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Trichoderma atroviride ISOLATED FROM MANGROVES OF THE EAST COAST OF PENINSULAR MALAYSIA EXHIBITED HIGH TOLERANCE AGAINST HEAVY METAL CADMIUM

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ABSTRACT

The toxicity of the heavy metal cadmium to organisms is known, with differing tolerance in different organisms. The ability of fungi to survive in sites polluted with heavy metals has led to its mechanism of heavy metal tolerance being widely investigated. However, little work has addressed cadmium tolerance in fungi isolated from mangroves, the heavy metal basin. Therefore, this study was carried out to isolate fungi and investigate their tolerance towards cadmium. Samples used were obtained from the mangroves in the east coast of Peninsular Malaysia. Aquatic fungal isolates were selected for cadmium-tolerance screening, in the range of 0 to 0.5 mM. The identification of the most tolerant fungus was confirmed using the molecular approach and used further to study its tolerance in higher concentrations of cadmium. A total of 25 fungi was isolated and seven isolates were classified as aquatic fungi. *Trichoderma* sp. was shown to be the most tolerant towards cadmium. The sequencing result of the amplified fungal gene confirmed its identity as *Trichoderma atroviride*. Further tolerance test showed that the fungus survived in 3.0 mM, the highest concentration tested, although at a slower growth rate and with affected sporulation. These results can be the baseline data for further investigations on the mechanism of cadmium detoxification in *T. atroviride*, thus enhancing its potential as a heavy metal bioremediator.

Key words: Mangrove fungus, east coast Peninsular Malaysia *Trichoderma atroviride*, tolerance, cadmium

INTRODUCTION

Heavy metals concentrations in the environment due to natural processes and human activities have increased. These concentrations cannot be degraded or destroyed and thus will remain in the environment and could affect it and the living organisms in it (Järup *et al.*, 2003; Morais *et al.*, 2012; Jaishankar *et al.*, 2014). At high concentrations, heavy metals can react to form toxic compounds in cells and tend to persist indefinitely in the food chain. Among the heavy metals, cadmium is a relatively rare earth element that is almost uniformly distributed in the earth crust and is probably the most biotoxic (Sadiq *et al.*, 1992). Cadmium occurs naturally everywhere in air, water, soils and foodstuffs and therefore is widely used in various industrial products and processes. The production of cadmium and cadmium products, their use and final disposal represent only a very small fraction of the total sources of all human cadmium exposure, which is less than 2% (ICdA *et al.*, 2009). However, cadmium has a biological half-life of about 20 years in the human body. At low-level long-term exposure, concentrations of cadmium in urine reflect in kidneys, where approximately one-third of the body burden is found (Friberg & Vahter,

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1983). Hence, it is very important to decontaminate cadmium and other heavy metals.

Heavy metals from the contaminated locations are removed through chemical and physical approaches which are generally effective in the treatment of high concentration of metals but less effective in the removal of metals in low concentration (10-100 ppm) (Simonescu & Ferdeş, 2012; Chen *et al*., 2017). A biological approach using certain organisms which can survive in high concentrations of metals and have the potential to accumulate different metals has been explored. As for cadmium, its tolerance in different organisms, especially in plant species, was reported many years ago (Coughtrey & Martin, 1977). Simon (1977) investigated the cadmium tolerance in higher plants, *Festuca ovina* and *Agrostis tenuis*, which was isolated from mining sites and an aerially contaminated site. Plants have developed defence systems that ensure efficient cadmium detoxification by complexation and vacuolar sequestration (Heiss *et al*., 2003). Several studies of cadmium tolerance in microorganisms such as *Schizosaccharomyces pombe* (Murasugi *et al*., 1981) and *S. cerevisiae* (Kneer *et al*., 1992) have been conducted. In bacteria, a wide range of efflux pumps has been shown to mediate metal detoxification (Silver & Phung, 1996). It has been suggested that different organisms have different mechanisms of cadmium detoxification depending on their biological reactions.

