Improvement of Salt Tolerance in Kentucky Bluegrass by Trinexapac-ethyl

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Abstract. Trinexapac-ethyl (TE) is a popular plant growth regulator in the turfgrass industry that inhibits gibberellic acid (GA) biosynthesis and effectively reduces leaf elongation and subsequent clipping production. This greenhouse sand culture experiment was conducted to determine effects of TE application on Kentucky bluegrass (Poa pratensis L.) responses to salinity stress. The five salinity levels (0, 20, 40, 60, and 80 mM NaCl) were applied in nutrient solutions and TE treatments (0, 1, and 1.7 g/100 m²) were applied twice at 4-week intervals. Under non-saline conditions and low level salinity conditions, application of TE at 1 g/100 m² (TE1) increased turf quality (TQ), leaf total non-structural carbohydrates (TNC), and chlorophyll (Chl) content. In high salinity, TE1 alleviated the decline in TQ, antioxidant enzyme activities, leaf TNC, Chl, and K+ content. In addition, treated turf with TE at 1 g/100 m² had lower proline, Na+, and malondialdehyde (MDA) contents. However, the adverse effects of high salinities were alleviated by TE, indicating that TE application can enhance salt tolerance of Kentucky bluegrass across a range of salinity levels.

Materials and Methods

Turfgrass culture and growth condition. ‘Bar-impala’ Kentucky bluegrass (Poa pratensis L.) was seeded (20 g m⁻²) in 15-cm-diameter × 30-cm-deep plastic pots filled with washed sand in September. Plants were grown in a greenhouse with average day/night temperatures under natural light (average: 800 μmol·m⁻²·s⁻¹ photosynthetically active radiation, 14-h photoperiod) at Okayama University. Pots were fertigated daily with half-strength Coic and Lesaint nutrient solution (pH 7.0) (Coic and Lesaint, 1975) until drainage occurred from the bottom of the containers for 4 months before initiation of treatments. Turf was hand-clipped weekly at a 5-cm height.

Treatments, experimental design, and data analysis. Five salinity treatments (0, 20, 40, 60, and 80 mM NaCl) were obtained by adding NaCl gradually (to avoid salinity shock) to nutrient solutions during a 5-d period. Trinexapac-ethyl [Primax Maxx; Syngenta Crop Protection, Inc., Greensboro, NC; active ingredient (a.i.): trinexapac-ethyl = 11.3%] was applied with a hand sprayer at 1 and 1.7 g/100 m² a.i., in addition to a non-TE-treated control, on two occasions, at the start of the treatments and 4 weeks after salt treatments were initiated. Grasses were exposed to salinity and TE treatments for a period of 8 weeks. During this period, all measurements except shoot and root growth were made every 2 weeks. First measurements were taken 1 d before initiation of treatments. The experiment was set out in a split-plot design with four replications for each treatment. Salinity levels and TE treatments were the main plots and subplots, respectively. This study was conducted at the Okayama University and was repeated at the University of Tehran with the same materials and methods. Representative data have been presented, except for antioxidant enzymes that only assayed at Okayama University. The data were statistically analyzed using the analysis of variance procedure (SAS Institute, 2001). Differences between treatment means were separated by Fisher’s protected least significance test at the 0.05 P level.

Measurements. During the treatment period, turf was clipped once every time week at a 5-cm height. Clipping yields were harvested weekly and dried at 70 °C for 48 h for dry weight determination. After the final clipping harvest after 8 weeks of salinity...
treatments, grass swards were harvested and divided into verdure and roots. Each fraction was dried at 70 °C for 48 h using a drying oven (DV41; Yamato Scientific Co., Tokyo, Japan) and then dry mass was measured. Shoot growth was calculated based on cumulative clipping and verdure dry weight (Qian et al., 2000).

Turf quality was visually rated on a scale of 1 to 9 based on color, density, and uniformity (Turgeon, 2002). Plants rated 1 were completely desiccated with a completely necrotic turf canopy. A rating of 9 represented healthy plants with dark green, turgid leaf blades and a full turf canopy. A rating of 6 was considered the minimal acceptable TQ.

