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Effects of 60-day NO_2 fumigation on growth, oxidative stress and antioxidative response in *Cinnamomum camphora* seedlings*

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Abstract: Objective: To study the oxidative stress and antioxidative response of *Cinnamomum camphora* seedlings exposed to nitrogen dioxide (NO_2) fumigation. Methods: Measurements were made up of the growth, chlorophyll content, chlorophyll fluorescence, antioxidant system and lipid peroxidation of one-year-old *C. camphora* seedlings exposed to NO_2 (0.1, 0.5, and 4 μl/L) fumigation in open top chambers over a period of 60 d. Results: After the first 30 d, 0.5 and 4.0 μl/L NO_2 showed insignificant effects on the growth of *C. camphora* seedlings. However, exposure to 0.5 and 4.0 μl/L NO_2 for 15 d significantly reduced their chlorophyll content (P<0.05), enhanced their malondialdehyde (MDA) content and superoxide dismutase (SOD) activity (P<0.05), and also significantly reduced the maximal quantum yield of PSII in the dark [the ratio of variable fluorescence to maximal fluorescence (F_v/F_m)] (P<0.05). In the latter 30 d, 0.5 μl/L NO_2 showed a positive effect on the vitality of the seedlings, which was reflected by a recovery in the ratio of F_v/F_m and chlorophyll content, and obviously enhanced growth, SOD activity, ascorbate (AsA) content and glutathione reductase (GR) activity (P<0.05); 4.0 μl/L NO_2 then showed a negative effect, indicated by significant reductions in chlorophyll content and the ratio of F_v/F_m, and inhibited growth (P<0.05). Conclusion: The results suggest adaptation of *C. camphora* seedlings to 60-d exposure to 0.1 and 0.5 μl/L NO_2, but not to 60-d exposure to 4.0 μl/L NO_2. *C. camphora* seedlings may protect themselves from injury by strengthening their antioxidant system in response to NO_2-induced oxidative stress.

Key words: *Cinnamomum camphora*, Fumigation, Growth, Chlorophyll content, Chlorophyll fluorescence, Antioxidant, Lipid peroxidation

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1 Introduction

Nitrogen dioxide (NO_2), one of the main traffic-related air pollutants, contributes to the forming of ozone via a photochemical reaction with hydroxyl radicals in the atmosphere (Takahashi *et al*., 2005). Hourly concentrations of NO_2 in Japan, UK and USA are reported to reach levels of 0.02–0.19, 0.25–0.4 and 0.01–0.12 μl/L (Takahashi *et al*., 2005). In China, atmospheric NO_2 concentration averagely in some large cities was recorded to be 0.017 μl/L in 2006 (Ministry of the Environmental Protection of the People's Republic of China, 2007).

Plants have been shown to metabolize dissolved NO_x through an NO_3^- assimilation pathway to form amino acids and proteins (Zeevaart, 1976; Marie and Ormrod, 1984). Stimulated growth has been reported in the presence of low concentrations of NO_2 (Sabaratnam *et al*., 1988; Okano *et al*., 1985; Marie and Ormrod, 1984). However, negative effects on plant health were observed under either high concentration

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and short-term NO_2 exposure (Sabaratnam and Gupat, 1988; Qiao and Murray, 1998) or low concentration and long-term NO_2 exposure (Ashenden, 1970; Ashenden *et al*., 1990; Maggs and Ashmore, 1998). Thus, the effect of NO_2 on plants depends on the experimental conditions such as the plant species studied, NO_2 concentration and stress duration.

Uptake of NO_2 results in a reduction in photosynthesis, which can be explained by competition for nicotinamide adenine dinucleotide phosphate (NADPH) between the processes of nitrite reduction and carbon assimilation in the chloroplast, and thereby leads to the generation of reactive oxygen species (ROS) (Sabaratnam and Gupat, 1988; Clyde Hill and Bennet, 1970; Shimazaki *et al*., 1992). When the production rate of ROS exceeds the elimination rate, lipid peroxidation and DNA injury may occur, and the organism may suffer from oxidative stress (Pan *et al*., 2006). However, plants have their own defense mechanisms including antioxidants and antioxidant enzymes (Wu and Tiedemann, 2002; Lai *et al*., 2007). For instance, peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) and the ascorbate-glutathione cycle (AGC) protect various vital physiological processes from injury by ROS produced under stress (Rai *et al*., 2004; Wu and Tiedemann, 2002). Studies have been carried out on the growth, chlorophyll content, photosynthesis, nitrogen content and enzymes related to nitrogen metabolism of plants exposed to NO_2, including nitrite reductase, glutamine synthetase and glutamate synthase (Takahashi *et al*., 2005; Yu *et al*., 1988; Sabaratnam *et al*., 1988; Qiao and Murray, 1998; Okano *et al*., 1985). However, little is known about the antioxidative response of plants and the regulation of photosynthesis.

