

Full Length Research Paper

# Effects of nitric oxide on the growth and antioxidant response of submerged plants *Hydrilla verticillata* (L.f.) Royle

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To investigate the effect of exogenous Nitric oxide (NO) on submerged plants, *Hydrilla verticillata* (L.f.) Royle was treated with 25 - 400  $\mu\text{M}$  sodium nitroprusside (SNP, a NO donor) for 0.5 - 4 d. The alterations in plant growth,  $\text{H}_2\text{O}_2$ , total chlorophyll and malondialdehyde (MDA) content and the antioxidant response were assayed. The results showed that 25 - 100  $\mu\text{M}$  SNP increased the plant growth and total chlorophyll content and reduced the level of  $\text{H}_2\text{O}_2$ . However, an increase in  $\text{H}_2\text{O}_2$  and MDA content and a decrease in total chlorophyll were detected in plants exposed to 200 - 400  $\mu\text{M}$  SNP. The activity of catalase and peroxidase decreased in plants exposed to 25 - 400  $\mu\text{M}$  SNP; and for superoxide dismutase, its activity was suppressed by the 25 and 50  $\mu\text{M}$  SNP. The content of ascorbic acid and dehydroascorbate were also determined. The results provided the evidence that 25 - 100  $\mu\text{M}$  SNP increased the growth of *H. verticillata* by alleviating the oxidative stress and 200 - 400  $\mu\text{M}$  SNP might enhance the oxidative stress in plant cell. The results suggested that NO has dual role on the growth and ROS metabolism of *Hydrilla verticillata* (L.f.) Royle.

**Key words:** Nitric oxide, *Hydrilla verticillata* (L.f.) Royle, chlorophyll, growth, malondialdehyde.

## INTRODUCTION

Nitric oxide (NO) has been considered as an important second messenger in organism. In animals, NO can be produced by the activity of NO synthases (NOS) and involves in many physiological processes including vaso-relaxation, platelet inhibition, neurotransmission, cytotoxicity, and immunoregulation (Brunori et al., 1999). In plants, NOS activity has also been detected (Ninnemann and Maier, 1996; Durner et al., 1998; Leshem et al., 1998) and inhibitors of NOS have been shown to reduce this activity (Ninnemann and Maier, 1996; Delledonne et al., 1998), but up to date NOS gene has not been identified

in the sequenced genomes of plants (Yamasaki, 2000) or their ancestors (Mallick et al., 2000). In fact, NO can be produced from  $\text{NO}_2^-$  in a non-enzymatic way or catalyzed by nitrate reductase (NR) (Yamasaki, 2000) in plants. By reacting with many targets (Wink et al., 1993; Beligni and Lamattina, 2001; Wendehenne et al., 2001), NO can involve in the regulation of physiological responses including photomorphogenesis, seed germination, de-etiolation, hypocotyl elongation (Leshem and Haramaty, 1996; Beligni and Lamattina, 2001), organ maturation and senescence (Leshem, 1996; Leshem et al., 1998; Tu et al., 2003). NO is also involved in many processes as antioxidant agent in plants in responses to abiotic stresses from heavy metals and UV-B-radiation (Beligni and Lamattina, 1999a; García-Mata and Lamattina, 2001; Uchida et al., 2002).

Aquatic plants have important roles in releasing oxygen, providing food and shelter for animals and in maintaining the health of aquatic ecosystems (Wang et al., 2008). However, they have markedly decline or disappeared

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**Abbreviations:** NO, Nitric oxide; SNP, sodium nitroprusside; MDA, malondialdehyde; POD, peroxidase; CAT, catalase; DHA, dehydroascorbate; TCA, trichloroacetic acid; AsA, ascorbic acid; ROS, reactive oxygen species; APX, ascorbate peroxidase.

in eutrophicated water column, for example in the Yangtze River basin, china (Wang et al., 2008). The decline of submerged macrophytes was closely associated with the fast-growth of phytoplankton including algae, which contributes to the water bloom, low light intensity and low level of soluble oxygen (Schriver et al., 1995). Many phytoplankton including harmful red tide phytoplankton *Chattonella marina* (Kim et al., 2006) and freshwater algae *Anabaena doliolum* (Mallick et al., 1999) can not only produce NO but also release NO into the water column. Recently, Zhang et al. (2005) found that exterior NO stimulated the growth of marine algae and it is involved in the regulation of the algae density. They suggested that NO can be released from marine algae and the high level of exterior NO is associated with the outbreak of plankton. These data demonstrates that NO have roles in the regulation of the algae growth (Mallick et al., 1999). However, the effects of NO on aquatic macrophytes remain unclear. Since high level of NO can cause damage to plant cell due to its active activity (Beligni and Lamattina, 1999a), it is necessary to investigate whether NO is involved in the decline of aquatic macrophyte.

