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Roles of multiwall carbon nanotubes in phytoremediation: cadmium uptake and oxidative burst in *Boehmeria nivea* (L.) Gaudich†

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Multiwall carbon nanotubes (MWCNTs) have been widely used recently and their interaction with heavy metals or plants will influence the phytoremediation of heavy metal contaminated sites. This study aimed to investigate the effects of MWCNTs on cadmium (Cd) accumulation and Cd-induced oxidative damage in plants. *Boehmeria nivea* (L.) Gaudich (ramie) seedlings were cultivated in Cd-contaminated river sediments with exposure to 100, 500, 1000 and 5000 mg kg^{-1} MWCNTs. Results showed that MWCNTs at 500 mg kg^{-1} promoted the accumulation and translocation of Cd in ramie seedlings and alleviated Cd-induced toxicity by stimulating plant growth, reducing oxidative stress, activating antioxidant enzyme activities and increasing specific antioxidant content. In contrast, plant growth inhibition, Cd accumulation reduction and oxidative damage aggravation were observed in ramie seedlings exposed to 5000 mg kg^{-1} MWCNTs. These findings demonstrated that the application of MWCNTs at suitable levels could improve the phytoremediation efficiency in the restoration of heavy metal contaminated river sediments, and the inevitable release of MWCNTs at high levels would exacerbate metal-induced toxicity to plants. This study provides a novel method to facilitate the phytoremediation of heavy metal contaminated sediments using nanomaterials and explores the potential risks of nanomaterials to the biological system.

Environmental significance

With the progressive production and inevitable release of multiwall carbon nanotubes (MWCNTs) in the environment, the interactions among MWCNTs, plants and heavy metal contaminants will affect the process of phytoremediation. It is imperative to understand the effects of MWCNTs on metal accumulation and metal-induced toxicity to plants. This study demonstrated that a certain level of MWCNTs could facilitate the phytoremediation of metal-contaminated sites, and this finding provides a potential novel application of MWCNTs for environmental remediation. Aggravated metal-induced toxicity to plants was also observed due to the negative effects of MWCNTs. This study will provide new insights into the novel application of MWCNTs and the potential risks of MWCNT release in large amounts.

Introduction

Heavy metals in waters can precipitate or adsorb on sediment particles.[1,2] Thus, river sediments serve as major sinks for heavy metals in the water system.[3,4] Recently, the pollution of heavy metals in river sediments has become increasingly serious due to the fast-growing sewage discharge from industry, agriculture and human daily life.[5] Once the water environment changes, the immobilized heavy metals in river sediments may release back to the water and cause serious water pollution.[6,7] Cadmium (Cd) is a common contaminant in river sediments, and it can be adsorbed by aquatic organisms and bioaccumulated through the food chain, therefore leading to potential threats to the natural ecosystem and human health.[8–10] Thus, there is a crucial need to remediate Cd-contaminated river sediments. Phytoremediation is an environment-friendly and inexpensive *in situ* remediation technique, which can directly remove heavy metals from the polluted sites. This technique has been used to treat polluted sediments,[11,12] and it could be a potentially efficient method for the remediation of Cd-contaminated sediments.

Nanomaterials (NMs) are defined as materials with at least one dimension of 100 nm or less. Due to their unique size

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and extraordinary properties, NMs have potential applications in electronics, manufacturing, medicine, energy, *etc.*[13,14] The widely used NMs will inevitably discharge into waters and precipitate in river sediments. Since the sediment system is extraordinarily complicated, the present NMs may interact with heavy metals, thus influencing the fate and toxicity of those heavy metals.[15–17] In addition, NMs occupy a major position in shaping modern agriculture with controlled delivery or release of nutrients, fertilizers and pesticides for plant growth and disease prevention.[18,19] Interactions between NMs and plants are likely to influence the uptake of heavy metals in plants and plants' responses to metal-induced toxicity.[20–22] Investigations focused on the effects of NMs on the phytoremediation of heavy metal contaminated sediments are scarce.

Carbon nanotubes (CNTs) are cylindrical molecules composed of carbon atoms. Recent developments increased the potential applications of CNTs in the field of environmental remediation.[23–25] For example, Zhang *et al.* used CNTs to treat sediments contaminated with organochlorine pesticides, and they found that CNTs could prevent dichlorodiphenyltrichloroethane and hexachlorocyclohexane from being released from sediments.[26] Matos *et al.* observed the immobilization of copper, zinc and lead in contaminated soils by CNTs.[27] The interactions between CNTs and contaminants will influence the bioavailability of contaminants to organisms. Xia *et al.* reported the decreased bioaccumulation of perfluorochemicals by *Chironomus plumosus* larvae with treatments with multiwalled carbon nanotubes (MWCNTs).[28] Conversely, Oloumi *et al.* found that MWCNTs at 10 mg L^{-1} increased the accumulation of Cd and Pb in *Cannabis sativa* L.[29] In addition, previous studies have confirmed the differential roles of CNTs in plants' responses to environmental stress. Some studies reported the alleviation effects of CNTs on contaminant toxicity to the plants. For instance, Fan *et al.* demonstrated that MWCNTs protected *Arabidopsis thaliana* against paraquat toxicity by stimulating the photosynthesis, antioxidant effect and nutrient assimilation.[30] However, a number of other reporters have observed the escalation of contaminant toxicity to plants by CNTs. For example, the aggravated toxicity of the herbicide diuron induced by CNTs was observed in *Chlorella vulgaris*.[31] The potential effects of CNTs on plants under environmental stress could be complex. CNTs induced metal accumulation enhancement and metal toxicity alleviation will be beneficial to the phytoremediation of metal-contaminated sites. However, it is unclear whether CNTs could promote the accumulation of heavy metals in plants, and how CNTs can influence metal-induced toxicity to plants.

