Physiological Response of European Hornbeam Leaves to Nitrogen Dioxide Stress and Self-recovery

Qianqian Sheng

Co-Innovation Center for Sustainable Forestry in Southern China, College of Landscape Architecture, Nanjing Forestry University, Nanjing 210037, People's Republic of China

Zunling Zhu¹

Co-Innovation Center for Sustainable Forestry in Southern China, College of Landscape Architecture, Nanjing Forestry University, Nanjing 210037, People's Republic of China; and College of Art and Design, Nanjing Forestry University, Nanjing 210037, People's Republic of China

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ABSTRACT. Plant leaves absorb atmospheric nitrogen dioxide (NO₂) primarily via the stomata. Studies of changes in plant growth and physiology after exposure to NO₂ are limited. Therefore, this study investigated the physiological response of *Carpinus betulus* (European hornbeam) chloroplasts after NO₂ exposure using fumigation equipment that was able to control timing and record NO₂ concentrations. The NO₂ concentration was 6 µL·L⁻¹. Seven treatment durations (0, 1, 6, 12, 24, 48, and 72 hours) were designed. After fumigation, plants recovered for 30 days under greenhouse conditions. The physiological response, stomatal behavior, thicknesses of palisade and spongy tissues, and chloroplast ultrastructure were measured. In the 48-hour and 72-hour NO₂ treatment groups, the chloroplast contents and net photosynthesis rates of the leaves decreased, palisade and spongy tissues thickened, and chloroplast thylakoids swelled; however, the 1-hour NO₂ treatment did not have a noticeable toxic effect on *C. betulus* leaves. After 30 days of recovery, the plants returned to their natural growth level by increasing the chloroplast content and enhancing net photosynthesis. Short durations and high concentrations of NO₂ exposure had significantly negative impacts on the physiological response of *C. betulus*; however, this toxic effect of high NO₂ concentrations on *C. betulus* can be recovered by restoration of unpolluted air. The results of this study may provide a scientific reference and an additional choice of plants species for the application of *C. betulus* in functional gardening design and ecological green space construction.

As one of the most common pollutant gases and a major threat to ecosystems, NO₂, which is a brown-red gas with a pungent odor, is an atmospheric pollutant that originates primarily from vehicle emissions, fuel burning in power plants, and industrial production of nitric acids (Chai et al., 2002). NO₂ serves as a precursor of ozone and atmospheric particles, and it is an essential atmospheric pollutant for the formation of acid rain (Bermejo-Orduna et al., 2014; Rahmat et al., 2013). In recent decades, the emission of NO₂ has increased in many countries worldwide (Frati et al., 2008; Munzi et al., 2009), particularly in China and other Asian countries (Hu et al., 2015; van der A et al., 2008). In China, the national concentration threshold for NO₂ pollution is 0.0199 µL·L⁻¹, and the atmospheric concentration of NO₂ near highways reaches 0.0255–0.058 µL·L⁻¹ (Nanjing Environmental Protection Bureau, 2016). Furthermore, with the progression of urban industrialization and automation, the NO₂ concentration may continue to increase (Munzi et al., 2009). Therefore, it is critical to determine ecologically and environmentally friendly methods to reduce atmospheric NO₂ concentrations.

Injury to plants is primarily due to the corrosive and high oxidative properties of NO₂, which influence various physiological and biochemical processes after entering plants via the stomata (Lu et al., 2002). NO₂ can cause reductions in growth, and the amount of damage experienced by a plant varies according to different factors, such as the concentration and length of exposure, plant age, edaphic factors, light, and humidity. Symptoms are often divided into “visible” and “invisible” (or hidden) injuries, which comprise an overall reduction in growth but no obvious signs of visible damage (Wellburn, 1990) and mainly affect the physiological metabolism of plants.

Chlorophyll (Chl) is the most important plant pigment because it has a crucial role in photosynthesis (Croft et al., 2017). Chen et al. (2010) performed NO₂ fumigation of *Cinnamomum camphora* seedlings at low-volume fractions for 60 d and found that 4.0 µL·L⁻¹ of NO₂ significantly decreased the Chl content in the leaves of seedlings. Changes in the leaf color that resulted in yellow spots or yellow leaves were attributable to the change in Chl content. Liu et al. (2015) found that *Arabidopsis thaliana* initiated stress-resistant protective mechanisms triggered by superoxide free radicals under NO₂ stress. Hu et al. (2015) reported that the net photosynthesis of *Populus alba* × *P. berolinensis* hybrid leaves significantly decreased after 14 and 48 h of exposure to 4 µL·L⁻¹ of NO₂, thus demonstrating that inhibition of photosynthesis may be responsible for the suppression of growth by NO₂. Furukawa et al. (1984) found that exposure to 2 and 4 µL·L⁻¹ of NO₂ for 2 h decreased the net photosynthesis rate by 20% and

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¹Corresponding author. E-mail: zhuzunling@njfu.edu.cn.

