

Research Article

Comparative Analyses of Physiological Responses of *Cynodon dactylon* Accessions from Southwest China to Sulfur Dioxide Toxicity

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Sulfur dioxide (SO₂), a major air pollutant in developing countries, is highly toxic to plants. To achieve better air quality and landscape, planting appropriate grass species in severe SO₂ polluted areas is very critical. *Cynodon dactylon*, a widely used warm season turfgrass species, has good SO₂-tolerant ability. In this study, we selected 9 out of 38 *C. dactylon* accessions from Southwest China as representatives of high, intermediate SO₂-tolerant and SO₂-sensitive accessions to comparatively analyze their physiological differences in leaves under SO₂ untreated and treated conditions. Our results revealed that SO₂-tolerant *C. dactylon* accessions showed higher soluble sugar, proline, and chlorophyll a contents under both SO₂ treated and untreated conditions; higher chlorophyll b and carotenoid under SO₂ treated condition; lower reactive oxygen species (ROS) level, oxidative damages, and superoxide dismutase (SOD) activities under SO₂ treated condition; and higher peroxidase (POD) activities under SO₂ untreated condition. Further results indicated that SO₂-tolerant *C. dactylon* accessions had higher sulfur contents under both SO₂ treated and untreated conditions, consistent with higher SO activities under both SO₂ treated and untreated conditions, and higher SiR activities under SO₂ treated condition. Taken together, our results indicated that SO₂ tolerance of *C. dactylon* might be largely related to soluble sugar, proline and chlorophyll a contents, and SO enzyme activity.

1. Introduction

Sulfur dioxide (SO₂), a gaseous pollutant with bad odor in the atmosphere, is mainly emitted from anthropogenic sources. It is estimated that more than 70% of global SO₂ is emitted from anthropogenic sources, half of which is from combustion of fossil fuel [1]. With rapid development of economy in developing countries, emission of SO₂ into the atmosphere has been increasing quickly. As the biggest developing country in the world, China is leading the world as the biggest SO₂ emitter, contributing to about one-fourth of the global emission and more than 90% of East Asia emission since the 1990s [2]. Total SO₂ emission in China increased by 53%, from 21.7 Tg (1 Tg = 10¹² g) in 2000 to 33.2 Tg in 2006, at an annual growth rate of 7.3%. The SO₂ emission began to decrease after 2006 mainly due to the widespread application of flue-gas desulfurization (FGD) devices at all newly built

thermal power units in order to implement a comprehensive national policy strategy of energy conservation and emission reduction since 2005. However, the total SO₂ emissions are still very high (27.7 Tg) in 2010 due to the dramatic growth of industrial production and energy consumption [3]. Thereafter, high level of SO₂ in the atmosphere will be a major concern in developing countries in a long period of forthcoming time.

In the atmosphere, when gaseous SO₂ meets with water, considerable amounts of SO₂ are converted to sulphurous acid, which is the important component of acid rain. Sulfur is well known to be a basic constituent of sulfur-containing amino acids, iron-sulfur clusters, cofactors, polysaccharides, and lipids for all living organisms. SO₂ can enter plants via their stomata by the process of photosynthesis and respiration [4]. Plant has the ability to incorporate this kind of inorganic sulfur into sulfur-containing amino acids, proteins, and

glutathione (GSH) and sulfur can also serve as the sulfur precursor of sulfur-containing secondary products in plant. However, above a certain threshold, both SO_2 and acid rain are highly toxic to plants, causing many visible symptoms in the plant like yellowing, chlorosis, bleaching, and even killing foliage depending on the dosages [4]. Because of the harmful effects of SO_2 , some plants cannot grow robustly and even die in severe polluted urban or industrial districts, creating “dead zones” without greenery. To achieve better air quality and landscape effect in such polluted areas, the plants with high resistance to SO_2 should be selected out for use. Tree species tolerant to SO_2 were selected out or developed for planting in air polluted areas [5–7].

Turfgrasses were extensively used in a sole manner or in combination with trees for environmental greening. Importantly, grass plants are more resistant to SO_2 than woody plants, because the former have a higher S:C ratio than the latter and therefore can take up more SO_2 from the atmosphere [8]. Turfgrasses can be generally classified as cool season, warm season, or evergreen types. A few studies on tolerance to SO_2 of cool-season grass populations in polluted areas have been carried out in the past decades. These studies mainly focused on identification of tolerant populations from cool-season species of *Dactylis glomerata*, *Festuca rubra*, *Holcus lanatus*, *Lolium perenne*, and *Phleum bertolonii* [9]; comparison of stomatal morphology and resistance, membrane permeability, and the uptake and metabolism of $^{35}\text{SO}_3$ and $^{35}\text{SO}_2$ in cool-season species of *D. glomerata*, *F. rubra*, *H. lanatus*, and *L. perenne* [10]; investigation on the rate of development of tolerance in cool-season species of *F. rubra*, *L. multiflorum*, *L. perenne*, *P. pratense*, and *Poa pratensis* [11]; and genetic nature of tolerance in cool-season species of *L. perenne* [12]. In such studies, *Cynodon dactylon*, a warm season perennial grass species, is not included, which is widely used as turfgrass on sports fields, golf courses, roadsides, and lawns in city or industry districts in warm season. Recently, our comparative study on physiological and growth performances found that *C. dactylon* displayed the highest resistance to SO_2 among four warm season turfgrasses including *C. dactylon*, *Eremochloa ophiuroides*, *Paspalum notatum*, and *Zoysia japonica* [13]. In the present study, we firstly compared influences of SO_2 on leaves of 38 wild *C. dactylon* accessions from Southwest China. Based on injury rate of SO_2 to leaves, nine *C. dactylon* accessions representing high SO_2 -tolerant, intermediate SO_2 -tolerant, and SO_2 -sensitive to SO_2 accessions were selected to comparatively study relationships between SO_2 tolerance and several physiological parameters. This study gained some insights into understanding the genetic and molecular mechanisms of *C. dactylon* to SO_2 and provided guideline for selection and development of *C. dactylon* variations for planting in SO_2 polluted urban or industrial areas.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions. Thirty-eight wild *C. dactylon* accessions used in this study were sampled from Sichuan Province, Chongqing municipality, Yunnan



FIGURE 1: The collection sites of the 38 samples of wild *C. dactylon* in Southwest China. Provinces where samples were collected are illustrated with number of *C. dactylon* accessions in parentheses underneath in map.

Province, Guizhou Province, and Tibet Autonomous Region in Southwestern China between years 2011 and 2012. A complete list of accession descriptions and geographical origins was provided in Table 1 and Figure 1. The wild *C. dactylon* accessions were collected originally from roadside, riverside, floodland, fieldridge, wasteland, hillside, or city park. All wild accessions used in this study were determined to be *C. dactylon* based on morphological characteristics as described by Harlan [14].

The experiments were carried out between April and August, 2013, at Experimental Station of Grass Science, Sichuan Agricultural University, Ya'an, Sichuan Province, China. The experimental location is 600 m in altitude with a humid subtropical climate. Mean annual precipitation, annual temperature, and relative air humidity in the area are 1800 mm, 16.2°C, and 79%, respectively. All the *C. dactylon* accessions were planted in plastic pots (18 cm in top diameter, 14 cm in bottom diameter, and 15 cm in depth) filled with typical sandy loam soil in the local place in April. Each *C. dactylon* accession was replicated six times. All *C. dactylon* grasses were grown under natural conditions for 2 months with regular watering every day and fertilizing and cutting every four weeks prior to the experimental treatment.

