Journal of Environmental Management 132 (2014) 129–134

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Trichoderma virens PDR-28: A heavy metal-tolerant and plant growth-promoting fungus for remediation and bioenergy crop production on mine tailing soil

A. Giridhar Babu [a], Jaehong Shim [a], Keuk-Soo Bang [b], Patrick J. Shea [c], Byung-Taek Oh [a,*]

[a] Division of Biotechnology, Advanced Institute of Environment and Bioscience, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan 570-752, Republic of Korea
[b] Department of Oriental Medicine Resources, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan 570-752, Republic of Korea
[c] School of Natural Resources, University of Nebraska-Lincoln, Lincoln, NE 68583-0817, USA

ARTICLE INFO

Article history:
Received 24 April 2013
Received in revised form
25 July 2013
Accepted 9 October 2013
Available online 30 November 2013

Keywords:
Bioenergy
Bioleaching
Mine
Phytoremediation
Phytostabilization
Trichoderma

ABSTRACT

A heavy metal-tolerant fungus, *Trichoderma virens* PDR-28, was isolated from rhizosphere soil and evaluated for use in remediating mine tailing soil and for plant biomass production. PDR-28 exhibited plant growth-promoting traits, including 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, acid phosphatase and phytase activity, siderophore production, and P solubilization. HMs were more available in mine tailing soil inoculated soil with PDR-28 than in uninoculated soil; the order of HM bioleaching was Cd > As > Zn > Pb > Cu. PDR-28 effectively removed HMs in the order of Pb > Cd > As > Zn > Cu from liquid media containing 100 mg HM L^{-1}. Inoculating HM-contaminated mine tailing soil with the fungus significantly increased the dry biomass of maize roots (64%) and shoots (56%). Chlorophyll, total soluble sugars (reducible and nonreducible), starch, and protein contents increased by 46%, 28%, 30%, and 29%, respectively, compared to plants grown in uninoculated soil. Inoculation increased heavy metal concentrations in maize roots by 25% (Cu) to 62% (Cd) and in shoots by 35% (Cu) to 64% (Pb) compared to uninoculated plants. Results suggest that PDR-28 would be beneficial for phytostabilization and plant biomass production as a potential source of biofuel in the quest for renewable energy.

1. Introduction

Mining, smelting, and associated activities cause environmental degradation and human health problems throughout the world. South Korea has a long history of mining and more than 1000 abandoned mines, most left unmanaged (Jung, 2008). The heavy metals (HMs) As, Cu, Cd, Pb, Ni and Zn, common in mine waste, have been dispersed downslope by surface erosion, wind action, and effluent drainage.

Vegetation promotes remediation for sustainable development of mine waste sites. Plants with a moderate to high accumulation capacity gradually reduce the fraction of available HMs in soil. Aside from extracting metals and stabilizing sites, some species also have potential for renewable energy production (Sheng et al., 2012). Such plants can provide biofuel, in the form of ethanol, biodiesel, and biomass for biogas production. This approach has high public acceptance and can be cost-effective on marginal or contaminated land. Olivares et al. (2013) showed how castor bean can be used for phytoremediation and oil production on mine tailing soil; Van Ginneken et al. (2007) similarly suggested rapeseed, wheat and maize as bioenergy crops for remediating HM-contaminated soil.

Phytoextraction uses plants to move contaminants from soil to aboveground biomass. Plant uptake of HMs can be enhanced by adding chelating agents, such as EDTA, DTPA, EGTA and NTA, to increase bioavailability or through the action of the associated microbial community (Meers et al., 2010; Van Ginneken et al., 2007). Chemically inducing HM phytoaccumulation is impaired by high cost and secondary pollution of soil, and leaching of chelating agents may result in groundwater contamination (Meers et al., 2010; Van Ginneken et al., 2007). Therefore, scientists have been investigating the use of HM-solubilizing (bioleaching) microorganisms to facilitate phytoextraction and enhance plant biomass production in HM-contaminated soils.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EC, electric conductivity; HM, heavy metal; IAA, Indole acetic acid; MIC, minimal inhibitory concentration.

