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# Lead accumulation and biochemical responses in *Rhus chinensis* Mill to the addition of organic acids in lead contaminated soils†

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Adding organic acid is an effective approach to assist phytoremediation. The effects of organic acids on phytoremediation efficiency are unknown in *Rhus chinensis*. This study aimed to evaluate the effect of citric acid (CA) and oxalic acid (OA) on the lead phytoremediation potential of *R. chinensis* with significantly inhibited growth in Pb-contaminated soil. The experimental pot culture study evaluated the long-term physiological response and metal accumulation patterns of *R. chinensis* grown in varying Pb-treated soil, and examined the effects of 0.5 and 1.0 mmol L<sup>-1</sup> CA and OA on the growth, oxidative stress, antioxidant system, and Pb subcellular distribution of *R. chinensis* grown in pots with 1000 mg kg<sup>-1</sup> Pb. Compared with the control, the biomass, leaf area, root morphological parameters, and chlorophyll concentration of *R. chinensis* decreased, whereas the carotenoid, malondialdehyde, H<sub>2</sub>O<sub>2</sub>, and O<sub>2</sub><sup>•-</sup> concentrations, and superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activity increased under Pb stress. A copious amount of Pb was taken up and mainly stored in the cell walls of the roots. The application of CA and OA increased plant growth. The highest shoots and roots biomass increase recorded was 44.4 and 61.2% in 1.0 mmol L<sup>-1</sup> OA and 0.5 mmol L<sup>-1</sup> CA treatment, respectively. The presence of CA and OA increased SOD, POD, and CAT activities and decreased the H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>•-</sup> and malondialdehyde content. A concentration of 0.5 mmol L<sup>-1</sup> CA significantly increased the Pb concentration in the organs. The other organic acid treatments changed root Pb concentrations slightly while increasing shoot Pb concentrations. The translocation factor values from organic acid treatments were increased by 38.8–134.1%. Our results confirmed that organic acid could alleviate the toxicity of stunted *R. chinensis* and improve phytoremediation efficiency.

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## 1. Introduction

With the rapid development and modernization of industry and population growth, serious soil contamination with heavy metals has increased rapidly, becoming a global problem.<sup>1,2</sup> Heavy metals cannot be degraded, which makes these toxic elements persistent in the environment and more difficult to control.<sup>3,4</sup> Lead (Pb), which is considered the second most toxic contaminant after arsenic,<sup>5</sup> exists widely in the ecosystems.<sup>6,7</sup> Excessive Pb in the environment has harmful effects on plants and animals and threatens human health throughout the food chain.<sup>8,9</sup> In China, Pb pollution is a serious environmental problem, and its concentration exceeds the recommended limits in 1.5% of agricultural land.<sup>10</sup> Therefore, the

development of efficient techniques is becoming a critical need for solving the problem of metal(loid) pollution.<sup>10,11</sup>

Compared with conventional methods, phytoremediation is an advancing technology that uses plants to remove or extract pollutants from the environment.<sup>12,13</sup> Moreover, phytoremediation is an *in situ* remediation, simple, low-cost and green technology.<sup>14,15</sup> For soil pollution, phytoextraction and phytostabilization are the two main phytoremediation strategies and are the most widely used.<sup>16</sup> In recent years, hyperaccumulators have been widely used for soil remediation and have attracted remarkable attention.<sup>17,18</sup> Among these species, a few hyperaccumulators are available for Pb-contaminated soil remediation.<sup>19,20</sup> Due to the low bioavailability of Pb in soil, many species cannot uptake large quantities of Pb from the soil and translocate it from the roots to the aerial parts. Furthermore, low biomass and remediation efficiency are bottlenecks for the practical application of phytoremediation.<sup>11,17</sup> Therefore, looking for new species for heavy metal phytoremediation is the focus of researchers.<sup>4</sup> An ideal species for phytoremediation can tolerate and accumulate heavy metals, grow fast, and produce large aboveground biomass and deep and developed root systems. Nevertheless, relatively few species possess all of these

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properties.<sup>21,22</sup> Generally, fast-growing woody species have a large aboveground biomass and deep root system compared with herbaceous plants.<sup>23,24</sup> In addition, some woody species can accumulate considerable levels of heavy metals and translocate them to their aboveground parts.<sup>12,23</sup> However, many researchers have indicated that Pb can be strongly bound by soil particles, especially in alkaline environments, and thus, its mobility is significantly affected.<sup>25,26</sup> Hence, it is difficult for plants especially woody species, to absorb Pb directly from the soil and translocate it to their shoots.<sup>27</sup>

To further improve Pb removal efficiency, many chelators have been used to increase the bioavailability of Pb in soil.<sup>17,28,29</sup> Compared with other chemosynthetic chelators, low-molecular-weight organic acids (LMWOAs) are natural compounds and are favored in metal-mobilizing agents when they are at low toxicity, readily biodegradable and eco-friendly.<sup>30–32</sup> Such LMWOAs may increase metal mobilization in soils by promoting soil acidity and forming metal-carboxylic acid complexes,<sup>33,34</sup> thereby enhancing heavy metal uptake and accumulation.<sup>31</sup> Moreover, they can alleviate oxidative stress by improving the enzyme activity of the antioxidative defense system.<sup>35,36</sup> Therefore, LMWOAs may enhance phytoremediation efficiency by promoting heavy metals uptake and accumulation by plants and improving their tolerance to heavy metals.<sup>19,20</sup> Cai *et al.* reported that citric acid, malic acid, and tartaric acid application can significantly increase the Pb phytoremediation efficiency of centipede grass.<sup>19</sup> Similar results were also found in *Solanum nigrum*<sup>31</sup> and *Typha latifolia*.<sup>37</sup> However, some studies pointed out that LMWOAs could not significantly promote the uptake of Pb by plants.<sup>38,39</sup> Moreover, the application of high concentration LMWOAs can significantly reduce the plant biomass.<sup>40</sup> These results suggested that their efficiency rests on the concentration, method, period of use, and physiological state of the plant.<sup>11,41</sup> Therefore, the rational application of LMWOAs can improve the efficiency of phytoremediation effectively.<sup>19</sup>

