Salt Stress-induced Injury is Associated with Hormonal Alteration in Kentucky Bluegrass

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Abstract. Plant hormones play an important role in plant adaptation to abiotic stress, but hormonal responses of cool-season turfgrass species to salt stress are not well documented. This study was carried out to investigate the responses of hormones to salt stress and examine if salt stress-induced injury was associated with hormonal alteration in Kentucky bluegrass (KBG, *Poa pratensis* L.). The grass was grown in a chamber for 6 weeks and then subjected to salt stress (170 mM NaCl) for 28 days. Salt stress caused cell membrane damage, resulting in photosynthetic rate (Pn), chlorophyll (Chl), and turf quality decline in KBG. Salt stress increased leaf abscisic acid (ABA) and ABA/cytokinin (CK) ratio; reduced trans-zeanin riboside (ZR), isopentenyl adenosine (IPA), and indole-3-acetic acid (IAA), but did not affect gibberellin A4 (GA4). On average, salt stress reduced ZR by 67.4% and IAA by 58.6%, whereas it increased ABA by 398.5%. At the end of the experiment (day 28), turf quality, Pn, and stomatal conductance (g_s) were negatively correlated with ABA and ABA/CK ratio, but positively correlated with ZR, IPA, and IAA. Electrolyte leakage (EL) was positively correlated with ABA and ABA/CK and negatively correlated with ZR, IPA, IAA, and GA4. GA4 was also positively correlated with turf quality and g_s. The results of this study suggest that salt stress-induced injury of the cell membrane and photosynthetic function may be associated with hormonal alteration and imbalance in KBG.

Introduction

Salinity is a major factor limiting plant growth and development of plants in many areas of the world. As population and potable water demand increase, water shortage is a major problem in many parts of the world (Huang et al., 2014; Marcum and Pessarakli, 2010; Sun et al., 2015). Turfgrasses increasingly experience salt stress because of the accelerated salinization of agricultural lands and increasing demand on the use of reclaimed or other secondary saline water for irrigation of turfgrass landscapes (Huang et al., 2014; Jiang et al., 2013; Sun et al., 2015). Salt stress may reduce turfgrass growth and quality by osmotic stress-induced injury. In many areas with limited fresh water resources, reclaimed water has been applied on golf courses and other turf surfaces to save water.

The complex regulatory processes of plant salt adaptation involve control of water flux, cellular osmotic adjustment, and hormonal regulation (Goldack et al., 2014; Ryu and Cho, 2015). The decline of cell *g* _s_  under salt stress may induce stomatal closure (Hu et al., 2013). However, excess accumulation of ions such as Na^+_ under salt stress may cause toxicity to cells. In addition, salt stress may damage plant physiological processes by over accumulation of reactive oxygen species, impairment of antioxidant defense systems and photosynthetic function, and imbalance of hormones (Hu et al., 2012, 2015; Kim et al., 2016).

Plant hormones function as central integrators that link and reprogram the complex developmental and stress adaptive signaling cascade (Goldack et al., 2014; Llanes et al., 2016; Ryu and Cho, 2015; Strivastava, 2002). ElevatedABA may help plants to acclimate to low water availability by closing stomata (Man et al., 2011; Zhang et al., 2015). Cytokinins (such as ZR and IPA) essentially regulate various plant developmental processes, including cell division and enlargement, chloroplast biogenesis, nutrient mobilization, leaf senescence, vascular differentiation, and apical dominance (Ryu and Cho, 2015). Cytokinins facilitate the responses to delay both stomatal closure and leaf senescence under abiotic stresses (Ryu and Cho, 2015; Zhang et al., 2015). Auxins such as IAA can promote root initiation and also delay plant senescence (Zhang et al., 2009). Among the 136 known GAs, only GA1, GA3, GA4, GA5, GA6, and GA7 have intrinsic biological activity (Davies, 2010). Bioactive GAs such as GA4 are involved in plant growth and development such as leaf expansion, stem elongation, and flowering (Ryu and Cho, 2015). GA4 is two orders of magnitude more active in delaying leaf senescence of *Alstroemeria hybrida* than GA1 (Davies, 2010). It may be possible that GA4 may delay plant senescence under salt stress. There is cross-talking between GA action and other hormones signaling during abiotic stress to control plant growth and development (Ryu and Cho, 2015). It has been reported that salt stress induced an increase in ABA of maize (*Zea mays*) plants (Jia et al., 2002) and a reduction in IAA of maize (Fahad and Bano, 2012) and CKs in barley (*Hordeum vulgare* L.; Kuiper et al., 1990). However, limited research has been reported on the relationship of hormonal responses to salt stress with photosynthetic function and visual quality in cool-season turfgrass species.

