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BRIEF COMMUNICATION

Enhanced chilling tolerance in *Zoysia matrella* by pre-treatment with salicylic acid, calcium chloride, hydrogen peroxide or 6-benzylaminopurine

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Abstract

Following leaf application of salicylic acid (SA), calcium chloride, hydrogen peroxide and 6-benzylaminopurine (BA), Manila grass (*Zoysia matrella*) plants were exposed to day/night temperature of 7/2 °C for 120 h in a growth chamber. The lower content of malondialdehyde (MDA) and H_2O_2 and higher activities of ascorbate peroxidase (APX), guaiacol peroxidase (POD) and catalase (CAT) during exposure to low temperature in pre-treated plants in comparison with control plants demonstrated that these compounds improved the chilling tolerance of Manila grass.

Additional key words: antioxidant enzymes, cold stress, Manila grass, oxidative damage, signalling compounds.

Manila grass (*Zoysia matrella*) is one of the most popular warm-season turf grasses in subtropical cities. However, the lower temperature in winter is a major factor restricting its utilization in these regions. Therefore, it is necessary to study how to enhance the chilling tolerance of Manila grass. Chilling can lead to the overproduction of reactive oxygen species including hydrogen peroxide (Anderson *et al*. 1995). In order to resist oxidative damage plants possess many antioxidative enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2) (Zhao and Blumwald 1998). Previous works have shown that pre-treatment of plants with salicylic acid (SA) or calcium chloride up-regulated the activities of antioxidant enzymes and increased tolerance of plants (Kang *et al*. 2003, Tasgin *et al*. 2006, Larkindale and Huang 2004, Nayyar and Kaushal 2002). Recently, it has been considered that H_2O_2 of low concentration may regulate the expression of numerous genes encoding antioxidants enzymes as a stress signalling molecule (Chen and Song 2006). H_2O_2 pre-treatment induced salt-tolerance of maize plants and pretreatment of seed with H_2O_2 also improves salt tolerance of wheat seedlings by alleviation of oxidative damage (Wahid *et al*. 2007). Transgenic tall fescue expressing a gene of isopentenyl transferase (*ipt*), which is a key enzyme in the biosynthetic pathway of cytokinins, increased cytokinin production and enhanced the tolerance to lower temperatures (Hu *et al*. 2005). In this study, we tried to enhance the chilling tolerance of Manila grass by the pre-treatment with SA, $CaCl_2$, H_2O_2 and 6-benzyl-aminopurine (6-BA).

Manila grass (*Zoysia matrella* L. Merr) plants were collected from a 1-year field plots at the Horticulture Cultivation Center, Nanjing University. They were grown in plastic pots filled with sand/organic/vermiculite (3/1/1, v/v/v) mixture in a greenhouse at day/night temperature of 32/26 °C for one month. They were clipped to a height of 10 cm, watered daily and fertilized weekly with Hoagland's solution. Prior to the experiment, the plants were transferred to growth chamber (temperature 32/26 °C, relative humidity about 70 %, irradiance 200 μmol m^{-2} s^{-1} and 14-h photoperiod) for two weeks. Based on a preliminary experiment, the optimum concentrations for pretreatments were: 0.5 mM SA, 10 mM H_2O_2, 10 mM $CaCl_2$ and 30 μM BA. Plants treated with similar volume of distilled water were used as controls.

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Abbreviations: APX - ascorbate peroxidase; BA - 6-benzylaminopurine; CAT - catalase; GR - glutathione reductase; GSH - glutathione; GSSG - oxidized glutathione; MDA - malondialdehyde; NBT - nitroblue tetrazolium chloride monohydrate; POD - guaiacol peroxidase; PSB - phosphate saline buffer; SA - salicylic acid.
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The plants were firstly sprayed by 50 cm^3 of the appropriate solution or water and then were transferred to the chamber with temperature of 7/2 °C and other conditions *ibidem*. To determine activities of POD, CAT, APX and GR leaves were sampled after 0, 12, 24, 48, 72 and 120 h and the contents of malondialdehyde (MDA) and H_2O_2 after 0, 24, 48, 72 and 120 h of chilling.

