Exogenous Nitric Oxide Pretreatment Enhances Chilling Tolerance of Anthurium

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ABSTRACT. Nitric oxide (NO) is well known for its multifaceted physiological roles as a signaling molecule in plants. Previous studies have indicated that exogenous application of NO may be useful for alleviating chilling injury (CI) in fruits and vegetables. However, the potential role and mechanism of NO in mitigating chilling stress in anthurium (*Anthurium andraeanum*) remain unclear. In this study, physiological and biochemical analysis were performed to investigate the effects of exogenous NO in alleviating CI in anthurium. Anthurium seedling plants were treated with the NO donor sodium nitroprusside (SNP) at four concentrations (0, 0.2, 0.4, and 0.8 mM) and stored at 12/5 °C (day/night) for 15 day. The results showed that exogenous SNP mitigated the adverse effects of chilling on anthurium, and the most effective concentration was 0.2 mM. In addition, NO effectively improved the CI index, malondialdehyde (MDA) content, electrolyte leakage, photochemical efficiency (F_v/F_m), and chlorophyll loss of anthurium during low temperatures. Pretreatment with SNP also increased the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX); the content of antioxidants including glutathione (GSH), ascorbic acid (AsA), and phenolics; and reduced the accumulation of hydrogen peroxide and O_2^- . SNP pretreatment at 0.2 mM also significantly promoted the accumulation of proline, increased the activity of $\Delta 1$ -pyrroline-5-carboxylate synthetase (P5CS), and reduced the activity of proline dehydrogenase (PDH), when compared with control (0 mM SNP → Chilling) under chilling stress. These results indicated that NO could enhance the chilling tolerance of anthurium by elicitation of an antioxidant response and proline accumulation for maintaining cell membrane integrity.

CI is one of the main restrictive factors affecting the growth and use of tropical plants in temperate and subtropical areas. Under chilling stress condition, physiological and biochemical processes, including growth rate, photosynthesis, respiration, nutrient absorption, and transport, are impaired (Aghdam and Bodbodak, 2013; Kratsch and Wise, 2000). It also caused membrane system dysfunction and accumulation of reactive oxygen species (ROS) such as H_2O_2 and superoxide (O_2^-) (Aghdam and Bodbodak, 2013; Rui et al., 2010). For example, cell membrane phase from a fluid liquid crystalline shifted to a rigid solid gel under chilling temperature, leading to diminishing membrane selective permeability (Aghdam et al., 2013). The excessive ROS associated with the incidence of CI are crucial sources of oxidative stress (Ruelland et al., 2009; Suzuki and Mittler, 2006). They can rapidly react with various molecules, resulting in membrane lipid peroxidation, protein degradation, and nucleic acid damage (Scandalios, 1993; Sharma et al., 2012). To defend against these damaging effects, plants have evolved two kinds of antioxidative systems to scavenge ROS. One is the antioxidant enzymes, including SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), POD (EC 1.11.1.7), and APX (EC 1.11.1.11) (Hossain et al., 2010). The other is the nonenzymatic antioxidants, such as proline, reduced GSH, AsA, and phenolics (Blokhina et al., 2003; Szabados and

Savouré, 2010; Tsantili et al., 2010). It is well recognized that both osmoprotective mechanism and antioxidation mechanism contribute in CI resistance of plants, even at extreme low temperatures (Javadian et al., 2010; Xu et al., 2012).

Some signaling molecules, such as NO, salicylic acid (SA), jasmonic acid, and hydrogen sulfide have been found to regulate the physiological processes of plants (Luo et al., 2015; Promyou et al., 2012; Siboya et al., 2014). NO is acclaimed to be involved in the modulation of various physiological processes during the entire life of the plant (Mur et al., 2012). For example, it was found to play a crucial role in the stimulation of seed and pollen germination, modulation of plant growth and development, regulation of cell elongation, floral regulation, mediation of stomatal movement, photosynthesis regulation, ripening of fruit and senescence of organs, and so on (Procházková et al., 2015). Moreover, NO is demonstrated as one of the key components of plant responses to abiotic stress including CI (Qiao and Fan, 2008; Siddiqui et al., 2011). Using fumigation with NO gas or treatments with NO-releasing chemicals, several studies have correlated the capacity of NO to prevent CI with the upregulation of the antioxidant defense (Singh et al., 2009; Xu et al., 2012). For example, it has been demonstrated that NO can regulate plant antioxidant systems, enhance the removal of ROS, stimulate proline accumulation, reduce the cellular membrane damage caused by low temperatures, and improve chilling tolerance (Wang et al., 2013; Wu et al., 2014). In addition, NO has also been selected as a promising strategy for combating chilling stresses encountered by various horticultural plants, such as peach (*Prunus persica*), cucumber (*Cucumis sativus*), and chinese cabbage (*Brassica campestris* ssp. *chinensis*) (Fan et al., 2014; Yang

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et al., 2011; Zhu et al., 2010). Nevertheless, the understanding of the interaction between NO and chilling stress tolerance remains scarce.

