Polyamine Content Changes in Creeping Bentgrass Exposed to Salt Stress

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ABSTRACT. Salt stress is a major problem in turfgrass management. Investigation of metabolites, such as polyamines (PAs) that may improve salt tolerance of turfgrass species, is needed. Two independent growth chamber studies were conducted to evaluate physiological characteristics and changes in PAs, such as putrescine (Put), spermidine (Spd), and spermine (Spm), in response to salt stress in ‘Penncross’ and ‘PsgSLTZ’ creeping bentgrass (*Agrostis stolonifera*). The study also aimed to determine a method of PA extraction to improve PA yields from creeping bentgrass. Salt solutions were drench applied to plants growing in pure sand daily in a stepwise manner for ≈70 days in both studies. For both cultivars, salt stress caused an increase in leaf Na⁺ content, percent of electrolyte leakage (EL), and canopy temperature depression (CTD) while it caused a decrease in turf quality (TQ), osmotic potential (Ψ_s), and K⁺ and Ca²⁺ content compared with controls. In the early stages of salt stress, Put content increased in salt-stressed plants compared with controls. Spd content did not change significantly while a transient increase in Spm was observed in the later stage of salt stress. The PA quantification method used in this study included using formic acid during the extraction process, which exhibited enhanced quantification of PAs from creeping bentgrass compared with other methods previously published. Salinity stress upregulated the content of Put and Spm in leaf tissue, which may be involved in salinity tolerance in creeping bentgrass, while Spd accumulation may not be a major salt tolerance mechanism; supplementation with these biochemical compounds could be an alternative to improve creeping bentgrass salt tolerance.

Salt stress is a major issue for turfgrass management around the world. Use of reclaimed, nonpotable water that may contain high levels of salts is becoming a common management practice (Duncan et al., 2008). Additionally, numerous turf areas are located in salt-prone environments, such as shorelines or areas with salt-afflicted soils. The primary damage to plants caused by salinity is due to toxic ion accumulation of Na⁺ in plant tissues and water deprivation from decreased external Ψ_s in the soil solution (Hasegawa et al., 2000). Creeping bentgrass does not possess major salt resistance mechanisms to rid plants of salt, such as salt glands, that are found in other grass species within Poaceae (Marcum, 2001). Since adequate avoidance mechanisms are not possessed by creeping bentgrass, the species must rely on other salt tolerance mechanisms for survival under salt stress conditions. Evaluating mechanisms that may promote tolerance to salt stress is necessary to develop salt-tolerant creeping bentgrass germplasm. Cultivar variation in salt tolerance of creeping bentgrass does exist, but the major metabolite pathways that may play a role in differential salt tolerance mechanisms between cultivars have not yet been fully elucidated. Additionally, knowledge of plant metabolites important in salt stress tolerance could lead to the development of new plant-protective chemical products.

A relatively unexplored biochemical process in turfgrass species is the PA biosynthetic pathway. The common PAs

associated with growth regulation and abiotic stresses are Put, Spd, and Spm. PAs are synthesized from arginine or ornithine decarboxylation pathways (Janowitz et al., 2003). PAs may respond differently to abiotic stress based on plant species, plant tissue type, and developmental stage (Capell et al., 2004). In *Arabidopsis thaliana*, there was a shift in PA biosynthesis pathways toward accumulating Put, whereas in the resurrection plant (*Craterostigma plantagineum*) this shift was toward accumulating Spm (Alcazar et al., 2011). Conversely, after salt stress in broad bean [*Vicia faba* (Sadak and Abdelhamid, 2015)] and foxtail millet [*Setaria italica* (Sudhakar et al., 2015)], all three types of endogenous PA concentration increased significantly compared with the control. In a salt-tolerant rice (*Oryza sativa*) cultivar, Put and Spm increased more compared with the sensitive one (Do et al., 2014). In maize (*Zea mays*) leaf blade elongation zone (Rodriguez et al., 2009), an increase of Spd content was observed in response to salt treatment. Recently, Li et al. (2015a) found that PAs were generally upregulated in white clover (*Trifolium repens*) under artificially induced drought stress in hydroponic cultures. In creeping bentgrass, Spd content decreased due to drought stress, whereas Put and Spm content increased compared with control plants (Li et al., 2015b). All these results show the importance of PA in salt stress responses. However, it is not yet fully clear whether differences in PA fluctuation during stress is due to species differences, plant developmental stage, or difficulties in comparing across studies.