2.2. Stress Treatment and Experimental Design. After two-month growth, three pots of grass plants with nearly the same crown from each accession were chosen from six replications (as mentioned above) for SO_2 stress treatment. All of the selected pots of grass plants were fumigated with SO_2 at a concentration of 3.75 mg/L in a custom-made fumigation chamber (85 cm × 85 cm × 40 cm) for 3 h per day over 7 days as described in our previous study [13]. The day when SO_2 fumigation started was designated as day 0. In order to achieve a uniform environment in the chamber, a fan was attached to the chamber ceiling to mix the SO_2 . A SO_2 gas detector (Z-1300, Environmental Sensors Co., Boca Raton,

TABLE 1: Geographical origin of 38 wild *C. dactylon* accessions used in this study.

Order	Accession number	Origin	Habitat	Altitude (m)	Mean annual temperature (°C)
1	SCI102	Pixian, Sichuan	Roadside	560	18.5
2	SCI105	Longquan, Sichuan	Hillside	750	16.5
3	SCI106	Shuangliu, Sichuan	Riverside	510	16.3
4	SCI109	Guangyuan, Sichuan	Roadside	490	16.1
5	SCI115	Zitong, Sichuan	Roadside	610	16.5
6	SCI119	Guanghan, Sichuan	Riverside	420	16.3
7	SCI201	Leshan, Sichuan	Floodland	420	17.4
8	SCI203	Wuhou, Sichuan	City park	540	16.7
9	SCI208	Luxian, Sichuan	Riverside	380	17.8
10	SCI209	Nanchong, Sichuan	Hillside	520	17.4
11	SCI211	Guang'an, Sichuan	Roadside	450	17.1
12	SCI213	Panzhihua, Sichuan	Roadside	1150	20.3
13	SCI217	Meishan, Sichuan	Fieldridge	460	17.1
14	CQ1101	Jiangjin, Chongqing	Roadside	590	18.4
15	CQ1102	Yongchuan, Chongqing	Floodland	340	18.2
16	CQ1107	Bishan, Chongqing	Roadside	530	18.3
17	CQ1108	Jiangbei, Chongqing	Roadside	440	17.5
18	CQ1109	Dianjiang, Chongqing	Fieldridge	420	17.0
19	CQ1112	Liangping, Chongqing	Roadside	520	16.6
20	CQ1116	Wanzhou, Chongqing	Riverside	180	17.7
21	YN1102	Kunming, Yunnan	City park	1910	15.0
22	YN1105	Chuxiong, Yunnan	Roadside	1790	15.8
23	YN1106	Dali, Yunnan	Roadside	1980	15.1
24	YN1107	Lijiang, Yunnan	Riverside	2360	15.8
25	YN1110	Baoshan, Yunnan	Wasteland	2410	16.0
26	YN1201	Yuxi, Yunnan	Roadside	1890	18.2
27	YN1205	Pu'er, Yunnan	Roadside	1750	17.7
28	YN1208	Xishuangbanna, Yunnan	Roadside	890	21.0
29	GZ1103	Guiyang, Guizhou	Riverside	970	15.3
30	GZ1104	Anshun, Guizhou	Roadside	860	14.0
31	GZ1106	Liupanshui, Guizhou	Fieldridge	1810	13.5
32	GZ1109	Zunyi, Guizhou	Roadside	960	15.1
33	GZ1110	Qianlan, Guizhou	Hillside	1050	16.1
34	XZ1205	Lhasa, Tibet	Roadside	3120	7.5
35	XZ1206	Nyingchi, Tibet	Roadside	3430	8.7
36	XZ1208	Bomi, Tibet	Riverside	2680	8.7
37	XZ1209	Baxoi, Tibet	Roadside	3250	10.4
38	XZ1213	Chamdo, Tibet	Floodland	3170	7.6

FL, USA) was used to measure the concentration of SO₂ and to keep the gas concentration constant in the chamber during the experiment. After fumigation treatment, grass plants were taken out and grown under natural conditions with regular watering every day. The remaining three pots of grass plants without SO₂ treatment from each accession served as control. Based on injury rate of SO₂ to leaves after 7-day treatment of SO₂, three high SO₂-tolerant, three intermediate SO₂-tolerant, and three SO₂-sensitive *C. dactylon* accessions were selected out for physiological studies. Leaves 2 cm above the soil from *C. dactylon* plants treated by SO₂ after 7 days were collected and brought back to laboratory for analysis. Leaves

from *C. dactylon* plants without SO₂ treatment at day 0 served as control.

2.3. Measurement of Total Soluble Sugars. The total soluble sugars were determined using the anthrone method as previously described by Lu et al. [15] with some modifications. Briefly, 0.2 mg dried leaf samples were extracted in 5 mL of 80% (v/v) ethanol at 80°C for 40 min and centrifuged at 15,000 ×g for 10 min. The pellets were further extracted twice with another 5 mL of 80% (v/v) ethanol. The supernatants were combined together and depigmented by activated charcoal at 80°C for 30 min. For the determination

of soluble total sugars, 0.2 mL of the filtrate was mixed with 3 mL of 0.15% (w/v) anthrone reagent (0.3 g anthrone was dissolved in 200 mL of 7.74 M H_2SO_4) and then heated at 90°C for 20 min. Finally, soluble total sugar level was determined at 620 nm of absorbance using a UV/VIS spectrophotometer Model 723PC (Jinghua Instruments, Shanghai, China).

2.4. Measurement of Proline Content. Proline content was estimated according to the method based on proline's reaction with ninhydrin described by Bates et al. [16] with modification. Briefly, 0.2 g leaf samples were ground in 5 mL 3% (w/v) sulfosalicylic acid and then filtered through 0.45 μ m filter paper. Two microliters of filtrate was mixed with equal volumes of ninhydrin reagent and glacial acetic acid. Well mixed solutions were boiled at 100°C for 1 h. The reaction was terminated in an iced bath and the chromophore was extracted with 4 mL toluene and its absorbance at 520 nm was determined using a UV/VIS spectrophotometer Model 723PC (Jinghua Instruments, Shanghai, China).

2.5. Estimation of Chlorophyll and Carotenoid. Photosynthetic pigments from the leaves were extracted as described by Lichtenthaler and Wellburn [17] with modification. Leaf samples (~0.2 g) were ground in 2 mL of 80% acetone and ethyl alcohol (1:1), using a mortar and pestle, and then filtered through 0.45 μ m filter paper. Absorbance of the resulting extracts was measured at three wavelengths 663, 646, and 470 nm for chlorophyll a, chlorophyll b, and carotenoids, respectively, using a UV/VIS spectrophotometer Model 723PC (Jinghua Instruments, Shanghai, China). The amounts of pigments were calculated according to the equations developed by Lichtenthaler and Wellburn [17]. Total chlorophyll was obtained from the sum of chlorophylls a + b.

2.6. Determination of H_2O_2 Level. For grass protein extraction, about 0.2 g fresh leaves were ground with liquid nitrogen and then homogenized in extraction buffer (50 mM sodium phosphate buffer, pH 7.8). After centrifugation at 15,000 \times g for 15 min at 4°C, the supernatant was used for determination of H_2O_2 levels as described by Hu et al. [18]. Briefly, 1 mL of the supernatant was mixed with 1 mL of 0.1% titanium sulphate in 20% H_2SO_4 (v/v) thoroughly for 10 min. After being centrifuged at 15,000 \times g for 10 min at room temperature, the absorbance of the supernatant was measured at 410 nm using a UV/VIS spectrophotometer Model 723PC (Jinghua Instruments, Shanghai, China).

