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In vitro RESPONSE OF FUNGI ISOLATED FROM ORCHIDS IN BRIS, SETIU WETLAND AND MANGROVE IN MORIB, TO DIFFERENT CONCENTRATIONS OF LEAD

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ABSTRACT

Interaction of plants with a mycorrhizal partner is known to mediate plant tolerance towards pollution of heavy metal minerals in soil. For orchids, their relationship with orchid mycorrhizal fungi may enable this largest flowering plant family to adapt to various extreme habitats. On the other hand, mangrove plants are also recognized to have this ability as mangroves are a known sink for heavy metal accumulation. Thus, this study aims to isolate and identify mycorrhizal fungi from two habitats; orchids in beach ridges interspersed with swales (BRIS) soil in Setiu Wetland, Terengganu and from mangrove plants in Morib, Selangor; then investigate their tolerance towards lead (Pb) stress. Isolation from BRIS orchids yield two *Rhizoctonia* species and *Penicillium chrysogenum* while *Penicillium pinophilum*, *Aspergillus fumigatus* and *Aspergillus niger* where isolated from mangrove plants in Morib. *In vitro* tolerance to lead tested with concentrations of 100 mg/L, 500 mg/L and 1000 mg/L of Lead (II) nitrate prepared in potato dextrose agar (PDA), showed that all the isolated fungi from mangrove were able to tolerate Pb even at the highest concentration of 1000mg/L but with a slower growth rate. Further studies must be carried out for the discovery of novel species of mycorrhizal fungi that are able to tolerate heavy metal stress to improve plant adaptation for agriculture.

Key words: Heavy metal tolerance, mangrove, mycorrhiza, orchid

INTRODUCTION

Industrial waste and agricultural practices such as the excessive use of fertilizers contribute to deteriorating soil health especially increase in soil salinity and heavy metal pollution (Savci, 2012). This negatively impacts the soil environment and eventually obstructs plant growth. To this end, mycorrhizal fungi or fungi that live in a symbiotic relationship with plants are known to have the ability to mediate plant growth in heavy metal stressed condition (Singh, 2006; Khade & Adholeya, 2007). Mycorrhizal fungi are able to alleviate heavy metal toxicity in plants by reducing metal translocation from root to shoot (Leyval *et al*., 2002) and assist in acquiring nutrients from contaminated substrates otherwise inaccessible to plants (Finlay, 2008).

For orchids, their relationship with orchid mycorrhizal fungi may enable this largest flowering plant family to adapt to various extreme habitats. One such extreme habitat is Beach Ridges Interspersed with Swales (BRIS) soil in Setiu Wetland where it is categorized as infertile land; sandy soil with harsh physical-chemical properties and low nutrient content (Lah *et al*., 2011). Micronutrients; Cu, Zn, Fe, and Mn tend to be deficient in a coarse textured soil such as sandy soil (Noulas *et al*., 2018) and BRIS has been found to be deficient in metal minerals (Toriman *et al*., 2009). Despite this, epiphytic and even terrestrial orchid species have been found to be able to adapt and live in these conditions.

On the other hand, mangroves can also be considered an extreme habitat. Mangroves are situated on coastlines and river estuaries with plants tolerant to fluctuating high saline conditions. Mangroves are known as a dump for waste materials, including heavy metals (Weis & Weis, 2004).

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Shazili *et al.* (2006) reported high lead concentrations in mangrove sediment in West Coast of Peninsular Malaysia. This is not surprising as urbanization and industrialization, in Selangor especially, contribute to the rapid increase in heavy metal waste including lead to the mangroves of Selangor (Ismail *et al.*, 2016). As such, the mangrove plants are still able to tolerate and adapt to the pollution.

As mentioned before, the respective association with specific mycorrhizal fungi may contribute to the adaptive ability of orchids and mangrove plants to heavy metal pollution. Identification of unique mycorrhizal species with tolerance capabilities may contribute to the development of biofertilizers and bioremediators that can enhance agricultural practices and reduce soil pollution. Thus, this study aims to isolate and identify mycorrhizal fungi from two extreme habitats; orchids in BRIS soil in Setiu Wetland, Terengganu and from mangrove plants in Morib, Selangor; and further investigate their *in vitro* tolerance towards lead (Pb) stress.