Total non-structural carbohydrates were measured according to the method of Lyons et al. (2007) that was a modified method of Ting (1956). Briefly, leaves were dried at 60 °C for 24 h. After a 24-h incubation period, 0.5 mL of 0.6 N HCl was incubated at 37 °C for 24 h. After a 24-h incubation period, 0.5 mL of 0.6 N HCl was added to the solution for an additional 18 h. The solution was then neutralized with 10 N NaOH and diluted to 50 mL with distilled water and filtered. Reducing sugars were measured by taking 1.0 mL of the solution and adding 1.5 mL of alkaline ferricyanide solution. The mixture was heated for 10 min in a 100 °C water bath and quickly cooled under running water. The pH of the solution was partially neutralized with 3.0 mL of 2 N H2SO4. Finally, 1.2 mL of arsenomolybdate solution was added and the total volume was adjusted to 25 mL with distilled water. Absorbance of the solution was measured at 515 nm and compared with a standard curve to determine TNC content.

Chlorophyll was extracted by soaking 0.1 g of fresh leaf sample in 20 mL of dimethyl sulfoxide in the dark for 72 h (Hiscox and Israelstam, 1979). Absorbance of the extract at 663 and 645 nm was measured with a spectrophotometer and total Chl concentration was calculated using the formulas described by Arnon (1949).

Proline content was measured according to the method of Bates et al. (1973). A 0.1-g sample of fresh leaves was homogenized in 1.5 mL of 3% aqueous sulfo salicylic acid and the residue was removed by centrifugation at 15,000 g for 20 min. Then, 1 mL of extract was mixed with 2 mL of 20% trichloroacetic acid containing 5% thiobarbituric acid. The mixture was heated at 100 °C for 30 min, quickly cooled, and then centrifuged at 10,000 g for 10 min. The absorbance of the supernatant was monitored at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the concentration of MDA was calculated using an extinction coefficient of 155 mmol–1·cm–1 (Heath and Packer, 1968).

To determine K+ and Na+ contents, leaves were rinsed thoroughly and dried at 70 °C for 2 d. Ground samples were dry-ashed at 550 °C for 4 h, mixed with hot 2 M HCl, filtered, and then brought to a final volume of 50 mL with distilled water. K+ and Na+ contents were determined in these digests using an Eppendorf flame photometer (Chapman and Pratt, 1982).

For enzyme extracts and assays, 0.1 g of leaves were frozen in liquid nitrogen and then ground in 3 mL of buffer (for superoxide dismutase, peroxidase, and ascorbate peroxidase; 71.4 mM KH2PO4 buffer pH 7.0; for catalase: 62.5 mM Tris-base buffer pH 8.0; for glutathione reductase: 56.8 mM Tris-base buffer pH 7.5). The homogenate was centrifuged at 15,000 g, for 20 min at 4 °C, and the supernatant was collected for enzyme assays. Superoxide dismutase (SOD) [electrical conductivity (EC): 1.15.1.1] activity was measured by using an SOD Assay Kit-WST (Dojing Molecular Technologies, Inc., Kumamoto, Japan) as described by Hoque et al. (2007a). Peroxidase (POX) (EC: 1.11.1.7) activity was assayed by following the increase in absorbance at 470 nm for 30 s (Nakano and Asada, 1981). The reaction buffer solution contained 50 mM KH2PO4 buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H2O2, and 10 mM guaiacol. Ascorbate peroxidase (APX) (EC: 1.11.1.11) activity was measured as a decrease in absorbance at 290 nm for 1 min (Nakano and Asada, 1981). The assay mixture consisted of 50 mM KH2PO4 buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H2O2, and 0.25 mM ascorbate. Catalase (CAT) (EC: 1.11.1.6) activity was calculated from the decrease in absorbance at 240 nm for 1 min (Aebi, 1984). The reaction mixture contained 50 mM Tris-base buffer (pH 8.0), 0.125 mM EDTA, and 10 mM H2O2. Glutathione reductase (GR) (EC: 1.6.4.2) activity was calculated from the decrease in absorbance at 340 nm for 2 min (Foster and Hess, 1980). The reaction buffer solution contained 50 mM Tris-Cl buffer (pH 7.5), 3 mM MgCl2, 0.12 mM NADPH, and 0.5 mM oxidized glutathione. One unit POX, APX, and CAT activities were defined as the μmol (nmol for GR) substrate metabolized per minute.