* Corresponding author. Tel.: +82 63 850 0838; fax: +82 63 850 0834.
E-mail addresses: anamgiri@gmail.com (A.G. Babu), btoh@jbnu.ac.kr (B.-T. Oh).

0301-4797/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.jenvman.2013.10.009

Filamentous fungi have a distinct advantage over bacteria because of their high tolerance for metals and ability to grow under extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations (Anand et al., 2006). While inoculating soil with bacteria can promote plant growth and HM accumulation (Ma et al., 2011; Sheng et al., 2012), growth-promoting saprophytic fungi have received less attention. Fungi tolerate and detoxify metals by extracellular precipitation, complexation and crystallization, chemical transformation, biosorption to cell wall and pigments, decreased transport or impermeability, efflux, intracellular compartmentation, and sequestration (Gadd, 1993). *Trichoderma atroviride*, *T. harzianum* and *T. pseudokoningii* directly link the soil to plants, increasing HM availability via solubilization while promoting plant growth and reducing metal toxicity (Adams et al., 2007; Bareen et al., 2012; Cao et al., 2008). However, research is limited on the use of *Trichoderma* spp. to enhance plant growth, HM removal and bioaccumulation by plants in mine tailing soil containing multiple HMs. The present research was conducted to determine: (1) HM tolerance and plant growth-promoting traits of a *T. virens* isolate from HM-contaminated mine tailing soil, (2) its HM bioleaching potential and removal efficiency, and (3) maize growth and HM accumulation in inoculated soil.

2. Materials and methods

2.1. Soil collection and characterization

Mine tailing soil was obtained from an abandoned mine site in South Korea. Samples were collected at 0–20 cm depth using sterilized stainless steel implements and sterile bags, mixed, air-dried and passed through a 2-mm sieve before physicochemical analysis. Soil pH was 6.0 (1:2.5 w/v water) and electrical conductivity (EC) was 0.5 dS m^{-1}; soil contained 0.61% organic carbon and 5.3 mg available P kg^{-1}. The mine tailing soil was digested with a mixture of concentrated HNO_3, $HClO_4$ and HF (5:1:1, v/v/v) and HM concentrations were determined by inductively coupled plasma analysis (ICP; Leeman Labs, Inc., Hudson, NH, USA). Concentrations of As, Cd, Cu, Pb, and Zn were 356, 93, 6428, 2904, and 921 mg kg^{-1}, respectively.

2.2. Isolation, identification, and metal tolerance of fungus

Rhizosphere soil containing 175 mg As, 69 mg Cd, 1270 mg Cu, 1105 mg Pb, and 370 mg Zn kg^{-1} was collected from *Pinus koraiensis* growing near the site. A pure culture of HM-tolerant fungus was isolated from the soil by conventional dilution and supplementing potato dextrose agar (PDA) with 100 μg chloramphenicol (bacteriostat) and 100 μg Cu per mL. Cu was used because its concentration was highest among HMs in the mine tailing soil. The fungus was tentatively indentified at the genus level by microscopic observation (Gilman, 1957; Smith, 1969). Species was identified by 18S rDNA (ITS1-ITS4 region) sequencing; DNA isolation, cloning and sequencing was as described by Cao et al. (2008). ABI PRISM (Model 3700, Foster City, USA), was used for automated sequencing analysis. Sequence similarities were determined with NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi); the sequence was deposited in the NCBI database (accession number JX424333).

T. virens PDR-28 was further screened for HM tolerance using 1/7 strength PDA medium containing As, Cd, Cu, Pb or Zn at 100–3500 mg L^{-1}. The lowest HM concentration preventing fungus growth was considered the minimal inhibitory concentration (MIC). Plates without HMs were used as controls. Dilute medium was used to reduce precipitation of metal salts and maintain limited nutrient conditions. Plates were incubated at 30 °C for 3–5 d and growth assessed by mycelial growth zone development. Treatments were conducted in triplicate.