*Rhus chinensis* Mill is a traditional Chinese medicine and a small deciduous tree with the properties of fast growth and strong environmental adaptability.<sup>42,43</sup> It is also a pioneer species and can grow in Pb/Zn mine tailing areas.<sup>44</sup> Furthermore, *R. chinensis* is a Pb-tolerant species with the capacity to absorb and accumulate Pb in moderate or higher Pb-contaminated areas.<sup>42,43</sup> Therefore, *R. chinensis* is a potential plant for Pb phytoremediation. However, previous studies mainly focused on hydroponic and short-term (one month) pot experiments.<sup>42,43</sup> Therefore, the growth performance and Pb accumulation capacity of *R. chinensis* for longer periods in soil containing higher Pb concentrations are unknown. Previous studies have also demonstrated that the majority of Pb is stored in roots and that plant growth is significantly inhibited under higher Pb concentration treatments.<sup>42,43</sup> To achieve high phytoremediation efficiency for high Pb-contaminated areas, adding LMWOAs is an effective approach. A preliminary study also confirmed that citric acid (CA) and oxalic acid (OA) were the main components of root exudates.<sup>42</sup> Their application, along with phytoremediation, can immediately enhance lead accumulation and mitigate toxic effects. However, the effect of CA and OA on the lead phytoremediation potential of *R. chinensis*

with significantly inhibited growth in Pb-contaminated soil are unknown. Therefore, the main objective of this work was to (1) evaluate the physiological response and metal accumulation patterns of *R. chinensis* grown in high Pb-treated soil for long-term; and (2) investigate the effects of different concentrations of CA and OA on the phytoremediation efficiency and subsequent biological responses of *R. chinensis* with significantly inhibited growth.

## 2. Materials and methods

### 2.1 Plant materials and growth conditions

Seeds were collected from Hangzhou, China (30°057'N, 119°56'E) and sown in a pot (diameter 4 cm × height 8 cm) containing perlite:peat (1:3, v/v). When the shoots and root systems of seedlings developed well, seedlings with a height of 50–60 cm and a ground diameter of 0.4–0.5 cm were selected for the experiment.

### 2.2 Experimental method

Red soil (0–30 cm) was collected from Fuyang District, Hangzhou (30°03'43"N, 119°57'12"E). Red soil is more viscous, and the effective and total lead concentrations were 24.0 and 40.8 mg kg<sup>-1</sup>, respectively. The concentrations of available phosphorus, hydrolyzed nitrogen, and available potassium were 3.53, 56.3 and 124.3 mg kg<sup>-1</sup>, respectively, and the pH value was 5.59. This soil was air-dried, sieved, and then used in the subsequent pot experiments. Based on the results of preliminary tests and field investigations in Pb/Zn mining areas, soil Pb concentrations of 0, 500, 750, 1000, 1250 and 1500 mg kg<sup>-1</sup> were designed. Pb(NO<sub>3</sub>)<sub>2</sub> solution was prepared and sprayed on the soil, and then the soil was equilibrated for one month. Due to losses during the experiment, the final Pb concentration was lower than the design concentration. The final Pb, available nitrogen, phosphorus and potassium concentrations and pH are listed in Table 1. In May 2016, uniform seedlings with their nutrient medium were transplanted into black cylindrical plastic pots with 3 kg of soil, and each pot contained one seedling. After 3 months of the trial, similar toxic symptoms were found in plants treated with high Pb concentrations (≥1000 mg kg<sup>-1</sup>), and seedling growth was significantly inhibited. Hence, 0.5 and 1.0 mmol L<sup>-1</sup> CA and 0.5 and 1.0 mmol L<sup>-1</sup> OA, respectively, were added to the 1000 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub> treatment to verify whether organic acids could alleviate the toxic effects of seedlings and improve the efficiency of phytoremediation. Therefore, there were 10 treatment groups in this experiment, and the 10 treatments were as follows: T0 (0 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub>), T1 (500 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub>), T2 (750 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub>), T3 (1000 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub>), T4 (1250 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub>), T5 (1500 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub>), T6 (1000 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub> and 0.5 mmol L<sup>-1</sup> CA), T7 (1000 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub> and 1.0 mmol L<sup>-1</sup> CA), T8 (1000 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub> and 0.5 mmol L<sup>-1</sup> OA), T9 (1000 mg kg<sup>-1</sup> Pb(NO<sub>3</sub>)<sub>2</sub> and 1 mmol L<sup>-1</sup> OA). Each treatment had 9 seedlings per replicate, for a total of 27 seedlings of *R. chinensis* per treatment. The experiment lasted 150 days.



**Table 1** Chemical characteristics of test soil. Each value represents the mean of three replicates  $\pm$  SD. The same below

Pb/(mg kg <sup>-1</sup> )	Available Pb/ (mg kg <sup>-1</sup> )	Total Pb/ (mg kg <sup>-1</sup> )	Hydrolysable nitrogen/ (mg kg <sup>-1</sup> )	Available phosphorus/ (mg kg <sup>-1</sup> )	Available potassium/ (mg kg <sup>-1</sup> )	pH
0	24.0 $\pm$ 4.7	40.4 $\pm$ 1.1	56.3 $\pm$ 2.5	3.53 $\pm$ 0.06	124.3 $\pm$ 0.6	5.59 $\pm$ 0.02
500	254.7 $\pm$ 9.9	409 $\pm$ 32.5	92.7 $\pm$ 2.1	5.62 $\pm$ 0.03	130.0 $\pm$ 1.0	5.66 $\pm$ 0.02
750	429.0 $\pm$ 11.8	622.3 $\pm$ 40.0	83.5 $\pm$ 3.1	5.54 $\pm$ 0.02	136.7 $\pm$ 0.6	5.43 $\pm$ 0.02
1000	630.3 $\pm$ 32.5	867.7 $\pm$ 19.1	76.8 $\pm$ 1.0	5.51 $\pm$ 0.02	135.0 $\pm$ 3.0	5.39 $\pm$ 0.02
1250	738.3 $\pm$ 28.4	1051.7 $\pm$ 51.9	72.9 $\pm$ 1.7	5.37 $\pm$ 0.02	133.7 $\pm$ 1.2	5.35 $\pm$ 0.01
1500	939.7 $\pm$ 24.9	1232.3 $\pm$ 33.5	74.9 $\pm$ 1.7	5.47 $\pm$ 0.02	134.0 $\pm$ 2.6	5.31 $\pm$ 0.02

