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RESEARCH ARTICLE

Tolerance performance of the cool-season turfgrass species *Festuca ovina*, *Lolium perenne*, *Agrostis tenuis*, and *Poa trivialis* to sulfur dioxide stress

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Tolerance of four cool-season turfgrass species sheep fescue (*Festuca ovina*), perennial ryegrass (*Lolium perenne*), colonial bentgrass (*Agrostis tenuis*) and rough bluegrass (*Poa trivialis*) to SO_2 was studied by investigating their injury and physiological responses under SO_2 stress for 14 days. Results showed that sheep fescue and perennial ryegrass had better SO_2 tolerance than rough bluegrass and colonial bentgrass. Chlorophyll a and carotenoid contents were significantly higher in the SO_2-tolerant turfgrass species (sheep fescue and perennial ryegrass) than in the SO_2-sensitive turfgrass species (rough bluegrass and colonial bentgrass). Relatively lower levels of reactive oxygen species (ROS), malondialdehyde, and electrolyte leakage (EL) were observed in SO_2-tolerant turfgrass species. Gradually increased antioxidant enzyme activities and proline content in SO_2-tolerant turfgrass species could counteract such damages and harmful effects caused by ROS and EL, respectively. Sulfite reductase played an important role in sulfur metabolism and subsequently reduced oxidative pressure on SO_2-tolerant turfgrass species.

Keywords: antioxidant enzymes; cool-season turfgrass; osmoprotectants; photosynthetic pigments; stress response; sulfur dioxide

Introduction

With the rapid development of both economy and manufacturing industries, sulfur dioxide (SO_2) is becoming the most common and important air pollutant in China, especially in the industrial areas and big cities (He 2009; D'Amato et al. 2010). Generally, SO_2 influences the biological systems of plant through acidification and oxidation. The acidification can directly lead to the phaeophytinization through substituting Mg^{2+} by H^+ in chlorophyll. The oxidation of SO_2 is the dominant process, which causes a series of damages to plants, such as pigment bleaching and the efflux of malondialdehyde (MDA) and potassium (Kong et al. 1999). The atmospheric SO_2 can be absorbed by the foliage and dissolved in the cellular cytoplasm to form H_2SO_3 and HSO_3^- (Singh et al. 2012). These sulfite and bisulfite will be enzymatically or non-enzymatically oxidized to SO_4^{2-} and then transported into the vacuole where they are poorly accessible for remobilization (Yang et al. 2006). During the oxidation process, superoxide radicals, hydroxyl radicals, and other free radicals are produced, which can influence many aspects of plant physiology, including biosynthesis, growth and development, energy consumption, etc. (Tan & Liu 1981; Heyneke et al. 2012).

With the diversified development of urban landscaping, turfgrass industry has entered into a fast development period in China (Guo et al. 2011). Turfgrass is widely used as urban landscaping plant in the modern cities due to its multiple functions. Turf surfaces can decrease the dust and noise, absorb the toxic gas, mitigate the violet radicalization, and reduce the urban heat island effect. Additionally, turfgrass adds aesthetical value to the environment and provides playing ground for entertainment and leisure (Jim and Chen 2009; Pickett et al. 2011). Turfgrass species display different tolerances to air pollutants. Some species can survive in severe air-polluted conditions, and return to normal growth level once the air pollutants are eliminated. However, others are sensitive to air pollutant, which can be damaged at lower concentration of air pollutant and cannot recover even if the air pollution is eliminated (Honour et al. 2009; Leung et al. 2011).

There are two types of turfgrasses: warm-season and cool-season. Warm-season grasses are primarily used in warm region (such as South China) and cool-season grasses are the dominant types used in cold region (such as North China). Both warm- and cool-season species can be cultivated in transition region (such as Central China). Compared with warm-season grasses, cool-season grasses turn green faster in the spring and stay green longer into autumn due to their preference for cooler temperatures. Tolerance to SO_2 of grass species has been briefly studied by estimating changes in growth, injury rate, and symptoms (Brennan & Halisky 1970; Ayazloo & Bell 1981). Recently, we compared the physiological responses of warm-season turfgrass bermudagrass (*Cynodon dactylon*) accessions from Southwest China to sulfur dioxide (Li et al. 2014). However, tolerance and physiological responses of cool-season

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turfgrasses to sulfur dioxide have not been reported in details till now. In order to achieve good afforestation and landscaping effects, selection of cool-season turfgrass species with strong resistance to air pollution for use in polluted urban and industrial areas is practically desired in North and Central China in winter.

Sheep fescue (*Festuca ovina*), perennial ryegrass (*Lolium perenne*), colonial bentgrass (*Agrostis tenuis*), and rough bluegrass (*Poa trivialis*) are the four typical cool-season turfgrass species, which naturally distribute in north and central provinces in China and are also used for landscaping widespread. In the present study, we studied the physiological responses of these four cool-season turfgrass species to sulfur dioxide stress. This study gained some insights into the mechanisms of cool-season turfgrass coping with SO_2 toxicity, and provided useful guidelines for selection of turfgrass species suitable for use in SO_2 polluted urban and industrial areas in cool and transition regions.

Materials and methods

Plant material

Four cool-season turfgrass species, including sheep fescue, perennial ryegrass, colonial bentgrass, and rough bluegrass, provided by the Beijing office of the Netherlands Royal Barenbrug Group, were used as research objects in this study. Grass seeds were surface disinfected with 0.05% $KMnO_4$ for 20 min, and sowed in large plastic pots (29 cm in diameter at the top, 17 cm in diameter at the bottom, and 22 cm in height) filled with sandy loam soil supplemented with organic fertilizer on April 2013, and 20 pots were used for each turfgrass species. The resulting seedlings were placed in outdoor conditions with timely watering, and monthly weeding and fertilizing.

Experimental design

The experiment was carried out at the Teaching Practice Base of the College of Landscape Architecture (103 49' 58" E, 30° 41' 48" N, 550 m a.s.l.), Sichuan Agricultural University, Wenjiang District, Sichuan Province, China. Average annual precipitation, annual temperature, and relative air humidity in this area are 972 mm, 15.9°C, and 84%, respectively. After one-month outdoor growth, 15 pots of grass plants with approximately the same growing stage were selected for SO_2 treatment. Five levels of sulfur contents, including CK (0 mg/m^3), S1 (1.30 mg/m^3), S2 (2.60 mg/m^3), S3 (3.90 mg/m^3), and S4 (5.20 mg/m^3), were applied in this study. Grasses were fumigated with incense containing sulfur in artificial fumigation chamber (0.289 m^3, 85 × 85 × 40 cm) for 3 h per day over 14 days. The day when SO_2 fumigation started was designated as day 0. Treatment was applied to three replicated pots for each turfgrass species for each SO_2 concentration. The fumigation chamber is an enclosed automatic cabinet, and SO_2 concentration is monitored by a Z-1300 gas detector (Environmental Sensors Co., Boca Raton, FL, USA). To increase the uniformity of gaseous mixture, a fan was attached to the ceiling to mix the SO_2 in the chamber. Leaves 2 cm above the soil from grass plants were collected before SO_2 treatment and after 14-day treatment with SO_2 and brought to laboratory for physiological analysis. Leaves from the same turfgrass species lacking SO_2 treatment served as negative control. Foliar injury was scored by assessing the visible leaf damage excluding senescent leaves after 14-day SO_2 fumigation treatment.

