

Metal (Pb, Cd, and Cu)-induced reactive oxygen species accumulations in aerial root cells of the Chinese banyan (*Ficus microcarpa*)

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Abstract The current study evaluated the toxicity of three heavy metals to aerial roots of the Chinese banyan (*Ficus microcarpa*), which is a tree species native to China. In a laboratory experiment, segments of aerial roots cut from trees were treated with 0, 25, 50, 100, and 200 μM of lead, cadmium, or copper (Cu). The contents of these heavy metals in cells increased and root cell viability decreased with increases in treatment concentration. High levels of reactive oxygen species accumulated in the aerial root sections after heavy metal treatment. Both biochemical assay and histochemical localization showed that $\text{O}_2^{\bullet-}$, which is a precursor of H_2O_2 accumulated in root sections and that the amount accumulated was positively related to heavy metal concentration, especially for Cu-treated samples. Histochemical staining with diaminobenzidine (DAB) and a fluorometric scopoletin oxidation assay indicated that the amount of H_2O_2 accumulated was positively related to heavy metal concentration in the treatments; the scopoletin fluorescence assay was more sensitive and efficient than DAB staining for detection and quantification of H_2O_2 . The results indicate that aerial roots are sensitive to heavy metal-induced oxidative damage and that aerial roots have the potential to be used as indicators of heavy metal pollution in urban areas.

Keywords Heavy metal toxicology · Oxidative stress · Pollution indicator · Tree decline

Introduction

Heavy metals and other hazardous substances have been increasingly released into the atmosphere as a result of the mining, smelting, and manufacturing associated with rapid industrialization (Onder and Dursun 2006). Heavy metals in industrial dust and automobile exhaust are important contributors to air pollution in China (Ministry of Environmental Protection of People's Republic of China 2011), and deposition of atmospheric sulfur, nitrogen, and heavy metals has led to the decline of indigenous tree species in Pearl River Delta, South China (Sun et al. 2009; Guan and Wen 2011).

Heavy metals like lead (Pb), cadmium (Cd), and copper (Cu) are toxic to plants and other organisms (Danesino 2009). Pb toxicity in plants is manifested as damage to cell membranes, disturbance of mitosis, inhibition of DNA synthesis, and inactivation of enzymes (MacFarlane 2003; Liu et al. 1994; Van Assche and Cliisters 1990; Islam et al. 2008). Toxic levels of Pb alter root morphology and reduce seed germination, root growth, photosynthesis, and transpiration (Wierzbicka and Obidzinska 1998; Islam et al. 2008; Liu et al. 2010; Rossato et al. 2012). Cd can inhibit water and nutrient uptake by roots, destroy chloroplast functions, and inactivate enzymes, leading to reduced growth of roots and leaves, and to leaf chlorosis (Sanita di Toppi and Gabrielli 1999; Benavides et al. 2005; Ďurčková et al. 2007). Unlike Pb and Cd, Cu is an oligonutrient that is required for plant growth but Cu can be toxic if concentrations exceed $20\text{--}30 \mu\text{g g}^{-1}$ DW (Robson and Reuter 1981). High levels of Cu alter membrane permeability, chromatin structure, and the activities of enzymes involved in photosynthesis and respiration (Alaoui-Sossé et al. 2004; Srivastava et al. 2006; Monferrán et al. 2009).

Previous research has demonstrated that Pb, Cd, and Cu toxicity to plants involves oxidative damage. Cu is a redox

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metal, and the redox cycling between Cu^{2+} and Cu^+ catalyzes the production of reactive oxygen species (ROS) such as superoxide ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet\text{OH}$) through Fenton reactions (Aust et al. 1985; Kappus 1985). As non-redox metals, Pb and Cd cannot directly participate in biological redox reactions with oxygen. Under Pb and Cd stress, however, ROS levels in cells are elevated because these two metals may inhibit protective mechanisms including the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, peroxidase (POD), glutathione reductase, and glutathione peroxidase (Zhang and Kirkham 1996; Sandalio et al. 2001; Islam et al. 2008; Garnier et al. 2006). The responses of these antioxidant enzymes to metal stress differ depending on plant species, tissue type, and metal (Mazhoudi et al. 1997).