As motorized traffic is one of the main sources of atmospheric NO_2, we should pay more attention to roadside species when studying the effects of NO_2 on plants. *Cinnamomum camphora*, the camphor tree, one of the most important trees in the subtropical evergreen broadleaf forest, is widely distributed in the south of China along the Yangtze River valley, and is also found in Korea, Japan and Vietnam (Zheng, 1983). It has been widely planted in cities throughout southern China because of its high landscaping value and disease and pollution resistance (Tian *et al*., 2007). Takahashi *et al*. (2005) reported that *C. camphora* showed a high assimilation capacity at 0.1 μl/L NO_2 and a low assimilation capacity at 4.0 μl/L NO_2, and was therefore classified in a group showing low resistance and high assimilation to NO_2 among 70 taxa of woody plants used as roadside trees.

In this study, 60-d NO_2 fumigation was carried out to study oxidative stress and the antioxidative response of *C. camphora*. The results will be helpful in determining the resistance of *C. camphora* to NO_2 and in providing a base for future studies of environmental toxicology in plants.

2 Materials and methods

2.1 Plant material and treatment

The study was conducted on one-year old *C. camphora* seedlings in a suburban area of Hangzhou, located in the southeast of China at 30°14′ N latitude, 120°09′ E longitude and 102 m above sea level. Seeds were germinated in a sand bed on Mar. 20, 2007 and seedlings were transplanted into 1-L pots (one plant per pot) after two leaves had grown. The culture medium was a mixture of turf (organic matter:crude ash=42:58, w/w), vermiculite and perlite in the proportions 4.5:4.5:1 (v/v/v). Seedlings were separated into four treatments: exposure to 0.1, 0.5 or 4.0 μl/L NO_2 or unfiltered air (the control, CK). One hundred seedlings per treatment were transferred to open-top chambers (OTC) on July 12, 2007. Seedlings were treated for a period of 60 d during daytime hours (10 h/d, 7 d/week). Measurements of their growth, chlorophyll fluorescence, chlorophyll content, antioxidant system and lipid peroxidation were made every 15 d.

2.2 OTC and the experimental environment

Potted seedlings were placed in eight OTCs. Each OTC was a hexagonal prism of 1.85 m height and 1.16 m diameter. A board with 1 200 holes (each 12 mm in diameter) was inserted in the chamber at a height of 30 cm from the bottom. Air was pumped continuously through the chamber from the bottom. NO_2 from a compressed source was ventilated into the bottom of the chamber through a Teflon tube connected by a solenoid valve. The concentration of the gas in the chamber was monitored using a system consisting of a solenoid valve, an online-sensor, a single chip micyoco (SCM) and a computer. The

concentration was sensed by an electrochemical sensor, converted to digital signal, and then sent to a computer by the SCM. The computer database was compared against the target concentration and used to send a command to the SCM to control the solenoid valve to open or close each second. The solenoid valve was in mic-flux and low-frequency pulse width modulation (PWM). The fluctuation of the gas concentration in different parts of the chamber was measured and found to remain within 5% of the target concentration. Natural sunlight was used as the light source. The measured data of temperature, humidity and light density inside the chamber during the experiment are shown in Table 1.

2.3 Growth measurement

Every 15 d, eight seedlings from each treatment were collected. Ground diameter and height were measured and the ratio of height/ground diameter was calculated. The seedlings were washed and heated to 80 °C and the above-ground dry weight and below-ground dry weight were recorded until they reached a constant level.

2.4 Chlorophyll content determination

Fully expanded leaves from area around the middle of the trunk of each of eight seedlings per treatment were randomly selected for measurement every 15 d. The petiole and the primary veins were removed from the leaves and discarded. Three independent chlorophyll measurements were made for each leaf sample. Portions of the samples were also used for measurements of the antioxidant system and lipid peroxidation determinations.

Frozen leaf tissue was homogenized in 80% (v/v) ice-cold acetone in the dark. The supernatant was separated by centrifugation at 2000×*g* for 10 min. Chlorophyll content was determined by measuring the absorbance of the supernatant at 646 and 663 nm, as described by Lichtenthaler (1987).