2.7. SOD, POD, CAT, SiR, and SO Enzyme Assays. Fresh leaf sample (~0.5 g) was homogenized in 5 mL of 0.1 M phosphate buffer (pH 6.8) containing 1 mM EDTA, 1 mM dithiothreitol, and 2% (w/v) polyvinylpyrrolidone (PVP) using a chilled mortar and pestle on ice. The homogenate was centrifuged at 15,000 \times g for 15 min at 4°C, and the supernatant was used for enzyme activity. Soluble protein content was determined following the Bradford method [19] with BSA as standard. Superoxide dismutase (SOD) activity was determined spectrophotometrically at 560 nm based on the measurement

of inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) [20, 21]. Peroxidase (POD) activity was determined by the guaiacol oxidation method [22]. Catalase (CAT) activity was determined by measuring the rate of decomposition of H_2O_2 at 240 nm, as described by Aebi [23]. Sulfite reductase (SiR) activity was estimated by the coupled SiR/OASTL assay [24, 25] with the addition of NADPH and tungstic acid [26]. Sulfite oxidase (SO) activity was determined by measuring sulphite disappearance using OH-mediated discolouring of fuchsine according to Pachmayr's report [27].

2.8. Estimation of MDA Content. Malondialdehyde (MDA) content was determined using the method described by Fu and Huang [28]. Fresh leaf sample (0.2 g) was homogenized with 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) using a chilled mortar and pestle on ice. The homogenate was centrifuged at 15,000 \times g for 20 min, at 4°C, and the supernatant was used for lipid peroxidation analysis. A total of 4 mL of 0.5% thiobarbituric acid (TBA) in 20% 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 \times g for 20 min. Absorbance was measured at 532 and 600 nm, and MDA concentration was estimated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm.

2.9. Estimation of Sulfur Content. For sulfur (S) determination, the turbidimetric method described by Reyes-Díaz et al. [29] was applied. Biomass of whole plant dried for 48 h was treated with 95% magnesium nitrate and ashed at 500°C for 8 h. Then the ashed samples were digested in 10 mL of 2 M HCl at 150°C for 60 min. After addition of barium chloride ($BaCl_2$) and Tween-80 into the solution, its absorbance was immediately measured using a UV/VIS spectrophotometer Model 723PC (Jinghua Instruments, Shanghai, China) at 440 nm.

2.10. Statistical Analysis. All experiments in this study were repeated at least three times. Statistical analysis (mean \pm standard error) was performed and chart was created using relative tools of Microsoft Excel 2010. All data were analyzed by ANOVA using SPSS 13.0 software package (SPSS Inc., Chicago, USA), and then LSD method was used to detect possible differences among the accessions. Asterisk symbols above the columns in the figures indicate significant differences at $P < 0.05$ (Student's t -test).

3. Results

3.1. Leaf Injury under SO_2 Stress Condition. After 7-day fumigation treatment by SO_2 , injury symptoms appeared on leaves of all the 38 *C. dactylon* accessions. The visible symptoms consisted of bifacial, marginal, or interval necrosis and chlorosis on leaves at the full stage of development (Figure 2). The necrotic areas ranged from white to brown in color, and the margins of the necrotic areas are mostly irregular and occasionally dark in color. Injury rate of leaves

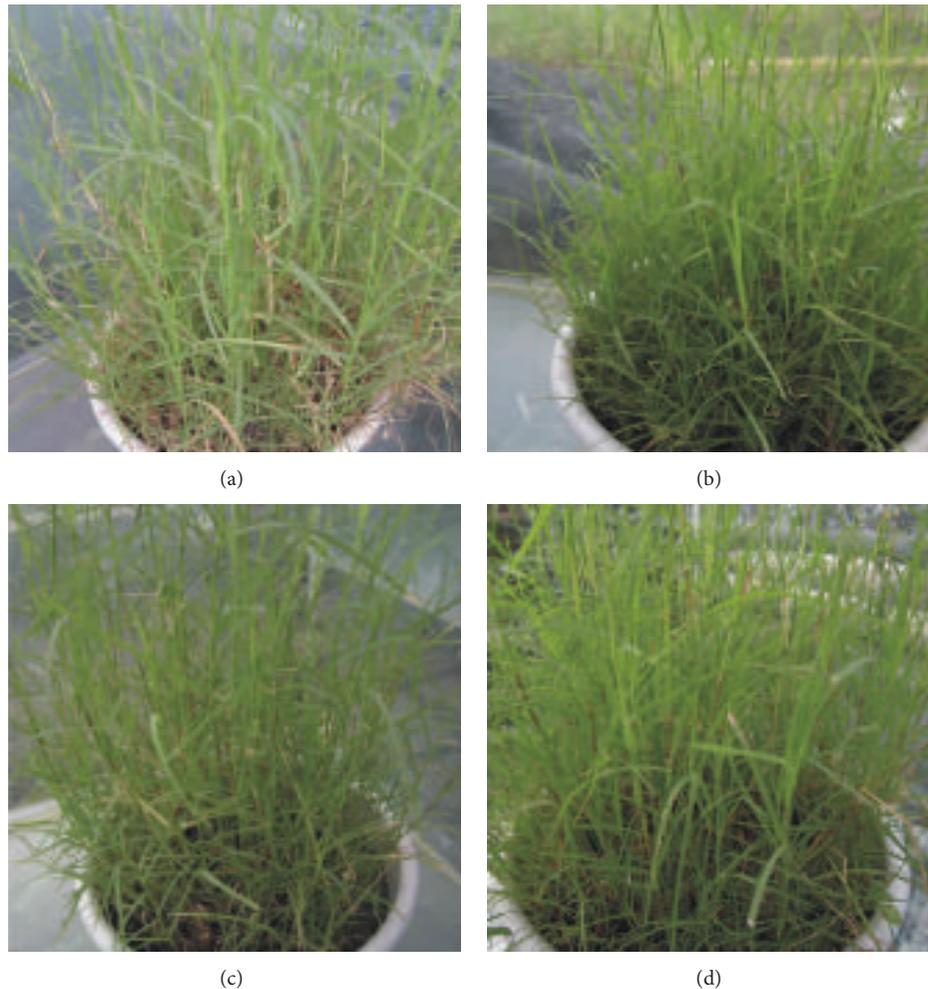


FIGURE 2: Symptoms of *C. dactylon* accessions in response to SO_2 . (a) SO_2 -sensitive representative *C. dactylon* accession CQ1116, (b) intermediate SO_2 -tolerant representative *C. dactylon* accession SC1217, (c) high SO_2 -tolerant representative *C. dactylon* accession SC1203, and (d) *C. dactylon* accession CQ1116 without SO_2 treatment as a control.

varied in *C. dactylon* accessions from 38.3% in accession YN1205 to 13.3% in accession SC1203 (Table 2). It seemed that accessions originated from city park and hillside had higher SO_2 tolerance than other habitat origins (Tables 1 and 2). To further study the physiological response of *C. dactylon* to SO_2 , we selected three accessions of SC1203, SC1209, and GZ1110 as high SO_2 -tolerant representatives, three accessions of SC1217, YN1110, and XZ1206 as intermediate SO_2 -tolerant representatives, and three accessions of YN1205, CQ1116, and SC1208 as SO_2 -sensitive representatives based on the injury rate of leaves and the geographic distribution (Tables 1 and 2).