Submerged plants *Hydrilla verticillata* (L.f.) Royle distributes widely in freshwater in China. To investigate the adaptive mechanism of *H. verticillata*, the alterations in relative growth rate, total chlorophyll content, oxidative stress and antioxidant response were assayed in plants exposed to 25 - 400  $\mu\text{M}$  SNP.

## MATERIALS AND METHODS

### Materials and treatment conditions

Plants of *H. verticillata* were purchased from a market in Hangzhou, Zhejiang Province, China. For acclimation, plants (7 cm of the tip portion) were cultured in 1% Hoagland's solution. After acclimation for two weeks, plants of *H. verticillata* were cultured in 1% Hoagland's solution with the addition of 0, 25, 50, 100, 200 and 400  $\mu\text{M}$  SNP. Plants were harvested after treatments for 0.5, 1, 2 and 4 days. Harvested plants were washed with double distilled water, blotted and frozen immediately in liquid nitrogen for storage at -80°C.

Plants were cultured with a photoperiod of 10:14 (light/dark) at 23( $\pm$ 1)°C and irradiance of 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in laboratory conditions. The solutions were renewed every day. Each treatment was performed in triplicates.

### Determination of relative growth rate and photosynthetic pigments

The plants were washed with distilled water for two times, blotted and then weighted before or after treatments with 0 - 400  $\mu\text{M}$  SNP, and weight of plant before and after treatments with 0 - 400  $\mu\text{M}$  SNP for 4 days were expressed as  $W_1$  and  $W_2$ , respectively. Relative growth rate of plants were calculated using the formula: relative growth rate =  $(W_2 - W_1) * 100\% / W_1$ .

To determine the content of chlorophylls, a 0.2 g of plant sample was ground in liquid nitrogen, and then extracted with 10 mL 96% ethanol for 24 h in dark. After centrifugation at 10,000 $\times g$  for 10 min, absorbance of the supernatant was taken at 649 and 665 nm. The

concentration of chlorophyll was calculated according to Wang et al. (2008).

### Determination of H<sub>2</sub>O<sub>2</sub>

The level of H<sub>2</sub>O<sub>2</sub> was determined as described by Jana and Choudhuri (1981). A 0.5 g sample of plant material was powdered in liquid nitrogen, and then homogenized in 3 mL 50 mM sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 8000 $\times g$  for 20 min. For the determination of H<sub>2</sub>O<sub>2</sub>, 750 mL of the supernatant was mixed with 2.5 mL of 0.1% titanium sulfate in 20% (v/v) H<sub>2</sub>SO<sub>4</sub> and centrifuged at 8000 $\times g$  for 10 min. The absorbance of supernatant at 410 nm was measured, and the absorbance values were calibrated against a standard curve generated with known concentrations of H<sub>2</sub>O<sub>2</sub>.

### Lipid peroxidation

The level of lipid peroxidation in plant leaves was determined by estimation of the malondialdehyde (MDA) content based on the method of Heath and Packer (1968). Briefly, 0.5 g of plant material was ground with 5 mL of 1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 $\times g$  for 10 min, then 0.5 mL of the supernatant was mixed with 2 mL of 0.5% thiobarbituric acid in 20% TCA. The mixture was heated at 96°C for 30 min, stopped at 0°C and centrifuged at 10,000 $\times g$  for 10 min. The amount of MDA equivalent of thiobarbituric acid-reactive substance was calculated from the difference in absorbance at 532 and 600 nm using the extinction coefficient  $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

