Isolation, identification, and heavy metal tolerance of fungi from rice growing area contaminated with cadmium and lead in San Manuel, Pangasinan, Luzon Island, Philippines

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Abstract Heavy metal contamination of rice paddy is one of the problems in the rice growing areas. High amounts of cadmium and lead are hazardous to the health of humans, animals, plants, and the environment. The present study isolated, identified, and evaluated the potential mycoremediators from the rice growing area contaminated with heavy metals at Sitio Namangonan, Guiset Norte, San Manuel, Pangasinan, Luzon Island, Philippines. The soil sample contained 0.42 mg/kg cadmium and 57.80 mg/kg lead. Four species of fungi namely: *Trichoderma koningii, Penicillium janthinellum, Penicillium resticulosum,* and *Penicillium nigricans* were isolated from the soil contaminated with cadmium and lead. *Penicillium janthenellum* had highest occurrence with 42.85% among the four identified species of fungi. Furthermore, *Trichoderma koningii, Penicillium resticulosum,* and *Penicillium nigricans* are tolerant up to 100 mg/kg cadmium (Cd) concentration while *Penicillium janthinellum, Penicillium janthinellum, Penicillium janthinellum, Penicillium janthinellum, Penicillium janthinellum, Renicillium janthinellum, Penicillium janthinellum, Renicillium janthinellum, and tolerate 10 mg/kg Cd concentration. Moreover, <i>Trichoderma koningii, Penicillium janthinellum, Penicillium resticulosum,* and *Penicillium nigricans* can withstand 1000 mg/kg lead (Pb) concentration. Hence, the different isolates are heavy metal tolerant in rice paddy soil contaminated with cadmium and lead.

Keywords: Cadmium, Contaminated soil, Fungal isolate, Heavy metal tolerance, Lead

Introduction

Elements with higher density than water are so called heavy metals (HMs) and normally exist in the soil (Hakeem *et al.*, 2015). Several human activities contributed to the deposition of heavy metals in soils namely: mining, use of fertilizer and pesticides, disposal of metal wastes, amelioration of manure, product of wastewater, etc. (Tasrina *et al.*, 2015; Wuana and Okieimen, 2011).

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Intake of heavy metals has adverse effects on plants and humans. Contaminated soil with HMs is one of the primary environmental challenges in the world which can result to negative outcome on the ecosystem, crop yield and human health (Kayamura and Esposito, 2010; Li *et al.*, 2013). These HMs include mercury, lead, chromium, cadmium, and arsenic. Toxic HMs are harmful to humans because of their accumulation in vital organs like brains, liver, bones, and kidneys, they may also cause serious carcinogenic health consequences even at low concentrations (Kabata-Pendias, 2011; Bello *et al.*, 2019). Although HMs are certainly available in the soil, geologic and human-caused actions intensify the concentration of these elements that are destructive to plants, animals, and humans (Alloway, 2009; Masindi and Muedi, 2018).

Cadmium and lead are the two of the toxic non-essential heavy metals that are commonly present in rice paddy soils (Fu et al., 2008). Cadmium is a highly poisonous element and one of the main concerns due to its significant increase because of anthropogenic activities worldwide. In 1960's cadmium is the core cause of 'itai-itai' illness, a disorder due to exposure or intake of food and water contaminated with cadmium. Too much exposure to cadmium was the reason for the development of lung insufficiency, renal disturbances, and osteomalacia. Moreover, development of high blood pressure and different kinds of carcinoma are caused by the exposure to cadmium (Bernard and Lauwerys, 1986). The reason for the cadmium exposure is the manufacturing of cadmium and other chemicals, inhalation of fumes including dusts, and eating or burning cigarettes with contaminated hands (Piscator et al., 1976; Satarug and Phelps, 2020). Lead on the other hand, is the most abundant heavy metal in nature. It is so much toxic element usually present in air, water, and soil. It is derived from fossil fuel, paints, and other industrial wastes. Exposure to lead can result to cancer, kidney failure, brain damage, rise in blood pressure, reproductive disease, and heart disease (Moshin, 2022).

Bioremediation, specifically the mycoremediation, is an intermediate biotechnology through the process of bioaccumulation that eliminates (binds) toxic compounds in dead (inactive) bioparticles removed from the microbes or flora (De Filippis, 2015). According to Das (2012), bio-absorption is an appropriate and economical technique for the elimination of metals and radionuclides using their biomass.