Photosynthetic pigment analysis

Photosynthetic pigments from turfgrass leaves were extracted as described by Lichtenthaler and Wellburn (1983) with modification. Fresh leaves were ground in 2 ml of 80% (v/v) acetone and ethyl alcohol (1:1) using a mortar and pestle, and then filtered through 0.45 μm filter paper. Absorbance of the resulting extracts was measured at three wavelengths 663, 646, and 470 nm for chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car), respectively, using a ultraviolet-visable spectrophotometer Model 723PC (Jinghua Instruments, Shanghai, China). The contents of pigments were calculated according to the adjusted extinction coefficients (Lichtenthaler & Wellburn 1983). Total chlorophyll contents were obtained from the sum of Chl a + Chl b.

Estimation of H_2O_2 content and $O_2^{-\cdot}$ generation rate

Fresh leaves were ground in liquid nitrogen using a mortar and pestle and then homogenized in extraction buffer (50 mM sodium phosphate buffer, pH 7.8). The homogenate was centrifuged at 15,000×*g* for 10 min at 4°C, and the supernatant was used for analyses of H_2O_2 content and $O_2^{-\cdot}$ generation rate. H_2O_2 content was determined according to the method described by Hu et al. (2012). Briefly, 1.0 ml of the supernatant was thoroughly mixed with 1.0 ml of 0.1% titanium sulfate (w/v) in 20% H_2SO_4 (v/v) for 10 min. After centrifugation at 15,000×*g* for 10 min at room temperature, the absorbance of the supernatant was measured at 410 nm. $O_2^{-\cdot}$ generation rate was determined according to the method described by Elstner and Heupel (1976). Briefly, 1 ml of the supernatant was mixed with 1.0 ml of 65 mM sodium phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylammonium chloride, 0.1 ml of 7.5 mM xanthine, and 0.3 ml xanthine oxidase (0.2 unit/ml), and incubated at 25°C for 20 min. Then 0.5 ml of 1.0% (w/v) a-naphthylamine and 0.5 ml of 19 mM sulfanilic acid were added to 0.5 ml of above reaction mixture, and incubated at 25°C for 20 min. The absorbance of the mixture was measured at 530 nm.

Protein extraction and enzyme activity assay

Fresh leaves were ground and extracted with 5 ml of 0.1 M sodium phosphate buffer (pH 6.8) containing 1 mM ethylenediaminetetraacetate, 1 mM dithiotreitol, and 2% (w/v) polyvinylpyrrolidone. The homogenate was

centrifuged at 15,000×*g* for 15 min at 4°C, and the supernatant was used for enzyme activity. Soluble protein content in the supernatant was determined using the modified Bradford (1976) method with bovine serum albumin as protein standard.

Superoxide dismutase (SOD; EC 1.15.1) activity was measured spectrophotometrically at 560 nm based on inhibition of the photochemical reduction of nitroblue tetrazolium (Beauchamp & Fridovich 1971). Catalase (CAT; EC 1.11.1.6) activity was measured by following the decomposition of H_2O_2 at 240 nm according to the method described by Aebi (1984). Peroxidase (POD; EC 1.11.1.7) activity was recorded by measuring the increase of absorbance at 470 nm due to guaiacol oxidation as described by Curtis (1971). Glutathione reductase (GR; EC 1.8.1.7) activity was determined by measuring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm as described by Tanaka et al. (1988). Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined by measuring the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (Habig et al. 1974). Sulfite reductase (SiR; EC 1.8.99.1) activity was determined by the coupled SiR/OASTL assay with the addition of NADPH and tungstic acid (Brychkova et al. 2012). Sulfite oxidase (SO; EC 1.8.3.1) activity was determined by reduction of ferricyanide at 420 nm (Eilers et al. 2001).

Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of MDA following the method described by Heath and Packer (1968). Fresh leaves were ground and homogenized with 2 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000×*g* for 20 min at 4°C, and the supernatant was used for lipid peroxidation analysis. A total of 4 ml of 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA was added to 1 ml of the supernatant. The mixture was incubated in hot water (95°C) for 30 min and cooled immediately on ice to stop the reaction and centrifuged at 15,000×*g* for 20 min. Absorbance was measured at 532 and 600 nm, and MDA content was estimated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm.

Determination of electrolyte leakage (EL) and osmoprotectants

For the EL assay, fresh leaves were rinsed for three times with deionized water to remove surface electrolytes, and then incubated in 10 ml of deionized water at 25°C with shaking for 6 h. The initial conductivity (C1) was determined using an electrical conductivity meter (Leici-DDS-307A, Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China). The samples were then boiled for 20 min and subsequently incubated at 25°C for 1 h. The total conductivity (C2) was determined using the Leici-DDS-307A conductivity meter. Ion leakage was calculated using the following equation:

$$\text{Relative EL}(\%) = \left(\frac{C1}{C2}\right) \times 100$$

The soluble sugar content was measured by the anthrone method as described by Irigoyen et al. (1992). Briefly, fresh leaves were extracted with 80% (v/v) ethanol at 80°C for 40 min. Total soluble sugars were analyzed by treating 0.2 ml of the alcoholic extract with 3 ml of freshly prepared 0.15% (w/v) anthrone reagent (0.3 g anthrone in 200 ml 72% (v/v) H_2SO_4). This mixture was placed in a boiling water bath for 10 min. After the mixture was cooled, total soluble sugar content was determined according to absorbance at 620 nm.

Free proline was extracted from the fresh leaves and analyzed according to the method described by Bates et al. (1973). Fresh leaves were homogenized and mixed with 3% (w/v) sulfosalicylic acid. The extracted solution was reacted with equal volumes of glacial acetic and ninhydrin reagent at 100°C for 1 h. The reaction was terminated by placing the tube in an ice bath, and the chromophore was measured at 520 nm.

Sulfur accumulation

For sulfur (S) content determination, the turbidimetric method described by Reyes-Díaz et al. (2011) was applied. Biomass of whole plants were dried for 48 h, treated with 95% (w/v) magnesium nitrate, and then ashed at 500°C for 8 h. The ashed samples were digested in 10 ml of 2M HCl at 150°C for 1 h. After addition of barium chloride ($BaCl_2$) and Tween-80 into the solution, its absorbance was immediately measured at 440 nm.