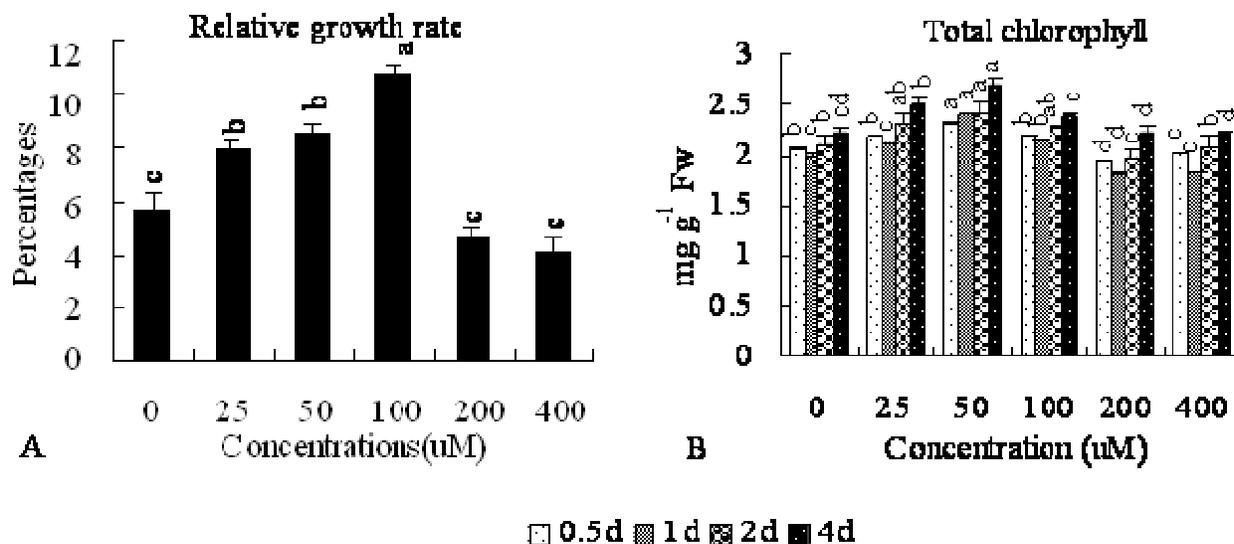
### Assay of antioxidant enzymes

A 0.5 g sample of plant material was ground in liquid nitrogen, and then homogenized with 4 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of ascorbic acid (AsA; 1 mM final concentration) for the APX assay. The homogenate was centrifuged at 10,000 $\times g$  for 20 min at 4°C, and the supernatant was used for the enzyme assays.

APX activity was determined essentially as described by Nakano and Asada (1981) but with a few modifications. The homogenate was centrifuged at 10,000 $\times g$  at 4°C for 30 min. The reaction solution contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5mM AsA, and 0.1 mM H<sub>2</sub>O<sub>2</sub>. The reaction was started by adding 50 - 100  $\mu\text{L}$  of enzyme extract. The decrease in absorbance at 290 nm was measured and activity was calculated using the extinction coefficient  $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ . One unit of APX activity was defined as the amount required to decompose 1  $\mu\text{M}$  ascorbic acid oxidized  $\text{min}^{-1} \text{ mg protein}^{-1}$ .

CAT activity was determined by measuring the change of absorbance at 240 nm that accompanied the consumption of H<sub>2</sub>O<sub>2</sub> (Aebi, 1974). The 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 20 mM H<sub>2</sub>O<sub>2</sub> and 100 - 150  $\mu\text{L}$  of enzyme extract. The decrease of absorbance at 240 nm was measured. The activity was calculated using the extinction coefficient  $\epsilon = 0.0394 \text{ mM}^{-1} \text{ cm}^{-1}$ . One unit of CAT activity was defined as the amount required decomposing 1 nmol H<sub>2</sub>O<sub>2</sub>  $\text{min}^{-1} \text{ mg protein}^{-1}$ .

POD activity was determined as oxidation of guaiacol by H<sub>2</sub>O<sub>2</sub> (Upadhyaya et al., 1985). The reaction mixture was 2.5 mL of 50 mM potassium phosphate buffer (pH 6.1), 0.1 mL of 3% H<sub>2</sub>O<sub>2</sub>, and 0.2 mL of 1% guaiacol and 10 - 20  $\mu\text{L}$  of enzyme extract. The increase in absorbance at 420 nm was measured. Activity was calculated using the extinction coefficient  $\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ . One unit of POD activity was defined as the amount required to decompose 1  $\mu\text{M}$  guaiacol  $\text{min}^{-1} \text{ mg}^{-1} \text{ protein}$ .



