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Woody species *Rhus chinensis* Mill. seedlings tolerance to Pb: Physiological and biochemical response

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ABSTRACT

Screening potential plant species is a crucial consideration in phytoremediation technology. Our previous study demonstrated that *Rhus chinensis* Mill. seedlings had potentials for phytoremediation of Pb contaminated soil. However, its bioaccumulation and tolerance characteristics remain unclear. Seedling growth, LMWOAs secreted by roots, Pb subcellular distribution and chemical forms, and mineral elements in *R. chinensis* tissues were evaluated under different Pb concentrations (0, 25, 50, 100, 200 and 400 mg/L) in culture solution at 14 days after planting. *R. chinensis* did not show visual symptoms of Pb toxicity under lower Pb treatments; however, Pb significantly declined the growth of seedlings under higher Pb treatments. Higher Pb stress also decreased the concentrations of nitrogen in leaves, but increased the concentrations of P and K in roots. Pb stress also decreased Mn concentrations in leaves. A great quantity of Pb was uptake and mostly retained in *R. chinensis* roots. Nonetheless, *R. chinensis* can still concentrate 459.3 and 1102.7 mg/kg Pb in leaves and stems, respectively. Most of Pb in *R. chinensis* tissues was stored in the cell wall with HAc-, HCl-, and NaCl-extractable form. LMWOAs secreted by *R. chinensis* roots showed a strong positive correlation with Pb concentrations in all plant tissues and with P in roots. Our results suggested that Pb deposited in the cell wall and integration with phosphate or oxalate might be responsible for the tolerance of *R. chinensis* under Pb stress in short period.

Introduction

It is generally known that lead (Pb) is one of the most toxic elements. Sources of Pb contamination in the environment mainly originated from extensive uses in petrol, paints, mining, sludge, industrial wastes, and agricultural activities (Gupta et al., 2013). Pb may enter in human body through the food chain and causes serious diseases (Tripathi et al., 2016). Obviously, the remediation of Pb-polluted soils has drawn much attention.

Phytoremediation is a relatively cheap and environmentally friendly technique (Zaier et al., 2010), and can be employed to treat large areas due to the relatively low cost (Cheng et al., 2015; Houben et al., 2013). Selecting the

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appropriate plant species is a crucial consideration in phytoremediation technology (Shukla et al., 2011; Solãs-Dominguez et al., 2012; Zhang et al., 2014). Most of the heavy metal hyperaccumulators are associated with low biomass production (Zaier et al., 2010). So far, few studies have conducted to explore the potential of woody species for phytoremediation of contaminated soil (de Souza et al., 2012; Pottier et al., 2015; Pulford and Watson, 2004). Woody species generally have a deep root system (Mendez and Maier, 2008). Furthermore, trees have a certain extent capacity to accumulate and translocate heavy metals to the aboveground parts (Shukla et al., 2011; Unterbrunner et al., 2007; Zhou et al., 2015b). Thus, understanding the growth response and metal accumulation patterns in candidate woody trees would contribute greatly to assess their potential use in phytoremediation in the field.

As is well known, under Pb stress, metabolism and physiological processes of plants were affected (Israr et al., 2011; Tripathi et al., 2016). Pb can cause oxidation of macromolecules, leading to inhibit the growth performance of plants (Malar et al., 2014b; Uveges et al., 2002; Zhou et al., 2015a). However, plants have developed some mechanisms for Pb tolerance. Chemical form and subcellular distribution of Pb have been proved to be associated with the Pb tolerance of plants (Li et al., 2016; Zhou et al., 2015b, 2016a). For example, Pb in root cells of *Neyraudia reynaudiana* was mainly present in the cell walls (Zhou et al., 2016a). Similar result was observed in *Iris halophile* (Han et al., 2016). Therefore, Pb ion deposition in root cell wall has been regarded as a possible explanation for Pb tolerance (Li et al., 2016). In addition, the chemical form of Pb is closely related to its biotoxicity (Verbruggen et al., 2009). For instance, Pb extracted HCl-extractable form was the dominant fraction in *Morus alba* leaves supported that the low mobility of Pb in plant tissues may be crucial for detoxification (Zhou et al., 2015b). Therefore, it is of great importance to understand the subcellular distribution and chemical form of Pb in plants related to the Pb tolerance mechanisms of plants.

In general, secretion of low molecular weight organic acids (LMWOAs) by plant roots is commonly increased under heavy metals stress (Chiang et al., 2011; Zhan et al., 2016). Numerous studies suggested that LMWOAs may alter element mobility and bioavailability, thereby increasing accumulation process in many species (Khan et al., 2016; Shakoor et al., 2014; Xin et al., 2015). However, some studies showed that citric acid could prompt Pb immobilized in soils and reduced the absorption and accumulation of Pb in plants (Kim et al., 2013). Whether the LMWOAs varied in assisting in Pb uptake is still not clear, especially in woody species.

Rhus chinensis Mill, one species in the genus *Rhus* (Tourn.) L. emend. Moench, is a pioneer woody species characterized with strong adaptability, fast growth, high drought and barren stress tolerance (Zhou et al., 2017). Our previous study suggested that *R. chinensis* have considerable Pb tolerance and phytoremediation potential in moderate Pb-contaminated areas (Shi et al., 2016). However, the knowledge of Pb subcellular distribution and chemical forms in plants *R. chinensis* was unknown. Furthermore, the relationship between ion mobility and LMWOAs secreted by root of *R. chinensis* remains unclear. Therefore, comprehensive studies of Pb subcellular distribution and chemical forms, mineral elements, root exudation, physiological indexes of *R. chinensis* under Pb stress will greatly improve our understanding of the physiological and biochemical response of *R. chinensis* against Pb stress. The aim of this study was to (1) determine the characteristics of Pb subcellular distribution and chemical forms in *R. chinensis* and (2) evaluate the effect of root-secreted LMWOAs on Pb accumulation, and their implication on Pb toxicity and the plant tolerance.

1. Materials and methods

1.1. Plant growth conditions

Seeds of *R. chinensis* were collected from Hangzhou (China) (30°057′N, 119°956′E) and sown in a pot (diameter 4 cm × height 8 cm) that contained perlite:peat (1:3, *V*/*V*) growing nutrient medium. When the shoots and root systems of seedlings developed well, uniform one-year seedlings (height of seedling 50–60 cm and ground diameter 0.4–0.5 cm) were selected for the experiment. Seedlings were transferred into hydroponic pots with tap water for 2 weeks in order to acclimate in a hydroponic environment and then 10 L aerated Knop's solution was added in each pot (Wang et al., 2014).