90%, respectively, but had no significant effect on reducing transpiration.

Ultrastructural changes associated with invisible injuries have often been linked to decreased transpiration and photosynthesis (Fink, 1988; Huttunen and Soikkeli, 1984), and some occurred in both mitochondria and chloroplasts. Four-year-old Norway spruce trees (*Picea abies*) were fumigated with $1 \mu\text{L}\cdot\text{L}^{-1}$ of NO_2 (low concentration) for 3 weeks, resulting in swollen thylakoids, decreased grana stacks, and enhanced starch contents compared with controls (Schiffgens-Gruber and Lütz, 1992). Mature bean (*Phaseolus vulgaris*) tissue was exposed to 1 and $2 \mu\text{L}\cdot\text{L}^{-1}$ of NO_2 for 1 and 2 h and compared with similar untreated tissues; the NO_2 -exposed bean tissue showed swelling of the thylakoids within the chloroplasts but no extrachloroplastic damage, and the thylakoid swelling could be reversed by removing the pollutant from the air flow or changing the rate of gas flow within the tissue (Wellburn et al., 1972). Chaparro-Suarez et al. (2011) found that NO_2 was not absorbed by plants when the stomata were closed, and that species-dependent differences in uptake rates could be clearly related to stomatal behavior. Despite the results obtained by the aforementioned studies, considering that NO_2 resulting from traffic pollution is a primary source of pollution in urban areas, and considering that landscape plants comprise an important part of the urban ecology, studies of the effects of NO_2 on plants that occupy an important position in urban green spaces should be performed. However, most previous reports have focused on field crops and herbs rather than urban landscape plants, particularly trees.

The species *Carpinus betulus* originates from Turkey and Ukraine (Jia, 2002). It is a deciduous broad-leaf tree in the family Betulaceae. *Carpinus betulus* has an attractive shape, with dense, delicate leaves in summer and golden leaves and unique fruit in autumn (Jia, 2002). Various cultivars of this species can be used for landscaping, and it has been grown in many countries. Shi et al. (2015) investigated the photosynthetic characteristics of *C. betulus* from different regions and the influence of salt stress on the photosynthetic and fluorescent characteristics of *C. betulus* seedlings. However, studies of the effects of NO_2 stress on the physiological response of *C. betulus* have not been reported (Taylor and MacLean, 1970).

Based on the aforementioned results, this study aimed to address the following hypotheses: 1) NO_2 treatment has a negative influence on the photosynthesis, stomatal behavior, and chloroplast ultrastructure of *C. betulus* leaves; and 2) *C. betulus* returns to normal growth after the removal of NO_2 stress. The results of this study may provide useful reference data for the selection of plant species for atmospheric absorption and a scientific reference for plant selection for urban gardening and the construction of ecological green space landscapes.

Materials and Methods

NO_2 FUMIGATION EQUIPMENT.

Equipment able to control the timing

and record NO_2 concentrations was used (Fig. 1). The outlet of the NO_2 gas cylinders was connected to a solenoid valve with a pressure-reducing valve. A certain volume of NO_2 was input into the microcomputer timer switch system. An NO_2 sensor was set in the fumigating chamber to detect the gas concentration. The other end of the sensor was connected to the air inlet pipe of the NO_2 gasometer. The gasometer was connected to the computer terminal via an RS-485 interface. Gas-monitoring software installed in the computer was used to record the variations in NO_2 concentrations of the fumigating chamber. This equipment was able to detect real-time changes in the gas in the fumigating chamber and could accurately control the volume of gas entering the chamber.

MATERIALS. One-year-old *C. betulus* seedlings were acquired from Hungary, and the experiment was conducted in the garden plant laboratory and greenhouse of Nanjing Forestry University in Nanjing, Jiangsu Province, China (lat. 32.07°N , long. 118.8°E). In Mar. 2017, healthy seedlings (free from pests and diseases) were selected and transferred to the greenhouse for substrate cultivation. The substrate was mixed soil that contained peat, vermiculite, and perlite at a ratio of 1:1:1 by volume. The seedlings were planted in plastic pots with an upper diameter, lower diameter, and height of 30, 20, and 15 cm, respectively. The pot had a drainage hole and was placed on a tray. In each pot, 500 g of dry soil was loaded, and two plants were planted. The plants grew under the same conditions [25 to 28°C , 60% to 70% air relative humidity (RH), $1500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination intensity] and were regularly managed. During cultivation, the plants were watered two to three times per week. One liter of Hoagland nutrient solution was applied every 2 weeks. After 2 months, the NO_2 stress experiment was performed.

