Responses of photosynthesis and long-term water use efficiency to ambient air pollution in urban roadside trees

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Abstract
We conducted on-site studies in Kyoto City, Japan, to evaluate the effect of air pollution by automobile gas exhaust on the leaf photosynthetic functions of four urban roadside tree species. Nitrogen oxides (NO and NO2) are major air pollutants that are related to automobile gas exhaust. The species-specific response of leaf photosynthesis to air pollution was obtained for single-year data, in which at the high air pollution sites, *Rhododendron × pulchrum*, *Rhaphiolepis indica*, and *Prunus × yedoensis* had a higher restriction of maximum photosynthesis ($A_{\text{max}}$), while the opposite trend was obtained for *Ginkgo biloba*. When the data were pooled across the years from 2007 to 2019 in *R. pulchrum*, the dose-dependent effect of NO and NO2 on photosynthesis became obvious, in which they decreased $A_{\text{max}}$ and increased the long-term leaf water use efficiency. A spatial variability map for *R. pulchrum* showed a lower $A_{\text{max}}$ and higher water use efficiency at the heavy traffic areas in Kyoto City, which suggests that *R. pulchrum* increased tolerance to air pollution and water stress at the expense of the leaf photosynthesis. This study revealed the importance of the evaluation of the species-specific response of photosynthesis to air pollution for the efficient use of urban trees, even in regions with relatively low atmospheric pollution levels such as <40 ppb of NO or NO2.

Keywords Stable carbon isotope discrimination · Maximum carboxylation rate · Stomatal limitation · Nitrogen oxide · Vehicle emissions

Introduction
The urban environment imposes distinct multiple environmental stresses on trees compared to trees grown in non-urban areas. A high level of ambient air pollution caused by traffic exhaust (Van Wittenberghe et al. 2013; Iqbal et al. 2015), as well as urban water stress caused by less shade, the poor water permeability of paved roads, and the small volume of tree pits (Fini et al. 2009), imposes significant impacts on urban trees. Leaf photosynthesis is a key factor for the growth and survival of trees, and the sequestration of CO2 is the major role of urban trees (Nowak and Crane 2002). Therefore, tree species with high leaf photosynthesis are favorable as urban trees (Xu et al. 2019). However, the leaf photosynthetic function of trees is particularly sensitive to both air pollution (Dhir 2016) and water stress (Drake et al. 2017), in which the interspecific differences are remarkable.

Air pollution by traffic exhaust reduces photosynthesis in *Pinus densiflora* (Kume et al. 2000), *Platanus occidentalis* (Woo and Je 2006), and *Rhododendron × pulchrum* (Kiyomizu et al. 2019), and increases photosynthesis in *Fex rotunda* (Takagi and Gyokusen 2004), *Acer rubrum* (Lahr et al. 2018), and *Ginkgo biloba* (Kiyomizu et al. 2019). Such diverse responses are affected by species-specific responses to multiple air pollutants. Nitrogen oxides (NO and NO2) are among the major air pollutants emitted as traffic exhaust, where previous laboratory exogenous applications of NO2 showed both negative (Hu et al. 2015) and positive effects.
(Wang et al. 2019) on the photosynthesis of urban trees. Although exogenous NO reduced leaf photosynthesis, NO has important roles in protection of chlorophylls against the harmful effects of heavy metals (Procházková et al. 2013). Another important air pollutant ozone (O$_3$) imposes significant negative effects on the leaf photosynthesis of trees (Xu et al. 2019; Hoshika et al. 2020). Although urban roadside trees are expected to improve air quality by capturing particulate air pollutants, such as PM2.5 (Grzędzicka 2018; Zhao et al. 2018), PM2.5 potentially reduces the leaf photosynthesis of urban trees; the heavy pollution of PM2.5 reduced the photosynthesis of a crop, wheat, up to 87% in
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NO2 levels from 2007 to 2019. The points represent the means (se) obtained from April to November. (c) Cluster analysis of the 14 monitoring stations, based on the NO and NO2 levels and traffic volumes in the years 2005, 2010, and 2015, using the database provided by the Japanese government. Three levels, low-, medium-, and high-pollution levels, were detected. (d) Diurnal variations in the atmospheric pollutants, NO, NO2, PM2.5, and O3 in Kyoto City. Data from the year 2018 were used, which was the means of hourly data from April to November for the three pollution levels \((n=932–1816)\). (e) Dependence of annual NO and NO2 levels on traffic volume. The points represent the means of low-, medium-, and high-pollution levels of each year, in which the data from the years 2005, 2010, and 2015 were used \((n=15)\). The annual NO and NO2 data used for the analysis were obtained from April to November. (f) Historical changes of NO and NO2 levels from 2007 to 2019. The points represent the means (se) for low-, medium-, and high-pollution levels, which were calculated using the data from April to November \((n=3−6)\).

wheat-planted farmland \((Gu et al. 2018)\). However, the levels of both O3 and PM2.5 are not directly related to the amount of traffic \((Hagenbörk et al. 2017; Kiyomizu et al. 2019)\). O3 is both produced and destroyed by NO and NO2, which are the major air pollutants related to the amount of traffic \((Hagenbörk et al. 2017)\). The concentration of PM2.5 in the western part of Japan is strongly affected by PM2.5 from foreign sources, i.e., the relative contribution of foreign sources is estimated to be 50–60% \((Ikeda et al. 2015)\).