1.2. Experimental method

R. chinensis seedlings were cultured in nutrient solution for 2 weeks, and then the different concentrations of $Pb(NO_3)_2$ (0, 25, 50, 100, 200 and 400 mg/L) were added in nutrient solution for 14 days. Each treatment was replicated three times and each replicate consisted of one pot containing eight seedlings. These pots were arranged in a completely randomized design. Other matters needing attention in experiment were according to the description of Wang et al. (2014). The treatment solutions were replaced twice a week to ensure consistency. The phytotoxicity symptoms were recorded throughout the experiment. After 14 days, all seedlings were harvested.

1.3. Biomass measurements

After harvesting, all samples were washed thoroughly with distilled water. The roots were immersed in 20 mmol/L Na_2-EDTA for 15 min to remove metals adhering to the root surface. Then the leaves, stems, and roots were excised and separated. The dry biomasses of samples were measured after drying at 80°C for 72 hr. The tolerance index (TI) based on biomass (leaf, stem, root and all plant) was calculated as TI = B_t / B_c (Metwally et al., 2005), where B_t (g) is the treatment biomass and B_c (g) is the control biomass. High values indicate high tolerance by plants.

1.4. Determination of Pb and mineral elements

All samples were ground into powder and 0.2 g of each sample was digested with a mixture of 4 mL HNO_3 and 1 mL $HClO_4$. The elements (Pb, Ca, K, Mg, P, Cu, Fe and Mn) were determined using atomic absorption spectroscopy (SOLAAR M6, Thermo Fisher Scientific, Inc., Waltham, MA, USA). N was determined by the Kjeldahl digestion (Madejón et al., 2003).

The translocation factor (TF) which can be used to estimate a plant's ability to translocate metals from the roots to the shoots was calculated as TF = A_s / A_r (Deng et al., 2004), where A_s (mg/kg) is the total heavy metal accumulated in leaves (TF1) or stems (TF2) and A_r (mg/kg) is the total heavy metal accumulated in roots.

1.5. Sub-cellular distribution of Pb

In this study, the low-level Pb concentration (25 and 50 mg/L) and the high-level concentration of (100, 200 and 400 mg/L) treatments were carried out and the similar performance of *R. chinensis* seedlings were observed in each level treatment, consequently, the Pb concentrations at the subcellular level in the leaves, stems and roots from 0, 25, and 100 mg/L Pb treatment were determined according to Zhou et al. (2016a). Fresh samples (0.5 g) were homogenized with a mortar and a pestle in liquid nitrogen. Then 20 mL of pre-chilled extraction buffer (containing 50 mmol/L Tris–HCl (pH 7.5), 250 mmol/L sucrose, 1.0 mmol/L dithioerythritol, 5.0 mmol/L ascorbic acid and 1.0% (W/V) crosslinking polyvingypyrrolidone) was added. The homogenate was centrifuged at 300 ×*g* for 15 min at 4°C. The pellet was considered as the cell wall fraction (F_I). The filtrate was centrifuged at 2500 ×*g* for 15 min, and the pellet was the chloroplast (leaf) or trophoplast (root and stem) fraction (F_{II}). Then the supernatant was centrifuged at 15,000 ×*g* for 30 min. The pellet was the organelle fraction (F_{III}) and the supernatant was the soluble fraction (F_{IV}).

1.6. Chemical form of Pb

The chemical form of Pb in the leaves, stems and roots under different treatments (0, 25, and 100 mg/L) were sequentially extracted by the designated solution according to Zhou et al. (2016b). Fresh samples (0.5 g) were homogenized with a mortar and a pestle in liquid nitrogen, diluted at a ratio of 1:50 (W/V) with extraction solution and then shaken for 24 hr at 25°C. The homogenate was centrifuged at 5000×*g* for 10 min. The supernatant was collected and transferred to the triangle bottle. Subsequently, the precipitate was washed twice by the respective extraction, shaking at 25°C for 2 hr, and centrifuging at 5000×*g* for 10 min. The supernatant from each of the three repetitions was then pooled. The designated extraction solution in the following order: 80% ethanol (F-Ethanol), distilled water (F-dH_2O), 1 mol/L sodium chloride (F-NaCl), 2% acetic acid (F-HAc), 0.6 mol/L hydrochloric acid (F-HCl).

1.7. Extraction of LMWOAs from root rhizosphere

The seedling root samples were washed thoroughly with ultrapure water and aquae sterilisata by three times, then the seedlings were placed in the triangular flask filled with the 0.5 mmol/L $CaCl_2$ at room temperature for 4 hr. After that seedlings were taken out and the roots were washed three times with aquae sterilisata. The solution was filtered with 0.42 μm filters. Organic acids from the water solution were extracted according to Magdziak et al. (2011). Samples were analyzed by high-performance liquid chromatography (1290 Infinity, Agilent, USA).

1.8. Estimation of photosynthetic pigment concentrations and chlorophyll fluorescence parameters

The concentration of chlorophyll *a*, *b* and carotenoids was determined according to Zhou et al. (2015b). Fresh samples (0.1 g) were homogenized with a mortar and a pestle in liquid nitrogen. Then 10 mL of 80% acetone was added. After 24 hr, the absorbance of the supernatant was measured at 470, 645, and 663 nm with a spectrophotometer.