In this study, phytoremediation was used to remediate Cd-contaminated river sediments. MWCNTs, a typical CNT material, were used to investigate the effects of CNTs on the phytoremediation of Cd-contaminated sediments. *Boehmeria nivea* (L.) Gaud. (ramie) was chosen as the remediation plant due to its efficient Cd enrichment ability and large biomass.[32] Plant dry biomass, Cd content and the subcellular distribution of Cd in different ramie tissues were determined. Oxidative stress, a well-known metal induced toxic effect, was analyzed. The potential effects of MWCNTs on the modulation of the activities of antioxidant enzymes (*i.e.*, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)) and the content of the specific antioxidant (glutathione) in ramie seedlings under Cd stress were also investigated. The main objective of this study was to explore the roles of MWCNTs in the metal accumulation and metal-induced oxidative burst in plants. This study will provide new insights into the interactions between MWCNTs, plants, and metal contaminants, and provide a reference to the potential noval application of NMs for environmental remediation. In addition, this work also conducted nano–bio interaction investigations and could provide a reference to the risks of NM application.

Materials and methods

Sediment preparation

Cd-contaminated sediments were obtained from the Xiangjiang River in Changsha, China. The sampled sediments were air-dried, ground and passed through 10 mesh sieves (<2 mm). The basic characteristics, such as pH, cation exchange capacity and metal contents, were determined according to a previous study.[33]

MWCNTs were purchased from Chengdu Organic Chemical Co., LTD., Chinese Academy of Sciences. A chemical vapor deposition method was used to synthesis the MWCNTs. The morphology of MWCNTs was examined using a scanning electron microscope (SEM). X-ray photoelectron spectroscopy (XPS) was carried out to determine the chemical structure of MWCNTs. A Malvern Zetasizer instrument was used to determine the hydrodynamic diameter and zeta potential of MWCNTs. In this study, different amounts of MWCNTs were added into the Cd-contaminated sediments to obtain 100, 500, 1000 and 5000 mg kg^{-1} MWCNT- amended Cd-contaminated sediments. The selected concentrations of MWCNTs were based on many previous studies and our pre-experiments.[34,35]

Plant cultivation

Ramie seedlings were obtained from the Ramie Institute of Hunan Agriculture University. The ramie seedlings were transplanted into Cd-contaminated sediments amended with different concentrations of MWCNTs and cultivated in a growth chamber with a 14 h photoperiod (6:00 a.m. to 8:00 p.m.), 24 ± 5 °C/18 ± 3 °C day/night temperature, and 65 ± 5% relative humidity. Seedlings grown in only Cd-contaminated sediments were used as controls. After two months of cultivation, the ramie seedlings were harvested. Plant leaves, stems and roots were collected separately and dried in an oven at 60 °C to achieve constant weight to determine the dry biomass.

Cd determination

Cd content in different plant tissues was determined using graphite furnace atomic absorption spectrometry (GFAAS).

Specifically, ramie leaves, stems and roots were collected and washed with ultrapure water thoroughly. To get rid of the adhered ions, ramie roots were soaked in Na_2EDTA (10 mmol L^{-1}) for 10 min and then rinsed with ultrapure water. The washed plant tissues were dried till constant weight and digested in a graphite digestion apparatus using HNO_3–$HClO_4$ (3 : 1 v/v). The Cd concentration in ramie leaves, stems and roots was determined using an atomic adsorption spectrometer (AAS, PEAA700, PerkinElmer). The accumulation of Cd (A-Cd) in each plant and the translocation factor (TF) of Cd in ramie seedlings were calculated as follows:

$$\text{A-Cd} = Cd_L \times B_L + Cd_S \times B_S + Cd_R \times B_R \quad (1)$$

$$TF = \frac{Cd_L \times B_L + Cd_S \times B_S}{(B_L + B_S) \times Cd_R} \quad (2)$$

where Cd_L, Cd_S, and Cd_R referred to the concentration of Cd in ramie leaves, stems and roots, respectively. B_L, B_S, and B_R represented the dry biomass of leaves, stems and roots, respectively.

The subcellular distribution of Cd in ramie seedlings was determined by a differential centrifugation method.[36,37] Fresh ramie leaves, stems and roots were collected separately and homogenized with 50 mmol L^{-1} Tris-HCl (pH 7.5) which contained 250 mmol L^{-1} sucrose and 1 mmol L^{-1} dithiothreitol. The obtained homogenate was firstly centrifuged at 3000 r min^{-1} for 15 min. This centrifuged deposit represented the cell wall portion. The supernatant was further centrifuged at 15 000 r min^{-1} for 30 min. The deposit and supernatant obtained at this time referred to the organelle and soluble fractions, respectively. To extract Cd, the cell wall and organelle fractions were further digested with HNO_3–$HClO_4$ (3 : 1 v/v). The digested solutions and cell soluble fraction were used to determine the Cd content in different cell fractions using an atomic adsorption spectrometer (AAS, PEAA700, PerkinElmer).

Oxidative stress analysis

The oxidative stress in ramie seedlings was estimated by measuring the contents of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA).

The content of H_2O_2 was determined by the potassium iodide (KI) method described by Velikova *et al.*[38] Specifically, plant tissues were ground with 0.1% trichloroacetic acid (TCA) and then centrifuged at 12 000 r min^{-1} for 20 min. Phosphate buffer (100 mmol L^{-1}) and KI (1 mol L^{-1}) were added to the supernatant. The supernatant mixture was used to determine the H_2O_2 content using an ultraviolet spectrophotometer (UV-2550, Shimadzu, Japan) at the absorbance of 390 nm. The extinction coefficient of 0.28 μM^{-1} cm^{-1} was used to calculate the H_2O_2 content, and the concentration unit of H_2O_2 was nmol g^{-1} FW (plant fresh weight).

MDA was measured according to the thiobarbituric acid (TBA) method.[39] To be specific, MDA was extracted from ramie seedlings using 10% TCA. TBA (0.5%) was added to the MDA extracting solution. The mixed solution was heated at 95 °C for 35 min and cooled rapidly for MDA determination. The content of MDA was determined using an ultraviolet spectrophotometer (UV-2550, Shimadzu, Japan), and the concentration unit of MDA was nmol g^{-1} FW.