### 2.3 Biomass measurements

After harvest, the leaves (leaflet and petiole), stems, roots (taproot and lateral roots) were separated. Biomass measurement and calculation of the tolerance index (TI) are detailed in a previous paper.<sup>42</sup>

### 2.4 Measurement of root traits and leaflet area

The traits of taproots and lateral roots from an individual plant were determined as described by Shi *et al.*<sup>45</sup> The leaf area was scanned using a CI-203 laser area meter (CID Bio-Science Inc, USA).

### 2.5 Estimation of photosynthetic pigment concentrations

The concentrations of chlorophyll (Chl), Chl a, Chl b and carotenoids (Car) were estimated according to Poursattari and Hadi.<sup>46</sup>

### 2.6 Hydrogen peroxide, superoxide and MDA concentrations and antioxidative enzyme activity

The hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>•-</sup>), and malondialdehyde (MDA) concentrations in the fresh leaflets and lateral roots (0.2 g) of the control and treated seedlings were estimated according to Shi *et al.*<sup>47</sup> Fresh leaflets and lateral roots (0.5 g) were used to determine antioxidative enzyme activity. The superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities of leaflets and lateral roots were determined as described by Amjad *et al.*<sup>48</sup>

### 2.7 Determination of Pb and sub-cellular distribution

The plant samples were cut and ground with a ball mill, and then passed through a 0.149 mm sieve to determine the Pb concentration. Each dried powdered sample (0.2 g) was digested with a 5 mL mixture (4 : 1 (v/v), 65% HNO<sub>3</sub> and 70% HClO<sub>4</sub>). The Pb concentration was determined using inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer NexION300D, USA).

Fresh leaflets and lateral roots (0.5 g) were used to determine the subcellular distribution. The Pb concentrations in the cell wall fraction (F1), the nucleus rich fraction (F2), the mitochondrial fraction (F3) and the soluble fraction (F4) were determined as reported by Zhou *et al.*<sup>43</sup>

The bioconcentration factor (BCF) and translocation factor (TF) are two important indicators for evaluating

phytoremediation efficiency. They were calculated according to Li *et al.*<sup>17</sup>

### 2.8 Determination of EC<sub>50</sub>

The EC<sub>50</sub> (concentration for 50% of maximal effect) is the metal concentration that resulted in a 50% reduction in plant biomass compared to the control.<sup>49</sup> Critical toxicity thresholds were estimated according to Wang *et al.*<sup>49</sup>

### 2.9 Soil indicators

Soil samples were passed through 2 and 0.149 mm sieves. The former was used to determine the soil pH and the concentration of available soil Pb and K, and the latter was used to determine the total Pb concentration. The available soil Pb and K were extracted with DTPA (The components are 0.005 mol L<sup>-1</sup> DTPA-0.01 mol L<sup>-1</sup> CaCl<sub>2</sub>-0.1 mol L<sup>-1</sup> TEA) and then measured using ICP-MS. The total Pb and K concentrations were determined using ICP-MS after extraction with aqua regia. The pH value of the soil sample (soil : distilled ratio of 1 : 2.5, v/v) was measured using a Metro-pH320 pH meter (Mettler Toledo Instruments Ltd, China). Soil organic matter, available phosphorus and hydrolyzed nitrogen were determined following the K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> external heating method, alkaline hydrolysis-diffusion method and Molybdenum blue colorimetric method, respectively.<sup>50</sup>

### 2.10 Statistical analysis

Statistical software SPSS 22.0 was used for variance analysis to test the significance of differences with Fisher's least significant difference (LSD) procedure. Pearson's correlation analysis was performed to investigate the relationship between biomass and other studied parameters. The results were standardized and subsequently calculated using R (Ver. 4.2.0, package "FactoMineR" and "factoextra") for principal component analysis (PCA). The data were visualized using OriginPro (Ver. 9.5).

## 3 Results and discussion

### 3.1 Plant growth and tolerance index

In this study, the heights of *R. chinensis* did not increase after a 2 months trial in T3, T4, and T5, and toxicity symptoms, such as yellowing from the leaf tip and a reduction in leaf area, were also observed (Fig. S1†). Compared to T0, the dry biomass was significantly reduced in all Pb treatments ( $p < 0.01$ ,  $R^2 = 0.865$ –0.937, Table S1†), and the highest reduction recorded was



73.2% in T5 (Table 2). Compared with T0, the reduction in lateral root biomass under different treatments was lower than that in other organs and reduced by 7.9% (T1), 37.2% (T2), 39.9% (T3), 44.4% (T4) and 48.2% (T5), respectively. The reduction of the other organs' biomass was greater than 50% in all treatments, except T1. The TI values for plants in all Pb treatments were only 0.27–0.60 (Table 2). In addition, the Pb toxicity thresholds were determined for the leaves, stems, and roots (Table S2†). All EC<sub>50</sub> values were 709.0 mg kg<sup>-1</sup> (roots), 565.4 mg kg<sup>-1</sup> (stems) and 756.4 mg kg<sup>-1</sup> (leaves). Pb is not an essential nutrient for plants and its threshold value in plants is very low.<sup>51</sup> Thus, it is not surprising that the biomass of *R. chinensis* decreased and that visual damage under high Pb stress was observed in this study. Correlation analysis also verified that dry weight yield was negatively correlated with Pb concentrations in all organs (Fig. S2†). The results were similar to the findings of the dry biomass in *Chenopodium quinoa*,<sup>48</sup> *Sophora alopecuroides*,<sup>2</sup> and *Festuca arundinacea*<sup>6</sup> which decreased at a higher Pb stress. However, compared to the other species, the TI values were lower, especially under a higher Pb concentration.<sup>20,52,53</sup> This might be due to the soil pH value in this study being 5.31–5.66, making the available Pb concentration significantly higher than that in other studies. Generally, the availability of heavy metals is very important for the phytoremediation of contaminated soils.<sup>54</sup> However, the high mobility of Pb in soil can also affect plant growth.