Among the microbes, bioremediation by fungi has become the preferred method because fungal biomass has a high percentage of cell wall materials which mainly consist of polysaccharides, proteins, and lipids, with many functional groups that can be used in metal bonding (Simonescu & Ferdeş, 2012). They also exhibit versatile biosorbent characteristics as they can grow under extreme conditions of pH, temperature and nutrient, apart from high metal concentration (Anand *et al*., 2006). However, little work has addressed the resistance towards cadmium in fungi isolated from mangroves, an ecosystem which has been considered as a heavy metal basin. This study was carried out to isolate fungi from mangroves of the east coast of Peninsular Malaysia, screen their tolerance towards cadmium and investigate its growth pattern in the presence of cadmium.

MATERIALS AND METHODS

Sampling sites

Sampling for mangrove fragments was conducted in the mangrove areas at Tok Bali, Kelantan and Universiti Malaysia Terengganu (UMT), Terengganu. The root, stem, and leaf samples from intertidal and aerial parts of *Avicennia alba, Rhizophora apiculata,* and *Sonneratia caseolaris* were collected and washed with seawater to remove adhering mud before fungal isolation in the laboratory.

Isolation of fungi associated with mangroves

The isolation of fungi was carried out using Direct Plating Technique (Jones & Hyde, 1988) that is commonly applied to isolate endophytic fungi. The roots, stems and leaves were cut into small fragments of approximately 3 cm in length. Then, the samples were washed with 95% (v/v) ethanol for 1 min followed by 5% (v/v) hypochlorite solution for 30 sec and rinsed with distilled water for 1 min to remove any possible contamination from the surface of the samples. The sterile samples were then placed onto Potato Dextrose Agar (PDA) and Sea Water Agar (SWA) incorporated with 0.5 g/L antibiotics. The samples were incubated in the dark, at room temperature for at least 5 days to induce growth and sporulation. After five to seven days, the colonies that had grown on the agar plates were isolated to obtain a pure culture.

Morphological identification of fungal isolates

The identification of fungi was conducted using the simple Slide Culture Technique (Riddel *et al*., 1950). Slide cultures of the fungal isolates were then observed using the light microscope for their morphology which includes the color of hyphae, appearance of spores, and texture of cultures. The morphology observed was then referred to the identification keys based on several references by Kohlmeyer and Kohlmeyer (1979), Sarma and Hyde (1999), Jones and Alias (1997), Barnett and Hunter (1998), Deacon (1997), Gupta (2004), Dighton *et al*. (2005), Ellis *et al*. (1992) and Dix and Webster (1995).

Screening for cadmium-tolerant aquatic fungi

The screening for aquatic fungi which are tolerant to cadmium toxicity was carried out by growing the marine fungi on SWA and freshwater fungi on PDA containing different concentrations of $CdCl_2$ in the range of 0 to 0.5 mM (Min *et al*., 1997). Seven species of fungi previously identified as aquatic fungi were placed on the agar plates and were incubated in the dark at 27°C for seven days. The growth of fungi on treatment medium was observed as weak growth (mycelia covering only a small part of media; +), moderate growth (mycelia covering three-quarter of media; ++), good growth (mycelia covering the whole media; +++) or no growth (-) (Luo *et al*., 2005). The fungus that tolerated the highest concentration of cadmium was selected for further experiment.

Molecular identification of the most tolerant fungus

Molecular identification was performed to further confirm the identification of the selected fungus. Firstly, the fungus was grown in 100 mL PDB and incubated for seven days at 27°C with shaking at 120 rpm in a dark condition. Following a modified method of Jaeckel *et al.* (2005), the culture was filtered through Whatmann No. 54 filter paper using vacuum filtration to collect the mycelia, washed twice with distilled water, and tamped dry between filter papers. The dried mycelia were stored at -80°C for one day.

