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Inoculation of paddy soils with *Rhodopseudomonas palustris* enhanced heavy metal immobilisation

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Abstract: To investigate the effect of microbial inoculum on soil heavy metal immobilisation, pot experiments were conducted with paddy soils contaminated by cadmium (Cd), lead (Pb), arsenic (As), and mercury (Hg), respectively. The results showed that the inoculation of *Rhodopseudomonas palustris* was more effective in the immobilisation of Pb and Cd in soils than the composite of *R. palustris* and *Bacillus subtilis*. Interestingly, a lower dosage of inoculum immobilised significantly more heavy metals than the higher dosage, potentially due to the competition of bacteria with limited nutrients. The heavy metal contents in rice grains also supported this finding, as less Pb and Cd were accumulated under the lower dosage. However, there were limited effects of microbial inoculations on the immobilisation of Hg and As. In general, our study indicated the effectiveness of *R. palustris* in immobilising Pb and Cd in soils and highlighted the importance of determining the optimal dosage of inoculum in bioremediation.

Keywords: co-inoculation; toxic element; *Oryza sativa* L.; biosorbent; contamination

Heavy metals are introduced into agricultural soils through anthropogenic sources, such as fertiliser and pesticide use, wastewater irrigation, and mining activities, posing enormous threats to the ecosystem and human health (Puga et al. 2015). Excessive heavy metals in the soil can inhibit plant respiration and photosynthesis, thus reducing productivity (Zeng et al. 2020). Rice is the staple food for most Asian populations with high yields, while paddy soils encounter serious contamination with heavy metals (Liu et al. 2011). Jallad (2015) reported high levels of lead (Pb) and mercury (Hg) in rice grains sampled from the USA, China, and Germany.

Metal-resistant and plant growth-promoting microbes have been extensively used for decreasing metal availability in soils and plant metal accumulation (Peng et al. 2018). Both pot and field experiments provided evidences of the effectiveness of bioremediation. For example, Wang et al. (2014) revealed that *Bacillus subtilis* was a high-quality biosorbent for the adsorption of various metals, including Pb, Hg, and chromium (Cr). Although a large number of researches indicated the broad prospect of bioremediation, most studies focused on the effects of single strains on metal availability in soils and plant metal accumulation (Cheng et al. 2020). However, it might be difficult for a single strain to survive in the indigenous environment (Haskett et al. 2020); thus, the degradation of the contaminants or the immobilisation of heavy

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metals is limited in that case. Thus, effective assembly of several strains may be a potential solution, as more niches can be occupied in the habitats. To data, whether the microbial consortia was more effective in the soil remediation of heavy metal is still not clear.

Photosynthetic bacteria (PSB) are a class of special physiological groups of prokaryotes that resistant to heavy metals and able to bio-accumulate metals (Pei and Xu 2011). *Bacillus* can effectively decompose complex organic matters (Abriouel et al. 2011). Ndeddy and Babalola (2016) showed that the seeds germination of *Brassica juncea* and the enrichment of heavy metals (Cd, Ni, and Cr) were positively affected by the inoculation of bacteria. Although PSB and *Bacillus* play important roles in geochemical cycling, plant health, and bioremediation (Lakshmi et al. 2014), little has been reported on their effects of metal resistance and metal accumulation of crops in metal-contaminated soils. Thus, we aimed to evaluate the effects of single-strains and co-inoculation of *Rhodopseudomonas palustris* and *B. subtilis* on the immobilisation of heavy metals (i.e., Pb, Cd, Hg, and As) in soil and the accumulation of heavy metals in rice grain (*Oryza sativa* L.) with different dosage of microbial inoculum.

MATERIAL AND METHODS

Experimental design. The soils for experiments were collected from the surface layer (0 ~ 20 cm) of a long-term rice field in Changzhou, China. Soil samples of 2.5 kg were used for each treatment. The metals were added to the soils by the addition of the metal salts solution separately: $Pb(NO_3)_2$, $CdCl_2 \cdot 2.5H_2O$, $HgSO_4$, and $NaAsO_2$. The final content of Pb, Cd, and Hg was 40 mg/kg, and the final content of As was 20 mg/kg. The amended soils were watered and allowed to equilibrate for 30 days.

