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Myco-phytoremediation of arsenic- and lead-contaminated soils by *Helianthus annuus* and wood rot fungi, *Trichoderma* sp. isolated from decayed wood

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ABSTRACT

In the present study, *Helianthus annuus* grown in arsenic- (As) and lead- (Pb) contaminated soil were treated with plant-growth promoting fungi *Trichoderma* sp. MG isolated from decayed wood and assessed for their phytoremediation efficiency. The isolate MG exhibited a high tolerance to As (650 mg/L) and Pb (500 mg/L), and could remove > 70% of metals in aqueous solution with an initial concentration of 100 mg/L each. In addition, the isolate MG was screened for plant-growth-promoting factors such as siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole acetic acid (IAA) synthesis, and phosphate solubilisation. Phytoremediation studies indicated that treatment of *H. annuus* with the isolate MG had the maximum metal-accumulation in shoots (As; 67%, Pb; 59%). Furthermore, a significant increase in the soil extracellular enzyme-activities was observed in myco-phytoremediated soils. The activities of phosphatase (35 U/g dry soil), dehydrogenase (41 mg TPF/g soil), cellulase (37.2 mg glucose/g/2 h), urease (55.4 mg N/g soil/2 h), amylase (49.3 mg glucose/g/2 h) and invertase (45.3 mg glucose/g/2 h) significantly increased by 12%, 14%, 12%, 22%, 19% and 14% in As contaminated soil, respectively. Similarly, the activities of phosphatase (31.4 U/g dry soil), dehydrogenase (39.3 mg TPF/g soil), cellulase (37.1 mg glucose/g/2 h), urease (49.8 mg N/g soil/2 h), amylase (46.3 mg glucose/g/2 h), and invertase (42.1 mg glucose/g/2 h) significantly increased by 11%, 15%, 11%, 18%, 20% and 14% in Pb contaminated soil, respectively. Obtained results indicate that the isolate MG could be a potential strain for myco-phytoremediation of As and Pb contaminated soil.

1. Introduction

Metals, especially arsenic (As) and lead (Pb), accumulating in soil and/or water via various natural routes as well as anthropogenic activities (e.g., mining and smelting) exerts a significant impact on human health and other living organisms in the ecosystem (Pan et al., 2009). As and Pb are the metal elements without any known biological function and one of the most toxic heavy metals. Soil and/or water contamination with these metals are widespread and pose a substantial threat to the environment. As and Pb contaminated soils are not suitable for feed-crop cultivation and require remediation to reduce risk associated with them (Marques et al., 2013). Most physicochemical methods to remove As and Pb are expensive, inefficient, and labour-intensive (Xiao et al., 2010).

The application of biological remediation techniques, such as phytoremediation appears as an excellent cost-effective alternative, which uses metal tolerant or hyperaccumulating energy-crops. This process may become a promising alternative for renewable energy source (Mlezeck et al., 2010; Antonkiewicz et al., 2016). However, most of the plants produce very less biomass and exhibit stunted growth in metal-contaminated soils. Hence, it is important to propose effective phytoremediation strategy for heavy-metal-contaminated soils (Rajkumar et al., 2009; Weyens et al., 2009). Recently, interaction between plants, microbes, and metals has attracted much attention because of the physiological potential of microorganisms to remove metals directly from contaminated soil and the possible role of microorganisms in promoting plant-growth in metal-contaminated soils (Deng et al., 2011).

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It has been reported that soil-extracellular enzme activities are likely to be affected by As, Pb as well as microbial biomass (Bhattacharyya et al., 2008). Koo et al. (2012) identified that As exerts a strong inhibitory effect on soil enzyme activities. Boshoff et al. (2014) observed that the microbial biomass is the major source of enzymes in soil and is highly susceptible to disruption by As and Pb contamination. The bioaugmentation of specific metal resistant microorganisms capable of metals speciation may enhance the microbial biomass in the soil, which subsequently influences soil enzyme activities (Tripathi et al., 2015; Wang et al., 2015). Accordingly, analysis of soil-extracellular enzyme activity will be useful in assessing the quality of contaminated soils after bioaugmentation with specific metal-resistant microorganisms.

The anamorphic *Trichoderma* sp. is imperfect filamentous fungi belonging to the ascomycete division. *Trichoderma* sp. are among the most frequently isolated soil-fungi and are well known for their biocontrol activity and plant-growth enhancement (Harman et al., 2004; Hoyos-Carvajal et al., 2009a, 2009b). *Trichoderma* sp. can influence the plant growth by producing siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole acetic acid (IAA), acid phosphatase under biotic or abiotic stresses (Babu et al., 2014a, 2014b). *Trichoderma* plays an important role in the ecosystem by taking part in decomposition of plant residues, as well as in biodegradation of anthropogenic chemicals. *Trichoderma* can also uptake a variety of metals from soil and/or water even under an extreme pH, temperature or nutrient shortage (Anand et al., 2006). Recently, *Trichoderma* sp. has been reported for its abilities of heavy-metal resistance, speciation and transformation (Zeng et al., 2010; Su et al., 2009). Thus, the present study describes the influence of plant growth promoting *Trichoderma* sp. on enhanced phytoremediation of artificially As- and Pb- contaminated soil.