The current study concerns the effects of Pb, Cd, and Cu on the Chinese banyan (*Ficus microcarpa*), which is a common evergreen tree species in urban, subtropical China (Ren et al. 2010). *F. microcarpa* produces aerial roots that grow along the surface of branches and drop downward from the branches to the soil. In China, *F. microcarpa* growing in areas with heavy traffic and industry is suffering the effects of in situ atmospheric pollution, and the damaged symptoms include leaf chlorosis and reduced aerial root density. Because the aerial roots of *F. microcarpa* directly contact air pollutants, their growth restriction is thought to be closely related to pollutant toxicity (Kong et al. 2003). To our knowledge, however, little information is available concerning the effects of Pb^{2+} , Cd^{2+} , and Cu^{2+} on oxidative damage to the cells of aerial roots of any plant species. The specific objectives of the current research were to localize and quantify the effects of Pb, Cd, and Cu treatments on ROS in *F. microcarpa* aerial roots. An improved method for quantifying H_2O_2 in plant tissues is also described. Finally, the potential *F. microcarpa* aerial root to serve as ecological indicators of pollution is suggested.

Materials and methods

Plant material

Chinese banyan, *Ficus microcarpa* Linn. f. (Moraceae), is a native ornamental tree and is one of the most common street trees in South China (Ren et al. 2010). In April 2011, newly sprouted aerial roots were obtained from five mature (15–20 years old) trees growing in the South China Botanical Garden, Guangzhou, China. The aerial roots were removed from the tree by cutting the roots 5 cm from the root tip, i.e., each root segment used in this study was 5 cm long and had a root tip on one end. The root segments were mixed, rinsed with distilled water, and wiped dry. A

total of 30 root segments were used for each measurement described in the following sections.

Metal treatment

The aerial root segments were infiltrated under vacuum for 30 min with aqueous solutions (30 mL) of Pb (CH_3COO)₂ (PbAC_2 for short), CdCl_2 , or CuCl_2 at concentrations of either 0 (distilled water as control), 25, 50, 100, or 200 μM . Vacuum infiltration was used to reduce differences in the rates at which Pb^{2+} , Cd^{2+} , and Cu^{2+} penetrated the root segments. According to our previous studies and our preliminary experiments, 200 μM PbAC_2 , CdCl_2 , or CuCl_2 represented moderate to high concentrations of Pb, Cd, and Cu in air-polluted sites in the Pearl River Delta, South China (Liu et al. 2010; Sun et al. 2009). After 24 h of incubation in the light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25 °C, the root tips of segments treated with metals became brown while those of segments treated with distilled water were near-white. A razor blade was used to obtain sections of root tips (3 cm from the root tip). The sections were used to determine metal content, cell viability, and H_2O_2 and $\text{O}_2^{\bullet-}$ production and localization as described in the following sections.

Metal content

The aerial root sections were oven dried (0.1 g DW per section) and subjected to microwave (Anton Paar, Multiwave 3000) wet digestion in 10 mL of concentrated HNO_3 ; the microwave was programmed to gradually heat from 0 to 700 W for 10 min, 700 W for 10 min, 700–1,000 W for 5 min, and 1,000 W for 20 min, followed by cooling to room temperature. The concentrations of Pb, Cd, and Cu in the sample (final volume of 25 mL) were measured with an atomic absorption spectrophotometer (GBC932AA, GBC Ltd., Melbourne, Australia).

Cell viability indicated by Evans blue absorbance

Cell viability was measured by Evans blue staining (Liu et al. 2010). After metal treatment, root sections were immersed in 0.25 % aqueous Evans blue solution for 10 h. Then, the root sections were picked out and quickly washed by distilled water to remove external Evans blue solution from root surface. The dyed root sections were chopped into small pieces (<0.5 cm) and were solubilized with 25 mL of 1 % SDS solution for 24 h to completely extract blue color. The blue precipitate, which is present in dead cells but not in living cells, was quantified with a UV spectrophotometer (Lambda 650, Perkin-Elmer, CT, USA) at 600 nm. The level of absorbance at 600 nm was positively correlated with dead cell and considered a measure of survival cell viability in tested aerial root tissue.

