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# Isolation of heavy metal-resistant fungi from contaminated soil and co-culturing with rice seedlings

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**Environmental pollution from toxic heavy metals is a growing problem throughout the world due to the expansion of industrialisation. The use of microbes and biotechnological processes can provide alternative or complementary methods for the removal or recovery of heavy metals through bioremediation. The present study isolated, identified and characterised heavy metal-resistant fungi from cadmium- and chromium-contaminated paddy soil from Daye City, Hubei Province, China. Eight metal-resistant fungal species were obtained using an acclimation to concentration gradient approach. Five strains tolerated cadmium ($Cd^{2+}$) to a maximum concentration of 16 mM, four strains could endure 1000 mg/L chromium ($Cr^{2+}$), and *Fusarium oxysporum* could withstand high levels of both metals. Morphological examination and 18S rDNA sequence analysis identified the strains. Metal-resistant fungi were co-cultured with 5-day-old rice (*Oryza sativa*) seedlings in 1/2 MS medium for 7 days and growth parameters were compared with control rice not incubated with microbes. In the obtained strains, *Metarhizium anisopliae* had no influence on plant height or root length, and *Saccharomyces cerevisiae* had no effect on these parameters or on fresh weight. Heavy metal-resistant fungi such as those identified in this study could prove useful for the bioremediation of heavy metal-contaminated environments.**

**Key words:** Heavy metal resistance, co-cultivation, filamentous fungi, acclimation of concentration gradient, rice, bioremediation.

## INTRODUCTION

Heavy metals are released from urban areas, metalliferous mines and major road systems, and pollution is an increasing problem due to the rapid growth of populations, industrial activities and technological development (Alloway et al., 1995). Industrial activity in particular continually discharges heavy metals such as cadmium, lead, chromium, copper and nickel (Hemambika et al., 2011), eventually causing toxic contamination to soils, sediments and surface and ground water, which presents a serious threat to human health.

Heavy metal pollution has been widely reported in arable fields in South China in recent years, due to the

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rapid expansion of mining and metallurgical activities, sewage irrigation, and the application of pesticides and fertilizers (Chen et al., 2014; Gong et al., 2009; Zeng et al., 2013). Around 2500 $hm^2$ of farmland has been contaminated by Hg and Cd, and by As, Cr and Cu to a slightly lesser extent (Xu et al., 2014). There are more than 329000 tons of chromic salt produced by only 25 companies every year, accompanied by 450000 tons of Cr residue. Approximately 5000 tons of Cr residue has been deposited along the riverside of Nanpanjiang in Yunnan Province each year for the last 15 years, which has resulted in concentrations of Cr (VI) in the river itself that are 2000 times higher than acceptable national standards (Gao et al., 2011). In 2002, the Ministry of Agriculture found very high levels of cadmium in 10% of rice samples, while the Guangdong provincial government found that 44% of rice sample contained excessive cadmium in a 2013 investigation. However, in general research on cadmium contamination has been minimal (Kevin et al., 2014).

Several conventional physicochemical approaches have been used for the removal and treatment of heavy metal pollution sites, including electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Kadirvelu et al., 2002; Luo et al., 2010). However, these methods are uneconomical due to high reagent and energy requirements, or ineffective at removing all metal contamination, and they can generate large quantities of toxic sludge (Hemambika et al., 2011). In contrast, bioremediation offers a cleaner and more economical option for treating heavy metals in contaminated sites (Iskandar et al., 2011). The use of microorganisms and solid-liquid separation (Yang et al., 2012) has several advantages, not least the highly efficient removal of heavy metals from dilute solutions (Kapoor et al., 1999; Cruz et al., 2004; Fan et al., 2008).

Some fungi are known to be particularly tolerant of heavy metals (Gavrilesca, 2004; Baldrian, 2003), and many species can adapt and grow under conditions of extreme pH, temperature, nutrient availability and high metal concentrations (Anand et al., 2006). Fungi tolerate and detoxify metals through various mechanisms involving valence transformation, extra- and intracellular precipitation or active uptake (Mala et al., 2006; Turnau et al., 2006). Scientists are currently exploring the use of microbes and associated biota residing in ecosystems for the bioremediation of pollutants through degradation or accumulation (Khan et al., 2000), and numerous strains isolated from contaminated sites possess such abilities. Vadkertiova and Slavikova (2006) studied metal tolerance in yeasts isolated from polluted environments and found inter- and intraspecific variation in metal tolerance among tested strains. Similarly, Zafar et al. (2007) reported promising biosorption of Cd and Cr using *Aspergillus* sp. and *Rhizopus* sp., filamentous fungi isolated from metal-contaminated agricultural soil. Given the types of mechanisms underpinning metal resistance, screening of metal-tolerant fungi is likely to identify strains with improved capacity for metal accumulation (Bai and Abraham, 2003).

