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Isolation of *Mucor circinelloides* Z4 and *Mucor racemosus* Z8 from heavy metal-contaminated soil and their potential in promoting phytoextraction with Guizhou oilseed rap

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Abstract: Fungi Z4 and Z8, isolated from the heavy metal polluted soil, have strong resistance to Cd and Pb. The strains were identified on the base of their morphology and internal transcribed spacers (ITS) region. Pot experiments were conducted to study the effect of two strains (Z4 and Z8) on the growth and accumulation of Cd and Pb of Guizhou oilseed rape. The results show that strains Z4 and Z8 belong to *Mucor circinelloides* and *Mucor racemosus*, respectively. The heights of Guizhou oilseed rape inoculated with strain Z8 increase by 47.90% than the control. The highest fresh mass is found in the plant with Z4/Z8, which is enhanced by 160.81%. Pot experiments show that Z4/Z8 inoculums can accelerate accumulation of heavy metals in the plant. The contents of Cd and Pb are increased by 117.60% and 63.48%, respectively. Meanwhile, the heavy metal concentrations in potting soil with the two strains are found to be lower than those of the control, and the concentrations of Cd and Pb are decreased by 60.57% and 27.12%, respectively.

Key words: phytoextraction; fungus; cadmium; lead; oilseed rape

1 Introduction

Heavy metal contaminated soil originates from rapid industrialization, irrational utilization of water and land resources, and inappropriate waste disposal methods [1]. The heavy metal pollution is a serious environmental problem, especially in Zhuzhou, Hunan, south of China, because of excessive discharging of industrial wastes [2]. Cd and Pb pollution is responsible for many negative consequences like affecting the growth of plants and animals, and causing harm to human health [3]. It also becomes a major hidden obstruction for the safety of surrounding site and water. So, remediation of heavy metal contaminated soil in this area has been paid much attention.

Phytoextraction is the most relevant in heavy metal remediation which can overcome some shortcomings of physical and chemical remediation methods, such as the destruction of soil-structure, secondary pollution and huge costs. In phytoextraction, metal-accumulating plants are used to concentrate heavy metal in harvestable parts [4]. Phytoextraction is still not extensively applied because of long growth cycle and low biomass of metal-accumulating plants. Plant combined with fungus has been proposed as a promising technique to remediate heavy metal contaminated soil which is characterized by promoting plant stress tolerance to metal toxicity, increasing biomass of plants, sometimes showing sufficient metal accumulation and sometimes showing sequestration [5].

Guizhou oilseed rape was selected for this study because of its capacity to tolerate and accumulate heavy metals, ability of rapid growth, deep roots and being easy to harvest. Heavy metal-resistant fungi were isolated from soil near Zhuzhou Smelter, China. It is an effective method for isolation of metal-resistant strain from the metal-polluted sites [6]. To establish different concentrations of heavy metal-contaminated soil, pot experiment was used by adding heavy metal to uncontaminated soil. However, this work is conducted to investigate the influence of fungus on the efficiency of Guizhou oilseed rape in phytoextraction of Pb and Cd contaminated soil, and to establish whether fungus

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combined with Guizhou oilseed rape can be successfully applied to the remediation of heavy metal contaminated soil.

2 Material and methods

2.1 Samples

Soils were sampled near Zhuzhou Smelter (sampling depth is 0−20 cm) in the Qingshuitang industry zone. This area has been contaminated by Cd and Pb due to industrial sewage and atmospheric deposition of metal-bearing dust. It is also the main pollution source of Xiangjiang River [7]. The properties of the soil samples are as follows: pH 7.2; organic matter 0.89%; total Cd content 38.432 mg/kg and total Pb content 315.183 mg/kg.

2.2 Screening of heavy metal-resistant fungi

To isolate heavy metal-resistant fungus from the soil, the conventional plate method was used. Soil suspension was added to the solid PDA medium containing 400 mg/kg cadmium ion and 800 mg/kg lead ion. The cultures were incubated in biochemical incubator at 30 °C for 3−7 d and then spread on plates. In order to isolate the strains with higher heavy-resistant ability, the cadmium and lead concentrations of medium were gradually increased. A series of solid media with different heavy metal contents from Cd 400 (Pb 300) to 1200 (Pb 1000) mg/kg were prepared and fungi were orderly inoculated and cultivated from low concentration to high level. The two fungi Z4 and Z8 with relatively high heavy-resistant were selected as the study object in this work.