Hydrogen peroxide (H₂O₂) accumulation

Five root sections per metal treatment were homogenized in phosphate buffer (20 mM, pH 6.0), the homogenates were centrifuged at 10,000×*g* and 4 °C for 10 min, and the supernatants (extracts) were collected. The extracts were incubated in 3 mL of phosphate buffer (20 mM, pH 6.0) containing 5 μM scopoletin and 3 μg/mL horseradish POD for 10 min. The fluorescence emission spectra from 350 to 550 nm were recorded using a fluorescence spectrophotometer (LS 55, Perkin-Elmer, USA) with an excitation wavelength of 346 nm (Schopfer et al. 2001). A decrease in fluorescence emission at 430 nm was used as an indicator of increased H₂O₂ accumulation.

O₂^{•-} accumulation

Extracts were obtained as described in the previous section. The extracts were then incubated in 1 mL of phosphate buffer (20 mM, pH 6.0) containing 500 μM of XTT (Na,39-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate) in darkness for 3 h according to Schopfer et al. (2001). In the presence of O₂^{•-}, XTT-formazan is formed. Changes in levels of XTT-formazan were detected by measuring absorption at 470 nm with a UV spectrophotometer (Lambda 650, Perkin-Elmer, CT, USA).

Histochemical localization of H₂O₂ and O₂^{•-}

H₂O₂ and O₂^{•-} were localized as previously described (Liu et al. 2010). After metal treatment, root sections were infiltrated with 0.5 mg/mL diaminobenzidine (DAB)-phosphate buffer (50 mM, pH 5.8) for histochemical detection of H₂O₂ or with 0.5 mg/mL nitroblue tetrazolium (NBT) with 10 mM NaN₃ in 50 mM HEPES–NaOH buffer (pH 7.6) for O₂^{•-} histochemical localization. Infiltration was conducted under vacuum for 30 min. The root sections were then held at room temperature until a blue (NBT–O₂^{•-}) or brown (DAB–H₂O₂) color became visible. The stained root sections were photographed with a digital camera (DSC-F717, Sony, Japan). The NBT-stained roots were then subjected to paraffin sectioning (longitudinal or cross sections). The sections were photographed with an imaging microscope (Axioplan Z, Zeiss, Germany).

Data analysis

Results were shown as mean ± SE. One-way analysis of variance was used to compare metal content, cell viability, and O₂^{•-} accumulation in root segments treated by the three metals at the same concentrations. Tukey's test was used for post hoc multiple comparisons. All statistical

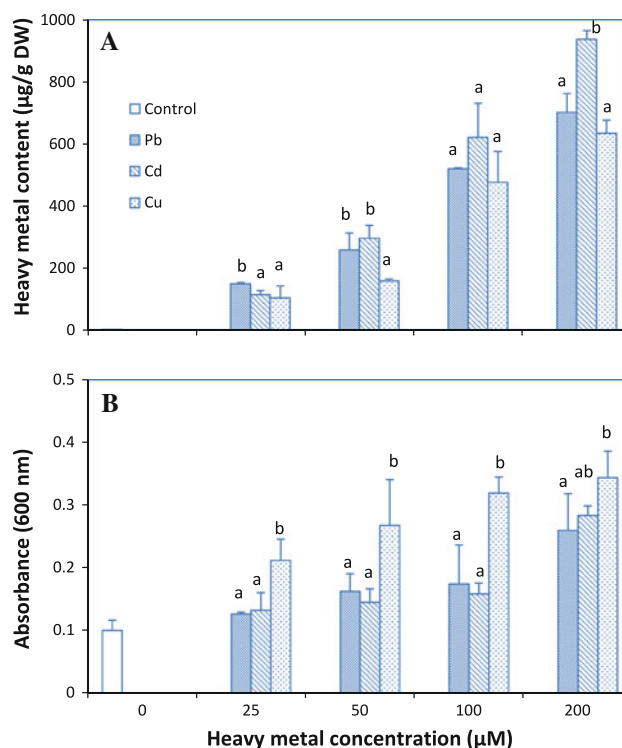


Fig. 1 Metal content (A) and cell viability indicated by Evans blue absorbance (B) in aerial roots of *F. microcarpa* treated with purified water (0) or PbAC₂, CdCl₂, or CuCl₂ at 25, 50, 100, and 200 μM. Data are shown as mean ± SE; *n* = 6. Values of each bar not sharing the same letters are significantly different at *P* < 0.05

analyses were carried out by SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Differences were considered significant at the 0.05 level.

