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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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Materials and methods

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 (leaflet and petiole), stems, roots (taproot and lateral roots) were separated. The dry biomasses of samples were measured after drying at 80 °C for 72 hours. The tolerance index (TI) based on biomass was calculated for each treatment as TI = B_t/B_c, where Bt (g plant^{-1}) is treatment biomass and B_c (g plant^{-1}) is control biomass. High values indicate high tolerance by plants.

Measurement of root traits

All roots from an individual plant were scanned while fresh using a root positioning system/STD4800 scanner (Regent Instruments Inc., Québec, Canada) at the end of the experiment. From the images obtained, the characteristics of roots (mean total length, mean surface area, mean volume, mean diameter, and mean number of root tips) were analyzed using WinRHIZO Pro 2005b software (Regent Instruments).

Estimation of photosynthetic pigment concentrations

Weigh 0.1 g of the leaf and place it in a porcelain mortar. After adding some acetone (80%), the leaf pieces were completely ground, and their volume was increased to 10 mm with acetone. The solution was used to measure photosynthetic pigments. For this purpose, the absorption of the solution was recorded using a spectrophotometer at 645, 663, and 470 nm. Chlorophyll and carotenoids were obtained based on the following equations:

Chlorophyll a = (19.3 × A633 − 0.86 × A645)V/100W

Chlorophyll b =(19.3 × A645 − 3.6 × A663)V/100W

Carotenoid = (1000A470 − 1.82Chlorophyll a − 85.02chlorophyll b)

Hydrogen peroxide, superoxide and MDA concentrations

A total of 0.2 g of fresh leaf and root tissue were weighed and rubbed in a mortar containing 5 ml of 0.1% trichloroacetic acid (TCA). The extract was centrifuged at 10,000 g for 5 min. The 1 ml of the supernatant from the centrifuge was added to 5.4 ml of 20% TCA solution containing 0.5% thiobarbituric acid (TBA). The resulting mixture was heated in a warm water bath for 95 min at 95 °C. It was immediately cooled in ice and centrifuged again for 10 min at 10,000 g. The absorbance of this solution was read using a spectrophotometer at 532 nm. The material to be absorbed in this wavelength

is the MDA-TBA red complex. The absorbance of the other nonspecific pigments was determined at 600 nm and subtracted from this value. The MDA concentration was calculated using the extinction coefficient of 155 cm^{-1} mM^{-1} , and the results from the following relationship were calculated and presented in terms of unit/g fresh weight (FW).

H_2O_2 was extracted by homogenizing 0.2 of leaf and root tissue with 3 ml of 50 mM phosphate buffer at pH = 7. Then, the homogenate solution was centrifuged at 6000 g for 25 min. A 3 ml of the extracted solution was mixed with 1 ml of titanium sulfate 0.1% in 20% (V/V) H_2SO_4 , the resulting mixture was centrifuged at 6000 g for 15 min. The yellow color intensity supernatant was measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient of 0.28 μmol^{-1} cm^{-1}.

The fine powder of leaf and root tissue (0.2 g) was homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7.8) and centrifuged (10000 g, 4°C, 10 min). One millilitre of the supernatant was mixed with 0.9 ml of 50 mM potassium phosphate buffer (pH 7.8) and 0.1 ml of 10 mM hydroxylamine hydrochloride. Subsequently, the reaction mixture was incubated at 25°C for 20 min before adding 1 ml of 17 mM p-aminobenzene sulphonic acid and 1 ml of 7 mM α-naphthylamine. After further incubation (25°C, 20 min), the absorbance of the mixture was recorded spectrophotometrically at 530 nm.