The present work reports the identification and characterisation of metal-resistant microorganisms isolated from polluted environments and the selection of strains with improved heavy metal resistance. Contaminated fields are the ideal environment in which to find organisms that have evolved survival mechanisms to adapt to polluted conditions that could be used for bioremediation of heavy metals in other contaminated locations.

## MATERIALS AND METHODS

### Soil sampling and analysis of heavy metal content

Soil samples were collected from rice paddies near Chenjiazuiwan village Luojiaqiao Towns Daye City, Hubei Province, China (30°08′47.63’’N, 114°57′23.20’’E, 21 m a.s.l.). Soil samples (1000 g) were taken from the surface down to a depth of 20 cm using a wooden spatula and half was stored at 4°C for fungi isolation, while the other half was air-dried for physicochemical analysis. Plant residues and stones were removed by hand, and soil samples were passed through a 1 mm sieve prior to analysis of heavy metal content (Shazia et al., 2013). Cr and Cd concentrations were determined using atomic absorption spectrometry (AAS 6200, SHIMADZU).

### Screening and selection of heavy metal-resistant fungi

Purified isolates were screened on the basis of their tolerance to $Cr^{6+}$, $Pb^{2+}$, $Hg^{2+}$, $Cd^{2+}$ and $Cu^{2+}$. A disk of mycelium was inoculated aseptically on Martin medium plates supplemented with different concentrations of individual heavy metal potassium dichromate, lead carbonate, mercury bichloride, cadmium sulfate and cupric sulfate salts, respectively. Inoculated plates were incubated at 25°C for at least 7 days. The effect of heavy metals on growth was estimated by measuring the radius of the colony extension (mm) compared with controls (medium lacking heavy metals) and determination of the index of tolerance (the ratio of the extension radius of treated vs. untreated colonies). Isolates showing resistance to $Cr^{6+}$, $Pb^{2+}$, $Hg^{2+}$, $Cd^{2+}$ and $Cu^{2+}$ were selected for subsequent experiments.

### Isolation of heavy metal-resistant fungi

Martin media was used to culture fungi for isolation experiments (Iram et al., 2011). Soil samples were processed and fungi isolated using the soil dilution plate method (Waksman, 1922). After incubation, individual colonies were counted and species identified on the basis of macroscopic (colony morphology, colour, texture, shape, diameter and appearance) and microscopic (septation of mycelia, presence of specific reproductive structures, shape and structure of conidia, presence of sterile mycelia) characteristics. Pure cultures were identified with the help of the existing literature (Domsch et al., 1980, Barnett and Hunter, 1999).

### Molecular identification of heavy metal-resistant fungi

Fungal DNA was extracted for amplification using the thermolysis

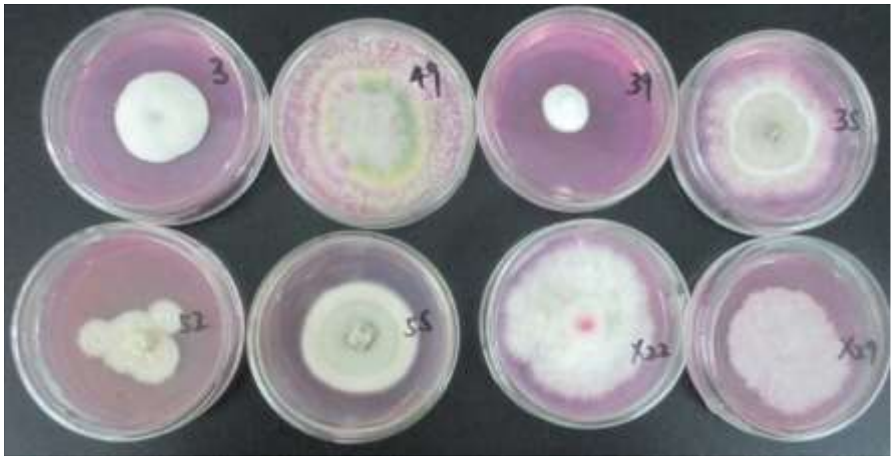

**Figure 1.** Colony morphology of the eight identified fungal strains.

method devised by Zhang et al. (2010), and amplification was performed as described previously (Onn, 2012). Primers (18S-1, 5'-GTAGTCATATGCTTGTCTC-3'; 18S-2, 5'-TCCGCAGG TTCACCTACGG A-3') were designed and used with the following cycling conditions: An initial denaturing step at 94°C for 2 min, followed by 25 cycles of amplification at 94°C for 30 s, 45°C for 30 s and 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were separated by 1% agarose gel electrophoresis and a band of ~1700 bp was gel-extracted, ligated, transformed, sequenced and aligned with sequences in GenBank.

