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Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria

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Abstract

This study focuses on the characterization of four bacterial isolates from heavy metal-polluted rhizosphere in order to examine their plant growth promoting (PGP) activity. The PGP activity on the canola (*Brassica napus*) of the strains which showed cadmium resistance and multiple PGP traits was assessed in the presence and in the absence of Cd^{2+}. The strains, *Pseudomonas tolaasii* ACC23, *Pseudomonas fluorescens* ACC9, *Alcaligenes* sp. ZN4 and *Mycobacterium* sp. ACC14 showed 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity. They also synthesized ACCD enzyme *in vitro* when 0.4 mM Cd^{2+} was added to the growth medium. The presence of the metal, however, reduced the ACCD activity in *Alcaligenes* sp. ZN4 and *Mycobacterium* sp. ACC14, while it did not affect the ACCD activity of *P. tolaasii* ACC23 and *P. fluorescens* ACC9. ACC9 and ACC23 produced indole acetic acid (IAA) and siderophores, while ACC14 produced only IAA. IAA and siderophores were produced more actively under Cd-stress.

Root elongation assays conducted on *B. napus* under gnotobiotic conditions demonstrated increases (from 34% up to 97%) in root elongation of inoculated canola seedlings compared to the control plants. Subsequently, the effect of inoculation with these strains on growth and uptake of Cd^{2+} in roots and shoots of canola was studied in pot experiments using Cd-free and Cd-treated (15 μg Cd^{2+} g^{-1} dw) soil. Inoculation with *P. tolaasii* ACC23 and *P. fluorescens* ACC9 and *Mycobacterium* sp. ACC14 promoted the growth of plants at concentrations of 0 and 15 μg Cd^{2+} g^{-1} soil. The maximum growth was observed in the plants inoculated with *P. tolaasii* ACC23. The strains did not influence the specific accumulation of cadmium in the root and shoot systems, but all increased the plant biomass and consequently the total cadmium accumulation.

The present observations showed that the bacterial strains used in this study protect the plants against the inhibitory effects of cadmium, probably due to the production of IAA, siderophores and ACCD activity.

Keywords: Plant growth promoting rhizobacteria (PGPR); ACC deaminase; Cadmium; Phytoremediation

1. Introduction

Heavy metals are continuously being added to soils through various agricultural and industrial activities such as the use of agrochemicals and the long-term deposition of urban sewage sludge on agricultural soils, waste disposal, waste incineration and vehicle exhausts. All these sources cause accumulation of these elements in agricultural soils and pose a threat to food safety and potential health risks. Among heavy metals, cadmium is relatively mobile in soils and is one of the most toxic. In plants, cadmium inhibits root and shoot growth, affects nutrient uptake and homeostasis, and is frequently accumulated by important crops consumed by animals and humans (Sanita di Toppi and Gabrielli, 1999). Contamination of soil with cadmium also negatively affects biodiversity and the activity of soil microbial communities (Liao et al., 2005).

The threat of heavy metal pollution has led to an increased interest in developing systems that can remove or neutralize its toxic effects in soils, sediments and wastewaters. Phytoremediation, i.e. the use of plants that have constitutive and adaptive mechanisms for tolerating or accumulating large concentrations of metals in their rhizosphere and tissues, is emerging as a potential *in situ* technology employed to clean up soils polluted by organics and heavy metals (Khan et al., 1998; Hayes et al., 2003).

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Since phytoextraction is a long-term technology, fields undergoing phytoremediation need to be kept productive to achieve economically viable and socially acceptable decontamination. The use of energy and/or bio-diesel crops (i.e. *Brassica napus*) as heavy metal phytoextraction plants would give contaminated soil a productive value and decrease remediation costs (Kos et al., 2003).

Currently, there are a number of reports available which describe metal-accumulating plants that are used in the removal of toxic metals from soil (Belimov et al., 2005; Rajkumar et al., 2006; Safronova et al., 2006; Zaidi et al., 2006). Elevated levels of heavy metals, however, lead to impaired metabolic activity and result in reduced plant growth. The interactions between plants and beneficial rhizosphere microorganisms can enhance biomass production and tolerance of the plants to heavy metals, rendering microorganisms an important component of phytoremediation technology (Wenzel et al., 1999; Glick, 2003).