**Figure 1.** Effect of different concentrations of sodium nitroprusside (SNP) on relative growth rate (A) and total chlorophyll contents (B) of *H. verticillata*.

#### Assay of antioxidants

AsA and total ascorbate (AsA+Dehydroascorbate (DHA)) were determined according to the method of Hodges et al. (1996). 2 g powdered sample was extracted in 5 mL 5% (v/v) m-phosphoric acid. The homogenate was centrifuged at 12,000 $\times$ g for 15 min. For determination of total ascorbate, 0.1 mL supernatant were added to 0.5 mL of 100 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7.4) containing 5 mM EDTA and 0.2 mL 10 mM dithiothreitol (DTT) were prepared. After reaction at room temperature for 15 min, 0.2 ml 0.5% (w/v) N-ethylmaleimide, 0.8 mL 10% (w/v) TCA, 0.8 mL 44% (v/v) phosphoric acid, 0.8 mL  $\alpha$ ,  $\alpha'$ -dipyridyl in 70% (v/v) ethanol and 0.4 mL 30 g/L  $\text{FeCl}_3$  were added and well mixed in sequence. AsA was assayed in a similar manner except that 0.2 mL of ddH<sub>2</sub>O was substituted for DTT. The absorbance of the mixture at 525 nm was recorded after incubation at 40°C for 1 h. The difference between total ascorbate and ascorbic acid (AsA) was considered to represent the content of DHA.

#### Statistical analysis

Each result shown in the figures is the mean of at least five replicate treatments. All the data were analyzed by ANOVA and Tukey's test,  $p < 0.05$ .

## RESULTS

#### Effect of NO on relative growth rate and total chlorophyll

As shown in Figure 1, relative growth rate was 5.46, 7.54, 6.98, 10.77, 4.63 and 4.11%, respectively, in plants after exposure to 0, 25, 50, 100, 200 and 400  $\mu\text{M}$  for 4 days. These demonstrated that compared with control plants (0  $\mu\text{M}$ ) relative growth rate increased in plants treated with 25 - 100  $\mu\text{M}$  SNP but was inhibited at 200 and 400  $\mu\text{M}$  SNP.

Compared with control supplementation of 25, 50 and 100  $\mu\text{M}$  SNP markedly increased the total chlorophyll content at 1-4 days (Figure 1B), and the top value in total chlorophyll content was detected in plants exposed to 50  $\mu\text{M}$  SNP for 4 days. Whereas, the total chlorophyll contents did not alter or decreased markedly in plants exposed to 200 and 400  $\mu\text{M}$  of SNP from 0.5 - 4 days as compare with the control.

#### Effect of NO on levels of ROS and malondialdehyde content

Effects SNP on the levels of  $\text{H}_2\text{O}_2$  were studied in the leaves of *H. verticillata* plants and a concentration-dependent manner were obtained (Figure 2A). Compared with control plants (0  $\mu\text{M}$  SNP),  $\text{H}_2\text{O}_2$  levels decreased in plants exposed to 25, 50 and 100  $\mu\text{M}$  SNP, while it increased in plants treated with 200 and 400  $\mu\text{M}$  SNP.

Figure 2B shows that MDA content slightly decreased in plant treatments with 25 - 100  $\mu\text{M}$  SNP at 0.5 - 4 days compared with control plants, while significant increase was detected in plants exposed to 200 and 400  $\mu\text{M}$  SNP for 1 - 4 days.