Chlorophyll fluorescence parameters in 25 min dark-adapted mature leaves from plants of the control and each treatment were measured with a portable fluorometer (Image-PAM, Walz, Effeltrich, Germany). The minimal fluorescence level in the dark-adapted state (F_0), the maximal fluorescence level in the dark-adapted (F_m) and light-adapted ($F_{m'}$) states was determined. The steady-state value of fluorescence (F_s) under actinic light was also recorded. Using both light- and dark-adapted fluorescence parameters, we calculated the maximum efficiency of photosystem II (PSII) photochemistry (F_v / F_m) in the dark-adapted state, the quantum yield of PSII electron transport as $\Phi_{PSII} = (F_{m'} - F_s) / F_{m'}$. Other fluorescence parameters were measured using the equations of Baccio et al. (2014). Photochemical quenching as $qP = (F_{m'} - F_s) / (F_{m'} - F_{0'})$; non-photochemical quenching as $qN = (F_v - F_{v'}) / F_v = 1 - (F_{m'} - F_{0'}) / (F_m - F_0)$. The quantum yield of regulated energy dissipation (Y_{NPQ}), the quantum yield of nonregulated energy dissipation (Y_{NO}), and electron transport rate (ETR) were calculated using the following formulas: $Y_{NPQ} = 1 - Y(II) - 1 / (NPQ + 1 + qL(F_m / F_0 - 1))$; $Y_{NO} = 1 / (NPQ + 1 + qL(F_m / F_0 - 1))$; ETR = Yield × PAR × 0.84 × 0.5, respectively, where Yield is the effective quantum yield corresponding to each light intensity (PAR); 0.84 is an assumed value for PAR absorptance, and 0.5 is a factor that accounts for 50% of the absorbed quanta being distributed to PSII (Dao and Beardall, 2016).

1.9. Hydrogen peroxide, superoxide and malondialdehyde (MDA) concentration

Root and leaf samples (0.5 g) were homogenized with a mortar and a pestle in liquid nitrogen, and 5 mL 0.1% (W/V) TCA was added. The homogenate was centrifuged at 10,000 ×*g* for 15 min. Then 0.5 mL 10 mmol/L potassium phosphate buffer (pH 7.0) and 1 mL 1 mol/L KI was added to the collected supernatant (0.5 mL). The absorbance of the supernatant was measured at 390 nm. The concentration of H_2O_2 was given on a standard curve (Velikova et al., 2000).

Root and leaf samples (0.5 g) were homogenized with a mortar and a pestle in liquid nitrogen, and 2 mL of 50 mmol/L potassium phosphate buffer (pH 7.8) was added. The homogenate was centrifuged at 13,000 ×*g*, 4°C, 10 min. Then 0.5 mL supernatant was mixed with 0.9 mL of 50 mmol/L potassium phosphate buffer (pH 7.8) and 0.1 mL of 10 mmol/L hydroxylamine hydrochloride. The mixture was incubated at 25°C for 60 min. After that, 1 mL of 17 mmol/L *p*-aminobenzene sulphonic acid and 1 mL of 7 mmol/L α-naphthylamine was added and incubated at 25°C for 30 min. The absorbance of the mixture was read at 530 nm. The concentration of $O_2^{\cdot-}$ was given on a standard curve by sodium nitrite (He et al., 2011).

Root and leaf samples (0.5 g) were homogenized with a mortar and a pestle in liquid nitrogen, and 5 mL of 0.1% (W/V) TCA was added. The homogenate was centrifuged at 10,000 ×*g* for 20 min. Then 2 mL of 0.5% (W/V) TBA in 20% TCA was added to the collected supernatant (0.5 mL). The mixture was incubated in boiling water (90°C) for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Then the mixture was centrifuged at 10,000 ×*g* for 5 min, and the absorbance of supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex was calculated from the extinction coefficient 155 mmol/(L·cm) (Tripathi et al., 2016).

1.10. Statistical analysis

The data are expressed as the mean of at least three replications ± standard error. All statistical tests were performed with SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). For all experimental variables, one-way analysis of variance was applied and the least significant difference test was used for comparisons of means. When necessary, analytical data were transformed using logarithms to assure normal distribution (Parra et al., 2014). Differences were considered significant when the *p* value of analysis of variance *F*-test was <0.05.

2. Results

2.1. Plant growth and tolerance index

After 7 days of Pb exposure, higher Pb concentration treatments (200 and 400 mg/L) induced toxicity symptoms in seedlings' leaves, such as yellowing from the leaf tip and wilting, while seedlings at control and lower concentration treatments (25 and 50 mg/L) did not show such symptoms. The Pb significantly decreased the total dry biomass in *R. chinensis* seedlings at higher Pb concentration treatments (100, 200 and 400 mg/L) compared to control, and the maximum reduction of biomass recorded was 33.7% under 400 mg/L Pb treatment. The biomass of root and leaf under 400 mg/L Pb treatment was lower than that in other treatments ($p < 0.05$), and reduced by 52.7% and 32.8% respectively, while the maximum reduction of stem biomass was recorded under 200 mg/L Pb treatment. The relative biomass allocation of *R. chinensis* was stem > root > leaf under the 0–50 mg/L Pb treatments, and the proportion was showed no statistical differences between the treatments. The root proportion *R. chinensis* was decreased with the increase of Pb concentration (≥100 mg/L). However, the stem proportion showed the opposite trend. The leaf proportion of all treatments was changed slightly (Fig. 1).

Table 1 showed the TI values of *R. chinensis* under different Pb concentration treatments. For *R. chinensis*, the TI values of 100, 200, and 400 mg/L Pb treatments were lower than those in the lowest treatment with 25 mg/L Pb, respectively, whereas in 50 mg/L Pb treatment, it was only slightly reduced. The TI values of 25 mg/L Pb treatment were ranged from 0.99 to 1.07. Stems had the highest TI values under higher concentration Pb treatments, while the lowest TI occurred in the roots.

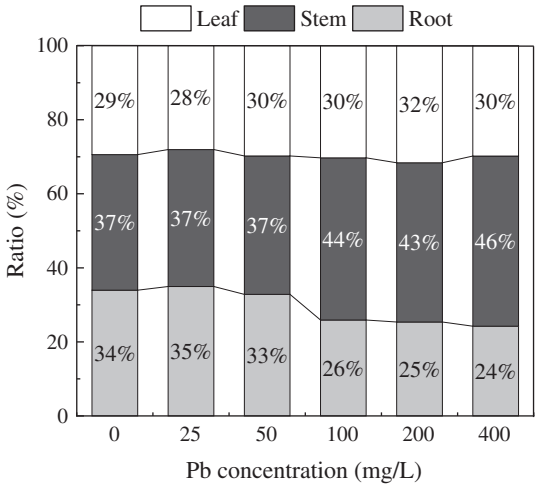

Fig. 1 – Biomass allocation of *Rhus chinensis* under Pb stress.