2.3 Morphological characterization and ITS sequence amplification of fungi

The fungi Z4 and Z8 were inoculated on solid PDA medium. The mycelia were observed at visual inspection. Mycelia, cysts and sporangia of fungi were observed with a scanning electron microscope (SEM).

The fresh mycelia were harvested and rapidly ground in a mortar under liquid nitrogen (N_2) for 5−10 min. The genomic DNA was extracted from the mycelia. The internal transcribed spacers (ITS) region sequence was amplified by the fungal universal primers ITS-4(5’-TCCTCCGCTTATTGATATGC-3’) and ITS-5 (5’-GGAAGTAAAAG-TCGTAACAAGC-3’) [8]. The polymerase chain reaction (PCR) conditions were as follows: 94 °C for 5 min; 32 cycles: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; 72 °C for 10 min and 4 °C pause.

The above PCR products were sequenced in Shanghai Sangon Biotechnologies Co. Ltd., China. The ITS sequences of the fungi were submitted to GenBank and analyzed with the basic local alignment search tool (BLAST). The phylogenetic trees were constructed on the basis of ITS sequence alignment.

2.4 Pot experiments

Soil samples were collected from Zhuzhou Smelter and autoclaved for 20 min at 121 °C. Guizhou oilseed rapes were treated by inoculating fungus Z4 and Z8 alone, Z4/Z8 inoculum and no-inoculate. Each treatment was performed in triplicates. Pots with a height of 15 cm, a top diameter of 23.5 cm and a bottom diameter of 16 cm were used. A plastic bag with three small holes at the bottom to prevent anaerobic conditions in the potted soil was placed in each pot to prevent roots from growing out of the pot. Each pot was filled with 2.5 kg of soil, and placed on an individual saucer. About 10 seeds of Guizhou oilseed rape were sown on the soil surface of each pot. Two weeks later, the plants were thinned to 4 plants per pot for analyses. One week later, the strains Z4 and Z8 which were cultivated in solid media were eluted by sterile water at a concentration of 7×10^{10} mL^{-1}. 26 mL spore suspension was sprayed on the soil surface of each pot. The type of Z4/Z8 inoculum was mixed with Z4 and Z8 in a solution, and the total volume of fungi suspensions was 26 mL (V(Z4):V(Z8)=1:1). The same amount of deionized water was added to the non-inoculated control treatment plants. The experiments were conducted in a chamber under condition of natural light, a relative humidity of 80 %, and temperature of 12 °C in light and 4 °C in darkness. Pots were watered daily with demineralized water.

2.5 Metal determination

Pot experiments were harvested after 90 days. Roots were cut just below the soil surface. Roots of plants were collected by shaking off soil and thoroughly washing with tap water followed by washing with demineralized water. The root samples were ground to powder after naturally air dried to constant mass. Soil samples were air-dried to constant mass and sieved through a 0.149 mm sieve. The root samples were digested in a solution containing $V(HNO_3):V(HClO_4)=6:1$. The soil samples were digested in a solution containing $V(HF):V(HClO_4):V(HCl):V(HNO_3)=1:1.6:2:4$. The concentrations of heavy metals were determined using the atomic absorption flame emission spectrophotometer (AA Mode Z-2000, Hitachi, Japan).

2.6 Statistical analysis

The data were subjected to one-way ANOVA using SPSS 17.0 software. Means and standard derivations were calculated based on three replicate values. Means were compared by Duncan’s Multiple Range Test at 0.05 significance level [9].

3 Results and discussion

3.1 Heavy metal-resistant fungi

Among the strains that can grow on the heavy metals, Z4 and Z8 with relatively higher heavy metal-resistant can tolerate 1000 mg/kg cadmium and 400 mg/kg lead on plate. When the cadmium content reaches 1200 mg/kg, the fungus Z4 fails to grow after culturing for 3 d, and 1200 mg/kg is the minimum inhibitory concentration (MIC) of cadmium. The cadmium MIC of Z8 is 1100 mg/kg. The lead MICs of Z4 and Z8 are 500 mg/kg and 400 mg/kg, respectively. Fungi Z4 and Z8 showing the highest degree of cadmium and lead resistance are selected and identified based on morphology. Strains Z4 and Z8 appear yellowish brown and white density mycelia respectively after being cultivated on PDA solid medium for 3−5 d (Fig. 1). SEM micrographs show that the developed mycelia of Z4 have no rhizoids (Fig. 2(a)), and sporangia grow out from aerial mycelia and present oval shape (Fig. 2(b)). SEM micrographs show that the developed mycelia of Z8 have no rhizoids (Fig. 3(a)), and sporangiospores are

