Differential Effects of Glycine Betaine and Spermidine on Osmotic Adjustment and Antioxidant Defense Contributing to Improved Drought Tolerance in Creeping Bentgrass

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ABSTRACT. Glycine betaine (GB) and spermidine (Spd) are known to play roles in plant adaptation to stresses that induce dehydration, including drought stress. The objectives of this study were to examine whether improved drought tolerance by exogenous application of GB or Spd is associated with the increases in the endogenous accumulation of those solutes under drought stress in cool-season turfgrass species, and to determine the relative effects of those solutes on osmotic adjustment (OA) and antioxidant metabolism for improving drought tolerance. Creeping bentgrass (Agrostis stolonifera cv. Penncross) plants were treated with 200 mM GB or 0.1 mM Spd for 3 weeks by weekly foliar application before the exposure to drought stress; those plants were subsequently subjected to drought stress by withholding irrigation. The endogenous content of GB and Spd increased significantly through the exogenous application of either compound, to a greater magnitude for GB. The comparison of GB- or Spd-treated plants to untreated plants exposed to drought stress for growth [turf quality (TQ)] and physiological responses (water relations, membrane stability, and antioxidant metabolism) demonstrated that both compounds led to significant improvement in drought tolerance in creeping bentgrass. The improved drought tolerance by exogenous GB could be due to its contribution to OA by significant accumulation of endogenous GB, and activation of antioxidant enzymes with the greatest effects on ascorbate peroxidase (APX). Exogenous Spd did not cause increases in leaf OA despite of the increased endogenous accumulation, but significantly enhanced antioxidant enzyme activities, with the most pronounced effects on catalase (CAT). This study demonstrated that GB and Spd had different effects on OA and activated different antioxidant defense pathways, protecting plants from drought damages in creeping bentgrass.

Drought injury in turfgrass is characterized by leaf desiccation, which is associated with excessive water loss from leaves and disruption of cell membranes due to oxidative stress induced by water deficit (Nilsen and Orcutt, 1996). One mechanism for plants to survive drought stress is OA through accumulating solutes that allow plants to maintain leaf cellular hydration and sustain metabolic activities during drought stress, and OA level is positively correlated to whole-plant drought tolerance in many plant species, including turfgrasses (DaCosta and Huang, 2006; Qian and Fry, 1997; White et al., 1992). Another strategy to maintain survival of leaves from drought damages is the activation of antioxidant defense systems, which involve antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), CAT, and APX, to scavenge excessive reactive oxygen species accumulated in plants during drought stress (Nilsen and Orcutt, 1996).

One convenient and effective approach to protecting plants from drought damages is exogenous application or enhanced endogenous production of some organic solutes that may enhance OA and antioxidant defense. Glycine betaine, a quarternary ammonium compound, is known to accumulate in chloroplasts in response to dehydration under drought or salinity stress, but the endogenous amount of GB may be not sufficient to ameliorate the adverse effects of dehydration stress in many plant species (Ashraf and Foolad, 2007; Rhodes and Hanson, 1993). Therefore, exogenous application of GB to low-accumulating or nonaccumulating plants is greatly beneficial for plant defense against the adverse effects of environmental stresses, but the effectiveness may vary with plant species due to the difference in endogenous amount of GB in the plant species (Ashraf and Foolad, 2007; Farooq et al., 2008; Gupta et al., 2013). A number of studies reported that GB-enhanced
drought tolerance is due to its involvement in OA in some plant species (Ashraf and Foolad, 2007; Burgess and Huang, 2013), whereas other studies found that GB-induced salinity tolerance was related to the enhanced antioxidant defense systems (Demiral and Türkan, 2004; Hoque et al., 2008; Okuma et al., 2004). Polyamines, including Spd, putrescine, and spermine, are ubiquitous aliphatic amines, which tend to accumulate in response to dehydration stress and have also been found to play positive roles in improving drought tolerance; among the three major polyamines, Spd is more effective in many cases (Bouchereau et al., 1999; Yamaguchi et al., 2007). The beneficial effects of polyamines are manifested by the reduction in electrolyte leakage (EL) (Amri and Shahsavari, 2010), increases in relative water content (RWC) and photosynthetic capability (Yin et al., 2014), as well as the activity of major antioxidant enzymes (Amri and Shahsavar, 2010; Kubiš, 2003).

