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Journal of Environmental Management xx (2008) 1–7

Journal of Environmental Management

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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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Received 13 July 2007; received in revised form 22 December 2007; accepted 29 January 2008

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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doi:10.1016/j.jenvman.2008.01.014

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

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non-inoculated seeds were planted in plastic pot (top diameter 120 mm, bottom 100 mm and height 90 mm) containing 300 g of soil. The plants were grown in a glasshouse at 25 °C and a 16:8 h day/night regime. After 45 days the plants were carefully removed from the pots and the root surface was cleaned several times with distilled water. Growth parameters such as shoot length, root length, fresh weight and dry weight of the plants were measured. The accumulation of total copper in root and shoot system was also quantified following the method of Freitas et al. (2004).

2.5. Plant growth promoting features of Cu-resistant PGPB

2.5.1. Production of indole acetic acid (IAA)

The amount of IAA produced by bacterial strain was determined as described by Bric et al. (1991). Briefly, an aliquot of 2 mL supernatant obtained from bacterial culture grown in LB medium with L-tryptophan (500 μg mL^{-1}) was mixed with 100 μL of 10 mM orthophosphoric acid and 4 mL of reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$). The absorbance of pink colour developed after 25 min incubation was read at 530 nm. The IAA concentration in culture was determined using a calibration curve of pure IAA as a standard following the linear regression analysis.

2.5.2. Solubilization of phosphate

The bacterial culture was grown in modified Pikovskayas medium (Sundara-Rao and Sinha, 1963) with 0.5% of tricalcium phosphate at 27 °C for 8 days at 175 rpm. The culture supernatants were collected by centrifugation at 10,000 rpm for 15 min. The soluble phosphate in the culture supernatant was estimated according to the method of Fiske and Subbarow (1925).

3. Results and discussion

3.1. Isolation and characterization of Cu-resistant PGPB

Metals exert their toxic effects on microorganisms through various mechanisms. Even at micromolar concentration, copper inhibits the growth of most wild type bacteria and is tolerated by only a minority of microoraganisms. During the initial screening, 37 colonies were selected from initial (50 mg L^{-1}) level Cu supplemented LB medium. After secondary screening, 13 bacterial strains showing high level of Cu-resistance were picked up from São Domingos mine soil. In order to isolate the plant growth promoting bacteria, the Cu-resistant strains were tested for the ability to grow on DF salts minimal medium with ACC. Among the 13 strains tested, Ax10 grew in DF salts minimal medium with ACC as a sole N source. In the absence of ACC, Ax10 showed a limited growth (Fig. 1). Bacterial strains utilizing ACC as a sole source of nitrogen possess ACC deaminase which hydrolyses ACC and enhance the elongation of plant roots (Glick et al., 1998). Certain heavy metal resistant bacterial strains potentially hydrolyse ACC and promote the plant growth. Nickel resistant *Kluyvera ascorbata* isolated from soil contaminated with Ni and other heavy metals has been shown to promote plant growth (Burd et al., 2000). Similarly, Belimov et al. (2005) isolated cadmium resistant *Variovorax paradoxus* from the rhizosphere of *B. juncea* for promoting plant growth.

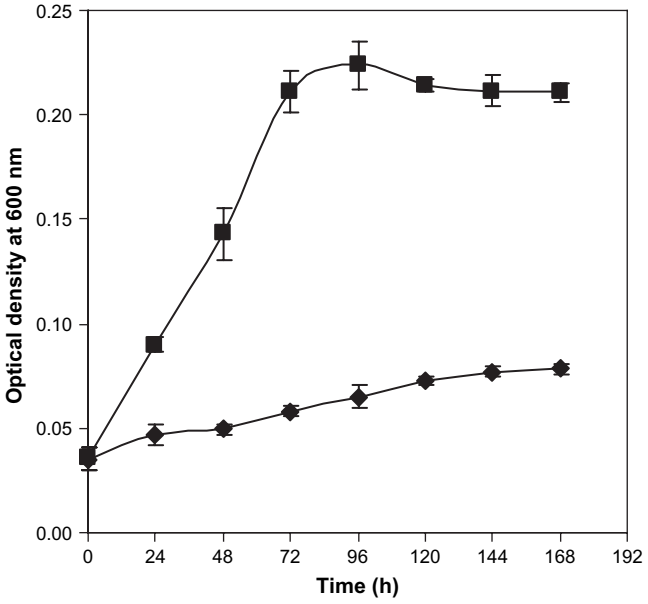

Fig. 1. Growth of *A. xylosoxidans* Ax10 on DF salts minimal medium. Strain Ax10 with ACC (■), strain Ax10 without ACC (◆). Each value is the mean of triplicates. Error bars represent standard deviation.

