Effects of nitrogen dioxide and its acid mist on reactive oxygen species production and antioxidant enzyme activity in *Arabidopsis* plants

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ABSTRACT

Nitrogen dioxide (NO_2) is one of the most common and harmful air pollutants. To analyze the response of plants to NO_2 stress, we investigated the morphological change, reactive oxygen species (ROS) production and antioxidant enzyme activity in *Arabidopsis thaliana* (Col-0) exposed to 1.7, 4, 8.5, and 18.8 mg/m^3 NO_2. The results indicate that NO_2 exposure affected plant growth and chlorophyll (Chl) content, and increased oxygen free radical (O_2^-) production rate in *Arabidopsis* shoots. Furthermore, NO_2 elevated the levels of lipid peroxidation and protein oxidation, accompanied by the induction of antioxidant enzyme activities and change of ascorbate (AsA) and glutathione (GSH) contents. Following this, we mimicked nitric acid mist under experimental conditions, and confirmed the antioxidant mechanism of the plant to the stress. Our results imply that NO_2 and its acid mist caused pollution risk to plant systems. During the process, increased ROS acted as a signal to induce a defense response, and antioxidant status played an important role in plant protection against NO_2/nitric acid mist-caused oxidative damage.

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Introduction

Nitrogen dioxide (NO_2) is one of the most common and harmful air pollutants. Due to its massive discharge from motor vehicle exhaust and stationary sources such as electric utilities and industrial boilers, the concentration of NO_2 in the atmosphere has gradually increased in many areas of the world during the past few decades. Therefore, NO_2 has been a strong indicator in atmospheric environment monitoring and a potential risk factor in adverse effect exploration. As reported, peak levels of up to 0.8–8 mg/m^3 have been encountered in the outdoors, particularly along curbsides in downtown areas with heavy motor vehicular traffic (Pathmanathan et al., 2003). However, due to the development of industrialized production and the continuous rise of automobile exhaust emissions, NO_2 concentration will be likely to further increase in the future. Following the increase of NO_2 environmental concentration, another serious issue is the occurrence of nitric acid precipitation (nitric acid mist), which results from its combining with atmospheric moisture and causes adverse ecological effects.

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Recently, epidemiologic data and experimental results have linked NO_2 exposure with a wide range of effects on human health, including increased mortality risk, increased rates of hospital admissions and emergency department visits, exacerbation of chronic respiratory conditions (*e.g.*, asthma), and decreased lung function (Samet and Krewski, 2007). Therefore, it is expected that plants might be affected after exposure to atmospheric NO_2 and its acid mist pollution. Some studies have demonstrated that NO_2 could enter leaf mesophyll tissue through stomata, combine with water and then convert to nitric acid, which burns plant tissue. The symptom appears as lesions visible on both sides of the leaves, which initially occurs between leaf veins or along leaf edges (Li et al., 2007; Miao et al., 2008). A deeper level of toxicity was also studied for some plants such as *Brassica campestris* seedlings, *Cinnamomum camphora* seedlings and tomato plants (Ma et al., 2007a,b; Pandey and Agrawal, 1994; Teng et al., 2010). NO_2 could induce change to growth and photosynthetic activity, causing oxidative damage accompanied by changes in the antioxidant defense system (Chen et al., 2010; Ma et al., 2007a; Takahashil et al., 2011).

However, whether these toxic effects of NO_2 apply to other plants or whether similar symptoms will occur under the stress of nitric acid mist is not yet clear. Hence, *Arabidopsis thaliana*, an internationally recognized model plant, was chosen in this study to evaluate the specific plant response to NO_2 and nitric acid mist exposure, and the research results will be extended to other plants.