Antioxidative enzyme activities and antioxidant content assays

The activities of SOD, POD and CAT were determined according to a previous study.[13] The activity of SOD was measured by the xanthine oxidase method, and one unit of SOD was defined as the amount of SOD that inhibited the oxidation of hydroxylamine by 50%. The activity of POD was measured by guaiacol colorimetry. One unit of POD was defined as the amount of enzyme that catalyzed 1 μg guaiacol per min. The activity of CAT was estimated by molybdate colorimetry. One unit of CAT was defined as the amount of enzyme which broke down 1 μmol H_2O_2 per second.

The concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined using a commercial reagent kit purchased from Nanjing Jiancheng Bioengineering Institute, China. The measuring method was based on the dithionitrobenzene cyclic reaction method, and the specific operating approach was described by a previous study.[13] The concentration unit of GSH or GSSG was nmol g^{-1} FW.

Statistical analysis

All the experimental results are presented as means ± standard errors of four replicates. The significant difference among all the treatments was analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Science (SPSS). Statistical significance was based on a probability of $p < 0.05$ according to the Duncan test.

Results and discussion

Characterization of MWCNTs and sediments

Table S1† shows the basic characteristics of MWCNTs. The diameter was 10–30 nm and the length was 10–30 μm. MWCNTs were negatively charged (Fig. S1†) with an average hydrodynamic diameter of 8.5 μm at pH 7. The SEM images (Fig. S2†) show the fiber-like structure of MWCNTs. The XPS spectrum (Fig. S3†) indicated the existence of carbon and oxygen on the MWCNTs. As shown in Fig. S4,† the high-resolution C1s spectrum could be fitted into four peaks. The peaks observed at 286.5 eV and 289.12 eV were associated with C–O and C=O bonds, whereas the peaks at 285.4 eV and 284.84 eV were attributed to C–C and C=C bonds.[40–42] The O1s spectrum could be deconvoluted into two peaks with binding energies of 533 eV and 531.5 eV, which were attributed to C–O and O–C=O bonds.[40,43]

The basic characteristics of the sediments are presented in Table 1. As shown in this table, the pH of the sediments was 6.76 ± 0.09, and the cation exchange capacity was 11.65 ± 1.23 cmol kg^{-1}. Cu, Pb, Cr and Cd were detected in the sediments with average concentrations of 49.18 ± 2.87, 194.91 ± 6.28, 158.12 ± 7.87, and 22.44 ± 1.26 mg kg^{-1}, respectively. The BCR sequential extraction result showed that the acid-soluble, reducible, oxidizable, and residual fractions of Cd in the sediments were 43.89 ± 3.28%, 22.44 ± 1.51%, 3.96 ± 0.28% and 29.71 ± 2.08%, respectively. According to the sediment quality guidelines by the EPA,[44] the Cd content in the sampled sediments massively exceeded the standard and it reached almost 23 times the sediment threshold effect level (0.99 mg kg^{-1}), while the contents of Cu, Pb and Cr in the sampled sediments were within or did not exceed much the normal limits. The results indicated that Cd was the most prevalent contaminant in the sampled sediments. The acid-soluble fraction of metal refers to metals in ionic form or bonded to carbonates, which is the most unstable metal fraction and can be easily absorbed by organisms. As shown in Table 1, Cd was mainly bound to the acid-soluble fractions, indicating that Cd in the sampled sediments was likely to be taken up by plants. In addition, the almost neutral sediment environment and suitable level of CEC in the sediments indicated that phytoremediation might be a potentially suitable method for remediating these Cd-contaminated sediments.

Seedling growth

To investigate the effects of MWCNTs on plant growth under Cd stress, the dry biomass of ramie leaves, stems and roots was determined. As shown in Fig. 1, MWCNTs at different concentrations had various effects on plant growth under Cd stress. Plant dry biomass in the leaves and stems of ramie under Cd stress decreased at the 100 mg kg^{-1} MWCNT treatments, but a slight increase was observed in the roots at these treatments. Relative to the control, MWCNTs at 500 mg kg^{-1} increased the dry biomass in ramie leaves, stems and roots by almost 20%, 33%, and 31%, respectively, while 1000 mg kg^{-1} MWCNTs increased the dry biomass in leaves by 28%, in stems by 21%, and in roots by 28%. However, compared with the control, there was a slight but not significant decrease in the dry biomass of ramie seedlings exposed to 5000 mg kg^{-1} MWCNTs ($p < 0.05$).

Growth inhibition is one of the most distinct toxic symptoms of plants in response to metal stress. In this study,

Table 1 Basic characteristics of the sampled sediments

Parameter	Mean ± standard deviation
pH	6.76 ± 0.09
Cation exchange capacity (CEC)	11.65 ± 1.23 cmol kg^{-1}
Copper (Cu)	49.18 ± 2.87 mg kg^{-1}
Lead (Pb)	194.91 ± 6.28 mg kg^{-1}
Chromium (Cr)	158.12 ± 7.87 mg kg^{-1}
Cadmium (Cd)	Total 22.44 ± 1.26 mg kg^{-1}
	Acid-soluble 43.89 ± 3.28%
	Reducible 22.44 ± 1.51%
	Oxidizable 3.96 ± 0.28%
	Residual 29.71 ± 2.08%

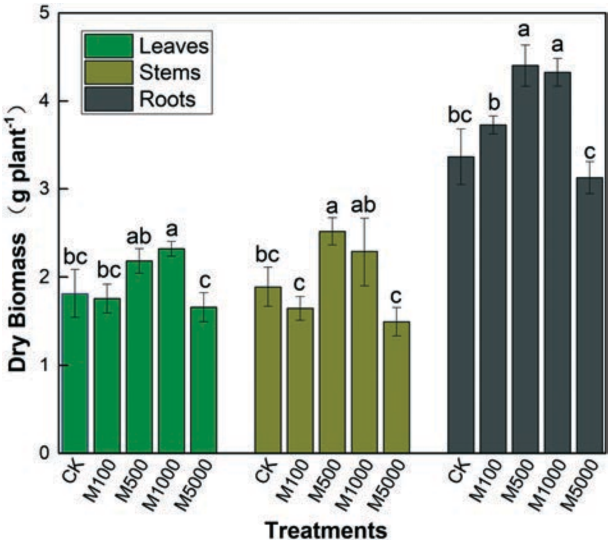

Fig. 1 Dry biomass of the leaves, stems and roots of ramie seedlings exposed to different concentrations of MWCNTs. CK, M100, M500, M1000, and M5000 represent 0, 100, 500, 1000 and 5000 mg kg^{-1} MWCNT treatments. Different letters (a, b, c) above the error bars indicate significant differences among treatments ($p < 0.05$) analyzed by one-way ANOVA.