FUMIGATION AND SELF-RECOVERY. Based on the literature (Liu et al., 2015; Pan et al., 2012), NO_2 at $6 \mu\text{L}\cdot\text{L}^{-1}$ (high-concentration treatment) was used in this study. The air current velocity was set to $1 \text{ L}\cdot\text{min}^{-1}$, with an illumination time of 13 h, an ambient temperature of 20 to 25°C , and 50% to 60% RH. Real-time NO_2 concentrations were monitored by an NO_2 automatic detector. Seedlings were divided according to different fumigation periods: 0 (control), 1, 6, 12, 24, 48, and 72 h. There were 30 pots in each treatment group.

Fig. 1. A fume exhauster device with timing control and nitrogen dioxide (NO_2) concentration recording capabilities for NO_2 fumigation: 1 = NO_2 gas cylinders; 2 = pressure-reducing valve; 3 = solenoid valve; 4 = microcomputer timer switch system; 5 = centrifugal fan; 6 = blower; 7 = air inlet pipe; 8 = NO_2 gas sensor; 9 = computer terminal; 10 = glass partition; 11 = acrylic sheet; and 12 = fume gas chamber.

After the fumigation experiment, the plants were removed from the fumigation chamber and then cultivated without NO₂ application in the greenhouse for 30 d of recovery; these plants comprised the recovery group (n = 30). Growth conditions were controlled to mimic environmental conditions of 25 to 28 °C, 60% to 70% RH, and 1500 μmol·m⁻²·s⁻¹ illumination intensity by an artificial light supplement. During cultivation, the plants were regularly managed and watered two to three times per week. The water content of the soil was maintained at 60% to 80% during the experiment.

All experiments were repeated three times and performed beginning on 15 May, 15 June, and 15 July 2017. Photosynthesis-related indices were measured immediately after treatment.

CHLOROPHYLL CONTENT AND CAROTENOID MEASUREMENT. Chl content was measured spectrophotometrically (Mittler, 2002). In each treatment group, the third young leaves were taken from five plants. The midribs were removed, and the leaves were cleaned and cut into pieces. Then, they were blended. Three replicates were set for each group. The sample (≈0.2 g) was placed into a mortar, and the homogenate was made with quartz sands and calcium carbonate powder in a small quantity with 2–3 mL of 95% ethanol. Approximately 10 mL of 95% ethanol was applied for further grinding until the tissue turned white. The sample was allowed to stand for 3–5 min. Then, a filter paper was placed in a funnel, and the paper was moistened with ethanol. The extraction solution was filtered into a 25-mL brown volumetric flask. The mortar, pestle, and residues were flushed with 95% ethanol and then filtered through the funnel. The chloroplast pigment on the filter paper was washed into the flask with a dropper. The filtered solution was diluted to 25 mL with ethanol and shaken well. The chloroplast pigment-extracting solution was poured into a cuvette with an optical path of 1 cm, and 95% ethanol was used as the control. Absorbance (A) was measured at wavelengths of 665, 649, and 470 nm. The contents of Chl a, Chl b, carotenoids, and total Chl and the Chl a/Chl b ratio were then calculated according to the method of Lichtenthaler (1987). The following equations were used: Chl a_c = 13.95A₆₆₅ – 6.88A₆₄₉, Chl b_c = 24.96A₆₄₉ – 7.32A₆₆₅, Carotenoids_c = (1000A₄₇₀ – 2.05 Chl a_c – 114.8 Chl b_c)/245, Total Chl_m = Chl a_c + Chl b_c, where c is the pigment concentration (milligrams per gram of extract).