MATERIALS AND METHODS

Sample Collection

Sample collection in Setiu Wetlands (BRIS area) was done in nine random plots of epiphytic (*Dendrobium crumenatum*) and terrestrial orchids (*Phalaenopsis pulcherrima* and *Bromheadia finlaysoniana*). Sample collection at mangrove site was done in Pantai Kelanang, Morib, where roots of *Rhizophora mucronata* were collected from three random plots.

Isolation and Identification of Orchid Mycorrhizal Fungi

Isolation of fungi was done according to Warcup and Talbot (1967). Root samples were cleaned off debris by washing under running tap water. Surface sterilization was done in 0.5% sodium hypochlorite for 3 mins, two washes with distilled water and later air dried. Isolation was done on 3.9% (w/v) Potato Dextrose Agar (PDA) with 0.01% (w/v) Penicillin at room temperature until fungal colonies arose. Growth rate, morphology and color of the colony were recorded. This was followed by microscopic and morphological observation by staining the mycelium with lactophenol cotton-blue solution and Acridine Orange and then examining under the fluorescent microscope (Olympus).

In vitro lead tolerance screening

In vitro lead tolerance screening was done according to a method by Iskandar *et al.* (2011) with treatments of $Pb(NO_3)_2$ in concentrations of 100 mg/L, 500 mg/L and 1000 mg/L in PDA, including plate without $Pb(NO_3)_2$ as a control. A hyphal disc from each isolate was inoculated on the treatments and incubated for 7 days. Fungal Pb tolerance was measured through growth rate and dry weight of fungal biomass (Fomina *et al.*, 2005).

Data analysis

Statistical analysis of fungal tolerance to different concentration of Pb was conducted using Statistical Package for the Social Sciences (SPSS) by applying one way ANOVA by comparing the means of growth rate and dry biomass of fungal in different Pb concentrations.

RESULTS AND DISCUSSION

The fungi isolated from mangrove roots in Morib, Selangor were identified as *Penicillium pinophilum, Aspergillus fumigatus* and *Aspergillus niger* while *Rhizoctonia* sp. *(A), Pencillium chrysogenum* and *Rhizoctonia* sp. *(B)* were isolated from orchids in BRIS, Setiu Wetland (Figure 1). Table 1 summarizes the morphological characteristics of all the isolates. Of all the identified species, only *P. pinophilum* has been previously reported as an arbuscular mycorrhiza (AM) and was shown to have a beneficial relationship with strawberry plants (Fan *et al.*, 2008). Typical mangrove mycorrhiza are the Glomeromycota such as *G. mossaeae* and *G. intraradices* that are distributed worldwide in the salt marches ecosystem (Wang *et al.*, 2010) while *Halocyphina villosa*, that have been isolated from mangrove in Morib. On the other hand, *Rhizoctonia* species are typical orchid mycorrhiza fungi while some *Penicillium* species have been known to be present in orchid roots as endophytes (Ma *et al.*, 2015).

In Vitro Lead Tolerance

In vitro lead tolerance assay showed the growth rate of *P. pinophilum* was significantly different between the highest concentration of Pb(II) concentration which is 1000 mg/L (1.414 ± 0.9243) as compared to the control (2.543 ± 0.2402). The growth rate in 100 mg/L (2.319 ± 0.2236) was also significantly different to 1000 mg/L. while for 500 mg/L (1.938 ± 0.1839), the difference was not significant. For *A. fumigatus*, the growth rate was significantly different between control (2.343 ± 0.204) to 1000 mg/L (1.500 ± 0.198) while the growth rate between control with 100 mg/L (2.038 ± 0.194) and 500 mg/L (1.971 ± 0.191) was not significantly different. For *A. niger*, the growth rate of control (2.657 ± 0.220) was significantly different with 100 mg/L (2.357 ± 0.196) and 1000 mg/L (1.557 ± 0.188). The fungal growth rate were compared using one-way ANOVA and Tukey Post