The strains of *R. palustris* and *B. subtilis* were purchased from Photosynthesis Biological Technology, Xinjiang, China. The strains were cultured in bioclean nutrient broth (pH 7.4 ~ 7.6), centrifuged at 12 000 rpm for 10 min, then rinsed with distilled water twice. The centrifuged cells were suspended in sterile normal saline to prepare bacterial inoculum (1×10^8 CFU (colony forming unit)/mL). Rice seeds (*Oryza sativa* L.) were surface-sterilised with 0.5% NaClO for 15 min, and then 15 seeds were sown in each pot. Each pot was treated with cell suspensions one week before seeding. Five treatments in triplicate were applied: control (no bacterial inoculation); *R. palustris*1 (3×10^{10} CFU of cells), *R. palustris*2 (4×10^{10} CFU

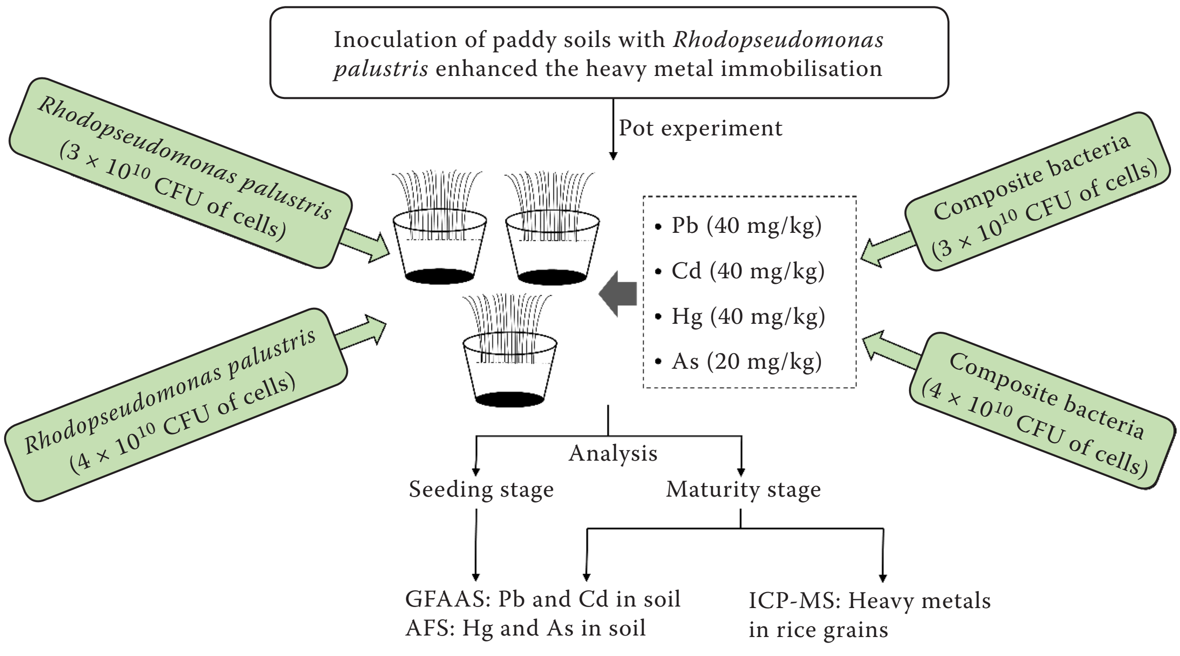

Figure 1. Experimental sketch of the effects of microbial inoculation on inhibition of heavy metal uptake and growth facilitation of rice. CFU – colony forming unit; GFAAS – graphite furnace atomic absorption spectrometry; AFS – atomic fluorescence spectrometry; ICP-MS – inductively coupled plasma massspectrometry

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of cells), *R. palustris* + *B. subtilis*1 (3×10^{10} CFU of cells, 1:1), and *R. palustris* + *B. subtilis*2 (4×10^{10} CFU of cells, 1:1). Soil samples were collected at both the seedling and maturity stages of the rice plant. The detailed experimental process is shown in Figure 1.

Measurement of heavy metals in soils and rice grains. To measure the heavy metal contents in soils, 0.50 g air-dried soil passed through a 0.2 mm sieve was treated with HNO_3:HF:$HClO_4$ (v:v:v = 3:1:1) in a microwave digestion system. Graphite furnace atomic absorption spectrometry was used to determine the content of Pb and Cd, and the As and Hg content was determined by atomic fluorescence spectrometry. 0.50 g rice grains sieved by 0.15 mm sieve were also treated with HNO_3:HF:$HClO_4$ for digestion and then diluted to 25 mL. The metal contents in the rice grains were determined by inductively coupled plasma massspectrometry (ICP-MS, iCAP-Q; Thermo Fisher Scientific, USA).