MEASUREMENTS OF PHOTOSYNTHETIC PARAMETERS. The photosynthetic photon flux density (PPFD) responses of the net photosynthetic rate (Pn), intercellular carbon dioxide (CO₂) concentration (Ci), transpiration rate (Tr), and stomatal conductance (g_s) of *C. betulus* leaves were measured on sunny days on 15, 16, and 17 of May 2007, 15, 16, and 17 of June 2017, and 15, 16, and 17 of July 2017 (corresponding to before fumigation, after fumigation, and after 30 d of recovery, respectively). Between 0900 HR and 1100 HR, nine seedlings were selected randomly from each treatment group. Three leaves below the top leaves were taken. Conjoined measurements were performed with a photosynthesis system (LI-6400XT; LI-COR, Lincoln, NE) under natural temperature, RH, and CO₂ concentration conditions (25 °C, 65%, and 350 μL·L⁻¹, respectively). Photosynthetic active radiation values at 1400, 1200, 1000, 800, 600, 400, 200, 100, 50, 20, and 0 μmol·m⁻²·s⁻¹ were used for photosynthesis rate measurements. Data at each light intensity value were collected for 5 min.

MEASUREMENT OF DIURNAL VARIATION IN PHOTOSYNTHESIS. Between 0800 HR and 1800 HR, nine seedlings were selected randomly from each treatment group. Three leaves below the

top leaves were taken. Conjoined measurements were performed with a photosynthesis system (LI-6400XT) under natural conditions (25 °C, 65% RH, 350 μL·L⁻¹ CO₂, and natural light). Three groups of data were recorded for each leaf, and measurements were performed once every 2 h. Measurements were performed for three consecutive days, and the averages were obtained. The indices, including Pn, Ci, and g_s, were measured repeatedly for all fumigation times, which were 0, 1, 6, 12, 24, 48, and 72 h and self-recovery.

SCANNING ELECTRON MICROSCOPY. After NO₂ stress, leaf samples were taken from different treatment groups. The leaves were sectioned transversely with a double-edged blade. Samples were fixed with 4% glutaral for 6 h and then washed with 0.1 M phosphate buffer (10 min for each washing). Then, 2% osmic acid was used for fixation until the sample turned black and transparent. The sample was washed with 0.1 M phosphate buffer (10 min for each washing). Dehydration was performed with 30% ethanol for 15 min, 50% ethanol for 15 min, 70% ethanol for 15 min, and 90% ethanol for 15 min. Finally, dehydration was performed twice with 100% ethanol for 15 min each. The sample was placed in a drying basket and dried with a critical point dryer. The dried sample was stuck to the sample table with electric conduction paste and then subjected to metal spraying (15 mA for 2 min). A scanning electron microscope (TM4000/TM4000Plus; Hitachi High-Technologies, Tokyo, Japan) was used for observation.

TRANSMISSION ELECTRON MICROSCOPY. Fresh samples were rapidly cut into 1- to 3-mm² pieces and then fixed in 4% glutaral. After complete precipitation (≈4 h later), the samples were washed three times with phosphate buffer (20 min for each washing). Then, the samples were fixed with 2% osmic acid until they turned black and transparent. The samples were washed with phosphate buffer three times (20 min for each washing). Gradient dehydration was performed with 30%, 50%, 70%, and 90% acetone (30 min for each gradient). Then, dehydration with 100% acetone was performed twice (30 min each). The samples were embedded and then incubated at 37, 45, and 60 °C overnight for polymerization.

Ultrathin sections were made with an ultramicrotome (LKB-V; LKB, Bromma, Switzerland) and had a thickness of 50–70 nm. Uranium–lead staining was performed. A transmission electron microscope (JEM-1400 Plus; Hitachi High-Technologies) was used for observation.

STATISTICAL ANALYSIS. Data are presented as the mean±standard deviation and were processed using SPSS software (version 24.0; IBM, Armonk, NY). The nonparametric Kruskal-Wallis one-factor analysis of variance was used for comparisons between and among groups. The level of significance was adjusted using the Bonferroni method. An adjusted *P* < 0.05 (*α'*) was considered significant.

Results

CHLOROPHYLL CONCENTRATIONS. Different NO₂ treatment groups exhibited noticeable differences in Chl concentration (Table 1). The concentrations of Chl a, carotenoids, and total Chl and the Chl a/Chl b ratio noticeably decreased with the prolongation of NO₂ fumigation. Compared with the control group (0 h), the 72-h NO₂ treatment group showed significant differences in the Chl a/Chl b ratio and the carotenoid concentration (both adjusted *P* = 0.008). In contrast, all indices of the recovery group (30 d after recovery) increased compared

with those of the NO₂ treatment groups and exhibited significant differences in the Chl a, Chl b, and total Chl concentrations compared with those of the 72-h treatment group (all adjusted $P = 0.008$). No significant differences in the concentrations of Chl a, carotenoids, and total Chl or the Chl a/ Chl b ratio were observed between the control group and the recovery group.