Statistical analysis

All data were presented as means ± SD from three replicates. Statistical analysis was performed by analysis of variance and least significant difference (LSD) test using the SPSS 18.0 (SPSS Inc., Chicago, IL). Significant difference was evaluated by LSD test at $p < 0.05$ level. Chart was created using the GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Foliar injury under SO_2 stress condition

Injury symptoms appeared on turfgrass leaves under SO_2 stress conditions after 14-day SO_2 treatment (Figure 1a). The visible symptoms consisted of marginal, interval, and/or bifacial chlorosis and necrosis on fully expanded leaves. The necrotic areas were irregular, with colors varied from brown to white and occasionally black. Leave injury symptoms were influenced by both SO_2 concentration and turfgrass species (Figure 1a and 1b). At low SO_2 concentration of S1–S2, effects of SO_2 on leaves of sheep fescue and perennial ryegrass were not noticeable. At high SO_2 concentration of S3–S4, although visible injury increased significantly along with the increase of

SO_2 concentration, the injury rates were relatively low (less than 20%) on leaves of both sheep fescue and perennial ryegrass (Figure 1b). However, significant effects of SO_2 on leaves of colonial bentgrass and rough bluegrass were observed at low SO_2 concentration of S1 and the injury degree significantly increased along with the increase of SO_2 concentration from treatment S1–S4, especially on leaves of rough bluegrass (Figure 1b). The injury rates on leaves were more than 50% under SO_2 treatment of S3 and nearly 70% under SO_2 treatment of S4 on leaves of colonial bentgrass and rough bluegrass (Figure 1b). Especially, the turfgrass species of rough bluegrass almost withered or died at SO_2 concentrations of S3 and S4 (Figure 1a).

Effect of SO_2 on photosynthetic pigment contents

Contents of photosynthetic pigments in leaves of the four cool-season turfgrass species showed a decreasing trend along with the increase of SO_2 concentration, but displayed different patterns with different pigment and grass species (Figure 2). Chlorophyll a content in leaves of sheep fescue decreased in a low degree at low SO_2 concentrations of S1 and S2 (Figure 2a). For the rest of three turfgrass species, significant decrease of chlorophyll a content appeared immediately from low SO_2 concentration of S1. At the highest SO_2 concentration of S4, chlorophyll a content decreased by 1.5, 2.0, 3.6, and 3.5-fold in leaves of sheep fescue, perennial ryegrass, colonial bentgrass, and rough bluegrass compared to their controls, respectively (Figure 2a). However, chlorophyll a contents in sheep fescue and perennial ryegrass plants maintained relatively higher levels compared to colonial bentgrass and rough bluegrass plants following S4 treatment. Chlorophyll b content decreased significantly under SO_2 stress conditions, but displayed no further decrease along with the increase of SO_2 concentration once it reached to a certain low content in leaves of sheep fescue, perennial ryegrass, and colonial bentgrass (Figure 2b). In leaves of rough bluegrass, significant

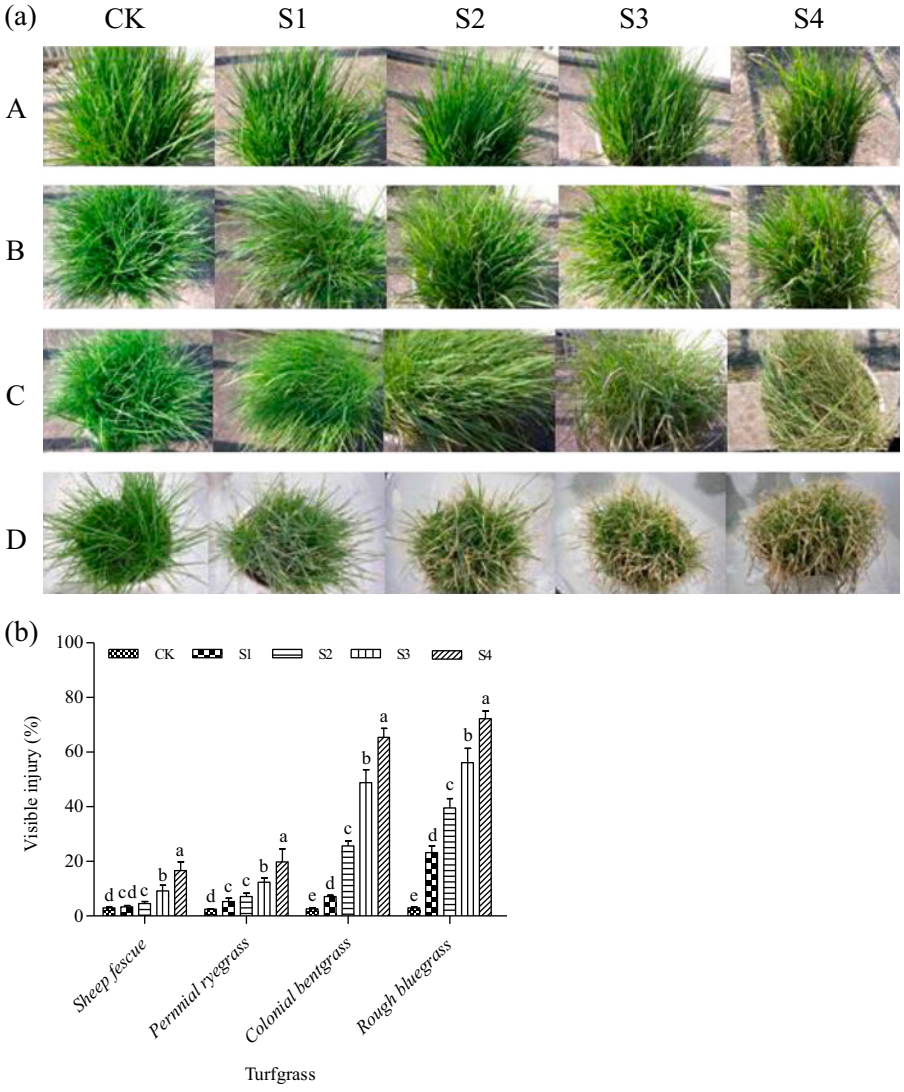

Figure 1. Effect of SO_2 on symptoms (a) and visible injury (b) of four cool-season turfgrass species. A, B, C, and D represent sheep fescue, perennial ryegrass, colonial bentgrass, and rough bluegrass, respectively.
Note: Mean values are presented with vertical error bars representing the standard deviations (n = 3). Different letters above the column within the same turfgrass species indicate significant differences among treatments ($P < 0.05$).

decrease of chlorophyll b content appeared at low SO_2 concentration of S1, and showed no significant differences from treatment of S1–S3, and then significantly decreased again from treatment of S3–S4 (Figure 2b). Content of total chlorophylls a + b showed a similar trend with chlorophyll a in all of the four cool-season turfgrass species under all SO_2-treated conditions (Figure 2c). Decrease of carotenoid contents showed a similar pattern in leaves of sheep fescue and perennial ryegrass, and its content decreased significantly along with the increase of SO_2 concentration from treatment of S1–S2, but did not decrease any further from treatment of S3–S4 (Figure 2d). In leaves of colonial bentgrass and rough bluegrass, carotenoid contents significantly decreased in a similar pattern along with the increase of SO_2 concentration from S1 to S4. Carotenoid contents in leaves of sheep fescue and perennial ryegrass were much higher than those in leaves of colonial bentgrass and rough bluegrass under SO_2-treated conditions of S3 and S4 (Figure 2d).

