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The effect of exogenous nitric oxide on alleviating herbicide damage in *Chlorella vulgaris*

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ABSTRACT

Herbicides present in the environment induce oxidative stress in plants. We investigated the roles of exogenous NO-regulated chlorophyll synthesis, antioxidant enzyme activity and gene expression in herbicide-treated unicellular green algae *Chlorella vulgaris*. Atrazine (100 μg/L) or glufosinate (10 mg/L) treatment alone or in combination with 20 μM sodium nitroprussiate (SNP, 10 μM with glufosinate) was administered to algae for a short time period of 48 h to observe changes in physiology and gene transcription and expression. Supplementation of atrazine or glufosinate with low SNP significantly reduced H_2O_2, reactive oxygen species (ROS) and malondialdehyde (MDA) induction by herbicides. Supplementation also increased chlorophyll content and antioxidant enzymes, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activity, as compared to herbicide treatment alone. This trend suggests an effect of NO on the scavenging of ROS. Furthermore, the expression of photosynthesis genes (*psb*C, *psa*B, *chl*B and *rbc*L) was also upregulated by supplementation of low SNP, thus maintaining the normal photosynthetic function. However, high concentration of SNP (100 μM) in combination with herbicides aggravated damage to algae, including increases in H_2O_2, ROS and MDA and decreases in chlorophyll content, antioxidant enzyme activity and photosynthesis genes transcription. 2-Phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), the NO scavenger, was also examined in this study; the results showed that PTIO could neutralise the effect of low SNP. Data also showed that an exogenous supply of NO protects *Chlorella vulgaris* against the toxicity of herbicides by protecting against oxidant substances and increasing the transcription of related genes.

1. Introduction

Nitric oxide (NO) is a diffusible multifunctional secondary messenger in animals that takes part in neurotransmission, vasorelaxation, smooth-muscle relaxation and immunoregulation of pathophysiological processes (Ignarro, 1990; Anbar, 1995; Gow and Ischiropoulos, 2001). Recently, reports have demonstrated that NO-mediated signal transfer also occurs in plants. Moreover, some studies postulate a role for NO as an inter- and intra-cellular signal molecule that can control plant metabolism ; regulate growth, flowering, maturation and apoptosis; and prevent infection. Thus, some researchers call NO as a "plant growth regulator" or "non-traditional plant hormone" (Beligni and Lamattina, 2001; Wendehenne et al., 2004).

NO is involved in the regulation of plant defense responses to biotic and abiotic stresses. Heavy metal pollution is a major environmental concern; some reports have shown that exogenous NO exhibited an antioxidative role in the context of Cu toxicity to *Chlorella vulgaris* (Singh et al., 2004) and rice leaves (Yu et al., 2005); NO also decreased Cd-induced oxidative damage in rice leaves (Hsu and Kao, 2004), sunflower leaves (Laspina et al., 2005) and wheat roots (Singh et al., 2008). Notably, salinity represents a major constraint of agriculture. NO stimulated ROS-scavenging (ROS, reactive oxygen species) enzymes and reduced accumulation of H_2O_2 in cucumber roots induced by NaCl (Shi et al., 2007), stimulated the germination of NaCl-treated *Suaeda salsa* seeds (Li et al., 2005), stimulated the expression of PM H^+-ATPase in reed under salt stress (Zhao et al., 2004), and dramatically promoted the germination of wheat seeds under osmotic stress by improving antioxidant capacity (Zhang et al., 2005). Recently, it has been observed that NO ameliorated UV-induced damage by lowering H_2O_2 content and ion leakage, as well as enhancing the activities of scavenging enzymes (Shi et al., 2005). Herbicides comprise another source of heavy pollution in the environment, and exogenous NO has been shown to reduce herbicide toxicity, like bipyridinium and paraquat (Beligni and Lamattina, 1999; Beligni and Lamattina, 2002; Hung et al.,

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2002). NO acts as an antioxidant and quenches ROS generated by stresses such as heavy metal, salinity, UV, herbicide, etc. NO upregulated the activity of antioxidant enzymes including superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR) to scavenge ROS, and it reduces oxidative damage and provides protection against cellular injury. These results implied the existence of a regulatory interplay between NO and ROS.

Further research is required to elucidate the exact molecular cascade and genes expressed in response to environmental stress-induced NO signalling. Given the essential role of NO in stress resistance, it is likely that this highly reactive and diffusible molecule affects the transcription of a battery of genes related to defence (Polverari et al., 2003). An important role for NO in regulating gene transcription has already been suggested by research in the medical field (Marshall et al., 2000; Pineda-Molina and Lamas, 2001) and may extend to several different transcription factors. Polverari et al. (2003) reported that exogenous NO modulates a variety of genes in *Arabidopsis thaliana* roots. These genes are involved in biotic and abiotic stresses, including ROS formation and scavenging, cellular defence and death. Noriega et al. (2007) proved that NO is cytoprotective against cadmium oxidative stress by an enhancement of heme oxygenase (HO) transcript levels in soybean, as it happens with genes associated with the antioxidant defence system.