2.2. Accumulation and translocation of Pb

The concentrations of Pb of *R. chinensis* in different tissues increased with increasing of the Pb concentrations in culture solution (Fig. 2a). The order of the Pb concentration of *R. chinensis* across tissues was roots > stems > leaves (100, 200 and 400 mg/L Pb treatments) and roots > leaves > stems (0, 25 and 50 mg/L Pb treatments). As shown in Fig. 2a, seedlings accumulated significantly a great quantity Pb at the 400 mg/L Pb treatment than that in other treatments. Similar to concentration of Pb in all tissues, the amounts of Pb in the leaves and stems under 400 mg/L Pb treatment were much higher than that in other treatments, though its biomass was the lowest among all treatments.

TF1 (Leaf/Root) values of Pb were increased with increasing of the Pb concentrations up to 50 mg/L Pb treatment and then decreased at higher Pb concentration treatments (Table 2). TF2 (Stem/Root) values of Pb were ranged from 0.17 to 0.57 among all Pb treatments.

Table 1 – Tolerance index (TI) of *Rhus chinensis* under Pb stress.

Treatment	Root	Stem	Leaf	Plant
25 mg/L	1.07 ± 0.20 a*	1.03 ± 0.02 a	0.99 ± 0.32 a	1.02 ± 0.20 a
50 mg/L	0.90 ± 0.27 a	0.94 ± 0.07 ab	0.89 ± 0.26 ab	0.89 ± 0.24 a
100 mg/L	0.56 ± 0.19 b	0.90 ± 0.02 b	0.73 ± 0.21 b	0.71 ± 0.21 b
200 mg/L	0.55 ± 0.18 b	0.83 ± 0.03 b	0.75 ± 0.37 b	0.69 ± 0.16 b
400 mg/L	0.52 ± 0.22 b	0.87 ± 0.14 b	0.71 ± 0.29 b	0.68 ± 0.15 b

* Each value represents the mean of three replicates ± SD. Different letters indicate significant difference between the treatments ($p < 0.05$).

2.3. Sub-cellular distribution of Pb

The sub-cellular distribution of leaves and roots from Pb treatments showed a similar trend with $F_I > F_{II} > F_{IV} > F_{III}$ (Fig. 2b). While, the order of sub-cellular distribution of stems was $F_I > F_{IV} = F_{II} > F_{III}$ (Fig. 2b). Under the control treatment, Pb in the leaves, stems and roots with 78.6%, 75.6%, and 79.2% were deposited in the cell wall (F_I) and 5.88%, 6.11%, and 5.90% of Pb in the organelle (F_{III}). Pb stress significantly reduced the proportion of Pb distributed in the cell wall (F_I) of the roots, stems and leaves (Fig. 2b). In addition, the proportions of Pb deposited in trophoplast (F_{II}) and soluble fraction (F_{IV}) were increased in all tissues (except for soluble fraction of stem).

2.4. Chemical forms of Pb

The chemical forms of Pb in roots, stems and leaves varied across the different treatments. When seedlings in the control treatment, the main extractable form of Pb in all tissues was HAc-extractable form (Fig. 2c). With an increase of Pb concentration in the growth medium, the ratios of Pb in 80% ethanol, H_2O and residual-extractable form were obviously decreased in all tissues (except H_2O-extractable form in root, $p < 0.05$). However, there was no statistical difference between the Pb treatments. The ratio of Pb in the HAc-extractable form was also reduced with the solution of Pb increased. Under the Pb stress, the ratios of HCl-extractable form and NaCl-extractable form Pb of leaves, stems and roots were increased, respectively (Fig. 2c).

2.5. Mineral elements

The concentrations of macro-elements (N, P, and K) showed significant difference among the treatments and tissues (Fig. 3). Moreover, the concentrations of N in leaves were increased and then decreased, the similar trend was observed in the concentrations of P (leaves and stems) and K (leaves and roots), while, the concentrations of N in stems and roots were changed slightly (except the concentration of stems at 400 mg/L Pb treatment). The current result also indicated that the concentrations of P were increased in the roots of treated seedlings, which showed the positive correlation with the concentrations of Pb in the culture solutions (Fig. 3). Pb stress reduced the concentrations of K in stems, especially at higher Pb concentration treatments (100, 200 and 400 mg/L). This phenomenon became more pronounced with the increasing Pb concentrations. Compared to the control, the Mg, Ca, and Cu concentrations of leaves were increased in Pb treatments (except 25 mg/L Pb treatment) (Fig. 4). The maximum significant increase in Mg, Ca, and Cu concentrations of leaves were observed at 100 mg/L Pb (Ca and Cu) and 200 mg/L Pb (Mg) treatment. Fe concentrations of leaves increased significantly under higher Pb stress ($p < 0.05$). An opposite trend was observed in Mn, where the concentrations

Table 2 – Translocation factor (TF) of *Rhus chinensis* under Pb stress.

Treatment	TF1 leaf/root	TF2 stem/root
25 mg/L	0.21 ± 0.03 c *	0.17 ± 0.04 d
50 mg/L	0.44 ± 0.05 a	0.38 ± 0.09 c
100 mg/L	0.37 ± 0.01 b	0.57 ± 0.05 a
200 mg/L	0.19 ± 0.01 c	0.33 ± 0.02 c
400 mg/L	0.19 ± 0.02 c	0.47 ± 0.08 b

* Each value represents the mean of three replicates ± SD. Different letters indicate significant difference between the treatments ($p < 0.05$).