Compared with other crop species, little information is available regarding the function, content, or regulation of PAs in creeping bentgrass or other cool-season turfgrass species, particularly during long-term salt stress conditions. For utility as an agricultural technology, determining how the common PAs, such as Put, Spd, and Spm, may respond to stress or play

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a role in turfgrass tolerance to stress deserves investigation. In addition, current PA quantification methods used in other crop species and turfgrasses did not prove to provide adequate recovery of free PAs from creeping bentgrass tissue in our preliminary tests. Therefore, the objectives of the study were to improve the methods of extraction and quantification of PAs from creeping bentgrass tissues, evaluate creeping bentgrass physiological health under salt stress, and quantify PAs from creeping bentgrass to achieve a better understanding of PA content regulation due to prolonged salt stress conditions.

Materials and Methods

PLANT PREPARATION AND STRESS TREATMENT. Two independent growth chamber studies (Expts. 1 and 2) were conducted. For both experiments, creeping bentgrass ‘PsgSLTZ’ and ‘Pennncross’ were used. Seeds of both cultivars were obtained from the Seed Research of Oregon (Tangent, OR). These cultivars showed good performance under salt conditions in their breeding efforts; however, PsgSLTZ rated higher in salt tolerance (Seed Research of Oregon, unpublished data). Seeds were directly sown into pots (12 cm²) at a rate of 4.88 g·m⁻² into pure sand. Expt. 1 was seeded on 18 Aug. 2013 and Expt. 2 on 14 Mar. 2014. The seedlings for both experiments were established in the greenhouse under 900 μmol·m⁻²·s⁻¹ of photosynthetically active radiation (*PAR*) for 14 h of daylength under 65% to 75% humidity and 24/18 °C (day/night) temperature for ≈2 months to attain dense and healthy turf canopy. Then, the fully established plants were transferred to an environmentally controlled growth chamber with 700 μmol·m⁻²·s⁻¹ *PAR* for 14 h of daylength, 65% to 70% humidity, and 23/18 °C (day/night) temperatures for both experiments.

Treatments were arranged in a randomized complete block design with four biological replications. All salt-treated plants were watered daily each week with 100 mL of salt solution at 0 (21 Dec. 2013), 12 (7 d), 12 (21 d), 16 (35 d), 16 (42 d), 20 (56 d), and 24 (70 d) dS·m⁻¹ for Expt. 1. The salt solution (Instant Ocean Aquarium Salt; Spectrum Brands, Blacksburg, VA) was made with half strength Hoagland solution (Hoagland and Arnon, 1950) twice per week. The salt solution was made from deionized water the rest of the 5 d within the week. The salt solution included 10.78 g·L⁻¹ Na⁺, 0.42 g·L⁻¹ K⁺, 1.32 g·L⁻¹ Mg²⁺, 0.4 g·L⁻¹ Ca²⁺, 0.008 g·L⁻¹ Sr²⁺, 19.29 g·L⁻¹ Cl⁻, 2.66 g·L⁻¹ SO₄²⁻, 0.2 g·L⁻¹ HCO₃⁻, 0.56 g·L⁻¹ Br⁻, and 0.001 g·L⁻¹ F⁻. The percentage of NaCl in the salt solution was 84%. The same amounts of salt solution were used in Expt. 2, which were applied at 0 (6 May 2014), 4 (28 d), 8 (35 d), 12 (49 d), 16 (63 d), and 20 (77 d) dS·m⁻¹.