Atrazine and glufosinate represent the most heavily used herbicides in the world. Our previous reports showed through real-time polymerase chain reaction (PCR) assay that atrazine and glufosinate inhibited photosynthesis by reducing the transcript levels of three photosynthetic genes (*psb*C, *psa*B and *rbc*L) in the aquatic unicellular algae *Chlorella vulgaris* (Qian et al., 2008a,b). In the present study, we performed experiments in algae with sodium nitroprussiate (SNP), a well-known NO donor, following herbicides administration. Here, we investigate the physiological role of NO as an antioxidant against short-term (48 h) exposure to herbicides by analysing the changes in chlorophyll content, as well as the activity of antioxidant enzymes (SOD, POD and CAT) and levels of the oxidative stress marker MDA. We also aim to elucidate the effect of exogenous NO on the photosynthesis of *Chlorella vulgaris* by analysing changes in *psb*C, *psa*B, *chl*B and *rbc*L at transcriptional level.

2. Materials and methods

2.1. Culture conditions

Chlorella vulgaris was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. The microalgae were cultured in sterilised freshwater and supplemented with shuisheng-4 medium (Zhou and Zhang, 1989). The algae were cultured at 25 ± 0.5 °C in flasks (250 mL) containing 50 mL of medium under cool-white fluorescent lights (approximately 2500 lx) with a daily light:dark cycle of 16:8 h. The cell density of cultures was monitored spectrophotometrically at 685 nm. The regression equation between cell density ($y \times 10^5$/mL) and OD685 (x) was calculated as $y = 162.1x + 1.3463$ ($r^2 = 99.34\%$).

The concentrations of atrazine (Changxing Chemical Company, Zhejiang, China) and glufosinate (AgrEvo) solutions in culture medium were 100 μg/L and 10 mg/L, respectively, and prepared when cultures reached the exponential growth. These two concentrations were determined by our preliminary research at acute toxicity (from 12 to 96 h) for *Chlorella vulgaris* (Qian et al., 2008a,b). At the same time, different SNP (Sigma Company) concentrations (5, 10, 20, 30, 40, 50 and 100 μM) were combined with herbicides to evaluate the presence of any neutralising effect. 2-Phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO, Sigma Company) was used as a NO scavenger, and it was administered together with SNP. Triplicate cultures were prepared for each treatment; samples were taken after 48 h for enzyme analysis and RNA extraction.

2.2. NO, H_2O_2 and ROS analysis

In this experiment, NO production in cells was measured according to the indication on the NO assay kit (Beyotime Institute of Biotechnology, Haimen, China). Thirty millilitres of each culture was centrifuged at 10,000 × *g* for 10 min to measure NO content. The protocol is based on the chemical diazotisation reaction that was originally described by Griess (1879). The level of H_2O_2 was analysed by a commercial Kit (Beyotime Institute of Biotechnology, China) and measured the absorbance at 560 nm using a microplate reader (Bio-TEK, USA). In this kit, ferrous ions (Fe^{2+}) were oxidative to ferric ions (Fe^{3+}) by H_2O_2. The Fe^{3+} then forms a complex with an indicator dye xylenol orange (3,3′-bis[N,N-di(carboxymethyl)-aminomethyl]-o-cresolsulfone-phthalein, disodium salt and XO) causing an increase in absorbance at 560–590 nm measurable as a purple colored complex (Deiana et al., 1999). ROS were measured according to the instruction of ROS kit instructions (Beyotime Institute of Biotechnology, Haimen, China). In this kit, the nonfluorescent probe 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) passively diffuses into cells and is deacetylated by esterases to form nonfluorescent 2′,7′-dichlorofluorescein (DCFH). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. The fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (Bio-TEK, USA). The intensity of fluorescence as compared to control was viewed as the increase of intra-cellular ROS.

2.3. Pigment analysis

Analysis of chlorophyll (a, b and total) content was carried out according to Inskeep and Bloom (1985). Ten millilitres of each culture was centrifuged at 10,000 × *g* for 10 min and 2.5 mL of N,N-dimethylformamide (DMF). Samples were kept in the dark at 4 °C for 1 day. The extracts were centrifuged at 5000 × *g*, absorption (OD) of the supernatant at 647 and 664.5 nm was measured against N,N-DMF. The following formulae were used for calculation of the chlorophyll content:

$$\text{chlorophyll } a = [12.7 \times \text{OD664.5}] - [2.79 \times \text{OD647}]$$
$$\text{chlorophyll } b = [20.7 \times \text{OD647}] - [4.62 \times \text{OD664.5}]$$
$$\text{total chl} = 17.9\text{OD647} + 8.08\text{OD664.5}$$

2.4. Enzyme and the transcription analysis

Fifty millitres of each culture was collected to extract enzymes. Separated algal cells were ground using a tissue grinder and homogenised with 1 mL of 20 mM phosphate buffer (pH 7.4) and 0.1 g of white quartz sand in a pre-cooled pestle and mortar. The extract was centrifuged at 12,000 × *g* for 10 min at 4 °C to obtain the supernatant for assays of the enzyme activity and lipid peroxidation. SOD activity was measured according to Alexander and Christine (2000). One unit of SOD activity was defined as the amount of enzyme inhibiting 50% of WST-1 photoreduction. CAT activity was measured according to Fossati et al. (1980). One unit of CAT activity was defined as the amount of H_2O_2 degraded per minute at 25 °C. POD activity was measured according to Andrews et al. (2000). One unit of POD activity was defined as the one absorbance unit of change per min. The lipid peroxidation level was determined in terms of MDA content according to Zhang and Kirkham (1994).