The genomic DNA was extracted using the Wizard Genomic DNA Purification protocol (Promega) according to the manufacturer's instructions. The quality of the DNA was evaluated using UV Vis Spectrophotometer (Shimadzu, Japan) at 260 nm and 280 nm and followed by 1.2% (v/v) agarose gel electrophoresis. The targeted DNA strands were amplified in a total Polymerase Chain Reaction (PCR) mixture of 25 µL using an Eppendorf Mastercycler Gradient. The reaction mixture was prepared by mixing 1.5 to 3.0 µM $MgCl_2$, 1× PCR buffer (1 mM Tris-HCl and 5 mM KCl), ~100 ng of template DNA, 200 µM dNTPs, 1 U Taq DNA polymerase, and 100 moles of each reversed and forward primers. The 28S ribosomal RNA gene identification was carried out using primer pairs: LROR 5'-ACCCGCTGAACTTAAGC-3' and LR7 5'-TACTACCACCAACATCT-3', which are normally used in genotypic identification.

The PCR was performed following the standard procedure: initial denaturation step at 95°C for 2 min followed by 35 cycles for each denaturation (94°C for 1 min), primer annealing (50°C for 1 min) and primer extension (72ºC for 1 min). The final extension step was at 72°C for 8 min and holding at 4°C. The PCR product was analysed on electrophoresis using 1.2% (v/v) agarose gel and visualized by staining with ethidium bromide. The amplicon was purified after ascertaining their integrity before it was sent for sequencing. The sequences were then aligned using the database retrieved from NCBI integrated database, GenBank, EMBL, and DDBJ using BLAST at http://www.ncbi.nlm.nih.gov/blast.

Higher cadmium-tolerance study

The selected fungus was cultured in 25 mL PDA supplemented with 0.25, 0.5, 0.75, 1.0, 1.25, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0 mM $CdCl_2$ and incubated at 30°C in the dark for 14 days. The control culture was grown without $CdCl_2$. The diameter of the fungal mycelia was measured at intervals of two days to determine its growth as described by Luo *et al.* (2005).

RESULTS AND DISCUSSION

Isolation and identification of mangrove fungi

Based on the three key features which are the color of hyphae, the appearance of spores, and texture of cultures, 11 species of fungi were isolated from the mangroves of Universiti Malaysia Terengganu and 19 were isolated from the mangroves of Tok Bali, Kelantan (Table 1). The species isolated from the mangrove area in UMT were *Absidia corymbifera, Acremonium* sp., *Chrysosporium* sp., *Curvularia lunata, Epicoccum nigrum, Fonsecaea pedrosei, Microsporum eqiunum, Mucor* sp., *Rhizopus* sp., *Savoryella paucispora,* and *Trichoderma* sp. Out of these, five species were found in both locations: *Acremonium* sp., *Epicoccum nigrum, Fonsecaea pedrosei, Microsporum eqiunum,* and *Rhizopus* sp. More marine fungi were recorded from the mangroves of Tok Bali, Kelantan. Final identification revealed a total of 25 species of fungi with seven species classified as aquatic fungi of which six were marine fungi: *Koralionastes augustus, K. violaceus, Haloguignardia cystoseirae, Pontogeneia calospora, Trematosphaeria mangrovei* and *Savoryella paucispora*, and one freshwater fungus, *Trichoderma* sp. (Table 2).

Fewer aquatic fungi were obtained and this could be due to the salinity factor. The samples were taken after a rainfall which had resulted in the flushing of freshwater and sediment to the mangrove habitat that led to lower salinity. Under low salinity, fresh water and terrestrial fungi were involved in litter conditioning while the activities of marine fungi were only supported when the salinity increased (Barnett & Hunter, 1998).

Screening for cadmium-tolerant aquatic fungi

The screening for Cadmium-tolerant aquatic fungi showed that *Trichoderma* sp. was the most tolerant fungus to Cd among the seven aquatic fungi tested (Table 3). This was followed by *P. calospora, S. paucispora, K. augustus, H. cystoseirae, T. mangrovei* and *K. violaceus*, indicating that the fungus is a good biosorption agent. *Trichoderma* genus comprises a great number of fungal strains that act as biological control agents and the antagonistic properties are based on the activation of multiple mechanisms (Benitez *et al.*, 2004).