MWCNTs at 500 and 1000 mg kg^{-1} alleviated plant growth inhibition by increasing the dry biomass in different ramie tissues. However, the growth inhibition was aggravated at the 5000 mg kg^{-1} MWCNT treatments. Similar concentration-dependent effects of MWCNTs on plant growth were observed in maize and mustard.[45,46] As reported by Tiwari *et al.*, MWCNTs enhanced the growth of maize seedlings at low concentrations, but a depression of seedling growth was observed at higher concentrations.[45] The authors ascribed the promotion of plant growth to enhanced water delivery and essential nutrient uptake (such as potassium and calcium) through the induction of fairly dense pore-like structures on the pericarp of maize seedlings by MWCNT perforation. In addition, Yan *et al.* found that CNTs could increase the expression of some genes associated with plant growth, thus accelerating maize seminal root growth.[47] The aggravated biomass reduction in ramie seedlings exposed to 5000 mg kg^{-1} MWCNTs indicated the nano-toxicity of MWCNTs at higher concentrations. This aggravated growth inhibition may be due to the aggregation of MWCNTs on the root surface, thus affecting root respiration and disturbing the interaction of ramie seedlings with the environment.

Cd accumulation and translocation

The effects of MWCNTs on Cd accumulation and translocation are shown in Fig. 2 and 3. Fig. 2 presents the concentration of Cd in ramie leaves, stems and roots. As shown in this figure, 100 and 500 mg kg^{-1} MWCNTs increased Cd concentration in ramie leaves and stems. Relative to the control, 100 and 500 mg kg^{-1} MWCNTs increased Cd concentration in ramie leaves by 14–17% and in stems by 27–

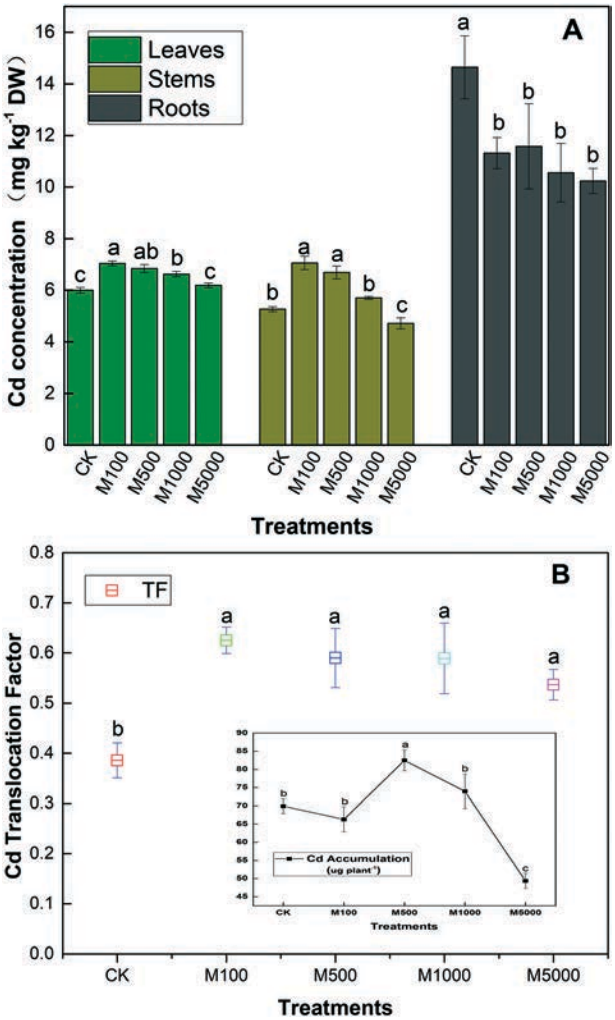

Fig. 2 Uptake and translocation of Cd in ramie seedlings treated with different concentrations of MWCNTs. A: Cd concentration in the leaves, stems and roots; B: Cd translocation factor and Cd accumulation in per plant. CK, M100, M500, M1000, and M5000 represent 0, 100, 500, 1000 and 5000 mg kg^{-1} MWCNT treatments. Different letters (a, b, c) above the error bars indicate significant differences among treatments (p < 0.05) analyzed by one-way ANOVA.

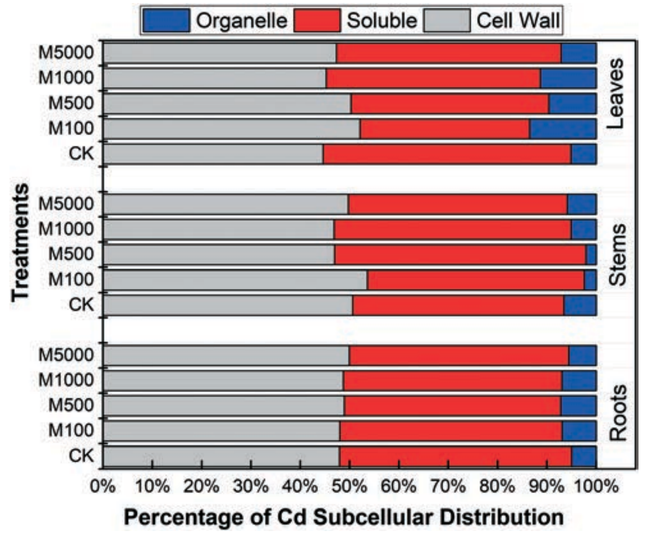

Fig. 3 Subcellular distribution of Cd in the leaves, stems and roots of ramie seedlings treated with different concentrations of MWCNTs. CK, M100, M500, M1000, and M5000 represent 0, 100, 500, 1000 and 5000 mg kg^{-1} MWCNT treatments.