There is ample evidence that reactive oxygen species (ROS) are crucial second messengers involved in the response to diverse abiotic and biotic stresses, and can confer a degree of cross-tolerance against distinct stresses (Apel and Hirt, 2004; Foyer and Noctor, 2005a). NO_2 is an oxidant pollutant, and induces oxidative damage to cell membranes, resulting in the generation of ROS (Mustafa and Tierney, 1978; Pathmanathan et al., 2003). Increased ROS can attack biomacromolecules and result in oxidative damage to nucleic acids, proteins and lipids (Foyer and Noctor, 2005b; Mittler et al., 2004; Yi et al., 2005). However, plants can scavenge excess ROS by invoking the antioxidant defense system to avoid oxidative damage (Inzé and Van-Montagu, 1995; Mittler, 2002). The induction of antioxidant enzymes has been thought to be a protective reaction of plants against NO_2 stress, but the exact defense mechanism was not clear.

In the present study, we characterized the changes in morphological features and physiological indexes in response to NO_2 and nitric acid mist in *Arabidopsis* shoots, and determined ROS production and antioxidant enzyme activities. Our research provides a particular insight into the capacity of NO_2/nitric acid mist to induce cellular ROS and antioxidant response in plant cells, and contributes to understanding the underlying mechanisms of toxicological reaction and plant adaptation to NO_2/nitric acid mist stress.

1. Materials and methods

1.1. Plant materials and NO_2/nitric acid mist treatment

Plants of *A. thaliana* (L.) ecotype Columbia (Col-0) were purchased from the Chinese Academy of Agricultural Sciences. The seeds were disinfected with sodium hypochlorite at 1% (V/V, containing Triton-100 at 0.01%), washed three times with running water, and soaked in the water. After vernalization for 3 days at 4°C, they were seeded in seedling trays about 4.5 × 3.0 × 4.0 cm, 5 plants per tray, with peat imported from Germany as substrate (a kind of pure moss peat, with proportions of N, P and K of 14/16/18, and pH 5.5–6.5). *Arabidopsis* plants were grown in a controlled growth chamber at 22 ± 1°C with a 16 hr photoperiod per day, 70% relative humidity and a photosynthetic photon flux density of 140 μmol/(m²·sec).

Four-week-old plants were exposed to 1.7, 4, 8.5, and 18.8 mg/m³ NO_2 at 6 hr/day for 7 days, respectively, while a control group was placed in another identical chamber, which was continually flushed with filtered air for the same period of time. The NO_2 gas was diluted with fresh air at the intake port of the chamber to yield the desired concentrations; then delivered to the plants through a tube positioned in the upper part of each chamber and distributed homogeneously *via* a fan. The NO_2 concentration within the chambers was measured every 30 min by the Saltzman colorimetric method using a spectrometer calibrated at 545 nm (Kumie et al., 2009). The desired NO_2 concentrations were controlled by the opening of a flow valve. For nitric acid mist treatment, four-week-old *A. thaliana* was exposed to nitric acid fog (pH 4.6–5.0), which was produced by misting HNO_3 solution for 4, 6 and 8 hr/day for 7 days, respectively. To obtain the misting HNO_3 solution, HNO_3 solution was diluted by distilled water to the proper pH value, and then sprayed on the leaf surface with a sprayer in a mist flow. Meanwhile, a control group was placed in another identical chamber, which was continually exposed to water fog for the same period of time.

1.2. Measurement of chlorophyll (Chl) content

Chl content was determined by the method reported by Bao (2005). Briefly, fresh shoots were pulverized with distilled water, and the homogenate was extracted by 80% acetone. Absorbance of the supernatant was measured at 663 and 645 nm using a spectrophotometer and Chl content was expressed as mg/g fresh weight (fw).

1.3. Measurement of oxygen free radical (O_2^-) production rate

O_2^- production rate was determined by the hydroxylamine method (Zhang and Qu, 2003). Briefly, fresh leaf sample was homogenized in phosphate buffer (0.05 mol/L, pH 7.8), and the homogenate was centrifuged at 10,000 g for 20 min. The supernatant was mixed with hydroxylamine hydrochloride, and then kept at 25°C for 1 hr. α-Naphthylamine and aminobenzenesulfonic acid were used as chromogenic agents. Absorbance of the supernatant was measured at 530 nm using a spectrophotometer.

1.4. Estimation of ascorbate (AsA) and glutathione (GSH) pool

The content of reduced AsA was determined spectrophotometrically according to the method of Kampfenkel et al (1995). The amount of reduced GSH was examined according to the method of Sgherri and Navari-Izzo (1995).