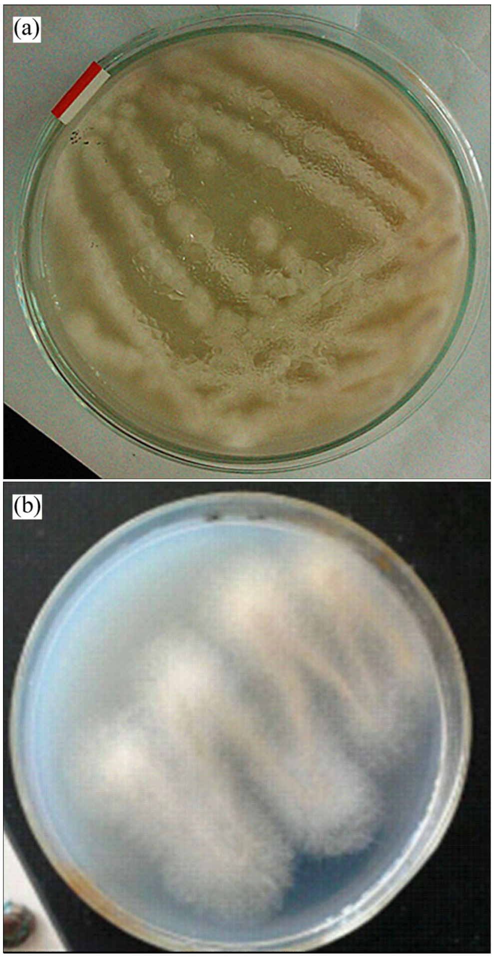

Fig. 1 Strains on PDA after 3 d at 30 °C: (a) Strain Z4; (b) Strain Z8

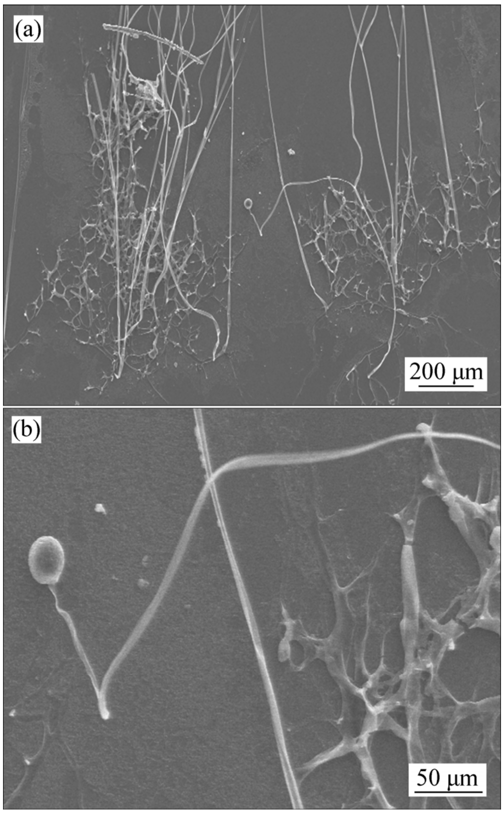

Fig. 2 SEM morphological characteristics of fungus Z4 cultured in PDA medium at 30 °C for 3 d: (a) Mycelia; (b) Sporangia