2.5 Chlorophyll fluorescence

Measurements of chlorophyll fluorescence were made every 15 d from the upper face of designated seedling leaves using an LI-6400 photosynthesis system (LI-COR Inc., Lincoln, NE, USA) fitted with an integral fluorescence chamber. Before each measurement, eight fully developed seedlings were taken and placed in the dark for 15 min at (22±1) °C. After recording the dark signal level, the minimum fluorescence F_0 was obtained by excitation of a probing light beam. The intensity of this light was sufficiently low so as to not produce any significant variable fluorescence [1.6 kHz, photon flux density (PFD)= 0.02 μmol photons/(m^2·s)]. A single saturating flash [1 s, PFD=4500 μmol photons/(m^2·s)] was then applied to reach maximal fluorescence (F_m). The yield of variable fluorescence (F_v) was calculated as F_m-F_0. Photosynthetic capacity (also termed the maximal quantum yield) was estimated by the ratio of $(F_m-F_0)/F_m$ (also termed F_v/F_m) for dark-adapted leaves (Genty *et al*., 1990; Frankart *et al*., 2002).

2.6 Antioxidant system analysis

SOD activity was assayed by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Dhindsa *et al*., 1981). The absorbance was recorded at 560 nm. It was expressed as U/g fresh weight, and 1 U was defined as the amount of enzyme causing 50% inhibition of NBT reduction under the assay conditions.

Ascorbic acid (AsA) content was determined following the procedure of Ella *et al*. (2003). Frozen leaf tissue was homogenized with 3 ml of 5% (w/v) metaphosphoric acid and then centrifuged at 20000×*g*. The mixture of 0.25 ml of 3 mmol/L 2,6-dichloroindophenol (DCIP) and 0.5 ml supernatant was kept at room temperature for 20 min, incubated at 50 °C for 1 h after adding 0.5 ml of 1% (w/v) thiourea in 5% metaphosphoric acid and 0.5 ml

Table 1 Environmental conditions of the experimental site during the experiment

Date	Mean temperature (°C)		Relative humidity (%)		PFD of the sunshine (μmol/(m^2·s))	
	Max	Min	Max	Min	Max	Min
July, 2007	34.0	28.7	42.9	28.2	180.6	6.45
August, 2007	34.1	29.4	39.8	22.8	182.4	7.23
September, 2007	26.8	24.9	54.0	41.5	135.3	4.01

of 10 mmol/L dinitrophenylhydrazone (DNPH), and then cooled in an ice bath for 15 min while adding 1.25 ml of ice-cold 85% (v/v) sulfuric acid (H_2SO_4). An aliquot of 0.5 ml of 20% (v/v) H_2SO_4 was added and absorbance at 520 nm was measured. Corrections were made for the oxidation of ascorbate by replacing DCIP with distilled water.

Glutathione reductase (GR) activity was assayed as described by Foyer and Halliwell (1976) and Liu *et al*. (2007). The reaction mixture consisted of 50 mmol/L phosphate buffered saline (pH 7.8), 0.15 mmol/L NADPH, 0.5 mmol/L oxidized glutathione (GSSG) and enzyme extract. The decrease in absorption at 340 nm [ε=6.2 $(\text{mmol/L}\cdot\text{cm})^{-1}$] due to NADPH oxidation was recorded over 2.5 min and was expressed as U/g fresh weight (1 U=1 mmol substrate activated during 1 min at 25 °C).

2.7 Lipid peroxidation determination

The degree of lipid peroxidation was estimated using malondialdehyde (MDA) as an indicator. The MDA content was determined using the thiobarbituric acid (TBA) method (Shalata and Tal, 1998). Samples were mixed with 1 ml of 10% (w/v) trichloroacetic acid (TCA) and 1 ml of 0.67% (w/v) TBA, and were then heated in a boiling water bath for 15 min. The amount of TBA reactive substance (TBARS) formed was determined by measuring absorbance at 535 nm and correcting for nonspecific absorbance at 600 nm. The MDA content was expressed as nmol/L MDA/g fresh weight.

2.8 Statistical analysis

Statistical data analysis was performed using SPSS version 10.0 software. Treatments were compared using one-way analysis of variance (ANOVA). Prior to the analysis, data were checked for normality and homogeneity of variance, and were square root transformed to equalize variances. Differences between treatments were considered significant at P<0.05.