Although the underlying physiological mechanisms for regulating the photosynthetic response to air pollution are still being studied, some new insights have been raised by recent studies. For the leaf photosynthesis, atmospheric CO2 is absorbed from the open stomata of the leaves, diffuses into the leaf mesophyll, and then is fixed to carbohydrates through biochemical reactions. The photosynthetic response to environmental variables is regulated by both stomatal and non-stomatal limitations, in which non-stomatal limitations are related to the leaf mesophyll anatomy and photosynthetic biochemistry \((Flexas et al. 2012)\). Although the stomatal response has been expected as a key for the photosynthetic response to air pollution \((Wittig et al. 2007)\), recent studies reported that for poplar and beech, the decline in photosynthesis caused by the atmospheric pollutant O3 is largely due to increased non-stomatal limitations \((Xu et al. 2019; Hoshika et al. 2020)\). Further and more extensive studies are needed to clarify the species-specific effect of air pollution on the photosynthesis of urban trees and its underlying mechanisms.

In recent years, urban drought in summer has been accelerated through global and urban warming in the temperate zone \((Savi et al. 2015; Meineke and Frank 2018)\), and efficient CO2 sequestration per water loss, e.g., high water use efficiency, is essential for the growth and survival of urban trees. Water use efficiency is used as a selection criterion for urban trees in Newcastle-upon-Tyne, UK \((Grote et al. 2016)\). We focused on the leaf carbon isotope ratio, \(\delta^{13}C\), which is a suitable measure for the evaluation of spatial and temporal variation in long-term averaged water use efficiency \((Kagotani et al. 2013)\). Plant \(\delta^{13}C\) has been used as a powerful tool for evaluating long-term averaged water use efficiency of the trees across multiple years, e.g., 150 years \((Brienen et al. 2017)\). In urban areas, leaf \(\delta^{13}C\) can be used as a metric of urban ecosystem services and has been used as an index of long-term water use efficiency in California, USA \((McCarthy et al. 2011)\).

We performed a series of studies in Kyoto City to clarify the photosynthetic response of roadside trees to air pollution. We used two data sets to address the four main purposes for our study. The first two main purposes of the present study were as follows: 1) to show how roadside trees in urban areas in Kyoto City are exposed to air pollution, 2) to compare the photosynthetic responses among the four major roadside tree species used in Japan, \(Rhododendron \times \text{pulchrum}\), \(Rhaphiolepis indica\), \(Ginkgo \text{biloba}\), and \(Prunus \times \text{venedoensis}\), and to clarify the contributions of stomatal and non-stomatal limitations to the photosynthetic response to air pollution. We used the first data set for these two purposes, which is detailed gas exchange measurements for the four tree species in a single year. The latter two main purposes are such as 3) to obtain the dependence of photosynthesis and water use efficiency on NO and NO2 in \(R. \text{pulchrum}\), which is the most commonly used roadside tree species in Japan, and 4) to evaluate the spatial variability of photosynthesis and water use efficiency of \(R. \text{pulchrum}\) in the central part of Kyoto City and clarify the response of trees to the urban environment. For these latter two purposes, we used the second data set, which is the pooled data across multiple years for \(R. \text{pulchrum}\). These analyzes will reveal the interaction of air pollution and drought on urban roadside trees.

Materials and methods

Site description, air pollution levels, and plant species

Kyoto City, Japan, is located in the Kyoto basin surrounded by mountains to the east, north, and west. The mean temperature, relative humidity, and cumulative precipitation during the growing season, from April to November, in an average year \((1981—2010)\) is 20.7 °C, 66.0%, and 1210 mm, respectively. Concentrations of air pollutants have been monitored continuously by the Kyoto government at 14 monitoring stations in Kyoto City since 1970, in which the data are obtained every hour \((http://www.nies.go.jp/igreen/td_down.html)\). We classified these 14 monitoring stations
into three levels of pollution, low, medium, and high, using cluster analysis. We selected 19 study sites in total for collecting branches from roadside trees (Fig. 1a, Table S1). The study sites were selected such that they had as wide a range of traffic volumes as possible within one species in one sampling year. The longitude, latitude, and altitude of the study sites were 135° 42'—49', 34° 56'—35° 03', and 20 – 80 m, respectively. The levels of air pollution of the study sites were determined by applying the pollution levels of the nearest monitoring stations. The traffic data of 12 h of daytime were obtained for the years 2005, 2010, and 2015 from the Ministry of Land, Infrastructure, Transport and Tourism. The data were obtained once a year at about 2000 sites in Kyoto Prefecture (https://www.mlit.go.jp/road/census/h17, h22-1, h27).

We used four major tree species planted as roadside trees in Kyoto Prefecture in this study. The tree species used were two evergreen shrub trees, *Rhododendron × pulchrum* Sweet ‘Oomurasaki’ (*R. pulchrum*, azalea) and *Rhaphiolepis indica* (L.) var. *umbellata* (Thunb.) H. Ohashi (*R. indica*, yeddo hawthorn), and the two broadleaved deciduous tall trees, *G. biloba* L. (*G. biloba*, ginkgo) and *Prunus × yedoensis* (Matsum.) Masam. et Suzuki ‘Somei-yoshino’ (*P. yedoensis*, cherry). *R. pulchrum* was the most common shrub species (62.7 × 10^6, 45% of the shrub trees) planted in Japan in 2017, followed by *R. indica* (9.7 × 10^6, 6.9%). *G. biloba* was the most common tall tree species used in Japan (5.5 × 10^5, 8%), followed by *P. yedoensis* (5.2 × 10^5, 7.8%, Iizuka and Funakubo 2018). The leaves at the top of the crowns of *R. pulchrum* and *R. indica* trees are considered annual leaves after July because they are pruned annually between May and June, and after that, new annual leaves then subsequently expand. *G. biloba* and *P. yedoensis* trees are neither watered nor pruned during the growing season by the Kyoto government. The phenology of *G. biloba* and *P. yedoensis* is such that the leaves expand in early April, fully expand in May, begin senescence in late November, and fall in December.