Helianthus annuus is an annual economic plant with food and energy values. Several studies have been reported the phytoremediation potential of *H. annuus* (Cindy et al., 2006; Niu et al., 2007; Fassler et al., 2010; Rojas-Tapias et al., 2012) and its growth on contaminated soil for simultaneous remediation and energy production (Madejon et al., 2003). It has been suggested that the application of integrative capability of phytoremediation along with fungal remediation would result in enhanced As and Pb removal and further improve soil fertility. Hence, the present study deals (i) isolation and identification of As- and Pb- resistant *Trichoderma* sp. MG from decayed wood collected from soil, (ii) assessment of the As- and Pb- removal efficiency of *Trichoderma* sp. MG in batch experiments, (iii) screen of the isolate MG for plant-growth promoting traits, (iii) assessment of the efficiency of the isolate MG in enhancing *H. annuus* growth and metal-accumulation, and (iv) evaluation of the extracellular enzyme activities in the myco-phytoremediated soil.

2. Materials and methods

2.1. Collection of decayed wood sample and isolation of fungi

A sample of decayed wood was collected from Pallipalayam, a municipality located in Namakkal District in the State of Tamil Nadu, India, where soil and water were contaminated with dyes and auxiliary chemicals associated with textile industrial wastes (Thangaraj et al., 2017). The wood sample was transported to the a laboratory and processed within 18 h. Fungi were isolated from decayed logs of wood using the pour-plate technique on potato-dextrose agar (PDA) supplemented with 30 µg/mL of chloramphenicol. The plates were incubated at 26 ± 2 °C for 8–16 d and observed for the fungal growth. The pure cultures of the isolates were transferred to PDA slants and maintained by sub-culturing every three weeks.

2.2. Identification of the isolate MG

The isolate MG was cultured in the potato-dextrose broth at 26 °C for 6–8 d. After incubation, mycelia mats were separated from the medium and the genomic DNA was extracted using Qiagen DNA extraction kit (QIAGEN, CA, USA), according to the manufacturer's protocol. The ITS region of the isolate was amplified using the primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR product was purified using a PCR purification kit (QIAGEN, CA, USA) and the amplicons were sequenced using an automated ABI PRISM 3700 sequencer (Foster City, USA). The sequences were compared using the BLAST program (http://www.ncbi.nlmnih.gov/BLAST) for identification of the isolate MG.

2.3. Metal tolerance and removal

The isolate MG was screened for metal-tolerance assay according to the agar dilution method. In brief, the fungal plug was aseptically inoculated onto the PDA medium supplemented with increasing concentration of metals (50–750 mg/L of As ($NaAsO_2$), Cu ($CuCl_2{\cdot}2H_2O$), Cr (Cr (VI), Ni (Ni^{2+}) and Pb (Pb ($NO_3)_2$). The plates were incubated at 26 ± 2 °C and observed for the fungal growth. PDA plates without metals were used as controls.

Metal removal experiments were performed according to Babu et al. (2014a, 2014b) with a minor modification. In brief, 10 mL of *Trichoderma*-spore suspension (10^8 cfu/mL) was inoculated into 100 mL of the potato-dextrose broth amended with different concentrations (100–500 mg/L) of As and Pb individually. The flasks were incubated in a shaking incubator (Thermo Scientific, Massachusetts, USA) (200 rpm) at 26 ± 2 °C for 7 d. After incubation, samples were collected and centrifuged (Kubota, Japan) at 10,000 rpm for 5 min. One mL of supernatant was immediately filtered through a 0.2 µm membrane, As and Pb concentrations were determined by inductively coupled plasma mass spectrometry (ICP, Leemans Labs, USA) and the metal removal by the fungus was determined by evaluating the difference from the initial and final metal concentrations.

2.4. Plant-growth promoting factors

Indole acetic acid production was determined according to the method described by Gordon and Weber (1951). Siderophore production was estimated using a modified chrome azurol S (CAS) agar medium (Milagres et al., 1999). Phosphate-solubilisation activity was estimated using the Pikoskoskaya medium (Pikovskaya, 1948). The ACC deaminase activity was measured according to Gravel et al. (2007).

2.5. Pot experiment

The natural soil was spiked with As and Pb solutions, generating a final concentration of 250 mg/kg, which was chosen according to the occurrence of As- and Pb-contaminated soils with the average concentration of a nearby site contaminated with heavy metals. The physico-chemical characteristics of the study soil are presented in Table 1. Soil samples were air-dried, sieved to < 2 mm, sterilized at 120 °C for 70 min for four consecutive days and dried in an oven at 40 °C for a week. The contaminated soil was incubated for 1 month, being irrigated with deionized water to 60% of the water holding capacity, to obtain a

Table 1
Physico-chemical characteristics of the study soil.

Soil properties	Values
Organic matter (%)	9.5 ± 0.14
pH	6.48 ± 0.03
EC ($m^{-s}\,m^{-1}$)	0.39 ± 0.023
N ($\mu g\,g^{-1}$)	24.3 ± 1.19
P ($\mu g\,g^{-1}$)	6.42 ± 0.85
K ($\mu g\,g^{-1}$)	23.9 ± 1.25