0) with 45 mM H_2O_2 and measuring the decomposition of H_2O_2 by CAT via spectrophotometer every 10 s for 1 min at a wavelength of 240 nm. One unit of CAT enzyme was expressed as the decrease in absorbance by 0.01 $U \cdot min^{-1}$. APX activity was analyzed using the methods of Nakano and Asada (1981), with certain alterations made to the procedure. To obtain an accurate measurement of APX activity, it was necessary to freeze fresh leaf tissue in liquid nitrogen, perform the extraction as detailed previously, and use the extract immediately. To quantify the oxidation of ascorbic acid by APX, the extract was mixed with 0.17 mM ascorbic acid, 100 mM acetic acid–sodium acetate buffer (pH, 5.8), 0.05 μM EDTA, and 0.08 mM H_2O_2; the absorbance at 290 nm was read on a spectrophotometer every 10 s for 1 min (Du et al., 2013). An APX activity unit was defined as a change in absorbance by 0.01 $U \cdot min^{-1}$.

To extract crude protein from each sample, 20% w/v trichloroacetic acid was added to the enzyme extract solution, and the mixture was rested at 4 °C for ≈1 h until protein precipitate condensed at the bottom of the tube. After centrifuging the sample at 11,500 g_n for 15 min, the supernatant was poured off and the pellet was air-dried and resuspended in 1.0 M sodium hydroxide. A standard curve was constructed to quantify protein content, where bovine serum albumin was used as a standard and was diluted serially using Coomassie Brilliant Blue G-250 Dye (Bio-Rad Laboratories, Hercules, CA). To quantify protein content, the absorbance of the sample for each dilution factor was read at a wavelength of 595 nm on a spectrophotometer, and the absorbance values were applied to the standard curve to calculate protein content, which was then used to quantify the activities of antioxidant enzymes.

To quantify lipid peroxidation, the content of MDA was measured using the thiobarbituric acid reactivity procedure of Heath and Packer (1968), and using some of the methods detailed by Dhindsa et al. (1981), with slight modification. A reaction solution (2 mL) consisting of 20% w/v trichloroacetic acid and 0.5% w/v thiobarbituric acid was added to 1 mL enzyme extract, and the solution was heated for 30 min in a water bath at 95 °C. After cooling the solution rapidly to room temperature in ice water, tubes were shaken to remove gas bubbles and then centrifuged for 10 min at 10,000 g_n. Using a spectrophotometer, absorbance of the supernatant was quantified at 532 and 600 nm, and MDA concentration was calculated using an extinction coefficient of 155 $mM^{-1} \cdot m^{-1}$ (Heath and Packer, 1968; Kwon et al., 1965).

STATISTICAL ANALYSIS. The significant effects of temperature and chemical treatments were determined by two-way analysis of variance using the general linear procedure with SAS (version 9.2; SAS Institute, Cary, NC). To define the differences between
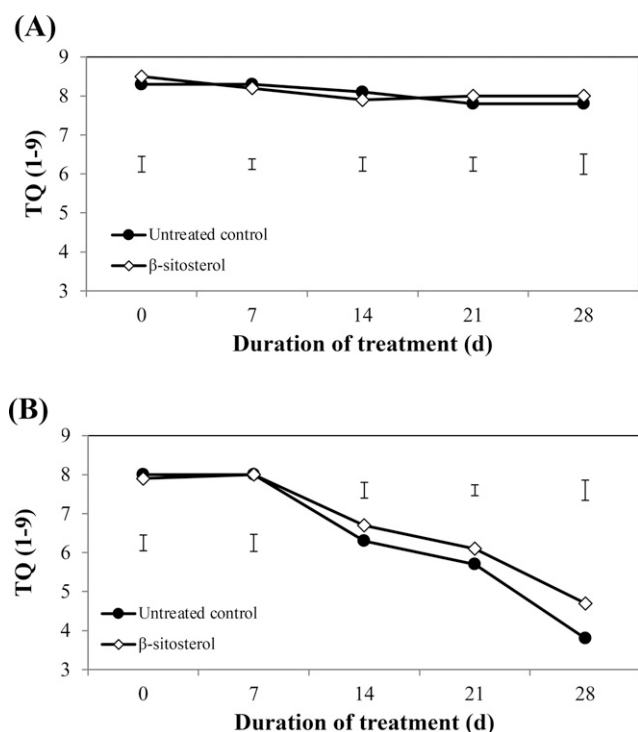