#### Effect of NO on APX, CAT and POD activity

The APX (ascorbate peroxidase) activity (Figure 3A) increased in all treatments at 0.5d and in plants exposed to 200 - 400  $\mu\text{M}$  SNP for 2 days compared with control, while it decreased significantly in plants exposed to 25 and 50  $\mu\text{M}$  SNP for 4 days. Compared with control plants, activity of CAT were inhibited in plants exposed to 25 - 400  $\mu\text{M}$  SNP for 0.5 - 4 days except that a significant

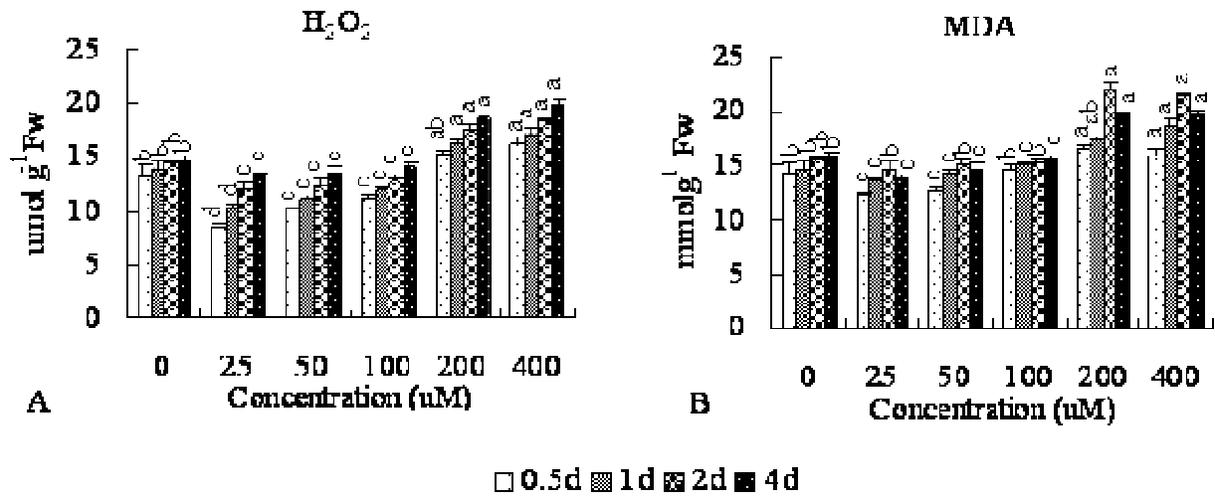


Figure 2. Effect of different concentrations of SNP on the H<sub>2</sub>O<sub>2</sub> levels and MDA contents of *H. verticillata*.

