

*Full Length Research Paper*

# Alleviating effect of exogenous nitric oxide in cucumber seedling against chilling stress

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**Nitric oxide (NO) is a bioactive and a multifunctional gaseous molecule signal playing a central role and mediating variety of physiological processes in plants. In this study, the protective effect against chilling stress of exogenously applied sodium nitroprusside (SNP, a NO donor) in *Cucumis sativus* L. cv ZND407 and cv ZND461 was investigated. The SNP was sprayed over cucumber seedlings using different doses at 4°C. The results indicated that malondehyde (MDA) content was decreased however, soluble sugar and chlorophyll content increased upon treatment with 1.0 mmol<sup>-1</sup> SNP. Further investigations showed that treatment with NO donor stimulated the activities of various enzymes such as, superoxide dismutase (SOD, EC1.15.1.1), glutathione reductase (GR, EC1.6.4.2), peroxidase (POD, EC.1.11.1.7) and catalase (CAT, EC.1.15.11). However, the soluble protein content did not change significantly under the NO treatment. The study indicated that exogenous NO at 1.0 mmol<sup>-1</sup> SNP enhanced chilling stress tolerance. In comparison with cvZND 461, cvZND407 had higher tolerance ability to chilling stress.**

**Key words:** Antioxidative enzymes, chilling stress, cucumber, nitric oxide (NO) osmotic adjustment; reactive oxygen species (ROS).

## INTRODUCTION

Chilling is one of the most important abiotic stress factors affecting plant growth, development, spatial distribution and productivity (Levitt, 1980; Chinnus et al., 2007; Xia et al., 2009). Interestingly, the plants are continuously exposed to natural chilling and have therefore evolved many adaptive mechanisms (Wu, 2009). Cold acclimation is one of the crucial mechanisms by which plants acquire freezing tolerance prior exposure to low non-freezing temperatures. Most temperate plants can cold-acclimate and acquire tolerance to extracellular ice formation in their vegetative tissues (Chinnus et al., 2007). Plants

suffering from chilling stress undergo common biochemical changes that involves the accumulation of reactive oxygen species (ROS) in mitochondria and chloroplast, which increases the cellular redox in favor of oxidized forms, thereby creating oxidative stress that can damage DNA, inactivate enzymes and cause lipid peroxidation (Shi et al., 2007). To protect cells and organelles from the damaging effects of ROS, complex antioxidant defense system have been evolved which comprises of enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione reductase (GR) (Lee et al., 2000; Farooq et al., 2008). A large body of evidence has demonstrated that antioxidant systems play important roles in protecting plants against oxidative damage induced by low temperature. It has been reported that chilling caused significant decrease in the growth of cucumber roots, which are associated with increases in free radical production and membrane damage resulting from decrease in the activity of antioxidant enzymes (Li et al., 2008; Zheng et al., 2009). The regulation of these antioxidants through an exogenous substance might mediate the activity of antioxidant

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**Abbreviations:** ROS, Reactive oxygen species; **ABA**, abscisic acid; **SOD**, superoxide dismutase; **CAT**, catalase; **POD**, peroxidase; **GR**, glutathione reductase; **CI**, chilling index; **MDA**, malondehyde; **SNP**, sodium nitroprusside.

enzymes in plant to improve chilling tolerance (Shi et al., 2007).

Nitrous oxide is a highly reactive gas which is a ubiquitous bioactive molecule that plays a central role as a signal in plant response to biotic and abiotic stresses (Nigel and Guo, 2005; Arasimowicz and Wieczorek, 2007). It has been increasingly evident that NO plays important roles in response to pathogen attack, programmed cell death, herbicides, and salt, drought and temperatures stress as well as heavy metal toxicity (Singh et al., 2008; Zhang et al., 2008). Diverse physiological processes are also regulated resulting from NO signalling in plants such as promotion in seed germination or reduction in seed dormancy, regulation of plant maturation and senescence, and mediation of stomatal movement for intermediate downstream of abscisic acid (ABA) signaling (Bethke, 2006; Leitner et al., 2009; Guo et al., 2003; Qiao et al., 2008). However, NO itself is a reactive nitrogen species and its effects on different types of cells have proven to be either as a potent oxidant or as effective antioxidant, depending on its concentration and location (Beligni and Lamattina, 1999; Qiao et al., 2008).