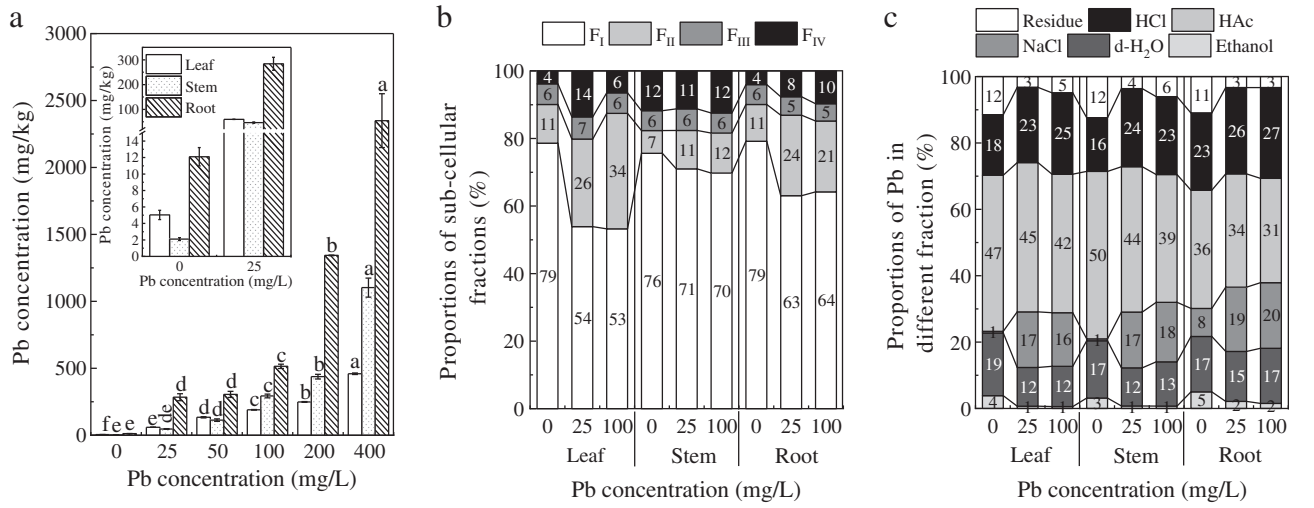

Fig. 2 – Average Pb concentrations in dry plant tissues of *Rhus chinensis* seedlings exposed to Pb (a) and proportions of sub-cellular fractions (b) and chemical form fractions of Pb in plant tissues (c) (F_I: cell wall; F_{II}: trophoplast; F_{III}: organelle; F_{IV}: soluble fraction). Each value represents the mean of three replicates ± SE. Different letters in panel (a) indicate significant differences between data derived from the same index ($p < 0.05$). Numbers in panels (b) and (c) indicate percentage of sub-cellular fractions and chemical form fractions of Pb in plant tissues, respectively.

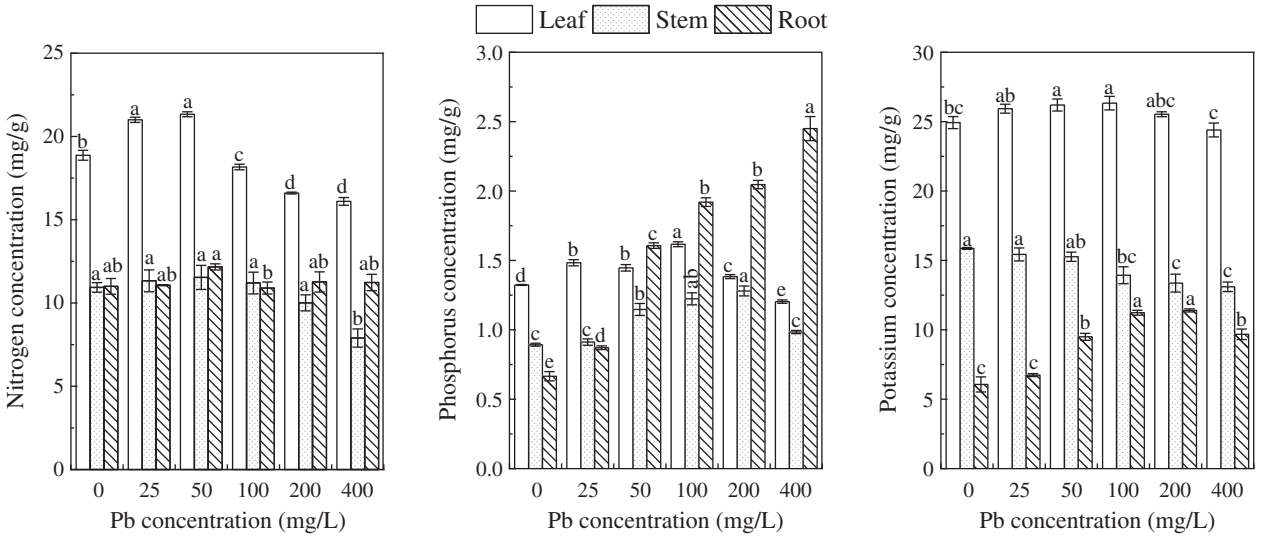

Fig. 3 – Average N, P, and K concentrations in dry plant tissues of *Rhus chinensis* seedlings exposed to Pb. Each value represents the mean of three replicates ± SE. Different lower case letters indicate significant differences between same tissue under various Pb treatments ($p < 0.05$).