The addition of different concentrations of CA and OA into the T3 growth medium ameliorated Pb toxicity in *R. chinensis*. However, the leaf area from T6 to T9 also decreased compared to T3, especially in T8 (Fig. S1†). Nevertheless, exogenous organic acids increased the leaflet biomass, indicating that they delayed the senescence process of *R. chinensis* under Pb stress. This phenomenon could also be explained by the fact that exogenous organic acids can compensate for the reduction of photosynthesis.<sup>1</sup> Furthermore, CA and OA application significantly increased the root biomass of seedlings compared to T3 (Table 2). The Pb-stressed seedlings treated with CA exhibited a decrease in the biomass of stem and petiole, respectively, compared with T3. The biomass of the stem under 1.0 mM OA treatments increased significantly ( $p < 0.05$ ), and those treated

with 0.5 mM OA were not significantly different from those of T3 (Table 2). The biomass under organic acid treatments was lower than that under T0 ( $p < 0.05$ ). The TI values of exogenous CA and OA treatments were 0.35–0.45, which were higher than those of the T3 (0.33) (Table 2). The biomass of *R. chinensis* was increased through the addition of CA and OA, although marginally relative to T3. This might be because CA and OA are the main constituents of *R. chinensis* root exudates and could decrease the toxic effects of free Pb ions as a result of organic acids providing vast amounts of small molecule ligands that induce metal chelation to form lower toxicity metal-organic complexes.<sup>32,55</sup> Moreover, the cause of this phenomenon probably lies in the fact that organic acids promote the uptake of nutrients in soils.<sup>11</sup> However, the results also showed that the organic acid treatments had different effects in mitigating toxicity. This difference might be due to the dose and type of organic acids.

### 3.2 Biomass allocation

After the experiment, the biomass allocation of *R. chinensis* was significantly different ( $p < 0.05$ , Table 2), and the lowest root-to-shoot ratio was found in T2. The root-to-shoot ratios in T6 and T7 were significantly higher than those in other treatments, and the values were 0.60 and 0.53, respectively. However, there were no statistically significant differences between the T3 and OA treatments according to the root-to-shoot ratio. Generally, plants under unfavorable conditions often adapt by altering their biomass allocation patterns.<sup>1</sup> In this study, the root-to-shoot ratio in Pb and organic acid treatments increased, except in T2. Thus, maintaining a certain level of biomass accumulation and steadily allocating more biomass to the root system is one of the reasons for *R. chinensis* to realize long-term adaptation in Pb-contaminated soil.<sup>1</sup> In the Pb treatments with combined CA and OA applications, there was a difference in the pattern of biomass allocation. After applying CA, a similar strategy of increasing root biomass allocation was found in this study. However, the root-to-shoot ratio decreased with increasing CA concentration, which might be related to the fact that a higher CA concentration was more likely to increase Pb availability in the soil, ultimately affecting root system

**Table 2** Mean harvest dry weights (g) of the component parts, the root-to-shoot ratio, and the tolerance index (TI) in *R. chinensis* under different treatments; different letters indicate significant difference between the treatments ( $p < 0.05$ ). The same below

Treatment	Petiole	Leaflet	Stem	Taproot	Lateral root	Root/shoot	TI
T0	2.28 ± 0.18 a	7.10 ± 0.46 a	9.73 ± 0.37 a	4.58 ± 0.05 a	1.57 ± 0.24 a	0.32 ± 0.01 bc	—
T1	1.05 ± 0.07 b	4.97 ± 0.21 b	5.11 ± 0.49 b	2.65 ± 0.79 b	1.45 ± 0.21 a	0.37 ± 0.06 b	0.60 ± 0.06 a
T2	1.07 ± 0.19 b	3.89 ± 0.93 bc	4.45 ± 0.55 b	1.49 ± 0.10 defg	0.99 ± 0.13 b	0.26 ± 0.02 c	0.47 ± 0.04 b
T3	0.98 ± 0.17 bc	1.81 ± 0.29 e	3.20 ± 0.60 c	1.30 ± 0.25 efg	0.94 ± 0.09 b	0.38 ± 0.06 b	0.33 ± 0.05 cde
T4	0.76 ± 0.18 cd	2.03 ± 0.42 de	3.04 ± 0.46 c	1.22 ± 0.28 fg	0.87 ± 0.16 b	0.36 ± 0.04 b	0.31 ± 0.06 de
T5	0.53 ± 0.10 d	1.71 ± 0.28 e	2.64 ± 0.39 c	1.05 ± 0.20 g	0.81 ± 0.14 b	0.39 ± 0.09 b	0.27 ± 0.02 e
T6	0.89 ± 0.17 bc	1.96 ± 0.32 de	3.16 ± 0.26 c	2.17 ± 0.30 bc	1.44 ± 0.08 a	0.60 ± 0.05 a	0.38 ± 0.04 c
T7	0.84 ± 0.08 bc	1.99 ± 0.09 de	3.00 ± 0.60 c	1.88 ± 0.10 cd	1.17 ± 0.16 ab	0.53 ± 0.08 a	0.35 ± 0.01 cd
T8	0.91 ± 0.17 bc	2.02 ± 0.07 de	3.27 ± 0.63 c	1.60 ± 0.15 def	0.93 ± 0.05 b	0.41 ± 0.03 b	0.35 ± 0.03 cd
T9	1.10 ± 0.18 b	3.13 ± 0.46 cd	4.42 ± 0.58 b	1.81 ± 0.13 cde	1.01 ± 0.11 b	0.33 ± 0.04 bc	0.45 ± 0.04 b
F	27.463	53.034	51.918	33.785	10.303	9.855	19.227
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001





development. When OA was added in T3, the opposite strategy of distributing more biomass to shoots was observed. This might be because OA could precipitate in the soil in the form of  $\text{PbC}_2\text{O}_4$ , which reduces free  $\text{Pb}^{2+}$  ions in soil, thus decreasing Pb concentrations in the roots to meet the demands of leaves for photosynthetic assimilates. Increasing the shoot biomass, especially leaflet biomass, compensates for the reduction in photosynthesis and thus promotes *R. chinensis* growth.<sup>1</sup>