Effect of SO_2 on reactive oxygen species (ROS) generation and lipid peroxidation

Exposure to SO_2 for 14 days caused an increase in ROS generation in leaves of the four cool-season turfgrass species. H_2O_2 contents in leaves of sheep fescue and perennial ryegrass showed a similar pattern under SO_2 stress conditions (Figure 3a). Overall, H_2O_2 contents did not show significant increase at low SO_2 concentrations of S1 and S2, especially in leaves of sheep fescue, but showed an increasing trend when SO_2 concentration increased from S2 to S4 in leaves of sheep fescue and from S1 to S4 in leaves of perennial ryegrass (Figure 3a). H_2O_2 contents significantly increased from low SO_2 concentration of S1 in leaves of colonial bentgrass and rough bluegrass, and reached the highest content under SO_2-treated condition of S3 in leaves of colonial bentgrass and S2 in leaves of rough bluegrass (Figure 3a). O_2^- generation rate did not show significant increase at low SO_2 concentrations of S1 and S2 in leaves of sheep fescue and at low concentration of S1 in leaves of perennial ryegrass (Figure 3b). Although O_2^- generation rate increased significantly in leaves of sheep fescue and perennial ryegrass at high SO_2 concentrations, their increasing degree was not high (about 1.4-fold and 2.0-fold increase, respectively) relatively to their controls. On the contrary, O_2^- generation rate increased significantly in leaves of colonial bentgrass and rough bluegrass from low SO_2 concentration of S1,

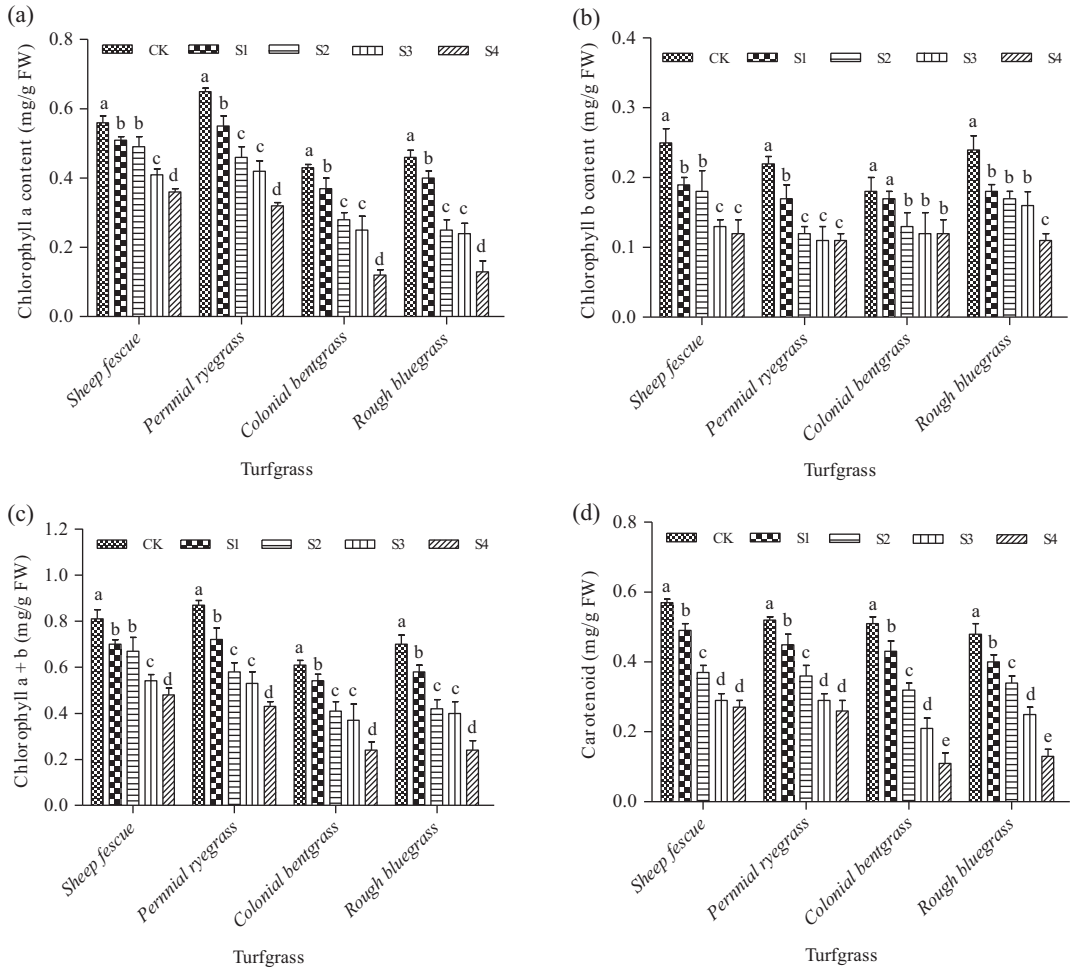

Figure 2. Effect of SO_2 on the chlorophyll a (a), chlorophyll b (b), chlorophyll (a + b) (c), and carotenoid contents (d) in leaves of four cool-season turfgrass species.

Note: Mean values are presented with vertical error bars representing the standard deviations (n = 3). Different letters above the column within the same turfgrass species indicate significant differences among treatments ($P < 0.05$).

and gradually increased along with the increase of SO_2 concentration. At the highest SO_2 concentration of S4, O_2^- generation rate increased by 4.7-fold in leaves of colonial bentgrass and 4.4-fold in leaves of rough bluegrass, respectively, when they were compared with their controls (Figure 3b). The level of lipid peroxidation in SO_2-treated grasses was determined according to the content of MDA in leaves of the four cool-season turfgrass species. As results, MDA contents did not increase significantly in leaves of sheep fescue and perennial ryegrass at low SO_2 concentration, but increased significantly in leaves of both species at high SO_2 concentrations (Figure 3c). However, MDA contents in leaves of colonial bentgrass and rough bluegrass increased significantly along with the increase of SO_2 concentration from S1 to S4, displaying 4.6-fold increase in the former and 5.3-fold increase in the latter species, respectively, when they were compared with their controls (Figure 3c).