PHYSIOLOGICAL TRAIT EVALUATION. TQ ratings were based on color, density, and uniformity of the grass canopy using a scale of 1–9 with 1 being dead plants and 9 being healthy and green plants (Beard, 2001). Percent EL was measured to estimate cell membrane stability of the leaves based on the method of Blum and Ebercon (1981). About 10 leaves were cut, briefly washed, and immersed in a 15-mL centrifuge tube with 7.5 mL of deionized water. They were then placed on a shaker for 24 h at 144 rpm. The conductivity of the solution containing the living tissue was measured using an electric conductivity meter (3200 conductivity meter; YSI, Yellow Springs, OH) as the initial conductivity (*C_i*) after shaking. The leaf tissues were boiled in a water bath for 5 h and then were placed on a shaker with the same speed for another 24 h. The electric conductivity of the

solution containing dead tissues was measured as the maximum conductivity (*C_{max}*). The percent EL was calculated as $C_i/C_{max} \times 100$.

The Ψ_s was determined as in the method of Qian and Fry (1997). About 10 leaves were taken from each plant and were immersed in deionized water in a petri dish for 3 h. Leaves were blotted dry, transferred to a 2-mL microcentrifuge tube, immediately frozen in liquid nitrogen, and then stored at –80 °C. Samples were ground with a micropestle and centrifuged at 20,817 g_n (Eppendorf 5430 R; USA Scientific, Ocala, FL) for 15 min at room temperature. A 10-μL leaf extract was placed on a piece of 0.5-cm-diameter filter paper, inserted into a vapor pressure osmometer (5520 VAPRO; Wescor, Logan, UT) to obtain leaf solute molarity. The conversion from molarity to MPa was calculated as $\Psi_s = -C \times R \times T$, where *C* is the leaf solute molarity, *T* the absolute temperature of 310 K, and *R* the constant (8.314 cm³ MPa·K⁻¹·mol⁻¹).

CTD is an indication of transpiration rate and has been widely used as a method to evaluate plant stress (Blum et al., 1982). Turf canopy temperatures were measured using an infrared thermometer (Davis Instruments, Vernon Hills, IL). The temperature depression was calculated by subtracting ambient temperature in the growth chamber from the plant canopy temperature.

MINERAL NUTRIENT DETERMINATION. For both experiments, 1 g of grass leaf tissue was harvested, dried at 60 °C in an oven for 3 d, and ground through a 1-mm screen within a stainless steel grinder (Wiley Mini-Mill Stainless Steel, 120V, 60 Hz; Thomas Scientific; Swedesboro, NJ) (Campbell and Plank, 1992). The same amount of ground leaf tissue was digested in 10 mL of 16 N HNO₃ for 30 min in a microwave, and the prepared sample volume was brought to 50 mL with deionized water before measurement (Jones, 2001). Na⁺, K⁺, and Ca²⁺ were measured using inductively coupled plasma mass spectrometer (ICP-MS) (Optima 3300 DV ICP-MS emission spectrophotometer; A & L Great Lakes Laboratories, Fort Wayne, IN) at wavelengths of 589.592, 766.490, and 317.933 nm, respectively. Final results were reported as milligrams per gram dry weight.

PA EXTRACTION. The method of Liu et al. (2011a) and Oefner et al. (1992) was used with modification to quantify the accumulation of free PAs in creeping bentgrass for both experiments with four biological replications. Modifications were necessary to improve the yield of PAs from creeping bentgrass samples in our preliminary studies. The major modification included the use of 5% formic acid (Sigma-Aldrich, St. Louis, MO) during PA extraction instead of perchloric acid. For PA extraction, a 250-mg sample of frozen leaf tissue was ground with liquid nitrogen to obtain a fine powder with a mortar and pestle. A 1-mL aliquot of 5% formic acid (prepared with water) was added to the ground leaf powder, and they were ground further until a fine slurry was reached. The ground sample slurry was collected in a 2-mL microcentrifuge tube and stored in a –80 °C freezer for further processing. To prepare the samples for derivatization, the samples were allowed to thaw at room temperature and were centrifuged for 30 min at 20,817 g_n at 4 °C. The supernatant containing PAs was transferred to a fresh, sterile 2-mL microcentrifuge tube. The pH of the supernatant was adjusted to be higher than 12.0 with 2 M NaOH (Fisher Scientific, Fair Lawn, NJ). The samples were then dried in a vacuum evaporator (79703-00 CentriVap; Labconco, KS City, MO) at room temperature.