Creeping bentgrass is drought-sensitive cool-season grass species widely used as turfgrass in high maintenance areas. This species may naturally produce low quantities of GB and Spd, and therefore, exogenous application may enhance drought tolerance by enhancing endogenous accumulation of these metabolites through influencing OA or antioxidant defense. Previous studies on creeping bentgrass reported the promotive effects of exogenous GB or Spd on drought tolerance (Burgess and Huang, 2013; Shukla et al., 2014; Yang et al., 2012), but whether it was related to the induction of endogenous accumulation of these solutes was not well documented. Despite of the known beneficial effects of GB and Spd in different plant species, whether the positive effects of GB and Spd are mainly due to their involvement in OA or/and antioxidant defense is not well understood. The objectives of this study were 1) to determine whether improved drought tolerance by exogenous application of GB or Spd is associated with the increases in the endogenous accumulation of those solutes under drought stress in creeping bentgrass, and 2) to determine the relative effects of those solutes on OA and antioxidant metabolism for improving drought tolerance in creeping bentgrass.

Materials and Methods

**Plant materials and growth conditions.** Sods of creeping bentgrass (cv. Penncross) were collected from field plots at the turfgrass research farm of Rutgers University in North Brunswick, NJ. Sods were washed free of soil with water, and transplanted into polyvinyl chloride (PVC) pots (10 cm diameter, 40 cm height) filled with a sterilized mixture of sand and loam soil (fine-loamy, mixed, mesic, Typic Hapludult) (1:3 v/v). A nylon screen covered the bottom of each PVC pot to allow to aeration of the soil and allow for drainage of water from the pots. Plants were maintained in the greenhouse at Rutgers University under 10- to 12-h (varying with the weather) natural light conditions with supplemental lighting provided by sodium lamp, and average temperatures of 21/13 °C (day/night) for 60 d during 6 Apr. to 6 May 2014. Plants were watered three times per week until drainage occurred from the bottom of the pots at each time of irrigation to maintain soil moisture at the pot capacity, and fertilized weekly with half-strength Hoagland’s solution (Hoagland and Arnon, 1950). All plants were hand clipped weekly to 3 to 4 cm height. After 60-d of plant establishment in the greenhouse, plants were moved to growth chambers that were controlled at 20/15 °C (day/night), 12-h photoperiod at photosynthetically active radiation of 650 µmol·m⁻²·s⁻¹, and 75% relative humidity. Plants were allowed to acclimatize to growth chamber conditions for 14 d before treatments were imposed.

**Treatments and experimental design.** Plants were sprayed with GB at 200 mM and Spd at 0.1 mM weekly for 3 weeks from 20 May to 11 June 2014, and then subsequently exposed to drought stress for 13 d by withholding irrigation, starting on 12 June 2014 (0 d of drought stress). The concentration used for each compound was selected based on a preliminary study, which was found to be most effective in enhancing turf performance under drought stress. For each treatment, sufficient solution (50 mL) was sprayed to evenly cover the turf canopy (78 cm² diameter) without dripping into the soil. Plants sprayed with water and exposed to drought stress served as the untreated control. Plants sprayed with water and maintained under well-watered conditions were used as the well-watered control.

The experimental design was a complete randomized design with four treatments (drought + GB, drought + Spd, drought, and watered control) and each treatment had four pots of plants as four replicates. Four replicates of each treatment were placed in four growth chambers, with one replicate of each treatment being placed in one growth chamber. Plants were relocated every 3 d among growth chambers to avoid potential confounding effects of variable environmental conditions in different chambers.

**Physiological analysis.** All physiological measurements were made on 0, 5, 9, and 13 d of drought stress. Turf quality was visually rated based on turf color and degree of leaf wilting on a scale of 1 to 9 (a rating of 1 indicated a completely desiccated brown turf canopy and a rating of 9 representing fully turgid, green, dense, and healthy turf canopy); TQ of 6.0 is considered as the minimal acceptable level (Turgeon, 2008).

For the measurement of leaf RWC, leaf samples were detached from the turfgrass and immediately weighed for fresh weight (FW). Leaf samples were placed into covered centrifuge tubes and soaked in water at 4 °C. After about 20 h, the leaf samples reach full hydration, and were taken out of tubes, then were blotted dry and weighed immediately for turgid weight (TW). DW is leaf dry weight after oven-drying the leaf samples for 72 h at 80 °C. Leaf RWC was calculated as: 100 × [(FW – DW)/ (TW – DW)].