3 Results

3.1 Growth

In the first 30 d of the experiment, all levels of NO_2 fumigation yielded insignificant differences in growth, including the height, ground diameter, above-ground dry weight, below-ground dry weight and ratio of height/diameter. By the end of the experiment, NO_2 fumigation still showed no significant effects on the height. However, by then 0.5 μl/L NO_2 had increased the ground diameter, above-ground dry weight and below-ground dry weight to the highest in the treatments, significantly higher than the corresponding control values. Treatment with 0.1 μl/L NO_2 also significantly promoted the growth of ground diameter, while 4.0 μl/L NO_2 significantly reduced the above-ground dry weight and below-ground dry weight (Table 2).

3.2 Chrolophyll fluorescence

Exposure to 0.5 and 4.0 μl/L NO_2 for 15 d brought about an enhancement in F_0 (P<0.05); however, in the subsequent treatment period, no statistical difference from the control was found. Exposure to 0.5 μl/L NO_2 had little impact on F_m but 0.1 μl/L NO_2 reduced F_m to 977.7 after 15 d (P<0.05). Exposure to 4.0 μl/L NO_2 reduced F_m significantly to 988.4 and 977.3 after 15 and 60 d, respectively. After 15 d of fumigation, NO_2 caused a significant reduction in the ratio of F_v/F_m at NO_2 concentrations of 0.5 and 4.0 μl/L. In subsequent periods, NO_2 showed insignificant effects on the ratio of F_v/F_m with the exception of 30 d×4.0 μl/L NO_2 and 60 d×4.0 μl/L NO_2 (Table 3).

3.3 Chlorophyll content

NO_2 fumigation at each concentration reduced the Chl a, Chl b and Chl (a+b) contents in the first 30 d (P<0.05), except that 0.5 μl/L NO_2 showed no statistical difference from the control in Chl a×30 d and Chl (a+b)×30 d (Fig. 1). In the second 30 d period, 0.1 and 0.5 μl/L NO_2 had little effects, while 4.0 μl/L NO_2 significantly reduced the chlorophyll contents. In the control samples, overall decreases were found of 18.36%, 15.32% and 17.53% in Chl a, Chl b and Chl (a+b) contents, respectively, compared with values obtained at the beginning of the experiment.

3.4 Lipid peroxidation

In every phase of the experiment, the MDA contents in NO_2 treatments were significantly higher than those of the control (Fig. 2). By the end of the experiment, the MDA contents in the 4.0, 0.5 and 0.1 μl/L NO_2 treatments were higher by 87.42%, 30.62% and 26.96%, respectively, than that of the control.

Table 2 Effects of different concentrations of NO_2 on the growth parameters of *Cinnamomum camphora* seedlings

NO_2 (μl/L)	Duration (d)	Ground diameter (cm)	Height (cm)	Above-ground dry weight (g)	Below-ground dry weight (g)	Ratio of height/ ground diameter
CK	0	1.92±0.124	15.18±1.22	0.2768±0.0357	0.2416±0.0250	79.036±9.78
	15	1.96±0.10a	17.60±1.23a	0.3792±0.0341a	0.2697±0.0247a	89.802±6.72a
	30	2.18±0.11a	21.21±0.93a	0.4389±0.0255a	0.2875±0.0309a	97.298±4.71a
	45	2.52±0.09a	22.18±1.87a	0.5134±0.0378a	0.3247±0.0351ab	88.027±4.07a
	60	2.66±0.10a	22.37±1.67a	0.5980±0.0486a	0.4531±0.0278a	84.104±9.23a
0.1	0	–	–	–	–	–
	15	2.01±0.16a	17.82±1.56a	0.3809±0.0350a	0.2875±0.0267a	88.662±9.07a
	30	2.34±0.16a	21.35±1.81a	0.4359±0.0323a	0.3525±0.0254a	91.242±5.16a
	45	2.74±0.17ab	22.69±2.11a	0.5695±0.0440a	0.3987±0.0381a	82.812±6.53a
	60	2.93±0.13b	23.68±1.01a	0.6563±0.0442a	0.4840±0.0452a	80.821±8.86a
0.5	0	–	–	–	–	–
	15	1.98±0.13a	18.21±0.56a	0.3154±0.0464a	0.2733±0.0298a	91.970±6.45a
	30	2.25±0.15a	21.72±1.25a	0.4251±0.0422a	0.3588±0.0398a	96.533±9.13a
	45	2.87±0.16b	23.05±2.03a	0.6391±0.0650b	0.4839±0.0365c	80.314±5.75b
	60	3.10±0.17b	24.06±0.86a	0.7493±0.0558b	0.6184±0.0372b	77.613±5.51b
4.0	0	–	–	–	–	–
	15	1.99±0.12a	17.93±1.39a	0.3322±0.0286a	0.2516±0.0369a	90.101±4.89a
	30	2.13±0.15a	21.06±1.71a	0.3769±0.0398a	0.2675±0.0449a	98.874±5.32a
	45	2.35±0.16a	21.66±1.08a	0.4682±0.0380a	0.2871±0.0465b	92.171±8.77a
	60	2.45±0.15a	21.73±1.59a	0.5028±0.0315c	0.3430±0.0350c	88.698±9.04a