Cucumber is one of the most popular members of the cucurbitaceae (vine crop) family. Like most cucurbits, the cucumber (*cucumis sativus* L.) is a warm-season horticultural crop and has little or no frost tolerance and is planted in the world. In Northern China, cucumber can only be grown in sunlight-heated greenhouse during winter and early spring, to prevent damage by chilling and low light conditions (Zhang et al., 2009). Previous investigations suggest that NO are able to alter the activities of some enzymes involved in scavenging ROS produced during different stress conditions such as drought, salinity, heavy metals and oxidative stress (Arasimowicz and Wieczorek, 2007; Zhao et al., 2004; Beligni and Lamattina, 2000). However, little is known about the responses of antioxidant enzymes against chilling stress which was induced by exogenous NO in vegetables, especially in cucumber. The objective of the present study is to assess the possible influence of various concentrations of exogenous NO treatment at 4 ( $\pm 0.5$ ) °C on antioxidant enzymes in leaves of two ecotype cultivars.

## MATERIALS AND METHODS

### Plant materials

Seeds of *cv ZND407* and *cv ZND461* were obtained from the Department of Vegetable Science, China Agricultural University. *cv ZND407* is a chilling tolerant cultivar while *cv ZND461* is sensitive to chilling. For all treatments, healthy seeds of similar sizes were used. Seeds were sterilized and allowed to germinate on filter papers under dark at 27°C for 48 h. Germinated seeds were sown in growth chambers in pots, filled with turf and vermiculite (2:1). The seedlings were developed at a photoperiod of 16 h (light intensity of 520  $\mu\text{molm}^{-2} \text{s}^{-1}$ ) at a temperature 24°C (day) and 20°C (night), and 70% humidity. Three-leaf seedlings were used for NO spraying at four concentrations (0, 0.5, 1.0, and 2.0  $\text{mmol}^{-1}$ ) 3 times every 24 h. After 3 days, the seedlings were exposed to a cold chamber at 4

( $\pm 0.5$ ) °C under the normal continuous light (520  $\mu\text{molm}^{-2} \text{s}^{-1}$ ) for 72 h with a photoperiod of 14 h per day. The leaf samples were collected for analysis at 48 h.

### Chilling damage index

Three-leaf stage seedlings were cultured at 4 ( $\pm 0.5$ ) °C. The chilling damage index was assessed visually every 12 h. The degrees of cold tolerance were measured based on the 6 grades criteria developed by Semeniuk et al. (1986). The different levels and their characteristics are as follows: Level 0: no symptom; level 1: chlorosis or crinkled at the edge of old leaves; level 3: chlorosis or crinkled at the edge of functional leaves with good new leaves; level 4: chlorosis or crinkled and wilting of functional leaves with damaged new leaves; level 5: severe damage of new leaves, plants wilt or dead. Samples of all the symptoms are shown in Figure 3. Finally, chilling index (CI) for each candidate was calculated according to the following formula:

$$\text{CI} = \frac{\sum (\text{each level} \times \text{number of plants with corresponding level})}{\text{the highest level} \times \text{total number of inoculated plants}}$$

### Physiological assay

Malondehyde (MDA) content was determined by the method based on Heath et al. (1968). Chlorophyll content was determined based on the protocol adapted from Winterlmans and Demts (1965). SOD activity was measured in terms of its capacity to inhibit photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich, 1971; Singh et al., 2008).