3.2. Changes of Sugar and Proline under SO_2 Stress Condition. The soluble sugar and proline contents in leaves from all of the nine *C. dactylon* accessions increased along with the increase of their SO_2 tolerability (Figure 3). Moreover, the soluble sugar and proline contents from all of the high SO_2 -tolerant *C. dactylon* accessions and intermediate SO_2 -tolerant *C. dactylon* accessions were significantly higher than those

from any of the three SO_2 -sensitive *C. dactylon* accessions at both 0-day time-point without SO_2 treatment and 7-day time-point after SO_2 fumigation treatment. However, the soluble sugar and proline contents from 7-day time-point after SO_2 fumigation treatment showed no significant change when they were compared with those from 0-day time-point in any *C. dactylon* accession, which indicates that both soluble sugar and proline are not induced or inhibited in *C. dactylon* under SO_2 stress condition (Figure 3).

3.3. Changes of Photosynthetic Pigments under SO_2 Stress Condition. Contents of photosynthetic pigments in leaves from all of the nine *C. dactylon* accessions decreased under SO_2 stress condition but showed different patterns with different pigment (Figure 4). Chlorophyll a contents from two intermediate SO_2 -tolerant *C. dactylon* accessions (YN1110 and XZ1206) and from all of the three high SO_2 -tolerant *C. dactylon* accessions were significantly higher than those from any of SO_2 -sensitive *C. dactylon* accessions in an increasing trend along with the increase of SO_2 tolerability at

TABLE 2: Leaf injury rate of 38 wild *C. dactylon* accessions under SO₂ stress condition.

Order	Accession number	Injury rate (%)
1	YN1205	38.3 ± 0.6
2	CQ1116	37.7 ± 1.2
3	SC1208	36.7 ± 0.6
4	SC1106	35.3 ± 1.5
5	GZ1109	34.3 ± 1.5
6	YN1107	32.0 ± 1.7
7	YN1208	31.7 ± 0.6
8	SC1213	30.3 ± 1.5
9	CQ1112	30.3 ± 1.2
10	XZ1209	28.7 ± 1.2
11	XZ1213	28.3 ± 0.6
12	GZ1104	27.3 ± 1.5
13	SC1119	26.3 ± 1.5
14	SC1201	26.3 ± 2.1
15	YN1106	25.7 ± 1.5
16	XZ1205	25.7 ± 2.5
17	CQ1102	25.3 ± 0.6
18	SC1217	25.3 ± 1.5
19	YN1110	25.0 ± 1.0
20	XZ1206	24.7 ± 0.6
21	SC1211	24.7 ± 2.5
22	YN1105	23.7 ± 2.1
23	XZ1208	23.0 ± 1.7
24	GZ1106	22.0 ± 1.0
25	SC1102	21.7 ± 2.1
26	SC1115	20.0 ± 2.6
27	CQ1108	20.0 ± 2.6
28	CQ1109	19.3 ± 1.5
29	YN1201	19.3 ± 0.6
30	SC1109	19.3 ± 1.5
31	GZ1103	19.0 ± 2.0
32	CQ1107	18.7 ± 1.5
33	CQ1101	17.7 ± 1.5
34	YN1102	17.3 ± 1.5
35	SC1105	16.3 ± 1.5
36	GZ1110	15.0 ± 2.0
37	SC1209	13.7 ± 0.6
38	SC1203	13.3 ± 1.5

Note: data are presented by means ± SE (n = 3).

0-day time-point (Figure 4(a)). Under SO₂ stress condition, chlorophyll a contents in leaves from intermediate and high SO₂-tolerant *C. dactylon* accessions reduced significantly less than those from SO₂-sensitive *C. dactylon* accessions. No significant differences of chlorophyll b contents were observed among the high SO₂-tolerant, intermediate SO₂-tolerant, and SO₂-sensitive *C. dactylon* accessions at 0-day time-point (Figure 4(b)). After 7-day stress treatment by SO₂ fumigation, chlorophyll b contents reduced in leaves from all of the nine *C. dactylon* accessions. The contents of chlorophyll b showed no significant differences between intermediate

SO₂-tolerant and SO₂-sensitive *C. dactylon* accessions, but significantly less reduction of chlorophyll b content was observed in high SO₂-tolerant *C. dactylon* accessions. As for total chlorophyll content, it showed a similar pattern with chlorophyll a in leaves from all of the nine *C. dactylon* accessions (Figure 4(c)). Carotenoid contents showed no significant differences among the high SO₂-tolerant, intermediate SO₂-tolerant, and SO₂-sensitive *C. dactylon* accessions at 0-day time-point but significantly less reduced along with the increase of SO₂ tolerability of *C. dactylon* accessions after 7-day SO₂ stress treatment (Figure 4(d)).

3.4. Changes of ROS Level and Antioxidant Enzyme Activities under SO₂ Stress Condition. As two major indicators for reactive oxygen species (ROS) level and oxidative damage, hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) contents were tested in this study. As shown in Figure 5, the high SO₂-tolerant, intermediate SO₂-tolerant, and SO₂-sensitive *C. dactylon* accessions displayed nearly the same levels of H₂O₂ and MDA in leaves at 0-day time-point without SO₂ treatment (Figures 5(a) and 5(b)). After 7-day SO₂ fumigation treatment, levels of both H₂O₂ and MDA increased in leaves from all of the nine *C. dactylon* accessions. When compared within all of the nine *C. dactylon* accessions, levels of both H₂O₂ and MDA in leaves from high SO₂-tolerant *C. dactylon* accessions and intermediate SO₂-tolerant *C. dactylon* accessions were significantly lower than those from SO₂-sensitive *C. dactylon* accessions (Figures 5(a) and 5(b)).

To address the relationship between the changes of ROS level and the antioxidant enzyme activities, three major antioxidant enzymes, including SOD, POD, and CAT, were analyzed for their enzyme activities. SOD activities showed no significant differences (about 30 U/g protein FW) in leaves from high SO₂-tolerant *C. dactylon* accessions, intermediate SO₂-tolerant *C. dactylon* accessions, and SO₂-sensitive *C. dactylon* accessions at 0-day time-point without SO₂ treatment (Figure 6(a)). After 7-day SO₂ stress treatment, SOD activities increased greatly in leaves from all of the nine *C. dactylon* accessions. However, the increase degree was in a decreasing trend along with SO₂ tolerability of the *C. dactylon* accessions, displaying highest activities in SO₂-sensitive *C. dactylon* accessions (more than 120 U/g protein FW) and lowest activities in high SO₂-tolerant *C. dactylon* accessions (more than 60 U/g protein FW) (Figure 6(a)). POD activities increased in leaves from all of the nine *C. dactylon* accessions after 7-day SO₂ stress treatment, but no significant differences were observed within high SO₂-tolerant *C. dactylon* accessions, intermediate SO₂-tolerant *C. dactylon* accessions, and SO₂-sensitive *C. dactylon* accessions (Figure 6(b)). However, we found that POD activities in leaves from SO₂-tolerant *C. dactylon* accessions were significantly higher than those from SO₂-sensitive *C. dactylon* accessions in an increasing trend along with an increase of SO₂ tolerability at 0-day time-point without SO₂ treatment, displaying nearly 1.4-fold increase (15433/11183 U/g protein FW) and 1.7-fold increase (18866/11183 U/g protein FW) of enzyme activities in intermediate SO₂-tolerant and