Fig. 1. Visual turf quality (TQ) ratings (1 = completely brown, desiccated, and dead; 9 = uniformly green and dense) for creeping bentgrass treated with β-sitosterol or untreated (control) under (A) nonstress or (B) heat stress conditions. Vertical bars define the SD of the means between treatments based on Fisher's protected least significant difference test (P = 0.05) for each treatment date, where n is defined as the number of measurements represented by each value (n = 4).
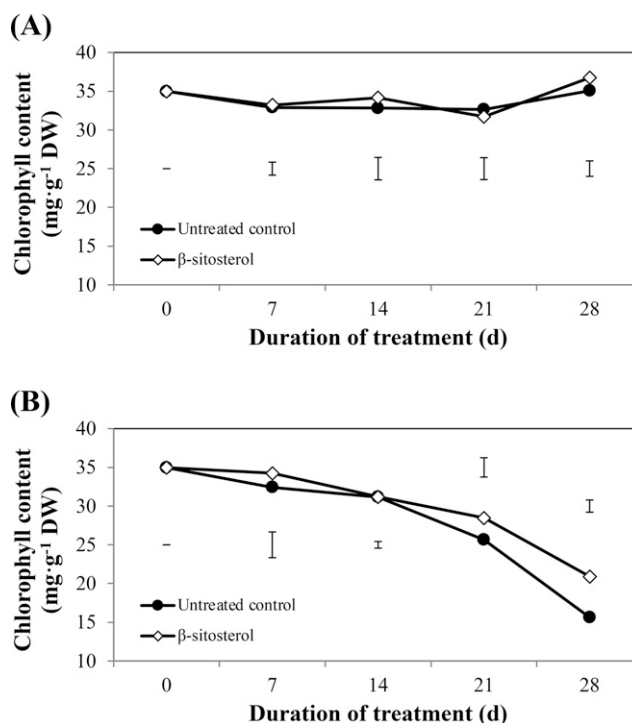

Fig. 2. Leaf chlorophyll content for creeping bentgrass treated with β-sitosterol or untreated (control) under (A) nonstress or (B) heat stress conditions. Vertical bars define the SD of the means between treatments based on Fisher's protected least significant difference test (P = 0.05) for each treatment date. where n is defined as the number of measurements represented by each value (n = 4). DW, dry weight.

the mean values of treatments, Fisher's least significant difference test was used at a 0.05 P level.

Results

Physiological effects of β-sitosterol on the suppression of heat-induced leaf senescence and improvement of heat tolerance in creeping bentgrass.

Three commonly used physiological parameters, TQ, leaf Chl content, and leaf EL were evaluated as indicators of the level of leaf senescence and overall turf performance associated with heat tolerance. All three parameters remained unchanged in plants under normal temperature conditions (nonstress control), regardless of whether plants were treated with β-sitosterol (Figs. 1A, 2A, and 3A). There were no significant differences in any of these physiological parameters between the β-sitosterol treatment and untreated control under nonstress conditions.

TQ and leaf Chl content declined whereas leaf EL increased during heat stress in both β-sitosterol-treated plants and untreated control plants (Figs. 1B, 2B, and 3B); however, the decrease in TQ and leaf Chl content and increase in leaf EL was less pronounced in β-sitosterol-treated plants compared with the untreated control plants (Figs. 1B, 2B, and 3B).

Under heat stress, TQ was significantly greater in β-sitosterol-treated plants at 21 and 28 d of heat stress (by 7.02% and 23.68%, respectively) in comparison with the untreated control (Fig. 1B). Leaf Chl content of plants treated with β-sitosterol was significantly greater than that of untreated controls at 21 d (by 10.98%) and 28 d (by 33.81%) (Fig. 2B).