**Leaf gas exchange measurements and photosynthetic limitations**

Sunlit healthy branches were harvested from three – four individual trees for each study site in the morning, one branch from one individual tree. The leaf numbers used for the photosynthetic measurement are summarized in Table S1. The branches were collected from the tops of the canopies for *R. pulchrum* and *R. indica* and from the sunlit crowns for *G. biloba* and *P. yedoensis*, avoiding rainy days. The ends of the branches were immediately immersed in water, brought back to the laboratory, and then photosynthesis measurements were performed for the healthy fully expanded mature leaves within 10 h. Leaf photosynthesis was measured using a photosynthesis system constructed in our laboratory (in 2007, Kagotani et al. 2013) or using Li-6400XT (Li-Cor, Lincoln, NE, USA, from the years 2014 to 2019), which had the same performance for leaf photosynthesis measurement. We previously confirmed that the leaf photosynthetic parameters of these four species were unchanged for 10 h after excision of the branches of the four tree species (p > 0.1, n = 4).

We obtained light-saturated photosynthesis (Amax) and stomatal conductance (gs) at ambient CO2 of 400 μmol mol⁻¹ and at 1000 μmol mol⁻¹ of PPFD (in 2007) or at 1500 μmol mol⁻¹ of PPFD (from 2014—2019). We confirmed that leaf photosynthesis was almost light-saturated at 1000 μmol mol⁻¹ for the four species, where the ratio to the maximum value was 94—95%. The photosynthetic biochemical parameters, such as the maximum carboxylation rate (Vcmax), were obtained from A/Ci curves (Ethier and Livingston 2004) using Li-6400XT (Kiyomizu et al. 2019; Kinoshita et al. 2021) for the years 2014 to 2019, where Ci means intercellular CO2. The measurements were conducted under 1500 μmol mol⁻¹ of PPFD. In these photosynthesis measurements, the leaf temperature and vapor pressure deficit (VPD) were set at 26—28 °C and 1.2—1.7 kPa, respectively.

We can evaluate the mechanisms of the effect of environmental stresses on leaf photosynthesis by performing a quantitative analysis of photosynthetic limitations (Flexas et al. 2012). If the reduction of photosynthesis is mainly due to stomatal closure, stomatal limitation is a dominant factor for restriction of photosynthesis. On the other hand, when some biochemical traits such as CO2 fixation enzymes are damaged by the stress, non-stomatal limitation becomes a dominant factor for the restriction of photosynthesis. The photosynthetic restriction, dA/A, is sum of contributions of stomatal (S), mesophyll (M), and biochemical limitations (B), in which the sum of mesophyll limitation and biochemical limitation is called “non-stomatal limitation” (Grassi and Magnani 2005):

\[ \text{Photosynthetic restriction, } dA/A = S + M + B \]

\[ \text{(1)} \]

\[ \text{Contributions of non-stomatal limitation} = M + B \]

\[ \text{(2)} \]

Stomatal limitation, Iw, was calculated using A/Ci curves obtained from the A/Ci curves, following a previous study (Grassi and Magnani 2005):

\[ \text{Stomatal limitation, } Iw = (g_{\text{tot}} / g_{\text{sc}} \cdot \partial A / \partial C_i) / (g_{\text{tot}} + \partial A / \partial C_i) \]

\[ \text{(3)} \]

where \( g_{\text{tot}} \) is the total conductance to CO2 between the leaf surface and carboxylation sites, \( g_{\text{sc}} \) is stomatal conductance to CO2, and \( C_i \) means chloroplast CO2. \( g_{\text{tot}} \) calculated as

\[ 1 / g_{\text{tot}} = 1 / g_{\text{sc}} + 1 / g_m \]

\[ \text{(4)} \]

where \( g_m \) is mesophyll conductance, which is calculated using A/Ci curves. The value of \( \partial A / \partial C_i \) can also be obtained from the A/Ci curves.
The difference in annual climate and sampling times should affect absolute values of leaf photosynthesis. To evaluate the effect of air pollution on the limitations of photosynthesis across the different years and sampling times, photosynthesis values need to be ‘normalized’ using a reference of each year across the study sites and sampling times. We obtained individual maximum value of $A_{\text{max}}$ for each year for each species as a reference value ($A_{\text{ref}}$), e.g., 11.5 μmol m$^{-2}$ s$^{-1}$ for $R$. pulchrum in 2017, following the previous study (Grassi and Magnani 2005). The restriction in $A_{\text{max}}$ in each year can then be estimated as follows:

$\text{Restriction in } A_{\text{max}} = (A_{\text{obs}} - A_{\text{ref}})/A_{\text{ref}}$ (5)

where $A_{\text{obs}}$ is the observed $A_{\text{max}}$. We defined that photosynthetic restriction, $dA/A$ in the Eq. (1), is the same as the restriction in $A_{\text{max}}$ in the Eq. (5).

We measured the SPAD values as an indicator of leaf chlorophyll content (Parry et al. 2014), because high levels of air pollutants cause a decline in the leaf chlorophyll content (Joshi and Swami 2009), which is related to the photosynthetic biochemistry. SPAD values were evaluated for the leaves on which leaf gas exchange measurements were conducted, as mean values of the three replicated measurements. The SPAD value was measured using a SPAD meter (SPAD-502 Plus, Konica-Minolta, Tokyo, Japan).