Statistical analysis. One-way analysis of variance and the Tukey's test ($P < 0.05$) were used to compare the averages of heavy metal contents of soils and rice grains in the presence of strains *R. palustris* and *B. subtilis* with those from the controls. Statistical analyses were carried out using SPSS, version 15 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Heavy metals in soils with microbial inoculations. The content of Pb, Cd, Hg, and As in the soil were determined at the rice seedling stage with the application of microbial inoculation (Figure 2A). With the application of microbial inoculations, we observed significant changes in the concentration of Pb ($P < 0.05$). Compared with the control group, the microbial inoculation of *R. palustris* (4×10^{10} CFU of cells) immobilised significantly more Pb and Hg in the paddy soils by 21.13% and 10.36% (Table 1). However, the co-inoculation seemed to have negative effects on the immobilisation of Pb in soils. The inoculation of *R. palustris* also promoted the immobilisation of Cd at the content of 3×10^{10} CFU of cells in the soils ($P < 0.05$). The lower dosage of *R. palustris* or the co-inoculation also showed no

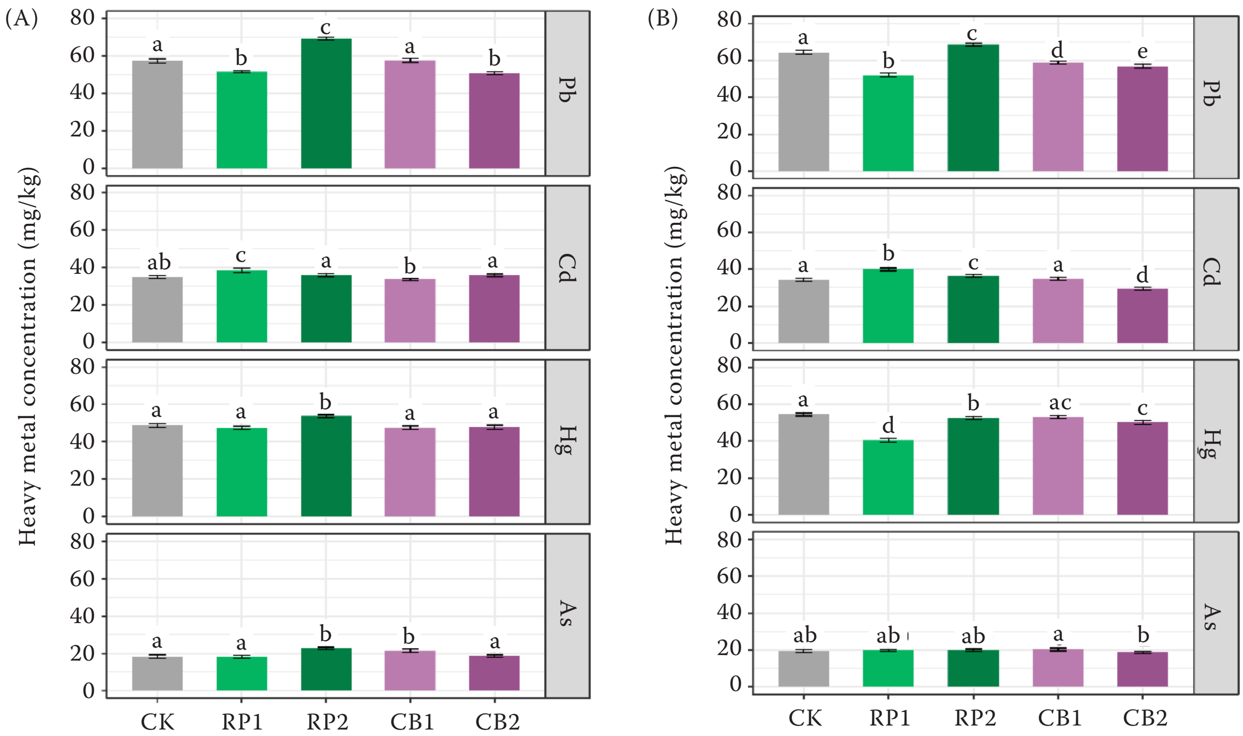

Figure 2. Soil concentrations of heavy metals at (A) the seedling stage and (B) in rice with microbial inoculations. CK – control group with no bacterial inoculation; RP – single-strain inoculation of *Rhodopseudomonas palustris*; CB – co-inoculation of *R. palustris* and *Bacillus subtilis*; 1 and 2 – inoculation of bacteria in soils with 3×10^{10} and 4×10^{10} CFU (colony forming unit) of cells, respectively. Means ± standard error followed by the same letters are not significantly different at $P < 0.05$