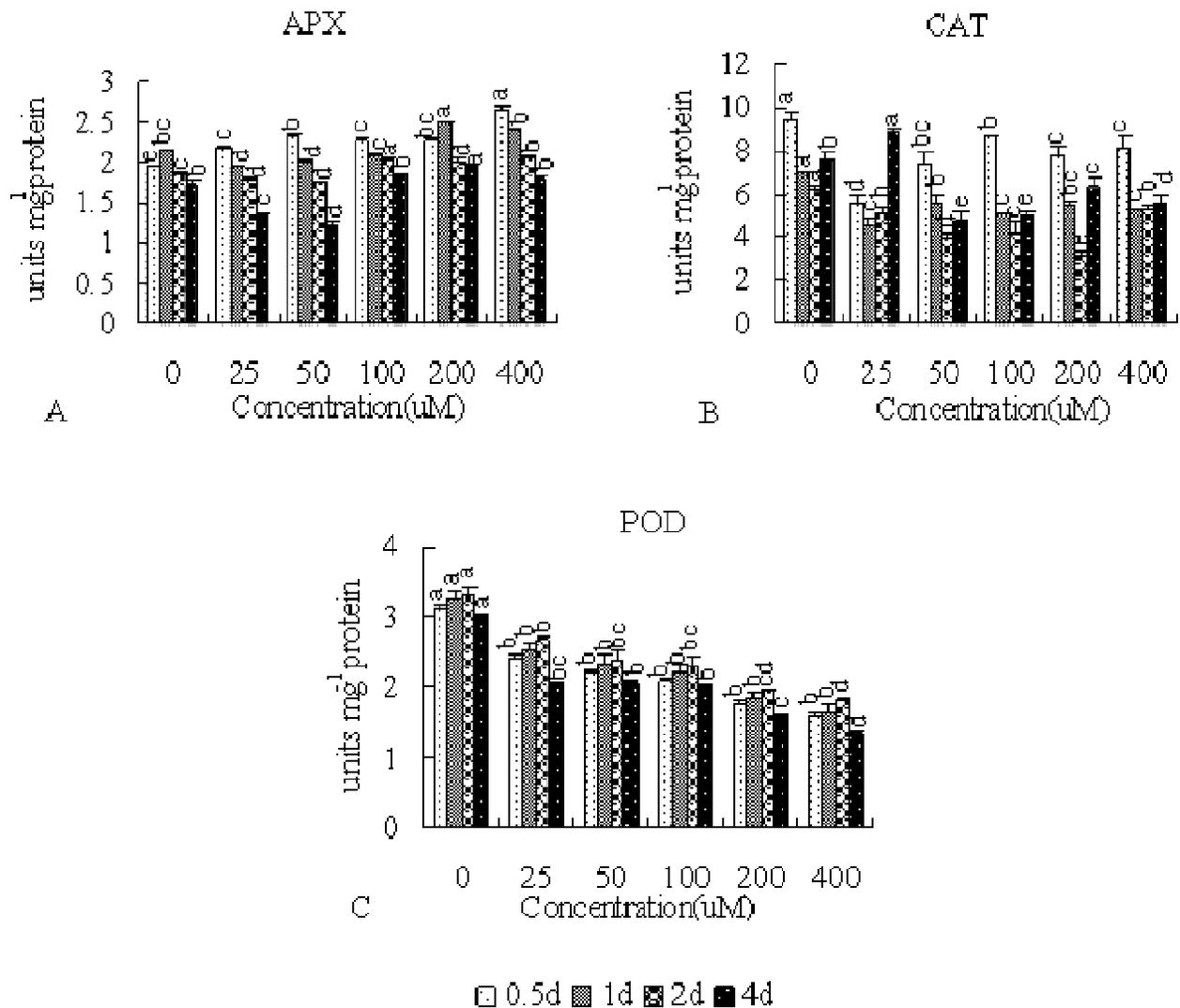
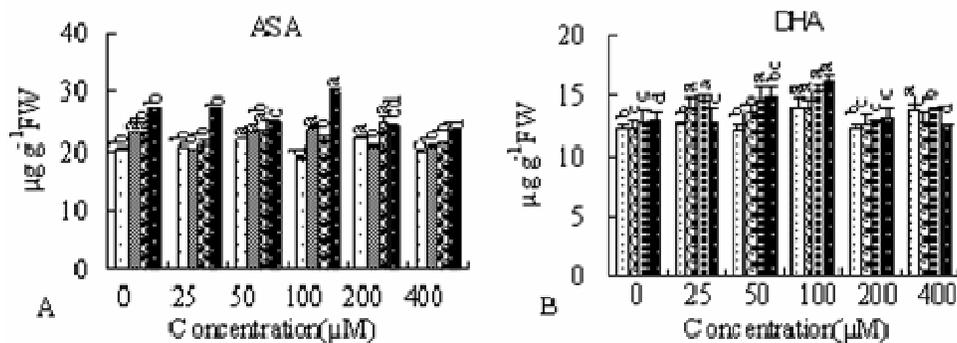


Figure 3. Effect of different concentrations of SNP on the activity of APX (A), CAT (B), POD(C) of *H. verticillata*.



**Figure 4.** Effect of different concentrations of SNP on the ASA (A) and DHA (B, Values for DHA  $\times 10$ ) of *H. verticillata*.

increase was detected at 25  $\mu\text{M}$  SNP for 0.5 days. The activity of POD were decreased in all the treatments at 0.5, 1, 2 and 4 days compared with control and the decrease in POD activity were somewhat in a concentration dependant way.

#### Effect of NO on antioxidants

Compared with control AsA concentration significantly increased at 50  $\mu\text{M}$  for 0.5 d and 100  $\mu\text{M}$  for 2 - 4 days but decreased at 100  $\mu\text{M}$  for 0.5 days, at 200  $\mu\text{M}$  for 1 and 4 d, and at 400  $\mu\text{M}$  for 1 - 4 days (Figure 4A). Figure 4B shows that DHA content increased in plants treatments with 25, 50 and 100  $\mu\text{M}$  SNP for 0.5-4 d compared with control.