Results

Shoot and root growth. In all salinity treatments, shoot growth was highest in non-TE-treated plants followed by TE1 and TE1.7 treatments, except for 80 mM NaCl-untreated plants exhibited lower shoot biomass than TE1 treatment. TE-treated plants at 1 g/100 m2 showed higher root growth than other treatments in all salinities. Treated turf at 1.7 g/100 m2 had lower root biomass than both untreated and TE1 treatments except at 20 and 40 mM NaCl in which there was no significant difference between untreated and TE1.7 treatments (Fig. 1).

Turf quality. In non-stressed plants, application of TE at 1.7 g/100 m2 increased TQ within the first 2 weeks of treatments and then decreased to below the control level, whereas TE-treated plants at 1 g/100 m2 had higher quality ratings than other treatments after 4

Fig. 1. Effects of trinexapac-ethyl (TE) and salinity on shoot and root growth of ‘Barimpala’ kentucky bluegrass. TE 1, application of TE at 1 g/100 m2; TE 1.7, application of TE at 1.7 g/100 m2. Vertical bars indicate ses.
weeks of TE application. In 20 mM NaCl, TQ in TE1 treatment increased during the first 2 weeks and then remained constant. Untreated TQ increased up to the level of TE1 treatment at 4 weeks and after 6 weeks dropped to the initial level. In TE1.7 treatment, TQ decreased after 4 weeks and plants had the lowest quality at the end of experiment. In 40 mM NaCl, TQ in untreated plants and treated turf at 1 g/100 m² increased during the first 2 weeks and then decreased, whereas TE1.7 treatment showed continuous decline in TQ. After 8 weeks, TE1 treatment, unlike other treatments, maintained an TQ equivalent to the initial level. In higher salinities, TQ declined with the progression of the salt stress and increasing salinity, whereas plants treated with TE at 1 g/100 m² maintained a higher quality and TE-treated plants at 1.7 g/100 m² showed greater reduction in TQ compared with untreated plants (Fig. 2).

Chlorophyll content. Under non-saline conditions, Chl content was not significantly different among TE treatments during the first 4 weeks. After 4 weeks, Chl content in untreated plants remained fairly constant, whereas TE-treated turfs showed a sharp increase, and highest Chl content was observed in TE1.7 treatment. Compared with non-stressed plants, in 20 and 40 mM NaCl, a more rapid and greater increase in Chl content was observed in TE-treated turfs, whereas little increase was detected in untreated plants with progression of stress. In 60 mM NaCl, Chl content increased in all TE treatments during the first 2 weeks. After 2 weeks, a severe reduction in Chl content was observed in untreated plants, whereas TE1.7 treatment remained constant. Chl content in TE1 treatment increased until 4 weeks and then decreased to below the TE1.7 treatment level. In 80 mM NaCl, a continuous decline in Chl content was observed in all TE treatments. However TE1 treatment maintained a significantly higher Chl content after 4 weeks (Fig. 2).

![Fig. 2. Effects of trinexapac-ethyl (TE) and salinity on turf quality (TQ), chlorophyll content, and total non-structural carbohydrates (TNC) of 'Barimpala' kentucky bluegrass. TE 1, application of TE at 1 g/100 m²; TE 1.7, application of TE at 1.7 g/100 m². TQ was rated 1 to 9 where 1 = poorest quality, 6 = lowest acceptable quality, and 9 = best quality. Vertical bars indicate least significant difference values (P = 0.05) for treatment comparisons at a given week of treatment.](image-url)
**Total non-structural carbohydrate content.** Leaf TNC content of non-TE-treated plants decreased with the progression of salt stress in all treatments, whereas TE-treated plants exhibited a steady increase in TNC content under non-saline conditions and 20-mM NaCl treatments. In 40 mM NaCl, a gradual decline in TNC content was observed in untreated turf and TE1.7 treatment, whereas TE1 treatment maintained significantly higher TNC content. A steady decline in TNC content in 60 mM NaCl was observed for all TE treatments. However, TE-treated plants at 1 g/100 m² slowed the decline in TNC content and the greatest decline was observed in TE1.7 treatment. In 80 mM NaCl, all plants exhibited a sharp decline in TNC content; however, untreated plants showed more TNC content at the end of experiment (Fig. 2).