Microorganisms use their enzymatic activity to purify the environmental problems. Moreover, they do this under a normal ecosystem with many other living microorganisms. The microorganisms that reduce the contaminants, also contribute to, or affect the development, as well as other microorganisms. Microorganisms also eliminate the many types of contaminants in the soil. Soil sediments develop as methane and turn into a greenhouse gas (Watanabe, 2001). Heavy metal contamination is one of the environmental problems in the Philippines. In 2001, due to heavy rains brought about by a typhoon, the mine tailing deposit of a mining company collapsed. The rice paddies in the municipalities of San Manuel and Binalonan, Pangasinan, Philippines were buried in one-meter-deep contaminated sediments with heavy metals (Alliance, 2007). These municipalities have a significant rice-growing area contaminated with heavy metals. Mycoremediation is one way to clean the environment from pollutants like heavy metals. Hence, the objective was to isolate, identify, and evaluate different species of fungi from heavy metal contaminated rice growing areas which can be used for mycoremediation.

Materials and methods

Collection of soil samples

Soil samples were collected from Sitio Namangonan, Guiset Norte, San Manuel, Pangasinan during the 2024 dry season. Collected soil samples were mixed to come up with approximately 9,000 grams (9.0 kg) composite samples from nine sampling location points with a depth of 20 cm. The composite soil samples were placed in a sterilized plastic tub and were instantly transported to the laboratory for analysis.

Analysis of cadmium and lead contents of soil samples

The method for extraction of soil samples was aqua regia or a combination of concentrated HNO₃ and concentrated HCl with 1:3 ratio. Concentration of heavy metals specifically cadmium and lead in the soil samples were evaluated through analytical methods of Shimadzu atomic absorption spectrophotometry (AAS).

Isolation of soil-borne fungi as bioremediators

Fungi existing in soil were isolated using serial dilution method. Ten grams (10 g) of naturally dried soil sample was weighed and mixed to 90 mL of sterilized distilled water (dH₂O). The suspension was shaken using vortex for 30 minutes. The one milliliter (1 mL) suspension was mixed in a 9 mL sterilized dH₂O using sterile pipette. One milliliter previous suspension was repeated to dilute five times in a 9 mL sterilized dH₂O. The serial dilutions were valued as 10^{-1} through 10^{-5} .

For the isolation of soil-borne fungi, 100 μ L of each of the five (5) suspensions (10⁻¹ through 10⁻⁵) were transferred and spread on sterilized Petri plates with three droplets of Streptomycin sulfate. The melted and warmed sterilized potato dextrose agar (PDA) was added in sterilized Petri plates with different suspensions (10⁻¹ through 10⁻⁵). The Petri plates with different suspensions were incubated at ambient temperature to favor the growth of the fungal colonies and incubated within 3 to 5 days. The distinct fungal colonies were aseptically picked using sterilized inoculating needle and transferred into sterilized Petri plates with PDA. These Petri plates served as stock culture of the fungal isolates.

Occurrence of fungal colonies

After the selection of desired suspension of serial dilution, percent fungal occurrence was determined using the formula:

Purification of fungal isolates

Fungal isolates were purified using three-point inoculation method. The fungal isolate with active portion was inoculated on the surface facing down on sterile PDA using flamed inoculating needle. The parafilm was used to seal the lid of the Petri plates. The Petri plates were incubated at room temperature to favor the growth of fungal isolate until profuse growth was observed. The pure culture of fungal isolates was photographed for documentation.

Identification of the fungal isolates

The growth of soil-borne fungi was observed using agar block culture. In this technique, the structural growth of fungal isolate was observed without disturbance or continuously moved as when the paper towel is lifted from the culture plate to the mounting slides. In agar block culture, the aluminum foil was bent and placed on a moist paper towel and was held on a glass slide in a sterile Petri plate. An agar block was cut aseptically with a square size from the PDA plate culture and was positioned on the surface of the glass slide. A cover slip was placed in the center of the inoculated agar block. Desired mycelia or spores of the fungal isolate were transferred in the four corners of the agar block. The Petri plate was enclosed and incubated until the desired development is attained. Sterile distilled water was added to the paper towel to maintain the moisture condition for the incubation period. When the mycelial growth of the fungal isolates is observed adhering to the glass slides or the coverslip, the cover slip was carefully raised and a small amount of lactophenol was added on a new clean slide at the area with the presence of mycelial growth. At the central portion of the fungal growth, another drop of lactophenol was used to observe the mounted glass of fungal isolates to identify morphologically using the books by Quimio and Hanlin (1999) and Watanabe (2010) as references.