Thirty millilitres algal cultures were collected to extract RNA. Separated algal cells were ground to a fine powder in liquid nitrogen in mortar, and then added to 500 μL RNAiso reagent (TaKaRa Company, Dalian, China) to extract RNA, according to the manufacturer's protocol. Nucleic acid concentrations were measured spectrophotometrically at 260 nm. The integrity and purity of the RNA were determined by 260/280 nm ratios and electrophoresis on a 1% agarose formaldehyde gel. Reverse transcription (RT) was carried out using an MMLV reverse transcriptase kit (TaKaRa Biochemicals, China). Four photosynthesis-related genes in a freshwater algae *Chlorella vulgaris* were selected as target genes. These genes are: (i) *psb*C (GeneID: 809130), which codes for an integral membrane protein component of photosystem II (PSII); the primers are forward 5′-CGGTGCTTGGCTTTTAGTTTG-3′, reverse 5′-GAACATCACCACCACCAGGA-3′; the product length = 80 bp; (ii) *psa*B (NCBI GeneID: 809130), which codes for the photosystem I (PSI) reaction centre protein; the primers are forward 5′-GCTGGTCAATCTTTGGCTTC-3′, reverse 5′-AAAGTCTCCGGTCCGATGGT-3′; product length = 90 bp; (iii) *chl*B (GeneID: 809139), which codes for light-independent protochlorophyllide reductase subunit B; the primers are forward 5′-AGCGAATTCCCGGTTTTGT-3′, reverse 5′-TTATGCTCCTACGGCTTCTTTTG-3′; product length = 124 bp; (iv) *rbc*L (accession number: AF499684), which codes for the large subunit of Rubisco; the primers are forward 5′-CTTGGACGACTGTATGGACTG-3′, reverse 5′-ATACCGTGAGGAGGACCTTG-3′; product length = 261 bp. The 18S rRNA gene (accession number: X13688) was used to standardise the results by eliminating variations in the quantity and quality of mRNA and cDNA; primers are forward 5′-TTGACGGAAGGGCACCA-3′, reverse 5′-CACCACCCATAGAATCAAGAAAGAG-3′; product length = 127 bp. Real-time PCR and relative quantification of gene transcription were performed as described in our previous report (Qian et al., 2008a,b).