Effect of SO_2 on antioxidant enzyme activities

To address the relationship between the changes of ROS level and antioxidant enzyme activities, five antioxidant enzymes, including SOD, CAT, POD, GR, and GST, were determined (Figure 4). Under control condition, CAT, POD, GR, and GST showed much higher enzyme activities in leaves of sheep fescue and perennial ryegrass than those in leaves of colonial bentgrass and rough bluegrass (Figure 4b–4e). Under SO_2-treated conditions, enzyme activities of all of the five antioxidants increased, but displayed different patterns with turfgrass species and SO_2 concentration. In leaves of sheep fescue and perennial ryegrass, enzyme activities of all of the five antioxidants increased gradually along with the increase of SO_2 concentration from S1 to S4. However, the enzyme activities in leaves of colonial bentgrass and rough bluegrass significantly increased at low SO_2 concentrations but did not increase or even decreased at high SO_2 concentrations. SOD, CAT, GR, and GST showed much higher enzyme activities in leaves of sheep fescue and perennial ryegrass than in leaves of colonial bentgrass and rough bluegrass, especially for SOD and GST at high SO_2 concentrations (Figure 4a–4e).

Effect of SO_2 on EL and osmoprotectants

Membrane permeability was estimated by measuring EL using a conductivity meter. EL did not show significant difference in leaves of sheep fescue and perennial ryegrass at low SO_2 concentrations, but increased significantly at high SO_2 concentrations (Figure 5a). In leaves of colonial bentgrass and rough bluegrass, EL increased significantly from low SO_2 concentration of S1 and showed a gradually increasing trend along with the increase of SO_2 concentrations from S1 to S4. EL degree in leaves of colonial bentgrass and rough bluegrass were much higher than those in leaves of sheep fescue and perennial ryegrass. Changes of osmotic adjustment

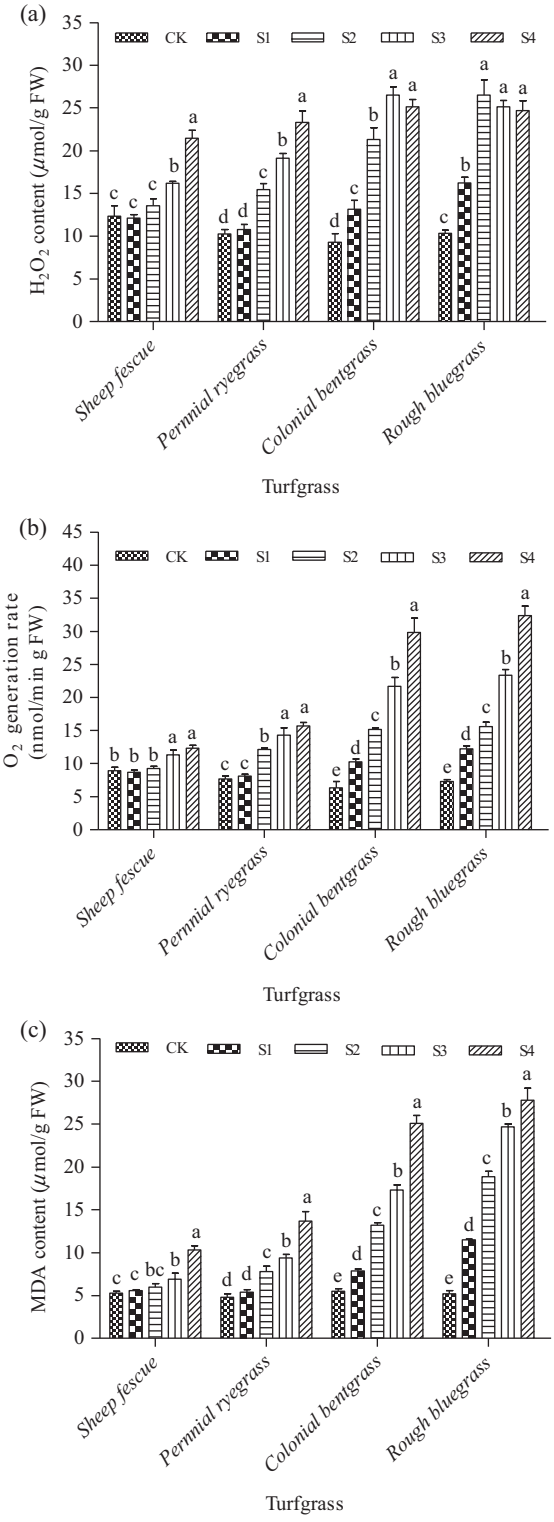

Figure 3. Effect of SO_2 on ROS levels reflected by H_2O_2 content (a), O^{2-} generation rate (b), and MDA content (c) in leaves of four cool-season turfgrass species.
Note: Mean values are presented with vertical error bars representing the standard deviations (n = 3). Different letters above the column within the same turfgrass species indicate significant differences among treatments ($P < 0.05$).

substances, including soluble sugars and proline, were measured in leaves of the four cool-season turfgrass species under SO_2 stress conditions. After 14-day SO_2 stress treatment, soluble sugar content remained steady

