

Cold Acclimation Treatment–induced Changes in Absciscic Acid, Cytokinin, and Antioxidant Metabolism in Zoysiagrass (*Zoysia japonica*)

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Abstract. Zoysiagrass (*Zoysia* spp.), a warm-season turfgrass species, experiences freezing damage in many regions. The mechanisms of its cold acclimation and freezing tolerance have not been well understood. This study was designed to investigate effects of cold acclimation treatment on leaf abscisic acid (ABA), cytokinin (transzeatin riboside (*t*-ZR)), and antioxidant metabolism associated with freezing tolerance in zoysiagrass (*Zoysia japonica*). ‘Chinese Common’ zoysiagrass was subjected to either cold acclimation treatment with temperature at 8/2 °C (day/night) and a photosynthetically active radiation (*PAR*) of 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over a 10-hour photoperiod or normal environments (temperature at 28/24 °C (day/night), *PAR* at 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 14-hour photoperiod) for 21 days in growth chambers. Cold treatment caused cell membrane injury as indicated by increased leaf cell membrane electrolyte leakage (EL) and malondialdehyde (MDA) content after 7 days of cold treatment. Cold treatment increased leaf ABA and hydrogen peroxide content and reduced *t*-ZR content. Leaf superoxide dismutase (SOD), ascorbate peroxidase (APX) activity, and proline content increased, whereas catalase (CAT) and peroxidase (POD) activity declined in response to cold treatment. Cold treatment increased freezing tolerance as LT₅₀ declined from –4.8 to –12.5 °C. The results of this study indicated that cold acclimation treatment might result in increases in ABA and H₂O₂, which induce antioxidant metabolism responses and improved freezing tolerance in zoysiagrass.

Zoysiagrass (*Zoysia* spp.) is a common warm-season turfgrass well adapted in use of lawns and golf courses in many regions because of its excellent heat tolerance, density, low pesticide requirements, and minimal maintenance inputs (Patton et al., 2007a, 2007b; Zhang et al., 2009). Expanded use of zoysiagrass could play an important role in making golf courses and home lawns more environmentally friendly and sustainable (Patton and Reicher, 2007). However, the use of zoysiagrasses has been limited by its relatively weak winter hardiness compared with some other C₄ turfgrass species, such as buffalograss, and freezing injury in many

regions. Freezing temperature may cause damage by forming ice crystals, which result in rupture of cell membranes and cell dehydration (Fry and Huang, 2004; Zhang et al., 2008). Injury during exposure to chilling temperature also results from reduced or defective metabolic defense functions (Karpinski et al., 2002; Thomashow, 1999).

Zoysiagrasses undergo cold acclimation in late fall as temperature drop and photoperiod gets shorter. It has been well documented that increased cold acclimation could improve freeze tolerance of plants, including turfgrasses (Anderson et al., 2003). Plants possess various adaptive mechanisms for surviving freezing temperature such as increases in certain sugars or amino acids, increases in ABA content, and antioxidant capacity (Patton et al., 2007a, 2007b; Rogers et al., 1977; Zhang and Ervin, 2008; Zhang et al., 2009).

Various metabolites (such as proline) may accumulate during cold acclimation (Zhang

and Ervin, 2008; Zhang et al., 2006, 2008). Proline, an amino acid, functions as osmoprotectant and antioxidant to protect cell membrane during dehydration. Patton et al. (2007a, 2007b) reported that proline content increased in response to cold acclimation in zoysiagrasses. Bermudagrass (*Cynodon* L.C. Rich) cultivars with higher stolon proline content exhibited greater freezing tolerance than those with lower proline during the winter (Munshaw et al., 2006).

Decreases in temperature and photoperiod in fall may create an imbalance, so that the energy absorbed through the light harvesting complex exceeds what can be dissipated or transduced by photosystem II (PSII; Karpinski et al., 2002; Zhang and Ervin, 2008). Excess energy may be directed to O₂ and result in accumulation of toxic reactive oxygen species (ROS). To protect from oxidative stress, plants have developed efficient antioxidant defense systems to scavenge ROS such as superoxide radicals (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (HO[•]) (McKersie and Bowley, 1997). The SOD (EC 1.15.11), a group of metalloenzymes, can convert O₂^{•-} to H₂O₂, and considered as the “primary defense” against ROS (Perl-Treves and Perl, 2002; Zhang et al., 2008). The H₂O₂ is further reduced to water by the antioxidant enzymes—CAT (EC 1.11.1.6) and APX (EC 1.11.1.11). The CAT, localized in peroxisomes, scavenges H₂O₂ produced by glycolate oxidase in the C₂ photorespiratory cycle (Perl-Treves and Perl, 2002). POD (EC 1.11.17) is also an important antioxidant enzyme for scavenging ROS. Antioxidant enzymes and metabolites have been shown to be associated with freezing tolerance in plants, including bermudagrass (Karpinski et al., 2002; Zhang and Ervin, 2008). Overexpression of a chloroplast Cu/Zn SOD gene increased resistance to chilling stress in tobacco (Gupta et al., 1993).