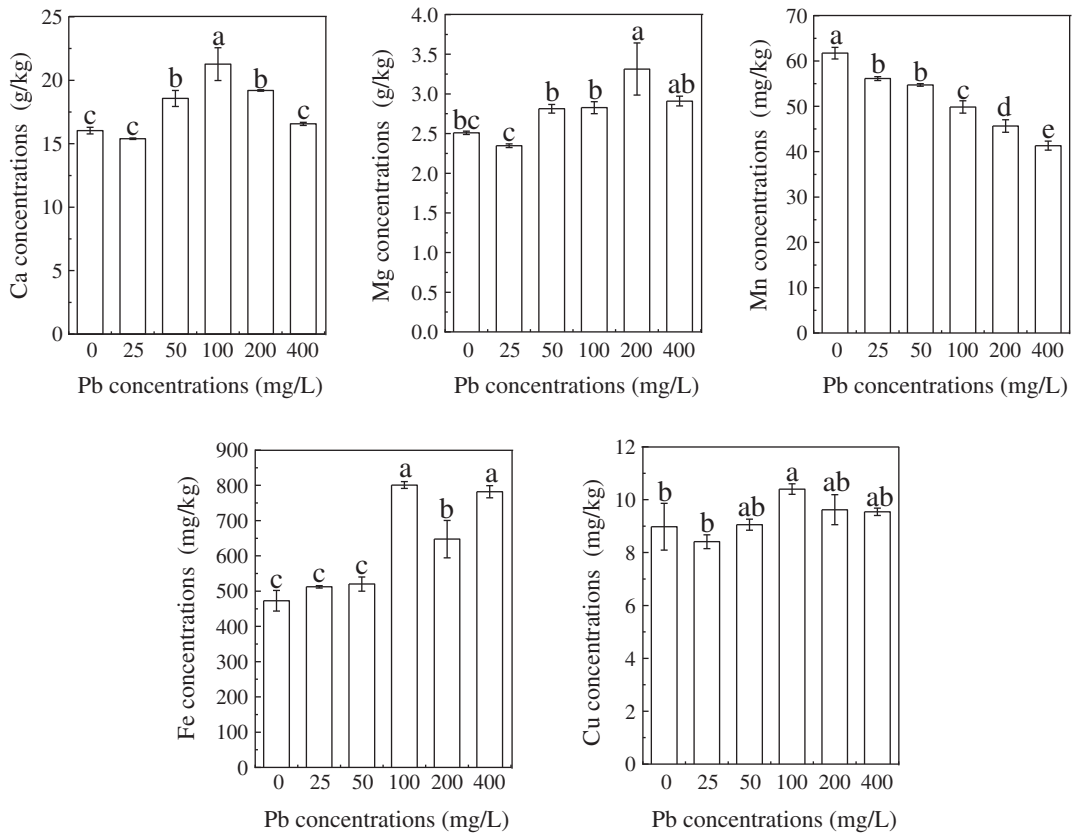

Fig. 4 – Average Ca, Mg, Mn, Fe, and Cu concentrations in leaf tissues of *Rhus chinensis* seedlings exposed to Pb. Each value represents the mean of three replicates ± SE. Different lower case letters indicate significant differences for a certain index between various Pb treatments ($p < 0.05$).

of Mn decreased significantly in the Pb treatments compared with that of control treatment ($p < 0.05$).

2.6. LMWOA concentrations

Six LMWOAs were obtained in the *R. chinensis* rhizosphere as succinic (SA), formic (FA), citric (CA), malic (MA), oxalic (OA) and propandioic acids (PAs) (Table 3). The succinic and formic acids were dominant, while the citric and malic acids were not detected under lower Pb concentrations and control treatments. Compared to control, the concentrations of succinic acid increased significantly at the highest Pb concentration treatment. Similarly, a gradual increase in the oxalic acid was observed in Pb treatment samples. Whereas, the concentrations of formic acid were decreased significantly at 25–400 mg/L Pb treatment to 47.9%–60.9% of the control treatment. Moreover, the addition of Pb had no impacts on the propandioic acids concentration. The malic and citric acids were detected only under 50–400 mg/L and 100–400 mg/L Pb treatments, and the concentrations were increased significantly with increasing of the Pb treatments ($p < 0.05$).

2.7. Photosynthetic pigment concentration

Chlorophyll (Chl) concentrations of *R. chinensis* were decreased significantly when it was exposed to Pb with higher concentrations (100, 200 and 400 mg/L), but slightly changes under lower concentrations (25 and 50 mg/L) (Appendix A Fig. S1). For *R. chinensis*, both Chl-*a* and Chl-*b* concentrations decreased and showed significant differences between different treatments ($p < 0.05$). The concentrations of carotenoid were also decreased when *R. chinensis* was exposed to Pb (except 25 mg/L Pb treatment).

2.8. Chlorophyll fluorescence parameters

The performance of chlorophyll fluorescence based parameters was similar to plant growth. The value of F_s decreased under higher Pb concentration treatments (100, 200 and 400 mg/L) (Appendix A Fig. S2), and the maximum reduction recorded was 22.9% at 400 mg/L Pb treatment. Similar results were also observed in F_v/F_m, Φ_{PSII}, qP and ETR (Appendix A Fig. S2). However, the value of qN was increased under the Pb stress. In this study, higher Pb stress caused a significant increase the value of Y_{NO} at the higher Pb treatments, while enhanced the value of Y_{NPQ} slightly (Appendix A Fig. S2).

2.9. H_2O_2, $O_2^{\cdot-}$ and MDA concentration

Under higher Pb stress, H_2O_2 and $O_2^{\cdot-}$ concentrations of leaves and roots were increased apparently ($p < 0.05$) compared with control (Appendix A Table S1). The $O_2^{\cdot-}$ concentrations were significantly elevated up to 810.8 and 931.2 μmol/g FW in roots and leaves of *R. chinensis*, respectively, under 400 mg/L Pb treatment. The H_2O_2 concentrations of roots and leaves were significantly increased by 114% and 95%, respectively. The total MDA concentrations were increased with increasing of the Pb concentrations, especially at the higher Pb concentration treatments (Appendix A Table S1).

3. Discussion

3.1. Heavy metal tolerance of R. chinensis

As is to know, Pb is not essential element in plant metabolism (Zaier et al., 2010). In the present study, *R. chinensis* can grow normally under the lower Pb stress (25 to 50 mg/L Pb). However, the physiological processes were inhibited under the higher Pb stress (≥100 mg/L) and the visual damage (leaf dehydration and chlorosis) to the seedlings was observed. Malar et al. (2014b) also reported that under the heavy metal stress, biomass is a good indicator for plant growth. The similar phenomenon was observed in this study. For example, the concentration of Pb in roots was strongly negative correlation with the root biomass (Pearson $r = -0.753$, $p < 0.001$, $N = 18$). And these symptoms were also reported by Islam et al. (2007), Malar et al. (2014a) and Zhou et al. (2017). This phenomenon was due to the oxidative stress induced by Pb^+ (Mahdavian et al., 2016). In this study, the concentrations of H_2O_2, $O_2^{\cdot-}$ and MDA in leaves and roots were increased apparently compared to control, especially at the higher Pb concentration treatments (Appendix A Table S1) and showed the negative correlation with plant biomass. These results suggested that *R. chinensis* is tolerant to lower and moderate Pb concentrations in this growth medium. It also demonstrated that *R. chinensis* can actively react to the environmental changes *via* a series of physiological and biochemical responses. In the current study, most of the Pb was sequestered in the roots, which suggested that the high bioavailability of heavy metals could be limited once inside the plant. This is because the endodermis Casparian strips in the roots would block Pb transport (Zhou et al., 2016a, 2017).

Table 3 – The low molecular weight organic acid (LMWOA) concentrations (mg/L) under Pb stress.