### 3.3 Photosynthetic pigment concentration

Both Chl a, Chl b and Chl concentrations were reduced with the increase in Pb concentrations ( $R^2 = 0.930\text{--}0.947$ , Table S1†), and the highest reduction recorded was 33.6, 56.3, and 42.3%, respectively, in T5. When CA and OA were added to 1000 mg  $\text{kg}^{-1}$  Pb, the Chl concentration increased except in T7 with respect to T3 (Table S3†). In this study, biomass was positively correlated with Chl concentration (Fig. S2†), indicating that chlorophyll is an important component of plant growth.<sup>29</sup> In this study, the decrease in the chlorophyll concentration of *R. chinensis* under Pb exposure was a manifestation of impaired photosynthesis.<sup>11,26</sup> However, there were no statistically significant differences between the organic acid treatments and T3 in terms of chlorophyll concentration (Table S3†). This signified that the addition of CA and OA could alleviate the toxic effects of plants whose growth had been severely inhibited, but the results were not satisfactory. In addition, based on the chlorophyll concentration, it is speculated that one of the reasons for the increased biomass accumulation may be that exogenous LMWOAs promote photosynthesis.<sup>19,56</sup> Simultaneously, the chlorophyll concentration of organic acid treatments was in the order of  $\text{T9} > \text{T8} > \text{T6} > \text{T7}$  (Table S3†). These results also further demonstrated that the application of oxalic acid could better promote plant shoot development.

Overall, the carotenoid concentration was increased with an increasing Pb dose in this study ( $R^2 = 0.899$ , Table S1 and S3†). Moreover, the carotenoid concentrations of T6–T9 were higher than those of T0. Biswas *et al.*<sup>32</sup> and González-Peña *et al.*<sup>57</sup> indicated that non-enzymatic antioxidants, including carotenoids, can scavenge oxygen species (ROS) under heavy metal stress. Therefore, the carotenoid concentration increased with increasing Pb dosage in this study. However, variance analysis also showed that there were no significant differences between the organic acid treatments and T3. These results indicate that the non-enzymatic antioxidant carotenoids play a lesser mitigating role with the addition of CA and OA.

### 3.4 $\text{H}_2\text{O}_2$ , $\text{O}_2^{\cdot-}$ and MDA concentration

Typically, Pb can induce excessive ROS production, causing severe oxidative damage in plants.<sup>4,51</sup> In the present study, the MDA,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2^{\cdot-}$  concentrations of leaflets increased with increasing Pb dosage, especially in T3–T5 ( $R^2 = 0.888\text{--}0.976$ , Table S1†). Overall, Pb stress (T1–T5) increased leaflet MDA,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2^{\cdot-}$  accumulation by 1.27–2.14 folds, 1.12–1.76 fold, and 1.13–1.88 fold, respectively, compared to T0. Similar to the leaflets, the MDA,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2^{\cdot-}$  concentrations of the lateral roots were also significantly elevated with Pb treatment alone

(Fig. 1). Furthermore, correlation analysis revealed a very significant positive correlation between the tissues for MDA,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2^{\cdot-}$  concentration and Pb concentration and a significant negative correlation with biomass (Fig. S2†). Most studies have shown that LMWOAs alleviate the toxicity of heavy metals in plants by reducing the level of membrane lipid peroxidation.<sup>19,58</sup> In the present study, this phenomenon was also observed. The MDA,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2^{\cdot-}$  concentrations of leaflets and lateral roots in T6–T9 decreased compared to T3, except in T7 and T8 in leaflets (Fig. 1), suggesting that CA and OA could ameliorate oxidative stress in plants through complexation with Pb.<sup>32,59</sup>

### 3.5 Antioxidant activities in *R. chinensis*

To protect against the damage caused by elevated ROS levels, plants possess certain defense mechanisms, such as anti-oxidative systems, which have enzymatic and non-enzymatic systems.<sup>51</sup> Among these, the enzymatic antioxidant system of plants is one of the most effective mechanisms for coping with the toxic effects of free radicals.<sup>6</sup> In this study, antioxidant activity (SOD, POD, and CAT) was significantly stimulated in solo Pb treatment (T1–T5) in relation to the control (T0), except for SOD and CAT activity of lateral roots in T1 (Fig. 1). Except for the POD activity of the leaves, all of them can be described using a linear equation ( $p < 0.05$ ,  $R^2 = 0.752\text{--}0.794$ , Table S1†). The highest values of SOD and CAT activity were found in T4, and the highest value of POD activity was observed in T3. Additionally, in this study, biomass was negatively correlated with antioxidant enzyme activities (Fig. S2†). These phenomena are in line with findings in *Festuca arundinacea*,<sup>6</sup> *Trifolium pratense*.<sup>9</sup> Furthermore, the activities of SOD, POD, and CAT showed a low–high–low trend with the increase in Pb concentration. This may be due to the oxidative stress induced by the high Pb concentration, which exceeds the ability of the antioxidant system to cope.<sup>11,60</sup> Many studies have indicated that under heavy metal stress, LMWOAs can help plants mitigate damage by improving the activity of antioxidant enzymes.<sup>32,36</sup> In this study, the application of organic acids also notably stimulated antioxidant activities in *R. chinensis* compared with T0 (Fig. 1). Although variance analysis also showed that there were no significant differences between organic acid treatments and T3 according to antioxidant enzyme activity, the POD and CAT activities in leaflets and lateral roots in organic acid treatments increased by 10–30% compared with T3 (Fig. 1). These results indicate that CA and OA can help *R. chinensis* mitigate damage by improving the activity of antioxidant enzymes.<sup>32,36</sup>