Free-living as well as symbiotic plant growth promoting rhizobacteria (PGPR) can improve plant nutrition and growth, plant competitiveness and responses to external stress factors (Burd et al., 2000; Egamberdiyeva and Hoflich, 2004; Mantelin and Touraine 2004). Ethylene is important for plant growth, but excessive ethylene promoted by stresses can depress growth. A prominent mechanism used by many PGPR to facilitate plant growth is to reduce the levels of ethylene stress by consuming 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene, through the synthesis of ACC deaminase (ACCD) (Glick et al., 1999). Thus, bacterial strains which have ACCD activity can partially prevent any reduction in the length of plant roots and shoots, and in biomass caused by high ethylene levels particularly those resulting from stressful conditions (Grichko and Glick, 2001; Penrose and Glick, 2003; Mayak et al., 2004a, b).

The principal aim of this study was to test the effect of Cd-resistant PGPR on plant biomass production and Cd-uptake by *B. napus* plants growing in Cd-contaminated soil. We approached the topic by measuring the effect of cadmium on plant growth promoting (PGP) activity, and by assessing the plant root elongation promotion (PREP) activity of PGPR on seedlings growing in presence of cadmium. Finally, we evaluated the effect of PGPR inoculation on growth, protein and leaf chlorophyll content and on Cd-uptake by *B. napus* growing in soil contaminated with the metal.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Four Cd-resistant strains, *Pseudomonas tolaasii* ACC23, *Pseudomonas fluorescens* ACC9, *Alcaligenes* sp. ZN4 and *Mycobacterium* sp. ACC14 were isolated from the roots of perennial *Graminaceae* grasses taken from a water meadow in the South of Milan polluted with cadmium, nickel and copper (Dell'Amico et al., 2005). The strains, identified on the basis of their 16S rDNA sequence, showed some PGP traits and optimal growth at 30 °C. In particular they utilized ACC as the sole nitrogen source and produced indole acetic acid (IAA) and siderophores (Dell'Amico et al., 2005). Dworkin and Foster (DF) mineral medium (Dworkin and Foster, 1958), LB broth medium or King B medium (King et al., 1954) were used as liquid media in which the bacterial strains were grown. If solid medium was required, 1.5% agar (w/v) was added.

The minimum inhibitory concentration (MIC) of Cd^{2+} was determined by observing the bacterial growth on Tris mineral medium at low phosphate content (0.12 g l^{-1} of Na_2HPO_4) to avoid heavy metal precipitation (Sadouk and Mergeay, 1993), supplemented with 0.6% (w/v) gluconate (TMMG) and containing different concentrations of $CdCl_2$ (0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM). For inoculation, the isolated strains were grown in LB medium. Cells in the exponential phase were collected by centrifugation at 6000 rpm for 10 min, washed with sterile distilled water and recentrifuged. Bacterial inoculum was prepared by suspending cells in sterile distilled water to get an inoculum density of ca 10^7 cells ml^{-1}. One ml of inoculum was seeded into duplicate 100-ml vials containing 19 ml of TMMG supplemented with the appropriate amounts of $CdCl_2$. After inoculation, vials were incubated at 30 °C for 5 days and the growth was measured as $OD_{600\,nm}$.

The strains were maintained in glycerol stocks at −70 °C. Prior to use, the strains were grown in LB at 30 °C with shaking to mid-exponential phase.

2.2. Screening of bacterial strains for ACCD gene (*acdS* gene)

To identify the gene which coded the ACCD enzyme, DNA was extracted according to Cavalca et al. (2000) and primers for PCR amplification were developed on the basis of consensus regions of known ACCD gene (*acdS*) sequences (*Enterobacter cloacae* UW4 (AF047840), *Pseudomonas* sp. 6G5 (M80882), *P. fluorescens* 17 (PFU37103) and *Ralstonia solanacearum* GMI100 (AL646080)). The primers were located at positions 514 and 980 of the reference nucleotide sequence, *acdS* gene from *E. cloacae* UW4. Amplifications were performed in a total volume of 25 µl, containing: 2.5 µl 10 × buffer (Invitrogen, Renfrew, UK), 2.5 mM $MgCl_2$; 200 µM dNTPs (Invitrogen), 0.25 µM forward primer 5′-cagcaggaaaaggat-3′ and 0.25 µM reverse primer 5′-actgaattgaacccg-3′ (Invitrogen), and 1 U Taq polymerase (Invitrogen). The amplification reaction was cycled as follows: 94 °C for 3 min followed by the addition of 1 U Taq polymerase, then 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) and a final extension step at 72 °C for 10 min. The amplicons were sequenced on both strands and searched for homology using the NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).