The values represent mean±SD (n=8). Different lower case letters following the values show significant differences (P<0.05) between treatments according to one-way ANOVA

Table 3 Effects of different concentrations of NO_2 on the chlorophyll fluorescence parameters of *Cinnamomum camphora* seedlings

NO_2 (μl/L)	Duration (d)	F_0	F_m	F_v/F_m
CK	0	165.7±5.1	922.8±34.94	0.820±0.004
	15	170.0±7.1a	1058.6±47.7a	0.839±0.014a
	30	175.7±7.2a	958.8±31.3a	0.817±0.004ab
	45	178.4±9.1a	974.7±32.2ab	0.817±0.005ab
	60	180.5±7.1a	1005.2±8.6a	0.820±0.009a
0.1	0	–	–	–
	15	163.7±6.5a	977.7±22.6c	0.832±0.011ab
	30	169.5±11.4a	892.4±47.8a	0.810±0.004a
	45	176.1±11.4a	1001.3±40.3a	0.824±0.009a
	60	178.3±13.5a	1028.2±14.5a	0.827±0.013a
0.5	0	–	–	–
	15	190.7±7.0b	1034.5±12.8ab	0.815±0.006b
	30	173.7±8.3a	968.5±32.0a	0.821±0.007b
	45	171.4±9.4a	958.8±43.8ab	0.821±0.004ab
	60	165.1±10.2a	964.9±38.9a	0.829±0.007a
4.0	0	–	–	–
	15	201.2±9.4b	988.4±12.6bc	0.797±0.007c
	30	182.0±9.3a	903.0±6.5a	0.799±0.005c
	45	183.5±9.7a	915.2±33.3b	0.799±0.009b
	60	174.9±7.7a	877.3±3.5b	0.801±0.009b

The values represent mean±SD (n=8). Different lower case letters following the values show significant differences (P<0.05) between treatments according to one-way ANOVA

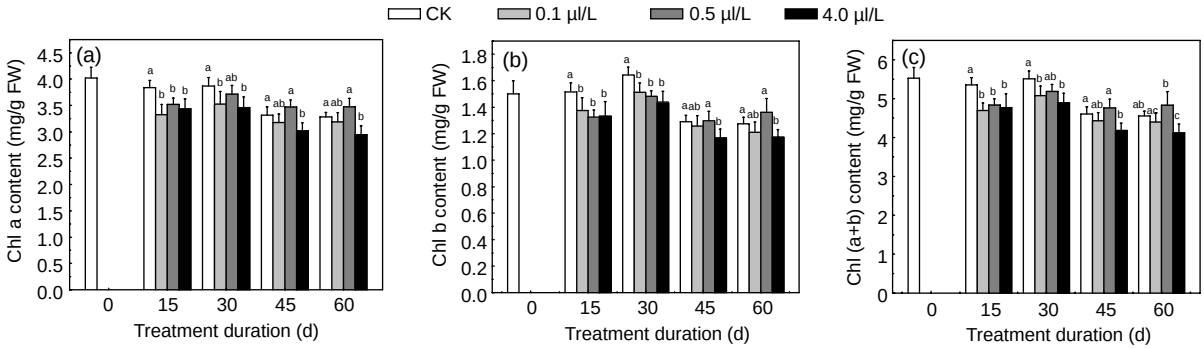

Fig. 1 Effects of different concentrations of NO_2 on chlorophyll a (a), chlorophyll b (b) and chlorophyll (a+b) (c) contents in leaves of *Cinnamomum camphora* seedlings. Data are expressed as mean±SD (n=8). Different lower case letters on bars show significant differences (P<0.05) between treatments according to one-way ANOVA