GR activity was determined by the oxidation of NADPH at 340 nm and based on the classic method adapted from Foyer and Halliwell (1976). POD activity was determined by the guaiacol method according to Zhang et al. (2008). CAT was measured as the decline in absorbance at 240 nm due to decline of  $\text{H}_2\text{O}_2$  extinction rate (Singh et al., 2008). Total soluble sugar was determined using anthrone reagent and glucose as standard (Ozaki et al., 2009). Soluble protein content was measured using bovine serum albumin (BSA) as standard according to the Bradford (1976). Standard curve was prepared using BSA as standard (Mandal et al., 2009).

### Statistics

Each treatment was replicated three times with 10 seedlings. Values presented are means  $\pm$  standard deviation (SD). Statistical analyses were performed by analysis of variance (ANVOA) using the Statistical Analysis System (SAS) software (SAS Instituted, Cary, NC). Differences between treatments were separated by the Duncan test at a 0.05 probability level.

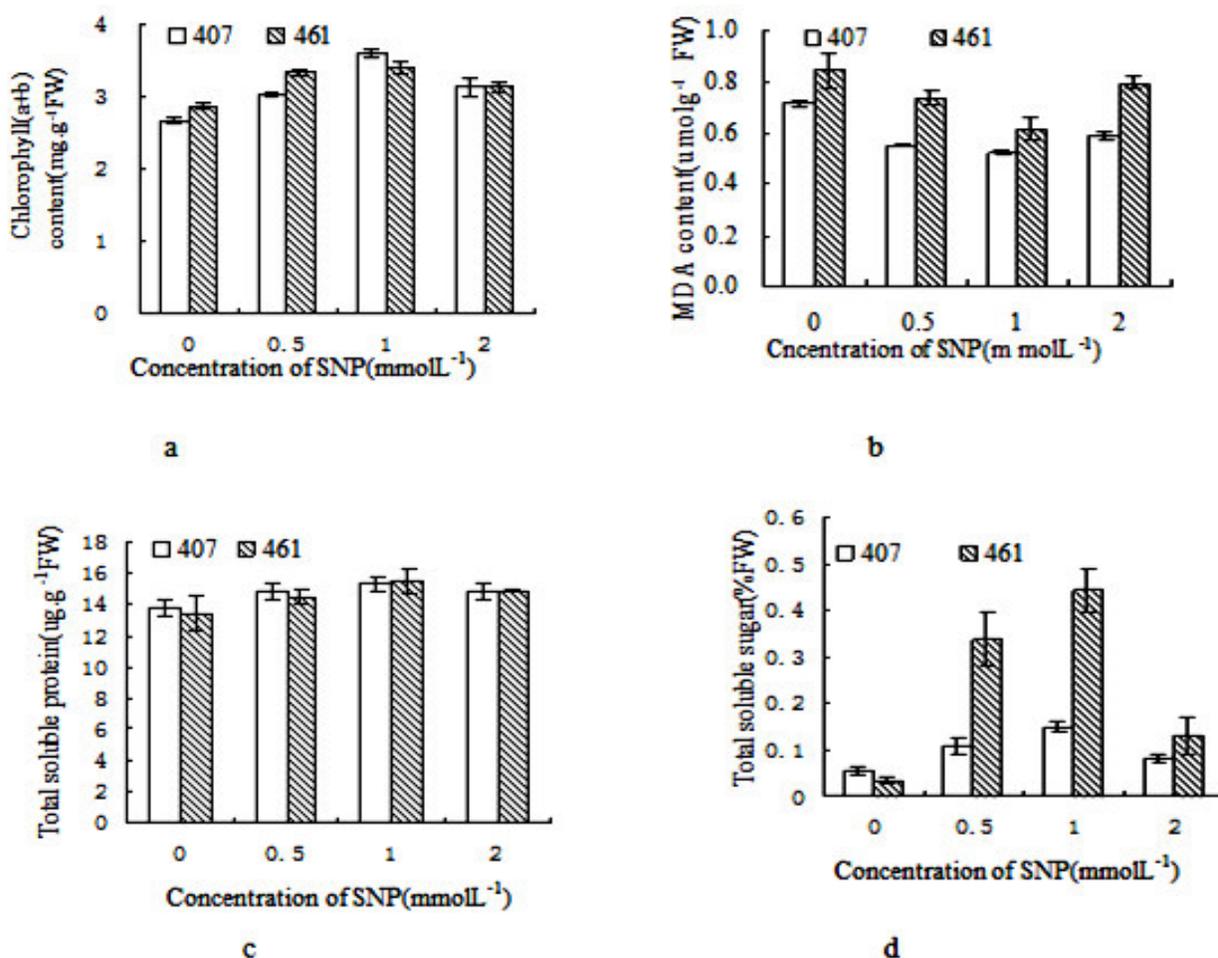