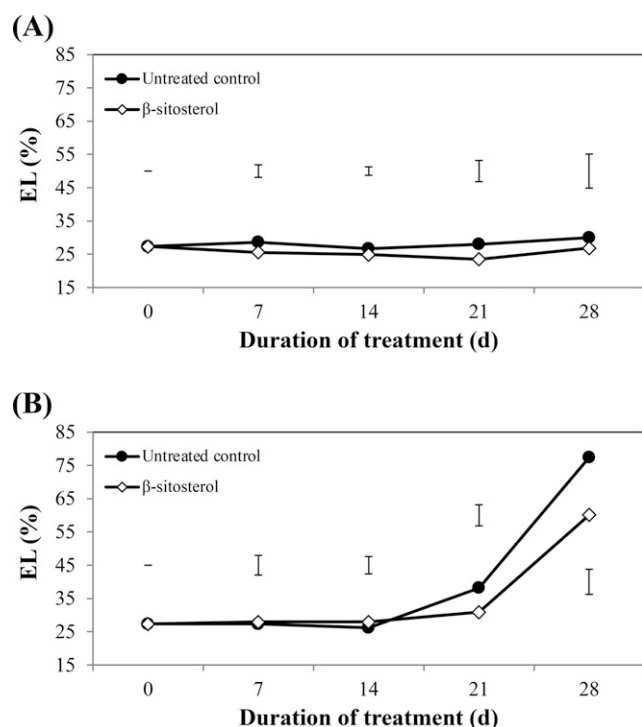Leaf EL was significantly less at 21 and 28 d of heat stress in plants treated with β-sitosterol (by 19.13% and 22.39%, respectively) (Fig. 3B).

Alleviation of heat-induced lipid peroxidation and activation of antioxidant enzymes by β-sitosterol in creeping bentgrass exposed to heat stress.

Leaf MDA content remained unchanged during the experimental period (28 d) in β-sitosterol-treated plants and untreated control plants under nonstress control conditions (Fig. 4A). The content of MDA in leaves was not significantly different between the β-sitosterol treatment and untreated control under nonstress conditions (Fig. 4A).

The content of MDA in leaves increased gradually through the duration of heat stress (28 d), but to a greater extent in untreated control plants compared with that of β-sitosterol-treated plants (Fig. 4B). Plants treated with β-sitosterol had significantly less MDA content in leaves compared with the untreated control at 21 d (by 23.53%) and 28 d (by 26.14%) of heat stress (Fig. 4B).

The activities of SOD (Fig. 5A), POD (Fig. 5B), CAT (Fig. 5C), and APX (Fig. 5D) in leaves were unchanged under nonstress control conditions in all plants regardless of β-sitosterol treatment, and were unaffected by β-sitosterol under nonstress control conditions. Heat stress caused a decline in the activity of all antioxidant enzymes in leaves, but to a lesser extent in plants treated with β-sitosterol (Fig. 5A–D).

Under heat stress–at 14, 21, and 28 d of heat stress treatment–β-sitosterol application resulted in significant increases

(A)

(B)

Fig. 3. Electrolyte leakage (EL) for creeping bentgrass treated with β-sitosterol or untreated (control) under (**A**) nonstress or (**B**) heat stress conditions. Vertical bars define the SD of the means between treatments based on Fisher's protected least significant difference test ($P = 0.05$) for each treatment date, where n is defined as the number of measurements represented by each value (n = 4).

(A)