The dried samples were resuspended in 500 μL of 4% benzoyl chloride (prepared in acetone; Sigma-Aldrich) and incubated at 30 °C for 2 h, with shaking. After incubation, the supernatant was centrifuged (20,817 g_n) and was mixed with 500 μL of a saturated NaCl solution and 1 mL of dichloromethane (99%; ACRO, Bridgewater, NJ) in a 15-mL centrifuge tube. Samples were centrifuged at 20,817 g_n for 5 min at room temperature. After centrifugation, the bottom layer containing derivatized PAs was collected with a pipette. Benzoyl chloride-derivatized PAs in dichloromethane (total 2 mL volume) were washed with an equal amount of water three times to get rid of salts by pipetting. Finally, samples were lyophilized in a freeze dryer (Genesis Pilot, Genesis 25L; SP Scientific, Warminster, PA) at room temperature. Dried samples were resuspended in 60% methanol for high-performance liquid chromatography (HPLC) MS analysis.

EXTERNAL STANDARD PREPARATION. Put, Spd, and Spm (Sigma-Aldrich) stock solutions of 0.5 $\text{mm}\cdot\text{L}^{-1}$ were prepared in 5% formic acid. Samples were evaporated to dryness at room temperature in a vacuum concentrator (Savant SPD111V; Thermo Fisher Scientific, Waltham, MA) and subjected to benzoyl chloride derivatization as described in PA Extraction section. Finally, standards were resuspended in 60% methanol to prepare a standard curve before loading onto the HPLC-MS in the same manner as the other analytes described below. The positive ions for detection of Put, Spd, and Spm were m/z 319.1, m/z 480.2, and m/z 641.3, respectively. The reproducibility of the instrument was validated by injection of the mixtures of those three standards at a concentration of 30 $\mu\text{M}\cdot\text{L}^{-1}$. A 60% methanol solvent was used as a blank between every six analytes to show that there was no carryover from the previous sample.

INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS. Both experiments were performed at room temperature based on the method of Liu et al. (2011a) with modifications. Benzoyl chloride-derivatized PA samples were analyzed using HPLC-MS (Quattro micro; Waters, Milford, MA) coupled with a binary pump (LC-20 AD) and auto-sampler (SIL 5000 Auto injector; Shimadzu, Columbia, MD). Derivatized PAs were injected into a C18 column (5 cm $\times$ 2.1 mm $\times$ 2.7 μm ; Ascentis Express, Sigma-Aldrich) at the gradient of 30% phase B and 70% phase A for the first 5 min, 74% B and 26% A for the next 10 min; 30% B and 70% A started at 10.01 min and was kept for another 3 min to get column equilibrium with the flow rate of 0.3 $\text{mL}\cdot\text{min}^{-1}$. The mobile phases A and B were water and methanol, respectively. The electrospray ionization positive mode and single ion recording (SIR) data type were used to acquire PAs Na adduct ions. The source and dissolution temperature was set at 100 and 350 °C, respectively. Capillary and cone voltage was 3.17 kV and 35 V, respectively. Dissolution gas flow was set at 600 $\text{L}\cdot\text{h}^{-1}$ and cone gas flow was 30 $\text{L}\cdot\text{h}^{-1}$.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS. Initially, all data of each trait were tested for their normality and homogeneity using the univariate procedure in SAS (version 9.4; SAS Institute, Cary, NC), and were normally distributed with uniform homogeneity. Therefore, all of the raw data were analyzed by using PROC MIXED based on the mixed linear model in SAS. Significant differences ($P \leq 0.05$) of means for each trait at each time point were separated by using Fisher's least significant difference test in the LSMEANS procedure.