## RESULTS

The 48 h NO treatment at 4 ( $\pm 0.5$ ) °C decreased chilling damage indices (CI) in both *cv ZND407* and *cv ZND461* at different extent compared to the control seedlings. However with increasing NO concentration, the CI increased initially and then decreased. At different NO concentrations, CI of *cv ZND407* decreased 22.96, 43.86 and 24.58%, respectively and that of *cv ZND461* decreased 34.17, 50.63 and 10.12%, respectively compared to the control (Table 1). However, the CI reached the lowest at

**Table 1.** Chilling damage index in cucumber leaves affected by NO spraying

Concentration of NO (mmol <sup>-1</sup> )	Chilling damage index	
	<i>cvZDN407</i>	<i>cvZND461</i>
0.00	0.57 ± 0.010a	0.79 ± 0.020a
0.50	0.44 ± 0.030bc	0.52 ± 0.003c
1.00	0.32 ± 0.010d	0.39 ± 0.264d
2.00	0.43 ± 0.009bc	0.71 ± 0.010b

Mean value ± SD (n = 3). Data with different letters are significantly different at the 5% level.



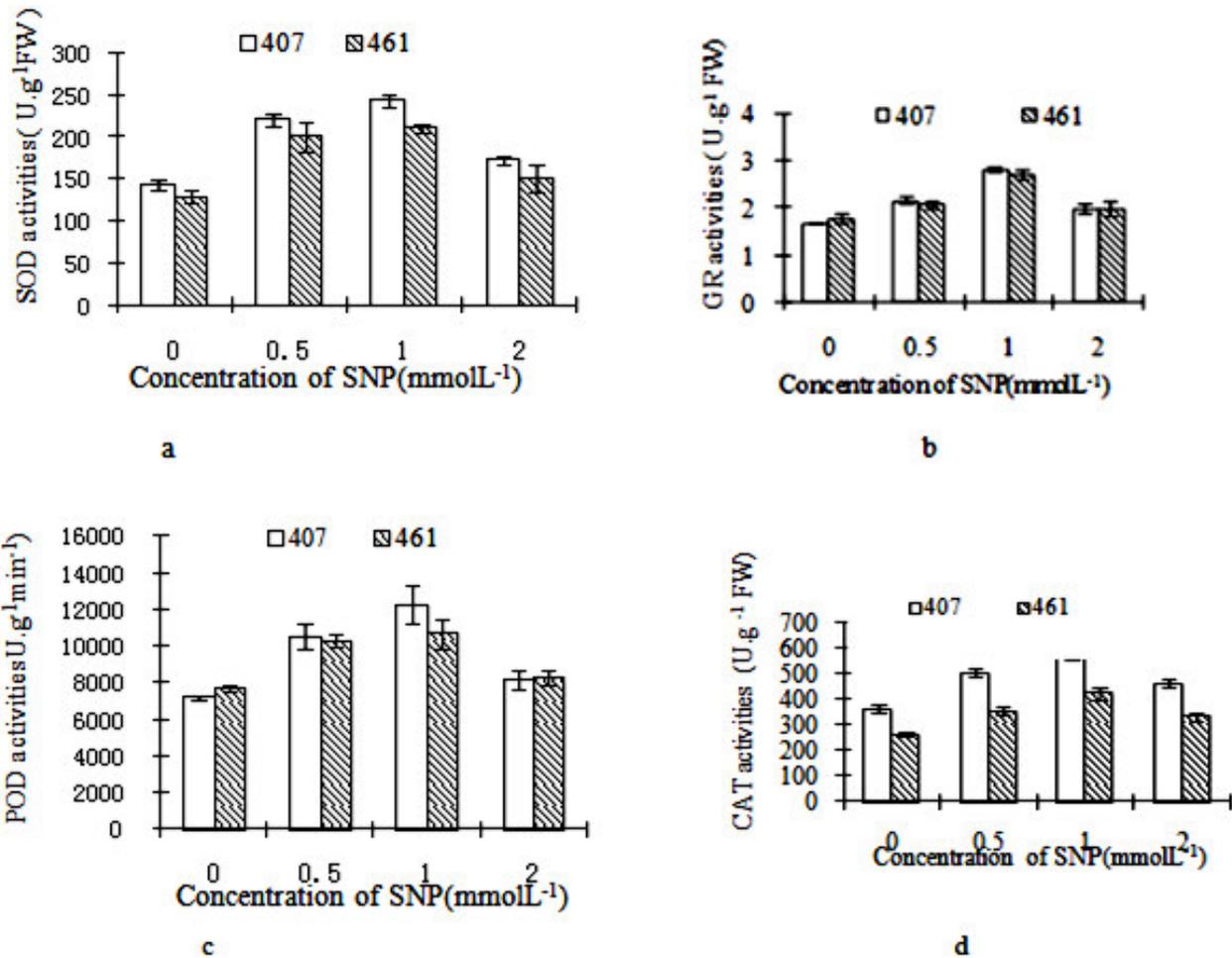
**Figure 1.** Effect of different concentration treatment with SNP on the content of Chlorophyll (a+b) (a), MDA (b), total soluble sugar (c) and total soluble protein in Cucumber seedling leaves under chilling stress.