(B)
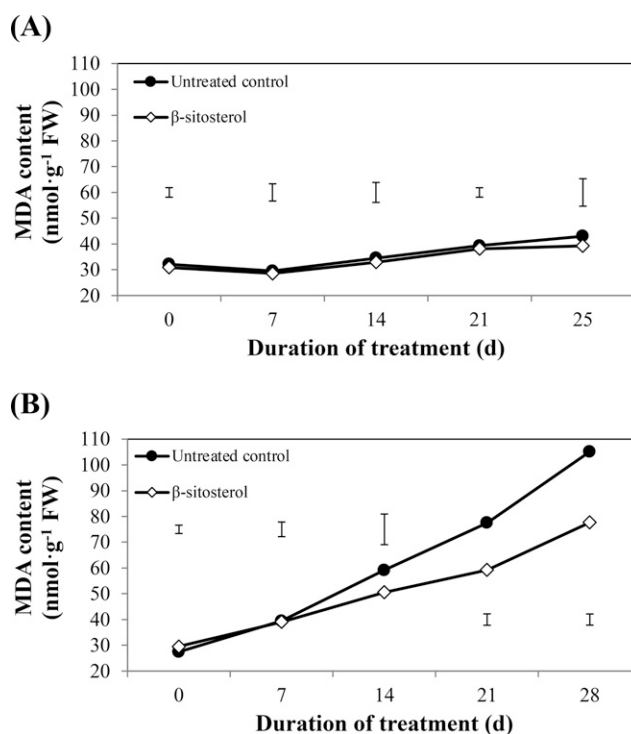
Fig. 4. Malondialdehyde (MDA) content as a measure of lipid peroxidation for β-sitosterol-treated or untreated (control) creeping bentgrass exposed to (**A**) nonstress or (**B**) heat stress conditions. Vertical bars define the SD of the means between treatments, and letters over bars indicate significant differences among chemical treatments at each treatment date based on Fisher's protected least significant difference test ($P = 0.05$), where n is defined as the number of measurements represented by each value (n = 4). FW, fresh weight.
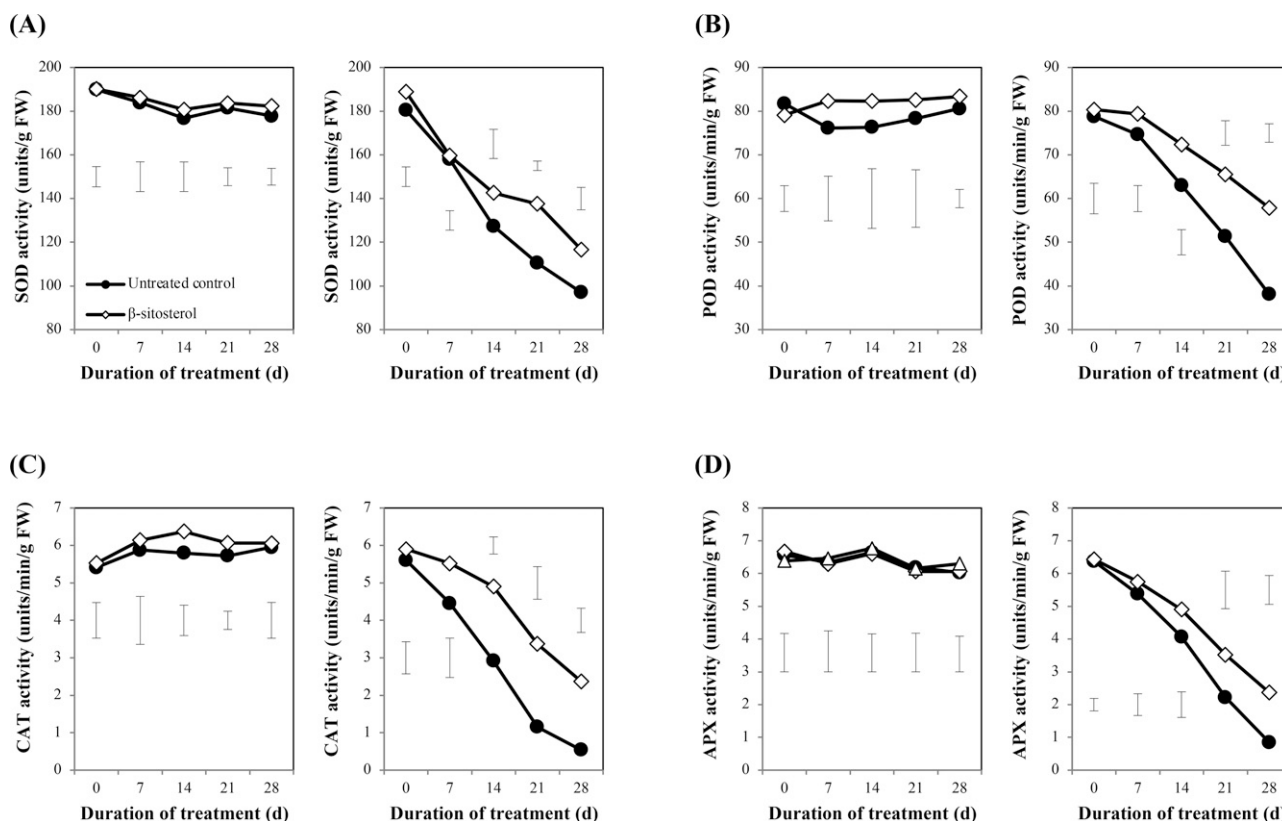
Fig. 5. Enzymatic activities for **(A)** superoxide dismutase (SOD), **(B)** peroxidase (POD), **(C)** catalase (CAT), and **(D)** ascorbate peroxidase (APX) in creeping bentgrass treated with β-sitosterol or untreated (control) under nonstress or heat stress conditions. Vertical bars define the sᴅ of the means between treatments based on Fisher's protected least significant difference test ($P = 0.05$) for each treatment date, where n is defined as the number of measurements represented by each value (n = 4).

in the activity of SOD [by 11.94%, 24.5%, and 20.23%, respectively (Fig. 5A)], POD [by 14.81%, 27.7%, and 52%, respectively (Fig. 5B)], CAT [by 67.99%, 191.59%, and 340.56%, respectively (Fig. 5C)], and APX [by 20.51%, 58.4%, and 178.88%, respectively (Fig. 5D)] .