Kentucky bluegrass (*Poa pratensis* L.) is one of the most important cool-season turfgrass species in temperate climates and widely used for home lawns, golf courses, urban landscapes, and other sports fields. However, the KBG quality declines because of salt stress in many areas (Huang et al., 2014). The objective of this study was to investigate the responses of hormones (IAA, ABA, ZR, IPA, and GA4) to salt stress and examine if salt stress-induced damage was associated with alteration of hormonal metabolism in KBG.

Materials and Methods

Plant materials and culture. This study was conducted in the growth chamber facility at Virginia Tech, Blacksburg, VA. Mature KBG (‘Wildhorse’, relatively salt tolerant) plugs (10 cm diameter, 5 cm deep) were collected from the field plots at Virginia Tech Turfgrass Research Center, Blacksburg, VA, on Dec. 2015. The grass was transplanted into pots (15 cm diameter, 15 cm deep, with eight holes on the bottom) filled with United States Golf Association (USGA)-specification sand with 10% peat by volume. A piece of plastic screen was placed at the bottom of the pot to prevent
sand from leaching. The grass was grown in growth chambers under optimum conditions (mean ± SD) at 22 ± 0.8/16 ± 0.6 °C (day/night), 70% ± 8% relative humidity, photosynthetically active radiation (PAR) of 400 ± 9 μmol m⁻² s⁻¹, and a 12-h photoperiod. Nitrogen was applied at 2 g m⁻² (from 28–8–18 complete fertilizer with micronutrients) at transplanting and then 1 g m⁻² biweekly on all treatments until the end of the trial. The grass was trimmed to 7 cm and irrigated two times a week to field capacity.

Salt treatments and sampling. Six weeks after transplanting, the grass was subjected to either no-salt (0 mM NaCl) or salt stress (170 mM NaCl) treatments. Based on the previous reports (Xu and Fujiyama, 2013) and our preliminary trial, the salt concentration (170 mM NaCl) was suitable for ‘Wildhorse’ KBG treatment. The salt solution was added in gradually increasing concentrations in aliquots of 45 mM every 12 h until the concentration of 170 mM was attained, and concentrations were maintained by measuring the conductivity of the growth media. Then each pot was placed in a plastic tray (15 cm diameter, 15 cm deep) filled with either the salt treatment solution or distilled water only. The grass receiving the same volume of water served as control.

Leaf samples were collected at 0, 4, 7, 14, 21, and 28 d after the initiation of stress treatment, and the samples were frozen with liquid N2 to powder. The sample (50 mg) was extracted in 1.6 mL Na-phosphate buffer (0.05 M, pH 7.0) containing 0.02% sodium diethyldithiocarbamate as an antioxidant, and the hormones were extracted for 1 h at 4 °C with shaking. The C13-labeled IAA (50 ng) was added into each sample as an internal standard. The pH of the samples was adjusted to 2.6 with 1.0 M HCl. The samples were diluted with 490 mM deionized H2O to be slurried two times with 1.5 mL dichloromethane for 30 min each at 4 °C (Edlund et al., 1995). The combined dichloromethane fractions were reduced to dryness with nitrogen gas. Then the samples were dissolved in 210 μL methanol and diluted with 490 μL deionized H2O containing 0.1% formic acid. The samples were filtered using an Acrodisc 13-mm syringe filter with a 0.2-mm nylon membrane (Fisher Scientific Company, Pittsburgh, PA).

Hormone analysis by liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS). An Agilent tandem LC-MS/MS system with an ESI sample introduction interface (Agilent, Santa Clara, CA), consisting of 1290 UPLC and 6490 QQQ, was used for analyzing IAA and ABA in extracts. High-performance liquid chromatography separation was performed on Agilent Zorbax Extend-C18 analytical (4.6 × 50 mm, 5 μm) and guard (4.6 × 12 mm, 5 μm) columns. The analytes were eluted with water (mobile phase A) and methanol (B) in 0.1% formic acid in a gradient: 0–4.5 min B increasing from 30% to 80%, 4.5–5 min B increasing to 100%, 5–7 min B at 100%, and B decreasing to 30% at 7.5 min. The injection volume was 10 μL and flow rate was 0.5 mL min⁻¹. The column temperature was 40 °C. The chromatography retention time (RT), precursor ion, fragmental reactions monitored, ionization mode, and collision energies used for each compound are given in Table 1. The C13-labeled IAA (IAA_C13) was used as an internal standard. The source parameters were: nebulizer pressure 310 kPa, dry gas temperature 250 °C, sheath gas temperature 200 °C, and gas flow 8 mL min⁻¹. The selected transitions were determined based on the RT, ion products, and standards of each compound.

