Pollutant-induced cell death and reactive oxygen species accumulation in the aerial roots of Chinese banyan (*Ficus microcarpa*)

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Industrial pollutants induce the production of toxic reactive oxygen species (ROS) such as O$_2^{-}$, H$_2$O$_2$, and OH in plants, but they have not been well quantified or localized in tissues and cells. This study evaluated the pollutant- (HSO$_3^-$, NH$_4$NO$_3$, Al$^{3+}$, Zn$^{2+}$, and Fe$^{2+}$) induced toxic effects of ROS on the aerial roots of Chinese banyan (*Ficus microcarpa*). Root cell viability was greatly reduced by treatment with 20 mM NaHSO$_3$, 20 mM NH$_4$NO$_3$, 0.2 mM AlCl$_3$, 0.2 mM ZnSO$_4$, or 0.2 mM FeSO$_4$. Biochemical assay and histochemical localization showed that O$_2^{-}$ accumulated in roots in response to pollutants, except that the staining of O$_2^{-}$ under NaHSO$_3$ treatment was not detectable. Cytochemical localization further indicated that the generated O$_2^{-}$ was present mainly in the root cortex, and pith cells, especially in NH$_4$NO$_3$- and FeSO$_4$-treated roots. The pollutants also caused greatly accumulated H$_2$O$_2$ and OH in aerial roots, which finally resulted in lipid peroxidation as indicated by increased malondialdehyde contents. We conclude that the *F. microcarpa* aerial roots are sensitive to pollutant-induced ROS and that the histochemical localization of O$_2^{-}$ via nitrotetrazolium blue chloride staining is not effective for detecting the effects of HSO$_3^-$ treatment because of the treatment’s bleaching effect.

China is experiencing serious pollution problems caused by petrochemical smelting, mining, manufacturing, and other activities associated with rapid industrialization. In 2015, China emitted an estimated 18.59 million tons of sulfur dioxide (SO$_2$), and 18.51 million tons of nitrogen oxides (NO$_x$), and had critical levels of soil pollution by heavy metal. Industrial pollutants, such as SO$_2$, NO$_x$, NH$_3$, and metal ions, are thought to directly or indirectly threaten the health of plants; the symptoms include damaged chloroplast ultrastructure, reduced cell viability, and Fe are involved in plant metabolism. Some metals such as aluminium (Al) are redox-active and have metabolic function in plants. Aluminiun ion (Al$^{3+}$) or its hydrated form AlCl(H$_2$O)$_6$ in acidic tropical soil is toxic to plants causing damage to the cell wall, cytosol, and root cytoskeleton. Unlike Al, redox-active metals like Zn and Fe are involved in plant metabolism. High levels of Zn can compete with iron, leading to decreased metabolisms in plants. Although plants require Fe, high levels of Fe in soil may cause deficiencies of other nutrients, including P, K, Ca, Mg, and Zn.

Some of the damage caused by industrial pollution to trees results from the induction of oxidative processes that reduce peroxodic bonds and that consequently catalyse the production of reactive oxygen species (ROS), such as superoxide (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH). SO$_x$ phytotoxicity is mainly attributed to the production of intracellular O$_2^{-}$, and its detoxification is primarily dependent on the oxidative conversion of SO$_3^{2-}$ and HSO$_3^-$ into non-harmful sulfate (SO$_4^{2-}$). Oxides of nitrogen (NO and NO$_2$) also cause

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Paraformaldehyde. After 6 times wash with 0.1 M phosphate buffer, they were dehydrated by alcohol steeply and ducted by fixing aerial root samples in 0.1 M phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 2.5% camera (DP50, Olympus, Japan). The sections were observed and photographed with a light microscope (AX70, Olympus, Japan) and a digital camera (DSC-F717, Sony, Japan) before semi-thin transverse sections (8 μm thick) were prepared. Semi-thin section was conducted by fixing aerial root samples in 0.1 M phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 2.5% Paraformaldehyde. After 6 times wash with 0.1 M phosphate buffer, they were dehydrated by alcohol steeply and edded in flat molds using EPON812 resin. Sections (2 μm) were cut by ultramicrotome (Leica, UC6, Germany). The sections were observed and photographed with a light microscope (AX70, Olympus, Japan) and a digital camera (DP50, Olympus, Japan).