**K⁺ and Na⁺ contents.** In non-stressed plants, the difference among TE treatments in K⁺ and Na⁺ contents was not significant. With increasing salinity and progression of salt stress, leaf Na⁺ content increased and K⁺ content decreased in all plants. In 20 mM NaCl, leaf Na⁺ content was not significantly different between TE-treated and untreated plants. In high salinities, highest Na⁺ content was observed in TE-treated turf at 1.7 g/100 m² and lowest Na⁺ content was detected in TE1 treatment. In contrast, TE-treated turf at 1 g/100 m² showed the highest K⁺ content and TE1.7 treatment exhibited the lowest leaf K⁺ content (Fig. 3).

**Proline content.** Under non-saline conditions, proline content increased in TE1.7 treatment after 2 weeks, whereas treated plants at 1 g/100 m² exhibited a gradual increase in proline content after 4 weeks. Leaf proline content increased with increasing salinity and progression of stress; however, in 20 and 40 mM NaCl, no remarkable difference existed in levels of proline among TE treatments. In 60 mM NaCl, the highest proline content at the end weeks of treatments was observed in TE1.7 treatment followed by untreated and TE1 treatment. Similarly, in 80 mM NaCl, the lowest leaf proline content

![Fig. 3. Effects of trinexapac-ethyl (TE) and salinity on Na⁺, K⁺, and proline content of 'Barimpala' kentucky bluegrass. TE 1, application of TE at 1 g/100 m²; TE 1.7, application of TE at 1.7 g/100 m². Vertical bars indicate least significant difference values (P = 0.05) for treatment comparisons at a given week of treatment.](image-url)
was detected in TE1 treatment; however, there was no significant difference between TE1.7 treatment and untreated plants (Fig. 3).

Malondialdehyde content. In non-stressed plants, applying TE increased leaf MDA content after 4 weeks. With increasing salinity and progression of stress, MDA content increased in all plants. However, in high salinity levels and end weeks of experiment, TE-treated plants at 1 g/100 m² had lower MDA content than other treatments (Fig. 4).

Antioxidant enzyme activities. Under non-saline conditions, the activities of all enzymes exhibited little change among TE treatments except for TE1.7 treatment that showed significantly higher SOD and POX activity than untreated plants at 8 weeks. In 20 mM NaCl, SOD activity in all TE treatments increased after 2 weeks and TE1 treatment showed lower enzyme activity than other treatments. Compared with 20 mM NaCl, a more rapid and greater increase in SOD activity was observed in 40 mM NaCl; however, enzyme activity decreased after 6 weeks in untreated turfs and treated plants at 1.7 g/100 m². In higher salinities, a pronounced rapid and sharp increase occurred in enzyme activities and then decreased with progression of the experiment and increasing salinity. Turf treated with 1 g/100 m² trinexapac-ethyl significantly maintained higher SOD activity at the end of treatments followed by untreated plants and TE1.7 treatment. A similar pattern was observed for POX; however, reduction in enzyme activity resulting from salinity was less than SOD. In 20 mM NaCl, APX, CAT, and GR activities slightly increased in all TE treatments during the entire period of the experiment. With progression of stress and increasing salinity, enzyme activities remarkably decreased in untreated plants and TE1.7 treatment. Whereas in 40 mM NaCl, treated plants at 1 g/100 m² still maintained an increase in enzyme activities and in higher salinities showed less reduction in all

Fig. 4. Effects of trinexapac-ethyl (TE) and salinity on malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, and peroxidase (POX) activity of 'Barimpala' kentucky bluegrass. TE1, application of TE at 1 g/100 m²; TE1.7, application of TE at 1.7 g/100 m². Vertical bars indicate least significant difference values (P = 0.05) for treatment comparisons at a given week of treatment.
enzyme activities than other TE treatments (Figs. 4 and 5).