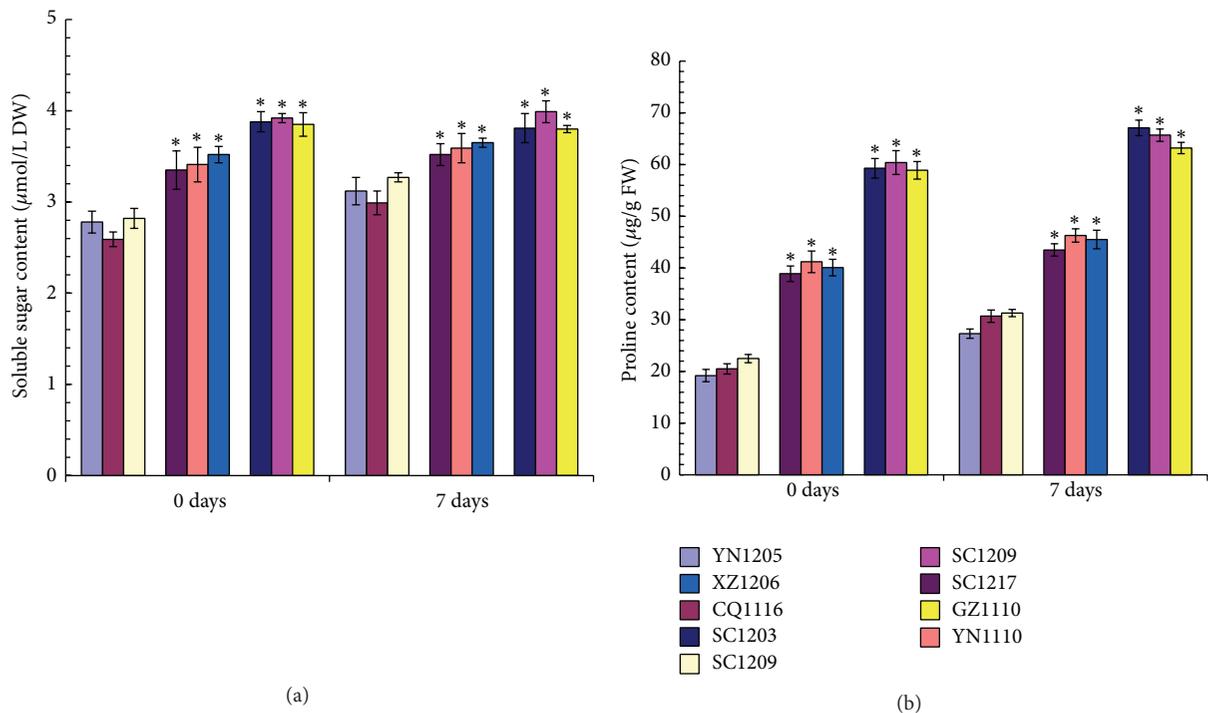


FIGURE 3: Comparison of soluble sugar (a) and proline (b) in leaves from nine selected *C. dactylon* accessions in response to SO₂ stress. Mean values are presented with vertical error bars representing the standard deviations ($n = 3$). The asterisk symbols indicate significant differences between SO₂-sensitive accessions and SO₂-tolerant accessions.

high SO₂-tolerant *C. dactylon* accessions, respectively (Figure 6(b)). CAT activities increased in leaves from all of the nine *C. dactylon* accessions after 7-day SO₂ stress treatment, but no significant differences were observed in leaves from high SO₂-tolerant *C. dactylon* accessions, intermediate SO₂-tolerant *C. dactylon* accessions, and SO₂-sensitive *C. dactylon* accessions either after 7-day SO₂ stress treatment or at 0-day without SO₂ treatment (Figure 6(c)).

3.5. Changes of Sulfur Content, SiR, and SO Enzyme Activities under SO₂ Stress Condition. Sulfur contents in leaves from two intermediate SO₂-tolerant *C. dactylon* accessions (YN1110 and XZ1206) and all of the three high SO₂-tolerant *C. dactylon* accessions were significantly higher than those from any of the SO₂-sensitive *C. dactylon* accessions at 0-day time-point without SO₂ stress treatment (Figure 7(a)). After 7-day SO₂ fumigation treatment, sulfur contents increased in leaves from all of the nine *C. dactylon* accessions in an increasing trend along with increase of SO₂ tolerability of the *C. dactylon* accessions. Moreover, sulfur contents in leaves from all of the high and intermediate SO₂-tolerant *C. dactylon* accessions showed significantly higher levels than those from any of the SO₂-sensitive *C. dactylon* accessions (Figure 7(a)). SiR activities were nearly in the same levels (about 5 U/mg protein FW) in leaves from all of the nine *C. dactylon* accessions at 0-day time-point without SO₂ treatment (Figure 7(b)). After 7-day SO₂ stress treatment, SiR activities increased about 2-fold (approximate 10 U/mg protein FW) in SO₂-sensitive *C. dactylon* accessions, 2.4-fold (approximate 12 U/mg protein FW) in intermediate SO₂-tolerant *C. dactylon* accessions, and

3.4-fold (approximate 17 U/mg protein FW) in high SO₂-tolerant *C. dactylon* accessions, respectively. More importantly, SiR activities showed significantly higher levels in leaves from high and intermediate SO₂-tolerant *C. dactylon* accessions than those from SO₂-sensitive *C. dactylon* accessions, displaying an apparent increasing trend along with SO₂ tolerability of the *C. dactylon* accessions (Figure 7(b)). SO activities in leaves from any of *C. dactylon* accessions after 7-day SO₂ fumigation treatment showed nearly the same level with those from 0-day time-point without SO₂ treatment (Figure 7(c)). However, SO activity levels were significantly higher in leaves from high and intermediate SO₂-tolerant *C. dactylon* accessions than those from SO₂-sensitive *C. dactylon* accessions, displaying an apparent increasing trend along with SO₂ tolerability of the *C. dactylon* accessions (Figure 7(c)).

4. Discussion

SO₂, a major air pollutant in developing countries, is highly toxic to plants once they are exposed to high doses of SO₂ above the threshold. *C. dactylon* is a widely used warm season turfgrass on sports fields, golf courses, roadsides, and lawns in city or industry districts. Our previous study indicated that growth rate of *C. dactylon* was affected and visible symptoms appeared on leaves under SO₂ stress condition; however this species has much better SO₂-tolerant ability among warm season turfgrasses [13]. *C. dactylon* is widely distributed in South America, Africa, Europe, and South Asia