Results

Metal content and cell viability

Atomic absorption analyses showed that contents of Pb, Cd, or Cu in the aerial roots segments increased as the treatment concentrations increased (Fig. 1a). The correlation coefficients between the treatment concentration and content in root tissues were 0.9391 for Pb, 0.9696 for Cd, and 0.9332 for Cu. The content in aerial root tissues was greater for Cd than for Pb (*P* < 0.05) in the 200-μM treatment and was greater for Cd than for Cu (*P* < 0.05) in the 50- and 200-μM treatments.

Based on the enhancement of Evans blue staining, cell viability decreased with treatment concentrations (Fig. 1b). Cell survival viability were similar for Pb and Cd treatments but were usually lower (*P* < 0.05) for the Cu treatment than for Pb or Cd treatments.

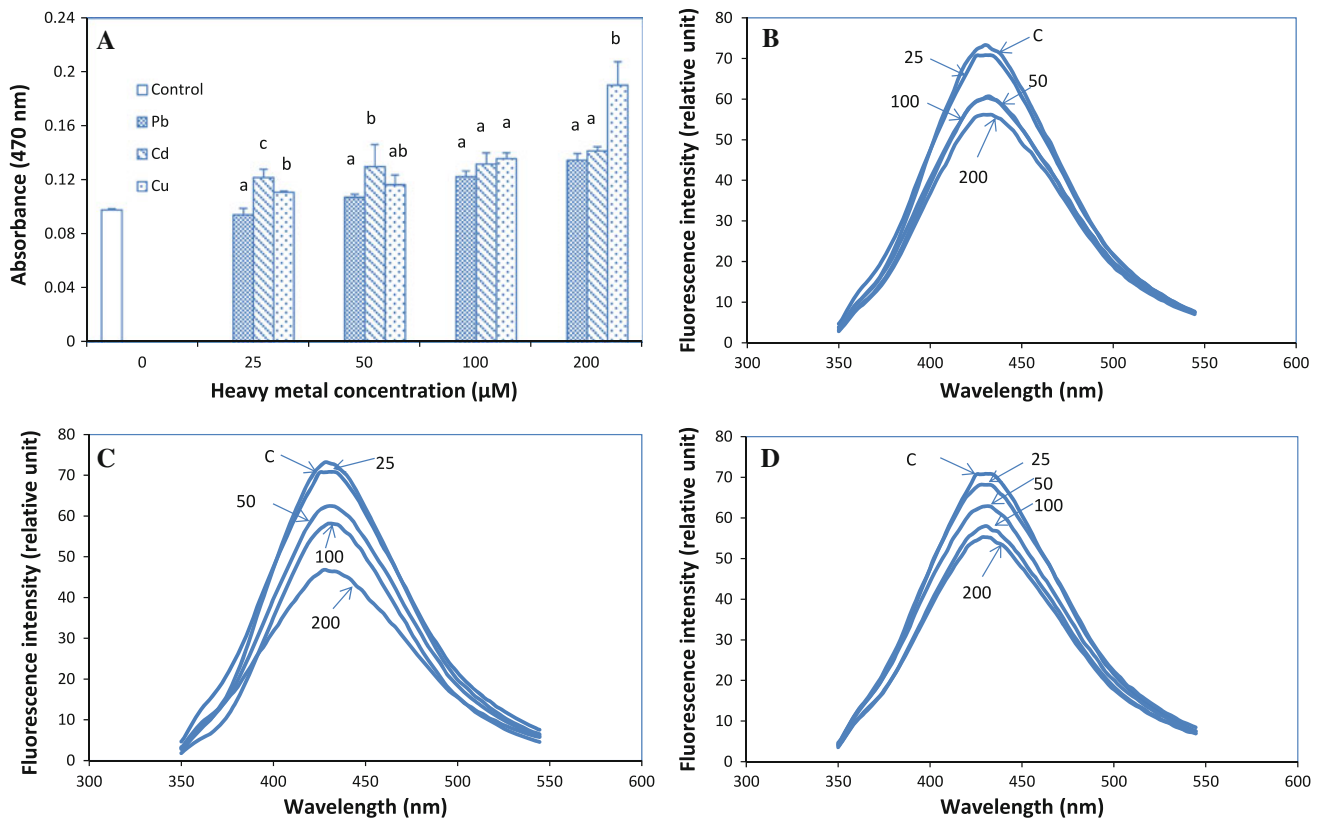


Fig. 2 $O_2^{\bullet-}$ and H_2O_2 accumulation in aerial root tissues of *F. microcarpa* treated with Pb, Cd, or Cu at 0, 25, 50, 100, or 200 μM . $O_2^{\bullet-}$ accumulation (A) as indicated by absorbance at 470 nm. H_2O_2 accumulation for Pb-treated roots (B) Cd-treated roots (C) and Cu-treated roots (D) as indicated by fluorescence intensity at

430 nm. Data are shown as mean \pm SE; $n = 6$. Values of each bar not sharing the same letters are significantly different at $P < 0.05$. Each curve is average of 6 replicates and associated numbers are concentrations in μM . C control

$O_2^{\bullet-}$ accumulation in aerial roots

$O_2^{\bullet-}$ content in root cells usually increased with an increase in treatment concentration (Fig. 2a). $O_2^{\bullet-}$ content tended to be greater with Cd than with Pb or Cu when the metals were applied at low concentrations (25 and 50 μM). When the metals were applied at 200 μM , $O_2^{\bullet-}$ content was greater ($P < 0.05$) with Cu than with Cd or Pb. Relative to the control, treatment with 200 μM Pb, Cd, or Cu resulted in an increase in $O_2^{\bullet-}$ content of 35, 41, or 91 %, respectively.