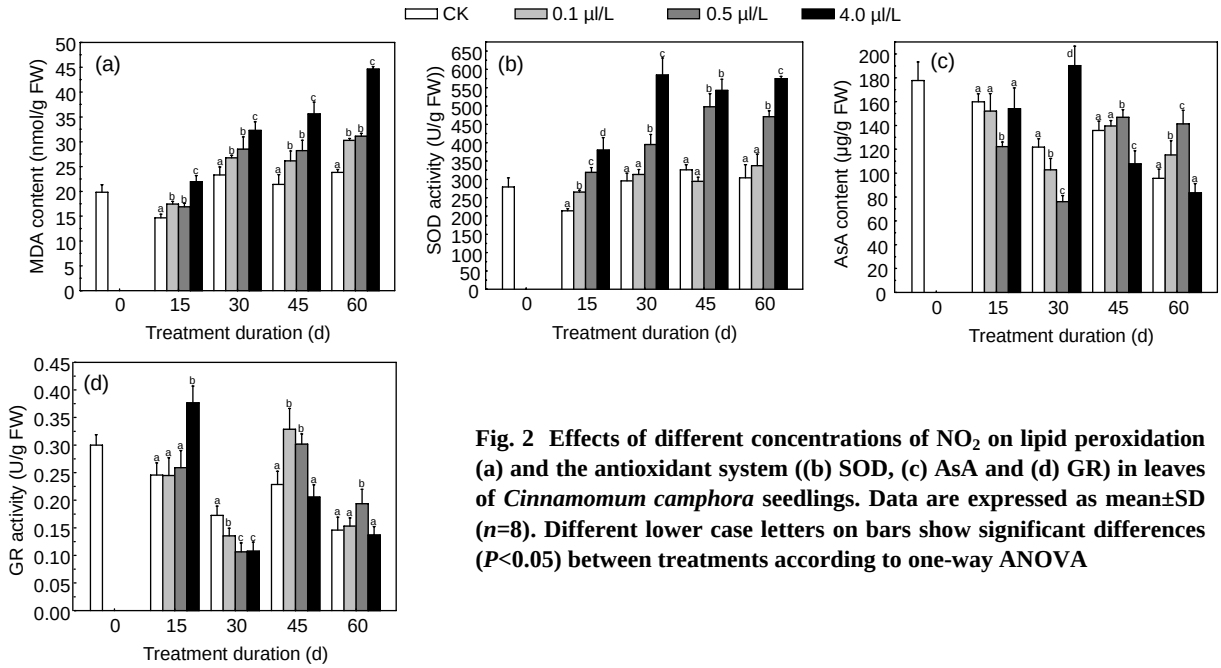

Fig. 2 Effects of different concentrations of NO_2 on lipid peroxidation (a) and the antioxidant system ((b) SOD, (c) AsA and (d) GR) in leaves of *Cinnamomum camphora* seedlings. Data are expressed as mean±SD (n=8). Different lower case letters on bars show significant differences (P<0.05) between treatments according to one-way ANOVA

3.5 Antioxidant system

As the experiment proceeded, the SOD activities first rose and then, in the 0.5 and 4.0 μl/L NO_2 treatments, stabilised at a level significantly higher than that of the control (Fig. 2). In the 0.1 μl/L NO_2 treatment, after the initial rise, levels fluctuated slightly. At the end of the fumigation period, SOD activities had risen in proportion to the concentration of NO_2. In the 4.0 and 0.5 μl/L NO_2 treatments, SOD activities were higher than that of the control by 88.81% and 54.80% (P<0.05), respectively.

Compared with the control, in the presence of 0.1 and 0.5 μl/L NO_2, the AsA contents decreased significantly in the first 30 d with the exception of 0.1 μl/L NO_2×15 d and increased significantly in the following 30 d with the exception of 0.1 μl/L NO_2× 45 d (Fig. 2). Treatment with 4.0 μl/L NO_2 increased the AsA content after 30 d to a much higher level than the other NO_2 treatments and 56.11% higher than the control (P<0.05), but reduced it sharply to a level significantly lower than the control after 45 d, and after 60 d showed an insignificant effect. The AsA content of control samples decreased by 46.16% from the start to the end of the experiment.