Effect of cadmium and lead concentration on fungal growth

Inoculum (10 mm disk) from the 14-day-old culture of the fungal isolates was inoculated on the center of PDA culture media containing different concentrations such as 0, 10, 100, and 1000 mg/L of cadmium (Cd) and lead (Pb) with four replications. All plates were incubated at 27°C for 7 days. The diameter of mycelial growth was measured 7 days after incubation period. Those fungi that survived in 100 and 1000 mg/L concentrations of heavy metals were categorized as tolerant species of fungi.

Statistical analysis

The data were analyzed using the Statistical Tool for Agricultural Research (STAR) of the International Rice Research Institute (IRRI) at a 5% significance level, and the significant difference among the treatment means was compared.

Results

Heavy metals concentration of soil

Total cadmium and lead in the soil samples were determined to confirm the presence of these two toxic heavy metals in the collection site. The soil sample contained 0.42 mg/kg cadmium and 57.80 mg/kg lead.

Cultural and morphological identification of fungal isolates

The isolates were identified based on their cultural and morphological characteristics. Four species of fungi namely: *Trichoderma koningii*, *Penicillium janthinellum*, *Penicillium resticulosum*, and *Penicillium nigricans*. The morphological and cultural characteristics of each isolate are presented.

Trichoderma koningii Oudem.

Conidiophores are transparent, firm, branches attached with spore masses at the tip of the phialides; phialides is elongated near the tip. Conidia are transparent, oval-shaped with narrowed ends, and one-celled. Chlamydospores are brownish and nearly globose (Figure 1a and b).

Pure cultures on PDA media are snowy color with a light yellow and green shade; and creamy white at reverse growth (Figure 1c).



Figure 1. Cultural and morphological characteristics of *Trichoderma koningii* grown on PDA, 7 DAI at room temperature. (A) Phialides (blue arrow), (B) Phialospore (black arrows), (C) Obverse, and reverse growth. (Magnification: (A) = 100x, (B) = 400x)

Penicillium janthinellum Biourge

Conidiophores are transparent, firm, branched resembling at the tips with whorl metula, end part phialides, and on each phialide has chainlike conidia, conidial heads had different forms. Phialides are small with shortly elongated tips. Conidia are transparent, light green with dark in mass, rounded or oval shaped, one-celled, smooth, and simple at one end (Figure 2a).

Pure cultures on PDA are smooth, light grayish green on the surface: reverse light yellowish brown (Figure 2b).



Figure 2. Cultural and morphological characteristics of *Penicillium janthinellum* grown on PDA, 7 DAI at room temperature. (A) Conidiosphores (black arrow) and phialides (red arrow), (B) Obverse, and reverse growth (Magnification: (A) = 100x)

Penicillium resticulosum Birkinshaw, Raistrick, & Smith

Conidiophores are transparent, firm, branched resembling at the tips with two to three metula, three to four whorl phialides, and on each phialide has chainlike conidia with compact cylinder-shaped light gray and dark green conidial heads; phialide is lace shaped. Conidia are light green, mass is dark brown color, oval-shaped, and closely with small spines or prickles on the surface (Figure 3a).

Pure cultures on PDA are cottony, tufted with packs of aerial hyphae, reddish brown with greenish color; and vibrant yellowish and reddish at reverse growth (Figure 3b).



Figure 3. Cultural and morphological characteristics of *Penicillium* resticulosum grown on PDA, 7 DAI at room temperature. (A) Conidiosphores (black arrow) and phialides (red arrow), (B) Obverse, and reverse growth. (Magnification: (A) = 100x)

Penicillium nigricans (Bainier) Thom

Conidiophores are transparent, firm, with little coarse, established from top of hyphae, branched resembling at the tips, with first or second matula, whorl phialides, and at each phialide has chainlike conidia, gray and green conidial head has cylindrical columnlike form; Phialides are small with polished tips. Conidia are transparent, light green, round or oval-shaped, one-celled, warty rough, or closely with small spines or prickles on the surface (Figure 4a).

Pure cultures on PDA are smooth, shiny grayish green with whitish color; and light yellowish brown at reverse growth (Figure 4b).