PHYSIOLOGICAL TRAITS EVALUATION. In both experiments, TQ declined due to salt stress whereas control plants did not decrease in TQ (Fig. 1). TQ decreased significantly after ≈ 42 d (16 $\text{dS}\cdot\text{m}^{-1}$) of salt treatment in Expt. 1 (Fig. 1A). However, in Expt. 2, the quality decreased in 'PsgSLTZ' later than in 'Pennncross', which happened after 49 d (12 $\text{dS}\cdot\text{m}^{-1}$) of salt treatment (Fig. 1B). Major differences in TQ were detected when comparing cultivars under salt treatment on two dates (35 and 70 d) in Expt. 1 (Fig. 1A). EL remained consistently low at $\approx 12\%$ for control plants in both experiments (Fig. 1C and D). In both experiments, EL levels began to significantly increase in salt-treated plants compared with controls of both cultivars at 42 d (16 $\text{dS}\cdot\text{m}^{-1}$) and 49 d (12 $\text{dS}\cdot\text{m}^{-1}$) of salt treatment (Fig. 1C and D). The EL levels of 'PsgSLTZ' were generally higher than in 'Pennncross' in the later stage of salt treatment in both experiments (Fig. 1C and D). The Ψ_s levels in both cultivars were maintained at ≈ -0.5 MPa for control plants throughout the duration of both experiments (Fig. 2A and B). About 28 d after salt treatment, a significant decrease in Ψ_s was observed in salt-treated plants from both experiments (Fig. 2A and B). However, the amount of decrease in 'PsgSLTZ' was significantly less than in 'Pennncross' on some sampling days in both experiments (Fig. 2A and B). No significant differences were detected for CTD between cultivars in both experiments under either control or salt-stressed conditions (Fig. 2C and D); however, control plants exhibited significantly lower CTD than the salt-stressed plants in both experiments (Fig. 2C and D).

MINERAL NUTRIENT CONTENT. Plant leaf Na^+ content increased significantly due to salt treatment in both cultivars of both experiments (Fig. 3). Under moderate salt stress conditions (21 d), both cultivars had similar Na^+ content accumulations in both experiments (Fig. 3A and B). After prolonged salt stress (70 d), 'PsgSLTZ' accumulated significantly less Na^+ than 'Pennncross' during both experiments (Fig. 3A and B). Plant leaf Ca^{2+} content decreased significantly compared with controls for both cultivars due to salt stress in both experiments (Fig. 3C and D). No significant differences were detected due to salt stress in Ca^{2+} content between the two cultivar types in the two experiments (Fig. 3C and D). Plant leaf K^+ content decreased significantly due to salt stress in both experiments (Fig. 3E and F). 'PsgSLTZ' had significantly higher K^+ content than 'Pennncross' on 35 and 70 d of salt stress in Expt. 1 (Fig. 3E) and on days 49, 63, and 77 in Expt. 2 (Fig. 3F).

PA CONTENT. Before optimizing the extraction, column size, solvent, and mobile phase described here, Spd and Spm were not resolved into separate peaks and therefore could not be quantified. The percent area after optimizing the method was 94% for the three PAs standards. PA extraction in formic acid instead of perchloric acid proved to be a more effective method for extraction of PAs in HPLC-MS detection from creeping bentgrass tissues. The method was validated by making a standard calibration curve with all three standards from a fixed concentration of 0.1, 1, 2, 5, 10, 25 $\mu\text{M}\cdot\text{L}^{-1}$ and the linearity correlation coefficient (r^2) based on the space between peak areas. The correlation coefficients for Put, Spd, and Spm were 0.983, 0.984, and 0.971, respectively. Residual carryover in the instrument was not detected. This was confirmed by placing 60% of methanol between every six analytes, and the standard deviations for all the analytes ranged from 0.0% to 0.5%.