Treatment	Succinic acid	Citric acid	Formic acid	Propandioic acid	Malic acid	Oxalic acid
0 mg/L	25.2 ± 4.9 b *	nd	39.2 ± 24.4 a	0.6 ± 0.3 a	nd	2.4 ± 0.8 d
25 mg/L	24.2 ± 5.6 b	nd	20.4 ± 12.9 b	0.7 ± 0.1 a	nd	2.8 ± 0.4 d
50 mg/L	31.9 ± 3.4 b	nd	17.2 ± 3.2 b	0.7 ± 0.2 a	0.8 ± 0.2 c	3.2 ± 0.7 cd
100 mg/L	32.8 ± 6.9 b	0.9 ± 0.3 c	18.7 ± 3.4 b	1.0 ± 0.4 a	1.0 ± 0.1 bc	4.1 ± 0.2 c
200 mg/L	37.1 ± 15.8 b	1.4 ± 0.5 b	18.4 ± 2.7 b	1.0 ± 0.6 a	1.3 ± 0.4 b	5.4 ± 0.8 b
400 mg/L	60.3 ± 19.1 a	3.4 ± 0.8 a	15.3 ± 2.6 b	1.0 ± 0.2 a	2.3 ± 0.5 a	8.4 ± 1.3 a

nd: not detected.

* Each value represents the mean of three replicates ± SD. Different letters indicate significant difference between the treatments ($p < 0.05$).

Furthermore, endothelial cells of the root tissue also blocked Pb transport to aboveground parts (Kopittke et al., 2008; Sharma and Dubey, 2005). Therefore, we speculated that the Pb accumulation in the roots is one of the Pb tolerance manifestations of *R. chinensis* seedlings.

Further analysis showed that at the sub-cellular level, most of the Pb in the *R. chinensis* tissues was stored in the cell wall, indicating that plants might have compartmentalized Pb to decrease its free levels to relieve the toxicity effect (Khan et al., 2016). Zhou et al. (2017) reported that the majority of Pb was deposited in the intercellular space or in the cell wall in the roots and leaves of *R. chinensis*, while it was compartmentalized into the vacuolar in the stems. Qiao et al. (2015) also reported similar results. In the current study, under Pb stress, the ratio of Pb in trophoplast was increased, while the ratio of Pb in the cell wall was decreased. This indicates that the concentrations of Pb in the cell wall reached 'saturation' or Pb damaged the cell wall structures (Brunner et al., 2008). We also observed that the biomass of plant was positively correlated with the ratio of Pb in cell wall, and negatively correlated with the ratio of Pb in trophoplast. This phenomenon was also found in *Brassica juncea* under Th stress (Zhou et al., 2016b). It signified that the cell wall might be playing an important role for the detoxification of Pb.

The metal chemical forms in tissues would affect plant growth and important physiological and biochemical processes (di Toppi and Gabbrielli, 1999). Our results showed that the concentration of H_2O-extractable form was increased with the concentration of Pb in the culture solution, which coincided with the toxicity stress of Pb to *R. chinensis*. Moreover, we also found that Pb in the roots, stems and leaves of *R. chinensis* existed in different chemical forms, with a larger part of Pb in less mobile forms (HAc-, HCl-, and NCl-extractable). Indeed, it was evident that alleviation of Pb toxicity was primarily by aggregation with peptide by phosphate ligands (HAc-extractable). Simultaneously, in this study, P concentrations of root were also increased with increasing of Pb concentrations in the culture solutions. Some experiments showed Pb immobilization by reaction with P (Hettiarachchi and Pierzynski, 2010; Jiang et al., 2012). It is consistent with most of Pb existed in HAc-extractable form in the present study, thus reducing Pb induced interference and damage to plant. Moreover, oxalate ligands (HCl-extractible) and peptide ligands (NaCl-extractible) appeared to be involved in counteracting Pb activity (Fig. 2c). In accordance with the current results, Pb combination with phosphate ligands was suggested as the first Pb detoxification strategy in *R. chinensis*. The fact was evidenced by reports (Kopittke et al., 2008; Zheng et al., 2012). This phenomenon was also observed in *Porphyra yezoensis* (Zhao et al., 2015) and *Brassica napus* (Mwamba et al., 2016) under Cd stress. However, Bovenkamp et al. (2013) reported that precipitation with a group ($-PO_4$) in roots and leaves of four plant families was not observed. The similar result was observed by Zhou et al. (2015b). This may be an indication that different plants showed different tolerance mechanisms that can explain the binding capacity of metal interaction with cellular ligands.

Though a great quantity of Pb remained in the roots, it might directly or indirectly cause damage to the shoots. In this study, higher Pb decreased the macro-element concentrations of leaves which might have contributed to Pb toxicity, and the concentrations of chlorophyll and carotenoid of leaves were also decreased gradually when increasing the concentration of Pb in the solution. However, *R. chinensis* could maintain its normal growth and tolerate Pb at the lower concentration treatments, which might be ascribed to the maintenance of an adequate nutrient uptake. The results of this study showed that the Pb stress has no impact on the N concentrations of roots, and the concentrations of P and K were increased compared to the control plants (Fig. 3). It was suggested that a large quantity of macro-elements was absorbed to alleviate the toxic effects on *R. chinensis* by Pb (Tripathi et al., 2016). That is a possible tolerance response of *R. chinensis* under Pb stress. Moreover, the chlorophyll fluorescence data also revealed that the seedlings of *R. chinensis* show relatively high tolerance to Pb. For example, the F_v/F_m and qP values were only significantly declined under higher Pb concentration treatments, indicating that Pb accumulation in leaves did not completely damage the photosynthetic system (He et al., 2011), and may be attributed to the retention of Pb in cell wall fractions and less mobile chemical forms in the leaves. Correlation analysis also showed that the concentrations of Mn were negatively correlated with Pb concentration ($r = -0.939$, $p < 0.01$, Fig. 5). Meanwhile, in the present study, the concentration of chlorophyll ($r = 0.870$, $p < 0.01$) and carotenoid ($r = 0.765$, $p < 0.01$), and electron transport rate ($r = 0.830$, $p < 0.01$) showed the strong positive correlation with the concentrations of Mn, respectively. As we know, Mn is a necessary element to maintain the normal structure of chlorophyll (Roosta et al., 2018). Therefore, it was suggested that the deficiency of Mn under the Pb stress was one of the main reasons of etiolation and photosynthetic decline of *R. chinensis* (Roosta et al., 2018).