ABA and H₂O₂ have been considered as signaling molecules for inducing plant antioxidant defense systems against abiotic stresses (Xiong et al., 2002). Cytokinins exhibit antisenesescence and antioxidant function. Heino et al. (1990) reported that ABA deficiency prevented development of freezing tolerance in *Arabidopsis thaliana*. Stolon ABA accumulation during cold acclimation is associated with freezing tolerance in bermudagrass (Zhang et al., 2008) and zoysiagrass (Zhang et al., 2009), and exogenous ABA increased freezing tolerance of bermudagrass (Zhang et al., 2008). Hu et al. (2006) found that ABA is a key inducer of H₂O₂ production in maize exposed to drought stress. Hsu and Kao (2010) indicated that ABA-induced leaf senescence of rice seedlings is due to H₂O₂ accumulation. Prasad et al. (1994) reported that accumulation of ABA and H₂O₂ protect mitochondria against CI in maize seedlings.

Cytokinins are adenine derivatives characterized by an ability to induce cell division in tissue culture (in the presence of auxin). They also promote shoot initiation, lateral

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bud growth, leaf expansion, nutrient mobilization, chloroplast differentiation, and activation of shoot meristems and delay senescence (Davies, 2010). Zeatin and zeatin riboside are some of the most important forms of cytokinins. Cytokinins are synthesized in root tips and developing seeds and transported from roots to shoots via the xylem (Davies, 2010). Taylor et al. (1990) measured cytokinin and ABA levels in field- and growth chamber-grown winter wheat plants. They found that ABA level increased, whereas cytokinin level declined during cold acclimation.

Research on changes of ABA, cytokinin, and H_2O_2 associated with antioxidant metabolism in zoysiagrass is lacking. Very few studies have been reported on antioxidant metabolism associated with cold acclimation and freezing tolerance in zoysiagrass. Investigations concerning the physiological responses of zoysiagrass to cold acclimation treatment would provide valuable selection information for turfgrass breeders and practitioners. The objectives of this study were to examine effect of cold acclimation treatment on the levels of ABA, cytokinin, and antioxidant enzyme activity and to investigate if cold treatment-induced changes of the hormones and antioxidants are associated with freezing tolerance in zoysiagrass.

Materials and Methods

Zoysiagrass culture and cold acclimation treatment. One zoysiagrass cultivar, Chinese common (relatively cold tolerant and widely used in China and some other regions), was used for this study (Patton and Reicher, 2007). Mature zoysiagrass plugs (25 cm diameter by 5 cm deep) were taken from field plots at the Turfgrass Research Institute, Beijing Forestry University, Beijing, China. The grass was transplanted on 15 June 2012 and grown in the pots (25.4 cm diameter \times 24.1 cm deep) filled with a mixture of top soil and sand (4:1, w/w) in a temperature-controlled greenhouse with temperature at 28/24 °C (day/night) and a *PAR* at 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with \approx 14-h photoperiod. The greenhouse was covered with a dark plastic sheet and artificial lamps (YZ28-T5; NVC Lighting Technology Corporation, Huizhou, China) were used to achieve uniform light environment. Nitrogen was applied at 45 $\text{kg}\cdot\text{ha}^{-1}$ with a 20 N–8.8 P–16.6 K soluble fertilizer 1 d after the plugs were transplanted and 23 $\text{kg}\cdot\text{ha}^{-1}$ every 4 weeks thereafter. The pots were irrigated by hand once every 3 d and trimmed at 4 cm height.

Two months after transplanting, each of five pots was placed in a growth chamber and subjected to cold acclimation at 8/2 °C (day/night) with a *PAR* of 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over a 10-h photoperiod for a period of 21 d (Zhang et al., 2006, 2008), and each of the other five pots was placed in another growth chamber with temperature at 28/24 °C (day/night) and *PAR* at 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over a 14-h photoperiod for a period of 21 d (Zhang et al., 2008). The two treatments (cold acclimation and non-acclimated) were replicated for five

times and a total of 10 growth chambers were used for this study. The irrigation was provided only in need to make sure no moisture stress occurred. No fertilization was provided during 21 d of cold acclimation treatment. The experiment was repeated from July through Oct. 2013.

Sampling collection. Leaf samples were collected from each pot at 0, 7, 14, and 21 d after cold acclimation treatment initiation. Leaf EL was measured immediately after sampling. Leaf samples were frozen with liquid nitrogen and stored at -80°C for analysis of ABA, ZR, proline, MDA, H_2O_2 , and antioxidant enzyme activity. In addition, stolons were sampled at 0 and 21 d and used for determination of LT_{50} . Stolon tissue was used for LT_{50} measurement because it is closely related to the plant survival of freezing temperature (Zhang et al., 2006, 2008).