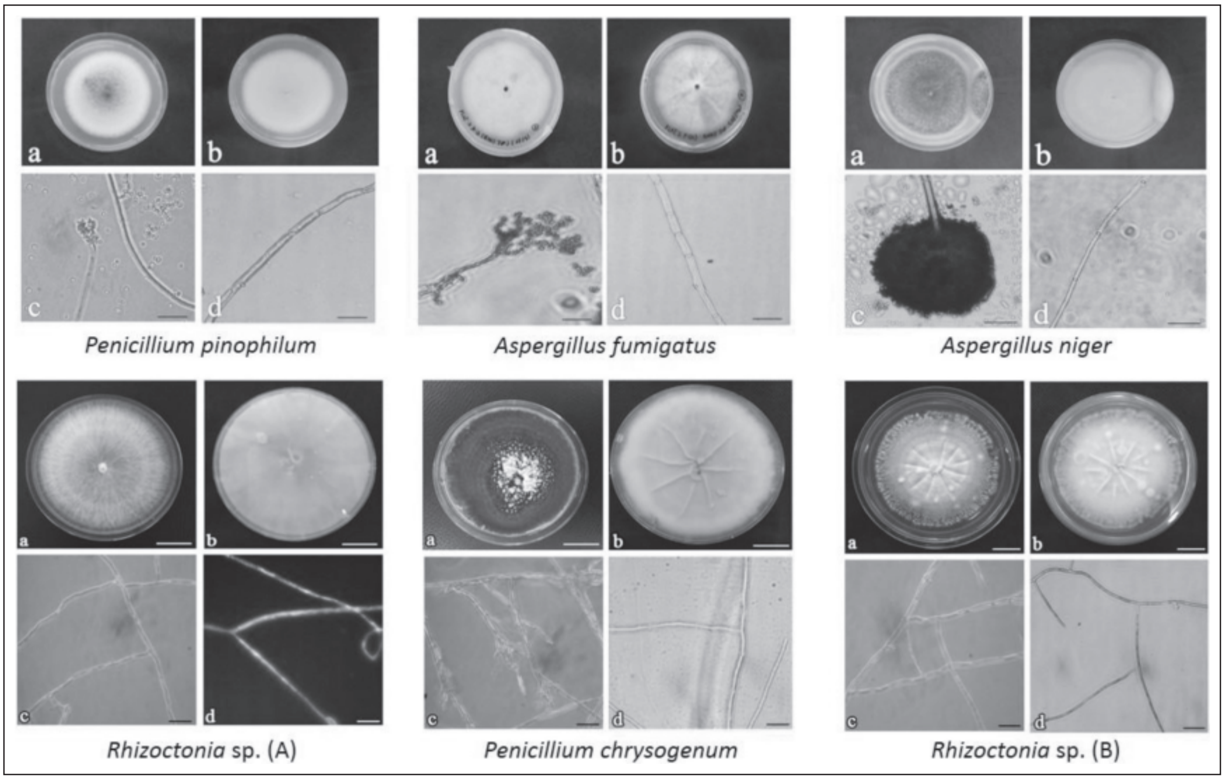

Fig. 1. Morphological characteristics of *Penicillium pinophilum, Aspergillus fumigatus, A. niger, Rhizoctonia* sp. (A), *P. chrysogenum*, and *Rhizoctonia* sp. (B). Surface of 7-day-old culture on PDA (A); Colony on reverse plate (B); Fungal hyphae under bright field or fluorescence of fluorescence microscope (C, D); Bars= 20 μm.

Table 1. Characterisation of fungi isolated from mangrove in Morib and from orchids in BRIS, Setiu Wetland

	Growth Rate (cm/day)	Colony Colour	Texture	Spore	Septa
Penicillium pinophilum	6.8	White	Smooth	Yes	Septate
Aspergillus fumigatus	7.0	White with dark spots	Smooth	Yes	Septate
Aspergillus niger	6.8	White with dark spots	Smooth	Yes	Septate
Rhizoctonia sp. *(A)*	3.7	White	Fluffy cotton	No	Septate
Penicillium chrysogenum	2.3	Dusky olive green	Smooth cotton, wrinkled	Yes	Septate
Rhizoctonia sp. *(B).*	1.9	White	Leathery, wrinkled	No	Septate

Hoc Test for different Pb(II) concentrations. Means with one letter (a and b) in each column are significantly different while column with letter more than one are not significantly different at $p \leq 0.05$.