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Table 1. Percentages of the effect of microbial inoculant on heavy metals in soils or rice grains against control

		Pb (%)	Cd (%)	Hg (%)	As (%)
Soils (seedling stage)	RP1	9.93 ↓	10.38 ↑	2.57 ↓	1.13 ↓
	RP2	21.13 ↑	2.92 ↑	10.36 ↑	23.61 ↑
	CB1	0.70 ↑	3.39 ↓	2.33 ↓	16.98 ↑
	CB2	11.50 ↓	2.54 ↑	1.84 ↓	1.17 ↑
Soils (maturity stage)	RP1	19.24 ↓	16.10 ↑	25.72 ↓	1.41 ↑
	RP2	6.71 ↑	6.18 ↑	3.69 ↓	2.37 ↑
	CB1	8.41 ↓	1.38 ↑	2.36 ↓	4.09 ↑
	CB2	11.81 ↓	14.10 ↓	7.66 ↓	3.60 ↓
Rice grains	RP1	0.56 ↓	0.45 ↑	nd	1.21 ↑
	RP2	0.13 ↓	3.00 ↑	nd	1.17 ↑
	CB1	0.58 ↓	–0.10 ↓	nd	0.95 ↑
	CB2	0.13 ↑	1.84 ↑	nd	1.51 ↑

↑ and ↓ – increase and decrease of heavy metals in soils or rice grains against control; nd – no data. RP – single-strain inoculation of *Rhodopseudomonas palustris*; CB – co-inoculation of *R. palustris* and *Bacillus subtilis*; 1 and 2 – inoculation of bacteria in soils with 3×10^{10} and 4×10^{10} CFU (colony forming unit) of cells, respectively

significant changes to the content of Hg in the soils. A similar pattern was observed for As in the paddy soils. These results indicated that *R. palustris* could effectively immobilise Pb, Cd, Hg, and As in paddy soils at the stage of seedling in rice.

Similar patterns were observed for the immobilisation of heavy metals with the microbial inoculations at the maturity stage in rice (Figure 2B, Table 1). The higher concentration of *R. palustris* immobilised significantly more Pb in paddy soils, with 6.71% more Pb immobilised compared to the control ($P < 0.05$). The lower dosage of *R. palustris* or the co-inoculation could not immobilise Pb in soil, which was consistent with that at the seedling stage in rice. Inoculation of *R. palustris* at the concentration of 3×10^{10} CFU of cells could also significantly immobilise Cd in the paddy soils, with 16.09% more Cd immobilised compared to the control ($P < 0.05$). However, the co-inoculation showed no significant changes to the content of Cd in the soils.

These results showed that significantly more Cd and Pb were immobilised in the soils inoculated with *R. palustris* compared to the *B. subtilis* and the co-inoculation. We speculated that *R. palustris* might be more competitive than the *B. subtilis* in paddy soils, as *R. palustris* is adaptive to anaerobic flooding conditions (Xiao et al. 2019). Moreover, some *B. subtilis* enter spore dormancy state, while the other part quickly consumes free oxygen in the internal environment of the soil to form a partial hypoxic environment, which prevented the effective fixation of heavy metals in soil (He et al. 2016). We also found that neither *R. palustris* nor co-inoculation showed a significant immobilised effect on Hg and As in paddy soils. Microbial strains that resistant to Hg and As and capable of immobilising Hg and As are urgent to be isolated.