the exogenous NO concentration of 1.0 mmol<sup>-1</sup> in both the ecotype seedlings.

The CI of *cv ZDN407* was lower than that of *cv ZND461*. Similarly, the cold tolerance of *cv ZDN407* was better than that of *cv ZND461* under treatment with exogenous NO. It was determined that 1.0 mmol<sup>-1</sup> exogenous NO treatment was the optimal concentration to enhance tolerance in cucumber seedlings.

#### Effect of NO on content of MDA in leaves of cucumber seedlings under chilling stress

The MDA is a measure of lipid peroxidation. The MDA values decreased significantly ( $p < 0.05$ ) after 48 h at 4 (± 0.5) °C treatment (Figure 1b). The MDA content in cucumber seedlings during chilling stress and sodium nitroprusside (SNP) treatment decreased significantly ( $p$



**Figure 2.** Effect of different concentration treatment with SNP on the content of SOD (a), GR(b), POD(c) and CAT(d) in cucumber seedling leaves under chilling stress.

< 0.05) when compared to those with chilling stress only, but without the SNP treatment. The values decreased by 27.2% and 27.6% in the two cultivars *cvZND407* and *cvZND461*, respectively. However, upon higher concentration of supplement with SNP (2.0 mmol<sup>-1</sup>), there was an increase in the MDA content. This could alleviate the effects of chilling stress but the effect was not significant at higher NO concentration. Both 0.5mmol<sup>-1</sup> and 1.0mmol<sup>-1</sup> SNP can protect the integrity of the plasma membrane from damage due to chilling-induced stress. However, the SNP concentration of 1.0 mmol<sup>-1</sup> SNP is found to be the best.