1.5. Measurement of lipid peroxidation level

The level of lipid peroxidation was estimated by measuring the concentration of malondialdehyde (MDA). MDA is a common product of lipid peroxidation and is a sensitive diagnostic index of oxidative injury (Janero, 1990; El-Moshaty et al, 1993). Fresh leaf sample was ground in 5% trichloroacetic acid (W/V) and the homogenate was centrifuged at 4000 g for 10 min. The supernatant fraction was added to an equal volume of 0.6% thiobarbituric acid (W/V). The mixture was heated at 100°C for 10 min and then centrifuged at 3000 g for 10 min after it was cooled. The absorbance of the supernatant was measured at 532 nm and lipid peroxidation level was expressed as μmol MDA/g fw.

Protein carbonyl (PCO) content is the most general indicator and marker of protein oxidative damage. In the present study, PCO level was tested by 2,4-dinitrophenylhydrazine (DNPH) spectrophotometry (Levine et al., 1990). Briefly, fresh leaf sample (0.5 g) was ground in phosphate buffer (0.05 mol/L, pH 7.0) and the homogenate was centrifuged at 10,000 g for 5 min at 4°C. The supernatant was used for DNPH reactions. PCO content was calculated by the absorbance at 370 nm, and the result was expressed as nmol carbonyl/mg protein.

1.6. Assay of antioxidant enzyme activities

Superoxide dismutase (SOD) activity was measured by Nitro Blue tetrazolium (NBT) spectrophotometry (Zhu et al., 1990). Fresh leaf sample (0.5 g) was homogenized in phosphate buffer (0.05 M, pH 7.8) and the homogenate was centrifuged at 1000 g for 20 min at 4°C. The supernatant was added to a reaction mixture containing 130 mmol/L DL-methionine (Met), 750 μmol/L NBT, 100 μmol/L EDTA-Na$_2$, and 20 μmol/L lactoflavin, in order to determine the absorbance at 560 nm. Inhibition of 50% of the reaction was defined as one unit of enzyme and the enzyme activity was expressed as U/g fw.

Catalase (CAT) activity was assayed according to the method of Zhang (1990), with some modifications. Fresh leaf sample (0.25 g) was homogenized in phosphate buffer (0.2 mol/L, pH 7.8) containing 1% polyvinylpyrrolidone K30. The homogenate was centrifuged at 4000 g for 15 min at 4°C and the supernatant was used for the enzyme assay. CAT activity was examined by measuring the decrease of absorbance at 240 nm in a reaction mixture containing 0.3 mL H$_2$O$_2$ (0.1 mol/L) and 0.1 mL extract. Results were expressed as U/(min·g) fw.

Peroxidase (POD) activity was determined using the guaiacol method (Maehly, 1955). Briefly, fresh leaf sample (0.1 g) was homogenized in phosphate buffer (0.1 mol/L, pH 6.0) and the homogenate was centrifuged at 4000 g for 15 min at 4°C. The supernatant was added to the reaction mixture, including 5 mmol/L guaiacol as the donor and 6.7 mmol/L H$_2$O$_2$ as the substrate. The rate of change in absorbance at 470 nm was measured and the activity was expressed as DA470/min/g fw.

1.7. Determination of dissolved protein

The amount of dissolved protein was determined by the Bradford method (Bradford, 1976). We used bovine serum albumin as the standard and results were expressed as mg/g fw.

1.8. Statistical analysis of data

Experiments were repeated three times, and all values were expressed as mean ± SE. In the study, all experiment groups and control group were placed in several identical chambers separately, which were kept at the same temperature, photoperiod, relative humidity, etc. The exposure to NO$_2$ and nitric acid mist were considered to be the single factor for NO$_2$ treatment and nitric acid mist treatment, respectively, which were adopted to perform single factor analysis. Therefore, the statistical differences (0.05, 0.01 or 0.001) among the negative control and a series of treated groups were analyzed by one-way analysis of variance (ANOVA), using the Origin 7.0 software package.