The strain Ax10 tolerated concentrations up to 600 mg Cu L^{-1}. This high tolerance to Cu could be attributed to the fact that the bacterium was isolated from a mine soil containing high levels of copper (457 mg kg^{-1}). Microorganisms isolated from natural environments contaminated with heavy metals often exhibit tolerance to multiple pollutants as they have adapted to such environments (Pal et al., 2005). Further, the effect of different concentrations of Cu on the growth of Ax10 was tested in liquid medium (Fig. 2). The growth pattern of Ax10 exhibited a variation in control compared to that of three concentration of Cu employed. During the initial 16 h, the maximum growth was observed in the control followed by that exposed to a concentration of 50 mg Cu L^{-1}. Further, higher concentrations of Cu (100 and 150 mg Cu L^{-1}) initially inhibited the growth rate of Ax10. However, after few hours Ax10 recovered its ability to grow in a Cu-polluted medium. The development of resistance against heavy metal ions is a generally observed phenomenon (Leyval et al., 1997).

The partial sequence (917 bp) of 16S rDNA of Ax10 obtained was matched against nucleotide sequences present in GenBank using the BLASTn program. The highest sequence similarity (99%) clearly indicates that Ax10 is a strain of *Achromobacter xylosoxidans*. The sequence was submitted in the NCBI databases under the accession number AM748708.

3.2. Plant growth promoting potential of A. xylosoxidans Ax10

The bacterial inoculated and non-inoculated plants were subjected to three levels of Cu in soil for 45 days responded

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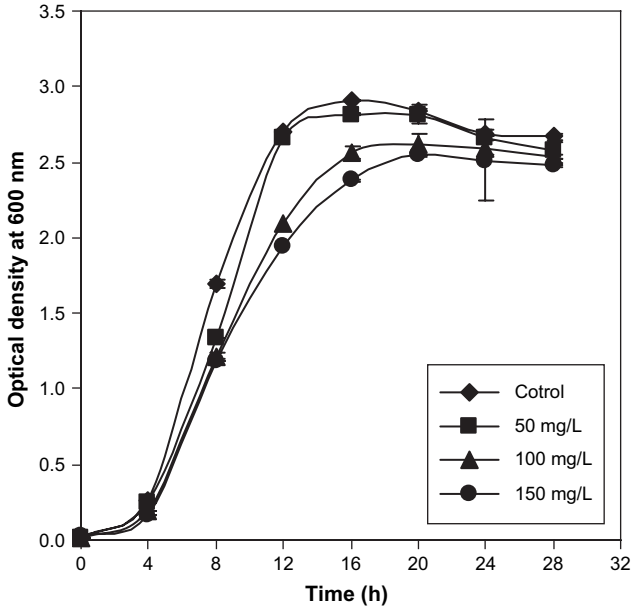

Fig. 2. Effect of Cu on the growth of *A. xylosoxidans* Ax10. Each value is the mean of triplicates. Error bars represent standard deviation.

differently in terms of plant growth (Table 1). In the absence of Cu, inoculation of *A. xylosoxidans* Ax10 showed an increase in root length, shoot length, fresh and dry weight of plant. The plant growth promotion by rhizosphere bacteria was observed by several authors (Belimov et al., 2005; Glick et al., 1998; Gupta et al., 2002) and reported to be due to the utilization of ACC, synthesis of phytohormones and solubilization of minerals. The non-inoculated plants exposed to different concentrations of Cu, showed a marked inhibition in the growth. With the increase in Cu concentration progressive decrease in root length, shoot length, plant fresh and dry weight was observed. At a concentration of 50 mg Cu kg^{-1} soil, the percent decrease was 5 for shoot and 15 for root; for 100 mg Cu, 11% and 16%; and for 150 mg Cu, 24% and 19%, respectively. Growth reduction at higher concentration may be due to the toxic effects of Cu on plants. It is known that Cu in excessive concentration inhibits plant growth and seed germination, induces chlorophyll degradation and interferes with a wide range of physiological and biochemical processes in cells (Caspi et al., 1999; Yruela et al., 1996).

Plants inoculated with *A. xylosoxidans* Ax10 exhibited an increase in shoot length, root length, plant fresh and dry weight in the presence of Cu. At a concentration of 50 mg Cu kg^{-1} soil, the percent increase was 52 for plant fresh weight and 97
for dry weight; for 100 mg Cu, 42% and 53%; and for 150 mg Cu, 23% and 6%, respectively. The increased growth response of plants caused by bacterial inoculation clearly indicates the potential of the organisms to survive and develop in the root zone. Doelman (1985) has reported that the efficiency of revegetation and phytoremediation of heavy metal-contaminated sites is closely related to the presence of higher proportions of metal resistant microbial populations in the soil, which likely conferred a better nutritional assimilation and protection effect on plants. It is known that contamination of soil with heavy metals increases the accumulation of ACC and ethylene in plant roots (Pennasio and Roggero, 1992). It may be speculated that increased accumulation of ACC in roots, caused by stressful growth conditions, can facilitate colonization of the rhizoplane with some metal-resistant forms of ACC-utilizing bacteria to some extent by providing the bacteria with additional sources of nitrogen, such as ACC.