The success of *Trichoderma* strains as bio-control agents is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. These properties have made

Table 1. Morphological descriptions of terrestrial fungi isolated from *Avicennia alba*, *Rhizophora apiculata* and *Sonneratia caseolaris* in UMT and Tok Bali, Kelantan

Species	Morphology			Location/Origin
	Colour of hyphae	Appearance of spores	Texture of cultures	
Absidia corymbifera	Grey	White	Fleshy	UMT
Acremonium sp.	White	White	Downy	UMT & Tok Bali
Apophysomyces elegans	White Cream	White	Fleshy	Tok Bali
Aspergillus sp.	White	Dark Green	Cottony	Tok Bali
Aureobasidium pulullans	Brown	Dark Brown	Mouldy	Tok Bali
Chaetomium globosum	White	White Cream	Downy	Tok Bali
Chrysosporium sp.	White	Dark Green and White	Cottony	UMT
Cladosporium herbarum	Dark Green	Dark Green	Downy	Tok Bali
Curvularia lunata	Black	Black	Downy	UMT
Epicoccum nigrum	Brown	Dark Brown	Mouldy	UMT & Tok Bali
Fonsecaea pedrosei	Black	Black	Mouldy	UMT & Tok Bali
Microsporum eqiunum	Bright Yellow	Cream	Slimy	UMT & Tok Bali
Microsporum sp.	Yellow Cream	Light Green	Downy	Tok Bali
Mucor sp.	Dark Grey	Dark Grey	Downy	UMT
Phialophora sp.	Dark Red	Dark Red	Downy	Tok Bali
Trichophyton sp.	Cream	White	Mouldy	Tok Bali
Trichophyton violaceum	Deep Violet	Deep Violet	Mouldy	Tok Bali
Rhizopus sp.	Greenish Grey	Light Green	Cottony	UMT & Tok Bali
Koralionastes augustus	Cream	Light Brown	Slimy	Tok Bali
Koralionastes violaceus	White Cream	Light Brown	Slimy	Tok Bali
Haloguignardia cystoseirae	Brownish Cream	Dark Brown	Slimy	Tok Bali
Pontogeneia calospora	Brown	Black	Mouldy	Tok Bali
Trematosphaeria mangrovei	Bright Yellow	Cream	Slimy	Tok Bali
Savoryella paucispora	Brown	Light Brown	Cottony	UMT
Trichoderma sp.	Light Green	Greenish White	Cottony	UMT

Table 2. Morphological descriptions of hyphae and microscopic view of fungal cultures isolated from *Avicennia alba, Rhizophora apiculata* and *Sonneratia caseolaris* in UMT and Tok Bali, Kelantan

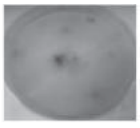
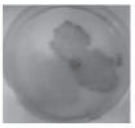
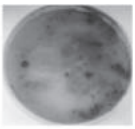
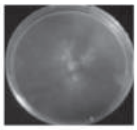
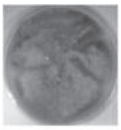
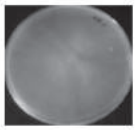
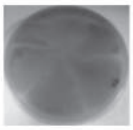
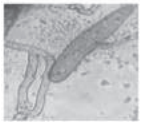
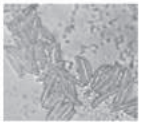
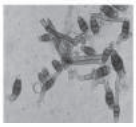
	Fungi						
	Koralionastes augustus	*Koralionastes violaceus*	*Haloguignardia cystoseirae*	*Pontogeneia calospora*	*Trematosphaeria mangrovei*	*Savoryella paucispora*	*Trichoderma* sp.
Top							
Bottom							
Microscopic (Magnification)							
	40x	40x	100x	100x	40x	40x	40x
Colour of hyphae	Cream	White Cream	Brownish Cream	Brown	Bright Yellow	Brown	Light Green