As an economically important tropical ornamental crop, anthurium stands out among most cultivated tropical flowers for its exquisiteness, durability, and long vase-life (Gantait and Mandal, 2010). Anthurium belongs to the largest genus of Araceae family that encompasses over 1500 species, of which 600 species are from tropical America (Venkat et al., 2014). Anthurium plants are particularly susceptible to low temperatures. They will stop growing when subjected to cold conditions and often exhibit additional symptoms including leaf etiolation, spadix wilting, and spathe browning (Aghdam et al., 2015). Besides, they are irreversibly injured by long-term exposure to temperatures lower than 6 °C. In temperate and cold areas, a much greater risk of cold stress occurs in the supply of anthurium plants, especially in the process of production and transportation (Tian et al., 2013). Thus, the improvement of chilling stress tolerance in anthurium may not only significantly increase its production and extend its shelf life but also reduce the economic loss.

Previously, the application of exogenous SA and γ -aminobutyric acid for improving the CI tolerance in anthurium has been studied (Aghdam et al., 2015; Promyou et al., 2012). Here, the beneficial effects of exogenous NO pretreatment on alleviating CI in anthurium plants were investigated. Our results might provide some valuable information of NO involved in chilling stress tolerance to help develop methods to reduce CI in anthurium.

Materials and Methods

PLANT MATERIALS AND TREATMENTS. Anthurium plants were obtained from commercial growers in Guangzhou province and preserved in greenhouses in the Anthurium Germplasm Resource Preserve Center (Jiangsu Academy of Agricultural Sciences, Nanjing, China). The experiment was performed from Sept. to Dec. 2015. Seedling plants (360) showing the same or similar sizes were selected and cultured in plastic pots (80 mm height $\times$ 90 mm diameter, with three plant seedlings per pot) containing peatmoss. The plants were placed in a growth chamber and fertilized every 1 week with 20N–4.4P–16.6K fertilizer solution (1:3000 dilution) for 20 d under the following conditions: temperature 25/18 °C (day/night), 12/12 h photoperiod (irradiance of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), and 70% relative humidity. Meanwhile, the plants were watered in 10 mL every 3 d. Subsequently, the plants (about 12–15 cm height) were randomly divided into four groups, and the whole plant seedlings in each group were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of SNP (a common NO donor) as a pretreatment for three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night) for the indicated time points (0, 3, 6, 9, 12, and 15 d). Then, 12–15 leaves (10–12 cm length, 5–6 cm width) at the same position of anthurium plants were harvested and stored at –80 °C immediately for the further analysis. The experiments were repeated at three times with similar results.

CI INDEX DETERMINATION. CI index was evaluated by visualizing the severity of necrotic tissues on the leaves of 30 individual plants using the following scale: 1 = no CI, 2 = mild injury (1% to 20% necrotic area), 3 = moderate injury (21% to 50% necrotic area), 4 = severe injury (51% to 80% necrotic area), and 5 = very severe injury (81% to 100% necrotic area), according to the

method of Aghdam et al. (2015). The CI index was calculated according to the following equation: CI index = Σ (number on CI scale $\times$ the number of plants at that number on the CI scale)/total number of plants.

PHOTOSYNTHETIC PIGMENTS AND CHLOROPHYLL FLUORESCENCE DETERMINATION. The chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll, and carotenoids (Car) were determined according to the method of Lichtenthaler (1987) with minor modifications. Twenty leaf discs (0.5 cm diameter) were excised from the uppermost fully expanded leaves of 10 plants selected randomly from each group. Then, 20 mL acetone (80%) was added for the extraction of photosynthetic pigments. After incubating 24 h in dark conditions, the absorbance of the extract was measured using a spectrophotometer (UH-5300; Hitachi, Tokyo, Japan) at 470, 645, and 663 nm. Concentrations of Chl *a*, Chl *b*, total Chl, and Car were then calculated.

Leaf chlorophyll fluorescence was measured by a chlorophyll fluorometer (Plant Efficiency Analyzer; Hansatech, Norfolk, UK). Readings were taken from 5-mm-diameter leaf disks after dark adaptation for 30 min. The ratio of F_v/F_m represents the maximum F_v/F_m of photosystem II (PS II), and was calculated according to Krüger et al. (1997).

