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Review

Effect of Zn-tolerant bacterial strains on growth and Zn accumulation in *Orychophragmus violaceus*☆

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ABSTRACT

Several Zn-tolerant bacterial strains were isolated from heavy-metal contaminated sludge, and their effects on root elongation, mobility, and accumulation of Zn in *Orychophragmus violaceus* were studied. The isolated strains included *Bacillus subtilis*, *B. cereus*, *Flavobacterium* sp. and *Pseudomonas aeruginosa* which were capable of stimulating root elongation in *O. violaceus* seedlings either in the presence or absence of Zn. The four bacterial strains significantly increased the concentration of water-extractable Zn compared with axenic soil. In addition, the four Zn-tolerant bacteria significantly increased the shoot biomass and Zn accumulation in *O. violaceus* compared to non-inoculated plants. The bacterial strains displayed different capacities to enhance plant Zn accumulation. *Flavobacterium* sp. was identified as the best candidate for enhancing Zn accumulation in plants, increasing Zn accumulation up to 1.21- and 1.19-fold in shoots and roots, respectively, compared to non-inoculated plants. It was indicated that Zn-tolerant bacteria played an important role in influencing the availability of water-soluble Zn in soil and Zn accumulation by plants. This study provides insight into the development of plant–microbe systems for phytoremediation.

Contents

1. Introduction

Metallic zinc has been used for a variety of applications such as galvanization, manufacture of brass and other alloys, and fabrication of batteries (Barceloux, 1999). The extensive use of

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zinc without its recovery caused contamination of soil and freshwater habitats by the divalent cation (Taniguchi et al., 2000). Although zinc is an essential trace element for animals, plants, and microorganisms, it is toxic to organisms at millimolar concentrations (Barceloux, 1999). Traditional methods for remediation of heavy-metal contaminated soils such as excavation or land filling are expensive, require specialized equipments and are mainly associated with declining soil biological activity (Glick, 2003). In recent years, intense research on the use of plants in the remediation process of the environment polluted with metals has been conducted. Being less expensive and less invasive to the environment, phytoremediation is considered an effective strategy for reclaiming metal contaminated soils (Medina et al., 2006; Glick, 2003).

Successful phytoremediation depends mainly on the bioavailability of metals in the soil, however the availability of metals for plants is usually restricted by the sorption to the solid soil fractions. Chelate-assisted phytoremediation involves the use of synthetic chelators, e.g. ethylenediamine tetraacetate (EDTA), and was used to enhance metal solubility in soil artificially and thereby increasing metal phytoavailability (Piechalak et al., 2003; Epstein et al., 1999; Sahi et al., 2002). Nevertheless, it is reported that many synthetic chelators are expensive and pose a threat to the quality of soil and groundwater (Blaylock et al., 1997; Huang et al., 1997; Kos and Leštan, 2003). To date studies on chelate-assisted phytoremediation are mainly focused on searching highly efficient chelators (Ernst, 1996; Raskin et al., 1997; McGrath et al., 2001). Soil microorganisms have higher activity and surface area-to-volume ratio because of their small size and therefore provide a large contact area. These features could have the potential as microbial chelators for phytoremediation (Karenlampi et al., 2000; Sitaula et al., 1999).

It was demonstrated that mycorrhizal fungi promote plant growth and nutrition, reduce metal translocation to shoots and alleviate metal toxicity (Colpaert and Vandenkoornhuyse, 2001; Vivas et al., 2003a,b). However, some bacteria associated with plant roots can not only increase plant growth and nutrition through a number of mechanisms such as nitrogen fixation, production of phytohormones and siderophores, and transformation of nutrient elements, but also can enhance tolerance and accumulation of metals in plants. Bacteria are considered to be an important component of phytoremediation technology (Wenzel et al., 1999; Glick, 2003).

The *Brassicaceae* is a family containing many metal-accumulating species (Broadley et al., 2001). Certain crop species of the *Brassicaceae* such as canola (*B. campestris)*, rape (*B. napus*) and mustard (*B. juncea*) can accumulate high amounts of metals (Sheng and Xia, 2006; Madhaiyan et al., 2007; Kumar et al., 2008), which can be considered a health risk. *O. violaceus* is a member of the *Brassiceae* tribe. This species is cultivated as an ornamental plant in China and its wild forms occur both in China and Korea (Luo et al., 1994). There is a great interest in employing it for phytoremediation of contaminated soils in above regions (Chen et al., 2004; Wu et al., 2004). However, with the superior oil quality and good yield components, Hu et al. (2002) suggested that the genes of O. *violaceus* be introduced into rape (*B. napus*) for the diversification of the oilseed rape gene pool. As a result, Zn accumulation in this plant may pose a health risk if the plant grown in Zn-contaminated soils, or in nearby soils with similar plant-available metal levels, was used for culturing or grazing of livestock.