Leaf EL measurement was performed to evaluate cellular membrane stability (Blum and Ebercon, 1981). Leaf samples (≤0.15 g) were cut to 0.5-cm-long segments, immersed in 25 to 30 mL of deionized water, and placed on a conical shaker for 12-h incubation. The initial level of EL (Cₒ) was measured using a conductance meter (Model 132; YSI, Yellow Springs, OH). Leaf samples were then killed at 120 °C for 15 min in an autoclave, incubated on the conical shaker for 12 h, and final level of conductance of the incubation solution (Cₘₙₐₓ) was measured. Leaf EL was calculated as (Cₒ/Cₘₙₐₓ) × 100 (Blum and Ebercon, 1981).

Leaf OA was determined as the difference in leaf osmotic potential at full turgor (Ψₒₚₒₒ) between well-watered and drought-stressed plants (Qian and Fry, 1997). Leaf Ψₒₚₒₒ was determined after soaking leaves in water for 8 h until reaching full hydration. Turgid leaf samples were frozen in liquid nitrogen and subsequently stored at −20 °C until analysis of Ψₒₚₒₒ. Frozen leaf samples were thawed and cell sap was pressed from leaves using a laboratory press (Fred S. Carver, Wabash, IN), which was subsequently analyzed for osmolality.
High-performance liquid chromatography (HPLC) was performed using a hydrophilic interaction liquid chromatography phase for HPLC analyses. GB was analyzed and quantified by HPLC. Solution of acetonitrile/water (50% v/v) was the mobile phase used for further analysis in conjunction with standard curve constructed with standard of GB. The peak areas were integrated and compared with a temperature maintained at 30 °C.

The content of Spd was determined according to the method described by Liu et al. (2002). Dry leaf tissue powder (0.1 g) was extracted in 2 mL of precooled perchloric acid, ice bath for 60 min, and centrifuged at 15,000 g, for 30 min. The supernatant was transferred into a centrifugal tube and stored at −20 °C. Polyamines (PA) contained in the supernatant were subjected to a benzoylation reaction in the alkaline medium. Benzoyl PA derivatives were extracted by diethyl ether. Ether fraction was evaporated to dryness and dissolved in methanol. High-performance liquid chromatography (HPLC) was performed on a liquid chromatograph (Thermo Fisher Scientific, Waltham, MA) using a silica column [5 μm, 250 × 4.6 mm (Diamonsil® C18(2); Dikma Technologies, Lake Forest, CA)]. 10 μL of benzoyl polyamine in methanol was injected into an autosampler (Surveyor; Thermo Fisher Scientific) every 35 min. Samples were eluted from the column with 70% methanol and with a temperature maintained at 30 °C, and the flow rate was 0.7 mL-min⁻¹. The Spd peaks were detected with an ultraviolet detector at 230 nm. Spd content was quantified using an internal standard.

GB was determined using the method described by Wang et al. (2010). Dry leaf tissue powder (0.1 g) was extracted in 12.5 mL water, shaken for 30 min, and then centrifuged at 14,000 g, for 5 min. The supernatant was filtered through glass fiber filters, and filtrate was transferred to solid-phase cartridges (Poly-Sery MCX; Organomation, Berlin, MA). Then extraction cartridges were rinsed by methanol/water (85:15 v/v) and methanol. Elution was completed by using a mixture of ammonia water/methanol (5:95 v/v) twice. The residue was condensed dry and diluted with acetonitrile/water (50% v/v) and filtered through a 0.45-μm membrane (Millipore; Merck, Darmstadt, Germany) for further analysis in HPLC. Solution of acetonitrile/water (50% v/v) was the mobile phase used for HPLC analyses. GB was analyzed and quantified by HPLC using a hydrophilic interaction liquid chromatography silica column [5 μm, 4.6 × 150 mm (Atlantis; Waters Corp., Milford, MA)]. The peak areas were integrated and compared with standard curve constructed with standard of GB.