The concentration of malondialdehyde (MDA) was measured by the thiobarbituric acid (TBA) method described by Xu *et al*. (2006). Fresh leaf samples (0.2 g) were homogenized with a mortar and pestle in 5 cm^3 10 % (v/v) trichloracetic acid and the homogenate was centrifuged at 12 000 *g* for 15 min. Two cm^3 supernatant was taken out, added 2 cm^3 of 0.6 % (v/v) TBA solution, heated in a boiling water bath for 30 min and cooled quickly in an ice bath. The mixture was centrifuged at 12 000 *g* for 15 min and the resulting supernatant was determined at 532 nm and 600 nm with UV-VIS spectrophotometer (*Beckman M36*, USA). Contents of H_2O_2 were estimated by forming a titanium hydroperoxide complex (Procházková *et al*. 2001). Fresh leaf material (0.2 g) was ground with 5 cm^3 cooled acetone in an ice bath and the homogenate was centrifuged at 6 000 *g* for 10 min. One cm^3 supernatant was taken out and 0.1 cm^3 5 % titanium sulfate and 0.2 cm^3 ammonia were added to this solution. The reaction mixture was centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was discarded and the precipitate was dissolved in 5 cm^3 2 mM H_2SO_4. The solution was analyzed at 415 nm. Content of H_2O_2 was determined using standard curve plotted with the known content of H_2O_2.

Pre-treated leaves (0.2 g) were harvested and frozen in liquid nitrogen at different times of treatment and stored at -80 °C. After the removal from the freezer, the samples were immediately ground with a mortar and pestle, in the ice bath, in 5 cm^3 of cold phosphate saline buffer (PSB, 100 mM, pH 7.6) containing 2 % polyvinylpolypyrrolidone, 1 mM EDTA and 0.5 mM ascoric acid (AsA). The homogenate was then centrifuged at 12 000 *g* at 4 °C for 20 min and the supernatant was used as the crude extract for the assays of antioxidant enzyme activity. By the methods of Larkindale and Huang (2004), APX activity was measured by monitoring the rate of oxidation of AsA at 290 nm for 1 min, and 3 cm^{-3} of reaction mixture was composed of 50 mM PSB (pH 7.0) containing 0.5 mM AsA, 0.06 % H_2O_2 dissolved in PSB (pH 7.0), and 0.1 cm^3 enzyme extract. POD activity was measured by monitoring the oxidation of guaiacol at 470 nm for 1 min, and 3 cm^3 of reaction mixture was composed of 50 mM PSB (pH 7.0), 1 % guaiacol, 0.3 % H_2O_2 and 0.05 cm^3 enzyme extract. CAT activity was measured according to the decomposition of H_2O_2 at 240 nm for 1 min, and 3 cm^3 of reaction mixture included 50 mM PSB (pH 7.0), 0.06 % H_2O_2, and 0.1 cm^3 of enzyme extract. GR activity was measured by the increase of the absorbance at 412 nm for 1 min according to Smith *et al*. (1988). The assayed mixture contained 100 mM PSB (pH 7.5), 0.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 2.0 mM NADPH, 2.0 mM GSSG and 0.1 cm^{-3} extract in a total of 3 cm^3, and the reaction was started by the addition of GSSG. The PSB of the same volume replaced the extract as blank in the measurement of these enzyme activities. One unit of POD, CAT and GR activity was defined as absorbance changed 0.01 in 1 min. Protein content was determined by the absorbance at 595 nm using bovine serum albumin as a standard (Bradford 1976). All data in the experiments were subjected to an analysis of variance and the least significant difference at 0.05 probability levels was performed (Student *t*-test).