PHOTOSYNTHETIC INDICES. Diurnal variations in Pn were bimodal. The Pn peak values were highest at 1200 HR and 1600 HR, and the “midday rest” phenomenon appeared at 1400 HR among the recovery, control, and NO₂ treatments (Fig. 2A). The Pn peak value showed a decreasing trend with increasing NO₂ treatment concentrations, but it was lower than the control and recovery values (detailed pairwise

comparisons among groups at different diurnal time points are shown in Supplemental Fig. 1).

The Tr values for all NO₂ treatment groups except for the 1-h group increased and then decreased, with the maximum value obtained at 1200 HR. The Tr values of the treatment groups were lower than those of the control group and recovery group (Fig. 2B); however, no significant difference was observed between the control group and recovery group (Supplemental Fig. 2).

The g_s values of the 6-, 24-, 48-, and 72-h treatment groups increased and then decreased, with the maximum value obtained at 1000 HR (Fig. 2C). All treatment groups except for the 6-h treatment group showed a noticeable difference compared with the control group and recovery group. Compared

Table 1. Leaf chlorophyll (Chl) a concentrations, Chl b concentrations, Chl a/Chl b ratios, carotenoid concentrations, and total chlorophyll concentrations after exposure of european hornbeam leaves to 6 μL·L⁻¹ of NO₂ for 0, 1, 6, 12, 24, 48, and 72 h and after self-recovery (n = 3).

Group	Mean ± SD				
	Chl a concn (mg·g ⁻¹ FW)	Chl b concn (mg·g ⁻¹ FW)	Chl a/Chl b (ratio)	Carotenoid concn (mg·g ⁻¹ FW)	Total Chl concn (mg·g ⁻¹ FW)
Control	15.13 ± 0.56	4.84 ± 0.15	3.12 ± 0.15A ^z	2.81 ± 0.01A	19.96 ± 0.01
1 h	10.47 ± 0.15	5.10 ± 0.10	2.13 ± 0.06	2.05 ± 0.04	15.52 ± 0.01
6 h	9.24 ± 0.05	4.26 ± 0.51	2.15 ± 0.01	1.72 ± 0.01	13.51 ± 0.01
12 h	9.02 ± 0.01	4.07 ± 0.50	2.25 ± 0.01	1.61 ± 0.01	13.04 ± 0.02
24 h	8.88 ± 0.01	3.72 ± 0.25	2.05 ± 0.05	1.35 ± 0.02	12.61 ± 0.01
48 h	8.87 ± 0.06	3.65 ± 0.15	1.87 ± 0.05	1.02 ± 0.01	12.51 ± 0.00
72 h	8.56 ± 0.01	3.31 ± 0.15	1.62 ± 0.01	0.72 ± 0.01	11.85 ± 0.01
Recovery	16.14 ± 0.05 A	7.16 ± 0.15 A	2.25 ± 0.15	2.27 ± 0.01	23.28 ± 0.02 A

^zData analysis was performed in the same column using Kruskal-Wallis one-factor analysis of variance, and A indicates adjusted $P = 0.008$ vs. the 72-h group in the same column, after Bonferroni correction.
FW = fresh weight.

Fig. 2. Diurnal change in photosynthesis [(A) net photosynthetic rate (Pn), (B) transpiration rate (Tr), (C) stomatal conductance (g_s), and (D) intercellular CO₂ concentration (C_i)] in leaves of european hornbeam grown under exposure to 6 μL·L⁻¹ of NO₂ for 0 [control (CK)], 1, 6, 12, 24, 48, and 72 h and after 30 d of self-recovery.

Fig. 3. Photosynthesis irradiance (PAR) responses [(A) net photosynthetic rate (P_n), (B) transpiration rate (Tr), (C) stomatal conductance (g_s), and (D) intercellular CO_2 concentration (C_i)] in leaves of european hornbeam grown under exposure to $6\ \mu\text{L}\cdot\text{L}^{-1}$ of NO_2 for 0 [control (CK)], 1, 6, 12, 24, 48, and 72 h and after 30 d of self-recovery; $PPFD$ = photosynthetic photon flux density.