MEMBRANE PERMEABILITY AND LIPID PEROXIDATION DETERMINATION. Membrane permeability, expressed by relative electrolyte leakage, was determined by a modified method (Promyou et al., 2012). Twenty discs (2 mm thick $\times$ 6 mm diameter) were excised from the leaves with a cork borer. After being rinsed three times in deionized water, they were incubated in 20 mL deionized water and shaken for 1 h at 100 cycles per minute. Conductivity of the solution was detected using a conductivity meter (DDSJ-308A; Leici, Shanghai, China). Total conductivity was determined after boiling the flasks for 20 min to release electrolytes. Relative electrolyte leakage was expressed as a percentage of total conductivity.

The level of lipid peroxidation was expressed as the MDA content using the thiobarbituric acid (TBA) method described by Hodges et al. (1999) with a slight modification. Leaves (0.5 g) were homogenized with 10 mL of 5% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 g_n for 15 min. TBA reactivity was determined by adding 2 mL of 0.67% TBA in 10% TCA to 2 mL of the supernatant. The mixture was held in a bath containing boiling water for 20 min, quickly cooled, and then centrifuged at 12,000 g_n for 10 min. Absorbance was measured at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm and interference generated by TBA-sucrose complexes at 450 nm. The MDA content was expressed as nanomoles per gram fresh weight (FW).

DETERMINATION OF ASA, GSH, TOTAL PHENOLS, AND PROLINE CONTENTS. To determine the contents of AsA and GSH, 0.5 g leaf was homogenized in 5% (w/v) ice-cold TCA and then centrifuged at 12,000 g_n for 20 min at 4 °C. The supernatant was used for the AsA and GSH assays. AsA and GSH contents were measured according to Tanaka et al. (1985) and Lin et al. (2012), respectively.

The total phenolics in the leaves were measured according to Chen et al. (2008) with modification. Briefly, 1 g plant material was homogenized and extracted with 8 mL of 80% methanol for 24 h in the dark. The solution was centrifuged at 10,000 g_n for 10 min. The total phenolic concentration in the supernatant was determined colorimetrically at 750 nm. The results were expressed as milligrams gallic acid equivalents per gram FW.

Proline was assayed using the method of Zhao et al. (2009). Leaf samples (0.5 g) were homogenized in 3% sulfosalicylic acid and centrifuged at 10,000 g_n for 10 min. Two milliliters of extract was incubated with 2 mL of 2.5% ninhydrin reagent and 2 mL of glacial acetic acid at 100 °C for 30 min. Then, toluene (4 mL) was added into the mixture after the solution was cooled. The absorbance of the organic phase was recorded at 520 nm. L-proline was used as the standard.

DETERMINATION OF O_2^- PRODUCTION RATE AND H_2O_2 CONTENTS. Leaf samples (1.0 g) were homogenized in 3 mL of cold 50 mM phosphate buffer (pH 7.0) containing 1% (w/v) ethylenediaminetetraacetic acid (EDTA), and then centrifuged at 12,000 g_n at 4 °C for 10 min. The O_2^- production rate was measured by monitoring nitrite formation from hydroxylamine in the presence of O_2^- as described previously (Xu et al., 2012). $NaNO_2$ was used as the standard.

The content of H_2O_2 was determined by the method of Ferguson et al. (1983). Leaf samples (0.5 g) were homogenized in 5 mL cold acetone and centrifuged at 12,000 g_n for 10 min. The supernatant (1 mL) was mixed with 0.1 mL of 20% $TiCl_4$ and 0.2 mL of concentrated NH_4OH solution. After shaking and reacting for 5 min, the mixture was then centrifuged (12,000 g_n) at 4 °C for 15 min, and the precipitates were washed repeatedly with cold acetone until the acetone was colorless. The precipitates were dissolved in 4 mL of 2 M H_2SO_4 . The absorbance was measured at 415 nm. H_2O_2 content was calculated from a standard curve prepared in a similar way.

MEASUREMENTS OF SOD, CAT, POD, AND APX ACTIVITIES. For the determination of the enzyme activities, 0.5 g leaves were homogenized with ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone (PVP) in a chilled mortar and pestle. The homogenate was centrifuged at 12,000 g_n for 20 min, and the supernatant was collected for determination of enzyme activities. Total protein content was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard sample.

SOD activity was analyzed by measuring its ability to inhibit the photoreduction of nitrobluetetrazolium (NBT) following the method of Beyer and Fridovich (1987). One unit of SOD activity was defined as the amount of enzyme that would inhibit 50% of NBT photoreduction at 560 nm. CAT activity was measured by following the decline in absorbance at 240 nm due to the consumption of H_2O_2 (Patra et al., 1978). One unit of CAT activity was defined as the amount of enzyme required to degrade 0.1 μ mol H_2O_2 per minute. POD activity was measured by following the change in absorption at 470 nm due to guaiacol oxidation (Polle et al., 1994). One unit of POD activity was defined as the amount of enzyme required for formation of 1 μ mol guaiacol per minute. APX activity was measured following the decrease in absorbance at 290 nm due to the oxidation of ascorbate (Nakano and Asada, 1981). One unit of APX activity was defined as the amount of enzyme that oxidized 1 μ mol of ascorbate per minute.