MDA is a product of peroxidation of unsaturated fatty acids in phospholipids and responsible for cell membrane damage (Xu *et al*. 2006). As chilling stress continued, MDA content in controls and pre-treated leaves was gradually increased. However, all pre-treated samples showed lower contents of MDA than controls during the cold stress (Table 1). It proved that pre-treatments alleviated and postponed oxidation damage resulted from chilling.

Biotic and abiotic stresses may induce overproduction of H_2O_2 in plant cells and H_2O_2 can lead directly to the damage of cell membrane, protein and nucleic acids. Our results showed contents of H_2O_2 in plants pre-treated with SA, $CaCl_2$, H_2O_2 and 6-BA significantly increased ($P < 0.05$) before the initiation of cold stress, however, these pre-treatments decreased the H_2O_2 content in the first 72 h of chilling (Table 1), which was in agreement with the changes in MDA content. In further cold stress, H_2O_2 was significantly accumulated ($P < 0.05$) because the over-production of H_2O_2 exceeded the capacity of antioxidative enzymes to eliminate it. However, H_2O_2 content in pre-treated plants was lower than in controls (Table 1).

It is well-known that there is a relationship between the improvement of antioxidant enzyme activity and chilling tolerance (Sala 1998, Zhao and Blumwald 1998, Shen *et al*. 1999). Activities of POD, CAT and APX changed rarely in controls, which may imply that chilling itself can not induce the activities of these enzymes in Manila grass (Table 1). However, increased CAT, APX and POD activities prior to the chilling initiation protected turf grasses against the subsequent chilling-induced damage (Horvath *et al*. 2007). All pre-treatments significantly increased POD, CAT and APX activity in first 72 h of chilling ($P < 0.05$). The CAT, APX and POD activities decreased during further chilling, but the activities were still greater than in controls. It is obvious that POD, CAT and APX played a primary role in preventing pre-treated plants from adverse effects of chilling. GR catalyzes the regeneration of GSH from GSSG and the activity of GR has been reported to associate with the alteration of the GSSG/GSH ratio which is more decisive in determining plant resistance to abiotic and biotic stresses than GSH content (Kómives *et al*. 1998). Our results showed that the GR activities in all pre-treated plants were similar to control in the first 24 h, and then they were increased in further chilling with some fluctuation, suggesting GR may be activated to regulate the oxidant-reduction status of GSH after it has been changed by chilling. Some studies showed that low temperature activates redox signalling

Table 1. Effects of pre-treatment with SA, $CaCl_2$, H_2O_2 and 6-BA on contents of MDA and H_2O_2 at 0, 24, 72 and 120 h and activities of POD, CAT, APX and GR at 0, 12, 24, 72 and 120 h of chilling (7/2 °C, day/night) in Manila grass. Control plants were pre-treated with distilled water and sampled at the same time. Means ± standard deviation ($n = 4$). Values marked by * differ significantly at $P < 0.05$ from corresponding ones measured on control plants according to *t*-test.