Analysis of antioxidant enzyme activity. Fresh leaves were sampled for the measurement of antioxidant enzyme activity at 9 d of drought stress when physiological effects were most significant. About 0.5–1.0 g leaf of fresh leaf tissue was randomly sampled from each plant, frozen in liquid nitrogen, and stored at −80 °C for further analysis. Extraction of antioxidant enzymes and malondialdehyde (MDA) was performed as previously described by Xu and Huang (2004). MDA is the final product of membrane lipid peroxidation, which is quantified as an indication of oxidative damages. For enzymes and MDA extraction, the frozen samples were homogenized with 7 mL of 50 mm phosphate buffer solution (pH 7.0), ground in a mortar on ice, and centrifuged at 20,000 g, at 4 °C for 25 min. The supernatant was used to evaluate enzyme activity and MDA content.

The activity of SOD was measured according to the method of Zhang and Kirkham (1996) and Xu and Huang (2004). One unit of SOD activity was defined as the amount of SOD required to cause 50% inhibition of nitroblue-tetrazolium reduction at 560 nm-min⁻¹. The activities of CAT, APX, and POD were determined based on the method of Chance et al. (1955) and Xu and Huang (2004). Enzyme activities were based on the absorbance change of the reaction solution per minute at a given wavelength for each enzyme: CAT at 240 nm, APX at 290 nm, and POD at 470 nm. MDA content was measured at 532 and 600 nm after reaction of the extraction solution with trichloroacetic acid and thiobarbituric acid using the method of Dhindsa et al. (1981). The formula used for calculation of MDA content was the absorbance at 600 nm (A₆₀₀) subtracted from the absorbance at 532 nm (A₅₃₂) multiplied by the extinction coefficient of 155 mm⁻¹·cm⁻¹ for MDA (Heath and Packer, 1968). All reaction solutions, nonreacted control solutions, and standards were analyzed at a given wavelength with a spectrophotometer (Spectronic Instruments, New York, NY).

Statistical analysis. Statistical significance of data was tested using the analysis of variance procedure (SAS, version 9.0; SAS Institute, Cary, NC). Differences between treatment means were separated by Fisher’s protected least significant difference test at the 0.05 P level.

Results and Discussion

Turf quality is a widely used parameter to evaluate overall turfgrass performance (Turgeon, 2008). In this study, TQ was maintained between 8.0 and 9.0 throughout the experimental period in the well-watered control, but exhibited a steady decline with the drought duration regardless of chemical treatments (Fig. 1). The application of GB or Spd resulted in increases in TQ compared with the untreated plants at 9 and 13 d of drought stress. These data suggested that exogenous application of GB...
or Spd promoted overall turf performance under drought stress. Previous studies that have examined the effects of individual compounds in different plant species found that TQ was improved under drought stress by GB in Kentucky bluegrass (*Poa pratensis* (100 mM)) and creeping bentgrass (200 mM) (Burgess and Huang, 2013; Yang et al., 2012) and by polyamines [5 mM Spd in bermudagrass (*Cynodon dactylon*) (Shi et al., 2013), 500 or 750 μM (Shukla et al., 2015)]. Our study found that foliar application of GB at 200 mM and Spd at 0.1 mM effectively improved TQ of creeping bentgrass exposed to drought stress. The results in this study with creeping bentgrass together with previous studies in other turfgrass species suggested that the effective doses of GB or Spd could vary with plant species.

Maintaining adequate water status in plants is critically important for maintaining high TQ and plant survival under drought stress (Rachmilevitch et al., 2006). In our study, leaf RWC was maintained at above 87.00% in the well-watered control throughout the experimental period (Fig. 2). All treatments exhibited a sharp decline in RWC below the well-watered control level during drought stress, but plants treated with GB and Spd showed significantly higher RWC than that of the untreated plants within 9 d of drought stress. However, Shukla et al. (2015) found foliar application of Spd (at 500 or 750 μM) had no significant effects on water relations of creeping bentgrass under drought stress. Our results suggested that exogenous application of GB or Spd could facilitate water retention in leaves to maintain cell turgidity during drought stress, which is critical for sustaining cellular functions under short-term drought stress.