Table 3. Growth of mangrove aquatic fungi in different cadmium concentrations

Fungi	Concentration of $CdCl_2$ (μM)										
	0	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50
Trichoderma sp.	+++	+++	+++	+++	+++	++	++	++	++	+	+
Trematosphaeria mangrovei	+++	++	++	++	++	+	+	+	+	–	–
Savoryella paucispora	+++	++	++	++	++	+	+	–	–	–	–
Koralionastes violaceus	+++	++	++	++	++	+	–	–	–	–	–
Koralionastes augustus	+++	++	++	++	++	+	–	–	–	–	–
Haloguignardia cystoseirae	+++	++	++	+	+	–	–	–	–	–	–
Pontogeneia calospora	+++	++	+	–	–	–	–	–	–	–	–

+ = weak growth; ++ = moderate growth; +++ = good growth; – = no growth.

Trichoderma a ubiquitous genus present in any habitat and at high population densities (Chet *et al.*, 1997). Benitez *et al.* (2004) have described the biocontrol mechanisms of various *Trichoderma* strains such as *T. harzianum, T. virens* and *T. hamatum* in different aspects especially as a biofertilizer and plant protector.

Molecular identification of the heavy metal tolerant fungus

The BLAST search further confirmed the identity of *Trichoderma* sp. as *Trichoderma atroviride,* where the sequence data of the isolated fungi shared 98% homology with the 28S ribosomal RNA gene of *Trichoderma atroviride* (EF591763). The gene was also highly identical to *Hypocrea rufa* but the morphological characteristics are slightly different compared to selected fungi screened previously. *H. rufa* was different in color and shape of spores but only *T. atroviride* matched both morphologically and genetically. Previously, *T. atroviride* was widely studied as an antifungal agent by the production of chitinase during mycoparasitism (Mach *et al.*, 1999; Benitez *et al.*, 2004) but no report on the studies of heavy metal detoxification in *T. atroviride* is available.