H_2O_2 accumulation in aerial roots

A fluorometric scopoletin oxidation assay was used to assess the H_2O_2 content of roots treated with Pb (Fig. 2b), Cd (Fig. 2c), or Cu (Fig. 2d). For all three metals, the decreases in fluorescence at 430 nm indicated that H_2O_2 content increased as the concentration of the metals in the treatment increased. Relative to the control, the increases in H_2O_2 accumulation (based on the drop in fluorescence intensity at 430 nm) induced by the 200- μM metal

treatments were 26.2 % for Pb, 52.2 % for Cd, and 22.2 % for Cu.

Histochemical localization of H_2O_2 and $O_2^{\bullet-}$

Sections from control and metal (200 μM)-treated root segments were dyed with DAB for H_2O_2 localization. Whereas the control roots were relatively light in color, the roots treated with metals were dark brown (Fig. 3). The intensity of the brown did not greatly differ among metal treatments or with distance from the root tip.

Control and metal (200 μM)-treated root segments were also dyed with NBT for $O_2^{\bullet-}$ localization. Compared to controls, Pb, Cd, and Cu-treated roots were dyed blue, indicating the presence of $O_2^{\bullet-}$ (Fig. 3, top). The blue color was especially evident in the first 2–3 cm behind the root tips. The blue color intensity was greater with Cu-treated root segments than with Pb- and Cd-treated root segments. Dyed sections provided additional information on $O_2^{\bullet-}$ localization. In contrast to the untreated root tips (Fig. 4a), the metal-treated root tips had large numbers of blue spots (indicating the presence of formazan and