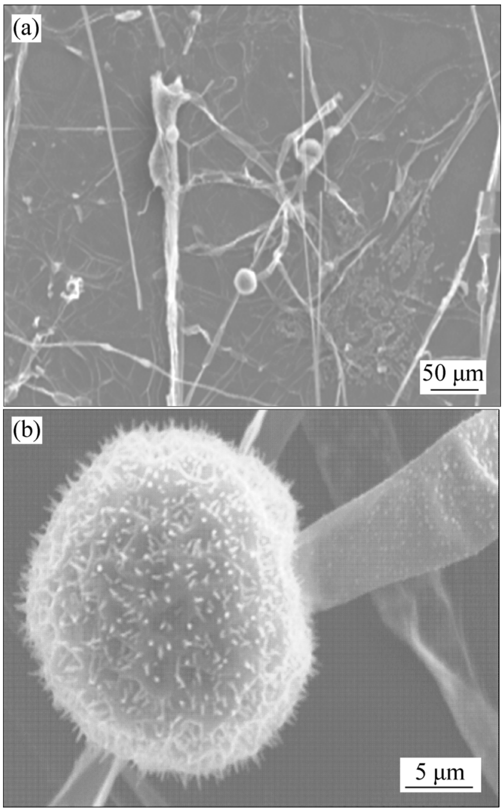

Fig. 3 SEM morphological characteristics of fungus Z8 cultured in PDA medium at 30 °C for 3 d: (a) Mycelia; (b) Sporangia

subglobose (Fig. 3(b)). The high heavy metals-resistant ability of fungi Z4 and Z8 possibly results from the long exposure to high heavy metal concentration environment.

3.2 ITS sequence analysis

In order to further study and apply the high heavy metal-resistant fungi in environmental bioremediation, it is very important to correctly identify the strains. In this work, the strain was identified by molecular biological methods. ITS region sequences were aligned with sequences published in GenBank by BLAST tool and the result suggests that 99% sequence of Z4 is similar to *Mucor circinelloides*, and 95% sequence of Z8 is similar to *Mucor racemosus*. The phylogenetic trees of ITS regions confirm the close relationship between the fungus Z4 and *Mucor circinelloides* (Fig. 4), Z8 and *Mucor racemosus* (Fig. 5). *Mucor racemosus* has great potential for use in heavy metals adsorption. *Mucor racemosus* can remove Cu(II) with the maximum specific uptake capacity of 213 μmol/g [10]. Some bio-sorbents such as *Mucor racemosus* which adsorbs Cr(VI) have also been reported [11]. To our knowledge, there are seldom reports about the resistance of *Mucor circinelloides* to heavy metals. This work gives information about the cadmium and lead resistance ability of *Mucor circinelloides* and *Mucor racemosus*. It would contribute to the enrichment of fungus species in bioremediation.