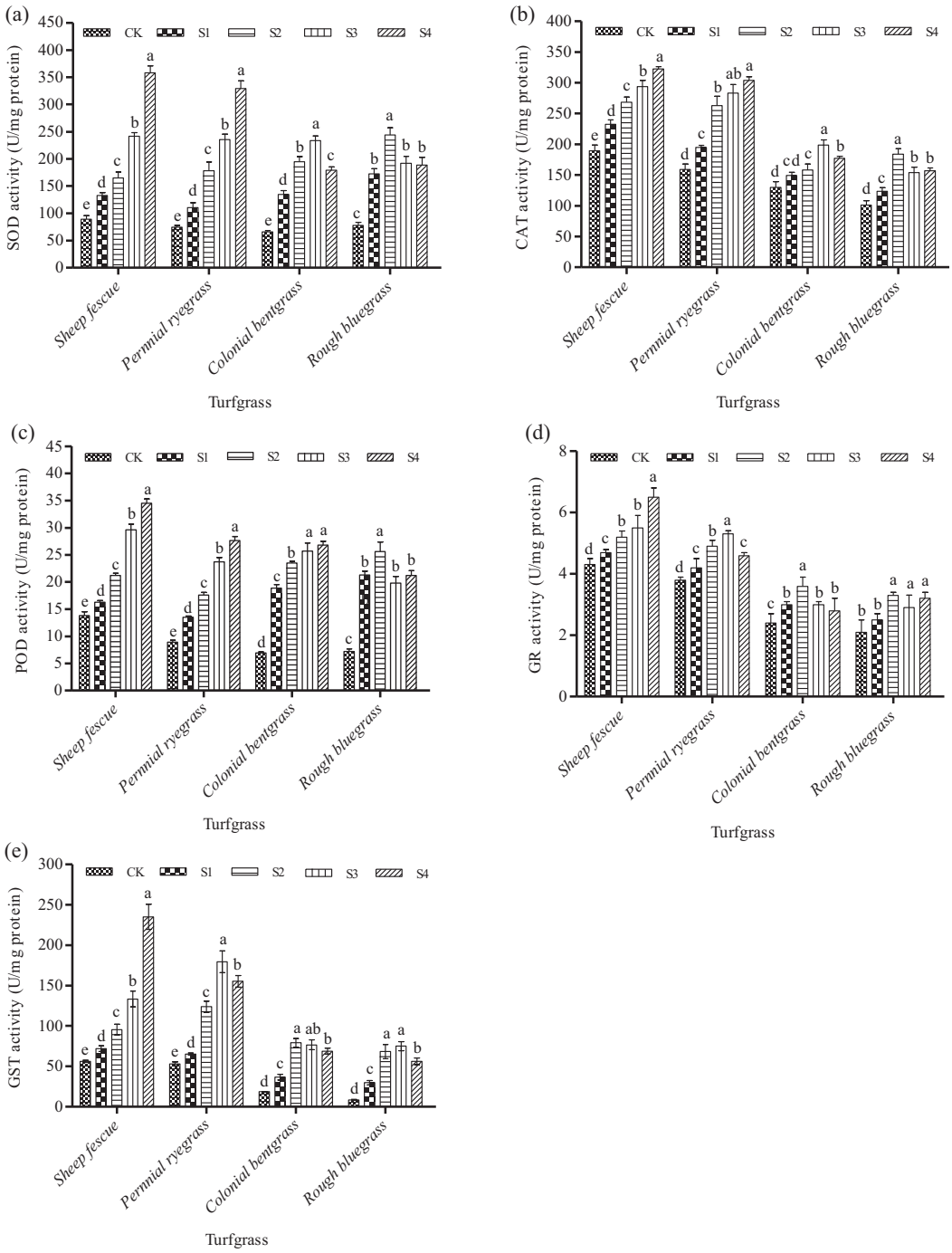

Figure 4. Effect of SO_2 on antioxidant enzyme activities of SOD (a), CAT (b), POD (c), GR (d), and GST (e) in leaves of four cool-season turfgrass species.

Note: Mean values are presented with vertical error bars representing the standard deviations (n = 3). Different letters above the column within the same turfgrass species indicate significant differences among treatments ($P < 0.05$).

in all of the four cool-season turfgrass species under all of the four SO_2-treated conditions (Figure 5b). However, proline, an osmotic adjustment substance, showed different responses to SO_2 stress in different turfgrass species (Figure 5c). Under SO_2 stress conditions, proline contents showed no significant changes in leaves of colonial bentgrass and rough bluegrass, but displayed an increasing trend along with the increase of SO_2 concentration in leaves of sheep fescue and perennial ryegrass, except for perennial ryegrass at SO_2-treated condition of S4 (Figure 5c).

Effect of SO_2 on sulfur content, and SiR and SO enzyme activities

Under the control conditions, no differences in sulfur contents were found in the four cool-season species (Figure 6a). After 14-day SO_2 stress treatment, sulfur contents showed an increasing trend along with the increase of SO_2 concentration from S1 to S4 in leaves of sheep fescue and perennial ryegrass. In leaves of colonial bentgrass and rough bluegrass, sulfur contents increased significantly at low SO_2 concentrations and then decreased at high SO_2 concentrations. Moreover,

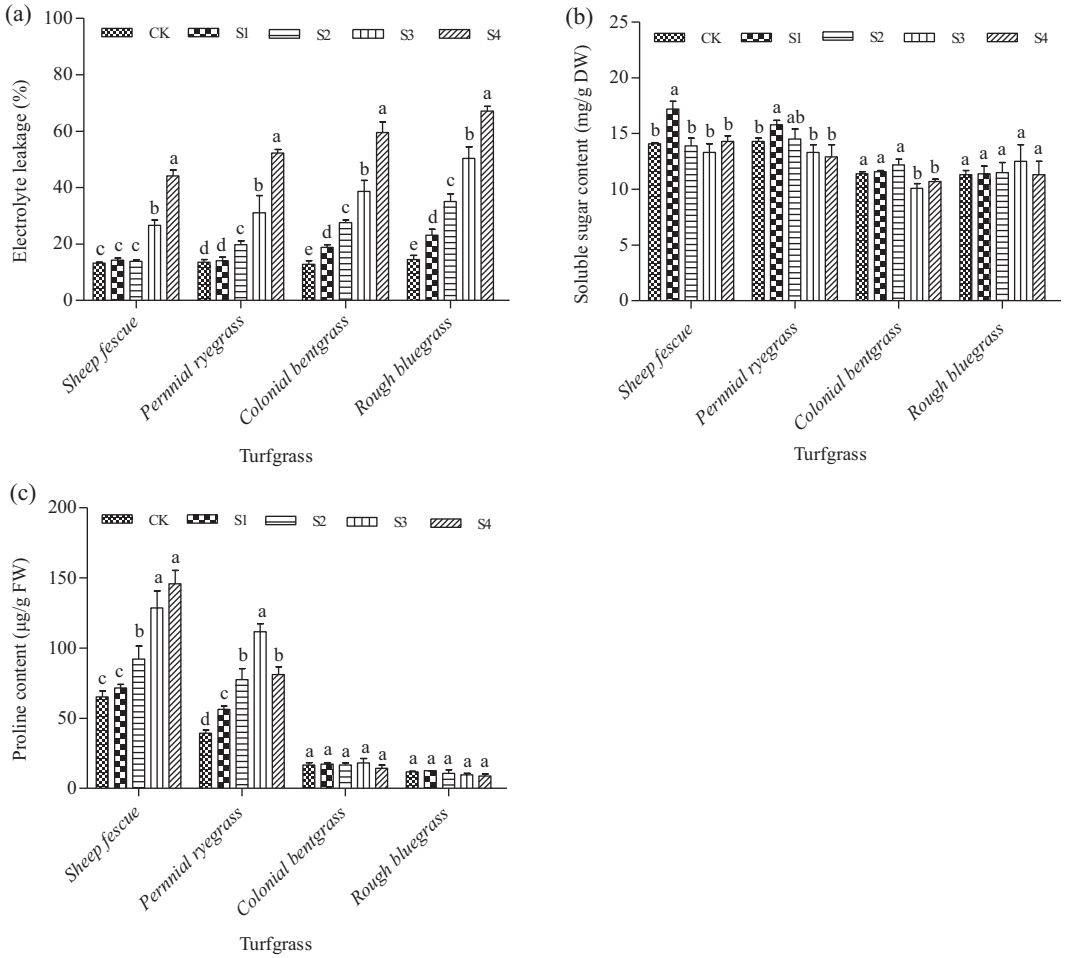

Figure 5. Effect of SO_2 on EL (a), soluble sugar (b), and proline contents (c) in leaves of four cool-season turfgrass species.
Note: Mean values are presented with vertical error bars representing the standard deviations (n = 3). Different letters above the column within the same turfgrass species indicate significant differences among treatments ($P < 0.05$).