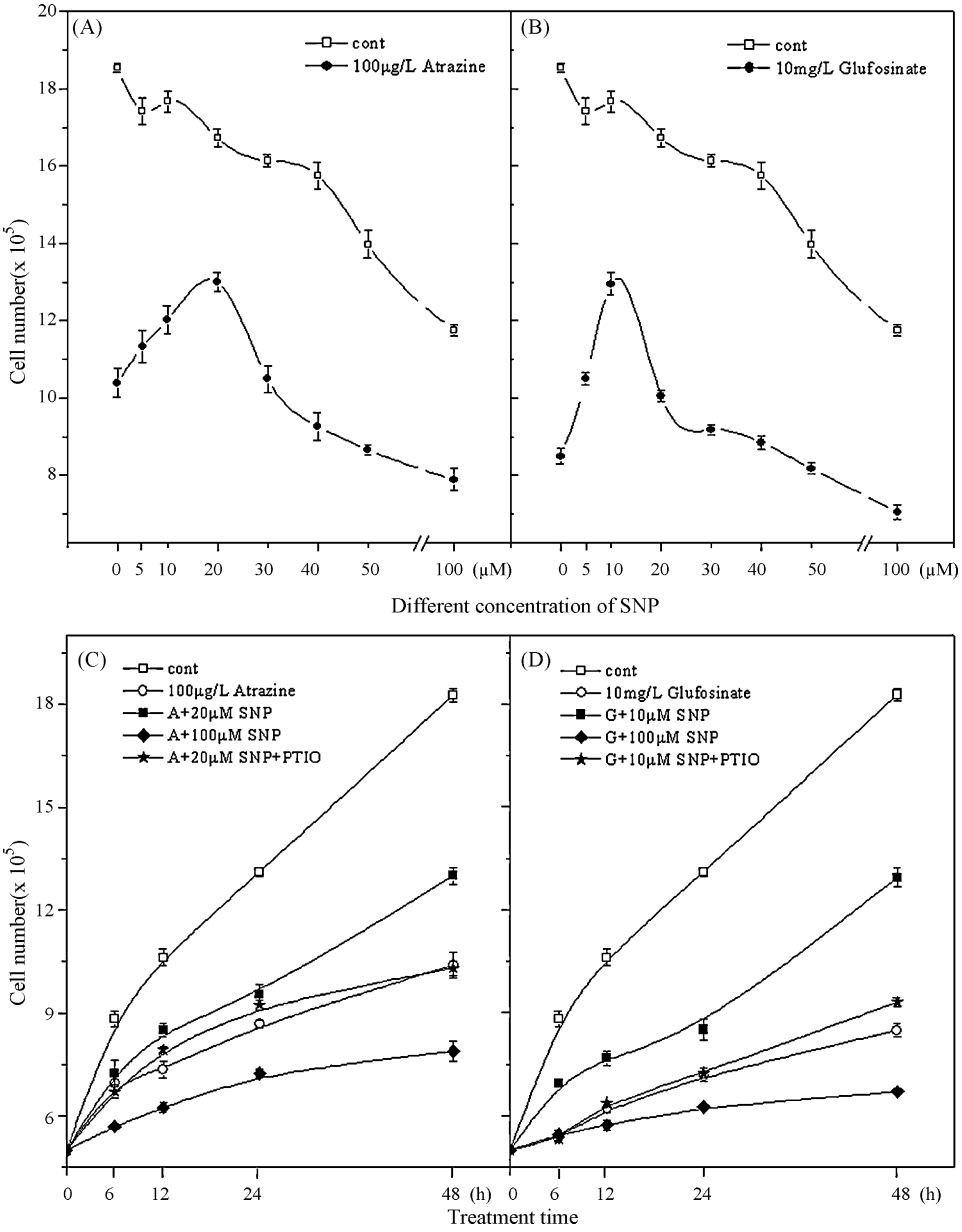

Fig. 1. The effect of SNP and PTIO on alleviating algae death caused by atrazine and glufosinate. (A) Algae were treated with 100 μg/L atrazine and different concentrations of SNP and kept for 48 h, (B) algae were treated with 10 mg/L glufosinate and different concentrations of SNP and kept for 48 h, (C) algae were treated with atrazine, SNP, and PTIO in some combination for 6–48 h and (D) algae were treated with glufosinate, SNP and PTIO in some combination for 6–48 h.

2.5. Data analysis

Data are presented as mean ± standard error of the mean (S.E.M.) and were tested for statistical significance using analysis of variance (ANOVA) followed by Fisher's post hoc test using the StatView 5.0 program (Statistical Analysis Systems Institute, Cary, NC, USA). Values were considered significantly different when the probability (p) was less than 0.05.

3. Results

3.1. Effect of SNP on algae growth

Exponential growth algae were prepared in flasks containing H_2O (control) or herbicides (100 μg/L atrazine and 10 mg/L glufosinate). Treatment with different concentrations of SNP, a nitric oxide donor, continued for 48 h. Cell numbers were recorded at different SNP concentrations. Fig. 1A shows that following treatment with atrazine, cell numbers had already decreased to 56.1% compared to the control. The low tested SNP concentrations (5, 10, 20 and 30 μM) were able to partially prevent algal death caused by atrazine; cell counts were 61.1%, 64.8%, 70.1% and 56.6% of control, respectively. Higher tested SNP concentrations (40, 50 and 100 μM) were found to be toxic, as cell numbers decreased much worse than in the treatment of atrazine without SNP. Fig. 1B shows that following treatment with glufosinate, cell count decreased to 45.8% compared to control. The low tested concentrations of SNP (5, 10, 20, 30 and 40 μM) were also able to partially prevent algal death caused by glufosinate, cell counts were 56.6%, 69.9%, 54.3%, 49.5% and 47.7% of the control, respectively. Higher SNP concentrations (50 and 100 μM) were still found to be toxic, as cell numbers decreased much worse than in the treatment of glufosinate without SNP. Under our experimental conditions, 20 and 10 μM SNP were the most effective doses and were selected to further test the effect of NO on alleviating the damage of atrazine and glufosinate.

In order to correlate decreased algal death with NO release from donor solutions, we used the NO scavenger PTIO. According to some reports (Beligni et al., 2002; Zhang et al., 2006), PTIO and SNP usages were equimolar. In this experiment, 10 and 20 μM PTIO was added together with 10 and 20 μM SNP, respectively. Fig. 1C and D shows that the SNP-mediated protection was arrested in the presence of the scavenger. Population density for atrazine (or glufosinate) + 20 μM (10 μM in glufosinate) SNP + 20 μM (10 μM in glufosinate) PTIO-treated algae was similar to population density following atrazine and glufosinate treatment.

3.2. Effects of SNP on the content of NO, H_2O_2 and ROS

Nitric oxide content did not significantly change with exposure to atrazine or glufosinate, but it increased 2.9- and 7.3-fold when exposure to low SNP and high SNP, respectively, were combined with atrazine. Nitric oxide content increased 2.1- and 5.3-fold when exposure to low SNP and high SNP, respectively, were combined with glufosinate. PTIO scavenged NO which was released by SNP; NO content subsequently dropped to control levels (Fig. 2A).

There was a significant increase in the H_2O_2 content of algal cells after 48 h of atrazine and glufosinate treatment as compared to untreated control (Fig. 2B, nearly 3.5- and 3.1-fold, respectively). However, upon addition of low SNP, H_2O_2 content dropped to 44.8% and 59.9% of levels observed after atrazine and glufosinate treatment, respectively. Nonetheless, H_2O_2 content still higher than that of control. The greatest increases in H_2O_2 content occurred in the atrazine or glufosinate stress plus high-SNP treatments, with respective increases of 6.9- and 6.7-fold as compared to control. Application of both PTIO and low SNP during herbicide stress

