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Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*

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Abstract

In this study, a copper-resistant plant growth promoting bacterial (PGPB) strain Ax10 was isolated from a Cu mine soil to assess its plant growth promotion and copper uptake in *Brassica juncea*. The strain Ax10 tolerated concentrations up to 600 mg Cu L^{-1} on a Luria–Bertani (LB) agar medium and utilized 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole N source in DF salts minimal medium. The strain Ax10 was characterized as *Achromobacter xylosoxidans* based on its 16S rDNA sequence homology (99%). The bacterium *A. xylosoxidans* Ax10 has also exhibited the capability of producing indole acetic acid (IAA) (6.4 μg mL^{-1}), and solubilizing inorganic phosphate (89.6 μg mL^{-1}) in specific culture media. In pot experiments, inoculation of *A. xylosoxidans* Ax10 significantly increased the root length, shoot length, fresh weight and dry weight of *B. juncea* plants compared to the control. This effect can be attributed to the utilization of ACC, production of IAA and solubilization of phosphate. Furthermore, *A. xylosoxidans* Ax10 inoculation significantly improved Cu uptake by *B. juncea*. Owing to its wide action spectrum, the Cu-resistant *A. xylosoxidans* Ax10 could serve as an effective metal sequestering and growth promoting bioinoculant for plants in Cu-stressed soil. The present study has provided a new insight into the phytoremediation of Cu-contaminated soil.

Keywords: Copper; *Brassica juncea*; Phytoremediation; Phosphate solubilization

1. Introduction

Soil contamination with heavy metals such as Cu has become a worldwide problem, leading to losses in agricultural yield and hazardous health effects as they enter the food chain. Moreover, the heavy metals cannot be degraded to harmless products and hence persist in the environment indefinitely. The sources of Cu in the soil are diverse, including the use of sludge or municipal compost, pesticides, fertilizers and emissions from municipal wastes incinerators, car exhausts, residues from metalliferous mine, and smelting industries (Yang et al., 2002). To clean up soils contaminated with Cu and other heavy metals by traditional physiochemical methods can be very costly, and, also destructive to the soil. Phytoremediation, an emerging low-cost and ecologically benign technology for decontamination of soils, is defined as the process of utilizing plants to absorb, accumulate and detoxify contaminants in soil through physical, chemical and biological processes (Wenzel et al., 1999). Currently there are a number of reports available on metal accumulating plants that are used in removing toxic metals from the soil (Delorme et al., 2001; Glick et al., 2003; Sheng and Xia, 2006). *Brassica juncea* is one of such plant species, which has attracted considerable attention because of its ability to grow in heavily polluted soil together with its capacity for metal ion accumulation (Blaylock and Huang, 2000).

The success of phytoremediation may not only depend on the plant itself but also on the interaction of the plant roots with bacteria and the concentrations of heavy metals in the soil (Wang et al., 1989). As reported by Terry (1981) elevated levels of heavy metals in the environment lead to impair of

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the metabolic activities and result in reduced plant growth. Hence the alternative ways to reduce the toxicity of heavy metals to plants is by using the rhizosphere microbes (Burd et al., 2000). Certain heavy metal resistant bacteria have exceptional ability to promote the growth of host plant by various mechanisms such as nitrogen fixation, solubilization of minerals, production of phytohormones and siderophores, and transformation of nutrient elements (Glick et al., 1999). Furthermore, the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, an enzyme that has no function in bacteria but modulates ethylene levels in developing plants (Glick et al., 1998), may further contribute to the heavy metal tolerance of plants. In addition, many microorganisms in the soil are able to solubilize 'unavailable' forms of heavy metal-bearing minerals by excreting organic acids (Abou-Shanab et al., 2003). Therefore, improvement of the interactions between plants and beneficial rhizosphere microbes can enhance biomass production and tolerance of the plants to heavy metals, and are considered to be an important component of phytoremediation technology (Glick, 2003). Although many soil bacteria are tolerant to heavy metals and play important roles in mobilization or immobilization of heavy metals, only a few attempts have been made to study their role in the tolerance to and uptake of heavy metals by plants.

Thus, the aim of this study is to (1) isolate and characterize a Cu-resistant soil bacterium capable of utilizing ACC as a sole N source, and (2) study the influence of Cu-resistant bacterium on plant growth and copper uptake in *Brassica juncea* under different concentrations of Cu in soil.