Carbon isotope discrimination

After the photosynthesis measurements were finished, the leaves were dried at 60—70 °C for 24 h using an oven (MOV-112, SANYO Electric, Osaka, Japan), and were stored in a desiccator at room temperature to analyze the leaf stable carbon isotope ratio ($\delta^{13}C$). Leaf carbon isotope discrimination, $\Delta^{13}C$, is a good indicator of long-term leaf water use efficiency (Broeckx et al. 2014), which can be used for the evaluation of air pollution effects (Kagotani et al. 2013). $\Delta^{13}C$ is determined as:

$\Delta^{13}C(\%) = (\delta_a - \delta_p)/(1 + \delta_p/1000)$ (6)

where $\delta_a$ is the $\delta^{13}C$ of atmospheric CO$_2$ and $\delta_p$ is leaf $\delta^{13}C$. We collected air samples of the study sites from 2007 to 2019 to obtain $\delta_a$, which depends on the air pollution level, and was used to calculate $\Delta^{13}C$. Air samples of 2 L near the tree canopies were collected in aluminum air bags using an air pump, and then $\delta_a$ was measured using an isotope mass spectrometer (Finnigan MAT 252, Thermo Fisher Scientific, MA, USA) or an isotope analyzer (G2101-i, Picarro, Inc., Santa Clara, CA, USA). The averaged values of $\delta_a$ were $-11.0$ (0.3) ‰, $-11.7$ (0.4) ‰, and $-12.0$ (0.4) ‰ for the low-, medium-, and high-pollution sites, respectively (mean (se), $n = 14 — 23$). In the years 2007 and 2014, $\delta_p$ was measured using powdered leaf samples using the combined system of an elemental analyzer (EA1108, Carlo-Erba, Italy), interface (Confl II, Thermo Fisher Scientific, MA, USA), and isotope mass spectrometer (Delta S, Thermo Fisher Scientific, MA, USA), and isotope mass spectrometer (delta V advantage, Thermo Fisher Scientific, MA, USA) at the Research Institute for Humanity and Nature.

To compare $\Delta^{13}C$ values across the different years, we calculated deviations in the $\Delta^{13}C$ value of each year using the maximum value as a reference ($\Delta^{13}C_{\text{ref}}$):

$\text{Deviations in } \Delta^{13}C = \Delta^{13}C_{\text{ref}} - \Delta^{13}C_{\text{obs}}$ (7)

where $\Delta^{13}C_{\text{obs}}$ is the observed $\Delta^{13}C$. The larger deviations in $\Delta^{13}C$ mean smaller $\Delta^{13}C_{\text{obs}}$, and in turn, higher long-term water use efficiency.

The two data sets used in this study

We used two sets of data for evaluation of the photosynthetic performance of urban trees (Table S1). In data set 1, leaf gas exchange data for the four tree species G. biloba, P. yedoensis, R. pulchrum, and R. indica in a single year were used to obtain the species-specific response to air pollution. Using data set 1, we compared the restrictions of photosynthesis, stomatal and non-stomatal limitations, and SPAD values that reflect the leaf chlorophyll content among low, medium and high pollution levels. In data set 2, we focused on the species R. pulchrum for the evaluation of photosynthesis and water use efficiency in response to atmospheric NO and NO$_2$ concentrations. The variation ranges in annual atmospheric NO and NO$_2$ concentrations from 2007 to 2019 were from 0.4 to 47 ppb and 6 to 37 ppb, which are larger than the ranges within a year, from 0.7 to 17 and 6 to 19 ppb, respectively (Japan National Institute for Environmental Studies, http://www.nies.go.jp/igreen/td_down.html). We can detect the photosynthetic response of roadside trees to a wider range of NO and NO$_2$ by using annual averaged data. The annual averaged values for the restriction of $A_{\text{max}}$ and deviations in $\Delta^{13}C$ were calculated in 2007 and 2014—2019. We obtained the dependence of leaf traits on NO and NO$_2$ concentration for the pooled data.

Finally, we made a map of photosynthetic restrictions and deviation in $\Delta^{13}C$ of R. pulchrum in Kyoto City, using the data set 2, traffic volumes, and NO$_2$ concentrations. First, the central part of Kyoto City, which is about 100 km$^2$, including the study sites, was divided into areas of a
1 km² mesh. We obtained 121 sections. Then, we obtained traffic volumes for each 1 km² section using traffic volume data of the road census stations from 2015 (http://www.mlit.go.jp/road/census/). If multiple census stations were included in a 1 km² section, the higher value was applied. Twenty three of the 121 Sects. (19%) contained no census station, so the value of the nearest station was applied to these sections. Then, the NO₂ concentration of each section was estimated using regression between the 12-h daytime traffic volumes and NO₂ as in Fig. 1e (n = 41):

\[ NO₂(ppb) = 4.93 + 0.521 \times 12 - h \ \text{daytime traffic volume (10³ cars)} \]  

(8)

Finally, restrictions of \( A_{\text{max}} \) and the deviation in \( \Delta^{13}C \) of each section were estimated using the correlations between NO₂ and restriction of \( A_{\text{max}} \) or deviation in \( \Delta^{13}C \) in Fig. 3 (n = 17—19):

Restrictions of \( A_{\text{max}}(\%) = 16.7 + 1.48 \times NO₂(ppb) \)  

(9)

Deviation in \( \Delta^{13}C(\%) = 0.700 + 0.115 \times NO₂(ppb) \)  

(10)