2. Results

2.1. Effect of NO$_2$ exposure on the morphology and Chl content in Arabidopsis shoots

To find the general toxic response, we first exposed *Arabidopsis* to NO$_2$ at different concentrations (1.7, 4, 8.5 and 18.8 mg/m^3) and observed their morphological changes. As shown in Fig. 1a, NO$_2$ at lower concentrations affected the plant growth, and induced the occurrence of yellowing leaf after 4 and 8.5 mg/m^3 exposure. However, when the NO$_2$ concentration increased to 18.8 mg/m^3, the plant appeared to have visibly acute injuries on the leaf and eventually died after treatment. Because of this, the 18.8 mg/m^3 exposure was not considered in follow-up experiments. Following this morphological change, we further determined Chl content in *Arabidopsis* shoots, which *in vivo* was an important kind of pigment involved in the processes of photosynthesis. Fig. 1b indicates that Chl content was the lowest after 4 mg/m^3 exposure, but there was no significant difference among the treated plants compared to the control.

2.2. Effect of NO$_2$ on ROS generation and antioxidants (AsA and GSH) in Arabidopsis shoots

Exposure to NO$_2$ caused an increase of ROS generation in *Arabidopsis* shoot cells. The O$_2^-$ generation rate enhanced with increasing NO$_2$ concentration, and a statistical difference was observed at all exposure concentrations (Fig. 2a).

AsA content tended to decrease with the enhancement of NO$_2$ concentration, and a statistical difference occurred at the 8.5 mg/m^3 group (Fig. 2b). On the contrary, GSH content showed an increasing tendency with increasing NO$_2$ concentration, and was significantly induced after 1.7 and 8.5 mg/m^3 exposure (Fig. 2c).

2.3. Effect of NO$_2$ on levels of lipid peroxidation and protein oxidation in Arabidopsis shoots

The levels of lipid peroxidation and protein oxidation in NO$_2$-treated *Arabidopsis* plants were measured from the contents of MDA and PCO. As presented in Fig. 3, MDA content and PCO level varied in a NO$_2$ concentration-dependent manner and statistical increases were observed at 4 and 8.5 mg/m^3,
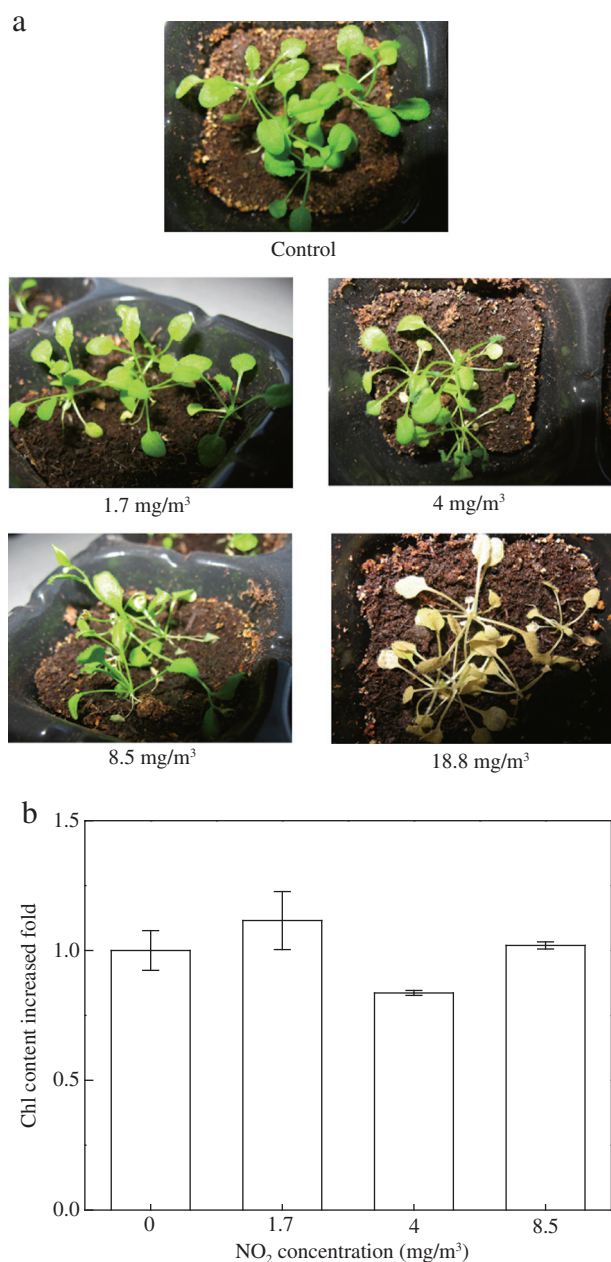
a