Leaf EL and LT_{50} determination. Leaf tissues (50 mg) were collected and rinsed with deionized water, and then cut into small sections (5 mm). The samples were placed in 50-mL tubes with 20 mL distilled water. The tubes were shaken for 24 h at 25 °C and an initial electrical conductance (EC1) was determined using a conductivity meter (Model DDSJ_308A; Shanghai Precision and Scientific Inc., Shanghai, China). The samples were autoclaved at 120 °C for 30 min and cooled down to 25 °C and final electrical conductance (EC2) was measured. The EL (%) is expressed as $(EC1/EC2) \times 100$ (Blum and Ebercon, 1981).

The concept of lethal temperature for 50% loss of electrolytes (LT_{50}) has been used as a measure of cold hardness and is defined as the predicted test temperature resulting in 50% or greater loss of total electrolytes (Zhang et al., 2008). At the beginning (0 d) and the end (21 d) of cold acclimation treatment, stolon LT_{50} was determined according to Zhang et al. (2008). Briefly, the soil was washed from the plants and the roots were removed. Turfgrass from each pot was then divided into five subsamples. Each subsample was wrapped in a wet paper towel and subjected to a series of freezing temperature. A freezing chamber was programmed to cool to -3 , -6 , -9 , -13 , and -17°C with a 2°C decline every 2 h at which time a subsample was removed for analysis. After thawing overnight at 4°C , stolons were collected and the EL was determined according to the procedure as described previously. Zoysiagrass freezing tolerance was evaluated based on predicted killing temperature from EL measurements in this study. The response curve between test temperature and EL was fitted to a sigmoidal response using SigmaPlot v.10 (Systat Software Inc., San Jose, CA). Response curves were developed for each treatment and each LT_{50} was determined from the inflection point of the curve (Tmid) based on the sigmoidal response equation.

Leaf MDA content. Lipid peroxidation of the leaf tissue was measured in terms of MDA content (Dhindsa and Matowe, 1981)

with some modifications. Frozen leaf tissues (50 mg) was ground to a powder with a mortar and a pestle in liquid nitrogen and extracted with 2 mL 10% trichloroacetic acid (TCA) on an ice bath. The mixture was centrifuged at 10,000 g_n for 10 min at 4°C . Supernatant (1.5 mL) was collected and mixed with 2 mL 0.67% thiobarbituric acid (TBA). The mixture was heated at 95°C for 15 min and cooled down in an ice bath. The mixture was centrifuged again at 10,000 g_n for 10 min at 4°C . The absorbance of supernatant was determined at 450, 532, and 600 nm. The formula for the calculation of MDA content was $\text{MDA content } (\mu\text{mol}\cdot\text{g}^{-1} \text{ fresh weight}) = [6.45(A_{532}-A_{600})-0.56A_{450}] \times v/w$, where v is the volume of cuvette and w is the weight of leaf tissues in each sample.

Leaf ABA and *t*-ZR content. The ABA in leaf tissues was extracted and purified according to the procedure described by Man et al. (2011). Briefly, leaf tissue (400 mg) was ground with a mortar and a pestle in liquid nitrogen and homogenized in 5-mL Na-phosphate buffer (0.05 M, pH 7.0) containing 0.02% sodium diethyldithiocarbamate as an antioxidant and the hormones were extracted by continuous shaking for 2 h at 4°C . The samples were transferred into test tubes after extraction and pH was adjusted to 2.6 with 1.0 M HCl. The sample was slurried with Amberlite XAD-7 (Sigma, St. Louis, MO) for 30 min. After removal of the buffer, the XAD-7 was washed two times with 4 mL of 1% acetic acid before being slurried two times with 5 mL dichloromethane for 30 min. The combined dichloromethane fractions were reduced to dryness with nitrogen gas. Then samples were dissolved in methanol (30 μL) and diluted with 0.6 mL of 50 mM tris-buffered saline (pH 7.0), and filtered using an acrodisc 13-mm syringe filter with a $0.2\text{-}\mu\text{m}$ nylon membrane (Fisher Scientific Company, Pittsburgh, PA) before being analyzed.

t-Zeatin riboside was extracted according to the procedure by Zhang and Ervin (2004) with some modifications. Leaf samples (350 mg) were used for extraction. The *t*-ZR samples were dissolved in methanol (30 μL) and diluted with 0.6 mL of 50 mM phosphate-buffered saline (pH 7.0) and filtered with an acrodisc 13-mm syringe filter with $0.2\text{-}\mu\text{m}$ nylon membrane before being analyzed (Man et al., 2011).