Cellular hydration and turgidity is largely controlled by the endogenous accumulation of compatible solutes under dehydration stress (Blum and Ebercon, 1981). In this study, naturally produced GB was quantified, which was below 20 mg−1 under well-watered conditions, and increased only by 15% in response to drought stress (23 mg−1) in plants without exogenous GB treatment (Fig. 3), which may not be sufficient for stress defense (Ashraf and Foolad, 2007; Rhodes and Hanson, 1993). Exogenous application of GB resulted in a 6.5-fold increase in the endogenous GB content (148 mg·g−1) in plants exposed to drought stress (Fig. 3). Foliar-applied GB can penetrate into leaves within 1 h of application and is transported from leaves to roots and other organs within 1 h, contributing to improved stress tolerance (Mäkelä et al., 1996, 1998). Although it is not clear how GB enter the leaves, through stomata and/or the epidermal cells, it was found that including surfactants in the spray solution of GB enhanced its penetration into the leaf. GB is xylem-phloem-mobile and primarily transported in phloem to other organs (Mäkelä et al., 1996).

Endogenous Spd content declined by 25% under drought stress in creeping bentgrass without the exogenous Spd treatment. The exogenous application of Spd however caused a significant increase (by 30%) in endogenous Spd content. Spd application also caused 10% increase in GB content under drought stress compared with untreated plants. Our results suggested that the highly enriched endogenous GB or Spd in leaves could lead to the growth and physiological effects of exogenous application of those compounds on improving drought tolerance in creeping bentgrass that could not naturally produce sufficient amount of those stress-protective solutes.

The accumulation of compatible solutes in plant cells is positively correlated with the level of capacity of OA (Chaves et al., 2003). High capacity of OA in leaf during drought stress exhibited high drought tolerance in many plant species, including turfgrasses (DaCosta and Huang, 2006; Qian and Fry, 1997; White et al., 1992). Glycine betaine and polyamines are considered major compatible solutes for OA (Ashraf and Foolad, 2007; Rhodes and Hanson, 1993). In this study, OA in leaves of well-watered plants was maintained at a low level (0.05 MPa) during the experimental period (Fig. 4). The OA level increased under drought stress in all treatments. A significant OA level was observed in plants treated with GB during the entire drought period compared with the untreated plants under drought stress. However, exogenous application of Spd had no significant effects on OA in creeping bentgrass (Fig. 4). These results suggested that the positive effects of GB on improving drought tolerance could be related to their involvement in improving water relations and stress defense. This study suggested that endogenous GB or Spd could be considered important for maintaining high TQ and plant survival under drought stress.

![Fig. 2. Leaf relative water content of creeping bentgrass under well-watered (W) and drought stress without (D) and with glycine betaine (D + GB) or spermidine (Spd; D + Spd) treatments. Drought stress was imposed on 12 June 2014 (0 d). Vertical bars at the bottom are least significant difference values (P = 0.05) for cultivar comparisons at a given day of treatment.](image)

![Fig. 3. Leaf electrolyte leakage of creeping bentgrass under well-watered (W) and drought stress without (D) and with glycine betaine (D + GB) or spermidine (Spd; D + Spd) treatments. Drought stress was imposed on 12 June 2014 (0 d). Vertical bars at the bottom are least significant difference values (P = 0.05) for cultivar comparisons at a given day of treatment.](image)

![Fig. 4. OA response to drought stress (23 mg·g−1) in plants without exogenous GB treatment (Fig. 3), which may not be sufficient for stress defense (Ashraf and Foolad, 2007; Rhodes and Hanson, 1993). Exogenous application of GB resulted in a 6.5-fold increase in the endogenous GB content (148 mg·g−1) in plants exposed to drought stress (Fig. 3). Foliar-applied GB can penetrate into leaves within 1 h of application and is transported from leaves to roots and other organs within 1 h, contributing to improved stress tolerance (Mäkelä et al., 1996, 1998). Although it is not clear how GB enter the leaves, through stomata and/or the epidermal cells, it was found that including surfactants in the spray solution of GB enhanced its penetration into the leaf; GB is xylem-phloem-mobile and primarily transported in phloem to other organs (Mäkelä et al., 1996).](image)
in OA, whereas Spd was not a major contributor to OA in creeping bentgrass.