During the fumigation, GR activity showed a double-peak tendency in the 4.0 μl/L NO_2 treatment and a single-peak tendency in the 0.1 and 0.5 μl/L NO_2 treatments (Fig. 2). In the 4.0 μl/L NO_2 treatment, GR activity increased to a significantly higher level

than in the other treatments after 15 d, but weakened steeply to a level significantly lower than in the control after 30 d, and remained statistically insignificant from the control in the last period of the experiment. In the 0.1 and 0.5 μl/L NO_2 treatments, compared with the control, GR activities were insignificantly different after 15 d, significantly lower after 30 d, then significantly higher in the last phase with the exception of 0.1 μl/L NO_2×60 d. To sum up, the effects of NO_2 fumigation on GR activity differed in the changing phases of the experiment. The GR activity of control samples fell by 51.32% from the start to the end of the experiment.

4 Discussion

4.1 Growth

In this study, 30-d NO_2 fumigation caused insignificant differences in the growth of *C. camphora* seedlings. 60-d treatment with 4.0 μl/L NO_2 inhibited growth, but 60-d treatment with 0.5 or 0.1 μl/L NO_2 promoted growth (Table 2). This suggests that *C. camphora* lacks adaptation to NO_2 at the level of 4.0 μl/L but is adapted to levels of 0.1 and 0.5 μl/L. Pandey and Agrawal (1994) reported that the relative growth rate and net assimilation rate of tomato plants increased initially but then declined after exposure for longer periods to 0.2 μl/L NO_2. A lack of adaptation to long-term and low-concentration NO_2 was also reported for *P. pratensis* and Pakistan rice (Ashenden, 1970; Maggs and Ashmore, 1998). Differences in adaptability to NO_2 among plant species may be caused by their different metabolic and antioxidant systems (Liu *et al*., 2007). Similar adaptation responses of plants to long-term ozone have been reported (Walmsley *et al*., 1980; Mehlhorn *et al*., 1991).

4.2 Oxidative stress

The view is commonly held that uptake of NO_2 results in the generation of ROS in plants because of competition for NADPH between the processes of nitrite reduction and carbon assimilation in the chloroplast, and the strong radical nature of NO_2 (Sabaratnam and Gupat, 1988; Clyde Hill and Bennet, 1970; Shimazaki *et al*., 1992; Ramge *et al*., 1993). Although growth was not significantly affected by NO_2 in the first 30 d, variation occurred in lipid peroxidation, chlorophyll fluorescence and chlorophyll contents, each of which might be an indicator of air pollution injury (Tables 2 and 3, Fig. 1).

MDA, the decomposition product of polyunsaturated fatty acids (PUFA) of biomembranes, is a good indicator of the severity of cell injury during oxidative stress (Price *et al*., 1990). In this study, three concentrations of NO_2 increased the MDA contents in every treatment period, indicating the existence of oxidative injury induced by NO_2 during the experiment (Fig. 2).

In this study, F_0 was significantly increased after 15 d at 0.5 and 4.0 μl/L NO_2, indicating that modifications were induced at the antenna pigment level (Calatayud and Barreno, 2001) (Table 3). After the same duration, a significant decrease in the ratio of F_v/F_m in these two treatments indicated that the photochemistry of PSII and its functional efficiency were affected (Maxwell and Johnson, 2000) (Table 3). Similar results were observed by Barnes *et al*. (1988), Guidi *et al*. (1997) and Carrasco-Rodriguez and Valle-Tascon (2001) in higher plants exposed to ozone. In the subsequent time period, the photosynthetic efficiency recovered at 0.5 μl/L NO_2 but remained lower at 4.0 μl/L NO_2 than at the control, which was reflected by the F_m and the ratio of F_v/F_m (Table 3). Although it was not possible to conclude that the decline in photosynthesis was the result or cause of ROS accumulation, the inverse correlation between the decreased ratio of F_v/F_m and increased MDA contents in the 15 d×0.5 μl/L NO_2, 15 d×4.0 μl/L NO_2 and 60 d×4.0 μl/L NO_2 indicated a possible association between them (Table 3, Fig. 2).

Chlorophyll pigments are essential for photosynthesis and a decrease in chlorophyll content has been used as an indicator of air pollution injury (Darrall and Jäger, 1984). In this study, NO_2 reduced the Chl a, Chl b and Chl (a+b) contents in the first 30 d (Fig. 1). Similar phenomena have been observed in plants such as *Hedera helix* (Della-Torre *et al*., 1998) and *Avena sativa* L. (Pleijel *et al*., 1994), and may be caused by the disintegration of pigments by ROS (Sakaki *et al*., 1983). In the subsequent 30 d, 4.0 μl/L NO_2 significantly reduced pigmentation, but 0.1 and 0.5 μl/L NO_2 had an insignificant effect (Fig. 1). Ra *et al*. (2005) found that moderate levels of fertilizer air pollutants may permit higher protein synthesis rates, improve the ratio of intact chlorophyll to degraded