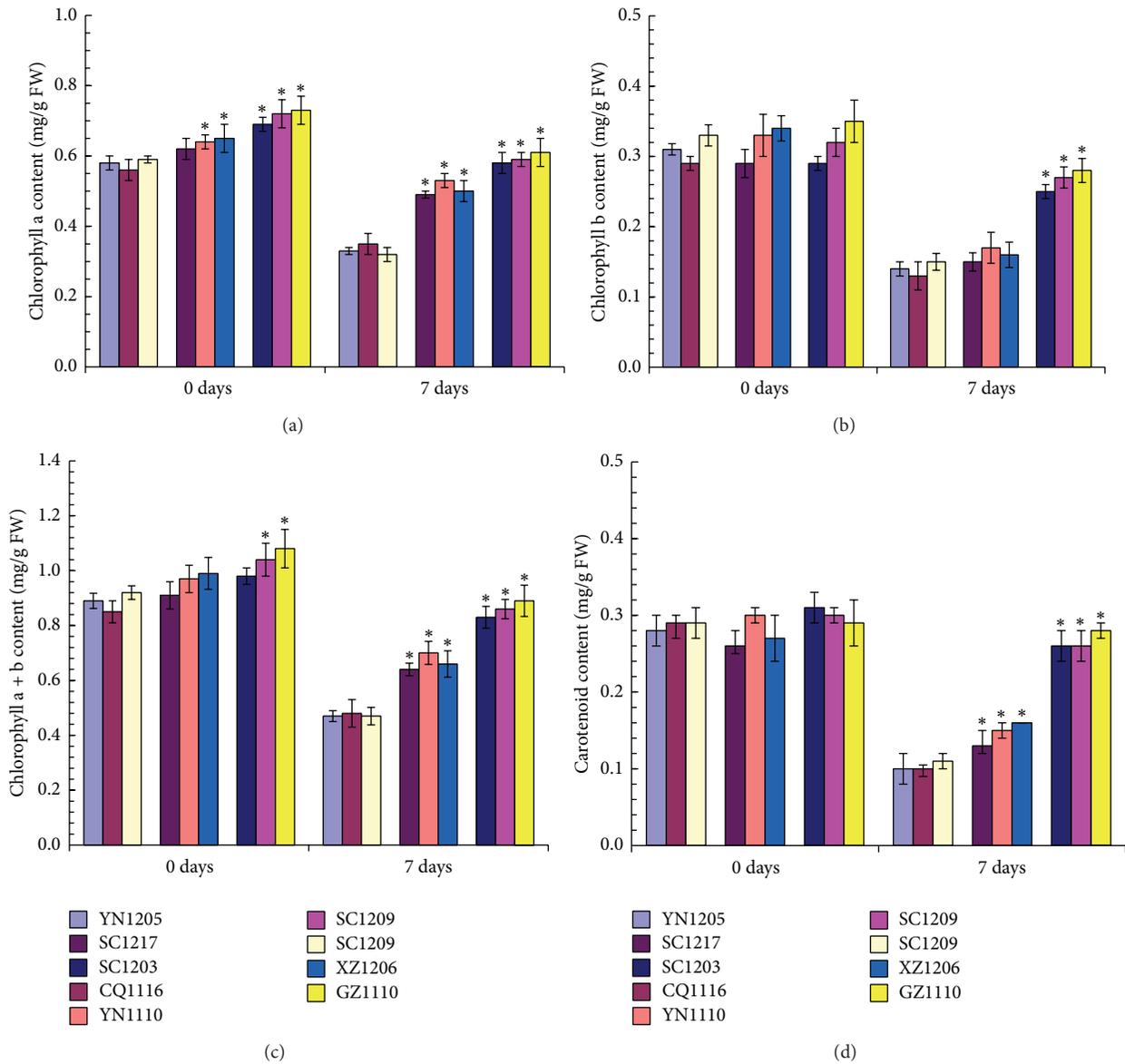


FIGURE 4: Comparison of chlorophyll a (a), chlorophyll b (b), total chlorophyll (c), and carotenoid (d) in leaves from nine selected *C. dactylon* accessions in response to SO_2 stress. Mean values are presented with vertical error bars representing the standard deviations ($n = 3$). The asterisk symbols indicate significant differences between SO_2 -sensitive accessions and SO_2 -tolerant accessions.

and displays abundant genetic diversities worldwide [30–33]. To achieve better air quality and landscape effect in SO_2 polluted areas, selection or development of high SO_2 -tolerant *C. dactylon* variations for planting in such regions is desired. In this study, we selected 9 out of 38 wild *C. dactylon* accessions from Southwest China as representatives of high, intermediate SO_2 -tolerant, and SO_2 -sensitive accessions based on the injury degree of SO_2 to leaves and the geographic distribution and then comparatively analyzed their physiological differences under SO_2 untreated and treated conditions. Our results indicated that SO_2 tolerance of *C. dactylon* might be largely related to soluble sugar, proline and chlorophyll a contents, and SO enzyme activities. To the best of our knowledge, this is the first comprehensive

study of physiological differences in *C. dactylon* accessions of warm season turfgrasses. This study gained some insights into understanding the genetic and molecular SO_2 -tolerant mechanisms of *C. dactylon* and provided guideline for selection and development of *C. dactylon* variations for planting in SO_2 polluted urban or industrial areas.

Soluble sugars and proline, as two major compatible solutes in the cytoplasm and organelle, play important roles under multiple stress conditions, such as drought and salinity [34, 35]. In this study, we observed that SO_2 -tolerant *C. dactylon* accessions showed significantly higher soluble sugar and proline contents under both SO_2 treated and untreated conditions (Figure 3), suggesting that both of them might be related to SO_2 tolerance. *C. dactylon* accessions originated

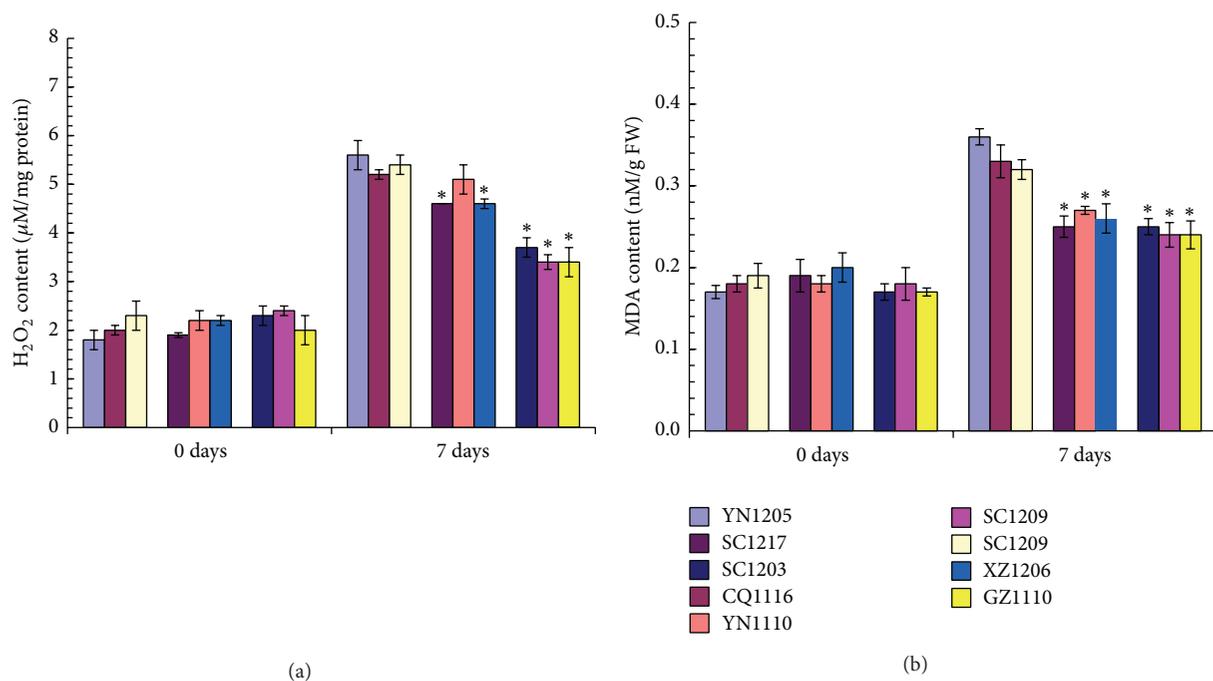


FIGURE 5: Comparison of ROS levels reflected by H_2O_2 (a) and MDA (b) contents in leaves from nine selected *C. dactylon* accessions in response to SO_2 stress. Mean values are presented with vertical error bars representing the standard deviations ($n = 3$). The asterisk symbols indicate significant differences between SO_2 -sensitive accessions and SO_2 -tolerant accessions.

from habitats of hillside and city park have much higher SO_2 tolerance than those from other habitats (Tables 1 and 2), suggesting that the increased soluble sugar and proline contents most probably evolved from drought and SO_2 stress adaptation. However, increased soluble sugar and proline contents in SO_2 -tolerant *C. dactylon* accessions are not likely involved in osmotic pressure but more likely involved in maintaining cell membrane stability, synthesis of other compounds, supply of energy, action as regulators of gene expression, and signal molecules based on their multiple functions [36]. Thereafter, soluble sugar and proline contents can be considered as marker for selection of *C. dactylon* variations with high SO_2 tolerability.