MEASUREMENTS OF PHENYLALANINE AMMONIA LYASE (PAL) AND POLYPHENOL OXIDASE (PPO) ACTIVITIES. PAL was extracted and assayed as described by Wang et al. (2013). Leaf samples (0.5 g) were homogenized in 5 mL of 50 mM phosphate buffer (pH 8.8) on ice and centrifuged at 12,000 g_n for 10 min at 4 °C. PAL activity was determined using the supernatant, 0.5 mL of which was added to a reaction mixture containing 1 mL of 200 mM L-phenylalanine and 3 mL of 50 mM borate buffer (pH

8.5). After incubating the mixture at 30 °C for 1 h, the reaction was stopped by adding 0.1 mL of 5 mM HCl. PAL activity was calculated from the absorbance of the assay mixture at 290 nm based on the production of cinnamic acid. One unit of PAL activity was defined as the amount of enzyme that produces 1 μ mol of cinnamic acid per hour. The PPO was extracted and assayed using the method of Nguyen et al. (2003). One gram of leaves was homogenized in 10 mL of phosphate buffer (100 mM, pH 7.8) with 1% PVP, and the solution was then centrifuged at 12,000 g_n for 10 min at 4 °C. The supernatant was collected as a crude PPO extraction. The reaction mixture contained 200 mM catechol in 50 mM phosphate buffer (pH 6.0). One unit of PPO activity was defined as the amount of enzyme that produces 1 μ mol of quinone per minute.

MEASUREMENTS OF P5CS AND PDH ACTIVITIES. The activities of P5CS and PDH were determined by the method of Shang et al. (2011). Leaf samples (0.5 g) were homogenized with ice-cold 50 mM Tris-HCl buffer (pH 7.4, containing 7 mM $MgCl_2$, 600 mM KCl, 3 mM EDTA, 1 mM DTT, and 5% PVP) in a chilled mortar and pestle. The homogenate was centrifuged at 12,000 g_n for 10 min (4 °C) and the supernatant was collected for the determination of enzyme activity. One unit of P5CS activity was defined as the amount of enzyme required to degrade 1 μ mol nicotinamide adenine dinucleotide phosphate per minute. One unit of PDH activity was defined as the amount of enzyme causing a change of 0.01 in absorbance per minute at 340 nm.

STATISTICAL ANALYSIS. All the experiments were performed with three biological replicates, and values were given as mean $\pm$ SD. The data were analyzed by one-way analysis of variance with the SPSS statistical software (version 13.0; IBM Corp., Armonk, NY). Significant differences were determined by Duncan's multiple range test at a level of $P < 0.05$.

Results

EFFECT OF NO ON CI IN ANTHURIUM. When subjected to chilling stress, the CI index of anthurium plants increased with the time of treatment. However, the increased extent of CI index was effectively delayed in anthurium plants pretreated with different concentrations of SNP (0.2, 0.4, and 0.8 mM) after 6 d of chilling stress, when compared with control [0 mM SNP $\rightarrow$ Chilling (Fig. 1)]. Among them, 0.2 mM SNP pretreatment was the most effective treatment for alleviating CI in anthurium ($P < 0.05$). At the same time, the protective effect of SNP pretreatment in anthurium under chilling stress decreased with increased SNP concentration.

MDA CONTENT AND ELECTROLYTE LEAKAGE. A continuous increment of MDA content in anthurium plants was observed during chilling stress period (Fig. 2A). Samples pretreated with SNP showed a significantly lower MDA content than the control ($P < 0.05$) at different treatment times. For example, at the end of the tested chilling stress (day 15), the MDA content in 0.2 mM SNP pretreated samples was 11.2% lower than the control samples. The changes of electrolyte leakage shared similar trends to the MDA content. A sharp increment was observed in the control samples, but the increment was delayed by the SNP pretreatments (Fig. 2B). Moreover, it also showed that the slowest increment in electrolyte leakage came from the samples pretreated with 0.2 mM SNP, which was 0.56 times that of controls at the end of the chilling stress period.