Fig. 4. Scanning electron microscope (SEM) images of the stomata of european hornbeam leaves of the control group [CK (0 h)] exposed to $6\ \mu\text{L}\cdot\text{L}^{-1}$ of NO_2 for 1, 24, and 72 h and after 30 d of self-recovery based on two scale bars (50 and 200 μm). (A) Microstructure of leaves without NO_2 treatment. (B) Normal stomatal state of the leaves without NO_2 treatment. (C-E) Increased number of closed stomata on abaxial surfaces with the increased timing of NO_2 treatment in comparison with the controls and self-recovered plants. (F) Stomatal state of the leaves after 30 d of recovery. UE = upper epidermis, PT = palisade tissue, ST = spongy tissue, LV = leaf vein, TR = trichomes.

with the other treatment groups, the 72-h group had the lowest g_s value (Supplemental Fig. 3). For all NO_2 treatment groups, the C_i value did not exhibit a significant difference between 0800 HR and 1400 HR (Fig. 2D; Supplemental Fig. 4); after 1400 HR, the C_i value decreased.

The bar charts of P_n , Tr , g_s , and C_i under eight light intensities based on the $PPFD$ responses are shown in Fig. 3. With the increased light intensity from 0 to $1400\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$,

the P_n value of all NO_2 treatment groups increased rapidly. When the light intensity reached $1400\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the P_n values of the 48- and 72-h treatment groups increased gradually to the maximum value and became stable. At a light intensity of $1400\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the P_n value of the 6-h treatment group increased rapidly to the maximum value of $15\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The values of the control and recovery groups increased slowly to the maximum values, which were 14 and $15.1\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively (Fig. 3A).

The Tr value varied with the NO_2 treatment time (Fig. 3B). With the increase in light intensity, the Tr values of the 1-h and 6-h treatment groups increased steadily and peaked at $1200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The Tr value of the 12-h NO_2 treatment group was higher than that of any other NO_2 treatment group, and no noticeable Tr variations were observed for the 24-, 48-, and 72-h NO_2 treatment groups.

The g_s values for all NO_2 treatment groups except for the 48- and 72-h groups increased and then decreased, and they peaked when the light intensity was $1200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Fig. 3C). Among all NO_2 treatment groups, the 6-h group had the highest g_s value. With the increased light intensity, the 48- and 72-h treatment groups did not exhibit noticeable changes in the g_s value.

The C_i value of all NO_2 treatment groups decreased and then increased, except for that of the 72-h treatment group, which did not show any noticeable change (Fig. 3D). At a light intensity of

Fig. 5. Scanning electron microscope (SEM) images of palisade tissue and spongy tissue of the european hornbeam leaves of the control group [CK (0 h)] exposed to $6 \mu\text{L}\cdot\text{L}^{-1}$ of NO_2 for 1, 24, and 72 h and after 30 d of self-recovery based on two scale bars (50 and 100 μm). (A) Normal palisade tissue and spongy tissue in the leaves without NO_2 treatment. (B–D) Thicknesses of the palisade and spongy tissues increased after NO_2 treatment compared with those of the controls and self-recovered plants. (E) Palisade tissue and spongy tissue of the leaves after 30 d of recovery.

$50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the Ci values of the 6-, 12-, and 24-h NO_2 treatment groups displayed the minimum value.

ANATOMICAL AND STOMATAL LEAF STRUCTURES. The stomatal sizes of the control, 1-, 24-, 72-h, and recovery groups are shown in Fig. 4B–4F. Wrinkles were observed in the guard cells around the stomata of the 1-, 24-, and 72-h treatment groups. Scanning electron microscopy showed that the stomata of *C. betulus* were on both surfaces. The opening size of the stomata decreased with the prolongation of NO_2 treatment, and the stomata closed after 72 h of NO_2 treatment. After 30 d of recovery, the stomata opened again and returned to the normal aperture level.

The thicknesses of the spongy and palisade tissues of the 1-, 24-, and 72-h NO_2 treatment groups were 106.9%, 112%, and 87.9% of those of the control group, respectively (Fig. 5A–E). In contrast, the thicknesses of the spongy and palisade tissues of the recovery group were 73% of those of the control group ($P < 0.05$), indicating that recovery had a positive effect on the leaf structure of *C. betulus*.