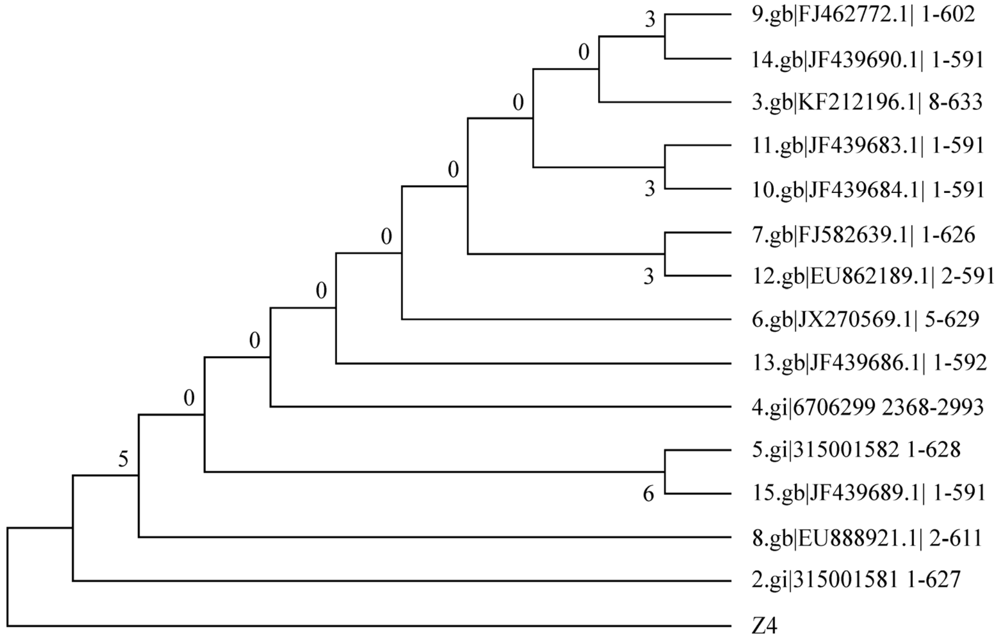

Fig. 4 Phylogenetic tree based on ITS sequence of fungus Z4

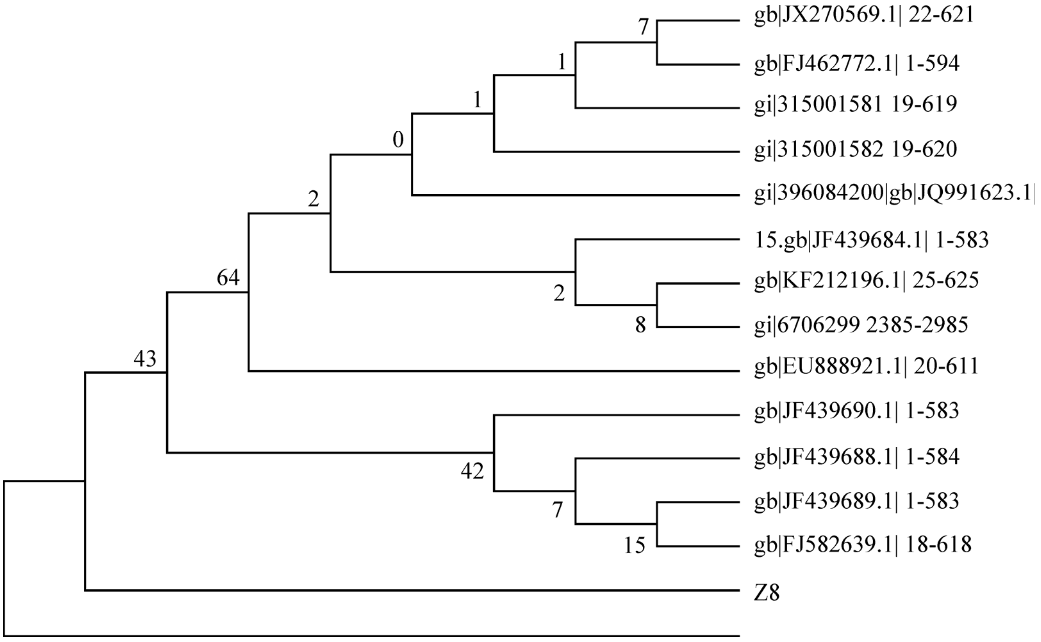

Fig. 5 Phylogenetic tree based on ITS sequence of fungus Z8

3.3 Plant biomass

There are no visible symptoms of heavy metal toxicity in Guizhou oilseed rape during germination. However, some leaves turn purplish, possibly due to P deficiency or the toxicity of the cadmium and lead [12−13]. Leaves recover to normal after Z4 and Z8 are added into the soil.

The effects of strains Z4 and Z8 on biomass of Guizhou oilseed rape are shown in Table 1. Fungi in soil have influence on the biomass of plant. Under the same conditions, the biomass is promoted to certain extent by inoculating strains Z4 and Z8, resulting in larger heights and larger fresh masses. A possible explanation for this enhancement could be that strains Z4 and Z8 supply plants with essential nutrients [14], and some heavy metals might also be absorbed by hyphae [15]. Compared with no-inoculation, heights of Guizhou oilseed rape with strain Z8 are significantly increased by 47.90%. The highest fresh mass-promoting effect is observed in Z4/Z8, which is enhanced by 160.81%. Similarly, Z8 enhances fresh mass by 103.38%. In general, the group with Z4/Z8 is the most effective, followed by Z8 and Z4 in the three groups. The positive interactions of Z4/Z8 may occur in Guizhou oilseed rape, and the result is consistent with HEIJDEN and VOSATKA [16] who proposed that mixed fungi inoculum is more beneficial to plant growth. These results are also similar to SONG et al [17]. The biomass of the plant is increased by inoculated Z4 and Z8, and the biomass is necessary for efficient bioremediation. This suggests that Z4 and Z8 play important role in heavy metals bioremediation of Guizhou oilseed rape, and Z4/Z8 colonization is the best way to increase biomass of Guizhou oilseed rape.

Table 1 Effects of different fungi on biomass of Guizhou oilseed rape grown in contaminated soil

Parameter	Z4/Z8	Z8	Z4	No-inoculate
Fresh mass of plant/g	3.86±1.10b*	3.01±0.65b	2.32±0.73a	1.48±0.26a
Plant height/cm	22.17±3.54b	22.54±0.87b	21.69±3.77b	15.24±1.21a

Within each row of fresh mass and plant height of Guizhou oilseed rape, * means the values with same letter are not significantly different at different inoculum according to Duncan’s Multiple Range Test at 5% level.