In summary, all isolates were able to tolerate the lead stress at 100 mg/L and 500 mg/L and 1000 mg/L Pb treatments, although the growth rate decreases with the higher lead concentration (Table 2). At the highest concentration of 1000 mg/L, *P. pinophilum*, *A. fumigatus*, *A. niger* and *Rhizoctonia* sp. (A) only showed a small decrease in growth rate at 1.414 cm/day from 2.543 cm/day, 1.500 cm/day from 2.343 cm/day, 1.557 cm/day from 2.657 cm/day and 3.89 cm/day from 4.611 cm/day respectively. *P. chrysogenum* and *Rhizoctonia* sp. (B) also grew on 1000 mg/L Pb concentration but at a very slow rate at 0.251 cm/day from 2.440 cm/day and 0.529 cm/day from 1.187 cm/day respectively. It is interesting to highlight that *Rhizoctonia sp. (A)*, an orchid mycorrhiza fungi isolated from orchids from BRIS soil, was able to successfully tolerate the 1000 mg/L lead treatment as it was expected that the mangrove isolates would better tolerate the lead stress since mangroves can grow in high lead conditions compared to the orchids in BRIS, which is an environment known to be lacking in mineral elements and nutrients. As such, this is the first report of *in vitro* lead stress tolerance of orchid mycorrhiza fungi. *Penicillium* species or

Table 2. Growth rate of isolated fungi on PDA at different Pb(II) concentration

Isolate	Concentration of Lead (II) Nitrate			
	0 mg/L (control)	100 mg/L	500 mg/L	1000 mg/L
P. pinophilum	2.543 ± 0.2402^{b}	2.319 ± 0.2236^{b}	1.938 ± 0.1839^{ab}	1.414 ± 0.9243^{a}
A. fumigatus	2.343 ± 0.204^{b}	2.038 ± 0.194^{ab}	1.971 ± 0.191^{ab}	1.500 ± 0.198^{a}
A. niger	2.657 ± 0.220^{b}	2.357 ± 0.196^{b}	1.943 ± 0.171^{ab}	1.557 ± 0.188^{a}
Rhizoctonia sp. *(A)*	4.611 ± 1.285^{b}	4.426 ± 1.237^{ab}	4.323 ± 1.212^{ab}	3.894 ± 1.131^{a}
P. chrysogenum	2.440 ± 0.619^{b}	2.400 ± 0.603^{b}	1.544 ± 0.364^{a}	0.251 ± 0.092^{a}
Rhizoctonia sp. *(B)*	1.187 ± 0.279^{b}	1.086 ± 0.230^{ab}	1.020 ± 0.222^{ab}	0.529 ± 0.200^{a}

more specifically *P. pinophilum* is not known to be a heavy metal tolerant fungi but is mostly found to be tolerant to low temperature, wide range of pH and salinity (Dhakar *et al.,* 2014; Velmurugan *et al*., 2010). However, Maity *et al.* (2014) suggested that *Penicillium* species may enhance the solubilization of potassium in the soil and thus the interaction will benefit plant growth.

For *A. fumigatus*, the result is consistent with Ramasamy *et al.* (2011) in which medium tolerance was observed with a decrease in fungal growth when lead concentration decreases. Al-Garni *et al.* (2002) stated that *Aspergillus* species are one of the most tolerant fungi to any type and quantity of industrial waste. This is the case for *A. niger* where is has been shown that the species is not only tolerant to lead, but also have high a tolerance to arsenate metal (Mukherjee *et al*., 2010).

In conclusion, isolation of fungi from two extreme habitats, namely orchid mycorrhiza fungi from orchids in BRIS, Setiu Wetland and mangrove in Morib, successfully discovered isolates that were tolerant to lead stress. Further identification and studies should be done on the *Rhizoctonia* sp. that is highly tolerant to lead concentrations. This may contribute to the development of biofertilizers and bioremediators that can enhance agricultural practices and reduce soil pollution.

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