Antioxidative enzyme activity

For leaf and root extract, 0.5 g of leaf samples was homogenized in potassium phosphate buffer (pH = 7) in pre-cooled mortar pestle. Samples were then centrifuged at 4 °C for 20 min at 10000 g and supernatant was collected for the determination of antioxidant enzymes

Superoxide dismutase (SOD)

The reaction mixture consisted of 0.05 µL of enzyme extract, 1.5 ml of 100 mM phosphate buffer, 0.1 ml of 200 mM EDTA, 0.1 ml of 200 mM methionine and 0.01 ml of 2.25 mM NBT, 1 ml of distilled water and 0.1 ml of 60 µL riboflavin. In order to start the reaction, the reaction mixture was kept under 30 W fluorescent lamp for 15 min, the distance between lamp and reaction mixture was 30 cm. The absorbance was measured at 560 nm using UV–VIS spectrometer.

Catalase (CAT)

Assay mixture was prepared by using 40 µL enzyme extract, 1 ml of 50 mM phosphate buffer and 5 µL of H_2O_2. The decomposition of H_2O_2 was measured by recording the absorbance at 240 nm for 1 min.

Peroxidase (POD)

Assay mixture was prepared by using 40 μL of enzyme extract, 15 μL of H_2O_2, 20 μL of guaiacol. The decrease in concentration of guaiacol was analyzed at 436 nm. The enzyme activity was expressed in μmol guaiacol per minute per milligram of protein.

Determination of Pb and sub-cellular distribution

The plant samples were cut and ground with a ball mill, and then passed through a0.149-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_3 and 70% $HClO_4$). The Pb concentration was determined using inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elemer NexION300D, USA).

Fresh leaflets and lateral roots (0.5 g) were used to determine the subcellular distribution. Frozen plant materials were homogenized using a chilled pestle and mortar with 20 mL of pre-chilled extraction buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM sucrose, and 1.0 mM dithioerythritol. The homogenate was transferred to a 50-mL centrifuge tube and centrifuged at 300 g for 10 min at 4 °C, and the pellet was collected as the cell wall fraction (F1). The filtrate was centrifuged at 2000g for 10 min, and the pellet was the nucleus rich fraction (F2). The supernatant was then centrifuged at 10,000 g for 30 min. The pellet was designated the mitochondrial fraction (F3) and the supernatant as the soluble fraction (F4). All homogenizations and subsequent fractionations were performed at 4 °C.

The bioconcentration factor (BCF) at the end of the experiment was calculated as BCF = $A_{tissues}/A_{soil}$, where $A_{tissues}$ (mg kg^{-1}) is the total heavy metal accumulated in roots or shoots, and A_{soil} (mg kg^{-1}) is the heavy metal concentration in the soil.

The translocation factor (TF) at the end of the experiment was calculated as TF = A_s/A_r, where A_s and A_r are total heavy metals accumulated in shoots and in roots, respectively (both in mg kg^{-1}).

Results and discussion

Root morphological

After exposure to Pb, the taproot systems of *R. chinensis* seedlings were significantly inhibited, especially in T3, T4 and T5. The root morphological parameters of taproot decreased significantly with the increase of Pb concentration compared to T0 ($p < 0.05$, Table S4), and the root length, the root surface area, average root diameter and root volume of T1 to T5 decreased by 24.7%-66.1%, 50.9%-80.5%, 43.8%-62.2% and 71.9%-92.0%, respectively. When organic acid was added in T3, the

morphological parameters of taproot were increased, except T8 (Table S4). Unlike the taproot, the lateral roots of *R. chinensis* seedlings developed better with treatment less than 1000 mg kg^{-1} (Table S4). In contrast with T0, the morphological parameters of lateral roots decreased to some extent in T3, T4 and T5, except root length in T3, with decreasing by -1.2%-22.3% (root length), 5.4%-23.5% (root surface area), 6.4%-23.8% (mean root diameter) and 12.7%-25.4% (root volume), respectively. Application of CA and OA markedly affected lateral root morphological parameters in *R. chinensis* (Table S4). In T6, T7 and T9, the root length increased compared to T0 and T3. However, the root length of T8 was impaired. Meanwhile, the change of the other root morphological parameters showed the same trend observed for root length described above.