sulfur contents were much higher in leaves of sheep fescue and perennial ryegrass than those in leaves of colonial bentgrass and rough bluegrass at high SO_2 concentrations of S3 and S4 (Figure 6a). At the highest SO_2 concentration of S4, sulfur contents increased by 2.5-fold in leaves of sheep fescue and 2.7-fold in leaves of perennial ryegrass, respectively. However, sulfur contents in leaves of colonial bentgrass and rough bluegrass increased by less than 1.5-fold under all SO_2-treated conditions (Figure 6a). SiR activities showed similar changes with sulfur content in all of the four cool-season turfgrass species under all SO_2-treated conditions (Figure 6a and 6b). In leaves of sheep fescue and perennial ryegrass, SiR activities increased by 6.5- and 4.6-folds, respectively, under the highest SO_2 concentration of S4; however, its activities in leaves of colonial bentgrass and rough bluegrass increased by less than 3.5-fold under all SO_2-treated conditions (Figure 6b). SO activities in all of the four cool-season turfgrass species showed no significant changes under any SO_2-treated condition compared with their control without SO_2 treatment (Figure 6c). However, SO activity levels in leaves of sheep fescue and perennial ryegrass were much higher than those in leaves of colonial bentgrass and rough bluegrass (Figure 6c).

Discussion

Sulfur is an essential element for growth and physiological function of plants. Under normal conditions, it is absorbed and assimilated into organic sulfur compounds to participate various biochemical processes in plant cells. SO_2 in the atmosphere can enter plant cells via their stomata during photosynthesis and respiration (Singh et al. 2012). After diffusion into mesophyll, sulfur is converted into sulfite and sulfate ions. Excessive accumulation of sulfate ions in leaf tissue will result in chronic injury, which is characterized by a general chlorotic appearance on leaves, or acute injury, which causes tissue death in marginal or intercostal areas of leaves (Darley & Middleton 1966). In the present study, injury of SO_2 was observed on leaves of all of the four cool-season turfgrass species, but showed different injury degrees. Rough bluegrass and colonial bentgrass were sensitive to SO_2 and visible injury immediately appeared on leaves at low SO_2 concentration of S1. In comparison, only a small amount of yellow leaves was observed in sheep fescue and perennial ryegrass, even at the highest SO_2 concentration. Photosynthetic pigments, especially the chlorophyll and carotenoids, play vital roles in absorbing and converting light energy into

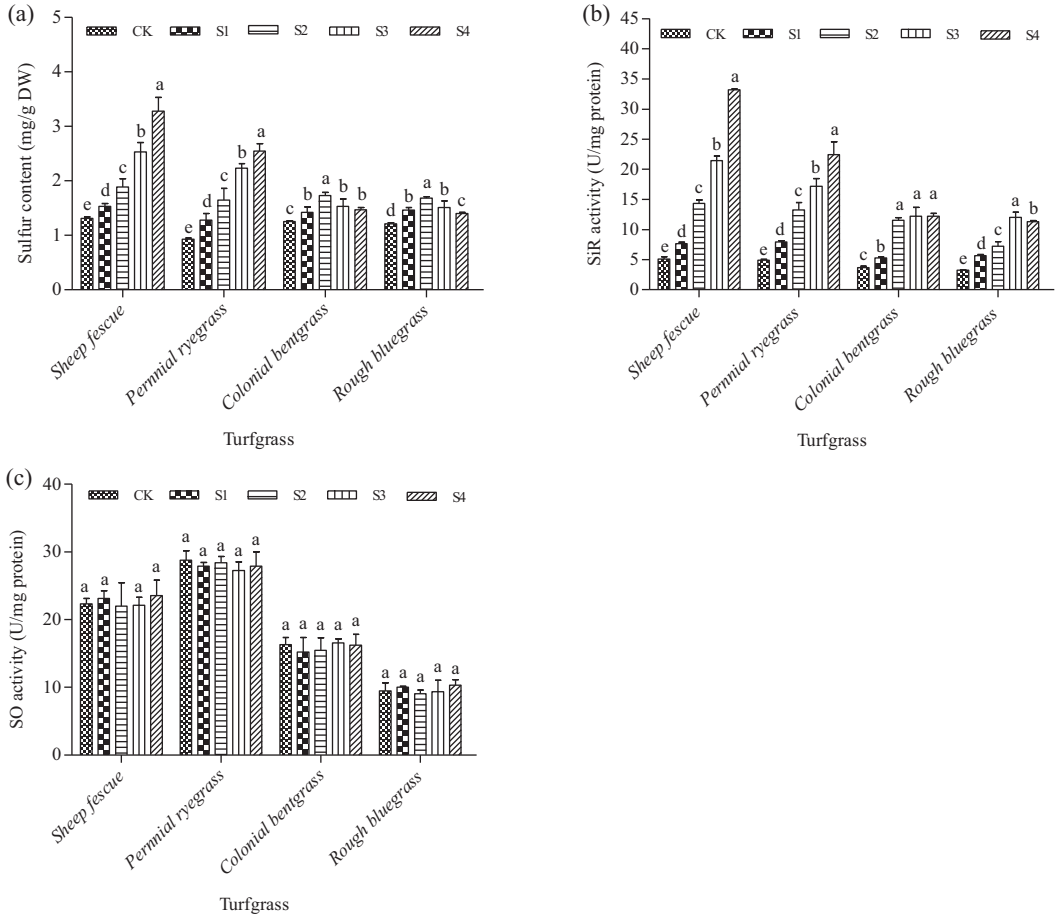

Figure 6. Effect of SO_2 on sulfur content (a), SiR (b), and SO activities (c) in leaves of four cool-season turfgrass species.
Note: Mean values are presented with vertical error bars representing the standard deviations (n = 3). Different letters above the column within the same turfgrass species indicate significant differences among treatments ($P < 0.05$).

biochemical energy in the process of photosynthesis (Pilon et al. 2013). Consistent with injury on leaves, we observed that chlorophyll a, chlorophyll b, and carotenoid contents decreased under SO_2-treated conditions in leaves of all of the four cool-season turfgrass species. However, the SO_2-tolerant turfgrass species (sheep fescue and perennial ryegrass) showed much higher contents of chlorophyll a and carotenoids than the SO_2-sensitive species (rough bluegrass and colonial bentgrass) at high SO_2 concentrations. Chlorophyll a is considered as the primary pigment for photosynthesis in plants, and carotenoids are called as accessory pigment because they cannot transfer sunlight energy directly to the photosynthetic pathway but must pass their absorbed energy to chlorophyll (Lodish et al. 2012). Thereafter, chlorophyll a and carotenoids could be considered as indicators for SO_2 tolerance.