Chlorophyll (including chlorophylls a and b) and carotenoid are known as the two important pigments in chloroplast of tree and grass plant leaves. The important role of pigments is to absorb certain wavelengths from sunlight and then convert the unusable sunlight energy into usable chemical energy during photosynthesis. Chlorophyll a is the primary pigment for photosynthesis in plants [37]. In this study, leaf injury of *C. dactylon* was observed under SO_2 stress condition (Table 2). As a consequence, chlorophyll a, chlorophyll b, and carotenoid contents decreased in *C. dactylon* under SO_2 stress condition, consistent with previous reports on grass and tree plants [13, 38, 39]. However, SO_2 -tolerant *C. dactylon* accessions showed significantly higher contents of chlorophyll a, chlorophyll b, and carotenoid under SO_2 treated condition, consistent with their less leaf injury SO_2 -tolerant *C. dactylon* accessions observed in this study. Moreover, SO_2 -tolerant *C. dactylon* accessions had significantly

higher content of chlorophyll a under SO_2 untreated condition. Now that chlorophyll a is the primary pigment for photosynthesis in plants, significantly higher contents of chlorophyll a in *C. dactylon* accessions under both SO_2 treated and untreated conditions indicate that SO_2 tolerance of *C. dactylon* might be largely related to content of chlorophyll a.

Early study showed that SO_2 gas after entering leaves of plant is converted into sulfite (SO_3^{2-}) and bisulfite (HSO_3^{2-}) once it is dissolved in cellular cytoplasm [40]. Furthermore, detoxification reaction of HSO_3^{2-} and SO_3^{2-} to sulfate (SO_4^{2-}) in plants leads to production of many kinds of ROS, such as superoxide radical ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^{\cdot}) [41]. Excessive ROS are highly reactive and toxic to plants, which could cause oxidative damage to membranes, DNA, proteins, photosynthetic pigments, and lipids [42]. To protect plant cells from ROS damage, plant developed antioxidant enzymes to deal with the excessive ROS in plant cells. SOD, POD, and CAT are considered as three major antioxidant enzymes. To analyze the oxidative effect of SO_2 on *C. dactylon*, we measured the ROS level and antioxidant enzyme activities in *C. dactylon* accessions. Although both ROS levels (reflected by H_2O_2 and MDA contents) and antioxidant enzyme activities (reflected by SOD, POD, and CAT) increased in all of the nine *C. dactylon* accessions under SO_2 stress condition, the SO_2 -tolerant *C. dactylon* accessions showed significantly lower ROS levels and SOD activities, indicating that the SO_2 -tolerant *C. dactylon* accessions have much stronger antioxidant ability and less damage occurs to them by SO_2 . Moreover, lower SOD activity was theoretically consistent with lower ROS

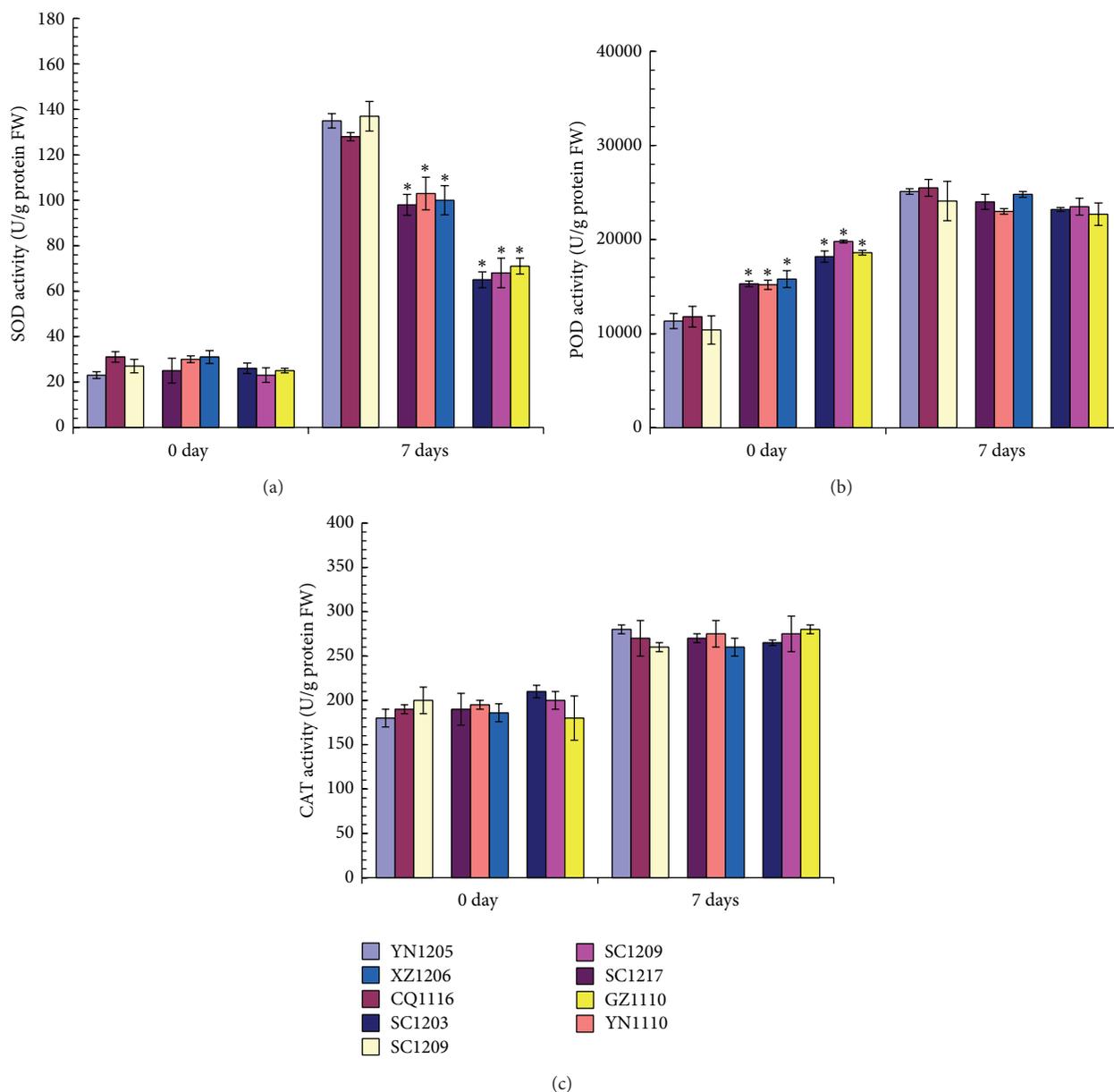


FIGURE 6: Comparison of antioxidant enzyme activities in leaves from *C. dactylon* accessions in response to SO₂ stress. Antioxidant enzyme activities of SOD (a), POD (b), and CAT (c) in leaves from nine selected *C. dactylon* accessions in response to SO₂ stress were compared. Mean values are presented with vertical error bars representing the standard deviations ($n = 3$). The asterisk symbols indicate significant differences between SO₂-sensitive accessions and SO₂-tolerant accessions.

level in the SO₂-tolerant *C. dactylon* accessions under SO₂ stress condition, which is in agreement with previous report [43]. Although POD activities were nearly at the same level in leaves from all of the nine *C. dactylon* accessions after 7-day SO₂ stress treatment, activities of this antioxidant enzyme from SO₂-tolerant *C. dactylon* accessions were significantly higher than those from SO₂-sensitive *C. dactylon* accessions. Taken together, we suggest that significantly higher activity of POD prior to SO₂ treatment might be devoted to the increased antioxidant ability in SO₂-tolerant *C. dactylon* accessions.