### 3.6 Accumulation and translocation of Pb

Linear regression analysis revealed that Pb accumulation was strongly dependent on Pb concentration in the soil in the case of each stem and root ( $R^2 = 0.861$  and  $0.872$ , respectively, Table 3 and S1†). The Pb concentration across organs decreased in the order lateral roots > petiole > stem > taproot > leaves. *Rhus chinensis* accumulated more Pb in T4 than in the other Pb treatments. Moreover, only a small quantity of Pb was translocated to the shoots, and the BCF of shoots and TF values of *R.*

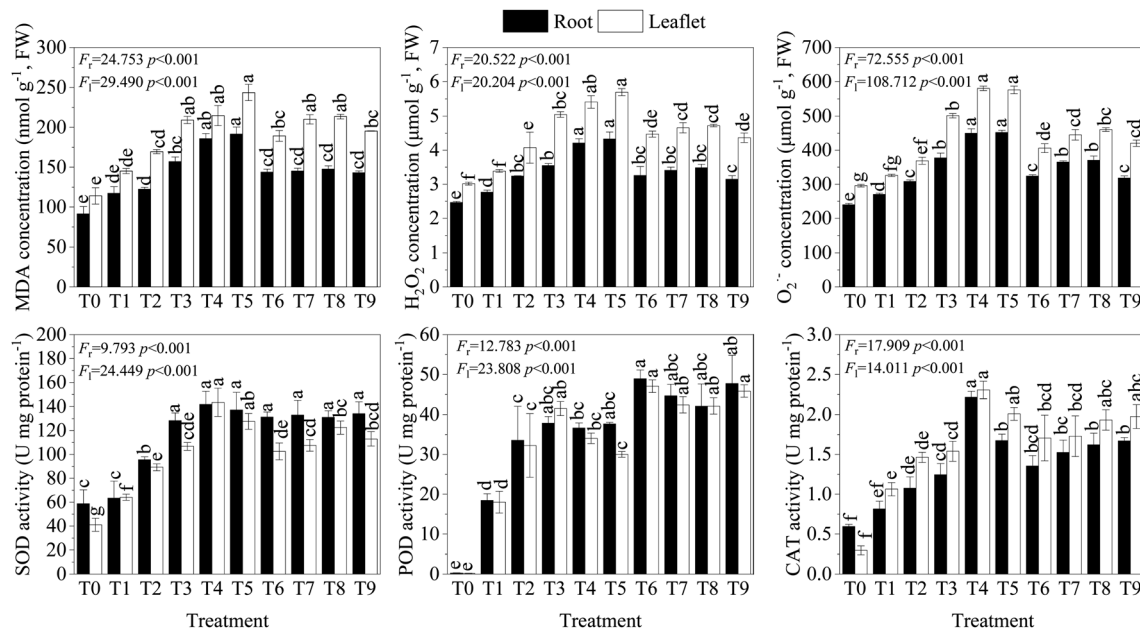


Fig. 1 Effects of different Pb and organic acid treatments on antioxidant enzyme (SOD, POD and CAT) activity and MDA,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2^{\cdot-}$  concentration in *R. chinensis*. Data over bars marked by the same letters are not significantly different at  $p < 0.05$ . Each value represents the mean of three replicates  $\pm$  SE. The same below.

*chinensis* were less than 0.2 and 1.0, respectively (Fig. S3†). Under similar Pb concentration treatments, the Pb concentration in the roots of *R. chinensis* was higher than that of other species.<sup>1,51,54,61</sup> This might be related to the lower soil pH in this experiment. Generally, higher Pb solubility with lower soil pH is therefore available for plant uptake.<sup>7,54</sup> Nevertheless, the highest Pb concentration did not exceed  $1000 \text{ mg kg}^{-1}$  in the lateral roots in T4 and T6, and the root BCF values were less than 0.5 in this study (Table 3 and Fig. S3†). However, previous studies have shown that *R. chinensis* can accumulate more than  $1000 \text{ Pb mg kg}^{-1}$  in roots under higher Pb stress in the relatively short term.<sup>42,43</sup> The difference indicated that it was difficult for *R. chinensis* to uptake a large amount of Pb under long-term stress with a high concentration of available Pb. In the current study, only a small quantity of Pb was translocated to shoots (Fig. S3†), which is similar to previous studies<sup>42,43</sup> and other non-

hyperaccumulator plants.<sup>4,6,9,31</sup> One possible explanation is the exclusion strategy in plants.<sup>29</sup>

The application of LMWOAs significantly increased the Pb concentration in plants.<sup>20,31,37</sup> However, many studies have suggested that LMWOAs have no significant effect on plant Pb uptake.<sup>38,39</sup> In this study, the Pb concentration in the lateral roots, stem, petiole, and leaflets in T6 increased significantly by 1.53, 1.59, 1.99, and 2.27 times compared with T3. The Pb concentration in T7 also increased significantly compared with T3, except in the lateral roots. The Pb concentrations of the aboveground parts of *R. chinensis* in T8 and T9 increased significantly compared with that of T3, while the Pb concentrations in the roots were slightly reduced in T9 and significantly decreased in T8. In addition, while CA and OA applications significantly increased the TF values compared to T3, the TF values were 0.32 (T6), 0.33 (T7), 0.54 (T8), and 0.42

Table 3 The Pb concentration ( $\text{mg kg}^{-1}$ ) in *R. chinensis* under different treatments