Fig. 1. Turf quality responses to salt stress in kentucky bluegrass. Bars represent se, and * indicates that the difference between the two treatments for the given sampling date is significant at P = 0.05.

Experimental design and statistical analysis. The treatments were arranged in a completely randomized block design with four replications. The data were subjected to

Table 1. Parameters for the analysis of leaf indole-3-acetic acid (IAA), C13-labeled IAA, abscisic acid (ABA), trans-zeatin riboside (ZR), isopentenyl adenosine (iPA), and gibberelin A4 (GA4) using liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS).

| Analyte   | Retention time (min) | Precursor ion   | Product ion   | Application | Collision energy | Mode |
|-----------|----------------------|-----------------|---------------|-------------|-----------------|------|
| IAA       | 3.9                  | 170.1           | 130.2         | Quantitative| 12              | +    |
| C13-IAA   | 3.9                  | 182.1           | 136.2         | Quantitative| 52              | +    |
| ABA       | 4.6                  | 265.2           | 229.2         | Quantitative| 52              | +    |
| ZR        | 2.3                  | 352.2           | 220.0         | Quantitative| 16              | +    |
| iPA       | 4.3                  | 336.0           | 204.0         | Quantitative| 32              | +    |
| GA4       | 6.0                  | 331.3           | 213.2         | Quantitative| 35              |      |
|           |                      |                 | 269.1         | Quantitative| 20              |      |
one-way analysis of variance using SAS software (v. 9.3 for Windows; SAS Institute, Cary, NC, 2010). Mean separations were performed using Fisher’s protected least significant difference test at the 0.05 P level. Pearson’s correlation coefficients of turf quality, EL, Pn, and $g_s$ with various hormones and ABA/CK were calculated using the SAS software.

**Results**

**Turf quality.** Salt stress reduced turf quality (Fig. 1). Turf quality started to decline at day 4 because of salt stress. At day 28, salt stress reduced turf quality rating by 64.9% relative to the control.

**Leaf EL.** Salt stress increased EL (Fig. 2). The EL differences between salt stress and no-salt treatments were observed from day 7 through day 28. At day 28, salt stress increased it by 273.2% relative to the control.

**Leaf Chl.** Salt stress reduced Chl content at days 14, 21, and 28 (Table 2). At day 28, salt stress reduced Chl by 74.5% relative to the control.

**Leaf Pn** and $g_s$. Salt stress reduced Pn (Fig. 3; Table 2). The Pn differences between salt stress and no-salt treatments were observed from day 4 through day 28. At day 28, salt stress reduced it by 90.7% relative to the control. Salt stress reduced $g_s$ (Table 2). The $g_s$ differences between salt stress and no-salt treatments were found from day 4 to day 28. At day 28, salt stress reduced it by 74.8% relative to the control.

**Leaf ZR and iPA content.** Salt stress reduced leaf ZR content (Fig. 4). The ZR differences between salt stress and no-salt treatments were observed from day 7 through day 28. At day 28, salt stress reduced it by 67.4% relative to the control.

Salt stress reduced leaf iPA content (Fig. 5). The iPA differences between salt stress and no-salt treatments were observed from day 14 through day 28. At day 28, salt stress reduced it by 36.0% relative to the control.

**Leaf IAA content.** Salt stress reduced leaf IAA content (Fig. 6). The IAA differences between salt stress and no-salt treatments were observed from day 14 to day 28. At day 28, salt stress reduced it by 58.6% relative to the control.

**Leaf ABA content and ABA/CK ratio.** Salt stress reduced leaf ABA content (Fig. 7). The ABA differences between salt stress and no-salt treatments were found from day 7 to day 28. At day 28, salt stress reduced it by 398.5% relative to the control. The ratio of ABA to CK (ZR + iPA) increased from 0.72 to 5.33 because of salt stress at day 28.

**Leaf GA4 content.** No difference in leaf GA4 between no-salt and salt stress treatments was observed regardless of the sampling date (Table 2).