Phytoremediation efficiency

R. chinensis is a small shrub and the biomass of per unit area (m^2) can be increased by dense planting, eventually improving the efficiency of phytoremediation. If two-year-old *R. chinensis* seedlings were used. According to our previous study, the biomasses of two-year-old *R. chinensis* seedlings were about 7.09 g (shoot) and 7.67 g (root) in Pb/Zn tailing. According to the data obtained in this study, the biomass of T3 can be set as 7.09 g (shoot), 7.67 g (root), T6 can be set as 7.09 g (shoot), 12.33 g (root) and T9 can be set as 10.21 g (shoot), 9.66 g (root), ultimately, *R. chinensis* could accumulate 21.28 (T3), 46.24 (T6), and 24.88 (T9) mg per unit area (m^2) $year^{-1}$ Pb from soil when the planting density was 8 plants per m^2. The results of this study revealed that rational application of exogenous organic acids could significantly improve the efficiency of phytoremediation in Pb heavily contaminated soil.

Table S1 Analysis of significance for linear regressions displaying relationships between Pb accumulation, growth and physiological parameters of *R. chinensis* response to Pb treatment (dependent variables) and metal concentration in cultivation medium (independent variable) at $\alpha = 0.05$ (R^2–coefficient of determination, *p*–empirical level of significance).

Dependent variables (y)	Linear regression analysis (Pb addition level as an independent variable x)		
	R^2	p	Regression equation
Root biomass	0.871	<0.01	y = -0.0029x + 5.5592
Stem biomass	0.865	<0.01	y = -0.0045x + 8.4810
Leaf biomass	0.937	<0.01	y = -0.0049x + 8.7632
Chl a	0.930	<0.01	y = -0.0003x + 1.3338
Chl b	0.945	<0.01	y = -0.0006x + 0.8001
Chl	0.947	<0.01	y = -0.0006x + 2.1339
Car	0.899	<0.01	y = 6E-05x + 0.0887
Leaf area	0.967	<0.001	y = -0.0202x + 40.351
Root length	0.377	0.195	y = -0.1999x + 1081.9
Root surface	0.477	0.129	y = -0.0431x + 215.79
Root diameter	0.430	0.157	y = -0.0003x + 1.6148
Root volume	0.547	0.093	y = -0.0007x + 3.4736
Pb concentration of leaf	0.617	0.064	y = 0.0359x + 15.036
Pb concentration of stem	0.861	<0.01	y = 0.0599x + 16.001
Pb concentration of root	0.872	<0.01	y = 0.2439x + 4.4993
Root MDA	0.945	<0.01	y = 0.0727x + 83.702
Leaf MDA	0.976	<0.001	y = 0.0887x + 108.86
Root $O_2^{\bullet-}$	0.953	<0.001	y = 0.0014x + 2.295
Leaf $O_2^{\bullet-}$	0.944	<0.01	y = 0.002x + 2.7774
Root H_2O_2	0.923	<0.01	y = 0.1612x + 215.45
Leaf H_2O_2	0.888	<0.01	y = 0.2218x + 256.72
Root SOD	0.877	<0.01	y = 0.0644x + 50.596
Leaf SOD	0.914	<0.01	y = 0.0683x + 38.46
Root POD	0.836	<0.05	y = 0.0257x + 6.0057
Leaf PDD	0.654	0.051	y = 0.0221x + 7.5779
Root CAT	0.752	<0.05	y = 0.001x + 0.4781
Leaf CAT	0.909	<0.01	y = 0.0013x + 0.3986

Table S2 Calculated EC_{50} toxicity thresholds, models, R^2 and P for root, stem and leaf of *R. chinensis* exposed to increasing levels of Pb.

	Regression equation	R^2	*p*	EC_{50} (mg kg^{-1})
Root	$y = 5.6812e^{-8E-04x}$	0.954	<0.001	709.0
Stem	$y = 8.9785e^{-9E-04x}$	0.965	<0.001	565.4
Leaf	$y = 9.4933e^{-0.001x}$	0.978	<0.001	756.4

Table S3 The photosynthetic pigment content (mg g^{-1}, FW) in *R. chinensis* under different treatments.