SO₂ gas after entering leaves of plant can be converted into either sulfate by SO to enter into oxidative pathway or sulfide by SiR to enter into reductive pathway [44]. Overexpression of both SO and SiR showed more tolerance to sulfur dioxide toxicity in *Arabidopsis thaliana* and/or tomato plants [44–47]. Transcriptional analyses indicate that SiR is induced by SO₂ but SO is constitutively expressed in natural plant [45, 46]. In this study, we found that SiR activity level was significantly increased under SO₂ stress condition but SO activity level had almost no change under SO₂ treated and untreated conditions in leaves from all of the nine

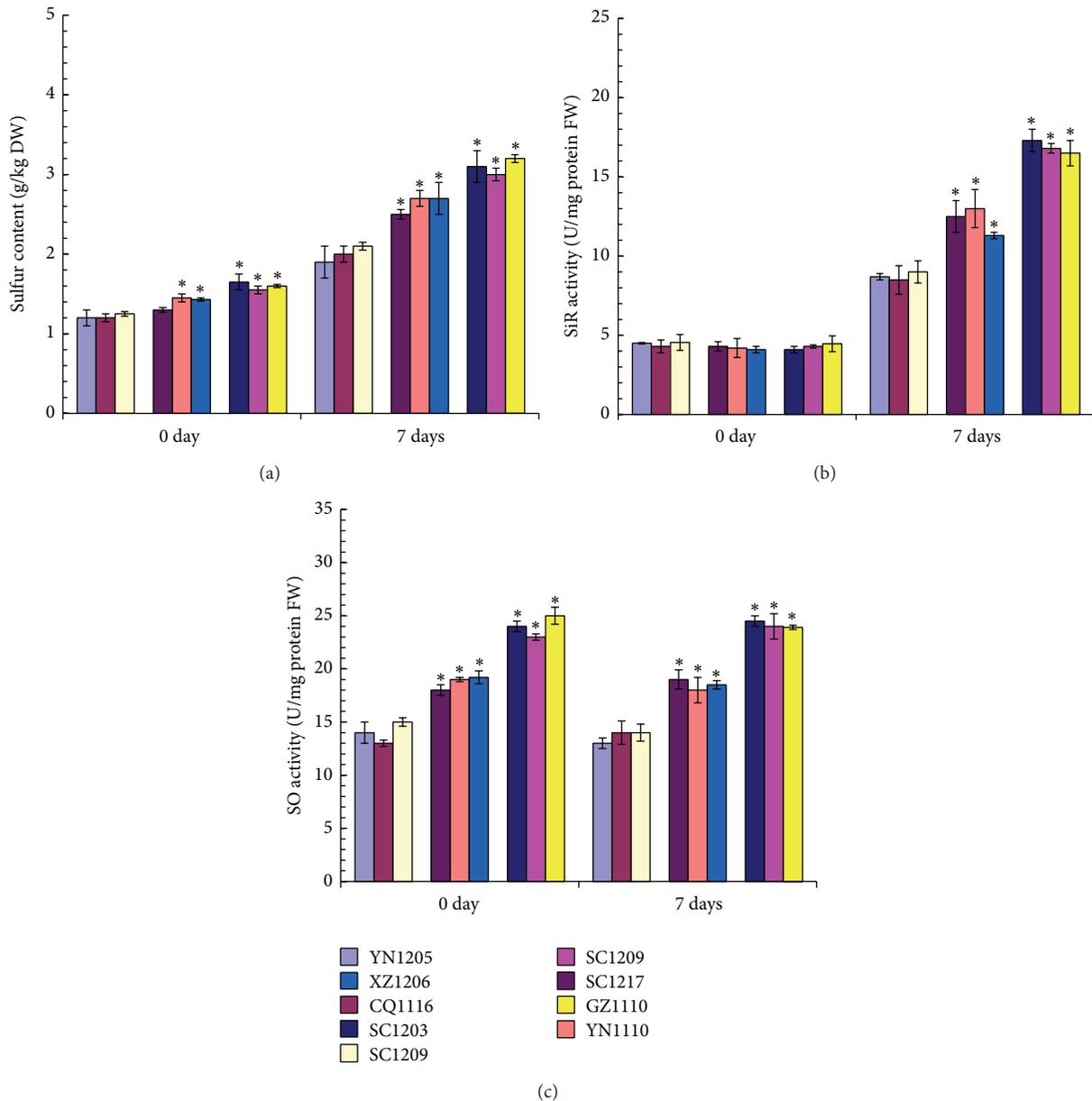


FIGURE 7: Comparison of sulfur contents (a), SiR activities (b), and SO activities (c) in leaves from nine selected *C. dactylon* accessions in response to SO_2 stress. Mean values are presented with vertical error bars representing the standard deviations ($n = 3$). The asterisk symbols indicate significant differences between SO_2 -sensitive accessions and SO_2 -tolerant accessions.

C. dactylon accessions, consistent with previous reports on other plant species [45, 46]. Under SO_2 stress condition, the SO_2 -tolerant *C. dactylon* accessions showed higher levels of both SiR and SO activities and contained higher sulfur content in leaves as corresponding consequence. More importantly, we found that the SO_2 -tolerant *C. dactylon* accessions showed significantly higher SO activities prior to SO_2 treatment, but no significant differences were observed among the nine *C. dactylon* accessions. Increased SO activity in SO_2 -tolerant *C. dactylon* accession could convert sulfite to nontoxic sulfate more efficiently than SO_2 -sensitive *C. dactylon* accession for storage, once highly toxic SO_2 gas enters into the *C. dactylon* cells, which indicates that SO

antioxidant enzyme plays an important role in SO_2 tolerance in *C. dactylon*.

5. Conclusion

C. dactylon, a warm season perennial grass species, is widely used as turfgrass on sports fields, golf courses, roadsides, and lawns in city or industry districts in warm season. Although this species has much better SO_2 -tolerant ability among warm season turfgrasses, its growth rate will be affected and visible symptoms like yellowing, chlorosis, bleaching, and even killing foliage will appear on leaves of *C. dactylon* in SO_2

polluted areas. To achieve better air quality and landscape effect in SO₂ polluted areas, selection or development of high SO₂-tolerant *C. dactylon* variations is desired. In this study, we selected 9 out of 38 *C. dactylon* accessions from Southwest China as representatives of high, intermediate SO₂-tolerant, and SO₂-sensitive accessions and then comparatively analyzed their physiological differences under SO₂ untreated and treated conditions. Our results indicated that SO₂ tolerance of *C. dactylon* might be largely related to soluble sugar, proline and chlorophyll a contents, and SO enzyme activities. This study gained some insights into understanding the genetic and molecular SO₂-tolerant mechanisms of *C. dactylon* and provided guideline for selection or development of *C. dactylon* variations for planting in SO₂ polluted urban or industrial areas.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xi Li and Ling Wang contributed equally to this paper.

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