34%, respectively. With the increase of MWCNT concentrations, this increasing trend tended to wane. MWCNTs at 5000 mg kg^{-1} even significantly reduced the Cd concentration in ramie stems compared with control ($p < 0.05$). Different from what were observed in ramie leaves and stems, Cd concentration in ramie roots decreased by 21–30% with the application of MWCNTs. In addition, the influence of MWCNTs on the TF of Cd and Cd accumulation in each ramie plant is shown in Fig. 3. Relative to the control, MWCNTs increased the TF of Cd by 39–62%. The highest Cd accumulation in each ramie plant was observed in ramie seedlings exposed to 500 mg kg^{-1} MWCNTs with an increase of 18% compared to control, while the 5000 mg kg^{-1} MWCNTs reduced the accumulation of Cd by 29% in ramie seedlings.

Results obtained from this study indicated that ramie could adsorb and translocate Cd from the contaminated environment, as reported previously.[32] Cd was particularly accumulated in ramie roots due to their direct contact with the contaminant. Most of the MWCNT treatments increased Cd concentration in ramie leaves and stems, whereas Cd concentration in ramie roots was significantly reduced with the application of MWCNTs ($p < 0.05$). These results indicated that MWCNTs promoted the translocation of Cd from the plant roots to the aerial parts. The increased TF value of all the MWCNT treatments further corroborated this hypothesis. The accumulation of metals in plants depends mainly on two aspects, *i.e.* metal concentration and plant biomass. Different from the effects of MWCNTs on Cd concentration in various ramie tissues, the promoted Cd accumulation was observed only in the 500 and 1000 mg kg^{-1} MWCNT treatments. This could have resulted from the plant growth stimulation at these treatments. The increased Cd concentration, enhanced TF value and promoted Cd accumulation in ramie seedlings suggested that a certain level of MWCNTs could facilitate the translocation and bioaccumulation of heavy metals in the plants. To further investigate the role of MWCNTs in Cd uptake by plants, a Cd adsorption experiment was conducted using different concentrations of MWCNTs (the detailed experimental procedure is available in ESI 1†). As shown in Fig. S5,† Cd could be removed from water by MWCNTs. The adsorption of Cd by MWCNTs might be due to the negatively charged surface and the existence of functional groups on the surface of MWCNTs. Moreover, the uptake of MWCNTs by plants was confirmed by Das *et al.* through quantitatively detecting the MWCNTs in lettuce tissues using a method of digestion coupled with programmed thermal analysis and a digestion-Raman analysis approach.[48,49] Thus, it was speculated that Cd adsorbed on MWCNTs could be accumulated by plants with the entrance of MWCNTs into plants, thus facilitating the translocation and accumulation of Cd in ramie seedlings. Similar promotion of Cd uptake was also observed in *Cannabis sativa* L., *Brassica napus* L. and *Helianthus annus* L. seedlings treated with MWCNTs.[29] A study conducted by Wild and Jones demonstrated that the movement of phenanthrene into the root cap and hair cells was facilitated by MWCNTs with the penetration of MWCNTs into wheat root cell walls.[50] Tang *et al.* found that nanomaterials could serve as Cd carriers to promote Cd uptake by *Microcystis aeruginosa*.[51] However, there is no consistent conclusion for the effects of MWCNTs on the behaviors of contaminants in the organisms. The accumulation of chlordane decreased in zucchini, corn, soybean and tomato with the application of MWCNTs at 500–5000 mg kg^{-1}.[52] Similarly, MWCNTs significantly reduced the bioaccumulation of contaminants in *Chironomus plumosus* and earthworms ($p < 0.05$).[28,53] In this study, although the removal efficiency of Cd in the water increased with increasing concentrations of MWCNTs from 1000 to 5000 mg L^{-1} (Fig. S5†), the 5000 mg kg^{-1} MWCNTs decreased Cd concentration in ramie stems and roots and significantly reduced Cd accumulation in the whole ramie seedlings ($p < 0.05$). It is expected that MWCNTs might agglomerate on the root surface at high concentrations, which decreased the bioavailability of Cd adsorbed on those MWCNTs. In addition, large amounts of MWCNTs adhering to the root surface would impede the entrance of Cd into plants. These results suggested that the effects of MWCNTs on the behaviors of heavy metals in the plants were strongly concentration dependent. As for this study, 500 mg kg^{-1} MWCNTs was the most suitable concentration for the enhancement of Cd uptake and translocation in ramie seedlings. Furthermore, except for the promotion of metal accumulation, it is interesting to find that all the MWCNT treatments accelerated the translocation of Cd from plant roots to the aerial parts. It was observed that MWCNTs at 5000 mg kg^{-1} increased the TF value of Cd with reduced Cd accumulation. Thus, the carrier role of MWCNTs for Cd in ramie seedlings is only partly the cause of the enhanced Cd translocation. The translocation of heavy metals is the determinant of phytoremediation and how to improve heavy metal translocation in plants is one of the major bottlenecks for the application of plants to remediate the contaminated environment. The improved translocation of Cd from the ramie roots to the aerial parts by MWCNTs observed in this study is of scientific value. To our knowledge, there are no confirmed specific channels for Cd to enter plants. The translocation of MWCNTs in plants has been demonstrated by many previous studies.[54,55] It is hypothesized that Cd might be translocated in plant tissues by the transporters and translocation channels for MWCNTs. However, the exact mechanisms are not clear. Given that the accumulation capacity of heavy metals in plant aerial parts obviously influences the phytoremediation efficiency, further research is needed to study the exact influence mechanisms of MWCNTs on heavy metal translocation in the plants.