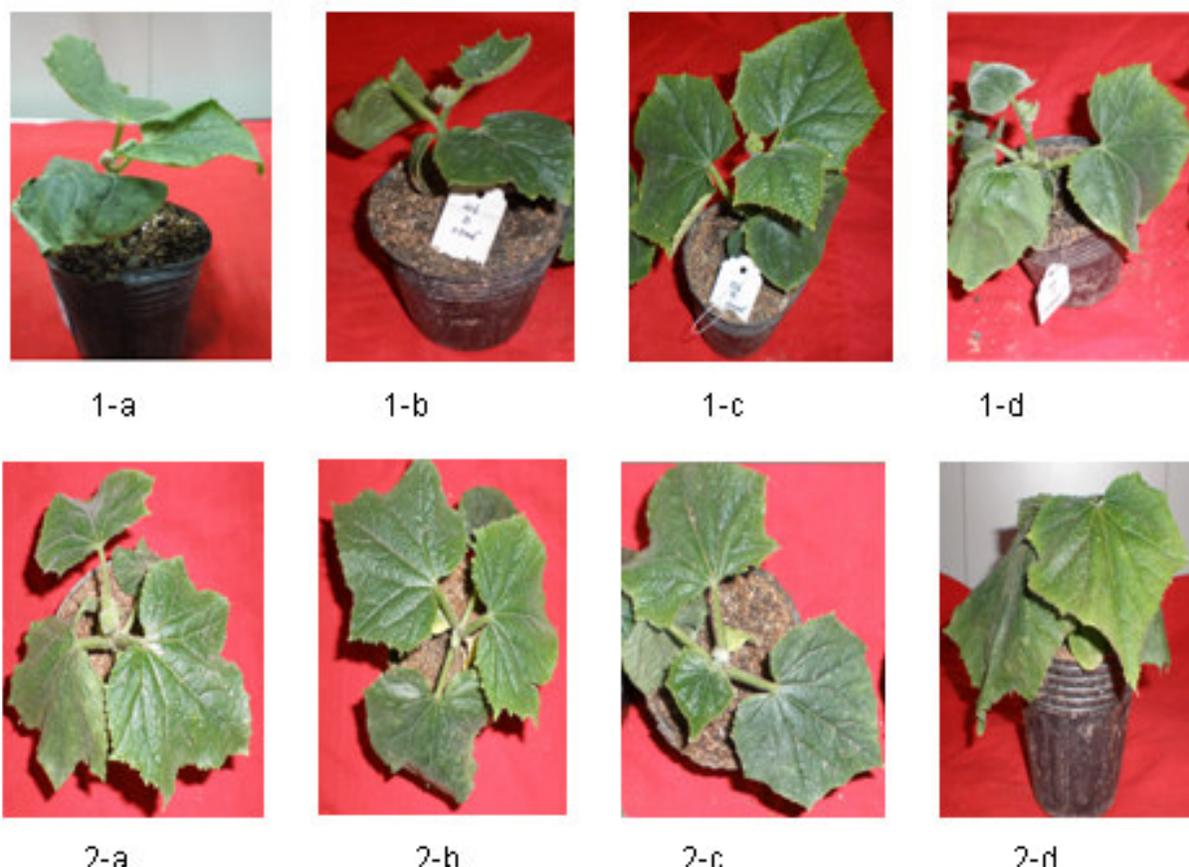
**Effects of NO on chlorophyll content in leaves of cucumber seedlings under chilling stress**

Under chilling stress, NO treatment slightly increased the chlorophyll (a+b) content (p < 0.05) (Figure 1a) (data of chlorophyll-a and chlorophyll-b were not shown). At 1.0

mmol<sup>-1</sup> level, the *cvZND407* and *cvZND461* increased 34.9%, 18.3% respectively compared to the non-treatment control. At the same time, NO treatment alleviated the effects of chilling stress on cucumber seedling leaves.

**Effects of NO on antioxidant enzymes in leaves of cucumber seedlings chilling stress**

There was a significant increase in the activities of antioxidant enzymes (SOD, GR, POD and CAT) in the leaves exposed to NO treatment (Figures 2a, b, c and d). In general, the induction of these enzymes after 0.5 mmol<sup>-1</sup> SNP to 2 mmol<sup>-1</sup> SNP treatments was higher in both *cvZND407* and *cvZND461* when compared to the untreated control. Activity of SOD was measured at 48 h after chilling stress. Upon SNP addition, the activity of SOD increased significantly only at 1.0 mmol<sup>-1</sup> SNP in both *cvZND407* and *cvZND461* (Figure 3a). The activity of SOD changed when *cvZND407* and *cvZND461* seedlings



**Figure 3.** Cucumber accession No.1 and 2 displayed after being treated with different concentrations of (a) NO and (b) CK.

were exposed to  $2.0 \text{ mmol}^{-1}$  SNP; however, the change in the activity was not significant.