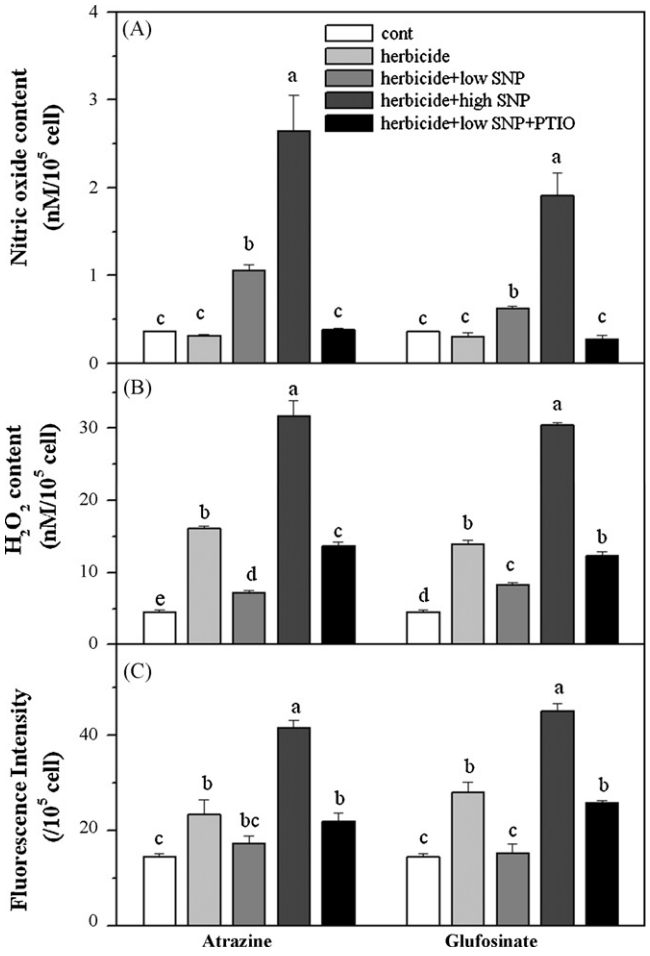

Fig. 2. Effects of SNP and PTIO on levels of NO (A), H_2O_2 (B) and ROS (C) in *Chlorella vulgaris* exposed to SNP and PTIO during a 48 h experiment. (A) NO content, (B) H_2O_2 content and (C) ROS content. Different letters represent statistically significant differences at $p < 0.05$.

increased H_2O_2 content as compared to low SNP during herbicide stress; H_2O_2 content in the latter treatment group was similar to herbicide treatment alone.

Herbicide stress also caused an expected increase of ROS; atrazine or glufosinate increased ROS levels to 1.6- and 1.9-fold of control (Fig. 2C). In contrast, significant decreases in ROS levels were seen in the algal cell treated with low SNP, as compared to herbicide. However, ROS levels exhibited significant increases of about 2.9- and 3.1-fold in algal cells treated with high SNP in the presence of atrazine and glufosinate, respectively. PTIO in the presence of herbicide and low SNP yielded ROS levels similar to those of herbicide stress alone.

3.3. Effects of SNP on chlorophyll content

Atrazine decreased chlorophyll content: Chla, Chlb and total chlorophyll content decreased, respectively, by 38.8%, 57.6% and 46.7% as compared to the control (Fig. 3). Experiments carried out in the presence of low SNP (20 μM) revealed that this concentration partially prevented chlorophyll loss following atrazine insult (33.0%, 43.9% and 37.8% decreases compared to control, respectively). On the other hand, 100 μM SNP caused greater chlorophyll loss, as Chla, Chlb and total chlorophyll decreased to only 14.2%, 29.1% and 19.8% of control. As shown in Fig. 3, the protective effect of 20 μM SNP could be reversed partly by the addition of 20 μM PTIO; the loss of chlorophyll was similar in samples treated with atrazine

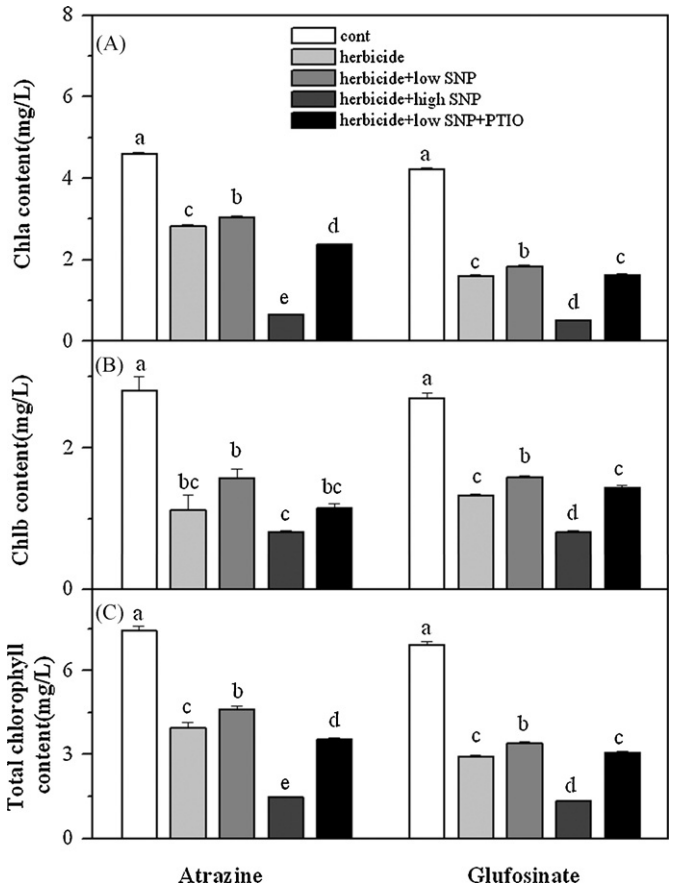

Fig. 3. Effects of SNP and PTIO on the inhibition of chlorophyll content in *Chlorella vulgaris* exposed to SNP and PTIO during a 48 h experiment. (A) chlorophyll *a*, (B) chlorophyll *b* and (C) total chlorophyll. Different letters represent statistically significant differences at $p < 0.05$.