Cd subcellular distribution

In order to further explore the influence mechanism of MWCNTs on plant metal accumulation at the cell level, the subcellular distribution of Cd in ramie leaves, stems and roots was determined. As shown in Fig. 3, the proportion of Cd accumulated in the cell wall was 45–54%, while that in the soluble portion was 34–48% in all the treatments. The subcellular distribution of Cd in ramie roots exposed to MWCNTs had no obvious difference compared with control, except a slight increase in the organelle fraction. As for the stems, MWCNTs increased the soluble fraction of Cd by 3–20% and decreased the proportion of Cd in the organelle fraction by 11–69%. The organelle proportion of Cd in ramie leaves increased significantly in the MWCNT treatments ($p < 0.05$). Specifically, in ramie leaves, the organelle proportion of Cd in the 100 mg kg^{-1} MWCNT treatments was 1.65 times higher than that in control. Different from what was observed in the stems, the soluble proportion of Cd in ramie leaves decreased by 11–64% compared with control, with the greatest degree of decline appearing in the 100 and 500 mg kg^{-1} MWCNT treatments.

According to the results shown in Fig. 3, Cd was mainly accumulated in the cell wall and soluble portion. These findings are consistent with previous studies. For instance, Mwamba *et al.* investigated the subcellular distribution of Cd in two *Brassica napus* cultivars (Zheda 622 and ZS 758), and they found that the percentage of Cd retained at the cell wall and soluble fraction was 70% for cultivar Zheda 622 and 88% for cultivar Zheda 622.[37] The plant cell wall, mainly consisting of polysaccharides, proteins, enzymes and fatty acids, acts as the first barrier to heavy metals entering the plant cells. The negatively charged groups in the cell wall could interact with the positive metal ions, thus immobilizing heavy metals in the plant cell wall. In addition, the proteins in the cell wall might bind with heavy metals and reduce their toxicity. As reported by Lai, most of the Cd in *Impatiens walleriana* was integrated with the pectates and proteins.[56] The relatively high amounts of Cd observed in the cell soluble fraction in this study were presumably due to the vacuolar compartmentation of heavy metals. Similarly, Fu *et al.* found that the majority of Cd in *Phytolacca americana* L. was located in the cell wall and soluble fractions, and they ascribed this phenomenon to the integration of Cd with pectates and proteins in the cell wall and the Cd compartmentation in the vacuole.[57] The influence of MWCNTs on the subcellular distribution of Cd varied in different plant organs. MWCNTs did not significantly change the subcellular distribution of Cd in ramie roots, whereas the soluble portion of Cd in ramie stems remarkably increased with the application of MWCNTs ($p < 0.05$). This increase resulted in a high Cd migration capacity and presumably could promote the translocation of Cd from the stems to the leaves. In addition, it is interesting to note that a portion of Cd in the soluble fraction was shifted to the organelle portion in ramie leaves with MWCNT treatments. This shift might be due to the increased binding of Cd to the chloroplast in the leaves. Zhang *et al.* found that MWCNTs increased the expression of genes associated with chloroplast development,[58] and this promoted chloroplast development by MWCNTs might lead to more Cd binding to the chloroplast in this study. Moreover, Zhai *et al.* certified the accumulation of MWCNTs in plant chloroplast,[59] and this accumulation presumably could immobilize MWCNT loaded Cd in the chloroplast of ramie seedlings. Furthermore, there were no significant differences in the subcellular distribution of Cd in ramie seedlings between control and 5000 mg kg^{-1} MWCNT treatments ($p < 0.05$). These results indicated that the influence of MWCNTs on the subcellular distribution of Cd in plants strongly depended on their concentrations. However, the specific influence mechanisms are not well understood. Further studies are needed to explore the related mechanisms by which MWCNTs differentially affect the subcellular distribution of heavy metals in various plant organs.

Oxidative stress

H_2O_2 and MDA were used as the indicators of the oxidative stress in ramie seedlings under Cd stress. The responses of H_2O_2 and MDA followed a similar pattern in all the treatments (Fig. 4). Relative to the control, MWCNTs increased H_2O_2 and MDA concentrations by 19–54% and 4–27%, respectively. The highest contents of H_2O_2 and MDA were observed in ramie seedlings treated with 100 mg kg^{-1} MWCNTs. With the concentration of MWCNTs increased from 100 to 500 or 1000 mg kg^{-1},

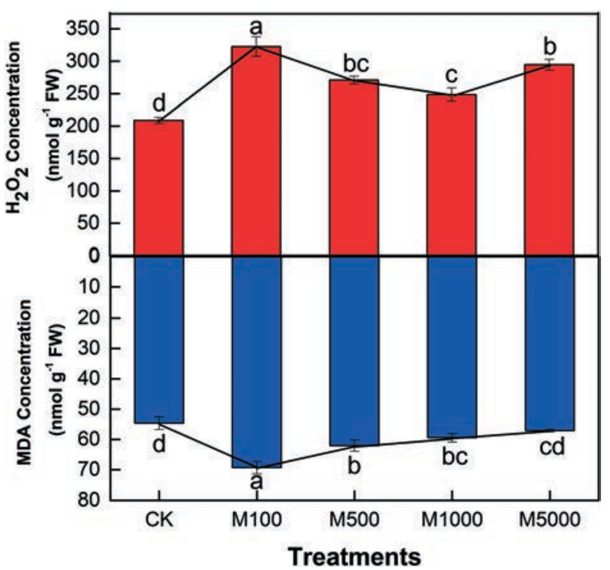

Fig. 4 H_2O_2 and MDA concentrations in ramie seedlings treated with different concentrations of MWCNTs. CK, M100, M500, M1000, and M5000 represent 0, 100, 500, 1000 and 5000 mg kg^{-1} MWCNT treatments. Different letters (a, b, c) above the error bars indicate significant differences among treatments ($p < 0.05$) analyzed by one-way ANOVA.

the concentrations of H_2O_2 and MDA decreased. Compared with the 1000 mg kg^{-1} MWCNT treatments, the application of 5000 mg kg^{-1} MWCNTs did not further change the content of MDA in ramie seedlings, whereas a significant increase (41%) of H_2O_2 content was observed ($p < 0.05$).

Reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and H_2O_2, are the by-products of normal aerobic metabolism processes in plants. The generation and scavenging of ROS are in equilibrium under temperate circumstances. However, this balance in the organisms can be disrupted by environmental stress, *e.g.*, salt, drought and heavy metals. The over generated ROS can interact with proteins, DNA and unsaturated fatty acids and cause oxidative damage to plants. H_2O_2 is a typical ROS, and MDA is the product of lipid oxidation.[60,61] The accumulation of excess H_2O_2 and MDA indicates the presence of oxidative stress in plants. In the present study, the application of MWCNTs increased the contents of H_2O_2 and MDA in ramie seedlings, indicating the aggravated oxidative stress by MWCNTs. This aggravated oxidative stress could result from the promoted Cd accumulation in ramie seedlings due to the application of MWCNTs. However, there was no statistically significant difference in the determined Cd concentration in ramie seedlings exposed to 100 and 500 mg kg^{-1} MWCNTs (Fig. 1), and the accumulation of Cd in the whole ramie seedlings treated with 500 mg kg^{-1} MWCNTs was significantly higher than that in the seedlings treated with 100 mg kg^{-1} MWCNTs ($p < 0.05$). With increasing MWCNT concentration from 100 to 500 mg kg^{-1}, the contents of MDA and H_2O_2 were decreased significantly ($p < 0.05$). These results indicated that the application of 500 mg kg^{-1} MWCNTs could alleviate Cd-induced oxidative stress in ramie seedlings by the reduction of MDA and H_2O_2 accumulation. Similar to what was observed in this study, Hatami *et al.* found that CNTs at 50 and 100 mg L^{-1} reduced the oxidative injury indices (MDA and H_2O_2) and significantly alleviated drought stress in *Hyoscyamus niger* seedlings ($p < 0.05$).[62] In addition, relative to the control, not only did 5000 mg kg^{-1} MWCNTs not increase the Cd concentration in different ramie tissues, but it also suppressed the accumulation of Cd in the whole ramie seedlings. However, the accumulation of H_2O_2 in ramie seedlings treated with 5000 mg kg^{-1} MWCNTs was significantly higher than that in the control ($p < 0.05$), indicating that MWCNTs at high levels could induce oxidative stress in plants. These results are in accordance with those observed in rice cells, wherein MWCNTs at 20 mg L^{-1} increased ROS accumulation and decreased cell viability.[63] The triggered oxidative stress by MWCNTs at high concentrations could be an indicator of nanomaterial-induced toxic effects, which could partly explain why the ramie seedlings exposed to 5000 mg kg^{-1} MWCNTs had the lowest dry biomass.

Antioxidative responses

The effects of MWCNTs on the enzyme activities in ramie seedlings are shown in Fig. 5. Relative to the control, a slight

(9–21%) but not statistically significant decrease of CAT activity was observed in ramie seedlings exposed to MWCNTs ($p < 0.05$). The activity of POD in the MWCNT treatments was significantly lower than that in the control ($p < 0.05$). MWCNTs at 100 mg kg^{-1} decreased the activity of POD by 60% compared with control. Relative to the 100 mg kg^{-1} MWCNT treatments, the 500 and 1000 mg kg^{-1} MWCNTs increased POD activity by 102% and 54%, respectively, whereas the 5000 mg kg^{-1} MWCNTs decreased this activity by 20%. As for the SOD activity, the MWCNTs increased it by 10–20%. The activity of SOD increased with the MWCNT concentration increasing from 100 to 500 mg kg^{-1}, and then it decreased as the MWCNT concentration further increased.

MWCNTs differentially influenced the accumulation of GSH and GSSG in ramie seedlings under Cd stress (Fig. 6). MWCNTs reduced the GSH content in ramie seedlings compared with the control. With the MWCNT concentration increasing from 100 to 1000 mg kg^{-1}, the GSH content increased from 89.37 to 128.10 nmol g^{-1} FW. MWCNTs at 5000 mg kg^{-1} reduced the GSH content by 41% compared with the control. Different from the changes of GSH, GSSG concentration in ramie seedlings treated with 100 and 5000 mg kg^{-1} MWCNTs increased by 53% and 77% compared with the control, whereas the 500 and 1000 mg kg^{-1} MWCNTs did not alter GSSG content significantly ($p < 0.05$). As for the ratio of GSSG/GSH, 100 mg kg^{-1} MWCNTs increased it by 169% compared with the control. With increasing the concentration of MWCNTs from 100 to 1000 mg kg^{-1}, the ratio of GSSG/GSH decreased. The highest value of the GSSG/GSH ratio (0.28) was observed in ramie seedlings exposed to 5000 mg kg^{-1} MWCNTs.

Plants have evolved well-equipped enzymatic and non-enzymatic antioxidant defense systems to fight against the oxidative burst. The enzymatic antioxidant system consists of a series of enzymes, which can scavenge the ROS excessively generated by heavy metals. SOD is the killer of oxygen free radicals that catalyze the conversion of $O_2^{\cdot -}$ into H_2O_2. CAT and POD are the major H_2O_2-scavenging enzymes, which accelerate the reduction of H_2O_2 to H_2O. In this study, MWCNTs increased SOD activity in ramie seedlings under Cd stress, which could promote the elimination of the representative ROS ($O_2^{\cdot -}$) and alleviate oxidative stress in the plants. The enhanced SOD activity could have resulted from the MWCNT induced modulation of stress-related protein expression. As confirmed by Yu *et al.*, MWCNTs at nontoxic doses could upregulate the expression of SOD-2 at both mRNA and protein levels.[64] Since H_2O_2 is one of the products of a SOD-participated reaction and the generation of H_2O_2 was promoted as the activity of SOD increased in ramie seedlings, the activities of POD and CAT were supposed to be enhanced to eliminate the generated H_2O_2. However, in the MWCNT treatments, those enzyme activities decreased. This could explain why the seedlings exposed to MWCNTs accumulated more H_2O_2 compared with control (Fig. 4). Heavy metal stress was acknowledged to produce ROS that was beyond the removal ability of plants, thus triggering the oxidative damage. In this study, ramie seedlings exposed to 500 mg kg^{-1} MWCNTs had an enhanced Cd accumulation ability, the highest SOD activity, and relatively lower H_2O_2 and MDA contents. These results indicated that a certain level of MWCNTs could promote Cd accumulation and help plants to better cope with Cd-induced oxidative stress. In addition, the lowest POD and CAT activities were observed in ramie seedlings treated with 5000 mg kg^{-1} MWCNTs, which could lead to an increase of H_2O_2 accumulation and aggravate Cd-induced oxidative damage to plants. This could be attributed to the