Treatment	Chla	Chlb	Chl	Car
T0	1.286 ± 0.146 a	0.784 ± 0.096 a	2.071 ± 0.240 a	0.084 ± 0.014 d
T1	1.214 ± 0.164 ab	0.649 ± 0.115 b	1.863 ± 0.278 ab	0.133 ± 0.003 bc
T2	1.180 ± 0.077 abc	0.637 ± 0.026 bc	1.817 ± 0.102 ab	0.120 ± 0.016 c
T3	1.023 ± 0.059 bcd	0.438 ± 0.024 de	1.461 ± 0.082 cd	0.144 ± 0.013 b
T4	0.978 ± 0.131 cd	0.422 ± 0.059 de	1.401 ± 0.190 cd	0.151 ± 0.015 b
T5	0.853 ± 0.129 d	0.343 ± 0.071 e	1.196 ± 0.199 d	0.178 ± 0.015 a
T6	1.111 ± 0.241 abc	0.463 ± 0.115 de	1.573 ± 0.355 bc	0.150 ± 0.021 b
T7	1.020 ± 0.061 bcd	0.429 ± 0.050 de	1.450 ± 0.104 cd	0.142 ± 0.018 bc
T8	1.116 ± 0.055 abc	0.473 ± 0.015 d	1.589 ± 0.070 bc	0.135 ± 0.005 bc
T9	1.132 ± 0.084 abc	0.527 ± 0.051 cd	1.658 ± 0.135 bc	0.143 ± 0.011 bc
F	2.913	10.615	5.043	8.862
p	**0.022**	**<0.001**	**<0.001**	**<0.001**

Table S4 The root morphological parameters of taproot and lateral root in *R. chinensis* under different treatments. Different letters indicate significant difference between the treatments ($p < 0.05$). Each value represents the mean of three replicates ± SD. The same below.

Treatment	Organ	Length (cm)	Surface area (cm^2)	Volume (cm^3)	Diameter (mm)
T0	Taproot	50.87 ± 2.78 a	99.65 ± 15.72 a	10.50 ± 3.10 a	4.00 ± 0.61 a
T1		38.28 ± 6.31 b	48.97 ± 9.98 b	2.95 ± 0.87 b	2.25 ± 0.33 b
T2		20.28 ± 5.09 c	27.11 ± 4.29 cd	1.83 ± 0.36 bc	2.12 ± 0.80 b
T3		20.88 ± 7.31 c	27.12 ± 3.45 cd	1.56 ± 0.30 bc	1.81 ± 0.62 b
T4		20.97 ± 7.00 c	25.12 ± 4.56 cd	1.17 ± 0.21 bc	1.54 ± 0.47 b
T5		17.27 ± 2.47 c	19.47 ± 2.50 d	0.84 ± 0.13 c	1.51 ± 0.20 b
T6		25.08 ± 1.73 c	34.57 ± 4.26 c	1.75 ± 0.41 bc	1.84 ± 0.23 b
T7		24.83 ± 1.49 c	30.36 ± 0.56 cd	1.59 ± 0.12 bc	1.93 ± 0.07 b
T8		16.07 ± 3.45 c	23.76 ± 3.86 cd	1.11 ± 0.23 c	1.57 ± 0.42 b
T9		23.67 ± 9.01 c	29.01 ± 7.42 cd	2.02 ± 0.29 bc	2.18 ± 0.61 b
F		12.318	33.841	22.391	6.670
p		**<0.001**	**<0.001**	**<0.001**	**<0.001**
T0	Lateral	898.50 ± 72.00 cde	184.60 ± 19.40 cde	3.04 ± 0.40 bcd	1.41 ± 0.19 cd
T1	root	1190.20 ± 108.80 ab	233.50 ± 36.90 ab	3.74 ± 0.84 ab	1.81 ± 0.31 ab
T2		1022.40 ± 132.80 bc	196.70 ± 20.80 bcd	3.05 ± 0.37 bcd	1.33 ± 0.20 cd
T3		909.90 ± 71.70 cde	174.64 ± 13.03 cdef	2.69 ± 0.19 cd	1.32 ± 0.18 cd
T4		772.60 ± 106.00 de	148.80 ± 20.70 ef	2.33 ± 0.33 d	1.25 ± 0.03 d
T5		698.40 ± 94.00 e	141.22 ± 11.35 f	2.29 ± 0.08 d	1.08 ± 0.13 d
T6		1375.80 ± 165.00 a	265.94 ± 10.73 a	4.19 ± 0.28 a	1.86 ± 0.14 a
T7		1074.00 ± 242.00 bc	208.30 ± 47.00 bc	3.23 ± 0.74 bc	1.45 ± 0.36 bcd
T8		755.40 ± 58.20 e	157.00 ± 1.09 def	2.64 ± 0.18 cd	1.36 ± 0.12 cd
T9		972.50 ± 86.60 cd	202.40 ± 25.00 bc	3.39 ± 0.53 bc	1.68 ± 0.39 abc
F		8.388	7.762	5.307	7.717
p		**<0.001**	**<0.001**	**0.001**	**<0.001**