#### DISCUSSION

The submerged plant *H. verticillata* has the character of fast-growth and inhabits freshwaters in a global scale, while it has been declined in Yangtze River basin due to the serious eutrophication. The effects of exterior NO on *H. verticillata* plants were investigated in this study. Plant growth rate is one of the most important indexes for the evaluation of plant growth status. In this study, the plant growth was stimulated in *H. verticillata* plants at 25 - 100  $\mu\text{M}$  SNP but was markedly inhibited at 200 - 400  $\mu\text{M}$  (Figure 1A). The growth of plant was associated with the chlorophyll content and photosynthetic rate, which determine the accumulation of dry or wet matter. Thus, the alterations in total chlorophyll content were determined and the results showed that 25 - 100  $\mu\text{M}$  SNP increased the total chlorophyll level while 200 - 400  $\mu\text{M}$  have no such effect or even markedly decreased the chlorophyll content. Accumulated data have showed that SNP application can inhibit the decrease of chlorophyll content in plants under pathogen infection or heavy metal toxicity (Hsu and Kao, 2004; Laxalt et al., 1997). Graziano et al. (2002) reported that the increase of chlorophyll content

may be attributed to the role of NO in maintaining the availability and/or delivery of metabolically active nutrient elements, which are necessary in chlorophyll biosynthesis and chloroplast within the plant, for example the active iron (Graziano et al., 2002). Similar to our results, Qian et al. (2009) showed that supplementation of low SNP (20  $\mu\text{M}$ ) increased chlorophyll content in *Chlorella vulgaris* but high level (100  $\mu\text{M}$ ) SNP inhibited the chlorophyll content. They also showed that the increase and decrease of chlorophyll content were associated with the enhanced- and inhibited-expression of photosynthesis genes, respectively.

Increase of ROS is one of the main causes of lipid peroxidation which is related to plasma membrane permeability and is considered as an important indicator of cell membrane damage (Beligni and Lamattina, 1999b).

In this study, application of 25 - 100  $\mu\text{M}$  SNP decreased  $\text{H}_2\text{O}_2$  level in *H. verticillata* plants. Singh et al. (2009) have reported that the protective effects of SNP in wheat under Cd stress clearly ascribed to the release of NO, although they did not use any NO scavenger (or the metabolic products of SNP) to establish the protective role of SNP through the release of NO. The application of NO may contribute to the decrease of  $\text{H}_2\text{O}_2$  level, since inhibition of NO synthesis can increase the hydrogen peroxide, superoxide anion, oxidized lipid, and oxidized protein levels in *Arabidopsis* (Guo and Crawford, 2005). Xiao and Zhang (2004) reported that 0.01 - 0.1 mmol/l SNP contradict with ROS and the exogenous NO (SNP) that concentrations above 0.5 mmol/l can cooperate with ROS. Earlier study (Beligni and Lamattina, 1999b) has indicated that lower level of NO can protect plant by directly reacting with effectual molecule or changing the redox electricity potential difference of cell indirectly and by participating in the signal transmit process of the growth and accommodation of plant. Accumulated data showed that high NO can show toxic effect (Beligni and Lamattina, 1999a) because it can induce the generation of free oxygen ( $\text{O}^\cdot$ ) and oxide hydrogen and can further interact with oxide hydrogen in producing a mass of peroxy nitrite, ONOO<sup>-</sup>, resulting in the damage to structure

and function of the big biology molecular. Increased  $H_2O_2$  contents were detected in *H. verticillata* plants exposed to 200 - 400  $\mu M$  SNP. Increase of  $H_2O_2$  concentration can be ascribed to the inhibited CAT, POD and APX activity. Reports from Tu et al. (2003) also showed that exogenous NO has dual roles in regulating the process of aging in wheat leaves and suggested that NO plays roles in regulating the aging process of wheat leaves by adjusting the ROS level. MDA is the end product of lipid peroxidation and also used to evaluate the lipid peroxidation (Gong et al., 1989; Baker and Orlandi, 1995). The alteration trend of MDA content changed similarly to that of  $H_2O_2$ , demonstrating that concentration of  $H_2O_2$  can be used as an index of lipid peroxidation. Thus, regulation of NO on the growth of *H. verticillata* may be related to its interaction with ROS, and lower NO might act as an antioxidant in plant tissues.