forms, and increase overall concentrations of pigments and CO_2 uptake of lichen. The availability of nitrogen from 0.1 and 0.5 μl/L NO_2 may promote the synthesis of pigments and counteract the destruction by ROS, leading to a recovery in chlorophyll content. The continuously declining chlorophyll contents in the 4.0 μl/L treatment indicated that oxidative stress was still inhibiting the plant (Fig. 1). The tendency of chlorophyll contents to decrease in the experiment may have resulted from the increasing senescence of the seedlings as growth was taking place (Makino and Osmond, 1991).

4.3 Antioxidative response

SOD is essential for the elimination of ROS in plants because its enzymatic action dismutates superoxide (O_2^-) to H_2O_2 and O_2 (Meloni *et al*., 2003). A correlation between increased SOD activity and tolerance to air pollutants has been observed in many plants, and is considered to be an adjustment response to stress (Lee and Bennett, 1982). In this study, SOD activities increased with increasing NO_2 concentrations, suggesting a dose-dependent effect of NO_2 on SOD activity (Fig. 2).

AGC contributes greatly to antioxidant protection against H_2O_2 (Potters *et al*., 2002). Several enzymes and antioxidants are involved in these reactions including AsA and GR (Foyer and Halliwell, 1976; Smirnoff, 1996). In general, the biosynthesis of AsA and reduced glutathione (GSH, catalyzed from oxidized glutathione by GR) is stimulated when the cell encounters stress conditions (Foyer and Halliwell, 1976; Horemans *et al*., 2000; Potters *et al*., 2002). In the present study, 0.5 μl/L NO_2 reduced AsA contents and GR activities in the first 30 d, but induced them in the subsequent 30 d, indicating that AGC was efficiently activated to detoxify ROS in the second 30 d period (Fig. 2). Activated AGC, together with enhanced SOD, formed a stronger antioxidant system in the final 30 d. A new balance between the production and elimination of ROS was established to control the oxidative stress within an acceptable level, as shown by the recovered chlorophyll contents and growth promotion in the 0.5 μl/L NO_2 treatment (Table 2, Figs. 1 and 2). Alternatively, *C. camphora* has a substantial reserve of phenolics, which can act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans *et al*., 1996; Zhang and Lin, 2008). The production of phenolics is considered to be a response to injuries (Dixon and Paiva, 1995) and may have contributed to the recovery from injury at 0.5 μl/L NO_2, and thus the continued growth and early leaf senescence in *C. camphora* (Table 2, Figs. 1 and 2). Further studies on phenolic compounds should be carried out to test this possibility.

In the 4.0 μl/L NO_2 treatment, the AsA content and GR activity fluctuated widely and asynchronously (Fig. 2), indicating a low efficiency AGC and thus an unbalanced antioxidant system less able to contribute to the scavenging of ROS. A similar asynchronous tendency between AsA content and GR activity was reported for *Populus euramericana* (Edjolo *et al*., 2001). As AsA and GSH not only act as antioxidants, but also have other functions in physiological processes (Horemans *et al*., 2000; Potters *et al*., 2002), it was difficult to identify the mechanisms by which they were affected by NO_2 treatment. This needs further study at the cellular level. The general duration-dependent fall in AsA content and GR activity in the control suggests a physiological process of senescence in leaves, which is similar to time-dependent changes in GSH content found in chloroplasts of tomato (Kuźniak and Skłodowska, 2001).

5 Conclusion

In conclusion, according to the antioxidative responses and the parameters, there was an adaptation response of *C. camphora* seedlings after 60-d of 0.1 and 0.5 μl/L NO_2, but not after 60-d of 4.0 μl/L NO_2, suggesting that the critical toxic value of atmospheric NO_2 in inducing influential oxidative injury to *C. camphora* seedlings is between 0.5 and 4.0 μl/L. The seedlings may protect themselves from injury by concordantly strengthening their antioxidant system under NO_2-induced oxidative stress. The MDA content and SOD activity responded comparatively steadily to the dose of NO_2 and may be important indicators of oxidative stress caused by NO_2 in *C. camphora* seedlings. Further studies at the cellular or molecular level and on antioxidative compounds (phenolics and flavonoids) should be conducted to clarify the mechanisms involved in rebuilding a balanced antioxidant system after extended exposure to 0.5 μl/L NO_2.

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