Table S5 The Pb content (mg) in *R. chinensis* under different treatments.

Treatment	Petiole	Leaflet	Stem	Taproot	Lateral root
T0	0.003 ± 0.001 e	0.007 ± 0.002 e	0.012 ± 0.001 e	0.009 ± 0.003 d	0.007 ± 0.000 e
T1	0.115 ± 0.008	0.122 ± 0.009 b	0.345 ± 0.037 abc	0.063 ± 0.017 b	0.345 ± 0.009 d
T2	0.128 ± 0.021 c	0.104 ± 0.035 bc	0.274 ± 0.040 bcd	0.050 ± 0.009 cd	0.393 ± 0.049 d
T3	0.122 ± 0.021 c	0.068 ± 0.005 cd	0.235 ± 0.045 d	0.075 ± 0.008 bc	0.551 ± 0.055 bcd
T4	0.101 ± 0.025 c	0.086 ± 0.002 bcd	0.298 ± 0.067 bcd	0.099 ± 0.016 b	0.705 ± 0.075 b
T5	0.055 ± 0.012 d	0.048 ± 0.024 d	0.252 ± 0.068 cd	0.100 ± 0.034 b	0.459 ± 0.071 cd
T6	0.218 ± 0.042 a	0.173 ± 0.048 a	0.369 ± 0.031 ab	0.157 ± 0.070 a	1.285 ± 0.057 a
T7	0.115 ± 0.012 c	0.097 ± 0.011 bc	0.351 ± 0.049 ab	0.178 ± 0.014 a	0.666 ± 0.056 bc
T8	0.132 ± 0.031 bc	0.094 ± 0.006 bc	0.345 ± 0.114 abc	0.100 ± 0.009 b	0.334 ± 0.014 d
T9	0.171 ± 0.026 b	0.199 ± 0.039 a	0.427 ± 0.057 a	0.091 ± 0.011 bc	0.527 ± 0.035 bcd
F	19.374	16.126	11.632	10.076	45.844
p	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table S6 The eigenvalues and principal component extraction results of all test parameters.