Figure 4. Cultural and morphological characteristics of *Penicillium nigricans* grown on PDA, 7 DAI at room temperature. (A) Conidiosphores (black arrow) and phialides (red arrow), (B) Obverse, and reverse growth. (Magnification: (A) = 100x)

Percent occurrence of the fungal isolates

Four soil-borne fungi were isolated from heavy metal contaminated soil such as *Trichoderma koningii*, *Penicillium janthinellum*, *Penicillium resticulosum*, and *Penicillium nigricans*. Among the four fungal isolates, *Penicillium janthinellum* recorded the highest percent occurrence (42.85%) while *Penicillium resticulosum*, and *Penicillium nigricans* registered the lowest precent occurrence of 14.29% (Table 1).

Table 1. Percent occurrence of the *Trichoderma* sp. and *Penicillium* spp. fromsoil contaminated with heavy metals

Fungal Isolates	Percent (%) Occurrence
Trichoderma koningii	28.57
Penicillium janthinellum	42.85
Penicillium resticulosum	14.29
Penicillium nigricans	14.29

Mycelial growth and density on different concentration of cadmium

The fungal isolates from heavy metal contaminated soil were cultured on PDA containing different concentrations of cadmium. After seven days after incubation (7 DAI), *Penicillium janthinellum* and *Penicillium nigricans* produced maximum mycelial growth in PDA without cadmium, with means of 50.72 and 54.35 mm, respectively. In case of *Penicillium nigricans*, the highest mycelial diameter was observed in 10 and 100 mg/kg cadmium with means of 54.49 and 54.42 mm, respectively. In all species, no mycelial growth was observed in 1000 mg/kg cadmium. Statistical analysis showed no significance difference between the 0 mg/kg (control), 10 mg/kg, and 100 mg/kg of cadmium. Meanwhile very thick mycelial density was observed in all fungal species grown in the control and PDA with 10 mg/kg to 100 mg/kg cadmium (Table 2).

Mycelial growth and density on different concentrations of lead

The influence of various concentrations of lead on the mycelial growth and mycelial density of *Trichoderma koningii*, *Penicillium janthinellum*, *Penicillium resticulosum*, and *Penicillium nigricans* was evaluated. After seven days of incubation (7 DAI), it can be noted *Trichoderma koningii* and *Penicillium nigricans* exhibited maximum mycelial growth in 100 mg/kg lead with means of 45.65 and 54.42 mm, respectively. *Penicillium janthinellum* and *Penicillium resticulosum* recorded maximum mycelial growth in 1000 mg/kg lead with means of 50.83 and 54.36 mm, respectively. However, statistical analysis

revealed no significant different of the four species on different concentration of lead. Moreover, very thick mycelial density was noted in different species in all concentrations of lead. The findings of this study indicated these fungal species are tolerant of lead (Table 3).

Table 2. Mycelial growth diameter and mycelial density of the *Trichoderma* sp. and *Penicillium* spp. from contaminated soil with heavy metals on potato dextrose agar media with different cadmium concentration at seven days after inoculation (7 DAI)

Cadmium concentration	Mycelial diameter (mm)			
(mg/kg)	T. koningii	P. janthinellum	P. resticulosum	P. nigricans
0	$45.60\pm0.92^{\texttt{a}}$	$50.72\pm0.61^{\mathtt{a}}$	$45.72\pm0.42^{\mathbf{a}}$	$54.35 \pm 1.00^{\text{a}}$
(Control)	(++++)	(++++)	(++++)	(++++)
10	$45.66 \pm 1.03^{\text{a}}$	$50.71 \pm 1.25^{\mathbf{a}}$	$45.74\pm0.18^{\text{a}}$	$54.49\pm0.99^{\text{a}}$
	(++++)	(++++)	(++++)	(++++)
100	$45.84\pm0.26^{\text{a}}$	$50.75\pm0.20^{\mathbf{a}}$	$45.70\pm2.15^{\mathtt{a}}$	$54.42 \pm 1.56^{\text{a}}$
	(++++)	(++++)	(++++)	(++++)
1000	$10.00\pm0.00^{\text{b}}$	$10.00\pm0.00^{\text{b}}$	$10.00\pm0.00^{\text{b}}$	$10.00\pm0.00^{\text{b}}$
	(-)	(-)	(-)	(-)

Values are mean \pm SD. Means with the same letter are not significantly different using Least Significant Difference (LSD) Test (p < 0.05)

Mycelial density: no growth (-), very thin (+), thin (++), thick (+++), very thick (++++)