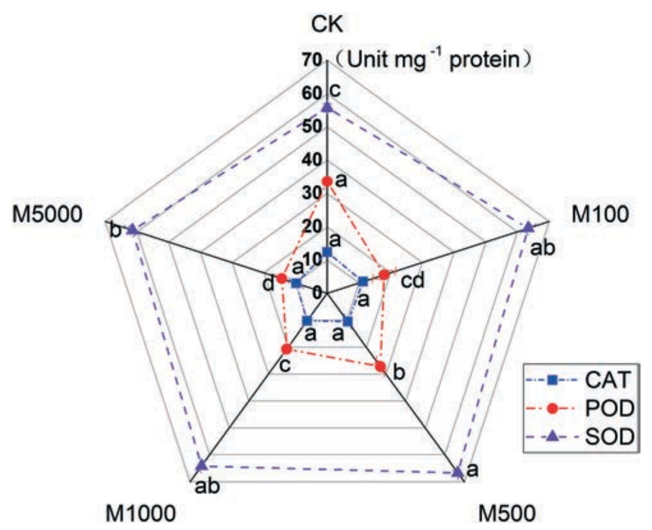

Fig. 5 Activities of CAT, POD, and CAT in ramie seedlings treated with different concentrations of MWCNTs. CK, M100, M500, M1000, and M5000 represent 0, 100, 500, 1000 and 5000 mg kg^{-1} MWCNT treatments. Different letters (a, b, c) above the error bars indicate significant differences among treatments ($p < 0.05$) analyzed by one-way ANOVA.

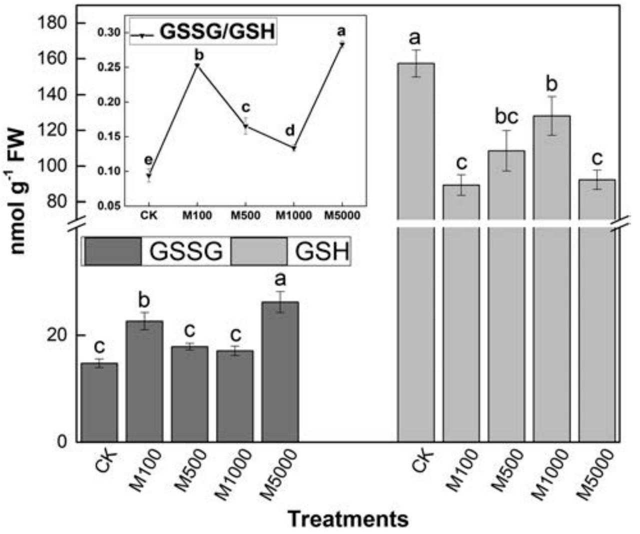

Fig. 6 GSH and GSSG concentrations, and the ratio of GSSG/GSH in ramie seedlings treated with different concentrations of MWCNTs. CK, M100, M500, M1000, and M5000 represent 0, 100, 500, 1000 and 5000 mg kg^{-1} MWCNT treatments. Different letters (a, b, c) above the error bars indicate significant differences among treatments ($p < 0.05$) analyzed by one-way ANOVA.

negative role of MWCNTs at high levels. Similarly, Hatami reported that MWCNTs might increase the toxic effects of drought stress by changing the activities of various cellular antioxidant enzymes such as POD, SOD and CAT.[65] The deterioration of metal-induced oxidative damage by MWCNTs was also observed in *Vicia faba* L. seedlings.[66]

GSH is one of the major ROS scavengers in the non-enzymatic antioxidant defense system, which can immobilize ROS and heavy metals by its sulfhydryl group and reduce their toxicity. In the ascorbate–glutathione (ASA–GSH) cycle, GSH can be oxidized to GSSG with the reduction of H_2O_2 to H_2O. The GSSG/GSH ratio indicates the redox status of plant cells. In this study, the reduced GSH content and increased GSSG/GSH ratio in ramie seedlings exposed to MWCNTs could be attributed to the increased Cd concentration at these treatments. With increasing MWCNT concentration from 100 to 1000 mg kg^{-1}, the GSH content increased, indicating that a certain level of MWCNTs could promote the accumulation of GSH in plants under Cd stress. Ghorbanpour and Hadian found that MWCNTs could act as an effective elicitor to stimulate the biosynthesis of valuable antioxidant compounds in the callus of *Satureja khuzestanica*.[67] In this study, MWCNTs at 500 and 1000 mg kg^{-1} perhaps could stimulate the biosynthesis of GSH in ramie seedlings under Cd stress, thus increasing the GSH content at these treatments and improving plant antioxidative capacity. The change in GSSG/GSH ratio corresponds to the altering trend of H_2O_2 in all the treatments. The raised GSSG/GSH ratio and increased GSSH content observed in the 5000 mg kg^{-1} MWCNT treatments suggested a more oxidized cellular redox status in the plants. To date, little information has been obtained about the effects of MWCNTs on the ASA–GSH cycle in plants under heavy metal stress, and further research is needed to explore the related influence mechanisms.

Conclusion

The presence of MWCNTs significantly influenced the bioaccumulation of Cd and Cd-induced toxicity in ramie seedlings. MWCNTs at high levels led to deterioration effects on Cd-induced toxicity to plants resulting from the ecotoxicity of nanomaterials. This nano–bio interaction between nanomaterials and plants is worthy of attention with the massive production and inevitable release of nanomaterials. Furthermore, the present results showed that a certain level of MWCNTs could increase the uptake of Cd, alleviate the oxidative damage and strengthen the antioxidant capacity in the plants. These results indicated that suitable levels of nanomaterials in the contaminated sites are beneficial to phytoremediation. The application of nanomaterials as amendments to strengthen phytoremediation is a novel finding, which is of environmental significance in the area of environmental remediation. Overall, this study provides new insights into the potential risks of nanomaterials to biological systems and explores the novel application of nanomaterials.

Conflicts of interest

There are no conflicts to declare.

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