Treatment	Petiole	Leaflet	Stem	Taproot	Lateral root
T0	$1.23 \pm 0.40$ h	$0.93 \pm 0.32$ f	$1.20 \pm 0.17$ d	$1.97 \pm 0.57$ f	$4.43 \pm 0.50$ e
T1	$109.87 \pm 3.23$ f	$24.60 \pm 2.38$ e	$67.39 \pm 3.19$ c	$23.73 \pm 1.64$ ef	$242.70 \pm 41.10$ d
T2	$119.87 \pm 3.11$ e	$27.10 \pm 8.90$ de	$61.47 \pm 3.01$ c	$33.90 \pm 8.43$ de	$396.80 \pm 61.10$ c
T3	$123.60 \pm 2.50$ e	$38.30 \pm 8.49$ cd	$73.54 \pm 1.44$ c	$58.53 \pm 4.81$ bcd	$583.90 \pm 93.00$ b
T4	$133.33 \pm 1.80$ d	$43.60 \pm 8.74$ c	$97.31 \pm 8.04$ b	$82.13 \pm 7.69$ ab	$808.80 \pm 33.10$ a
T5	$102.93 \pm 3.78$ g	$27.40 \pm 10.83$ de	$94.80 \pm 15.50$ b	$95.20 \pm 32.00$ a	$557.80 \pm 64.50$ b
T6	$246.13 \pm 2.41$ a	$87.10 \pm 9.61$ a	$117.08 \pm 12.03$ a	$72.40 \pm 30.30$ abc	$896.00 \pm 81.50$ a
T7	$136.80 \pm 1.74$ d	$48.70 \pm 3.06$ c	$118.03 \pm 7.51$ a	$94.67 \pm 2.41$ a	$575.10 \pm 96.10$ b
T8	$144.00 \pm 8.27$ c	$46.37 \pm 1.35$ c	$103.23 \pm 16.74$ ab	$62.53 \pm 1.01$ bc	$358.47 \pm 6.65$ c
T9	$155.47 \pm 1.67$ b	$63.20 \pm 3.00$ b	$96.75 \pm 1.21$ b	$50.00 \pm 4.85$ cd	$525.00 \pm 37.00$ b
F	865.021	35.767	44.482	13.316	55.609
p	<0.001	<0.001	<0.001	<0.001	<0.001

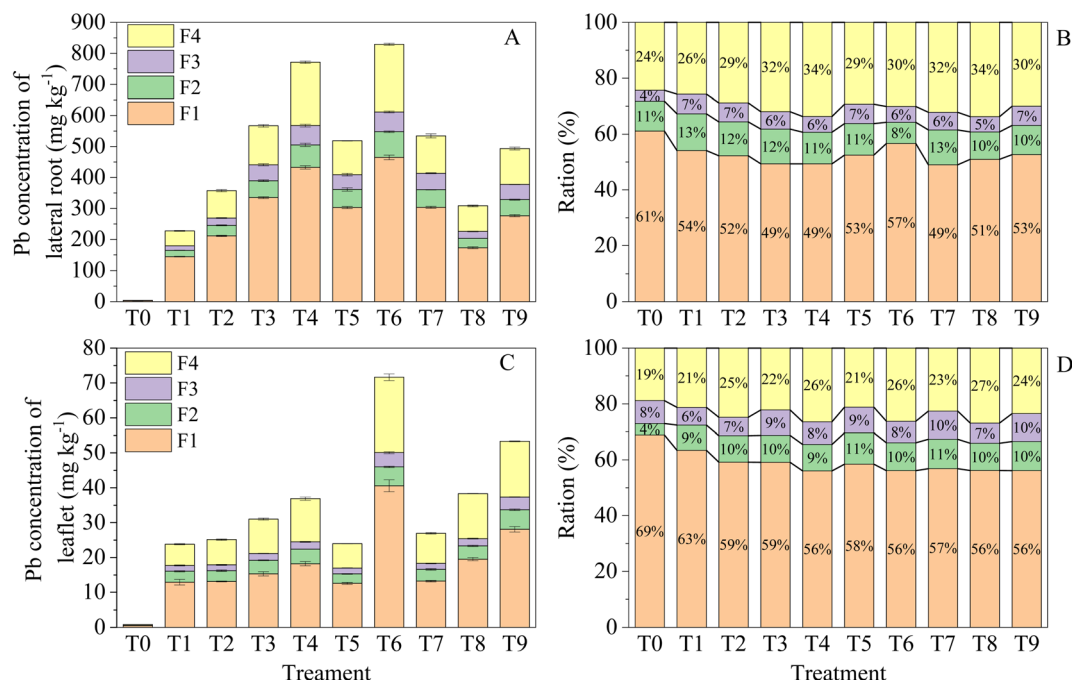


Fig. 2 Pb subcellular distribution in *R. chinensis* under different treatments. (A) and (C): Pb concentrations of four subcellular fractions in lateral root and leaflet tissues, respectively. (B) and (D): Pb distribution ratios of four subcellular fractions in lateral root and leaflet tissues, respectively. F1: cell wall fraction; F2: nucleus rich fraction; F3: mitochondrial fraction; F4: the soluble fraction.

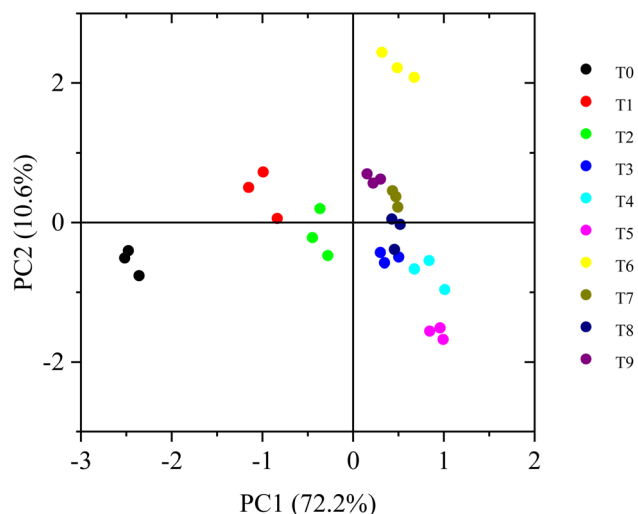


Fig. 3 Principal component analysis of physiological growth and Pb accumulation indexes under different Pb and organic acid treatments.