The maintenance of cellular membrane integrity is also a key factor for plant survival of drought stress that can interrupt membranes through oxidative damages of membrane lipids or cause changes in membrane chemical or structural or properties (Zhang and Kirkham, 1996). Electrolyte leakage is a widely used parameter for the estimation of membrane stability, which is negatively correlated with whole-plant stress tolerance (Jespersen et al., 2013; Marcum, 1998; Rachmilevitch et al., 2006). In this study, well-watered plants maintained low leaf EL (below 24.6%) throughout the duration of the study, whereas the other four treatments exhibited increases in leaf EL with the duration of drought stress (Fig. 5). Significantly lower EL was detected in Spd-treated plants at 9 d of drought and in GB-treated plants at 5, 9, and 13 d of drought compared with the untreated plants. The reduction in EL by GB or Spd has also been reported in other plant species (Quan et al., 2004; Yamada et al., 2007). Our results indicated that GB or Spd had protective effects on cell membranes of creeping bentgrass from drought damages, with GB having longer (13 d) effects than Spd (9 d). The mechanisms by which GB and Spd protect membranes from drought damages are still unknown. Those compounds may interact with phospholipids and proteins in the membrane to stabilize membrane structures.

Fig. 4. Osmotic adjustment of creeping bentgrass under well-watered (W) and drought stress without (D) and with glycine betaine (D + GB) or spermidine (Spd; D + Spd) treatments. Drought stress was imposed on 12 June 2014 (0 d). Vertical bars at the bottom are least significant difference values ($P = 0.05$) for cultivar comparisons at a given day of treatment.

Fig. 5. The content of glycine betaine (GB) of creeping bentgrass under well-watered conditions (W) and at 9 d of drought stress without (D) and with GB (D + GB) or spermidine (Spd; D + Spd) treatments. Columns marked with different letters indicate significant differences among genotypes for each parameter based on least significant difference test ($P = 0.05$).

Fig. 6. The content of spermidine (Spd) of creeping bentgrass under well-watered conditions (W) and at 9 d of drought stress without (D) and with glycine betaine (D + GB) or D + Spd treatments. Columns marked with different letters indicate significant differences among genotypes for each parameter based on least significant difference test ($P = 0.05$).

Fig. 7. The content of malondialdehyde (MDA) of creeping bentgrass under well-watered conditions (W) and at 9 d of drought stress without (D) and with glycine betaine (D + GB) or spermidine (Spd; D + Spd) treatments. Columns marked with different letters indicate significant differences among genotypes for each parameter based on least significant difference test ($P = 0.05$).
Antioxidant enzymes play key roles in preventing lipid peroxidation of cellular membranes and maintaining membrane stability (Chakraborty and Pradhan, 2011, 2012; Zhang and Kirkham, 1996). Leaf MDA content increased under drought stress in all treatments compared with the well-watered control, whereas the exogenous application of GB and Spd significantly reduced MDA content in creeping bentgrass exposed to drought stress (Fig. 6). The activity of all antioxidant enzymes decreased under drought stress (Fig. 7). The activity of all antioxidant enzymes decreased under drought stress (Fig. 7). Plants treated with GB or Spd had significantly greater activities of SOD, POD, CAT, and APX than untreated plants under drought stress, with the most pronounced increases for APX (165% higher) in GB-treated plants and for CAT (143% higher) in Spd-treated plants (Fig. 8). Enhanced activity of antioxidant enzymes by exogenous GB or Spd has also been reported in other plant species under salt stress (Hoque et al., 2008; Islam et al., 2009) or drought stress (Farooq et al., 2009; Li et al., 2016). Although the positive effects of GB and Spd on antioxidant enzyme activities are well known, whether GB or Spd directly or indirectly regulates those enzymes is not clear. Nevertheless, data from this study indicated that exogenous application of GB and Spd could improve the antioxidant defense system and maintain the membrane stability in creeping bentgrass exposed to drought stress, specifically with GB being most effective influencing APX, and Spd having most pronounced effects on CAT.

In summary, the improved drought tolerance by exogenous GB could be due to its contribution to OA by significant accumulation of endogenous GB and activation of antioxidant enzymes with the greatest effects on APX. Exogenous application of Spd did not cause increases in leaf OA despite of the increased endogenous accumulation, but significantly enhanced antioxidant enzyme activities, with the most pronounced effects on CAT. This study demonstrated that GB and Spd differentially affected OA and activated different antioxidant defense pathways, protecting plants from drought damages in creeping bentgrass. The underlying molecular mechanisms of how GB and Spd differentially affect antioxidant potential and OA deserve further investigation.

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