Fig. 1. Turf quality rating of creeping bentgrass ‘PsgSLTZ’ and ‘Pennncross’ exposed to salt stress treatment in (A) Expt. 1, which was started on 21 Dec. 2013, and (B) Expt. 2, which was started on 6 May 2014. Percent electrolyte leakage of leaves in (C) Expt. 1 and (D) Expt. 2. Treatment means were separated using Fisher’s least significant difference ($n = 4$) ($P \leq 0.05$), which is represented by the vertical bar. Rating was from 1 = poor to 9 = excellent based on turf color, uniformity, and density.

During these tests, no peaks were found at the designated retention time points.

Based on each linear curve of the PA standards, the range of quantities of the samples ranged from 263 to 5607 nmol·g⁻¹ FW. Salt stress caused an increase in Put production compared with control plants in both experiments (Fig. 4A and B); however, Put content induction in ‘PsgSLTZ’ was lower compared with ‘Pennncross’ on 7 and 21 d after salt treatment in Expt. 1 (Fig. 4A). In Expt. 2, a similar trend occurred except for on 28 d after salt treatment in which ‘PsgSLTZ’ had a high Put content compared with ‘Pennncross’ (Fig. 4B). The overall content of Spd production was greater in Expt. 1 (Fig. 4C) compared with Expt. 2 (Fig. 4D). Spd content in ‘PsgSLTZ’ was significantly higher compared with ‘Pennncross’ in the early stage (21 d) of salt stress in Expt. 1 (Fig. 4C). For the remaining time points measured for Spd, no significant differences were detected in salt-treated plants compared with the control plants from both experiments (Fig. 4C and D). Spm production was

generally induced by salt stress in both experiments (Fig. 4E and F). For Expt. 1, Spm content was significantly greater in salt-treated plants on 21 and 35 d (Fig. 4E). On these days, ‘PsgSLTZ’ had significantly greater Spm content than in ‘Pennncross’ (Fig. 4E). Under prolonged salt stress conditions (70 d), Spm content was at or below the level of control plants (Fig. 4E). A similar trend was observed for Expt. 2 (Fig. 4F). On 49 d of treatment, ‘PsgSLTZ’ had a greater level of Spm content, whereas on 63 d of treatment ‘Pennncross’ had significantly greater levels of Spm (Fig. 4F).

Discussion

The results of the physiological traits evaluated in this study indicate that both ‘PsgSLTZ’ and ‘Pennncross’ creeping bentgrass experienced similar levels of damage due to salt treatments in both experiments. Salt exposure of these two creeping bentgrass cultivars resulted in an accumulation of Na⁺ in plant

Fig. 2. Leaf osmotic potential of creeping bentgrass 'PsgSLTZ' and 'Penncross' exposed to salt stress treatment in (A) Expt. 1, which was started on 21 Dec. 2013, and (B) Expt. 2, which was started on 6 May 2014. Canopy temperature depression presented in (C) Expt. 1 and (D) Expt. 2. Treatment means were separated using Fisher's least significant difference at $P \leq 0.05$ ($n = 4$), which is represented by the vertical bar.

leaves and a reduction in Ca^{2+} and K^{+} content in both experiments. 'PsgSLTZ' exhibited significantly higher K^{+} (throughout salt treatment), significantly lower Na^{+} (on the last day of salt stress in both experiments), less change in Ψ_s (on most dates of both experiments) compared with 'Penncross'. This is relatively consistent with our previous studies of other creeping bentgrass cultivars differing in salt tolerance (Krishnan and Merewitz, 2015). Salt stress may reduce the content of K^{+} and Ca^{2+} and maintenance of K^{+} is associated with salt tolerance (Krishnan and Merewitz, 2015; Qian and Fu, 2005; Sairam et al., 2002). We cannot fully conclude whether the cultivars differed in salt tolerance from the results of this study, as more morphological and physiological data would be required. Further research and physiological characterization is needed to better understand potential salt tolerance attributes of various creeping bentgrass cultivars.