Discussion

Although positive effects of β-sitosterol on plant tolerance to abiotic stresses, including drought stress in white clover (Li et al., 2019) and wheat (Elkeilsh et al., 2019), salt stress in pepper and sunflower (Abu-Muriefah 2015; Fawzia et al., 2016), and heat stress in tomato (Gamel et al., 2017), have been studied previously, this is the first study investigating how foliar application of β-sitosterol ameliorated heat-induced leaf senescence in association with mitigation of oxidative damage in a cool-season turfgrass species exposed to heat stress. The findings of our study indicate that foliar application of β-sitosterol suppressed heat-induced leaf senescence or improved heat tolerance of creeping bentgrass, as exhibited by increased TQ and Chl, and decreased EL in β-sitosterol-treated plants. The repression of heat-induced leaf senescence mediated by β-sitosterol may be attributed to the regulation of enzymatic antioxidant metabolism.

Sterols accumulate endogenously in various plant species under abiotic stresses and offer protection to membranes. β-Sitosterol accumulated in drought-tolerant rice (*Oryza sativa*), corresponding to an increased membrane stability index in drought-treated plants (Kumar et al., 2015). Although the effects of β-sitosterol on

antioxidant enzymes are well-established in animal systems, documentation of the manners in which β-sitosterol contributes to the mitigation of abiotic stresses in plants is very limited. In an animal study, β-sitosterol was found to be an active antioxidant that prevented membrane lipid peroxidation and enhanced the levels of enzymatic antioxidants and nonenzymatic antioxidant compounds (Baskar et al., 2012). In wheat plants, it was reported that exogenous application of β-sitosterol alleviated drought stress by reducing MDA content and enhancing the activities of SOD, POD, CAT, and APX (Elkeilsh et al., 2019). In our study, β-sitosterol had no direct antioxidant effects when there was no oxidative stress, as antioxidant enzyme activities, MDA content, and EL in plants remained stable under nonstress conditions during the experimental period (28 d). However, β-sitosterol application increased the activities of SOD, POD, CAT, and APX in plants when heat stress reduced the activities of those enzymes in untreated controls. The enhanced activities of antioxidant enzymes corresponded to the β-sitosterol-reduced MDA content and EL under heat stress, indicating that β-sitosterol could have alleviated lipid peroxidation and improved antioxidant capacity when oxidative stress was induced by heat stress.

Among the antioxidant enzymes, CAT was most responsive to β-sitosterol, having the most pronounced increases in its activity during the entire heat stress period (by 3.4-fold at 28 d). APX also had increased activities to a large extent (by 1.8-fold at 28 d) in response to β-sitosterol application under heat stress. CAT, in the water–water cycle, and APX, in the ascorbate–glutathione cycle, play essential roles in reducing H_2O_2 into water. More specifically, CAT reacts directly with H_2O_2 to yield water and

oxygen in the water–water cycle, whereas APX catalyzes the conversion of H_2O_2 to water and monodehydroascorbate using ascorbate as a substrate in the ascorbate–glutathione cycle (Asada, 1999; Zhang and Kirkham, 1996). Our results imply that β-sitosterol was most effective in detoxifying H_2O_2 by enacting the water–water cycle and the ascorbate–glutathione cycle of antioxidant metabolism against heat-induced oxidative stress.

In summary, our study demonstrated that β-sitosterol had positive effects in suppressing heat-induced leaf senescence or improving heat tolerance in creeping bentgrass involving activation of enzymatic antioxidant metabolism mainly in the water–water cycle and ascorbate–glutathione pathway, providing protective roles when antioxidant enzymes were damaged or when activities of these enzymes were reduced by heat stress. Our study suggests that β-sitosterol may be used as an effective agent to improve turfgrass performance through antioxidant protection against heat-induced oxidative stress in cool-season turfgrass species in the summer in warm-climate regions.

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