PHOTOSYNTHETIC PIGMENTS AND CHLOROPHYLL FLUORESCENCE. The contents of photosynthetic pigments in control plants

Fig. 1. Effects of nitric oxide (NO) pretreatment on the chilling injury (CI) index in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP → Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests.

decreased gradually with the treatment time increasing, whereas SNP pretreatment delayed this tendency (Table 1). The decrease of photosynthetic pigment contents was mostly inhibited by 0.2 mM SNP pretreatment. Meanwhile, the contents of Chl *a*, Chl *b*, total Chl, and Car in 0.2 mM SNP-pretreated plants were 1.66, 1.62, 1.64, and 1.61 times higher, respectively, than those of controls at day 3. In addition, the maximum F_v/F_m of PS II showed a similar pattern to chlorophyll content (Fig. 3). Although chilling stress resulted in a significant decrease in F_v/F_m ($P < 0.05$), the values of F_v/F_m in SNP-pretreated anthurium plants were still higher than those of control. Moreover, the slowest decline of F_v/F_m was also observed in the samples when pretreated with 0.2 mM SNP.

O_2^- PRODUCTION RATE AND H_2O_2 CONTENT. The O_2^- production rate in all groups increased quickly during the first 9 d of low temperatures and then decreased progressively (Fig. 4A). Exogenous SNP pretreatment significantly inhibited the production of O_2^- during the entire chilling treatment ($P < 0.05$). The production of O_2^- was inhibited most by 0.2 mM SNP pretreatment, which was 88.11% of control at 9 d. The content of H_2O_2 increased progressively in control plants, which was evidenced by an ≈ 3.83 -fold increment within 15 d (Fig. 4B). The changes of H_2O_2 content in the control and SNP-pretreated anthurium plants showed the similar tendency. However, different concentrations of SNP pretreatment (0.2, 0.4, and 0.8 mM) decreased the content of H_2O_2 by 83.97%, 86.87%, and 95.19% of control, respectively, at day 15 after chilling stress treatment.

ACTIVITIES OF ANTIOXIDANT ENZYMES AND CONTENTS OF ASA AND GSH. The changes of antioxidant enzymes, such as SOD, CAT, POD, and APX, in anthurium plants under chilling stress were shown in Fig. 5. They showed the similar change tendency

Fig. 2. Effects of nitric oxide (NO) pretreatment on (A) malondialdehyde (MDA) content and (B) electrolyte leakage in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP → Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests.

under chilling stress condition. For example, they all exhibited a significant increment after the first 6 d of chilling treatment; thereafter, they decreased quickly until day 15. Exogenous SNP pretreatment exhibited a positive effect on the activities of these four antioxidant enzyme activity, and the most effective SNP pretreatment concentration was 0.2 mM.

The content of GSH in anthurium plants increased steadily during chilling stress treatment (Fig. 6A). SNP pretreatments significantly strengthened the increment of the GSH content under the stress condition. In addition, with the increasing concentration of SNP pretreatment applied, the content of GSH decreased significantly. By contrast, the content of AsA in both control and SNP-pretreated anthurium samples was steadily decreased after being treated with low temperatures (Fig. 6B).

Table 1. Effects of nitric oxide (NO) pretreatment on the pigment content in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d, and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates.

Time (d)	Treatment (mM → Chilling)	Pigment content [mean ± SE (mg·g ⁻¹ FW)] ^z			
		Chl <i>a</i>	Chl <i>b</i>	Total Chl	Car
0	0	29.84 ± 1.02 a ^y	16.71 ± 0.56 a	46.55 ± 1.58 a	8.29 ± 0.35 a
	0.2	29.64 ± 1.22 a	16.44 ± 0.79 a	46.08 ± 2.01 a	8.29 ± 0.18 a
	0.4	29.68 ± 1.12 a	15.69 ± 0.41 a	45.37 ± 1.53 a	8.34 ± 0.51 a
	0.8	29.19 ± 0.75 a	15.94 ± 0.66 a	45.13 ± 1.41 a	8.23 ± 0.15 a
3	0	16.31 ± 1.08 d	9.87 ± 0.99 d	26.18 ± 2.07 d	4.94 ± 0.45 d
	0.2	27 ± 1.05 a	16.02 ± 0.65 a	43.02 ± 1.70 a	7.97 ± 0.27 a
	0.4	24.52 ± 1.66 b	14.47 ± 0.91 b	38.99 ± 2.57 b	7.32 ± 0.29 b
	0.8	21.47 ± 0.37 c	12.85 ± 0.23 c	34.32 ± 0.60 c	6.49 ± 0.02 c
6	0	9.64 ± 0.10 b	5.86 ± 0.66 b	15.5 ± 0.76 b	3.29 ± 0.19 b
	0.2	11.37 ± 1.00 a	6.98 ± 0.07 a	18.35 ± 1.07 a	3.87 ± 0.27 a
	0.4	10.08 ± 0.71 b	6.18 ± 0.10 ab	16.26 ± 0.81 b	3.44 ± 0.30 ab
	0.8	9.76 ± 0.22 b	5.91 ± 0.22 b	15.67 ± 0.44 b	3.29 ± 0.21 b
9	0	8.96 ± 0.26 c	5.49 ± 0.15 b	14.95 ± 0.41 c	3.18 ± 0.06 b
	0.2	11.19 ± 0.12 a	6.79 ± 0.62 a	17.98 ± 0.74 a	3.75 ± 0.26 a
	0.4	9.34 ± 0.07 b	5.71 ± 0.45 b	15.03 ± 0.52 b	3.28 ± 0.13 b
	0.8	9.32 ± 0.19 b	5.61 ± 0.16 b	14.45 ± 0.35 bc	3.24 ± 0.11 b
12	0	7.99 ± 0.48 b	4.84 ± 0.24 b	12.83 ± 0.72 b	2.92 ± 0.20 b
	0.2	10.51 ± 0.56 a	6.50 ± 0.40 a	17.01 ± 0.96 a	3.63 ± 0.15 a
	0.4	8.88 ± 1.03 b	5.45 ± 0.64 b	14.33 ± 1.67 b	3.19 ± 0.23 b
	0.8	8.76 ± 0.23 b	5.31 ± 0.40 b	14.07 ± 0.63 b	3.12 ± 0.08 b
15	0	5.75 ± 0.60 c	3.56 ± 0.32 c	9.31 ± 0.92 c	2.23 ± 0.16 b
	0.2	10.24 ± 0.34 a	7.10 ± 0.55 a	18.34 ± 0.89 a	3.21 ± 0.29 a
	0.4	8.59 ± 0.72 b	5.34 ± 0.06 b	13.93 ± 0.78 b	2.94 ± 0.28 a
	0.8	8.08 ± 0.37 bc	5.07 ± 0.42 b	13.15 ± 0.79 b	2.87 ± 0.05 a