The ABA and *t*-ZR were analyzed using the SCL-10A VP HPLC system (Shimadzu, Japan) according to the methods described by Man et al. (2011). Briefly, an aliquot (10 μL) of the filtered samples was injected into a Shim-Park C18 VP-ODS chromatographic column (150 mm \times 4.6 mm, 5 μm) with isocratic elution at a flow rate of 0.7 mL min^{-1} at 30°C using methanol and 0.075% acetic acid as a mobile phase (45:55, v/v). Detection of ABA and *t*-ZR was carried out at 254 nm by a Shimadzu SPD-10A VP ultraviolet detector and cochromatography with authentic standards (Sigma). Identification of the compounds was carried out by comparison of retention times and characteristic

absorbance of the eluting peaks with authentic standard compounds.

Leaf H_2O_2 content. The H_2O_2 content was determined using the methods of Bernt and Bergmeyer (1974). Leaf sample (200 mg) was homogenized in 3 mL of 100 mM sodium phosphate buffer (pH 6.8) and the extracts were centrifuged at 16,000 g_n for 5 min at 4 °C. Then 0.17 mL of supernatant was added to 0.83 mL peroxidase reagent containing 83 mM sodium phosphate (pH 7.0), 0.005% (w/v) *o*-dianisidine, and 40 μ g peroxidase per mL. The mixture was incubated at 30 °C for 10 min, and 0.17 mL of 1 M perchloric acid was added to stop the reaction. The absorbance at 436 nm was read against a blank.

Leaf antioxidant enzyme activity. Frozen leaf tissues (250 mg) were ground to a powder with a mortar and a pestle in liquid nitrogen, and then the powder was homogenized with 4 mL cold extraction buffer [50 mM Na_2HPO_4/NaH_2PO_4 , pH 7, 0.2 mM ethylenediamine-tetracetic acid (EDTA), and 1% polyvinylpyrrolidone (PVP)]. The extracts were centrifuged at 12,000 g_n for 20 min at 4 °C. The supernatant was collected and stored in different tubes and used for the assay of enzyme activity (Zhang and Kirkham, 1996).

The activity of SOD was measured according to the method of Giannopolitis and Ries (1977) with modifications. A 3 mL reaction mixture was composed of 50 mM phosphate buffer (pH 7.8), 1.3 μ M Riboflavin, 13 mM methionine, 0.1 mM EDTA, 63 μ M nitro blue tetrazolium (NBT), and 0.1 mL enzyme extract. The test tubes containing the mixture were placed under light at 3,000 lx for 10 min, and absorbance at 560 nm was recorded. No purple color should appear in tubes without enzyme and NBT (CK 0). Tubes containing NBT but without enzyme should turn dark purple as the control (CK Max). One unit of SOD activity was defined as the amount of enzyme inhibited 50% photochemical reduction of NBT.

The CAT activity was determined through the decomposition of hydrogen peroxide (H_2O_2), which was measured by a decrease in absorbance at 240 nm ($\epsilon = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$) for 1 min (Chance and Maehly, 1955). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 , and 0.1 mL enzyme extract. The reaction was started with enzyme extract.

The POD activity was measured as an increase in absorbance at 470 nm ($\epsilon = 26.6 \text{ mm}^{-1} \text{ cm}^{-1}$) for 1 min following the oxidation of guaiacol (Rao et al., 1996). The reaction mixture contained 50 μ L of 20 mM guaiacol, 2.83 mL of 10 mM phosphate buffer (pH 7.0), 20 μ L of 40 mM H_2O_2 , and 0.1 mL enzyme extract. The reaction was started with the addition of H_2O_2 .

APX activity was determined by following the decrease in absorbance at 290 nm because of ascorbic acid oxidation ($\epsilon = 2.8 \text{ mm}^{-1} \text{ cm}^{-1}$) according to Nakano and Asada (1981) as described by Zhang and Kirkham (1996). The 1.5 mL reaction mixture