Control

1.7 mg/m^3 4 mg/m^3

8.5 mg/m^3 18.8 mg/m^3

b

Fig. 1 – Effect of Nitrogen dioxide (NO$_2$) exposure on the morphology (a) and chlorophyll (Chl) content (b) in *Arabidopsis* shoots. Data represent the mean ± SE of three different experiments.

indicating oxidative damage in plant cells after NO$_2$ exposure at higher concentrations.

2.4. Effect of NO$_2$ on antioxidant enzyme activities in Arabidopsis shoots

Fig. 4 indicates the changes of antioxidant enzyme activities in response to NO$_2$ exposure. SOD activity increased as a function of NO$_2$ concentration, and significant difference occurred at 4 and 8.5 mg/m^3. POD activity remained unchanged after 1.7 mg/m^3 treatment, and statistically increased when

the NO$_2$ concentration reached 4 and 8.5 mg/m^3, despite a slight decline at 8.5 mg/m^3. For CAT, it tended to be induced after the 8.5 mg/m^3 treatment, but no statistical difference was observed at all exposure concentrations.

2.5. Effect of nitric acid mist exposure on Chl content, lipid peroxidation and protein oxidation, and antioxidant enzyme activities in Arabidopsis shoots

To mimic the actually environmental exposure, we treated *Arabidopsis* with nitric acid mist for different exposure durations. Fig. 5a shows that with exposure to nitric acid mist, the Chl content significantly decreased with the increase of treatment time, and a statistical difference was observed after 6 and 8 hr/day exposure.

Following the result, we observed the induction of MDA and PCO contents after nitric acid mist exposure for different durations (Fig. 5b). The MDA level tended to increase, but no statistical difference occurred, while the PCO content increased with the enhancement of treatment time, and showed significant difference when the daily exposure time reached 8 hr/day. Also, we further tested the change of SOD, POD and CAT activities (Fig. 5c). SOD activity changed slightly in all groups of *A. thaliana*, but no statistical difference occurred. POD activity significantly increased after exposure to nitric acid mist. CAT activity remained unchanged after 4 and 6 hr/day treatments for 7 days, and was slightly but statistically enhanced after the daily exposure time reached 8 hr/day.

3. Discussion

NO$_2$ affected the growth and Chl content in *Arabidopsis* shoots, and the effects varied as functions of exposure concentration. Inhibited growth and Chl content in the present study might be attributed to the damaged defense system and consequently unbalanced metabolism.

In plant cells, ROS are unavoidable by-products of aerobic metabolism. Under normal growth conditions, amounts of ROS are modest and cells experience only mild oxidative stress, whereas many stresses enhance ROS production (Inzé and Van-Montagu, 1995; Mittler et al., 2004). The results of our present study clearly show that NO$_2$ triggered a rapid increase in O$_2^-$ generation rate in *Arabidopsis* shoots. The enhanced production of ROS under stress can pose a threat to cells, but it is also thought that ROS serve as signal molecules to activate the stress responses and defense pathways (Apel and Hirt, 2004; Knight and Knight, 2001; Mittler, 2002). Thus, NO$_2$-induced ROS can be viewed as cellular indicators of stress and as secondary messengers involved in the stress-response signal transduction pathway.