Heavy metals in rice grains with microbial inoculations. The potential mechanism of microbial inoculations in reducing heavy metals content in rice was further investigated (Figure 3, Table 1). Microbial inoculation of *R. palustris* and the co-inoculation (3×10^{10} CFU of cells) resulted in significantly less Pb in rice grains against the control ($P < 0.05$). Moreover, the Pb and Cd concentration in rice grains was the lowest when *R. palustris* was applied at the concentrations of 3×10^{10} CFU of cells. Interestingly, the co-inoculation and a higher dosage of *R. palustris* had a negative effect on the decrease of Cd concentration in rice grains. Compared to the control, the As concentration in rice grains inoculated with *R. palustris* or the co-inoculum was significantly higher ($P < 0.05$). The concentration of Hg was not detected in this study, indicating that Hg was rarely enriched in the rice grains.

The concentration of soil heavy metals directly affects the accumulation of metals in rice grains. Generally, the migration of heavy metals in rice grains increased with the heavy metal content in the soil (Yang and Deng 2005). Therefore, when *R. palustris*

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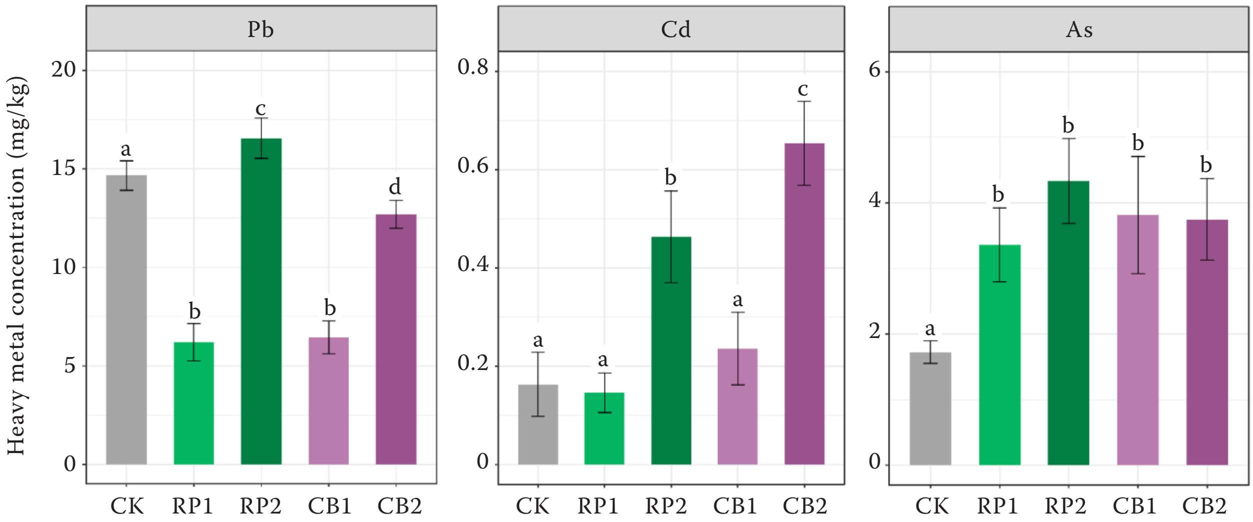

Figure 3. Heavy metal concentration in rice with the application of microbial inoculations. CK – control group with no bacterial inoculation; RP – single-strain inoculation of *Rhodopseudomonas. palustris*; CB – co-inoculation of *R. palustris* and *Bacillus subtilis*; 1 and 2 – inoculation of bacteria in soils with 3×10^{10} and 4×10^{10} CFU (colony forming unit) of cells, respectively. Means ± standard error followed by the same letters are not significantly different at $P < 0.05$

(3×10^{10} CFU of cells) were inoculated, the highest concentration of Cd in paddy soils at the maturity stage resulted in the lowest concentration of Cd in rice grains (0.14 mg/kg). Furthermore, heavy metals in rice grain were also indirectly affected by microbial inoculation. The microorganisms can alter the rhizosphere micro-environment and increase the absorption, volatilisation, or immobilisation efficiency of plants for heavy metals (Xie et al. 2019). Moreover, a lower dosage of microbial inoculation seems more capable of decreasing the heavy metal content in the grains than the higher dosage. This may be due to the excessive addition of stains under the condition of a certain soil capacity, space, and nutrition will cause competition for survival (Griffiths et al. 2004).

Our results indicated that the inoculation of *R. palustris* can effectively immobilise Pb and Cd in soils, and thus reducing their accumulation in rice grains. However, microbial inoculations of *R. palustris* and *B. subtilis* had limited effects on the immobilisation of Hg and As in soils. We also highlight the importance of determining the optimal dosage of inoculum when conducting bioremediation.

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