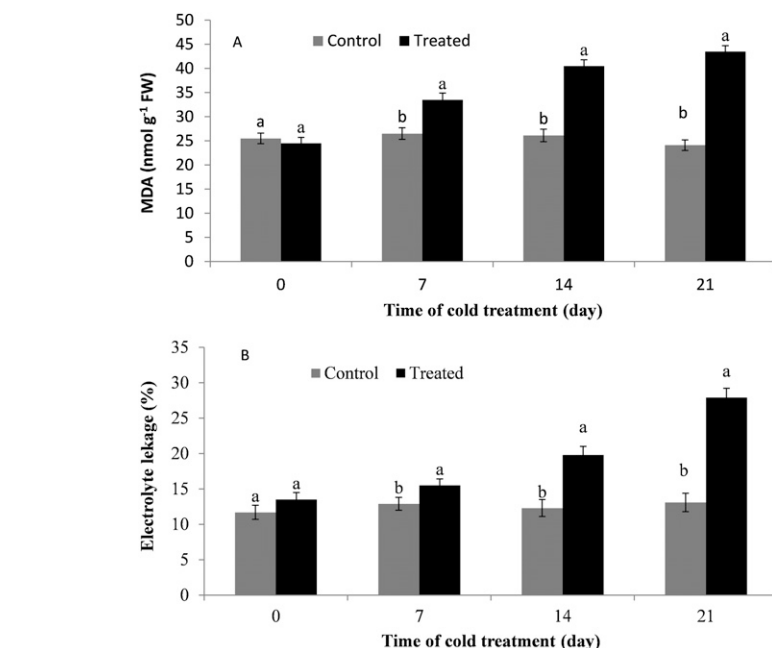


Fig. 1. Leaf electrolyte leakage (EL; mean \pm SE, $n = 10$) and malondialdehyde (MDA content) responses to cold acclimation treatment in 'Chinese Common' zoysiagrass. The treatments marked with same letters are not significantly different at the given sampling date at $P = 0.05$.

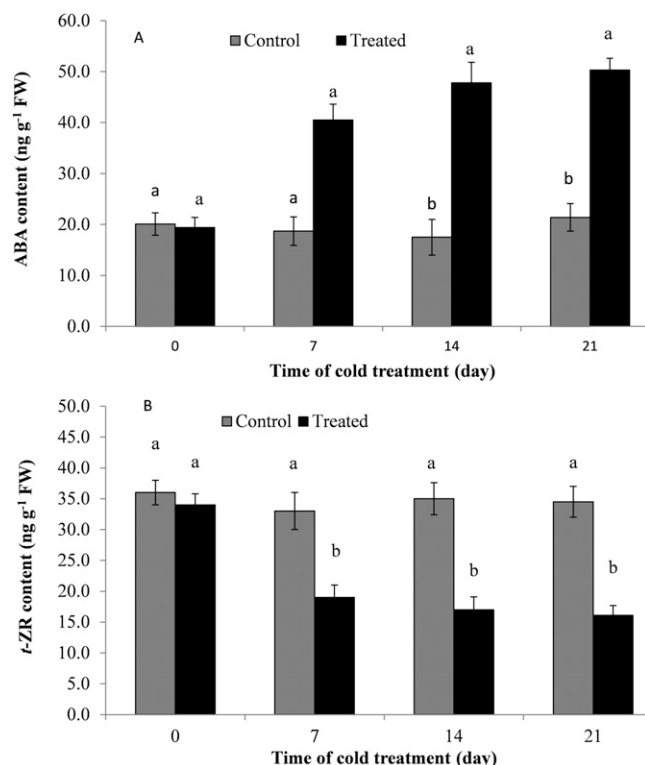


Fig. 2. (A) Leaf abscisic acid (ABA) and (B) transzeatin riboside (*t*-ZR) content (mean \pm SE, $n = 10$) responses to cold acclimation treatment in 'Chinese Common' zoysiagrass. The treatments marked with same letters are not significantly different at the given sampling date at $P = 0.05$.

consisted of 0.5 mM ascorbic acid, 0.1 mM H_2O_2 , 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.00), and 50 μ L enzyme extract. The reaction was initiated by adding H_2O_2 .

Leaf proline content. Leaf proline content was measured according to Bates et al.

(1973). Briefly, frozen leaf tissues (50 mg) were crushed with liquid nitrogen and extracted with a pestle in an ice-cold mortar with 1 mL of 3% 5-sulfosalicylic acid solution and the homogenate was transferred into 1.5 mL micro centrifuge tube. The tubes were vortexed for 15 s (four times), and

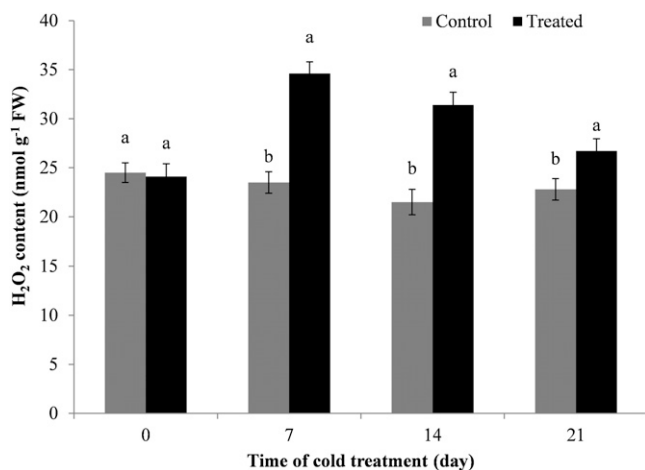


Fig. 3. Leaf hydrogen peroxide content (mean \pm SE, $n = 10$) responses to cold acclimation treatment in 'Chinese Common' zoysiagrass. The treatments marked with same letters are not significantly different at the given sampling date at $P = 0.05$.