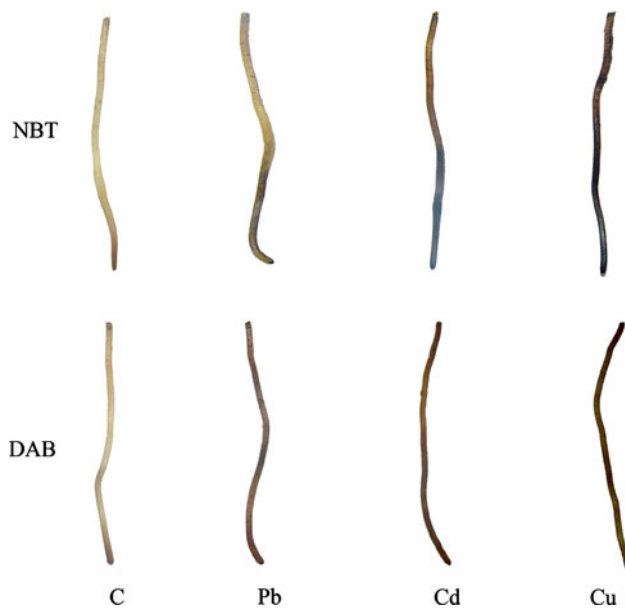


Fig. 3 Histochemical localization of H_2O_2 (bottom) and $\text{O}_2^{\bullet-}$ (top) in aerial roots of *F. microcarpa* treated with purified water (C) or PbAC_2 , CdCl_2 , or CuCl_2 at 200 μM . H_2O_2 accumulation is indicated by the formation of a red-brown color resulting from polymerization with DAB. $\text{O}_2^{\bullet-}$ accumulation is indicated by the formation of the blue compound. The bottom part of the root section is the root tip (Color figure online)

$\text{O}_2^{\bullet-}$ accumulation) in the root meristem, cortex, and pith cells (Fig. 4c, e, g, i). Observation of cross sections also indicated blue precipitation especially in the plasma membranes (Fig. 3d, f, h, j). Once again, these micrographs showed that Cu treatment resulted in the accumulation of large quantities of $\text{O}_2^{\bullet-}$ in root cells, and that $\text{O}_2^{\bullet-}$ accumulation was greater with Cu treatment than with Cd or Pb treatments. The micrographs also indicated that the 200- μM metal treatments did not damage root cell structure.