Variables	PC1	PC2	PC3	PC4
Biomass of taproot	-0.24	0.12	0.28	0.09
Biomass of lateral root	-0.18	0.36	0.42	-0.08
Biomass of stem	-0.25	-0.05	0.00	0.11
Biomass of petiole	-0.24	-0.01	-0.01	0.41
Biomass of leaflet	-0.25	-0.02	-0.02	-0.06
Chl	-0.20	0.16	-0.43	-0.34
Car	0.21	-0.03	0.28	-0.57
Leaflet area	-0.25	-0.02	0.02	0.01
Lateral root length	-0.23	0.08	0.37	-0.08
Taproot Pb	0.23	-0.04	0.27	-0.23
lateral root Pb	0.21	0.21	0.25	0.29
Stem Pb	0.23	0.24	0.08	-0.11
Petiole Pb	0.18	0.48	-0.05	0.03
Leaflet Pb	0.17	0.48	-0.02	0.28
Leaflet MDA	0.24	-0.15	0.02	-0.07
Leaflet H_2O_2	0.23	-0.23	0.14	0.28
Leaflet $O_2^{\bullet-}$	0.22	-0.30	0.23	0.20
Leaflet SOD	0.24	-0.11	-0.11	0.07
Leaflet POD	0.21	0.28	-0.28	0.00
Leaflet CAT	0.24	-0.03	-0.17	0.04
Proportion of variation (%)	72.22	10.63	3.98	2.70

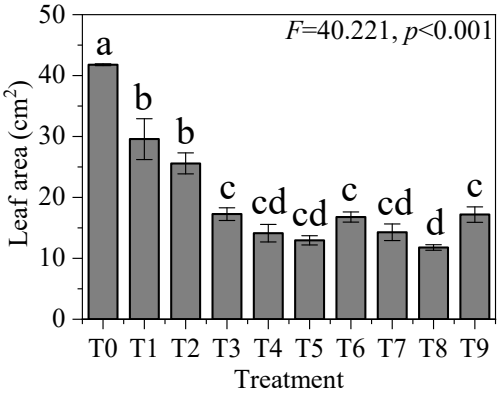

Fig. S1 The lea area of taproot and lateral root in *R. chinensis* under different treatments. Data over bars marked by the same letters are not significantly different at $p < 0.05$. Each value represents the mean of three replicates ± SE. The same below.