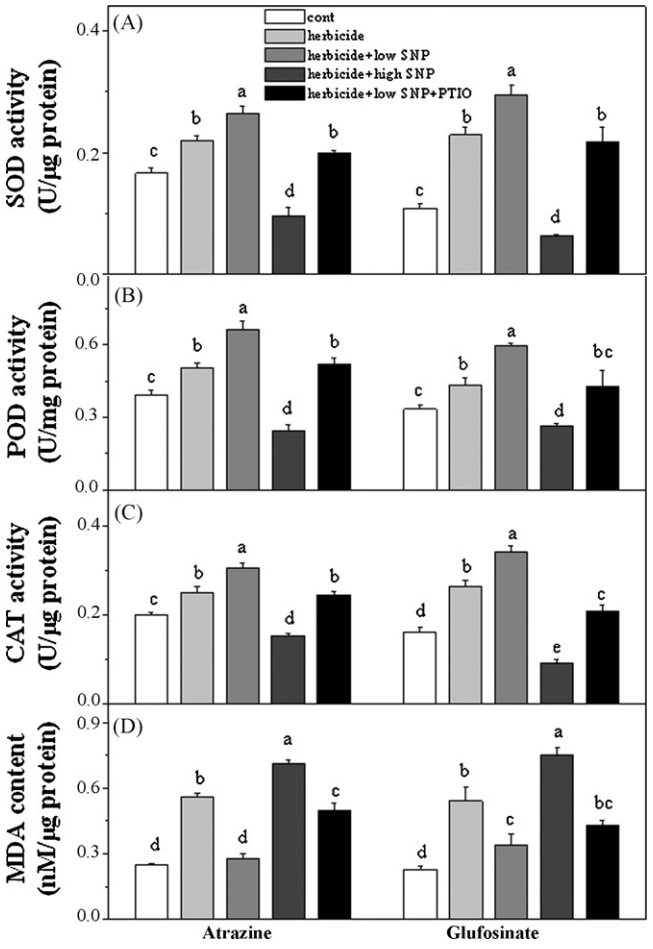

Fig. 4. Activities of superoxide dismutase (A), peroxidase (B), catalase (C), and the content of malondialdehyde (D) in *Chlorella vulgaris* exposed to SNP and PTIO during a 48 h experiment. *Y*-axis represents the activities of enzymes (or the content of MDA) expressed as mean ± S.E.M. of three replicate cultures. Different letters represent statistically significant differences at $p < 0.05$.

alone. Chlorophyll content variation during glufosinate treatment exhibited the same pattern as under atrazine treatment. Glufosinate decreased synthesis of Chla, Chlb and total chlorophyll by 61.9%, 50.8% and 57.6% of control levels. The low concentration of SNP (10 μM) also partially prevented chlorophyll loss due to glufosinate, while the high concentration of SNP exacerbated decreases in Chla, Chlb and total chlorophyll to 12.4%, 30.1% and 19.3% of control, respectively. The protective effect of low SNP in alleviating chlorophyll loss was also reversed by the addition of 10 μM PTIO.

3.4. Effects of SNP on SOD, POD, CAT activities and MDA content

SOD, POD, CAT activities, as well as MDA content, are shown in Fig. 4. Atrazine exposure resulted in 1.3-, 1.3-, 1.2- and 2.2-fold increases in the activity of SOD, POD, CAT and MDA content, respectively. These data suggest that atrazine stress could stimulate the activities of antioxidant enzymes to eliminate the damage of environmental stress, an inherent characteristic of plants. When exposure to atrazine was combined with low SNP (20 μM), the activity of SOD, POD and CAT increased 1.6-, 1.7- and 1.5-fold in comparison to control; MDA content decreased to control levels. Whereas antioxidant enzymes decreased following atrazine/high SNP (100 μM), the activities of SOD, POD and CAT were only 57.8%, 61.8% and 76.0% of control; MDA content increased 2.9-fold as compared to control. The NO scavenger PTIO was added together with 20 μM SNP and atrazine, and the SNP-mediated protection was arrested in the presence of the scavenger. In addition, antioxidant enzyme activity and MDA content were similar to levels observed after atrazine treatment.

Changes in antioxidant enzyme activity and MDA content with exposure to glufosinate were the same as those observed with exposure to atrazine. Glufosinate stimulated the activities of SOD, POD and CAT and the level of MDA 2.1-, 1.3-, 1.6- and 2.4-fold, respectively, as compared to control. Glufosinate combined with low SNP (10 μM) increased SOD, POD and CAT activities 2.7-, 1.8- and 2.1-fold, respectively, and reduced MDA content. High concentration SNP reduced the activities of antioxidant enzymes and increased the level of MDA. PTIO added together with 10 μM SNP and glufosinate prevented SNP-induced antioxidant enzyme activity and MDA returned to the levels observed with glufosinate treatment.