**Relationship of hormone level with turf performance.** At the end of the experiment (day 28), turf quality, Pn, and $g_s$ were negatively correlated with ABA and ABA/CK ratio, but positively correlated with ZR, iPA, and IAA (Table 3). The EL was positively correlated with ABA and ABA/CK and negatively correlated with ZR, iPA, IAA, and GA4. GA4 was also positively correlated with turf quality and $g_s$.

**Discussion**

The results of this study showed that salt stress (170 mM NaCl) caused significant damage to KBG. The salt stress damaged cell membrane integrity (higher EL) and photosynthetic function (lower Pn), resulting in decline in leaf Chl and turf quality. This is in general agreement with previous studies by Puyang et al. (2016) and Xu and Fujiyama (2013) with KBG. Hu et al. (2013) reported that salt stress may cause damage to perennial ryegrass through stomatal limitation. Salt-induced osmotic stress may cause stomatal closure and inhibits gas exchange, resulting in oxidative damage of cell membrane and photosynthetic function.

Our results showed that the decline in Pn and turf quality due to salt stress was observed as early as day 4. At the same time, $g_s$ declined rapidly (as measured at day 4) in response to salt stress. Liu et al. (2011) reported that salt stress (300 and 500 mM NaCl) reduced $g_s$ and Pn of centipedegrass [Eremochloa ophiuroides (Munro) Hack] beginning at day 4 of salt treatment. This suggests that hormones and other molecules may induce stomatal closure, resulting in cell membrane damage, and decline in photosynthetic function and visual quality as signal molecules at the early stage of stress.

Plant hormones play an important role in regulating plant tolerance to salt stress (Davies, 2010; Huang et al., 2014; Ryu and Cho, 2015). The results of this study indicated that salt stress increased ABA content and ABA/CK ratio, reduced levels of IAA, ZR, and iPA; and did not affect GA4. The differences in ABA content between salt stress and no-salt treatments were detected as early as day 4.

The ABA level and ABA/CK ratio were negatively correlated with Pn, $g_s$, and turf quality, but positively correlated with EL as measured at the end of salt stress. Gao and Li (2014) reported that salt-tolerant tall fescue exhibited slower ABA accumulation rate than sensitive cultivars during short-term salt stress. This suggests that the slow and low accumulation rate of ABA in leaves could be beneficial for the maintenance of Pn under salt stress.

The results of this study indicated that CKs were positively correlated with turf quality, Pn, and $g_s$, but negatively correlated with EL at day 28. Cytokinins may have antagonistic effects with ABA in controlling

**Table 2. Leaf chlorophyll (Chl), stomatal conductance ($g_s$), and gibberellin A4 (GA4) responses to salt stress in Kentucky bluegrass.**

| Treatment | 0        | 4        | 7        | 14       | 21       | 28       |
|-----------|----------|----------|----------|----------|----------|----------|
| Chl (mg·g⁻¹ FW) |          |          |          |          |          |          |
| No-salt   | 2.76 a   | 2.74 a   | 2.07 a   | 2.02 a   | 2.31 a   | 2.12 a   |
| Salt      | 2.66 a   | 2.61 a   | 1.99 a   | 1.16 b   | 0.80 b   | 0.54 b   |
| $g_s$ (mol H₂O/mol²/s) |          |          |          |          |          |          |
| No-salt   | 0.119 a  | 0.119 a  | 0.167 a  | 0.190 a  | 0.210 a  | 0.202 a  |
| Salt      | 0.147 a  | 0.069 b  | 0.069 b  | 0.038 b  | 0.025 b  | 0.051 b  |
| GA4 (ng·g⁻¹ FW) |          |          |          |          |          |          |
| No-salt   | 10.63 a  | 12.90 a  | 12.50 a  | 14.25 a  | 11.50 a  | 7.95 a   |
| Salt      | 38.40 a  | 10.70 a  | 9.80 a   | 11.73 a  | 9.78 a   | 7.55 a   |

Means followed by same letters within the same column for each data set are not significantly different at $P = 0.05$. FW = fresh weight.

**Fig. 3.** Leaf photosynthetic rate (Pn) responses to salt stress in Kentucky bluegrass. Bars represent SE, and * indicates that the difference between the two treatments for the given sampling date is significant at $P = 0.05$.

**Fig. 4.** Leaf zeatin riboside (ZR) responses to salt stress in Kentucky bluegrass. Bars represent SE, and * indicates that the difference between the two treatments for the given sampling date is significant at $P = 0.05$. 

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