Activity of GR, POD and CAT were enhanced after SNP treatment (Figure 2b, 2c and 2d). Compared to SOD activity, the variation of GR activity with various treatments showed similar changes. However, the activity of GR increased compared to SOD. The changes of POD activity (Figure 2c) was similar to GR. Noticeable changes were observed in POD activity among different treatments.

As shown in Figure 2d, the CAT activity in NO-treatment seedlings increased 60.7% in *cvZND407* and 60.1% in *cvZND461* under  $1 \text{ mmol}^{-1}$  SNP treatment, respectively, and at  $0.5 \text{ mmol}^{-1}$  SNP treatment they increased at 39.3 and 41%, respectively. Therefore, NO may alleviate the effect of antioxidant thereby enhancing the ability of eliminating free radicals in leaves.

#### **Effect of NO on total soluble sugars and proteins in leaves of cucumber seedlings under chilling stress**

The soluble sugar content increased significantly upon SNP treatment compared to the control (Figure 1c). Soluble

sugar content in *cvZND407* and *cvZND461* at  $1 \text{ mmol}^{-1}$  SNP treatment increased by 164 and 1100%, respectively compared to the control. However, at  $2 \text{ mmol}^{-1}$  SNP treatment, the soluble sugar content decreased significantly in both *cvZND407* and *cvZND461*.

The protein content (Figure 1d) in the leaves followed a similar pattern as the soluble sugar content. The protein in leaves increased with increasing concentrations (0, 0.5, 1.0 and  $2.0 \text{ mmol}^{-1}$ ). But no significant changes were observed in soluble proteins among different treatments.

#### **DISCUSSION**

SNP, a widely used exogenous NO donor (Delledonne et al., 1998), is one of the compounds with the ability to reduce the damaging effects of various plant stresses (Uchida et al., 2002). NO can alter various plant processes and against different stresses (Nigel and Guo, 2005; Bethke, 2006; Arasimowicz and Wiczorek, 2007).

Application of NO increased chlorophyll content in pea and potato (Leshem et al., 1997; Laxalt et al., 1997; Beligni and Lamattina, 2002). In the present work, 0.5 and  $1.0 \text{ mmol}^{-1}$  SNP obviously increased chlorophyll content

(Figure 1a), suggesting that the exogenous NO could markedly alleviate the oxidative stress generated by chilling stress on seedlings. NO protects chlorophyll retention by maintaining iron availability and by alleviating chlorophyll decay in leaves under osmotic and oxidative stress conditions.

Excessive ROS cannot only cause photo-oxidative damage to chlorophyll but also induce overproduction of MDA, a marker of lipid peroxidation or plasma membrane in plant cells (Zhang et al, 2010). Our results show that spraying NO can decrease the MDA content in *cvZND407* and *cvZND461* (Figure 1b). The results were consistent with Leshem et al. (1997), indicating a protective role of NO at low concentrations by reacting with lipid radicals and stopping the propagation of lipid oxidation.

Chlorophyll and MDA have been used as important indicators for several physiological and biochemical parameters of plants under diverse stresses due to a decrease in membrane integrity and lipid peroxidation (Zhang et al, 2009). Applying NO might not only influence membrane oxidation system to enable seedlings to be adaptive to chilling stress but also increase activity of oxidant enzymes, especially by GR and CAT.