3.5. Effects of SNP on the transcription of photosynthesis genes

We analysed the transcription of *psb*C, *psa*B, *chl*B and *rbc*L mRNA in algae cells following exposure to different combinations of SNP, atrazine (or glufosinate) and PTIO (Fig. 5). Real-time PCR revealed that atrazine (or glufosinate) inhibited the transcription of *psb*C, *psa*B, *chl*B and *rbc*L to 56.1%, 48.7%, 49.8% and 28.8% of control (45%, 55.2%, 55.1%, 66.6% by glufosinate). When low concentrations of SNP were added into algae cultures exposed to atrazine (or glufosinate), levels of *psb*C, *psa*B, *chl*B and *rbc*L returned to control levels (91.6%, 95.7%, 93.1% and 108.2% of control [atrazine] and 88.0%, 94.9%, 97.5% and 88.7% of control [glufosinate]). When the added concentration of SNP was high (100 μM in this study),

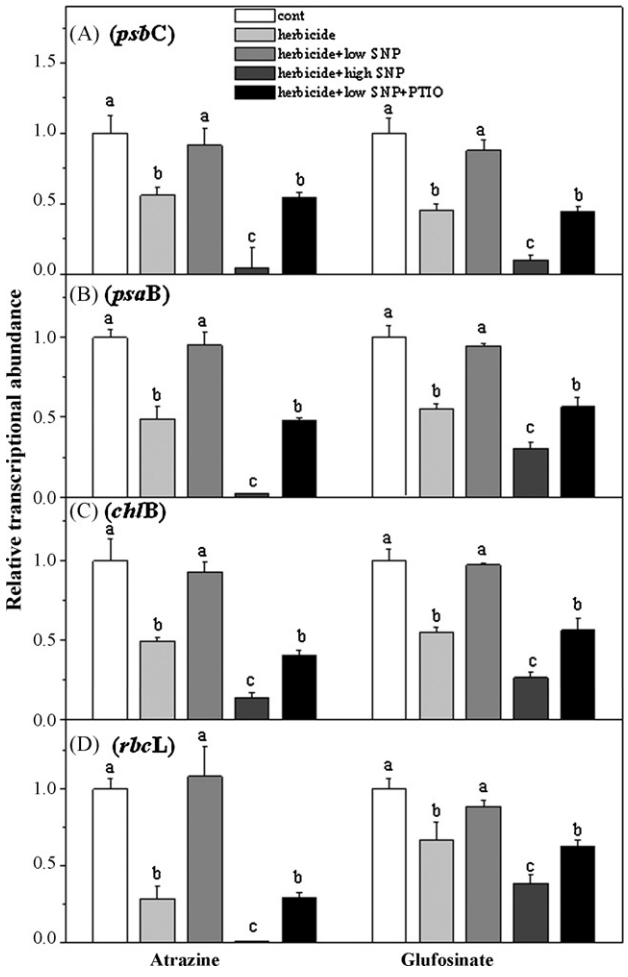

Fig. 5. Expression of *psb*C(A), *psa*B(B), *chl*B(C) and *rbc*L(D) in *Chlorella vulgaris* exposed to SNP and PTIO during a 48 h experiment. Values were normalised against 18S, a housekeeping gene, and represent the mean mRNA expression value ±S.E.M. ($n = 3$) relative to that of control. Different letters represent statistically significant differences at $p < 0.05$.

the transcription of photosynthesis genes was inhibited. Levels of *psb*C, *psa*B, *chl*B and *rbc*L were only 4.6%, 2.1%, 14.2% and 9% of the control following atrazine/high SNP treatment; levels were 10.1%, 30.3%, 26.7% and 38.7% of control following glufosinate/high SNP treatment. PTIO returned the transcription of photosynthesis gene to levels observed with atrazine or glufosinate treatment alone.

4. Discussion

Environmental stressors, such as bright light, drought, mechanical injury, temperature extremes, heavy metals, salinity, UV radiation, ozone, herbicide treatments and pathogens, lead to the production of free radicals in plants; these free radicals can destroy the organelles, damage the membrane system and inhibit related gene expression (Foyer et al., 1994; Gould et al., 2003). NO, one of the key reactive nitrogen species, represents a new and exciting field in plant biology. Decade-long investigations of NO functions in plants have shown that NO is involved in various physio-biochemical processes and plays a prominent role in the activation of various environmental stress responses in plant species (Delledonne et al., 2001). Despite the wealth of information gathered from the analysis of its functions in physiological processes, little was known about NOs impact on gene expression. Such activity may elucidate the mechanism of action for NO. Because of ignorance of the pathways comprising NO production in plants, researchers used the exogenous NO donors SNP, S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-DL-penicillamine (SNAP), as well as the NO scavengers PTIO and c-PTIO to analyse NO activity.