In plant cells, ROS are inevitably and continuously generated as by-products in aerobic metabolism (Cha & Lee 2004). The amounts of ROS are moderate under normal condition, and cells suffer only mild oxidative stress under such condition. However, many exogenous stresses can greatly stimulate generation of ROS, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radical (HO^-; Mohammed & Tarpley 2009), which disrupt physiological processes, and eventually result in cell death under severe stress conditions (Yan et al. 2003). Under such oxidative stress conditions, cell membrane lipids will be peroxidized to yield MDA (Xu & Huang 2009), which is usually considered as an indicator of oxidative-stress-related peroxidation of membrane lipids (Mahan & Mauget 2005). However, plants can scavenge excessive ROS by invoking the non-enzymatic (glutathione, ascorbate, α-tocopherol, and carotenoids) and antioxidant enzymatic defense system such as SOD, POD, and CAT (Darkó et al. 2009). In this study, we observed that ROS levels increased under SO_2 stress conditions. As corresponding defendant responses, activities of antioxidant enzymes increased under such stress conditions. SOD catalyzes the dismutation reaction of $O_2^{-}\cdot$ into H_2O_2 and O_2 (He et al. 2005), before H_2O_2 can be reduced to non-poisonous H_2O and O_2 by CAT and POD, thus preventing their destruction to the cell membrane by ROS (Xu & Huang 2009). Stress responses of antioxidant enzymes of SOD, CAT, and POD to SO_2 were studied on a warm-season turfgrass *C. dactylon* in our previous study, and enzyme activities of three of them were increased under SO_2 stress conditions (Li et al. 2014). In this study, we found that in addition to these three enzymes, GR and GST showed higher activities in all of the four cool-season turfgrass species under SO_2 stress conditions. Strong up-regulated

transcriptional responses of *GSTs* and *GRs* to SO_2 were observed on grape berry (Giraud et al. 2012). More importantly, enzyme activities of the five antioxidants showed an increasing trend along with the increase of SO_2 concentration in leaves of sheep fescue and perennial ryegrass. However, such defendant antioxidant responses in colonial bentgrass and rough bluegrass only appear at mild SO_2 concentrations but not under high SO_2 concentrations. Failure to scavenge excessive ROS by antioxidant enzymes in colonial bentgrass and rough bluegrass caused their accumulation in cells and then destroyed cell membrane at high SO_2 concentrations, as observed in this study and reported previously by others (Tseng et al. 2007, 2008; Li & Yi 2012). Therefore, we suggest that lower ROS levels together with higher antioxidant enzyme activities under SO_2 stress conditions, especially at high SO_2 concentrations, are important factors correlated with SO_2 tolerance in cool-season turfgrass species.

As discussed above, increased MDA level in cool-season turfgrass species indicated that ROS caused damage to membrane lipids by peroxidation under SO_2 stress condition. This membrane damage was indirectly reflected by increase of EL in this study. Soluble sugars and proline, as two major osmotic adjustment substances, play important roles in osmo-protection under multiple stress conditions. Under many stress conditions, soluble sugars such as glucose, sucrose, and fructose can stabilize proteins and membranes of plants by replacing hydrogen bonding through polar residues and preventing denaturation of protein and fusion of membranes (Iturriaga et al. 2009). It is well known that proline not only functions as an osmolyte for osmotic adjustment, but also contributes to stabilizing sub-cellular structure (such as membranes and protein), buffering cellular redox potential, and scavenging free radicals under adverse stress conditions (Ashraf & Foolad 2007). In this study, we found that soluble sugar did not increase significantly in all of the four cool-season turfgrass species, indicating its limited role on SO_2 stress tolerance. However, proline contents increased significantly in SO_2-tolerant turfgrass species (sheep fescue and perennial ryegrass) but not in SO_2-sensitive species (rough bluegrass and colonial bentgrass) at high SO_2 concentrations. Since growth and development of turfgrass species were inhibited at high SO_2 concentrations as discussed above, increased proline in turfgrass could not likely be used for protein biosynthesis but for other functions, such as osmo-protection. Therefore, proline content in turfgrass can be considered as an indicator for selection of SO_2-tolerant turfgrass species.

Once SO_2 in the atmosphere enters plant, it is rapidly converted to sulfite. Sulfite is a highly cytotoxic molecule, which can wreak havoc on the cellular organellae or even the entire plant. In plant cells, sulfite, as an intermediate substance, can be further converted to either sulfide by SiR to enter reductive pathway or sulfate by SO to enter oxidative pathway (Brychkova et al. 2013). Overexpression of SiR or SO in *Arabidopsis thaliana* and/or *Solanum lycopersicum* (tomato) plants increased their tolerance to sulfur dioxide toxicity (Brychkova et al. 2007; Lang et al. 2007; Yarmolinsky et al. 2013). SiR was rapidly induced by SO_2, whereas SO was constitutively expressed and was not significantly induced by SO_2 in *A. thaliana* and tomato plants (Brychkova et al. 2007). In this study, we found that SiR was also significantly induced by SO_2 and SO was constitutively expressed regardless of concentration changes of SO_2. More importantly, we found that SO_2-tolerant turfgrass species (sheep fescue and perennial ryegrass) showed much higher SiR activities than SO_2-sensitive turfgrass species (colonial bentgrass and rough bluegrass) under SO_2 stress conditions, especially at high SO_2 concentrations. As SiR catalyzes a reductive reaction, conversion of cytotoxic sulfite to sulfide by SiR will reduce oxidative stress on plant cells, and then contribute to SO_2 tolerance in turfgrass species. Consequently, SO_2-tolerant turfgrass species showed higher sulfur content in leaves.

Conclusion

In this research, we compared physiological responses of sheep fescue, perennial ryegrass, colonial bentgrass, and rough bluegrass to sulfur dioxide stress by estimating the visible injury, measuring the chlorophyll contents, testing the activities of antioxidant enzymes, measuring the contents of soluble osmoprotectants, and estimating sulfur content and metabolic enzyme activities. Our results showed that SO_2 causes injury symptoms on cool-season turfgrass species in different degree; SOD, CAT, POD, GR, and GST antioxidant enzymes contributed to SO_2 resistance; proline content in cool-season turfgrass species is correlated with SO_2 tolerance; and SiR plays important role on sulfite metabolism and then contributes to SO_2 tolerance. To the best of our knowledge, this is the first physiological study of effect of SO_2 on cool-season turfgrass in details. This study gained some insights into the tolerant mechanisms of cool-season turfgrass to SO_2 toxicity, and provided useful guidelines for selection of turfgrass species suitable for use in SO_2 polluted urban and industrial areas in cold and transition region in cold season.

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