GR activity has been documented to associate with the alteration of the oxidized/reduced glutathione (GSSG/GSH) ratio which is more decisive in determining plants that are resistant to abiotic and biotic stresses than GSH content (Wang et al., 2009). GSH has a redox thiol group which is involved in the redox regulation of the cell cycle and has therefore been considered to play a key role against oxidative stress (Lozano et al., 1996). GR activity is increased in leaves with exogenous NO treatment. Leaves showed high GR activity at 1.0 mmol<sup>-1</sup> SNP (fig 2b). The GR activities also increased at different levels of NO treatment with some fluctuation, suggesting GR may be activated to regulate the oxidant status of GSH after the plant was treated with low temperature-induced stress.

Catalase is one of the important enzymes involved in the removal of toxic peroxides (Verma and Dubey, 2003). Increase in the catalase activity was observed upon NO treatment followed by chilling stress, which suggests a possible removal of H<sub>2</sub>O<sub>2</sub> and toxic peroxides. The CAT activity was also reported to be increased in cucumber roots under salt stress (Shi et al, 2007), and in rice seedlings under Cd stress (shah et al, 2001) upon exogenous spraying of SNP. However, Ruan et al (2002) found that CAT activities in wheat leaves displayed a decline in their activity. Clark et al. (2000) speculated that NO reversibly inhibited tobacco CAT activity by directly interacting with the iron atom in the heme moiety, forming an iron-nitroso complex. The contradictory observations indicate the dual role NO might depend on the concentration of NO as well as environmental conditions.

Both CAT and POD functions as effective quenchers of ROS (Levitt, 1980). In this study, spraying of exogenous NO enhanced POD activity, however the increase was not significant in both *cvZND407* and *cvZND461* (Figure

2c) when compared to the increases in CAT activity. These results suggested that CAT can serve as a better intrinsic defense tool to resist low temperature-induced oxidative damage in cucumber seedlings than POD.

SOD is one of the important components of the reactive oxygen species scavenging system in the plant cell (Levitt, 1980). Our results showed that leaves exhibit significant increase in the activity of SOD compared to the control (Figure 2a). Similar results had been reported when plants were exposed to salt and stress (Shi et al., 2007; Singh et al., 2009) suggesting that NO could increase SOD which responds to stresses by affecting *de-novo* synthesis of the enzymic protein and reducing the injury from chilling stress. The elevated activities of GR, CAT, SOD and POD due to NO treatment enabled a more balanced redox state, thereby sustaining higher survival rate of seedlings under chilling stress. Thus, it could be concluded that NO regulated the antioxidant system by rapidly responding to chilling stress. Moreover, osmotic substances such as soluble sugar and protein also affect seedlings tolerance to stress. The soluble sugar content in cucumber leaves treated by exogenous SNP increased sharply. Interestingly, soluble sugar content in *cvZND407* at 1.0 mmol<sup>-1</sup> SNP treatment was 1.6-fold compared to the control and in *cvZND461* was 11-fold compared to control (Figure 1c). Similar results were observed in *Arabidopsis* rosettes, with a significant increase in the degree of freezing tolerance that occurs within 1 day at 2°C being positively correlated with soluble sugar content (Wanner and Junttila, 1999) and that the heterosis of leaf freezing tolerance generated by crossing between different ecotypes is positively correlated with leaf sugar content (Rohde et al., 2004).

Soluble protein metabolism is an important metabolic pathway against chilling stress (Sarhan and Perras, 1987). Our results showed that under the chilling stress soluble protein in the leaves treated with NO had no significant changes (Figure 1d), suggesting a limited regulatory role associated with the metabolic changes known to occur during the onset chilling stress.

In conclusion, exogenous NO at 1.0 mmol<sup>-1</sup> SNP is able to significantly improve the tolerance of cucumber seedlings subjected to chilling stress. NO exerted its protective effect through the activation of some anti-oxidative enzymes and osmotic adjustment substances. Application of exogenous NO during the growth of vegetable plants under chilling stress can be used to decrease plant stress conditions. However, the molecular mechanisms involved in the antioxidative adaptation are still poorly understood and the signaling pathways involved needs further investigation.

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