3.2. Metal accumulation and translocation

The high efficiency of phytoremediation depends on plant roots which accumulate a large metal and transport it to aerial parts quickly (Chen et al., 2016). However, many researches showed that Pb hyperaccumulation is a rare phenomenon in plants (Gupta et al., 2013; Zaier et al., 2010). In general, the TF value of Pb of woody species was lower (Zhou et al., 2015b). For example, *Erythrina speciosa* (TF = 0.37–0.56) and *Schizolobium parahyba* (TF: 0.27–0.47) (de Souza et al., 2012). Zhou et al. (2017) also reported that *R. chinensis* could transport small quantity metals to the shoots after 30 days of exposure to Pb-spiked soil. A number of studies also have shown that Pb was mainly concentrated in roots in herbaceous plants and crops (Li et al., 2016; Tripathi et al., 2016; Wierzbicka, 1999; Zhong et al., 2017; Zhou et al., 2016a). To date, only a few species were reported to accumulate Pb at high concentration in the shoots (Bhargava et al., 2012). However, in this study, *R. chinensis* seedlings concentrate far more than 1000 mg/kg Pb in the shoots at 400 mg/L Pb treatment with a certain degree growth inhibition. It suggested that *R. chinensis* could be classified as Pb "accumulator" species. Furthermore, plants having a TF greater than 1 are considered as hyperaccumulators (Mendez and Maier, 2008). Although the concentration of Pb in the shoots of *R. chinensis* was far more than 1000 mg/kg, TF value was lower than 1 (Table 2). This result suggests that *R.*

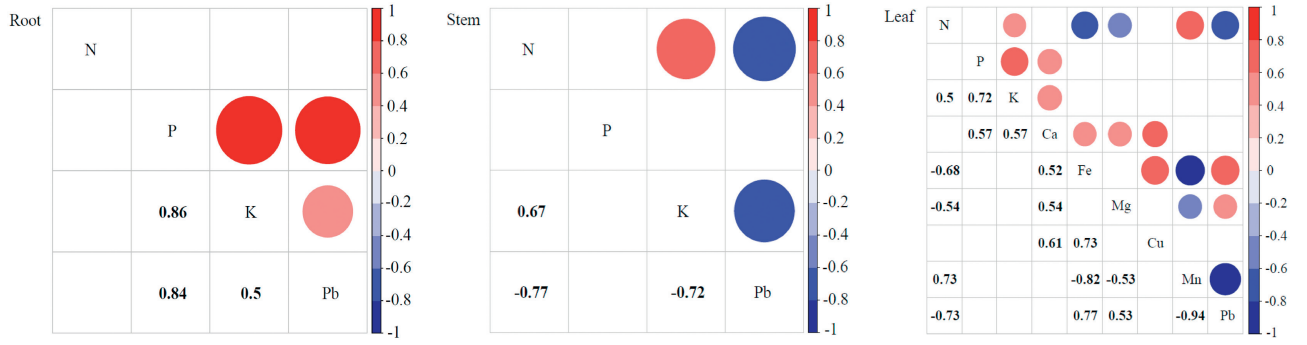

Fig. 5 – Element correlation analyses. Pearson's correlation analyses performed on element concentrations measured in the root, stem and leaf (positive correlations are displayed in red and negative correlations in blue color. Color intensity and circle size are proportional to the correlation coefficients. The significant level of correlation test which is less than 0.05 are shown in the figure).

chinensis plants might have the potential for effective phytoremediation of Pb under lower or moderate Pb stress.

Recently, many reports have highlighted the ability of LMWOAs for Pb phytoextraction (Khan et al., 2016; Shakoor et al., 2014). Current results showed that a certain amount of Pb was transported from roots to shoots, which exhibited the greater phytoremediation potential of *R. chinensis*. In the present study, the LMWOAs secreted by *R. chinensis* roots were increased significantly under the higher Pb concentration treatments (except formic), where the SA, OA, MA and CA showed a strong positive correlation with the concentrations of Pb in tissues. What is more, the concentration of H_2O-extractible form of Pb was increased with Pb in growth medium and the ratio in tissues accounted for 11.53% to 16.61%. Obviously, H_2O-extractible form of Pb has high capacity to migrate in plant tissues. Summing up, it suggested that *R. chinensis* could be used on different formed organic acids to chelate Pb ion and transport Pb to harvest parts. Simultaneously, some studies confirmed that citrate and malate probably play a role in heavy metal transport (Dresler et al., 2014). According to above statement, it speculated that citrate and malate in root exudation of *R. chinensis* played an important role in the activation of undissolved heavy metals. However, the correlation analysis also indicated that OA secreted by roots was a positive correlation with the HCl-extractible form of Pb. This may be an indication that oxalate ligands appeared to be involved in counteracting Pb activity and it is suggested that application with different LMWOAs may be beneficial in the field remediation project against lead toxicity. Overall, the results pointed out an important role of LMWOAs' phytoremediation potential in *R. chinensis*.

4. Conclusions

Pb significantly decreased the growth of *R. chinensis* seedlings under the higher concentration Pb treatments, which may be attributed to higher concentration of Pb in plant tissues and suppressed severe oxidative stress subsequently. In this study, *R. chinensis* seedlings concentrated far more than 1000 mg/kg Pb in the shoots at the highest Pb treatment with a certain degree growth inhibition. In addition, under 100 mg/L Pb treatment, *R. chinensis* had a certain extent capacity to transport Pb from roots to shoots, and the translocation factor values were 0.37 to 0.57. LMWOAs secreted by *R. chinensis* roots showed a strong positive correlation with Pb concentrations in plant tissues. These results suggested that *R. chinensis* plants might have the potential for phytoremediation of Pb, and LMWOAs might play an important role for the phytoextraction process. Moreover, most of the Pb in the *R. chinensis* tissues was stored in the cell wall and larger part of Pb existed in HAc-, HCl-, and NaCl-extractable. All those forms are low mobile and toxicity forms of Pb. Therefore, these observations may explain the tolerance manifestation of *R. chinensis* to Pb. Due to fast growth and high biomass production, *R. chinensis* could fulfill the purpose of phytoremediation from contaminated areas driven by repeated reaping.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2018.07.003.

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