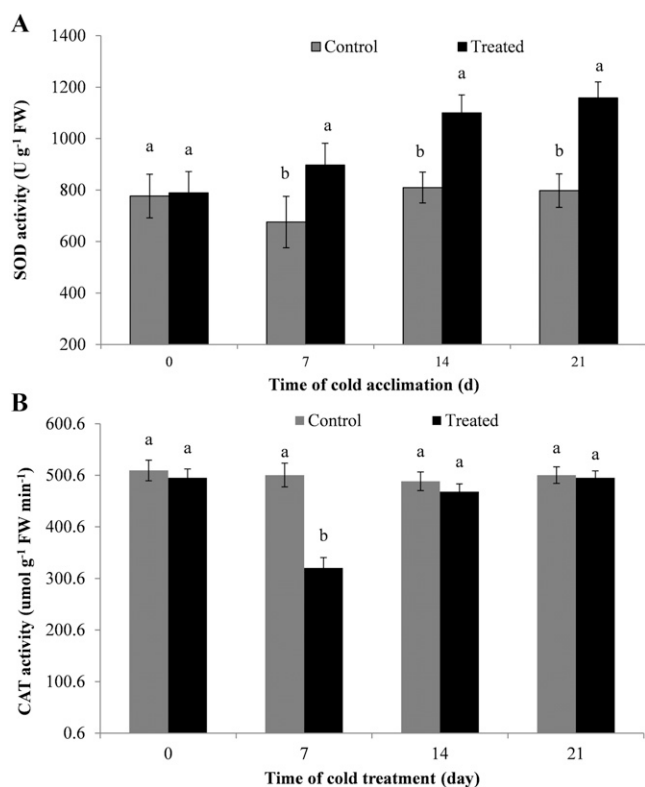


Fig. 4. (A) Leaf superoxide dismutase (SOD) and (B) catalase (CAT) activity (mean \pm SE, $n = 10$) responses to cold acclimation treatment in 'Chinese Common' zoysiagrass. The treatments marked with same letters are not significantly different at the given sampling date at $P = 0.05$.

then centrifuged at 16,000 g_n for 15 min. Supernatant (100 μ L) was collected for proline assay. The aliquots were mixed with 900 μ L deionized water, 0.1 mL acid ninhydrin, and 1.0 mL glacial acetic acid. The tubes were vortexed for 15 s and then incubated in an oven at 100 $^{\circ}$ C for 1 h, and cooled down in an ice bath. Toluene (4.0 mL) was added to each tube. After the tubes were vortexed for 20 s and allowed to stand for 5 min, 3.0 mL of the upper chromophore was transferred into test tubes and the absorbance was read at 520 nm in a spectrophotometer

(Spectrometer 2802s ultraviolet/VIS; Shanghai Precision and Scientific Inc.).

Experimental design and statistical analysis. A randomized complete block design was used with five replications. The data from the first and second experiment were analyzed using an ANOVA with two factors (experiment and cold treatment; SAS version 9.1.3) for each sampling date. Since there was no interaction between experiment and cold treatment, the data were reported as means of two experiments ($n = 10$). The mean separations were performed using

a Fisher's protected LSD test at 5% probability level.

Results

Leaf EL and MDA content. Cold treatment caused an increase in MDA content at 7, 14, and 21 d (Fig. 1A). At 21 d, cold treatment increased MDA by 80% when compared with the control.

Cold treatment increased EL as observed at 7, 14, and 21 d of treatment (Fig. 1B). EL gradually increased in response to cold treatment. At 21 d, cold treatment increased EL by 113% relative to the control.

Leaf ABA and t-ZR content. Cold treatment increased ABA content as measured at 7, 14, and 21 d (Fig. 2A). The greatest increase in ABA content occurred from 0 to 7 d. The ABA level in the cold treated grass was similar at 14 and 21 d. At 21 d, cold treatment increased ABA content by 135% relative to the control.

Cold treatment reduced t-ZR content as measured at 7, 14, and 21 d (Fig. 2B). The t-ZR level dropped sharply from 0 to 7 d and then remained unchanged. At 21 d, the t-ZR content in cold treated grass was only 58% of the control.

Leaf H₂O₂ content. H₂O₂ content increased sharply from 0 to 7 d and then declined from 7 to 21 d in response to cold treatment (Fig. 3). At 7 d, cold treatment increased H₂O₂ content by 47% relative to the control. A higher H₂O₂ level in cold-treated grass was found at 7, 14, and 21 d when compared with the control.

Leaf antioxidant enzyme activity. Leaf SOD activity gradually increased from 0 to 21 d due to cold treatment (Fig. 4A). A higher SOD activity in the cold-treated grass was found at 7, 14, and 21 d relative to the control. At 21 d, cold treatment increased SOD activity by 45% when compared with the control.

Cold treatment reduced CAT activity by 36% at 7 d of treatment (Fig. 4B). No difference in CAT activity was found between the treatment at 0, 14, and 21 d.