#### Co-cultivation of heavy metal-resistant fungi with rice tube seedlings

Rice seed (Guangliangyou, 272) was provided from the Hubei Academy of Agriculture, and sterile rice tube seedlings were obtained as previously described (Li et al., 2008). Sterile seedlings were grown for five days to a height of ~1 cm in 10 cm diameter petri dishes. Square culture bottles were filled with 1/2 MS medium and autoclaved at 121°C for 15 min. A quarter of the culture medium was cut vertically after cooling, and five fungi blocks of 0.5 cm in diameter were inoculated ~2 cm from the bottom of the culture bottle. The rice seedlings were then inoculated above the corresponding fungi blocks and incubated at 28°C with a 16/8 h (day/night) photoperiod (2000 lux light intensity). Rice plant height, root length and fresh weight were recorded after seven days. Control plants were inoculated onto Martin media agar blocks without fungal strains.

#### Statistical analysis

All experiments were performed with three replicates. Individual isolates were subjected to analysis of variance using SPSS 17 statistical software to compare resistance to metals.

## RESULTS AND DISCUSSION

### Detection of the heavy metal content

According to the evaluation standards for heavy metal pollution in soil in China (GB15618-1995), the concentration of Cd in the soil sample was 0.94 mg/kg, which corresponded to primary standard levels, while Cr was 60.2 mg/kg, which corresponded to second grade standard levels. The introduction of heavy metal compounds into the environment generally induces morphological and physiological changes in microbial communities (Vadkertiova and Slavikova, 2006), and exerts a strong selective pressure on the microbiota (Verma et al., 2001), making contaminated sites an ideal source of metal-resistant microorganisms (Gadd, 1993). Sampling polluted sites therefore increases the probability of isolating cadmium- and chromium-resistant strains that have evolved to adapt to heavy metal-rich niches.

### Screening and selection of heavy metal-resistant microorganisms

We identified eight resistant fungal strains through screening of cadmium- and chromium-contaminated paddy soil using an acclimation of concentration gradient approach. Following purification, the colony morphology of individual strains resistant to different heavy metals was assessed (Figure 1 and Table 1). Five strains could endure a maximum concentration of 16 mM $Cd^{2+}$, four strains could tolerate $Cr^{2+}$ up to 1000 mg/L, and strain X29 could withstand these maximum concentrations of both heavy metals.

### Identification of metal-resistant fungal species

The identity strains was confirmed using 18S rDNA sequence analysis following comparison with sequences in the NCBI, which also afforded their registration numbers (Table 2). The raw data have been submitted to a public repository (NCBI) under accession number KU350742-KU350749.The eight metal-resistant filamentous fungi were identified as *Metarhizium anisopliae*, *Phomopsis* sp., *Aspergillus* sp., *Trichoderma*

**Table 1.** Maximum concentration of heavy metals tolerated by resistant fungi.

| Strain No. | $Cd^{2+}$ (mM) | $Cr^{6+}$ (mg/L) | $Pb^{2+}$ (mM) | $Hg^{2+}$ (mg/L) | $Cu^{2+}$ (mM) |
|---|---|---|---|---|---|
| 3 | 16 | 800 | 30 | 450 | 4.0 |
| X22 | 12 | 1000 | 15 | 200 | 5.6 |
| X29 | 16 | 1000 | 20 | 150 | 3.0 |
| 35 | 10 | 1000 | 10 | 120 | 3.0 |
| 39 | 16 | 800 | 50 | 300 | 5.0 |
| 49 | 8 | 1000 | 30 | 250 | 4.0 |
| 52 | 16 | 800 | 40 | 300 | 3.0 |
| 55 | 16 | 800 | 40 | 120 | 7.4 |