Table 3. Mycelial growth diameter and mycelial density of the *Trichoderma* sp. and *Penicillium* spp. from contaminated soil with heavy metals on potato dextrose agar media with different lead concentration at seven days after inoculation (7 DAI)

Lead concentration	Mycelial diameter (mm)			
(mg/kg)	T. koningii	P. janthinellum	P. resticulosum	P. nigricans
0	$45.60\pm0.92^{\mathtt{a}}$	$50.72\pm0.61^{\mathbf{a}}$	$45.72\pm0.42^{\rm a}$	54.35 ± 1.00^{a}
(Control)	(++++)	(++++)	(++++)	(++++)
10	$45.61\pm0.34^{\text{a}}$	50.77 ± 0.68^{a}	$45.78\pm0.42^{\mathbf{a}}$	$54.37\pm0.37^{\mathbf{a}}$
	(++++)	(++++)	(++++)	(++++)
100	$45.65\pm0.23^{\mathbf{a}}$	$50.79\pm0.41^{\mathbf{a}}$	45.76 ± 0.36^{a}	$54.42\pm0.51^{\mathtt{a}}$
	(++++)	(++++)	(++++)	(++++)
1000	$45.63\pm0.32^{\mathbf{a}}$	$50.83\pm0.42^{\mathbf{a}}$	45.84 ± 0.46^{a}	$54.36\pm0.16^{\rm a}$
	(++++)	(++++)	(++++)	(++++)

Values are mean \pm SD. Means with the same letter are not significantly different using Least Significant Difference (LSD) Test (p < 0.05)

Mycelial density: no growth (-), very thin (+), thin (++), thick (+++), very thick (++++)

Discussion

The presence of Trichoderma koningii, Penicillium janthinellum, Penicillium resticulosum and Penicillium resticulosum in rice growing areas contaminated by lead and cadmium is documented in this study. This finding confirms earlier report that different species of fungi occurred in soil contaminated with heavy metals (Oladipo et al., 2018). In the present study, it was noted that different fungal isolates exhibited a noticeable tolerance to various concentrations of heavy metals such as cadmium and lead. The tolerance expressed by fungal isolates could be due to their ability to adopt to this kind of environment. Heavy metal tolerance may result from both intracellular and extracellular process of reducing heavy metal through binding of ligand or protein (Anahid et al., 2011). Moreover, lower fungi can detoxify heavy metals through biosynthesis (converting chemical substances into inactive state), bioadsoprtion (removal of pollutant substances), bioaccumulation (build-up in the cells of fungi), biomineralization (biotransformation from pollutant to mineral), bio-reduction (bioburden), bio-oxidation (microbial oxidation of the heavy metals), extracellular precipitation (remove heavy metals from a substrate), intracellular precipitation (intracellular accumulation), surface sorption (absorb pollutants from their environment through their cell walls), etc. which vary from species to species (Gadd, 2007; Vala and Sutariya, 2012).

Previous studies have confirmed the potential use of fungi in bioremediation. For instance, Madhi *et al.* (2021) reported that *Trichoderma koningii* is effective in bio-absorption with a 46.8% removal rate in 3 mg/kg Cd in soil. *Penicillium* spp. has high tolerance and thrives in Cd contaminated soil with a maximum removal rate of 32.2% (He *et al.*, 2023). Endophytic microorganism such as *Penicillium janthinellum* can reduce the Cd accumulation in tomato plants. Endophytic symbiosis can exert the negative effect of heavy metal stress in plants (Khan *et al.*, 2014). *Penicillium* sp. is tolerant to high concentration of Cd and Pb (Khodja *et al.*, 2018) while other soil-borne fungus like *Penicillium chrysogenum* removed 49% Cd with maximum resistance at 1000 mg/kg Cd concentration in the culture media (Din *et al.*, 2022). *Penicillium chrysogenum* is effective in the removal of Cd, Pb and other heavy metals present in the soils, water, and other environments (Niu *et al.*, 1993). Furthermore, *Penicillium* species could be utilized as for effective bioremediation for contaminated soil with Cd and Pb (Zehra *et al.*, 2018).

In summary, the present study reported the successful isolation of *Trichoderma koningii, Penicillium janthinellum, Penicillium resticulosum,* and *Penicillium nigricans* from rice paddy soil contaminated with cadmium and lead. The study also established the heavy metal tolerance at certain concentrations of cadmium and lead, which suggests their potential as mycoremediator or mycouptaker, which needs further investigation in future studies.

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