APX activity declined at 7 d and increased at 14 d due to cold treatment (Fig. 5A). At 14 d, cold treatment increased APX activity by 31% relative to the control. No difference in APX activity was found between the treatments at the end of cold treatment (21 d). POD activity dropped sharply from 0 to 7 d and then remained unchanged due to the cold treatment (Fig. 5B). At 21 d, cold treatment reduced POD activity by 21%.

Leaf proline content. Proline content increased gradually in response to cold treatment (Fig. 6). Higher proline content in the cold-treated grass relative to the control was observed at 14 and 21 d. At 21 d, cold treatment increased proline content by 3.1-fold relative to the control.

Stolon LT₅₀. LT₅₀ is a good indicator of freezing tolerance. Before cold treatment, the averaged LT₅₀ was -4.8 $^{\circ}$ C. After 21 d of cold acclimation treatment, LT₅₀ decreased from -4.8 to -12.5 $^{\circ}$ C (Fig. 7).

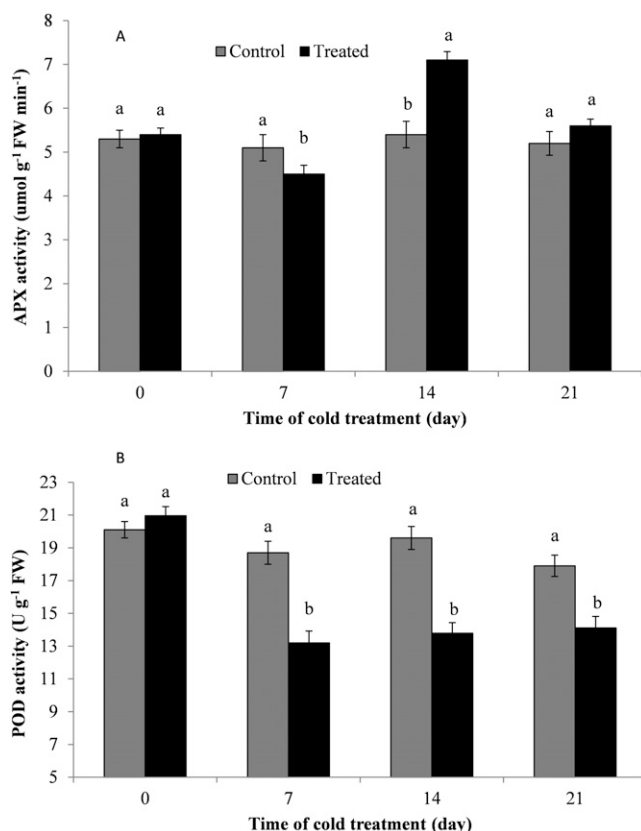


Fig. 5. (A) Leaf ascorbate peroxidase (APX) and (B) peroxidase (POD) activity (mean \pm SE, $n = 10$) responses to cold acclimation treatment in 'Chinese Common' zoysiagrass. The treatments marked with same letters are not significantly different at the given sampling date at $P = 0.05$.

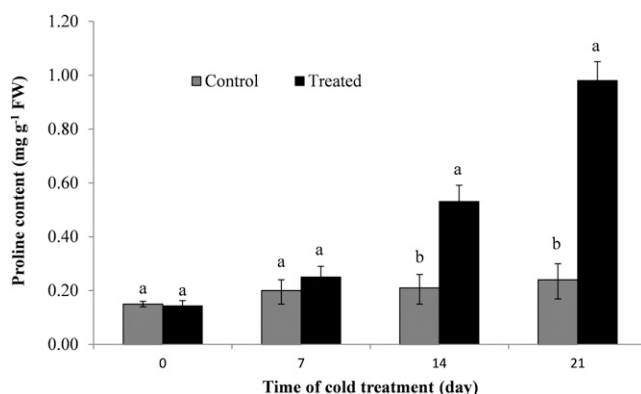


Fig. 6. Leaf proline (mean \pm SE, $n = 10$) responses to cold acclimation treatment in 'Chinese Common' zoysiagrass. The treatments marked with same letters are not significantly different at the given sampling date at $P = 0.05$.