The inhibitory effects of atrazine and glufosinate on physiology and photosyhthesis genes in *Chlorella vulgaris* have been previously analysed by our group (Qian et al., 2008a,b). This research shows that in algae, 100 μg/L atrazine and 10 mg/L glufosinate significantly inhibit functions such as algae growth and gene expression, and this treatment also increased MDA content in agreement with previous data (Qian et al., 2008a,b). The physiological relevance of NO in alleviating damage induced by atrazine and glufosinate was assessed by adding compounds that react with NO or inhibit its production. Low or high concentrations of SNP released NO in a concentration-dependent manner, in agreement with previous reports (Ederli et al., 2008). Low SNP also decreased levels of H_2O_2 and ROS, rapidly accumulated during atrazine or glufosinate treatment. At the same time, low SNP increased the activity of antioxidant enzymes (SOD, POD and CAT), supporting a report that exogenous NO increases the activity of SOD, ascorbate peroxidases (APX) and CAT in salt-stressed barley (Li et al., 2008); the activity was higher than that caused by herbicide stress. We also found that SNP decreased the level of MDA, which was used as an indicator of lipid peroxidation (Halliwell and Gutteridge, 1984). Supplementation of atrazine or glufosinate with SNP may reduce H_2O_2 and ROS levels by heightening the activity of antioxidant enzymes, thus decreasing the damage done by lipid peroxidation.

Chlorella vulgaris is one of the simplest eukaryotic organisms; photosynthesis is the most complex physiological process in its metabolism. Plants have two photosystems, photosystem I (PSI) and photosystem II (PSII), to absorb light energy and to provide the reducing equivalents and chemical energy by converting carbon dioxide into carbohydrates. *psbC* encodes a PSII chlorophyll (Chl)-binding protein involved in splitting, acting as an oxygen-evolving enzyme of photosynthesis(Murray et al., 2006). *psa*B encodes a protein in the core of the PS I reaction centre, which binds ~100 Chl a and 30β-carotene molecules. As the photochemical reaction centre, this protein coordinates most of the electron transfer cofactors and acts as an inner antenna (Murray et al., 2006). As is well known, green algae have a light-independent pathway for chlorophyll biosynthesis in which chlorophylls *a* and *b* can be synthesised in complete darkness (Burke et al., 1993). *chl*B is one of the chloroplast genes necessary for the light-independent pathway for chlorophyll biosynthesis in photosynthetic eukaryotes (Suzuki and Bauer, 1992). *rbc*L encodes the large subunit of Rubisco, which is the key enzyme of the Calvin–Benson–Basham cycle, catalysing the first step in which CO_2 is reductively assimilated into organic carbon. Therefore, the expression of these four genes is necessary for the transfer of photosynthetic electrons, as well as the synthesis of chlorophyll and carbohydrates. In the present study, exogenous NO also upregulated photosynthesis-related gene expression of *psb*C, *psa*B, *chl*B and *rbc*L to the normal level, even after expression was severely inhibited by herbicides. These results showed that NO increases gene transcriptional level associated with photosynthesis-related genes. Beligni and Lamattina (2002) also found that SNP increased the abundance of *rbcLS* and *psb*A, both of which decreased following diquat treatment. The study also showed that herbicide treatment (like diquat) caused general degradation of total RNA, while NO protected against RNA breakdown.

High SNP had the opposite effects, it decreased antioxidant enzyme activity, increased the levels of ROS, H_2O_2 and MDA, and further decreased the expression of photosynthesis-related genes. Thus NO acts in a concentration-dependent manner, and low concentrations of NO could act as an efficient scavenger in breaking

the oxidative chain. High NO concentrations could provoke a major oxidative insult, since this molecule is itself a nitrogen reactive species (Shi et al., 2005). NO is recognised both as a signaling molecule that regulates many enzyme activities but is also a reactive nitrogen species that could have a toxic effect. The cytotoxic effects of NO can be largely ascribed to peroxynitrite ($ONOO^-$), produced by the diffusion-limited reaction of NO and superoxide (O_2^-): $NO + O_2^{\bullet -} \rightarrow ONOO^-$. Therefore, when O_2^- is produced in plant cells under stress conditions, $ONOO^-$ is potentially formed as the result, $ONOO^-$ is one of the most cytotoxic radical species, which can oxidise and nitrate DNA and may potentially cause strand breaks through attack on the sugar-phosphate backbone (Asada, 1999). NO in biological systems can also be oxidated by molecular oxygen to form a powerful nitrosating agent, N_2O_3, which forms as follows: $2NO + O_2 \rightarrow 2NO_2$, $NO_2 + NO \rightarrow N_2O_3$. N_2O_3 can alkylate DNA through an indirect mechanism and directly nitrosate the primary amine functionalities of DNA bases, leading to deamination (Chen and Deen, 2001).

To further verify the physiological and gene transcriptional role of exogenous NO under herbicides, the NO scavenger PTIO was used to scavenge the NO released by SNP. Our work showed that treatments with PTIO clearly reduced the accumulation of NO content in algal cell. This result clearly suggests that PTIO indeed has the ability to scavenge NO. However, without enough NO, ROS and H_2O_2 levels increased to those of samples exposed only to herbicide stress, and antioxidant enzyme activity dropped. Therefore, ROS and H_2O_2 damaged the algal cell and inhibited the process of metabolism, as proven by the increase of MDA and the decrease of chlorophyll content (an index for chloroplast development). Furthermore, the expression of photosynthesis genes were also inhibited to the level observed with herbicide treatment. As the NO scavenger PTIO abolished all the protective effect of SNP, NO may alleviate herbicide damage in this experimental system.

In this study it was demonstrated that depending on its concentration, NO can not only increase the activity of antioxidant enzyme to protect against the oxidative insult caused by herbicide stress, but also increase the transcriptional level of photosynthesis-related genes (*psb*C, *psa*B, *chl*B and *rbc*L), which are inhibited by herbicides stress.

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