Histochemical and cytochemical localization of O$_2^-$.

O$_2^-$ was localized by staining with nitrotetrazolium blue chloride (NBT; N6876, Sigma)$^{38}$. The 3-cm-long subsamples (five per treatment) were immersed in HEPES-NaOH buffer (pH 7.6) containing 0.5 mg of NBT/ml and 10 mM NaN$_3$. The subsamples were vacuum infiltrated in this NBT solution for 30 min and were then held at room temperature until the blue colour (NBT-O$_2^-$) became visible. The NBT-stained roots were photographed with a digital camera (DSC-F717, Sony, Japan) before semi-thin transverse sections (8 μm thick) were prepared. Semi-thin section was conducted by fixing aerial root samples in 0.1 M phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 2.5% Paraformaldehyde. After 6 times wash with 0.1 M phosphate buffer, they were dehydrated by alcohol steeply and edded in flat molds using EPON812 resin. Sections (2 μm) were cut by ultramicrotome (Leica, UC6, Germany). The sections were observed and photographed with a light microscope (AX70, Olympus, Japan) and a digital camera (DP50, Olympus, Japan).

Histochemical localization of H$_2$O$_2$.

H$_2$O$_2$ was localized by staining with 3,3′,5,5′-Tetramethyl benzidine dihydrochloride hydrate (TMB, V900355, Sigma)$^{36}$. The 3-cm-long subsamples (five per treatment) were immersed in 10 mM sodium-citrate buffer (pH 4.0) containing 1 mM TMB at room temperature until the TMB-H$_2$O$_2$ formazan became visible. The stained roots were then photographed with a digital camera (DSC-F717, Sony, Japan).

·OH and H$_2$O$_2$ quantification.

·OH was quantified using terephthalic acid (TPA) as a hydroxyl radical dosimeter as described in previous studies$^{21,29}$. The 3-cm-long subsamples (five per treatment) were homogenized in phosphate buffer (50 mM, pH 7.0), and the supernatant was collected after centrifugation at 10000 g for 10 min at 4 °C. The 0.2-ml extracts were incubated in a 2-ml solution containing 0.2 ml of 50 μM TPA and 1.6 ml of phosphate buffer (50 mM, pH 7.0). After incubation for 10 min, the fluorescence emission spectra from 350 to 550 nm of monohydroxy terephthalate (TPA-·OH) was recorded with a fluorescence spectrophotometer (LS 55, Perkin-Elmer, USA) with an excitation wavelength of 326 nm.
H$_2$O$_2$ was detected using a fluorescence spectrophotometer (LS 55, Perkin-Elmer, USA) as previously described$^{18,31}$. The 3-cm-long subsamples (five per treatment) were homogenized in phosphate buffer (20 mM, pH 6.0). After the homogenate was centrifuged at 10000 $g$ for 10 min at 4 °C, 5 ml of the supernatant was collected. The 3-ml reaction mixture also included 0.2 ml of root extract, 5 $\mu$M scopoletin (S2500, Sigma), and 3 $\mu$g ml$^{-1}$ horseradish peroxidase. The fluorescence emission spectra were recorded from 400 to 550 nm with an excitation wavelength of 346 nm.

Detection of O$_2$\(^{-}\) accumulation. Root sample extracts were obtained after homogenization in phosphate buffer (20 mM, pH 6.0). The 0.2-ml extracts were then incubated for 5 h in the dark in 2 ml of phosphate buffer (20 mM, pH 6.0) containing 0.5 mM Na, 39- [1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT, X4626, Sigma). Formation of XTT-O$_2$\(^{-}\)-formazan was detected using a UV spectrophotometer (Lambda 650, Perkin-Elmer, USA) at 470 nm$^{18,31}$.