Creeping bentgrass does not readily exhibit salt escape or avoidance mechanisms (Marcum, 2001). Plants use salt tolerance mechanisms, such as accumulation of osmolytes, production of antioxidants, and other mechanisms to deal with salt accumulation (Fry and Huang, 2004). A tolerance mechanism

to various abiotic stresses in creeping bentgrass is proposed to be the regulation of gene expression and biochemical pathways controlling PA content homeostasis. Common PAs (Put, Spd, and Spm) are thought to act as regulatory molecules in plant cells by binding and modulating nucleic acids under abiotic stresses (Gill and Tuteja, 2010; Marco et al., 2011; Yang et al., 2007). Some research focuses on the function of conjugated PAs for protection in salt stressed tissues (Quinet et al., 2010; Yang et al., 2007); however, free PA is still the main form of this compound studied for its function under stress conditions. Free PA homeostasis depends on their synthesis, transport, degradation, and conjugation, which can be highly complex related to abiotic stress tolerance (Groppa and Benavides, 2008; Minocha et al., 2014). Whether PA content production may be a tolerance mechanism to abiotic stresses in turfgrass species has not yet been fully elucidated. Particularly, little information is available on the function, content, or regulation of these PAs in turfgrass species, such as high-value creeping bentgrass.

To quantify PAs in creeping bentgrass, current methods of PA quantification did not work well in our preliminary tests. The method described here is an economic and simple

Fig. 3. Leaf mineral content of creeping bentgrass 'PsgSLTZ' and 'Penncross' under salt stress. Leaf content of (A) Na⁺ (C) Ca²⁺, and (E) K⁺ in 'PsgSLTZ' and 'Penncross' from Expt. 1 (B) Na⁺ (D) Ca²⁺, and (F) K⁺ for Expt. 2. Treatment means were separated using Fisher's least significant difference at $P \leq 0.05$ ($n = 4$), which is represented by the vertical bar. Day 0 for Expts. 1 and 2 started on 21 Dec. 2013 and 6 May 2014, respectively.

technique that is a method modified from Liu et al. (2011a) and Oefner et al. (1992). After optimizing the method, 5% formic acid was used to extract free PA from creeping bentgrass while

Liu and Moriguchi (2011) used 10% HClO₄ as extraction buffer from human urine. This modification is more accommodating for HPLC-MS equipment due to its higher volatility compared

Fig. 4. Content of free polyamines in leaves of creeping bentgrass 'PsgSLTZ' and 'Penncross' under salt stress. Putrescine content in (A) Expt. 1 and (B) Expt. 2. Spermidine content in (C) Expt. 1 and (D) Expt. 2. Spermine content in (E) Expt. 1 and (F) Expt. 2. Treatment means were separated using Fisher's least significant difference at $P \leq 0.05$ ($n = 4$), which is represented by the vertical bar. Day 0 for Expts. 1 and 2 started on 21 Dec. 2013 and 6 May 2014, respectively.

with perchloric acid. Using HPLC-MS through electro-spray ionization positive mode and single ion recording (SIR) data type, based on the mass-to-charge ratio of the compound, is different from what has been used in other experiments (Li

et al., 2015a, 2015b). These experiments have used the method as described in the work of Duan et al. (2008), and it is based on ultraviolet detection within HPLC (Liu and Moriguchi, 2007). Compared with a ultraviolet detector, the MS detection