ULTRASTRUCTURE OF CHLOROPLASTS. In the control group, the chloroplasts had an oval shape and contained a large number of

Fig. 6. Transmission electron microscope (TEM) micrographs of chloroplasts from european hornbeam leaves of the control group [CK (0 h)], $6 \mu\text{L}\cdot\text{L}^{-1}$ NO_2 -treated (1, 24, and 72 h, respectively) and self-recovery plants. (A) CK plant with a discoidal chloroplast containing abundant well-compartmentalized grana. (B) Chloroplast with complete leaf structures in the 1-h treatment group. (C) Slightly changed chloroplasts with dilated thylakoid (yellow oval) in the 24-h treatment group. (D) Extensively damaged chloroplasts in the 72-h treatment group showing grossly dilated thylakoids (yellow oval). (E) Chloroplasts with complete leaf structures after 30 d of self-recovery. C = cytoplasm, M = mitochondria, P = plastoglobuli, Pl = primary leaf, S = starch grain, T = tonoplast, V = vacuole, CW = cell wall, G = grana stack.

basic granules and thylakoids (Fig. 6A). NO_2 treatment had a remarkable effect on the shape of chloroplasts. In the 1-h treatment group, the chloroplasts showed a normal ultrastructure in the basic granule arrangement and thylakoid morphology (Fig. 6B). In the 24-h treatment group, fewer than 10% of the chloroplasts presented with slight damage, such as slight loosening and swelling of the thylakoids (yellow oval in Fig. 6C). In the 72-h treatment group, a large number of chloroplasts presented with severely swelling thylakoids (yellow oval in Fig. 6D). Compared with the 24- and 72-h treatment groups, the 1-h group possessed many more thylakoids. In the recovery group, the chloroplasts showed a disc-like shape (Fig. 6E), most chloroplasts returned to their normal shape, and the negative effects of NO_2 stress on the functions of thylakoids nearly disappeared.

Discussion

In this study, we explored the effects of NO₂ treatment according to different exposure times on the photosynthesis, stomatal behavior, and chloroplast ultrastructure of *C. betulus* leaves. Then, we observed the recovery state of *C. betulus* under simulated natural growth conditions. Our results showed that although the severity of the effects of NO₂ treatment on the Chl content in *C. betulus* leaves varied according to exposure time, NO₂ stress generally caused a decrease in Chl content in the leaves. These results were consistent with the conclusions of Liu et al. (2015), who reported that NO₂ stress at different concentrations (3.48, 8.2, 17.4, and 38.5 $\mu\text{L}\cdot\text{L}^{-1}$) decreased the chloroplast content in the mesophyll tissue of *A. thaliana* shoots. Nitrogen is an indispensable element for Chl synthesis (Croft et al., 2017). We speculated that after NO₂ treatment, NO₂ around the leaves combined with moisture to form nitric acids or nitrous acids, which had an acidification effect on mesophyll cells. This process further affected the antioxidant defense system of the organism and inhibited the synthesis of Chl, resulting in difficulty in the absorption of nutrient elements (such as nitrogen and phosphorus) of the plant. Furthermore, NO₂ stress increased the activity of chlorophyllase, which expedited the decomposition of Chl and decreased the light energy absorption of chloroplasts.

In this study, we used scanning electron microscopy and transmission electron microscopy to analyze the changes in stomatal behavior, the thicknesses of palisade and spongy tissues, and the chloroplast structure of *C. betulus* leaves after NO₂ treatment. It has been assumed that the factors related to the decrease in Pn under adversity include stomatal factors and nonstomatal factors (Cai et al., 2013). Under NO₂ stress, both stomatal and nonstomatal factors exist; atmospheric NO₂ deposits on the surface of the leaves and enters the mesophyll cells via the stoma. Photosynthesis capability is an important index for judging the adaptability of plants to certain environments (Cai et al., 2013). Additionally, the response to extreme environments is another index for judging the adaptability of plants. This study showed that 6 $\mu\text{L}\cdot\text{L}^{-1}$ of NO₂ had an inhibitory effect on the Pn, g_s, and Tr values of *C. betulus* leaves (Fig. 2), which indicated that *C. betulus* performs a series of regulatory behaviors to decrease photosynthesis after long-term NO₂ exposure, such as closing the stoma to reduce NO₂ acidification.

To ensure normal photosynthesis within *C. betulus* leaves, the chloroplasts in the mesophyll cells must have a complete and normal configuration and structure, such as an integral outer shell with a clear boundary and developed thylakoids. In this study, the 24- and 72-h NO₂ treatment groups exhibited fewer thylakoids among the basic granules inside the leaves than did the 1-h NO₂ treatment group. In contrast, the control group and recovery group showed a better configuration of chloroplasts and basic granules, lower Pn, and lower pigment content than the 1-h NO₂ treatment group. According to the literature (Rantanen et al., 1994; Schiffgens-Gruber and Lutz, 1992), sulfur dioxide (SO₂) and NO₂ decrease the number of basic granules and cause thylakoids to swell. Our results are consistent with those reported in the literature. Presumably, the 1-h NO₂ treatment may have had a promotive effect on plant growth because the development of chloroplasts resorted to regulating the number of thylakoids and the grana lamella, whereas NO₂ treatment for more than 24 h harmed the physiological growth of the plants.