^zChl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*; Total Chl = total chlorophyll; Car = carotenoids; FW = fresh weight.

^yNumbers in a column not followed by the same letter for the same chilling stress treatment time are statistically different using Duncan's multiple range test at *P* < 0.05.

However, SNP pretreatment delayed this tendency, and the content of AsA showed a significant increment in 0.2 mM SNP-pretreated sample when compared with the control.

TOTAL PHENOL CONTENT AND ACTIVITIES OF PAL AND PPO. The content of total phenols was found to reach a peak after 3 d under chilling treatment, and then decreased (Fig. 7A). During the chilling treatment time, the content of total phenols was higher in SNP-pretreated samples than in controls. The increase in total phenol content was greatest in the pretreatment with 0.2 mM SNP. After 3 d of chilling stress, the content of total phenols in 0.2 mM SNP-pretreated plants was 1.79 times higher than that of control plants. SNP pretreatments induced the activities of PPO and PAL enzymes, which showed an initial increase followed by a decline (Fig. 7B and C). PPO activity peaked at 6 d in all groups. PAL activity peaked at 3 d in 0.2 mM SNP pretreatment, whereas this activity reached the highest level at 6 d in other groups. PPO in anthurium pretreated with 0.2 mM SNP showed lower levels than control plants during the whole treatment time. However, PAL in anthurium pretreated with 0.2 mM SNP showed higher levels than control plants during the whole treatment.

CONTENT OF PROLINE AND ACTIVITIES OF PDH AND P5CS. The content of proline increased steadily during the duration of the chilling stress treatment (Fig. 8A). SNP pretreatments enhanced the increase in proline, and the contents were respectively 2.17, 1.58, and 1.51 times higher than that of control plants after 15 d of chilling stress treatment.

Simultaneously, PDH activity was decreased in all plants during the chilling stress treatment (Fig. 8B). The activities of P5CS increased sharply first, reaching a peak on day 6, and then decreased to the end of the treatment (Fig. 8C). In all, exogenous SNP pretreatment decreased the activity of PDH, but enhanced the activity of P5CS, with chilling stress.

Discussion

As a plant signaling molecule, NO plays an important role in the regulation of many kinds of plant processes, especially in abiotic stresses (Sheokand and Kumari, 2015; Wink and Mitchell, 1998). However, little scientific information of NO involved in CI tolerance in anthurium has been shown so far. As a common NO donor, SNP has been widely performed to play protective effect in different plants under various abiotic stresses, ranging from 5 μM to 1 mM, and showing a dose-dependent manner in most cases (Leung, 2015). For example, 0.2 mM SNP treatment enhanced wheat seedling growth and kept high relative water content and alleviated the oxidative damage under drought conditions (Tian and Lei, 2007). In the present study, we determined that different concentrations of SNP pretreatment reduced the CI symptoms of anthurium in a dose-dependent manner under the chilling stress condition. Among them, 0.2 mM SNP pretreatment showed the most significant effect (Fig. 1). In addition, NO also reveals prooxidative cytotoxic property at its higher concentration. The

Fig. 3. Effects of nitric oxide (NO) pretreatment on photochemical efficiency (F_v/F_m) in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP → Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests.

excess of NO is able to inhibit plant growth and division (Corpas et al., 2011). Considering the highest NO⁺ concentration (6 μM) was recorded by the electrochemical method 2 h after constant illumination (120 μmol·m⁻²·s⁻¹) in 0.2 mM SNP solution (Ederli et al., 2009; Floryszak-Wieczorek et al., 2006), the detailed effects of NO released by its donor should be further illuminated in anthurium.