Malondialdehyde (MDA) quantification. Root samples were homogenized with 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid. The mixture was incubated at boiling water for 30 min and then quickly cooled in a refrigerator. After centrifugation at 1800 g for 10 min, the supernatant was used for MDA determination using a UV spectrophotometer (Lambda 650, Perkin-Elmer, USA)$^{32}$.

Data analysis. Results are shown as means ± standard deviations (SDs). One-way analyses of variance (ANOVA's) were used to determine the effects of treatment on Evans blue staining, XTT-O$_2$\(^{-}\) formation and MDA quantification. When effects were significant, means were compared with the Tukey's test. All statistical analyses were performed by SPSS 19.0 (SPSS, Inc., USA). Differences were considered significant at $P < 0.05$.

Results

Aerial root viability. The surface colour of roots incubated in NH$_4$NO$_3$, ZnSO$_4$, AlCl$_3$, or FeSO$_4$ became darker relative to the control, while the surface colour of roots incubated in NaHSO$_3$ became lighter (Fig. 1). As indicated by Evans blue staining, the pollutants reduced cell viability (Fig. 1). The blue staining mainly occurred in the root tips after treatment with NaHSO$_3$ but occurred throughout the 3-cm-long subsample following treatment with NH$_4$NO$_3$, ZnSO$_4$, AlCl$_3$, or FeSO$_4$. The absorbance of the blue extract (600 nm) confirmed that all of the pollutants significantly reduced the viability of the aerial roots ($P < 0.05$, Fig. 2A). Moreover, the viability was lower following NaHSO$_3$, ZnSO$_4$, and FeSO$_4$ treatment than following NH$_4$NO$_3$ or AlCl$_3$ treatment.

Histochemical and cytochemical localization of O$_2$\(^{-}\). When dyed with NBT, root segments treated with NH$_4$NO$_3$, ZnSO$_4$, AlCl$_3$, or FeSO$_4$ but not with NaHSO$_3$ became blue, indicating the presence of O$_2$\(^{-}\) (Fig. 1). The blue was most intense in roots treated with NH$_4$NO$_3$, ZnSO$_4$, AlCl$_3$, or FeSO$_4$. The absorbance of the blue extract (600 nm) confirmed that all of the pollutants significantly reduced the viability of the aerial roots ($P < 0.05$, Fig. 2A). Moreover, the viability was lower following NaHSO$_3$, ZnSO$_4$, and FeSO$_4$ treatment than following NH$_4$NO$_3$ or AlCl$_3$ treatment.
with the histochemical observations, the cytochemical localizations of O$_2^-$ indicated that accumulation of O$_2^-$ was greater following treatment with NH$_4$NO$_3$ and FeSO$_4$ than in the control. Although large quantities of ROS were generated in the root tips following treatment with the pollutants for 24 h, pollutant-induced damage to cell structure was not evident in the enlarged microscopic pictures (Fig. 3C,I,L,O,R).

**Histochemical localization and quantification of O$_2^-$ and OH.** As indicated by XTT-O$_2^-$-formazan absorbance at 470 nm, O$_2^-$ accumulation in aerial root cells was significantly higher in all of the pollutant treatments than in the control (P < 0.05, Fig. 2B). The significantly elevated O$_2^-$ accumulation induced by the pollutants is consistent with the NBT staining of root cross sections (Fig. 1).