**Table 2.** GenBank accession numbers for the eight identified strains.

| Strain no. | Generic name | Accession number | Strain no. | Generic name | Accession number |
|---|---|---|---|---|---|
| 3 | *Metarhizium anisopliae* | KU350742 | X22 | *Aspergillus* sp. | KU350749 |
| 39 | *Saccharomyces cerevisiae* | KU350743 | 49 | *Trichoderma* sp. | KU350745 |
| X29 | *Fusarium oxysporum* | KU350748 | 52 | *Penicillium* sp. | KU350746 |
| 35 | *Phomopsis* sp. | KU350744 | 55 | *Penicillium* sp. | KU350747 |

sp., *Fusarium oxysporum*, *Saccharomyces cerevisiae* and *Penicillium* sp., while Strain 35 and Strain 55 belonged to *Aspergillus* sp. Soils contaminated long-term with both organic compounds and heavy metals contain microbial communities that are structurally and functionally adapted to grow under the polluted conditions. These adapted microorganisms could in principal be used for bioremediation of organic compounds and heavy metals into non-toxic products at other polluted sites. Adaptation involves evolving catabolic activities that enables the organisms to utilize the contaminants as nutrients and energy sources (Atlas and Unterman, 1999; Boopathy, 2000). However, when allochthonous microorganisms are incorporated into new soils, they may not be fully capable of participating in and complementing the existing microbial community, unlike indigenous microorganisms that are more likely to be better adapted to the local conditions and hence more useful for bioremediation. Ezzouhri et al. (2009) identified 36 microorganisms in isolates from heavy metal-contaminated sites in Tangier, Morocco, belonging to *Aspergillus, Penicillium, Fusarium, Alternaria* and *Geotrichum* genera that demonstrated high levels of resistance to all metals tested. This and previous studies reveal fungi as promising agents for bioremediation of heavy metal-polluted soils, and biosorption of cadmium and chromium in particular.

## Co-cultivation of heavy metal-resistant fungi with rice seedlings

Heavy metal-resistant strains were co-cultured with rice seedlings in 1/2 MS medium for 7 days (Figure 2), and plant height, fresh weight, root length and root number were analyzed (Table 3). Plants co-cultured with strains 3 and 39 grew better than controls, whereas strains X22, X29, 35, 49, 52 and 55 grew significantly worse than controls.

Increasing attention is being paid to the use of growth-promoting soil microbes for bioremediation of heavy metal pollution in soils, and to the nature and benefits of specific plant-microbe interactions. Rice is the most widely planted grain crop grown in heavy metal-polluted farmland in Hubei province and other areas of China, and this important food crop can accumulate high levels of toxic Cd from contaminated soil. Reducing the transfer of heavy metals from the soil into plant material is a major research goal, and selection of appropriate functional microbes can facilitate phytoremediation of mining-contaminated soils. If such microbes are present or introduced, the slow or non-existent growth and low biomass often associated with mining wastelands can be overcome, and soil fertility and heavy metal toxicity can be improved and reduced, respectively (Wong, 2003). In order to survive in heavy metal-contaminated soils, functional microbes (Drake and Liu, 2008) have undergone adaptation of their metabolic activities to suit their particular environment (Kumar et al., 2009; Turgut et al., 2010). In this study, although plant height and root length were not enhanced by co-culturing with strain 3, the number and fresh weight of rice roots was significantly improved, compared to control plants. Similarly, strain 39 did not improve plant height, root length or fresh weight, but significantly increased root number. Strain 52 had the largest positive impact on rice