Since membrane lipids and proteins are the preferred targets of ROS in plants under environmental stress (Prassad, 1996), they are considered to be reliable indicators of controlled modulation of ROS levels and oxidative stress (Halliwell and Gutteridge, 1993). Therefore, we further investigated the levels of lipid peroxidation and protein oxidation by measuring MDA and PCO contents. In *Arabidopsis* shoots, the MDA level and PCO content increased, and oxidative stress occurred after
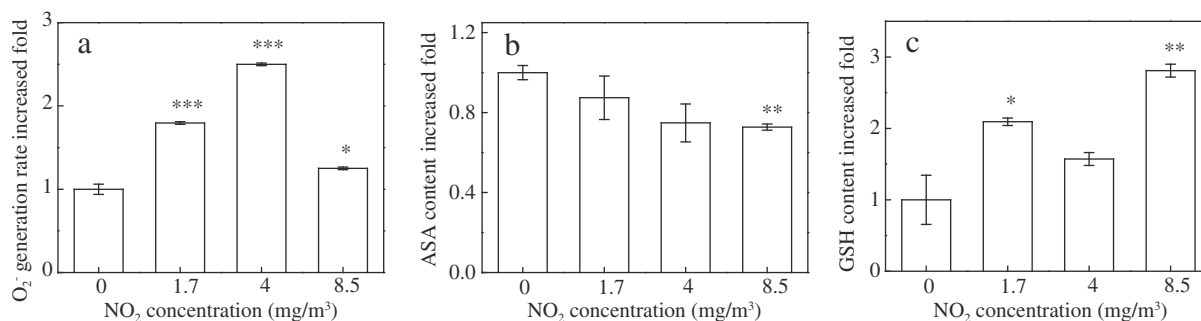
Fig. 2 – Effect of Nitrogen dioxide (NO_2) on reactive oxygen species (ROS) generation (a) and antioxidant contents (ascorbate (AsA) (b) and glutathione (GSH) (c)) in *Arabidopsis* shoots. Data represent the mean ± SE of three different experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* control.

4 mg/m^3 NO_2 exposure. This finding suggests that NO_2 at lower concentrations stimulated defense reactions, and these defense reactions might counteract ROS generated *via* elevated antioxidant defense systems in the tissue; whereas high concentration NO_2 exposure induced the production of ROS, and the capacity of the antioxidant system was exceeded, or inhibited. As a result, the excessive ROS attacked lipids and proteins and the plant experienced substantial oxidative stress.

Additionally, the activities of antioxidant enzymes (SOD, CAT and POD) were tested under the same treatment conditions. In this study, NO_2 enhanced the activities of several antioxidant enzymes including SOD, POD and CAT associated with increased ROS generation, suggesting an induction of antioxidant defenses regulated by a ROS-mediated signaling pathway. SOD is a protein class that contains metals and catalyzes the dismutation of superoxide radical anions into H_2O_2 and molecular oxygen (Scandalios, 1993). The elevated SOD activity in *Arabidopsis* plants suggests that the chemical induced SOD to increase the generation of H_2O_2. CAT and POD are the primary H_2O_2-scavenging enzymes in plant cells. In this study, NO_2 pronouncedly increased POD activity, whereas it had little effect on CAT activity. CAT has a high capacity but low affinity enzyme for H_2O_2, whereas POD has a high affinity for