(T9) (Fig. S3†). The cause of this phenomenon probably lies in the fact that exogenous LMWOAs promote radial transport of Pb in roots.<sup>19</sup> In general, the increase in Pb accumulation and translocation might be attributed to the acidifying soil pH. However, after the experiment, there was no statistically significant difference in pH among the treatments (pH: 4.75–4.86), which is consistent with the conclusion of many studies indicating that the application of LMWOAs has little effect on the pH of contaminated soil.<sup>31,62</sup> Therefore, the complexation of

CA and OA plays a major role in promoting the bioavailability and mobility of Pb in the soil. The current study showed that CA and OA have different performances in Pb accumulation and translocation in plants. Overall, the Pb concentration in the organs of *R. chinensis* in OA treatment was lower than that in CA treatment, especially in the roots. The difference in efficiency between CA and OA was due to the following three aspects: first, the different complexation ability of the two organic acids in Pb. Second, the different degree of H<sup>+</sup> provided by CA and OA, and then, the different effects on soil pH in the short term. Third, the heavy metal concentrations in the roots were significantly different in different order roots.<sup>63</sup> Generally, low-order roots are responsible for water and nutrient uptake.<sup>64</sup> In this study, the development of lateral roots under CA treatment was significantly better than that under OA treatment (Table 2 and S4†), which might explain why CA treatment generally had a higher Pb concentration than OA treatment in this study. Simultaneously, with the further increase in the CA concentration in the soil, the Pb concentration in organs decreased, which might be attributed to the fact that a high CA concentration increased phytotoxicity to plants.<sup>65</sup> OA showed the opposite trend. A low concentration of oxalic acid is easily adsorbed by aggregates and organic matter in soil and has a poor activation effect on Pb in soil.

### 3.7 Sub-cellular distribution of Pb

Compared to T0, the ratio of Pb distributed in F1 significantly decreased in the Pb treatment alone (Fig. 2B and D). In addition, the proportions of Pb deposited in the mitochondrial fraction and soluble fraction increased in the leaflets (Fig. 2B).

In the lateral roots, the proportions of trophoplasts and soluble fractions also increased with respect to T0 (Fig. 2D). Furthermore, a large amount of Pb remained and was immobilized in the cell wall or vacuole, and the biomass was closely related to the subcellular distribution ratio of Pb. Similar results were also found in *Eremochloa ophiuroides*<sup>49</sup> and *Sasa argenteostriata*<sup>66</sup> under Pb stress. Therefore, the storage of Pb in the cell wall and soluble fractions would help *R. chinensis* alleviate Pb toxicity.<sup>42,43</sup> Compared with T3, the organic acid treatment did not significantly change the subcellular distribution of Pb in the leaflets and lateral roots, except leaflets in T6 (Fig. 2). One reason could be that organic acids can be degraded in the soil in a shorter time.

### 3.8 Phytoremediation efficiency

Phytoremediation efficiency is related to biomass production and heavy metal concentrations in plants; the total content of heavy metals accumulated by plants is the more important factor for evaluating phytoremediation efficiency.<sup>4,20,67</sup> In this study, Pb accumulation in acidic soil without chelator application ranged between 0.91 and 1.29 mg per plant, and the highest Pb content was observed in T4 due to the highest concentration in tissues (Table S5†). Moreover, the highest Pb content in leaflets was found in T1 because its biomass production was significantly higher than that of the other treatments (Table S5†). Compared with other studies, the Pb content of *R. chinensis* in this study was relatively high.<sup>1,51</sup> The application of CA and OA increased Pb accumulation, except in T8 (Table S5†). This was mainly due to the biomass significantly increasing as the Pb concentration changed slightly. By comparing both chelators, 0.5 mM CA performed better in enhancing Pb accumulation, and the Pb content was 2.20 mg per plant. Exogenous 1.0 mM OA showed the best promoting effects on Pb transport, and the shoot Pb content was 0.79 mg per plant. Therefore, the application of 0.5 mM CA and 1.0 mM OA can obtain twice the phytoremediation efficiency.

### 3.9 PCA analyses

To discuss the responses of *R. chinensis* to exposure to Pb and Pb + organic acids, PCA analysis was performed using the biomass, leaf area, root length, ROS, antioxidant, and Pb concentration data from all organs. According to the PCA results, two principal components explained 82.85% of the total variance (Table S6†). Table S6† shows that the first component represented 72.22% of the total variance and was positively linked to the Pb concentration in the roots and stems, carotenoid concentration, membrane lipid peroxidation levels, and antioxidase activity in leaflets and negatively linked to biomass (except the lateral roots), leaf area, lateral root length, and chlorophyll concentration. PC2 accounted for 10.63% of the total variance and was positively linked to the biomass of the lateral roots, Pb concentration in leaflets and petiole. PC1 delineated the effect of Pb treatments, and PC2 reflected variations in the influence of Pb combined with organic acid treatments (Fig. 3). These results further indicate that exogenous LMWOAs actively regulate the physiological growth process of *R. chinensis* under Pb

stress in different ways. Their positive effects were reduced in the order of T6 > T9 > T7 > T8 (Fig. 3).

## 4. Conclusion

In short, the toxic effects of exogenous Pb exhibited a concentration-dependent pattern. *Rhus chinensis* allocates more biomass to the roots and promotes lateral root development. In addition, we found that *R. chinensis* retained a large amount of Pb in the cell walls of tissues, which probably improved the tolerance in Pb-contaminated soil. Furthermore, to protect against the damage, SOD, POD, and CAT were significantly stimulated in all treatments. Our findings indicate that organic acids can use two different strategies of biomass allocation and Pb accumulation and distribution to mitigate Pb stress. Citric acid application increased Pb accumulation in roots and shoots and promoted root system development. Oxalic acid improved shoot biomass production and the capacity for Pb translocation. The order of their promoting effects was 0.5 mM CA > 1.0 mM OA > 1.0 mM CA = 0.5 mM OA. Therefore, the combined application of 0.5 mM CA and 1.0 mM OA in *R. chinensis* could effectively remediate Pb-contaminated soil.

## Ethical statement

The authors declare that there is no conflicts of interest exists among fundings, and no Human Participants and/or Animals involved.

## Author contributions

Xiang Shi: conceptualization, methodology, original draft preparation. Shufeng Wang: methodology. Wenxiang He: investigation. Yangdong Wang: reviewing and editing.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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