**Discussion**

Salinity stress caused significant declines in grass quality and overall turf performance. Application of TE at 1 g/100 m² alleviated salt injury and had a positive impact on kentucky bluegrass survival of salt stress. However, the adverse effects of high salinity were more pronounced when turf was treated by TE at 1.7 g/100 m², suggesting that effects of TE on salt tolerance varied with its application rate and salinity levels.

A relatively high TE application rate may have resulted in phytotoxicity, leading to a decrease in overall visual quality (Gardner and Wherley, 2005). Furthermore, severe inhibition of shoot growth and consequently lower root mass may have increased plant sensitivity to salt stress. The mechanisms responsible for enhanced stress tolerance by TE are not well understood. Some studies have demonstrated that inhibition of GA₃ resulting from TE action increases leaf cell density resulting in more Chl content per unit area and darker green leaves (Ervin and Koski, 2001; Heckman et al., 2005). Our results also showed that TE application increased leaf Chl content and alleviated the decline in Chl during the stress period; however, in high salinity levels, Chl content decrease may be the result of an increase of Chl degradation or a decrease of Chl synthesis (Santos, 2004). In low levels of salinity and initial periods of stress, leaf Chl content increases may be the result of inhibition of growth or decrease in leaf water content, because Chl was measured on a fresh weight basis.

Chlorophyll content is an important factor in determining photosynthetic capacity. It has been shown that TE application increases canopy photosynthetic rate and photochemical

![Fig. 5. Effects of trinexapac-ethyl (TE) and salinity on ascorbate peroxidase (APX) activity, catalase (CAT) activity, and glutathione reductase (GR) activity of 'Barimpala' kentucky bluegrass. TE 1, application of TE at 1 g/100 m²; TE 1.7, application of TE at 1.7 g/100 m². Vertical bars indicate least significant difference values ($P = 0.05$) for treatment comparisons at a given week of treatment.](image-url)
efficiency (Ervin and Zhang, 2007; McCann and Huang, 2007), whereas maintenance respiration may be decreased (Heckman et al., 2001). Photosynthetic not used for leaf elongation must be stored or transported to other organs such as stems, roots, and leaves and would explain increased tillering (Ervin et al., 2002) and rooting (McCullough et al., 2005) that has been reported in previous research. 

Increased root/shoot ratio during a stress period is very important and promotes salinity tolerance by increasing the absorbing surface to take up more water (O’Toole and Bland, 1987). Baldwin et al. (2006) reported that application of TE increased root growth in two cultivars of bermudagrass. Parallel to these results we observed that a decrease in root mass resulting from salinity progressed more slowly in TE-treated plants.

In the present study, application of TE increased leaf TNC under non-saline conditions and slight or moderate salinity stress. Increased TNC content by TE has also been observed in different turfgrass species (Ervin and Zhang, 2007; Wang et al., 2006), although some research showing that TE application had no effects on TNC accumulation or reduced TNC content (Han et al., 1998; Richie et al., 2001). Total nonstructural carbohydrates in grasses provide a reservoir of energy in plants beyond the immediate requirements for growth and maintenance and have been described as a physiological measure of stress tolerance (Beard, 1973). Soluble sugars, as a component of TNC, are one of the major solutes contributing to osmotic adjustment in the leaves of many plants species (Morgan, 1984). In addition, TNC is a source of energy for salt tolerance processes such as active transport of Na+ and K+. Therefore, higher TNC accumulation may result in lower leaf Na+ content and increasing K+ levels.

Proline is one of the most common compatible solutes or osmoprotectant in that its accumulation has been correlated with salinity and tissue Na+ concentration for several turfgrass species (Lu et al., 2007; Razzmooj et al., 1998). Similarly, our data showed that in high salinity levels, treated turf with TE at 1 g/100 m2 had lower proline content than could be the result of less Na+ accumulation.