To assess the accumulation of OH, fluorescence spectra were detected by adding TPA to the root extracts. The TPA-·OH fluorescent emission curves peaked at 463 nm, and the intensities were much higher for roots treated with pollutants than for control roots (Fig. 4A). The peak of the relative fluorescent values of TPA-OH was higher for FeSO$_4$ than for the other pollutants.
Figure 3. Cytochemical localization of \( \text{O}_2^- \) in aerial root cells (cross section behind root tips) of *F. microcarpa* treated with purified water (A–C), 20 mM NaHSO₃ (D–F), 20 mM NH₄NO₃ (G–I), 0.2 mM ZnSO₄ (J–L), 0.2 mM AlCl₃ (M–O), or 0.2 mM FeSO₄ (P–R). (C,F,I,L,O,R) are enlarged pictures of corresponding (B,E,H,K,N,Q) by the order of 100 times, respectively. Co: cortex; Ep: epidermis; Pi: pith; Pm: plasma membrane.
Quantification of H$_2$O$_2$. The accumulation of H$_2$O$_2$ was assessed using fluorescence spectra by adding sco- poletin to the root extracts. H$_2$O$_2$ accumulation (based on relative fluorescence intensity at 433 nm) in aerial root segments did not substantially differ between the control and the other treatments (Fig. 4B). Different from fluorescent assays, the histochemical staining of TMB-H$_2$O$_2$ showed that root segments treated with NH$_4$NO$_3$, ZnSO$_4$, AlCl$_3$, or FeSO$_4$ was obvious, indicating the presence of H$_2$O$_2$. By contrary, the staining of TMB-H$_2$O$_2$ on NaHSO$_3$ treated root samples was not detected (Fig. 1).

Quantification of MDA. The MDA contents of pollutant-treated aerial root samples were mostly higher than that of controls. The significantly increased MDA levels were detected in all pollutant treated root samples, indicating higher oxidative damage and lipid peroxidation (Fig. 2C).

Discussion

In this study, aerial roots of Chinese Banyan obviously suffered from treatment with pollutants as indicated by darker root surfaces (except in the case of NaHSO$_3$), dehydration symptoms (especially in the case of FeSO$_4$), and accumulation of ROS. Bisulfite (HSO$_3^-$) is the byproduct of SO$_2$ in cells, and the derivative is directly and indirectly toxic to plant tissues$^2$. SO$_2$ and its derivate HSO$_3^-$ harm leaves by generating excessive quantities of ROS, resulting in the bleaching of photosynthetic pigments$^{33,34}$. Our study found, for the first time to our knowledge, that aerial root systems were also harmed by bleaching caused by HSO$_3^-$, the cell death caused by NaHSO$_3$ was confirmed by Evans blue staining (Fig. 1). Similarly, Evans blue staining in this study indicated that the viability of aerial root cells was decreased by NH$_4$NO$_3$, ZnSO$_4$, AlCl$_3$, or FeSO$_4$ (Fig. 1). The decrease of aerial root cell viability was mainly caused by the decrease of cell pH, imbalance of mineral assimilation, as well as injuries in cell wall, plasma membrane, and signal transduction pathways$^{11–16}$.

Under biotic and abiotic stress, plant cells produce ROS in several subcellular compartments$^{35}$. As revealed by previous studies, redox-active metals (e.g., Fe$^{2+}$ and Zn$^{2+}$) as well as redox-inactive metals (e.g., Al$^{3+}$) may induce the activity of plasma membrane-localized NADPH oxidase, which transfers electrons from cytosolic NADPH to O$_2$, and subsequently forms O$_2^{-}$.$^{36,37}$ In our study, the deep-blue staining of NBT-O$_2^{-}$-formazan in the aerial roots that were treated with metal pollutants was documented by histochemical staining and by cytochemical observation of micrographs; our cytochemical observations were consistent with previous reports that NBT-O$_2^{-}$ is mainly found in cells.$^{18}$ Thus, we infer that the increased absorbance by XTT-O$_2^{-}$ at 470 nm and the formation of NBT-O$_2^{-}$ can be attributed to the activation of NADPH oxidase by metal ions in aerial roots. High concentrations of NaHSO$_3$ and NH$_4^+$ have been reported to damage cells because HSO$_3^-$ detoxification and NO$_3^-$ assimilation cause the generation of free radicals$^{12,20}$. In accordance with these studies, our results showed
that all pollutants caused massive accumulations of $\text{O}_2^−$ in cells, as indicated by biochemical assay (Fig. 2B) and by histochemical staining (Fig. 3). The exception was that only low levels of $\text{O}_2^−$ were detected in NaHSO3-treated root segments; even though XTT-$\text{O}_2^−$ absorbance was high, NBT-$\text{O}_2^−$-formazan was almost undetectable by histochemical observation (Fig. 3D–F). NaHSO3 was usually used as an additive bleaching agent. Therefore, we suspect that the bleaching caused by HSO3− may result in the failure of NBT staining and that NBT-$\text{O}_2^−$ staining is not suitable for $\text{O}_2^−$ detection in $\text{SO}_2^−$- or HSO3−-treated tissues.