Discussion

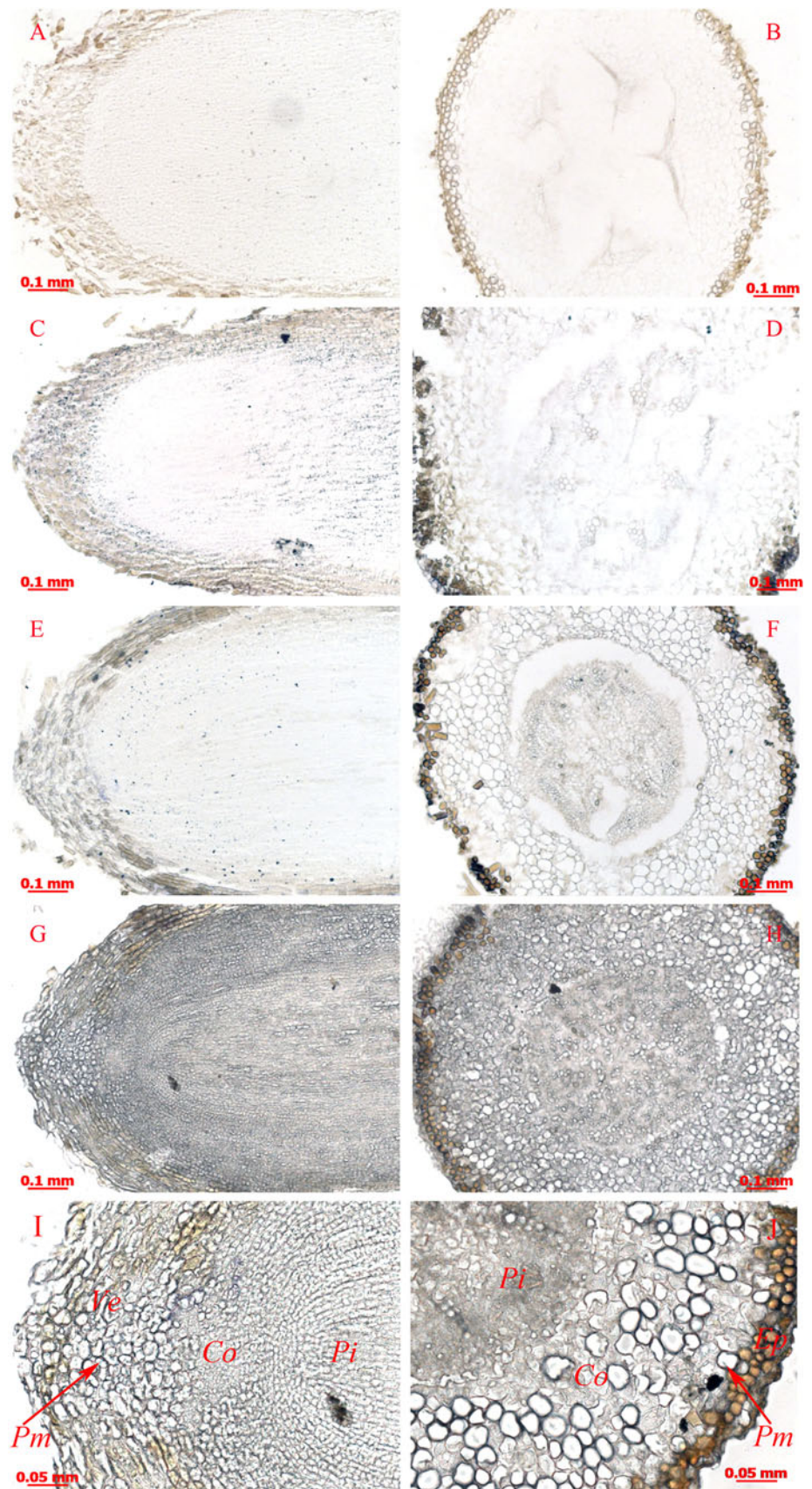
Most heavy metals lack metabolic function and are toxic to organisms (Weast 1984). Excessive uptake of heavy metals can interfere with cellular metabolism by increasing the production of ROS via auto-oxidation and the Fenton reaction or Haber–Weiss reaction, inhibiting essential biomolecular functional groups, and replacing important metal ions (e.g., Ca^{2+} , Mg^{2+}) in biomolecules (Prasad 1998; Schützendübel and Polle 2002). In the present study, high levels of Pb, Cd, or Cu (100–1,000 $\mu\text{g}/\text{g}$ DW) were detected in aerial roots of the Chinese banyan tree, *F. microcarpa*, after various metal treatments. Previous studies reported that the hyper-accumulation of metals (>50 $\mu\text{g}/\text{g}$ DW) caused profound oxidative damage, growth inhibition, and finally cell death (Khatun et al. 2008;

Madejón et al. 2009; Liu et al. 2010). As indicated by Evans blue staining in this study, the cell viability of *F. microcarpa* aerial roots decreased with increases in metal concentrations, and cell viability was lower with Cu than with Pb or Cd. Our previous study showed that Cd was much more damaging than Pb to leaf cells of *Alocasia macrorrhiza* (Liu et al. 2010), whereas the current study indicated that Cd and Pb caused equivalent levels of cell death in aerial roots of *F. microcarpa*, it is likely depending on plant species.

High concentrations of heavy metals generate oxidative stress as a result of increased ROS levels (Khatun et al. 2008; Liu et al. 2010). The best characterized ROS-generating enzyme is NADPH oxidase, which is localized on the plasma membrane (Foreman et al. 2003). This protein transfers electrons from cytosolic NADPH to O_2 to form $\text{O}_2^{\bullet-}$ and subsequently H_2O_2 under various stresses (Olmos et al. 2003; Lin et al. 2009). In the current study, $\text{O}_2^{\bullet-}$ accumulations (as indicated by the production of XTT-formazan) increased in metal-treated aerial roots based on measurement of extract absorbance at 470 nm (A_{470}). $\text{O}_2^{\bullet-}$ accumulation was generally greater in Cd- and Cu-treated samples than in Pb-treated samples. Cd has a strong affinity for membrane surfaces, and as noted earlier, NADPH oxidases that generate $\text{O}_2^{\bullet-}$ are also localized to the plasma membrane (Sagi and Fluhr 2006). Our histochemical observations of $\text{O}_2^{\bullet-}$ were consistent with these previous findings in that the $\text{O}_2^{\bullet-}$ –NBT was mainly found on the plasma membrane of aerial root cells. In addition, the increase in $\text{O}_2^{\bullet-}$ accumulation quantified spectrophotometrically that was caused by treatment with 200 μM Cu was confirmed by the deep blue staining of plasma membranes evident in micrographs of stained root sections. Consistent with our previous findings (Lin et al. 2009; Liu et al. 2010), the formation of $\text{O}_2^{\bullet-}$ –NBT and the increased absorbance at 470 nm can therefore be attributed to the activation of NADPH oxidase by Cd^{2+} , Pb^{2+} , and Cu^{2+} .