Statistical analysis

For the cluster analysis of the air monitoring stations, the data of nitrogen oxides (NO and NO₂) and traffic data for the years 2005, 2010, and 2015 were used, in which the traffic data of the nearest sites were applied to the air pollution monitoring stations. The NO and NO₂ data used for the analysis were monthly means from April to November (n = 1034). The levels of major atmospheric pollutants in Kyoto City from the years 2007 to 2019 (Table 1) showed that the daily and annual levels of SO₂, from 2 to 8 ppb and from 1 to 5 ppb, respectively, were low, and far below the environmental conservation standards, and furthermore, were much lower than the minimum dose for suppression of photosynthesis, 100 ppb (Smith and Lytle 1997). The daily levels of atmospheric NO₂ and fine particulate matter PM2.5, as well as the maximum of the hourly level of atmospheric O₃, exceeded the environmental conservation standards in some cases. The annual values from 2007 to 2019 in the present study are not so different from those observed in Europe between 2000 and 2010, NO₂ (0 - 12 ppb), SO₂ (3 ppb), O₃ (55 ppb), and PM2.5 (25 μg m⁻³) (Jiang et al. 2020). The annual levels of nitrogen oxides (NO and NO₂) strongly correlated with the 12-h daytime traffic volumes, while the annual SO₂, PM2.5, and O₃ did not (Table 1), which supports previous studies

Table 1 Ranges of air pollutant levels from 2007 to 2019 at the 14 monitoring stations, their environmental conservation standards in Kyoto City, and the results of regression analysis between the pollutant levels and 12 h-daytime traffic volumes

| Pollutant | Range of mean values | Environmental conservation standard | Linear regression to 12 h-daytime traffic volume |
|-----------|----------------------|------------------------------------|------------------------------------------------|
| SO₂ (ppb) | 2 – 8 (daily)        | 20 (daily)                         | -                                               |
|           | 1 – 5 (annual)       | NA                                 | -0.099 **                                     | 12 |
| NO (ppb)  | 6 – 100 (daily)      | NA                                 | -                                               |
|           | 1 – 36 (annual)      | NA                                 | 0.61***                                        | 28 |
| NO₂ (ppb) | 18 – 50 (daily)      | 40 (daily)                         | -                                               |
|           | 8 – 30 (annual)      | NA                                 | 0.58**                                         | 28 |
| PM2.5 (μg m⁻³) | 19.3 – 42.8 (daily) | 35 (daily)                         | -                                               |
|           | 8.5 – 18.1 (annual)  | 15 (annual)                        | 0.0061 *                                       | 22 |
| O₃ (ppb)  | 43 – 136 (max of hourly values) | 60 (hourly values) | -                                               |
|           | 28 – 40 (annual)     | NA                                 | -0.056 **                                      | 18 |

The ranges of pollutants were hourly, daily, or annual averaged values for the 14 monitoring stations. The environmental conservation standards are daily means of hourly values, “daily”; hourly values, “hourly”; or annual means, “annual”. NA means not applicable. Regression analysis was performed for the annual data for the years 2010 and 2015. ** The values were measured at the 4, 11, and 10 stations, respectively.

Results and discussion

Atmospheric pollution levels in Kyoto City

The levels of major atmospheric pollutants in Kyoto City from the years 2007 to 2019 (Table 1) showed that the daily and annual levels of SO₂, from 2 to 8 ppb and from 1 to 5 ppb, respectively, were low, and far below the environmental conservation standards, and furthermore, were much lower than the minimum dose for suppression of photosynthesis, 100 ppb (Smith and Lytle 1997). The daily levels of atmospheric NO₂ and fine particulate matter PM2.5, as well as the maximum of the hourly level of atmospheric O₃, exceeded the environmental conservation standards in some cases. The annual values from 2007 to 2019 in the present study are not so different from those observed in Europe between 2000 and 2010, NO₂ (0 - 12 ppb), SO₂ (3 ppb), O₃ (55 ppb), and PM2.5 (25 μg m⁻³) (Jiang et al. 2020). The annual levels of nitrogen oxides (NO and NO₂) strongly correlated with the 12-h daytime traffic volumes, while the annual SO₂, PM2.5, and O₃ did not (Table 1), which supports previous studies.
The trends were similar between NO and NO\textsubscript{2} into three classes, high-, medium-, and low-pollution levels at atmospheric pollutants, NO and NO\textsubscript{2} are major air pollutants related to automobile gas exhaust in Kyoto City, and that the differences in photosynthetic responses among study sites in Kyoto City would be strongly affected by the levels of NO and NO\textsubscript{2}.