In this study, several Zn-tolerant bacteria were isolated from heavy-metal contaminated sludge, and their effects on root elongation of *Orychrophragmus violaceus,* the mobility of Zn in soil, and accumulation of Zn by *O. violaceus* were studied.

2. Materials and methods

2.1. Characterization, sampling and treatment of soils

The soil was sampled to a depth of 0–20 cm from arboretum fields of Shanghai University. Its chemical and physical characteristics were as follows: pH (H_2O), 7.42; total carbon, 3.5%; total nitrogen, 1.89 g kg^{-1}; total phosphorus, 1.93 g kg^{-1}; total potassium, 3.5 g kg^{-1}; total zinc 73.2 mg kg^{-1}.

The soil used was supplemented with 500 mg Zn kg^{-1} dry soil, as $Zn(NO_3)_2{\cdot}6H_2O$ dissolved in water, immediately after sampling and incubated in sterile plastic pots in the dark at room temperature (20–25 °C) for one week before use. Soil moisture content was maintained at 60% of water holding capacity via addition of sterile tap water.

2.2. Isolation and identification of Zn-tolerant bacteria

The bacteria were isolated from sewage sludge of Taopu wastewater plant in Shanghai, in which the major metals concentrations (mg kg^{-1} dry sludge) were 1973 (Cu), 3532 (Zn), 20397 (Fe), 524 (Mn), 2.60 (Cd), 1109 (Cr).

The fresh sludge was diluted in sterile water (fresh sludge:sterile water = 1:5). Aliquots of diluted homogenates were plated onto Petri dishes (three replicates) in solid medium supplemented with 54.6 mg Zn L^{-1} (separately filter-sterilized and added to the autoclaved beef peptone agar medium). The medium contained beef extract, 0.75 g; peptone, 1.5 g; NaCl, 0.75 g; and agar, 3.0 g; in 150 mL of distilled water, the pH was adjusted to 7.6. The plates were incubated at 30 ± 1 °C for 18–24 h.

Four bacterial colonies were visible in the medium supplemented with 54.6 mg Zn L^{-1}. These phenotypically different bacterial colonies were picked from the medium using sterile inoculation needles and transferred to beef peptone agar medium for isolation and purification.

These isolates were assigned identities from their cellular fatty acid profiles using standard fatty acid methyl ester (FAME) extraction protocols, gas chromatography and the Sherlock Microbial Identification System (MIDI Inc.) (Bell et al., 2005). Four Zn-tolerant bacteria identified from FAME were *Bacillus subtilis*, *B. cereus*, *Flavobacterium* sp. and *Pseudomonas aeruginosa*.

2.3. Bacterial inoculum

Pure culture Zn-tolerant bacteria were grown in Luria–Bertani (LB) liquid medium (tryptone, 10.0 g L^{-1}; yeast, 5.0 g L^{-1}; NaCl, 5.0 g L^{-1}; adjusted to pH about 7.0), and placed on a shaker at 200 rpm and 30 ± 1 °C. After 48 h, the cultures were centrifuged at 8000 rpm for 10 min, washed twice in phosphate buffer (pH 7.0), resuspended, washed again in sterile water, re-centrifuged, and finally resuspended in 10 mL sterile distilled water. The final concentration of the bacterial suspension was about 10^{11} CFU mL^{-1} (CFU, colony forming units) enumerated on a plate count beef peptone agar medium. This suspension was diluted to 10^8 CFU mL^{-1} as inoculum.

2.4. Effect of Zn-tolerant bacteria on root elongation assay on filter paper culture

The plant root elongation promoting (PREP) activity of the Zn-tolerant bacteria was determined using the root elongation assay of Belimov et al. (2005). Eighteen milliliters sterile Hoagland (de Souza et al., 1999) nutrient solution lacking Zn was placed in glass Petri dishes with filter paper (Whatman Grade No. 41 Quantitative Filter Paper), and 2 mL of the bacterial suspensions with 10^8 CFU mL^{-1} or sterile Hoagland nutrient solution lacking Zn