2.3. Plant growth-promoting (PGP)traits

The fungus was screened for plant growth-promoting traits in the absence of HM stress. Indole acetic acid (IAA) production was determined using a modification of the method of Gordon and Weber (1951). Briefly, 4-mm diameter plugs of the fungus were inoculated into 1/7 strength potato dextrose broth (PDB) containing 100 μg filter-sterilized L-tryptophan (Sigma–Aldrich, St. Louis, USA) per mL and incubated for 7 d on a rotary shaker (170 rpm) at 30 °C. After incubation, 1 mL of filtrate was mixed with 2 mL of Salkowski's reagent, incubated at room temperature for 20 min, and absorbance measured at 535 nm. IAA was estimated by comparison with a standard curve prepared by serial dilution of solution containing 100 μg IAA mL^{-1} in 1/7 strength PDB. ACC deaminase activity was estimated (Gravel et al., 2007). Siderophore production was determined using modified chrome azurol S (CAS) agar medium, (Milagres et al., 1999). Solubilization of P was quantitatively estimated using Pikovskaya's broth supplemented with tricalcium phosphate equivalent to 0.25% P_2O_5 and 0.5 mL of homogenized mycelium suspension which had been grown for 7 d. Treated culture samples were incubated at 30 °C for 9 d with shaking at 160 rpm. Soluble P, acid phosphatase, and phytase activity were determined in culture filtrate (Singh and Reddy, 2011).

Soluble P was estimated by incubating 1 mL of filtrate in a test tube containing 2 mL of boric acid and 3 mL of mixed reagent (4 M sulfuric acid containing 4% ammonium molybdate, 1.75% ascorbic acid and 0.275% potassium antimony tartrate) at 30 °C for 1 h. Released phosphate was measured at 720 nm using a UV-VIS spectrophotometer. For acid phosphatase activity, 0.1 mL filtrate was added to 0.48 mL of 0.1 M universal buffer at pH 4.8 and 0.12 mL of 0.05 M *p*-nitrophenyl phosphate (pNPP) solution, and incubated 1 h at 30 °C. The *p*-nitrophenol liberated from pNPP was measured at 410 nm. Phytase activity was determined by incubating 1 mL of culture filtrate at 37 °C for 30 min in 0.2 M sodium acetate buffer (pH 5.5) containing 0.5% sodium phytate (Sigma, St. Louis, Mo. USA). After adding 2 mL of a coloring reagent, the sample was incubated for 15 min at 50 °C and P concentration was measured at 820 nm. An uninoculated treatment mixture was used as the control.

2.4. Mycoremediation of mine tailing soil

Inoculum was prepared by growing the fungus on PDA plates at 30 °C for 2 weeks until dense spores formed. Spores were mixed with sterile water or vermiculite at 10^8 spores mL water or g vermiculite. To determine HM mycoremediation efficiency, 5 mL of the spore suspension (10^8 cfu mL^{-1}) was inoculated into 100 g of autoclaved (121 °C for 15 min) mine tailing soil supplemented with 25 mL of 1/7 strength PDB. The pH of the media was adjusted with 0.1 M NaOH to 6.0 to mimic the mine tailing soil pH. The soil and fungal spores were mixed and incubated in an open environment (~27–30 °C) for 15 d. Treatments were conducted in triplicate. Water lost was replenished with distilled water (by weight) every 2 d. Controls consisted of mine tailing soil without fungal spores. After 15 d of mycoremediation, pH was measured and the soil was separated at 10,000 rpm for 30 min, followed by repeated washing with deionized water to remove soluble metal and determine the amount of HMs bioleached into aqueous solution. Bioleached HMs in filtrate were determined by ICP.

2.5. Fungus growth and HM removal

To determine HM removal, batch tests were performed and repeated twice. Briefly, 1 mL of fungal spore suspension (10^8 cfu mL^{-1})