In our study, $\text{H}_2\text{O}_2$ accumulation was not detected in pollutant-treated tissues by the fluorometric scopoletin oxidation assay (Fig. 4B). The histochemical staining, however, clearly indicated the production of $\text{H}_2\text{O}_2$ in pollutant-treated aerial root samples. Here, we infer that $\text{H}_2\text{O}_2$ detection by fluorescence intensity of $\text{H}_2\text{O}_2$-formazan may not always be effective, because the peroxide activity might be enhanced during the preparation of root extract, causing more consumption of $\text{H}_2\text{O}_2$ and reduced fluorescence intensity of $\text{H}_2\text{O}_2$-formazan. This result was also in agree with previous study that $\text{H}_2\text{O}_2$ is a versatile member of ROS network and that $\text{H}_2\text{O}_2$ increased in plant tissues under Al stress38–40.

$\text{OH}$ is among the most toxic of the ROS because of its capacity to initiate radical chain reactions that result in irreversible chemical modifications of various cellular components45. Because different pollutants ($\text{SO}_2$, $\text{NH}_4\text{NO}_3$, and metal ions) are all involved in the accumulation of ROS within plant cells2,20,29, the induced oxidative processes finally break the free radical chains of membrane lipids, causing membrane decomposition (increased MDA content, Fig. 2C) and cell death (decreased cell viability, Fig. 2A). In our study, TPA-$\cdot\text{OH}$ fluorescence was greatly increased by all five pollutants, indicating that these pollutants increased $\text{OH}$ accumulation in aerial root tissues (Fig. 4A). Because $\text{Fe}^{2+}$ is involved in the Fenton reaction, $\text{FeSO}_4$ treatment greatly increased $\cdot\text{OH}$ concentrations in aerial root tissues. $\text{NH}_4\text{NO}_3$ treatment also greatly increased $\text{OH}$ accumulation in aerial root tissue, which is consistent with previous findings that nitrate assimilation directly interferes with free radical metabolism and causes free radical-induced injury40.

Overall, the pollutant treatments in the current study caused ROS accumulation and profound oxidative damage, and finally cell death in aerial root tissues. Because $\text{O}_2^−$ is the initial ROS generated during $\text{O}_2$ metabolism in plant tissue, quantification of $\text{O}_2^−$ is vital for assessing ROS damage in plant tissues subjected to various stresses. In our study, we used both XTT and NBT to detect the accumulation of $\text{O}_2^−$. XTT is more sensitive than NBT, and XTT-$\text{O}_2^−$ can be quantitatively detected using spectrochemical methods. NBT staining may be suitable for the qualitative assessment of $\text{O}_2^−$ accumulation in plant tissues that have been subjected to most stresses but not to NaHSO3. The bleaching effect of HSO3− reduced the effectiveness of NBT staining in plant tissues. This study also showed that the aerial roots of $\text{Ficus microcarpa}$ are sensitive to various pollutants and that aerial roots may be good indicators of pollutants in industrially polluted regions.

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**Author Contributions**

N.L., C.C. and Z.S., analysed data and wrote the paper. Z.L. and N.L. designed the study, proposed the scientific hypothesis and supervised the project. N.L., C.C. and R.D. carried out the experiments. N.L. and C.C. collected and determined samples. N.L., Z.S., Z.L. and R.D contributed to the interpretation of the work. All authors discussed the results and reviewed the manuscript.

**Additional Information**

**Competing financial interests:** The authors declare no competing financial interests.

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