We then focused on the NO, NO\textsubscript{2}, and daytime traffic, to classify the atmospheric pollution levels in Kyoto City. Cluster analysis revealed that the levels of atmospheric pollution at the 14 monitoring stations (Fig. 1a) were classified into three classes, high-, medium-, and low-pollution levels (Fig. 1c). Diurnal variations of air pollutants revealed that the trends were similar between NO and NO\textsubscript{2}, which showed peaks that corresponded to the trend in traffic volume in Kyoto City (Fig. 1d). However, the diurnal patterns in PM2.5 and O\textsubscript{3} were different from those of NO and NO\textsubscript{2}, in which the differences between pollution level (low, medium, and high) were much less clear compared to those for NO and NO\textsubscript{2} (Fig. 1d). These results again indicate that among the atmospheric pollutants, NO and NO\textsubscript{2} are important pollutants that strongly relate to the traffic volume. The $r^2$ values for linear regression between the 12-h daytime traffic volume and the annual levels of NO/NO\textsubscript{2} from April to November were 0.49 - 0.53 for the data of 2005, 2010, and 2015 (Fig. 1e). The strong dependence of NO\textsubscript{2} on traffic volume supports our previous study (Kagotani et al. 2013) and the results reported in Beijing, China (Yang et al. 2019), indicating that areas with high traffic volumes in Kyoto City have high nitrogen oxide pollution. For the annual trends of these pollutants in Kyoto City, on the other hand, a significant reduction in NO and NO\textsubscript{2} with time was observed in the years 2007 to 2019, where the NO and NO\textsubscript{2} levels in 2019 became 40 - 60% of those in 2007 for the three pollution levels (Fig. 1f). The historical decreasing trend in NO\textsubscript{2} is in line with the reported decreasing trend of tropospheric NO\textsubscript{2} concentrations compared to those of NO\textsubscript{2} in Japan from 2000 - 2007, which is partly related to anatomical alterations in the leaf mesophyll cells by air pollution (Hoshika et al. 2020). Our previous study reported an increase in the mesophyll thickness of G. biloba at high-pollution sites, which induces increases in both mesophyll CO\textsubscript{2} diffusion and the biochemical capacities (Kiyomizu et al. 2019) that involves the reduction in non-stomatal limitations.

We then focused on the major urban shrub species R. pulchrum, which had the highest restriction of photosynthesis imposes a negative impact on the photosynthesis of these three species. In G. biloba, on the contrary, restrictions of $A_{\text{max}}$ were less at the medium- and high-pollution sites compared to the low-pollution sites by 17 - 35%. The contrasting results for R. pulchrum and G. biloba confirm our previous study obtained in autumn (Kiyomizu et al. 2019), which revealed that the photosynthetic response to urban NO and NO\textsubscript{2} is highly species-specific, even under a relatively low level of NO\textsubscript{2}, below 50 ppb. Such levels of NO\textsubscript{2} are generally observed in many cities including in East Asia (Kim and Guldmann 2015), Africa (Adon et al. 2016), Europe (Jiang et al. 2020), and the USA (Yli-Pelkonen et al. 2017).

The effects of air pollution on the restrictions of photosynthetic biochemical capacities, $V_{\text{cmax}}$ (carboxylation efficiency) and SPAD (reflects chlorophyll concentration), were overall in line with those of $A_{\text{max}}$, with the exception of R. indica at the high pollution site with a low restriction in SPAD (Table 2). These results confirm that the highly species-specific photosynthetic response to air pollution strongly depends on the biochemical trait. The higher restrictions in the SPAD value, i.e., the reduction in chlorophyll content under high atmospheric pollution, is reported in previous studies, in which a low chlorophyll content is often linked to a low photosynthetic performance (reviewed by Dhir 2016).

The analysis of photosynthetic limitations revealed that for all species, non-stomatal limitations were significantly larger than stomatal limitations ($p < 0.05$), in which the difference between non-stomatal and stomatal limitations at the high-pollution levels were larger for R. pulchrum, R. indica, and P. yedoensis (Fig. 2b). This indicates that non-stomatal factors are more important constraints than the stomata for leaf photosynthesis of these three urban tree species under high levels of NO and NO\textsubscript{2} within a single year. The importance of non-stomatal limitations for the reduction in photosynthesis by an air pollutant, O\textsubscript{3}, has been reported for poplar and beech (Xu et al. 2019; Hoshika et al. 2020). For G. biloba, on the other hand, the reduction in the difference between non-stomatal and stomatal limitations at the high-pollution levels (Fig. 2b) indicates less non-stomatal limitation at the high-pollution levels, which is partly related to anatomical alterations in the leaf mesophyll cells by air pollution (Hoshika et al. 2020). Our previous study reported an increase in the mesophyll thickness of G. biloba at high-pollution sites, which induces increases in both mesophyll CO\textsubscript{2} diffusion and the biochemical capacities (Kiyomizu et al. 2019) that involves the reduction in non-stomatal limitations.

Restrictions of photosynthesis by air pollution in the four urban roadside tree species

The ratio of atmospheric NO and NO\textsubscript{2} concentrations compared to the low pollution sites were 4 – 20-fold and 1.5 – 2.1-fold, respectively, at the medium-pollution sites, and 15 – 50-fold and 2.7 - 3.9-fold, respectively, at the high-pollution sites (Fig. 2a). The restriction of maximum photosynthesis ($A_{\text{max}}$) was higher at the high-pollution sites than those at the low-pollution sites for R. pulchrum, R. indica, and P. yedoensis by 19 – 34% (Fig. 2b), which indicates that air pollution restrictions in photosynthesis and deviations in long-term water use efficiency in response to air pollution across the years

We then focused on the major urban shrub species R. pulchrum, which had the highest restriction of photosynthesis
Fig. 2 NO and NO₂ levels, restriction of \( A_{\text{max}} \), and stomatal and non-stomatal limitations of photosynthesis for the four urban roadside tree species at three different pollution levels. The data were obtained in the year 2016 (P. yedoensis) and 2017 (R. pulchrum, R. indica, and G. biloba). (a) Mean (se) NO and NO₂ levels and (b) restriction of \( A_{\text{max}} \) and photosynthetic limitations by stomata and non-stomatal capacities. The significance of the difference between pollution levels were tested by ANOVA, Dunnett’s test, in which the values for the low-pollution level were treated as a control. The significant levels are * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \).