	Time [h]	Control	SA	$CaCl_2$	H_2O_2	6-BA
MDA content	0	4.08±0.37	2.19±0.84	5.07±0.45	5.38±0.62	3.30±0.65
[μmol g^{-1}(f.m.)]	24	6.52±0.32	2.07±0.68*	5.73±0.53	5.85±0.35	4.68±0.51
	72	7.93±0.79	2.24±0.83*	6.45±0.43*	5.89±0.23*	4.32±0.57*
	120	13.35±0.88	9.72±0.45	11.76±1.24	12.28±0.6	10.46±0.45
H_2O_2 content	0	1.27±0.27	2.74±0.22*	1.75±0.20	2.68±0.02*	2.64±0.05*
[μmol g^{-1}(f.m.)]	24	1.22±0.14	1.98±0.39*	1.62±0.47	1.34±0.37	2.19±0.14*
	72	1.16±0.36	1.67±0.34	1.52±0.30	1.17±0.28	2.18±0.45*
	120	3.80±0.31	3.34±0.27	3.06±0.49	3.52±0.16	3.17±0.37
POD	0	155.38±24.23	176.78±19.59	240.06±27.79*	177.49±29.76	256.18±22.52*
[U mg^{-1}(protein) min^{-1}]	12	203.71±18.27	274.77±17.28*	350.47±25.07*	379.50±26.21*	219.19±38.24
	24	182.04±28.47	252.63±20.30*	241.33±27.46*	324.03±32.09*	299.86±29.61*
	48	150.43±26.17	458.98±38.49*	255.77±16.49*	384.27±21.41*	259.42±33.42*
	72	153.59±17.02	447.78±28.17*	261.72±20.28*	316.81±32.87*	332.26±19.72*
	120	164.50±7.85	206.46±18.25	188.01±12.85	185.36±35.11	160.74±28.37
CAT	0	6.74±2.17	13.45±1.24*	15.10±1.89*	10.27±2.14*	13.53±2.39*
[U mg^{-1}(protein) min^{-1}]	12	8.81±1.60	37.07±3.39*	19.94±0.73*	21.19±0.69*	12.04±1.74*
	24	7.25±1.93	33.36±1.72*	16.01±0.99*	17.89±1.40*	11.85±0.31*
	48	5.80±1.02	50.63±2.17*	17.95±0.47*	18.66±1.26*	11.86±1.43*
	72	7.25±1.60	41.32±2.49*	14.52±2.06*	13.72±1.61*	13.09±2.70*
	120	7.82±0.51	11.15±0.10	8.20±1.08	8.83±0.73	7.58±0.44
APX	0	2.98±0.87	8.76±0.48*	6.32±0.37*	4.23±0.84*	13.34±0.89*
[mmol(AsA) mg^{-1}(protein) min^{-1}]	12	2.21±0.81	8.09±1.15*	8.72±0.49*	8.05±0.88*	9.70±1.34*
	24	4.45±0.70	7.60±0.36*	8.76±1.28*	5.77±1.28*	8.43±0.66*
	48	3.27±1.14	8.85±0.89*	7.58±0.52*	8.07±1.23*	8.54±0.53*
	72	3.62±0.89	8.74±1.15*	6.20±0.95*	5.78±0.99*	9.61±0.76*
	120	3.22±0.29	4.29±0.13	3.53±0.23	3.26±0.13	2.84±0.08
GR	0	4.82±0.52	3.37±0.45	5.67±0.73	2.82±0.37	5.07±0.35
[U mg^{-1}(protein) min^{-1}]	12	5.16±0.53	6.16±0.25	5.08±0.39	6.28±0.26	4.84±0.46
	24	4.98±0.51	5.69±0.46	4.25±0.41	4.83±0.32	5.24±0.38
	48	3.60±0.41	8.10±0.50*	4.84±0.69*	5.71±0.15*	4.92±0.44*
	72	3.39±0.36	7.28±0.32*	3.82±0.26	4.41±0.37	4.98±0.34*
	120	3.27±0.33	3.54±0.36	3.50±0.70	4.78±0.16	4.21±0.33

either directly *via* changes in H_2O_2 concentration and GSH/GSSG ratio or indirectly by affecting ABA, Ca^{2+} or SA contents, which then alter the GSH/GSSG ratio (Kocsy *et al*. 2001). Our results suggested that the change of H_2O_2 content and the GSH/GSSG ratio was possibly involved in signal transduction of these compounds (Table 1).

In conclusion, our results have demonstrated that SA, $CaCl_2$, H_2O_2 and 6-BA pre-treatments protected Manila grass from cold stress. The enhanced chilling tolerance may be associated, at least in part, with the control and/or prevention of oxidative damage and the increase of POD, CAT and APX activities played a primary role in preventing plants from adverse effects of chilling. From the four compounds tested, SA induced higher activities of CAT and GR than other compounds 24 h after chilling, as well as the lowest MDA content.

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