In the current study, the enhancement of H_2O_2 accumulation in metal-treated aerial roots was clearly revealed by DAB staining. Moreover, fluorometric scopoletin oxidation assay of H_2O_2 was clearly more sensitive and precise compared to histochemical localization method when analyzing the oxidative stress of root sections. These increases in H_2O_2 accumulation induced by metal treatment were accompanied by increases in $\text{O}_2^{\bullet-}$ accumulation. The concomitant increases in these two ROS is reasonable because $\text{O}_2^{\bullet-}$ produced by NADPH oxidase on the plasma membrane can be converted to H_2O_2 through non-enzymatic reaction or by superoxide SOD (Passardi et al. 2004). SOD is an induced enzyme in cells. Hence, we speculate that one reason of the elevation of H_2O_2 in Pb-, Cd-, and Cu-treated root tissue might be due to an induction of high SOD activity in response to metal stress. In a

Fig. 4 Cytochemical localization of $O_2^{\bullet-}$ in cells of aerial roots of *F. microcarpa* treated with purified water (a, b), 200 μ M $PbAC_2$ (c, d), 200 μ M $CdCl_2$ (e, f), and 200 μ M $CuCl_2$ (g–j). a, c, e, g, i: Longitudinal sections through root tips; b, d, f, h, j: Cross sections behind root tips. a–h Magnified for $\times 50$ and i, j are magnified for $\times 100$. *Pi* pith, *Co* cortex, *Ep* epidermis, *Pm* plasma membrane, *Ve* velamen



previous study, however, SOD activity was inhibited at higher Cu concentrations, and it failed to scavenge $O_2^{\bullet-}$ and to protect against cellular oxidative damage (Khatun et al. 2008). Our finding that treatment with a high concentration of Cu (200 μ M) caused a high level of $O_2^{\bullet-}$ and a lower level of H_2O_2 may reflect the inactivation of SOD. This is in accordance with the finding of Khatun et al. (2008). Moreover, $O_2^{\bullet-}$ and H_2O_2 act directly on most cell components and are also as the precursors of more cytotoxic or highly reactive oxygen derivatives, such as peroxynitrite or the $\bullet OH$, which is the most reactive and toxic ROS known (Yang and Guo 2001; Halliwell 2006). So the lower viability of cells in Cu-treated roots than in Pb- and Cd-treated roots in this study was mainly related to the high level of $O_2^{\bullet-}$ accumulation, which might result from the redox characteristics of Cu.

The current study used aerial root segments that were treated with metals via vacuum infiltration in the laboratory. Future research should determine whether data collected with this artificial system are consistent with data obtained from intact aerial roots in the field.

Conclusions

This work provides a starting point for understanding how aerial roots respond to heavy metal stress. This study has shown that: (1) heavy metals pollution induces oxidative stress and cell death in Chinese banyan aerial roots. With respect to oxidative damage, $O_2^{\bullet-}$ is the initial and important ROS in the cells of the root meristem, cortex, and even in the pith; (2) the scopoletin fluorescence-dependent assay for H_2O_2 is more sensitive and efficient than DAB staining, especially for tissues with background color; and (3) as a redox metal, Cu is more toxic to aerial roots than Pb or Cd. Moreover, the current study suggests that cell viability as well as ROS accumulation appear to be good indicators of heavy metal damages of Chinese banyan tree.

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