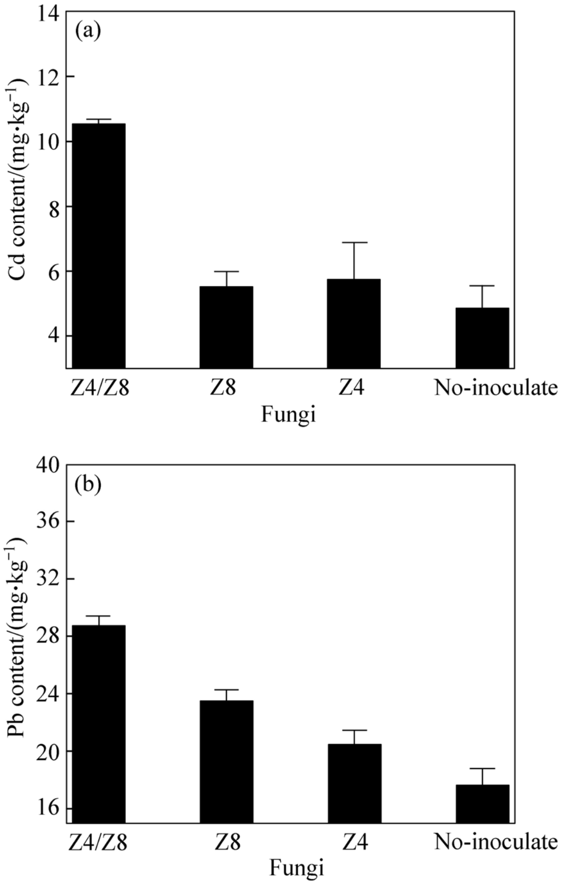

Fig. 6 Cd (a) and Pb (b) concentrations within underground tissue of Guizhou oilseed rape under different fungi inoculation

3.4 Plant heavy metal uptake

Cd and Pb contents in roots of Guizhou oilseed rape inoculated with different fungi for 3 months are shown in Fig. 6. The content of heavy metals in the roots is significantly increased when being inoculated with Z4 and Z8. Possible explanations for Z4 and Z8 increasing Guizhou oilseed rape tolerance to heavy metal are that the fungi can increase plant growth and absorb Cd and Pb to the chitin of the fungal. The Cd and Pb contents in the roots of inoculation with Z4/Z8 are increased by 117.60% and 63.48% compared with no-inoculation. The result is probably due to the fact that Z4 and Z8 may have biological barrier against translocation of heavy metals from roots [10]. The results agree with transformation of Cd to roots which is increased by inoculation with *G. intraradices* at low Cd soil [18]. For the single fungus inoculum, the plant accumulates higher concentration of Pb in the roots when it is inoculated with Z8 rather than inoculated with Z4. There are few significant differences between inoculated with Z8 and inoculated with Z4 in the Cd content of potting soil. There is obvious symptom of accumulation for Pb. Strains Z4 and Z8 have the potential to accumulate higher Pb concentration in underground parts. The symptom of accumulation is weaker for Cd than Pb. It seems that Guizhou oilseed rape under Z4/Z8 inoculated has higher tolerance to Pb. This indicates that fungi are selective for accumulation of toxic heavy metals. Compared to other strains, Z4 and Z8 show higher potentials to improve extraction efficacy of Cd and Pb by Guizhou oilseed rape. The content of Cd in *B. campestris* ssp inoculated with *Enterobacter* sp. CBSB1 in metal contaminated soil is increased by 19.2% compared to the uninoculated control [19]. Plants inoculated with *G.*

intraradices accumulates 45% more Cd than the nonmycorrhizal control [20]. SHENG and XIA [21] reported that the addition of *Bacillus* sp. to *Brassica napus* grown in metal contaminated soil significantly increases the plant uptake of Cd from 20% to 27% compared with the non-inoculation control.

3.5 Heavy metal content in potting soil

The effect of plant-fungus on heavy metal-contaminated soil is observed in this work. There are significant differences for Guizhou oilseed rape inoculated with different fungi species affecting heavy metal content in potting soil (Fig. 7) ($P<0.05$).