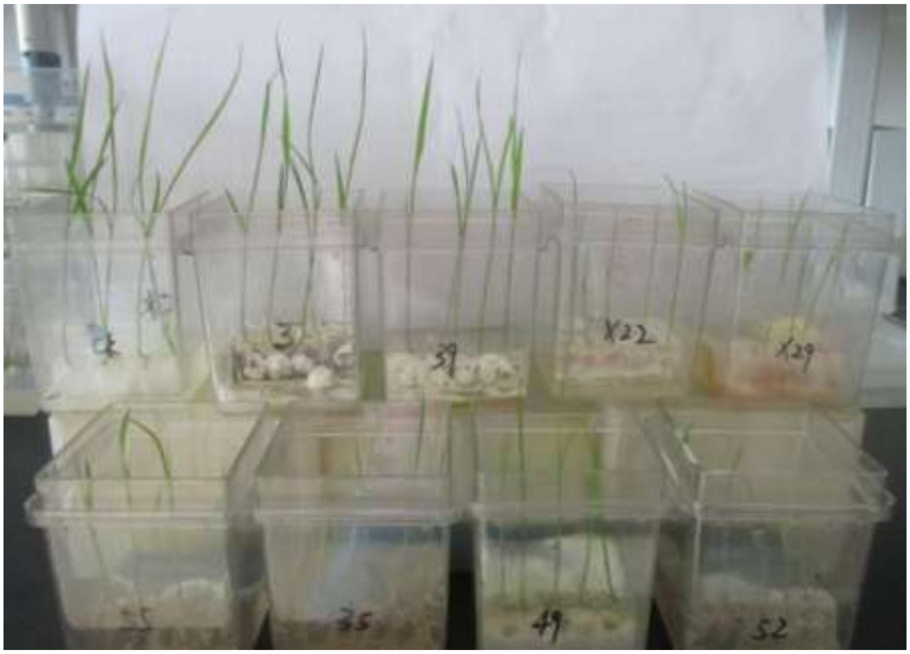


**Figure 2.** Co-cultivation of rice tube seedlings with heavy metal-resistant fungal strains.

**Table 3.** Biological characteristics of rice seedlings co-cultivated with heavy metal-resistant fungi for 7 days.

| Strain No. | Biological characteristics | | | |
|---|---|---|---|---|
| | **Plant height (cm)** | **Root number (pcs)** | **Root length (cm)** | **Fresh weight (g)** |
| 3 | 13.56 ± 0.47[a] | 5.47 ± 0.47[b] | 2.53 ± 0.16[a] | 0.11 ± 0.005[b] |
| 22 | 7.97 ± 0.32[c] | 5.27 ± 0.56[b] | 1.44 ± 0.10[bc] | 0.06 ± 0.002[c] |
| 29 | 6.20 ± 0.25[de] | 3.27 ± 0.44[c] | 1.02 ± 0.11[d] | 0.04 ± 0.003[d] |
| 35 | 7.21 ± 0.52[cd] | 1.67 ± 0.23[d] | 1.56 ± 0.12[b] | 0.06 ± 0.004[c] |
| 39 | 12.97 ± 0.48[a] | 6.33 ± 0.46[b] | 2.60 ± 0.14[a] | 0.12 ± 0.007[a] |
| 49 | 9.57 ± 0.44[b] | 2.00 ± 0.22[d] | 1.45 ± 0.17[bc] | 0.08 ± 0.004[c] |
| 52 | 5.93 ± 0.29[e] | 2.20 ± 0.30[cd] | 1.65 ± 0.15[b] | 0.06 ± 0.004[c] |
| 55 | 7.39 ± 0.36[c] | 1.80 ± 0.37[d] | 1.37 ± 0.14[bc] | 0.06 ± 0.003[c] |
| ck | 14.61 ± 0.30[a] | 9.00 ± 0.54[a] | 2.33 ± 0.10[a] | 0.12 ± 0.003[a] |

Values followed by different letter among the different treatment differ at p=0.05 (Tukey's HSD multiple comparison).

growth. The results suggest the combined use of heavy metal-adapted soil microbes and functional plants could be an effective approach for improving plant growth and lowering assimilation of heavy metals in crops grown on contaminated land.

## Conclusion

Our preliminary findings indicated that native fungi by screening from cadmium and chromium contaminated paddy soil could grow at high heavy metal concentrations. Five fungi (strain 3, strain X29, strain 39, strain 52 and strain 55) that showed high resistance to $Cd^{2+}$ (up to 16 mmol/L), four fungi (strains X22, X29, 35 and 49) that showed high resistance to $Cr^{6+}$ (up to 1000 mg/L), strain 39 showed high resistance to $Pb^{2+}$ (up to 50 mmol/L), strain 3 showed high resistance to $Hg^{2+}$ (up to 450 mg/L), and strain 55 showed high resistance to $Cu^{2+}$ (up to 7.4 mmol/L). Findings of the present study indicate that fungi populations isolated from heavy metal-contaminated sites have the ability to resist higher concentrations of metals.

Strains 3 and 39 growing on the surface was similar to contrast with co-cultured rice seedlings. Further investigations are needed to assess their biosorption capabilities and determine such things as the proteins involved in the biosorption process, the most efficient contact time and optimum growth conditions. Microbes can affect the availability of heavy metals through direct or indirect metabolic activities, which play a decisive role in maintaining soil function. The results indicate that native fungi play a more important role in the process of phytoremediation of heavy metal-contaminated soils.

## Conflict of interests

The authors have not declared any conflict of interest.

## ACKNOWLEDGMENTS

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