CI leads to the structural damage to cell, such as loss of cell membrane integrity, cell membrane permeability, or relative electrolyte leakage (Aghdam and Bodbodak, 2013; Cao et al., 2012). The increase in electrolyte leakage from plant tissue exposed to chilling temperatures has been widely used as an important qualitative indicator of oxidative damage on membranes during chilling stress (Marangoni et al., 1996; Zhang et al., 2013). Besides, MDA as the final product of lipid peroxidation is also often considered as an index of cell membrane damage under environmental stress (Hodges et al., 1999; Marangoni et al., 1996). The dysfunction of one or more cell membranes may be the primary cause of CI. In this study, it was observed that the MDA content and electrolyte leakage of anthurium seedlings were increased during chilling stress, whereas different concentrations of SNP pretreatments significantly decreased the cellular damage compared with the control treatment [0 μM SNP → Chilling (Fig. 2)]. This result suggested that NO might influence membrane oxidation to enable plants to adapt to chilling stress. Previously, the protective effect of NO on cell membrane under different abiotic stresses has also been found (Fan et al., 2014; Farooq et al., 2009). Moreover, the photosynthesis of plants is closely associated with lipid peroxidation and ROS production (Hung et al., 2002). It has been reported that the damage to the chloroplast membrane done by low temperatures can decrease

Fig. 4. Effects of nitric oxide (NO) pretreatment on (A) O₂⁻ production rate and (B) H₂O₂ content in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP → Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests. FW refers to fresh weight.

chlorophyll content, reduce the efficiency of PS II, and inhibit photosynthesis (Fedina et al., 1993). In the present study, the decrease in photosynthetic pigment contents (Table 1) and F_v/F_m (Fig. 3) of PS II in anthurium was significantly postponed by exogenous SNP pretreatments. This could be due to the NO application which reduces damage to the chloroplast membrane, leading to photosynthetic efficiency being maintained under low temperature conditions.

The imbalances in metabolic processes occurring in abiotic stresses including CI may lead to an increased accumulation of ROS, such as O₂⁻ and H₂O₂ (Ruelland et al., 2009). Antioxidant enzymes, such as SOD, POD, CAT, and APX, establish one of the most important parts of the ROS scavenging system in plant cells, which can reduce the toxicity caused by the production of

Fig. 5. Effects of nitric oxide (NO) pretreatment on the activities of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) peroxidase (POD), and (D) ascorbate peroxidase (APX) in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP → Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests.

O_2^- and H_2O_2 (Zhang et al., 2009; Zhou et al., 2006). Meanwhile, antioxidant enzymes have also been demonstrated to be important factors for plant tolerance to various environmental stresses (Wang et al., 2010). Many studies have confirmed that NO can improve plant tolerance to abiotic stresses by acting as a direct ROS scavenger or modulating components of antioxidative system (Sheokand and Kumari, 2015). For example, NO could serve as an antioxidant during several stress to interact with ROS in various ways, directly scavenging ROS, such as O_2^- to form peroxynitrite ($ONOO^-$) (Misra et al., 2011), comparatively less toxic than peroxides and thus restricts the cellular damage in plants. Moreover, NO also functions as a signaling molecule to maintain the cellular homeostasis and regulates the toxicity of ROS by its augmentation of antioxidant content and antioxidative enzyme activity, which affects cell wall components and regulates the expression of stress-related genes (Sheokand and Kumari, 2015). Here, our results clearly showed that the application of SNP

pretreatment could markedly decrease the rate of O_2^- production and content of H_2O_2 under chilling stress (Fig. 4). Meanwhile, the increased activities of SOD, POD, CAT, and APX were also observed by pretreatment with SNP (Fig. 5). Similar results were also observed previously (Chongchatuporn et al., 2013; Sheokand et al., 2010).