technique in HPLC-MS is considered to be more precise and free of background interference (Holcapek et al., 2012; Mayr and Schieberle, 2012) because of the polarities of PAs [Put²⁺, Spd³⁺, and Spm⁴⁺ (Vafaezadeh et al., 2016)]. Furthermore, the retention time (24 min) that Liu et al. (2011a) used in liquid chromatography quadruple time-of-flight MS was longer while our HPLC-MS used only 13 min for PA detection without carryover, which is more economical by saving analytic time. Besides, the mobile phase program for their method was 55% A (water) from 0 to 14 min and 74% A from 14 to 24 min, which was different from ours (see Material and Method section). Our mobile phase program is optimized to be beneficial for the mass analyzer to separate and detect PA ions. The flow rate, which affects the sensitivity of mass detector, in this method (0.3 mL·min⁻¹) was different from that of Liu et al. (2011a) (1 mL·min⁻¹). Also, the smaller column size compared with that of Liu et al. (2011a) is better for a higher peak resolution. It is necessary to optimize the method as technologies become more advanced. To our knowledge, only one other published study has evaluated PAs from creeping bentgrass ('Penn-A4') leaf tissue. Their study aimed to evaluate PA content after treatment with exogenously applied PAs in response to drought stress (Li et al., 2015b). The method described here allowed for detection of ≈100 times the level of PAs in response to salinity stress (Li et al., 2015b); however, it is worthy to note that this difference could also be related to differences in the cultivar used and experimental conditions.

In this study, we have found that the monocot, creeping bentgrass, may exhibit PA regulation in response to salt stress. An increase in Put, no significant changes in Spd and an increase in Spm were observed due to salt stress of both creeping bentgrass cultivars (Fig. 4). Similarly, in grape (*Vitis vinifera*) seedlings, Put and Spm content and PA biosynthesis gene expression was induced when grown in media with 200 mM NaCl (Liu et al., 2011b). Creeping bentgrass 'PsgSLTZ' overall exhibited less production of Put and higher production of Spm in response to salt stress (Fig. 4A and E), which could be associated with a greater salt tolerance. Zapata et al. (2004) studied free PA accumulation in seven crop species including spinach (*Spinacia oleracea*), lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), melon (*Cucumis melo*), pepper (*Capsicum annuum*), broccoli (*Brassica oleracea* var. *italica*), and beetroot (*Beta vulgaris*) under salinity stress. Slightly alternate results were observed in their study since, in most of these dicot species under salt stress, a decrease in Put and an increase in Spd and Spm was observed. They attributed a higher amount of Spd and Spm with a low amount of Put to be associated with salt tolerance. A similar trend was also found in seedlings of common wheat (*Triticum aestivum*), since Put decreased under saline stress while a significant increase in Spd and Spm occurred (Capell et al., 2004; El-Shintinawy, 2000; Maiale et al., 2004). However, the results found here were mostly consistent with a salt stress study in the monocot grass species rice, in which salt sensitivity of a rice cultivar was associated with accumulation of Put and low level of Spd and Spm (Krishnamurthy and Bhagwat, 1989). Creeping bentgrass 'PsgSLTZ' overall exhibited less production of Put in response to salt stress, which could be associated with a greater salt tolerance; however, based on physiological evaluation, the cultivar differences in salt tolerance were not fully clear. Thus, it seems that these two creeping bentgrass cultivars exhibit a trend in PA content changes that are associated with salt

sensitivity. Therefore, supplementation of PA biochemical pathways via exogenous application of PAs or by genetic modification may be beneficial for creeping bentgrass survival of salt stress.

In conclusion, both creeping bentgrass cultivars exhibited a significant decline in plant health due to salt treatment. PA content was also affected by salt stress treatment. Overall, Put increased due to salt stress while Spd was generally unaffected and Spm had a transient increase in content due to salt stress. Since creeping bentgrass exhibits PA regulation that may be associated with stress sensitivity, the enhancement of PA regulation in creeping bentgrass, such as through exogenous application or molecular techniques, may be a viable method of improving creeping bentgrass tolerance to salt. In our recent work, we have found that exogenous application of PAs did improve creeping bentgrass responses to drought stress (Shukla et al., 2015). More work is needed to elucidate the physiological effects of PAs in relation to abiotic stresses in turfgrass species.

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