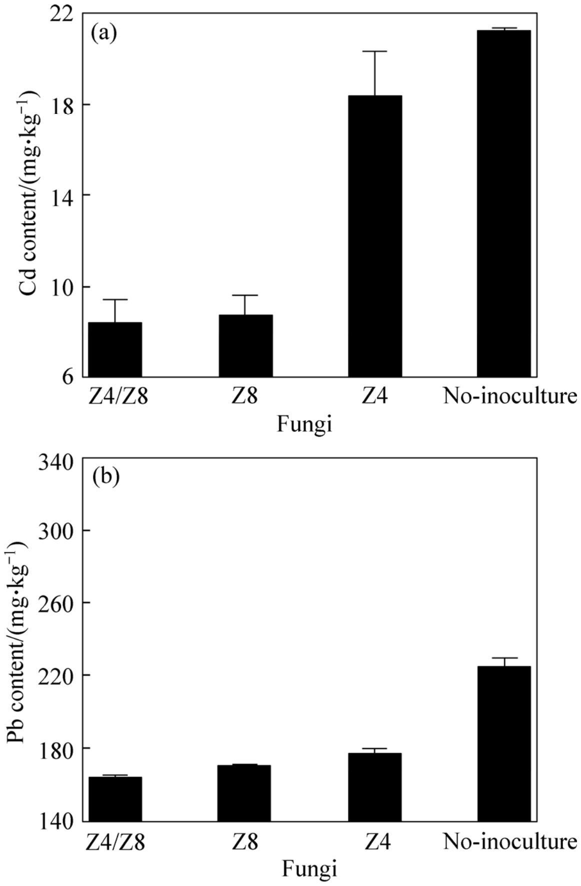

Fig. 7 Cd (a) and Pb (b) contents in potting soil of Guizhou oilseed rape under different fungi inoculations

In all treatments, the highest decrement of heavy metal content in potting soil is found for Z4/Z8, which reduces Cd and Pb contents by 60.57% and 27.12%, respectively, compared with non-inoculated plants. This is possibly due to the fact that Z4/Z8 inoculum carries both indigenous fungi which survive in metal contaminated site, and hence they are able to form a more efficient relationship with the soil. There is also a possibility of a vast amount of native fungi living in the soil samples. Positive interactions may occur to the plant such as nutrient acquisition. A synergistic effect may exist in fungi Z4 and Z8 such as increasing the surface area for nutrient absorption and enhancing chemical alternation in the soil [12]. The second highest decrement is observed in Z8, which decreases Cd and Pb contents by 58.78% and 47.52%, respectively. There are no significant difference between single strain Z8 and Z4/Z8 inoculated in the Cd concentration of potting soil. Figure 7 shows that being inoculated with Z4, the highest level of Pb and Cd contents is produced in strain-inoculated potting soil. The Cd and Pb contents are 18.33 and 177.24 mg/kg, respectively. Some heavy metals may be absorbed by hyphae cell wall [21]. However, the adsorption capacity of Z4 inoculum is possibly lower than that in other treatments. Heavy metal absorbed by plants from a heavy metal polluted soil probably differs from artificially contaminated soil with certain heavy metal contamination [22]. This may significantly affect the efficiency of fungi inoculum in the field. Fungi Z4 and Z8, isolated from the soil near the Zhuzhou smelter, have a tolerance for heavy metals, and they may play a role in the phytoremediation of the site. It is important to use indigenous fungi and plants, which are best adapted to actual soil and climatic conditions to produce site-specific fungus and co-remediate heavy metals polluted soil [23]. Accordingly, Guizhou oilseed rape inoculated with Z4/Z8 can normally grow and extract considerable heavy metals. Hence, they can effectively remedy contaminated soil, phytoextract and beautify the environment. Native strains Z4 and Z8 potentially which are useful for microbial-assisted phytoremediation are considered to be those present in contaminated sites, preferably more abundant in the contaminated ones. They negatively influence the ecosystem harmony, and other strains are hardly adapted to the new environmental conditions.

4 Conclusions

It is indicated that indigenous Z4 and Z8 are effective in removing heavy metal from contaminated soil. On the basis of the ITS regions analysis and the morphological characteristics, the fungi Z4 and Z8 can be identified as *Mucor circinelloides* and *Mucor racemosus*, respectively. Inoculation with Z8 and Z4/Z8 could be the most effective way in increasing the heights and the fresh mass of Guizhou oilseed rape, respectively. Strains Z4 and Z8 significantly increase the contents of Pb, Cd in underground of Guizhou oilseed rape. Pb and Cd contents in potting soil significantly decrease when Guizhou oilseed rape is inoculated by Z4/Z8.

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