Higher tolerance to cadmium study

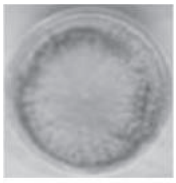
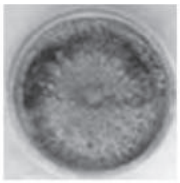
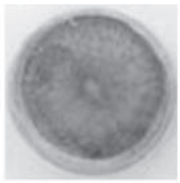
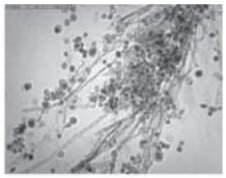
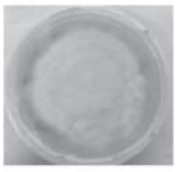
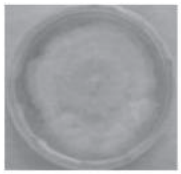
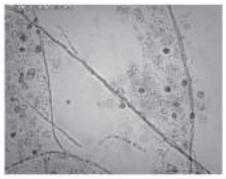
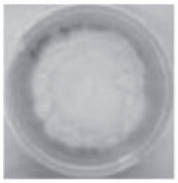
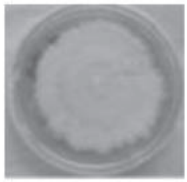
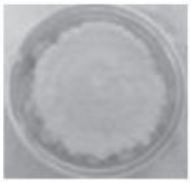
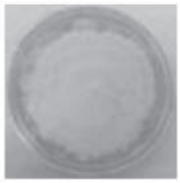
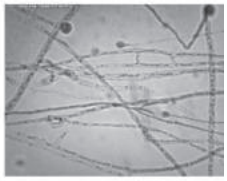
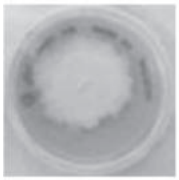
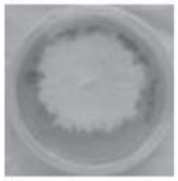
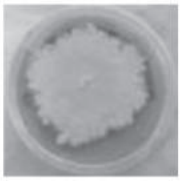
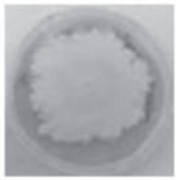
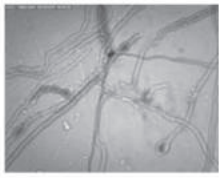
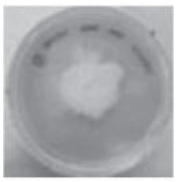
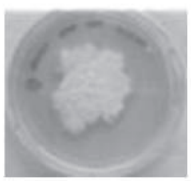
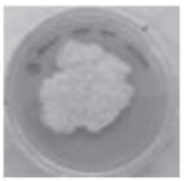
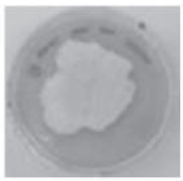
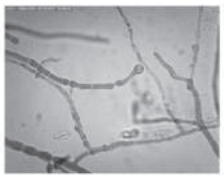
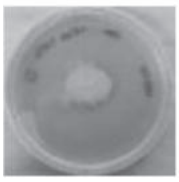
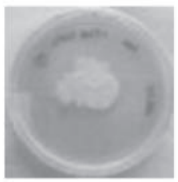
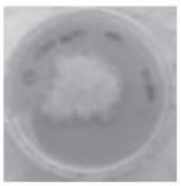
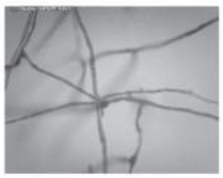
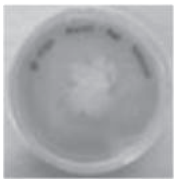
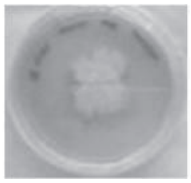
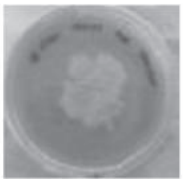
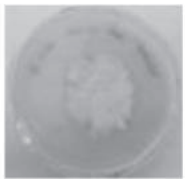
Trichoderma atroviride was found to be highly tolerant to Cd where growth was possible in up to 3.0 mM $CdCl_2$, although the diameter of the mycelial growth decreased with increased concentration of Cd, indicating weaker growth (Table 4). In the absence of Cd, growth of mycelia increased remarkably on day 2 of incubation and achieved almost full-blown growth on day 5. In the presence of Cd, the highest concentration of Cd in PDA that *T. atroviride* could tolerate to achieve full growth on day 14 was 0.75 mM $CdCl_2$. With the highest Cd in media (3.0 mM), the fungal growth was reduced to 58.5% on day 14.

According to Baldrian (2003), the hyphae aerial of fungus *Schizophyllum commune* in solid media containing Cd was increased and the growth segment was changed. Loops and connective filaments were developed together with an increase of hyphal branching and the extent of these changes reflected the increasing concentration of the metal ion. Additionally, the sporulation by *T. atroviride* growing in Cd was also affected, despite the tolerance to Cd. The control fungus started to sporulate around day 5 involving younger culture at the edge of the plate. As for the Cd-treated fungus, the culture remained whitish, indicating a lack of sporulation, as supported by the microscopic view which shows that the formation of spore decreased when the concentration of Cd was increased.

CONCLUSION

From the two mangroves areas in the east coast of Peninsular Malaysia – Tok Bali and UMT – a total of 25 species of fungi was isolated but only seven aquatic fungi were found. The only freshwater fungus isolated, *Trichoderma atroviride,* was found to be the most tolerant to the heavy metal cadmium, to up to 3.0 mM. The ability of this fungus to withstand high toxicity of cadmium provides a potential source of new bioremediation agent that could bring benefits to the waste-water treatment industry. It is recommended that studies be carried out in the future on the cadmium detoxification mechanism in *T. atroviride*, to provide greater insights on the roles of bioactive compounds and metabolites during the process. This would also lead to the identification of potential biomarkers to detect cadmium contamination in the environment.

Table 4. Effect of cadmium concentration on the growth of *T. atroviride* on PDA at 30°C

$CdCl_2$ (mM)	Day of incubation				
	8	10	12	14	Microscopic view (40x) at day 14
0.0					
0.75					
1.0					
1.5					
2.0					
2.5					
3.0					

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