Discussion

The results of this study indicated that the cold acclimation treatment resulted in an increase in ABA and decline in *t*-ZR content in zoysiagrass. The greatest change occurred during the first week of cold treatment. This is consistent with previous studies with warm-season turfgrass species (Zhang et al., 2009, 2011). Zhang et al. (2009) indicated that stolon ABA content in zoysiagrass increased from late fall to winter in the field conditions in Kansas state. Zhang et al. (2011) noted that cold

acclimation treatment increased stolon ABA content and reduced cytokinin content in bermudagrass. Plants may close stomata in responses to abiotic stresses such as cold (Zhang and Ervin, 2008). ABA and cytokinin can regulate stomatal opening and closure, and higher ABA may induce stomatal closure (Davies, 2010). Closure of stomata would inhibit gas exchange and may cause oxidative stress and accumulation of ROS such as H₂O₂. The results of our study indicated that H₂O₂ increased sharply during first week of cold treatment. In addition, EL and MDA content

increased in response to cold treatment. Lu et al. (2009) noted that H₂O₂ is involved in ABA-induced stomatal closure and enhanced activities of antioxidant enzymes in plants subjected to drought stress. This suggests that H₂O₂ and ABA may be involved in inducing defensive response during early stage of cold acclimation. Excess ROS may cause cell membrane lipid peroxidation (higher EL and MDA content). The results of our study suggest that cold acclimation may induce upregulation of ABA and H₂O₂ and mild cell membrane injury.

Increases in ABA and H₂O₂ may trigger plant defense systems such as certain antioxidant enzymes. It has been documented that ABA and H₂O₂ may serve as signal molecules in initiating plant defense systems during cold acclimation (Xiong et al., 2002). The results of this study indicated that cold acclimation increased antioxidant enzyme SOD and APX activity. SOD activity was higher in cold-treated grass relative to the control at 7, 14, and 21 d. This is consistent with previous studies (Zhang et al., 2006; Zhang and Ervin, 2008). Higher SOD activity may effectively scavenge O₂⁻ and convert it to H₂O₂, which can further be converted to H₂O and O₂ by CAT and APX. The decrease of CAT activity at 7 d may be due to H₂O₂ depletion. Cold acclimation treatment may cause excess ROS production, which is related to the decline of certain enzyme activity from 0 through 7 d. The increase in ROS level may trigger upregulation of SOD and APX activity as observed from d 7 to d 14. The SOD and other antioxidant enzymes and metabolites may suppress ROS and protect cells under low-temperature stress (Zhang and Ervin, 2008).

The results of this study also showed that the leaf proline content increased in response to cold acclimation treatment. This supports previous studies by Patton et al. (2007a) who reported a significant increase in proline content during cold acclimation in zoysiagrass. Several researchers also found that proline content increased during cold acclimation in other warm-season turfgrasses (Cai et al., 2004; Munshaw et al., 2006; Zhang et al., 2006, 2008). Proline functions as an osmoprotectant and regulates cell ψ_s and stabilizes cell membrane during abiotic stresses. Proline also functions as an antioxidant to suppress ROS. The higher proline content in cells may alleviate dehydration and protect cells during freezing stress.

Cold acclimation treatment increased zoysiagrass freezing tolerance as LT₅₀ decreased from -4.8 to -12.5 °C. Zhang et al. (2009) also found that LT₅₀ of zoysiagrass decreased from late fall to winter in field conditions. Increases in ABA, antioxidant enzymes (SOD, APX), and proline because of cold acclimation treatment may contribute to the improvement of freezing tolerance. The cold-induced mild oxidative stress may increase ROS, which initiates antioxidant defense systems.

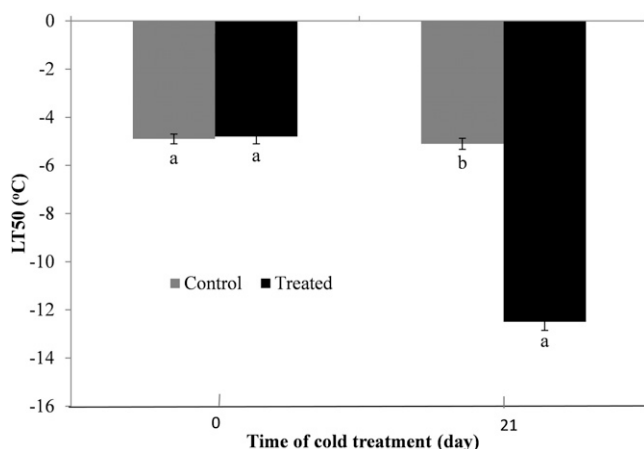


Fig. 7. Lethal temperature for 50% loss of electrolytes in stolons (LT₅₀; mean \pm SE, $n = 10$) responses to cold acclimation treatment in 'Chinese Common' zoysiagrass. The treatments marked with same letters are not significantly different at the given sampling date at $P = 0.05$.

Conclusions

The results of this study indicated that cold acclimation treatment induced an increase in ABA, H₂O₂, antioxidant enzyme (SOD and APX) activity, as well as in proline. ABA and H₂O₂ are involved in regulating plant antioxidant defenses. Cold treatment-induced upregulation of ABA, antioxidant enzyme (SOD and APX) activity, and proline may be closely associated with improvement of freezing tolerance (lower LT₅₀). The results suggest that the hormone (ABA), antioxidant enzymes (SOD and APX), and osmoprotectant (proline) are involved in cold acclimation process and associated with freezing tolerance and winter survival of zoysiagrass.

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