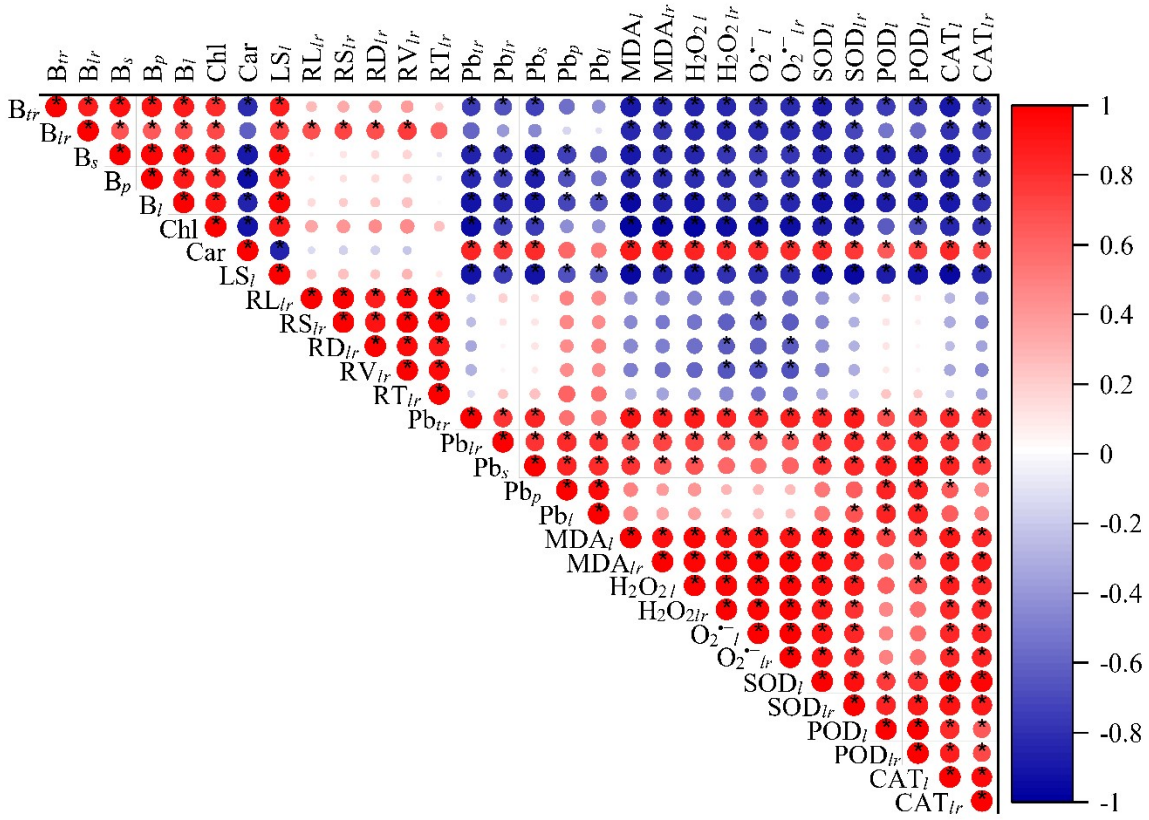

Fig. S2 Pearson's correlation of tissue Pb concentration, plant biomass and physiological growth parameters.

B_{tr}: Biomass of taproot, B_{lr}: Biomass of lateral roots, B_s: Biomass of stem, B_p: Biomass of petiole, B_l: Biomass of leaflet, Chl: chlorophyll, Car: carotenoids, S_l: leaflet area, RL_{lr}: root length, RS_{lr}: root surface area, RD_{lr}: root diameter, RV_{lr}: root volume, RT_{lr}: root tips, Pb_{tr}: Pb concentration of taproot, Pb_{lr}: Pb concentration of lateral roots, Pb_s: Pb concentration of stem, Pb_p: Pb concentration of petiole, Pb_l: Pb concentration of leaflet, MDA_{lr}: MDA of lateral roots, MDA_l: MDA of leaflet, H_2O_{2lr}: H_2O_2 of lateral roots, H_2O_{2l}: H_2O_2 of leaflet, $O_2^{\bullet-}{}_{lr}$: $O_2^{\bullet-}$ of lateral roots, $O_2^{\bullet-}{}_l$: $O_2^{\bullet-}$ of leaflet, SOD_{lr}: SOD of lateral roots, SOD_l: SOD of leaflet, POD_{lr}: POD of lateral roots, POD_l: POD of leaflet, CAT_{lr}: CAT of lateral roots, CAT_l: CAT of leaflet

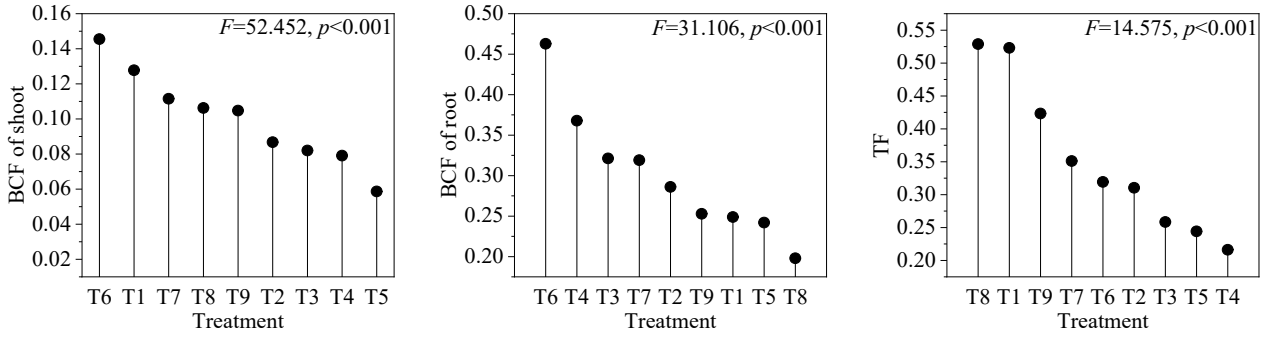

Fig. S3 The bioconcentration factor (BCF) and translocation factor (TF) of Pb in *R. chinensis* under different treatments. Each value represents the mean of three replicates.