Salt stress, like other abiotic stresses, induces oxidative stress, resulting from the increase in reactive oxygen species (ROS) production such as superoxide (O2−), hydroperoxide (H2O2) and hydroxyl radicals (OH) (Mittler, 2002). To scavenge ROS, plants have developed enzymatic and non-enzymatic systems. Several enzymes are involved in the detoxification of ROS. For example, SOD catalyzes the dismutation of superoxide to H2O2 and molecular oxygen. H2O2 is scavenged by CAT, APX, and POD. Glutathione reductase also can remove H2O2 through the ascorbate-glutathione cycle to maintain a high level of reduced ascorbate (Asada, 1999; Mittler, 2002).

A close correlation between salinity tolerance and antioxidant capacity has been reported in several works (Lu et al., 2007; Sairam and Srivastava, 2002; Seckin et al., 2010). Our data also showed that in non-TE-treated plants, the activity of SOD and POX increased in low levels of salinity and initial periods of stress and decreased as stress prolonged. A dramatic increase in MDA content, a product of lipid peroxidation, which is an indicator of free radical damage to cell membranes under stress conditions (Smirnoff, 1995), was parallel to a decrease in antioxidant enzyme activities. Compared with SOD and POX, the induced levels of enzyme activities were lower and the rate of decrease in enzyme activities resulting from salt stress were higher in APX, CAT, and GR, suggesting that SOD and POX can be more important to defend against salinity-induced oxidative damages than CAT, APX, and GR.

An increase in activities of antioxidant enzymes is a common adaptive response of plants to salt stress and has been reported for different enzymes (Lu et al., 2007; Seckin et al., 2010; Vaidyanathan et al., 2003). On the other hand, several investigations have shown that exposure of plants to salt stress leads to a decrease in activities of antioxidant enzymes (Hoque et al., 2007a, 2007b; Lee et al., 2001). The reduction in enzyme activities under salinity stress may have been the result of either reduced synthesis or enhanced degradation of the enzymes and it has been shown that salt stress can reduce protein synthesis in plants (Fidalgo et al., 2004).

In this study, compared with non-TE-treated plants, application of TE at 1 g/100 m2 decreased SOD and POX activity in plants, which had received 20 mm NaCl. This may be the result of less ROS production. In contrast, in high salinity levels, TE application alleviated the decline in activities of all antioxidant enzymes and increased plant protection from oxidative damage, which was proven by less MDA content. Enhanced antioxidant enzyme activities by TE could have resulted from a positive effect of TE application on TNC content that could be beneficial to protein synthesis. Ervin and Zhang (2007) reported that TE treatment increased leaf cytokinin content in creeping bentgrass, Kentucky bluegrass, and hybrid bermudagrass. Cytokinins are known as antisenescence agents and prevent oxidation of unsaturated fatty acids in membranes (Salisbury and Ross, 1992). Also, it has been reported that cytokinins enhanced stress resistance and it may be the result of protection from oxidative stress by preventing the formation of free radicals or by the regulation of antioxidant enzyme activities (Liu and Huang, 2002; Wang et al., 2006; Zhang and Schmidt, 2000a). So, in plants treated with 1 g/100 m2 TE, less MDA content might be correlated with a decrease in production of ROS and an increase in cell membrane stability. Increased endogenous SOD levels in TE-treated plants have also been reported by Zhang and Schmidt (2000b).

In summary, the results reported here suggest that TE treatment was beneficial for Kentucky bluegrass survival of salt stress, as manifested by improved TQ under stress conditions. Increased salt tolerance resulting from TE application could be related to effects of TE on the increased TNC content and consequently decreased in Na+ uptake as well as enhanced antioxidant enzyme activities. Our data indicate that a high rate of TE application might reduce salt tolerance. Therefore, additional studies are required to find proper TE application rates for various turfgrass species and cultivars in different salinity levels.

**Literature Cited**