In addition to antioxidant enzymes, nonenzymatic antioxidants also play critical roles in scavenging ROS in plants subjected to chilling conditions. For instance, GSH and AsA play key roles in reducing ROS levels in plants (Agastian et al., 2000). NO treatment has been shown to enhance GSH and AsA contents and increase the activities of antioxidant enzymes, while alleviating the production of H_2O_2 and O_2^- (Chen et al., 2014). Moreover, in the presence of O_2 , NO can react with GSH to form *S*-nitrosoglutathione (GSNO), a reactive nitrogen form, which is also a long-distance signaling molecule and a natural reservoir of NO (Airaki et al., 2012). In our study, higher AsA and GSH contents were also observed in SNP-pretreated anthurium plants

Fig. 6. Effects of nitric oxide (NO) pretreatment on (A) reduced glutathione (GSH) content and (B) ascorbic acid (AsA) content in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP→Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests.

during chilling stress (Fig. 6). This result indicated that NO pretreatment might enhance the AsA and GSH levels of plants, leading to lower ROS being maintained under chilling conditions. Besides, stress conditions often result in a much more phenolic production, which can be used to inhibit the initiation or propagation of oxidizing chain reactions and thus alleviate chilling damage (Pennycooke et al., 2005). PAL and PPO are two key enzymes in the synthesis and decomposition of phenolics and have been reported to protect plants against stress conditions by regulating phenolic products (Dixon and Paiva, 1995; Mayer, 2006). It has been reported that MJ and SA treatment could alleviate chilling damage in lemon (*Citrus limon*) fruit by increasing the synthesis of total phenolics and PAL while inhibiting the activity of PPO (Siboz et al., 2014). In the present

Fig. 7. Effects of nitric oxide (NO) pretreatment on (A) total phenolics content, (B) polyphenol oxidase (PPO) activity, and (C) phenylalanine ammonia-lyase (PAL) activity in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP→Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests.

Fig. 8. Effects of nitric oxide (NO) pretreatment on (A) proline content, (B) proline dehydrogenase (PDH) activity, and (C) Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) activity in anthurium under chilling stress. Anthurium plants used in this study were grown in a growth chamber for 20 d, and then pots with established plants were sprayed with different concentrations (0, 0.2, 0.4, and 0.8 mM) of sodium nitroprusside (SNP) three times at 2-d intervals. After that, chilling stress was performed by decreasing the temperature to 12/5 °C (day/night). The experiment included three biological replicates. The control (0 mM SNP $\rightarrow$ Chilling) was plants treated with double-distilled water. Calculation of the mean values and the SD used data from the three biological replicates. The bars show SD, whereas the different letters indicate the significant statistical differences at $P < 0.05$ among the treatments according to Duncan's multiple range tests.

study, higher total phenolic contents and PAL activity, with lower PPO activity were observed in SNP-pretreated anthurium plants (Fig. 7). This result suggested that the application of exogenous NO could enhance the chilling tolerance of anthurium by affecting the phenolic metabolism.

It was proposed that NO induction under cold stress played a role in freezing tolerance through proline synthesis (Zhao et al., 2009). As one of the osmotic regulators, proline also plays a pivotal role in protecting plant cells against abiotic stresses (Li et al., 2013). For example, proline plays important physiological roles in osmotic adjustment, regulation of the NAD^+/NADH ratio, stabilizing membrane structures, and scavenging ROS (Ashraf and Harris, 2004). Although the actual role of proline accumulation remains unclear, it has been indicated that proline may enhance plants stress tolerance by adjusting cellular osmosis, balancing the redox status of the cell, modifying the activity of antioxidant enzymes, eliminating ROS, and stabilizing the structure of biological macromolecules (Ben Ahmed et al., 2010; Iqbal et al., 2014; Ozden et al., 2009). In addition, the accumulation of proline is a phenomenon frequently occurring in plants under environmental stresses (Ashraf and Foolad, 2007; Szabados and Savaure, 2010). Moreover, some reports have shown that proline also seems to be involved in signal transduction pathways that regulate stress-responsive adaptive responses (Lehmann et al., 2010; Maggio et al., 2002). On the other hand, proline accumulation is also a complex, dynamic process, in which P5CS and PDH play decisive roles (Ruiz et al., 2002; Verbruggen and Hermans, 2008). During chilling stress, the accumulation of proline in plants is part of the defense response against CI (Shang et al., 2011). The application of exogenous SNP also protects plants from abiotic stress by boosting the contents of proline (Farooq et al., 2009). Our results showed a significant increase in proline contents with pretreatment of SNP after chilling stress (Fig. 8A). Meanwhile, the increased proline contents were associated with lower PDH activity (Fig. 8B) and higher P5CS activity (Fig. 8C), which is similar to the results reported before (Cao et al., 2012). These results indicated that NO pretreatment might enhance the chilling tolerance of anthurium by mediating proline metabolism.

In conclusion, our results suggest that NO could increase chilling tolerance in anthurium by stimulating the antioxidant defense system, decreasing the accumulation of ROS, and elevating antioxidant contents. These acquired results explain the beneficial role of NO in improving chilling tolerance in anthurium and will also provide in-depth information on different strategies which NO adopts in facing the detrimental effects of abiotic stress.

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