H_2O_2 (Mittler, 2002). After NO_2 exposure and shorter daily exposure to nitric acid mist, POD activity was significantly enhanced and eliminated part of the generated H_2O_2. As a result, the remaining H_2O_2 might not be enough to induce CAT activity. This was not the case for the longest daily exposure, which accumulated more H_2O_2 and induced CAT activity. Thus, POD, but not CAT, was the most efficient scavenging enzyme to decrease the cellular levels of H_2O_2 in plant cells under NO_2 stress. Moreover, the increase of POD activity can contribute to the overall cell resistance to NO_2 stress since POD participates in many other cell processes involved in the plant defense reaction (Valério et al., 2004). POD can initiate cell-wall toughening events such as phenolic cross-linking and lignification, which can strengthen leaf and stem tissues against potential damage (Polle et al., 1994). The roles that POD can play in cell wall toughening and in the production of secondary metabolites, and its simultaneous oxidant and antioxidant capabilities, make it an important factor in the integrated defense response of plants to NO_2 stress. Additionally the AsA–GSH redox system in chloroplasts is also an effective scavenger of O_2^- and H_2O_2 in plant cells (Foyer and Halliwell, 1976; Jiang and Chen, 2008). Thus as NO_2 concentrations increased, GSH content increased, but AsA content decreased, suggesting that
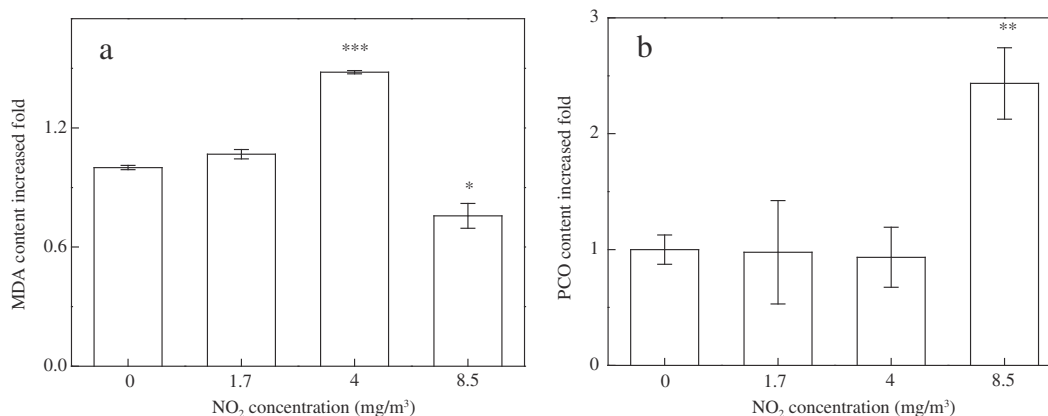

Fig. 3 – Effect of Nitrogen dioxide (NO_2) on levels of lipid peroxidation (a) and protein oxidation (b) in *Arabidopsis* shoots. Data represent the mean ± SE of three different experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* control.
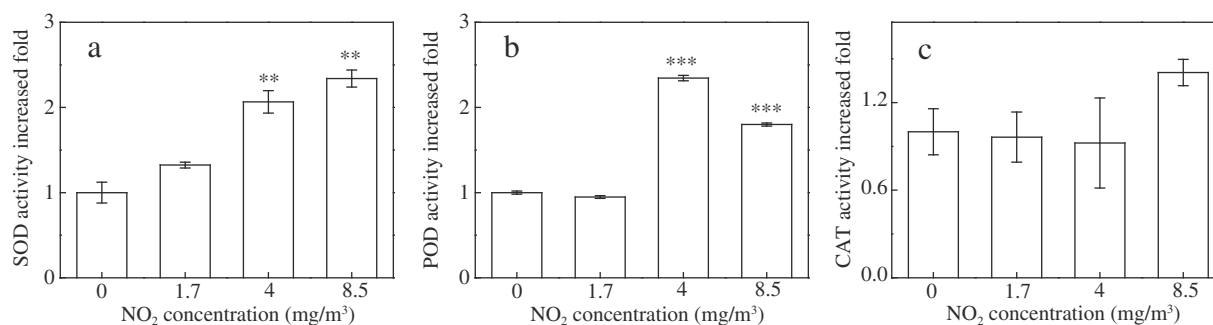
Fig. 4 – Effect of Nitrogen dioxide (NO$_2$) on antioxidant enzyme activities (including: Superoxide dismutases (SOD) (a), Peroxidase (POD) (b), Catalase (CAT) (c)) in *Arabidopsis* shoots. Data represent the mean ± SE of three different experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control.

the antioxidants also played an important role in plant defenses against NO$_2$ stress.

4. Conclusions

NO$_2$ exposure affected plant growth and chlorophyll level, and increased O$_2^-$ production rate in *Arabidopsis* shoots.