To date, among the studies of damage remission of plants after NO₂ exposure, most adopted foreign matters, such as hydrogen peroxide (H₂O₂) (Wu et al., 1995) and ascorbic acid (Shalata and Neumann, 2001). Studies of metabolism repair via autoregulation, without the addition of foreign matter, have not been reported. In this study, we found that natural repair of *C. betulus* benefited the thicknesses of palisade and spongy tissues, chloroplast content, and Pn in the leaves of NO₂-treated *C. betulus*. These results may provide new ideas for functional gardening and the construction of ecological green space landscapes.

In conclusion, 6 $\mu\text{L}\cdot\text{L}^{-1}$ of NO₂ damages the ultrastructure of the chloroplasts in *C. betulus* leaves. This condition has a direct negative effect on the Chl content and photosynthesis of the leaves. The reason for the decrease in the Chl content is that absorption of a large volume of NO₂ within a short time by mesophyll cells causes cellular acidification, which damages the cellular membrane and changes cell permeability, resulting in chloroplast damage. However, a certain period of natural recovery can strengthen the ultrastructure or biosynthesis of chloroplasts, enhance photosynthesis, and improve the stomatal aperture, thus increasing the physiological metabolism of *C. betulus*.

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Supplemental Fig. 1. Pairwise comparisons of the net photosynthetic rates among the control (0 h), 6 $\mu\text{L}\cdot\text{L}^{-1}$ NO_2 -treated (1, 6, 12, 24, 48, and 72 h), and self-recovery european hornbeam plants at different diurnal time points based on nonparametric Kruskal-Wallis one-factor analysis of variance. The yellow line indicates a significant difference between groups after Bonferroni correction: (A) 0800 HR; (B) 1000 HR; (C) 1200 HR; (D) 1400 HR; (E) 1600 HR; and (F) 1800 HR. 1 = control group; 2 = 1-h group; 3 = 6-h group; 4 = 12-h group; 5 = 24-h group; 6 = 48-h group; 7 = 72-h group; 8 = recovery group.

Supplemental Fig. 2. Pairwise comparisons of the transpiration rates among the control (0 h), 6 $\mu\text{L}\cdot\text{L}^{-1}$ NO_2 -treated (1, 6, 12, 24, 48, and 72 h), and self-recovery european hornbeam plants at different diurnal time points based on nonparametric Kruskal-Wallis one-factor analysis of variance. The yellow line indicates a significant difference between groups after Bonferroni correction: (A) 0800 HR; (B) 1000 HR; (C) 1200 HR; (D) 1400 HR; (E) 1600 HR; and (F) 1800 HR. 1 = control group; 2 = 1-h group; 3 = 6-h group; 4 = 12-h group; 5 = 24-h group; 6 = 48-h group; 7 = 72-h group; 8 = recovery group.

Supplemental Fig. 3. Pairwise comparisons of the g_s among the control (0 h), $6 \mu\text{L}\cdot\text{L}^{-1} \text{NO}_2$ -treated (1, 6, 12, 24, 48, and 72 h) and self-recovery european hornbeam plants at different diurnal time points based on nonparametric Kruskal-Wallis one-factor analysis of variance. The yellow line indicates a significant difference between groups after Bonferroni correction: (A) 0800 HR; (B) 1000 HR; (C) 1200 HR; (D) 1400 HR; (E) 1600 HR; and (F) 1800 HR. 1 = control group; 2 = 1-h group; 3 = 6-h group; 4 = 12-h group; 5 = 24-h group; 6 = 48-h group; 7 = 72-h group; 8 = recovery group.

Supplemental Fig. 4. Pairwise comparisons of the intercellular CO_2 concentrations between different diurnal time points in the same European hornbeam group. The yellow line indicates a significant difference between different diurnal time points after Bonferroni correction: (A) control group; (B) 1-h NO_2 -treated group; (C) 6-h NO_2 -treated group; (D) 12-h NO_2 -treated group; (E) 24-h NO_2 -treated group; (F) 48-h NO_2 -treated group; (G) 72-h NO_2 -treated group; and (H) recovery group. 1 = 0800 HR; 2 = 1000 HR; 3 = 1200 HR; 4 = 1400 HR; 5 = 1600 HR; 6 = 1800 HR.