2. Materials and methods

2.1. Isolation of Cu-resistant PGPB

The bacterial strains were isolated from soil collected at a Cu mine in São Domingos, south-east of Portugal. About 1 g of wet soil sample was serially diluted using 25 mM phosphate buffer and spread on Luria–Bertani agar medium (LB) amended with 50 mg Cu L^{-1} ($CuSO_4$). The plates were incubated at 27 °C for 48 h. From the Cu resistant colonies, different strains were picked and purified on LB agar medium containing 50 mg of Cu L^{-1} according to the procedure of Rajkumar et al. (2005). Purified colonies were gradually taken to higher concentration of Cu (50–1000 mg L^{-1}) and the same procedure was continued to isolate Cu resistant strains. The physicochemical properties of soil sample used for the isolation of Cu-resistant bacteria were: pH (1:1 w/v water) 4.36; organic matter 1.36%; copper 457 mg kg^{-1}; zinc 236 mg kg^{-1}; nickel 70 mg kg^{-1}. In order to isolate the PGPB, the Cu resistant strains were grown on DF salts minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC to provide a nitrogen source at 30 °C for 168 h at 175 rpm. The inoculated DF salt minimal medium without ACC was used as a blank. The bacterial growth was monitored at definite time intervals by measuring the optical density at 600 nm.

2.2. Bacterial growth under increasing Cu levels in the medium

The culture flask (250 mL) containing 20 mL LB broth supplemented with different concentrations of Cu, namely 0, 50, 100 and 150 mg L^{-1} medium, were inoculated with logarithmic-phase bacterial isolate. All the cultures including controls (in triplicate) were incubated at 27 °C for 28 h at 200 rpm. The bacterial growth was monitored at definite time intervals by measuring the optical density at 600 nm.

2.3. DNA isolation and PCR amplification of 16S rDNA for genetic characterization of bacterial strain

The bacterial strain was grown in LB broth in presence of 1 mM Cu at 30 °C. Cells were harvested after 20 h and processed immediately for DNA isolation using standard procedure (Sambrook et al., 1989). Amplification of 16S rRNA gene sequence was performed by PCR with the conserved eubacterial primers pA (5′-AGAGTTTGATCCTGG CTCAG; *Escherichia coli* bases 8–27) and pC5B (5′-TACCTTGTTACGACTT; *E. coli* bases 1507–1492) (Dunbar et al., 1999). Reaction conditions were as described by Branco et al. (2005). Each amplification mixture (5 μL) was analysed by agarose gel (1.5% w/v) electrophoresis in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) containing 1 mg mL^{-1} (w/v) ethidium bromide. For further sequencing reaction, the amplified DNA was purified from salts and primers using the PCR purification kit (Roche Diagnostics) according to the manufacturer's instructions. Automated sequencing of the purified PCR products was performed using the dRodamina terminator cycle sequencing kit and the ABI 310 DNA Sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Partial 16S rDNA sequences obtained were matched against nucleotide sequences present in GenBank using the BLASTn program (Altschul et al., 1997).

2.4. Influence of Cu-resistant PGPB on plant growth and Cu uptake

Soil samples were collected from the Botanical garden, Department of Botany, University of Coimbra, Coimbra, Portugal, previously described by Rajkumar and Freitas (in press). The soil was sieved (2 mm) and sterilized by steaming (100 °C for 1 h on three consecutive days). After sterilization the soil was amended with aqueous solution of $CuSO_4$ to achieve the final concentrations of 50, 100 and 150 mg Cu kg^{-1} and left in a greenhouse for a 3-week period (for metal stabilization). *B. juncea* seeds were surface sterilized in 2% $Ca(OCl)_2$ (2 h) and rinsed several times with sterile distilled water. For inoculation of the seeds, bacterial culture was grown for 18 h, cells harvested by centrifugation (6000 rpm, 10 min), washed twice with sterile distilled water, and resuspended in biological saline (0.85% KCl). The seeds were inoculated by soaking in a bacterial suspension containing 10^8 cells mL^{-1} for 1 h as detailed by Burd et al. (2000). Seeds soaked in sterile water were used as control. The inoculated and