Furthermore, NO$_2$ elevated the levels of lipid peroxidation and protein oxidation, accompanied by the induction of SOD, POD and CAT activities and the change of antioxidant contents. Following this, we mimicked nitric acid mist under experimental conditions, and confirmed antioxidant mechanisms of the plant in response to the stress. Our results imply that NO$_2$ and its acid mist caused pollution risk to plant systems, increased ROS acted as a signal to induce a defense
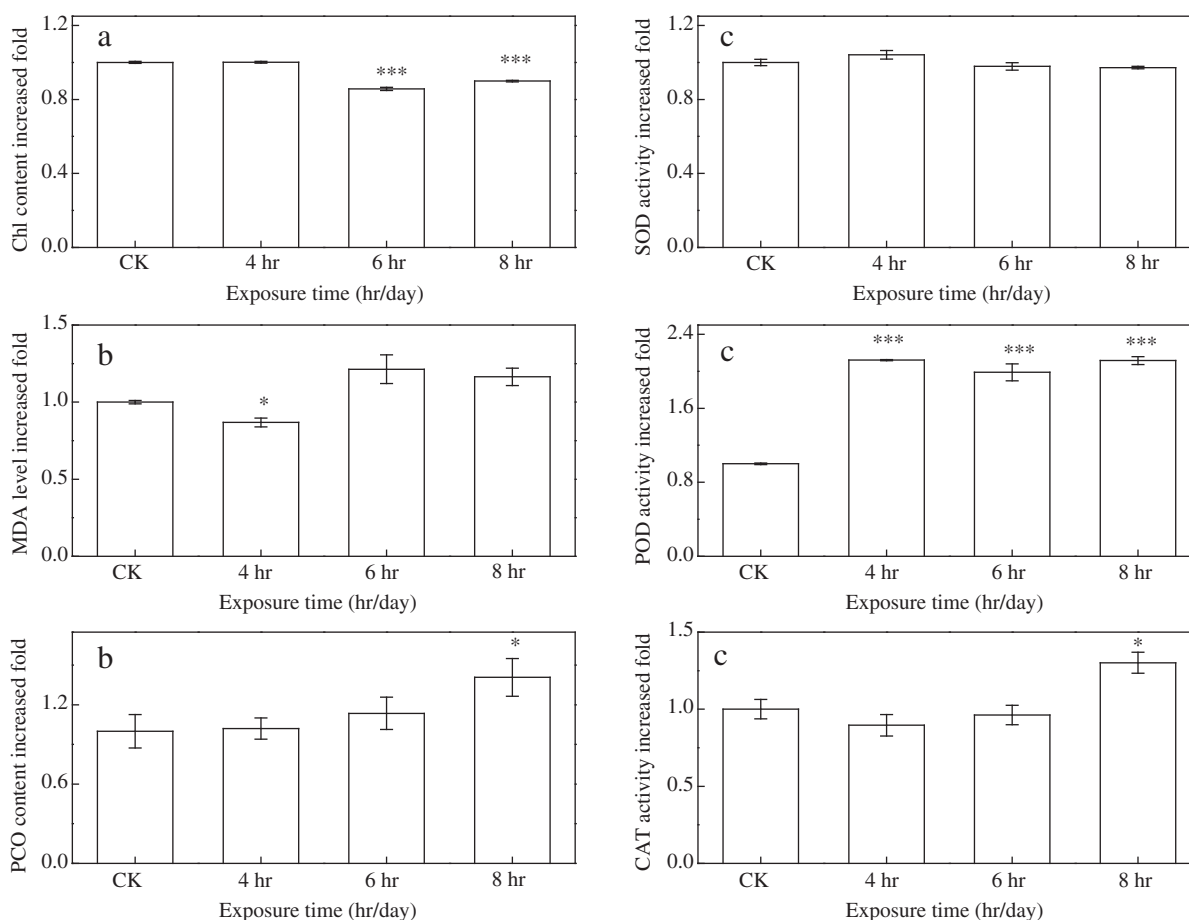

Fig. 5 – Effect of nitric acid mist exposure on chlorophyll (Chl) content (a), oxidative stress (b) and antioxidant enzyme activities (c) in *Arabidopsis* shoots. Data represent the mean ± SE of three different experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control.

response, and antioxidant status played an important role in plant protection against NO_2/nitric acid mist-caused oxidative damages.

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