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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.

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/(m^2·sec).

Four-week-old plants were exposed to 1.7, 4, 8.5, and 18.8 mg/m^3 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).