Because high ROS levels can damage lipids, proteins and DNA, ROS concentrations are normally well controlled by antioxidant system including the non-enzymic and enzymic antioxidants in plants (Apel and Hirt, 2004). CAT and POD are important antioxidant enzymes in detoxification  $H_2O_2$ . In the present study, CAT activity increased significantly in leaves of *H. verticillata* exposed to 25  $\mu M$  SNP at 4d while it was inhibited in high SNP or a longer exposition. Qian et al. (2009) also found that high SNP application decreased CAT activity. POD activity was inhibited in both SNP concentrations. Tu et al. (2002) found that SNP application could decrease the activity of POD significantly in detached wheat Leaves, but the effect on CAT is less. Many isoforms of CAT and POD contain haemachrome and NO can potentially regulate their activity by acting on the haemachrome directly (Clark et al., 2000). However, different isoforms may have different response to NO, for example, NO can irreversibly inhibit the activity of CAT in tobacco by combining with heme iron in CAT directly but have no effect on the CAT isozymes which have non-haemachrome (Clark et al., 2000). POD is believed to be involved in the lignification of secondary cell walls by catalyzing the polymerization of phenolics into lignin, and in the forming of covalent cross-links between ferulylated polysaccharides, hydroxyproline-rich glycoproteins and lignin (Zhang et al., 2007; Wang et al., 2008). Leshem and Haramaty (1996) think that NO acts on the lipid double molecule layer of membrane cell and other wall components directly by apoplastic, and then cell wall loosed that can enhance the fluid of cell membrane and accelerate the expansion of cell. In this sense, 25 - 100  $\mu M$  SNP may increase the plant growth by decreasing the level of lignification of cell wall and accelerating the expansion. However, high level of SNP may enhance the leakage of membrane due to the oxidative stress and the impaired cell wall, and thus inhibited the growth of plants.

Many antioxidants including ascorbic acid (AsA) play important roles in scavenging free radicals, preventing lipid peroxidation and protecting photosynthetic system.

The increase of AsA concentration may contributed to the ROS detoxification, since AsA has an important role in maintaining APX activity and can be consumed by APX in scavenging  $H_2O_2$  in ascorbate-glutathione pathway (Ye et al., 2006). Supplication of 100 - 400  $\mu M$  SNP can stimulate the increase of APX activity, while 25 - 50  $\mu M$  SNP decreased the APX activity. Hsu and Kao (2004) also detected that 100  $\mu M$  SNP decreased the APX activity in rice leaves under cadmium toxicity, while Singh et al. (2009) found that 50  $\mu M$  SNP increased APX activity in roots of *Oryza sativa*. Our results demonstrated that NO could regulate APX activity in different way. The increase of DHA can also suggest the important role of APX.

In this study, both the decrease of AsA content and the decrease of DHA concentration were detected in leaves of *H. verticillata*. Meanwhile, the ratios of DHA/AsA increased significantly at 25  $\mu M$  SNP for 1 day and 100  $\mu M$  SNP for 12h while normally increased at other treatments compared with control (data not shown). Ratio of DHA/AsA is an important indicator of the redox status of the cell, and increase of the ratio is always considered as one of the first signs of oxidative stress (Di Baccio et al., 2004). The increase of DHA/AsA ratio may ascribe to the increase of ROS, since excess NO and  $H_2O_2$  can cause oxidative stress in plant cell (Ye et al., 2006).

In conclusion, the results showed that 25 - 100  $\mu M$  SNP could increase total chlorophyll contents and enhance the relative growth rate of *H. verticillata*, whereas 200-400  $\mu M$  SNP decreased total chlorophyll contents and have no significant effect on the relative growth rate. The results indicating NO may be not the main cause of *H. verticillata* in entrophic water column, since 100  $\mu M$  SNP can cause damage to algae *Chlorella vulgaris* (Qian et al., 2009). The increased plant growth rate is associated with the decreased  $H_2O_2$  and MDA content. We also found NO (100 - 200  $\mu M$  SNP) application could decrease the activity of APX, CAT and POD which contributed to the accumulation of  $H_2O_2$  and thus induced the oxidative stress.

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