(Fig. 2b) and lowest photosynthesis rate among the four species at high-pollution levels (Table S2). The relationship between atmospheric NO or NO₂ and leaf traits across the years was analyzed using the pooled data from 2007 to 2019 (Fig. 3). Restrictions of \( A_{\text{max}} \) were increased with increasing NO or NO₂ for R. pulchrum, which indicates that photosynthesis becomes more inhibited by higher atmospheric nitrogen oxides, which confirms our previous results (Fig. 2, Table 2). Additionally, two-way ANOVA revealed that there was a small interaction between year and pollution level on \( A_{\text{max}} \) (Table S3), which indicates that the effect of pollution level on these variables was overall held across the years.

Deviations in annual carbon isotope discrimination from the maximum value of each year became higher as NO or NO₂ increased, i.e., \( \Delta^{13}C \) decreased with increasing nitrogen oxides (Fig. 3). This result confirms our previous study (Kiyomizu et al. 2019) and the study for Norway spruce in central Europe that reported decreased \( \Delta^{13}C \) with air pollution (Čada et al. 2016), which indicates that long-term water use efficiency becomes higher as nitrogen oxides increase (Kume et al. 2006). The evaluation of the effect of air pollution on long-term water use efficiency by using the leaf carbon isotope ratio (\( \delta_{\text{p}} \)) can be difficult because of the opposite effect of fossil fuel \( \delta^{13}C \) (\( \delta_{\text{a}} \)) and \( \Delta^{13}C \) on \( \delta_{\text{p}} \) (Eq. 2, Pataki et al. 2010). In fact, atmospheric NO₂ imposes a positive
effect (Kume et al. 2006) or no effect (Yang et al. 2018) on leaf δ¹³C (δp) of Pinus densiflora foliage in Japanese and Korean cities, respectively. In spite of these difficulties, we successfully obtained a clear trend of Δ¹³C in relation to atmospheric nitrogen oxides (Fig. 3) by both correction of the effect of fossil fuel δ¹³C and evaluation of relative changes in Δ¹³C (Eq. 2). The increase in long-term water use efficiency with the decrease in photosynthesis by nitrogen oxides in R. pulchrum suggests that at sites with high air pollution, stomatal closure becomes significant, which may contribute to reducing the absorption of atmospheric pollutants from the stomata, as well as to reduce water consumption. In this sense, R. pulchrum becomes more favorable when summer drought is more serious due to the effects of urban and global warming. The high water use efficiency has often been considered to be an advantageous trait in water-limited conditions (Medrano et al. 2009).

Finally, we performed mapping of the restriction of Aₘₐₓ and deviations in Δ¹³C for R. pulchrum in the central area of Kyoto City (Fig. 4), based on the relationships among the traffic volumes, NO₂ level, and restriction of Aₘₐₓ or deviations in Δ¹³C (Figs. 1 and 3). The restriction of Aₘₐₓ

| Table 2 Restriction (%) of the photosynthetic biochemical capacities Vcₘₐₓ and SPAD for the data set |
| Parameter | Pollution level | R. pulchrum | R. indica | P. yedoensis | G. biloba |
|------------|----------------|-------------|-----------|-------------|-----------|
| Restriction of maximum carboxylation efficiency, Vcₘₐₓ (%) | Low | 5.1(4.8) | 3.6(1.4) | 10.9(6.0) | 36.5(2.0) |
| Medium | 25.5(2.5)** | 28.1(2.7)** | 21.7(8.4) | 27.7(5.0) |
| High | 50.4(4.9)** | 21.6(3.0)** | 26.4(4.2) | 6.1(3.3)** |
| Restriction of SPAD, which reflects chlorophyll content (%) | Low | 10.8(3.9) | 18.2(0.8) | 5.9(2.7) | 21.7(6.2) |
| Medium | 27.3(0.8)** | 31.6(0.9)** | 7.2(3.8) | 18.2(2.3) |
| High | 30.4(3.3)** | 4.5(1.6)** | 17.9(1.5)* | 3.3(1.1)** |

Values are means (se), in which restrictions were calculated as % of the maximum value for each species. The significance of the difference between pollution levels was tested by ANOVA, Dunnett’s contrast, with data for the low pollution level treated as a control. The significance of the difference is shown as symbols ’ ’ P < 0.05, ** P < 0.01, and *** P < 0.001. No symbol means no significant difference.

Fig. 3 Dependence of photosynthetic variables, restrictions of Aₘₐₓ and deviations in Δ¹³C on NO and NO₂, for R. pulchrum. The points represent the annual means for 4 – 35 leaves from the years 2007 to 2019, which were collected from low-, medium-, and high-pollution sites (n = 14 – 19). Solid lines indicate regression lines with r² values and significance of the regression line shown. The significant levels are ** P < 0.01, and *** P < 0.001. Data from the years 2014 and 2015 were redrawn from the published data of our previous study (Kiyomizu et al. 2019). Error bars indicate se values.
Fig. 4 Maps of restrictions of $A_{\text{max}}$ (a) and deviations in $\Delta^{13}C$ (b) for *R. pulchrum* in the central area of Kyoto City. The central area of Kyoto City is about 100 km$^2$, and was divided into areas of 1 km$^2$ (122 sections) and then restrictions of $A_{\text{max}}$ and deviation in $\Delta^{13}C$ for each section were estimated using correlations among traffic volumes, $NO_2$, and restrictions of $A_{\text{max}}$ or deviations in $\Delta^{13}C$ (Eqs. 5, 6, and 7 in the text).
of *R. pulchrum* was higher in the area near Kyoto Station, 50 – 60%, compared to the northern or eastern part of Kyoto City, 20 – 30% (Fig. 4a). Although the spatial distributions of vegetation in urban areas were reported using remote-sensing in previous studies (Liu and Yang 2013; Zhong et al. 2019), few studies have shown the spatial distribution of gas exchange measurement-based photosynthesis in an urban area at this time. Our present study revealed that in some tree species, leaf photosynthesis is more strongly restricted at sites with higher traffic volumes in urban areas, indicating that such species have smaller production. From the viewpoint of CO₂ sequestration, such species are less suitable for urban areas with heavy traffic volumes.

The mapping of deviations in Δ¹³C in Kyoto City showed that the leaf Δ¹³C of *R. pulchrum* was small at the area near Kyoto Station (Fig. 4b), which indicates that the long-term intrinsic leaf water use efficiency is higher at the heavy-traffic area. This high water use efficiency at the heavy-traffic area may contribute to enhancing tolerance to air pollution and summer drought. The high long-term water use efficiency at the heavy-traffic area may affect the photosynthesis of roadside trees in urban areas. A species-specific response of leaf photosynthesis to air pollution was obtained. Within a single year, the three roadside tree species, *R. pulchrum*, *R. indica* and *P. yedoensis*, had a higher restriction of maximum photosynthesis (Amax) at the high air pollution sites. On the contrary, the leaf photosynthesis of *G. biloba* was enhanced at high air pollution sites. This result coincides with the lower stomatal limitation of *G. biloba* at the higher pollution sites (Table 3), which indicates that high pollution induces open stomata for *G. biloba*.

### Conclusions

Atmospheric NO and NO₂ are important air pollutants that affect the photosynthesis of roadside trees in urban areas. A species-specific response of leaf photosynthesis to air pollution was obtained. Within a single year, the three roadside tree species, *R. pulchrum*, *R. indica* and *P. yedoensis*, had a higher restriction of maximum photosynthesis (Amax) at the high air pollution sites. On the contrary, the leaf photosynthesis of *G. biloba* was enhanced at high air pollution sites. Non-stomatal limitations are key traits that involved these photosynthetic responses to air pollution within a year. When the data were pooled across the years, the effect of air pollutants on long-term leaf water use efficiency became apparent, in which NO and NO₂ increased the long-term leaf water use efficiency in *R. pulchrum*. It should be noted, however, that we are not able to conclude that NO and NO₂ are the only decisive factors that cause a reduction in Amax and increase in long-term leaf water use efficiency under high pollution levels; some interactions among air pollutants, such

### Table 3: Stomatal limitation and deviations in carbon isotope discrimination (Δ¹³C) for the data set 1

| Traits | Species          |污染水平 | R. pulchrum | R. indica | P. yedoensis | G. biloba |
|--------|------------------|----------|-------------|-----------|--------------|-----------|
|        |                  |          |            |           |              |           |
|        |                  | Stomatal limitation |          |           |              |           |
|        |                  | Low      | 0.142(0.014) | 0.176(0.026) | 0.303(0.013) | 0.184(0.006) |
|        |                  | Medium   | 0.125(0.012) ** | 0.167(0.018) ** | 0.307(0.024) ** | 0.148(0.016) ** |
|        |                  | High     | 0.090(0.016) † | 0.179(0.023) † | 0.382(0.015) † | 0.089(0.007) ** |
|        | Deviations (%) in carbon isotope discrimination (Δ¹³C) |          |            |           |              |           |
|        |                  | Low      | 2.73(0.93) | ND         | 0.37(0.32)  | 3.50(0.84)  |
|        |                  | Medium   | 0.46(0.35) *** | ND        | 1.00(1.04) *** | 1.30(0.77) *** |
|        |                  | High     | 3.78(0.92) ♂ | ND         | 1.49(0.48) ♂ | 1.49(0.19) ** |

Values are means (se). The effects of pollution level was tested using one-way ANOVA, Dunnett’s test, in which the significant levels are † P < 0.1, ** P < 0.01 and *** P < 0.001. n.s. means no significant difference. No data of Δ¹³C were obtained for *R. indica*.

Note that the trends in the deviations in Δ¹³C with pollution level were somewhat different between single-year and multiple-years. The high deviation in Δ¹³C at the high pollution site across the multiple years (Fig. 3) was not significant within a single year for *R. pulchrum*, as well as for *P. yedoensis* (data set 1, Table 3). The range of NO₂ within a single year in the present study, 5.3 to 20.7 ppb, may not be large enough to induce significant changes in long-term water use efficiency for *R. pulchrum* and *P. yedoensis*. For *G. biloba*, an opposite trend was obtained in the deviations in Δ¹³C, which suggests low long-term water use efficiency at the higher pollution sites. This result coincides with the lower stomatal limitation of *G. biloba* at the higher pollution sites (Table 3), which indicates that high pollution induces open stomata for *G. biloba*.
as particulate matters and O$_3$, may affect the photosynthetic response of urban trees in Kyoto City. Additionally, the present results were obtained using detached leaves; further study using attached leaves in the field will add more information on the photosynthetic responses of urban trees. R. pulchrum had a higher restriction in $A_{\text{max}}$ up to by 60% but had a higher water use efficiency at the heavy traffic areas in Kyoto City, which suggests that R. pulchrum has strategies to increase tolerance to air pollution and water limitation at the expense of the leaf photosynthesis rate. This study showed that the air pollution effects on urban trees act both positively and negatively, in which some tree species, such as G. biloba, may grow well even in areas where the effects of automobile exhaust gas are strong.

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Authors contribution MM, SY, LK, and YTH planned the experimental design. MM, SY, and LK performed the sample collection and physiological measurements. YTH and TK performed the data analysis. YTH, TK, and AK wrote the manuscript.

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Code availability Not applicable.

Declarations

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