In addition to ACC utilization, the metal resistant bacteria could exert their beneficial effects on host plant by several possible mechanisms. The mechanisms include: synthesis of siderophore which can solubilize and sequester iron from the soil (Glick et al., 1999); production of phytohormones which can enhance the growth of plants (Glick et al., 1998); and solubilization of phosphate (Gupta et al., 2002). In the present study, assessment of plant growth promotion parameters revealed the intrinsic ability of *A. xylosoxidans* Ax10 for the production of IAA and solubilization of phosphate. *A. xylosoxidans* Ax10 grown in LB medium supplemented with L-tryptophan exhibited a substantial production of IAA, indicating that the strain could utilized L-tryptophan as a precursor for growth and IAA production (Dell'Amico et al., 2005) (Fig. 3). The estimation of IAA in culture filtrate at different time intervals showed a non-linear and time-dependent increase and a maximum IAA production (6.4 μg mL^{-1}) after 168 h of incubation. An increase in shoot length, fresh and dry weight, generally promoted by IAA-producing rhizobacteria (Patten and Glick, 1996), was also observed in *A. xylosoxidans* Ax10 inoculated *B. juncea* plants (Table 1). The metal

Table 1
Influence of *A. xylosoxidans* Ax10 on the growth of *Brassica juncea* in Cu-amended soil

Cu^{2+} in soil (mg kg^{-1} dry soil)	PGPB strain	Shoot length (cm)	Root length (cm)	Fresh weight (mg $plant^{-1}$)	Dry weight (mg $plant^{-1}$)
Control	Blank	41.11[a] (±0.54)[b]	9.43 (±0.38)	10.31 (±2.42)	0.97 (±0.04)
	Ax10	51.67 (±0.44)	13.28 (±0.82)	17.01 (±1.81)	1.14 (±0.19)
50	Blank	39.22 (±0.82)	8.00 (±0.17)	9.05 (±1.30)	0.96 (±0.14)
	Ax10	47.11 (±0.69)	11.78 (±0.51)	13.78 (±1.47)	1.89 (±0.19)
100	Blank	36.61 (±0.92)	7.89 (±0.19)	9.85 (±2.49)	1.27 (±0.37)
	Ax10	50.67 (±0.33)	10.78 (±0.69)	13.99 (±2.59)	1.94 (±0.44)
150	Blank	31.44 (±0.87)	7.61 (±0.25)	9.64 (±2.21)	1.17 (±0.17)
	Ax10	34.11 (±0.69)	11.89 (±0.51)	11.83 (±2.99)	1.24 (±0.42)

[a] Values represent average of three replicates.
[b] Values in parentheses represent standard deviation.

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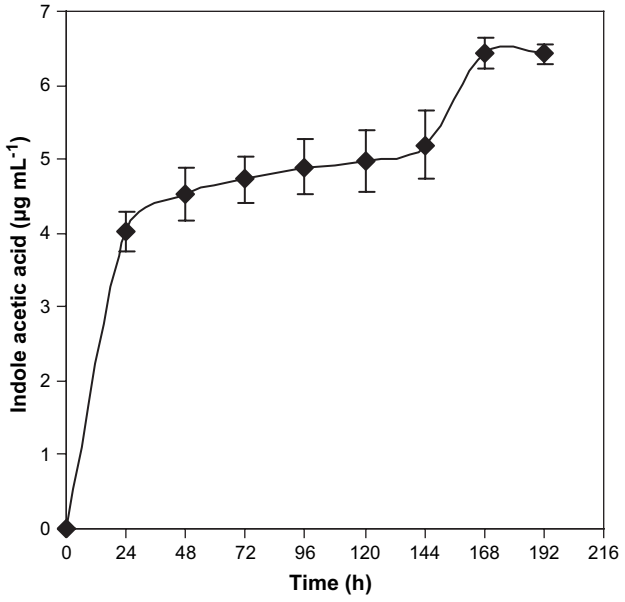

Fig. 3. Production of indole acetic acid by *A. xylosoxidans* Ax10. Each value is the mean of triplicates. Error bars represent standard deviation.

resistant bacteria belonging to different genera such as *Pseudomonas, Mycobacterium, Agrobacterium* and *Arthrobacter* were found to have plant growth-promoting features that can potentially promote plant growth and reduce stress symptoms in plants (Dell'Amico et al., 2005). Quantitative estimation of phosphorous solubilization was carried out in modified Pikovskaya's medium for 192 h (Fig. 4). *A. xylosoxidans* Ax10 utilized tricalcium phosphate as the sole source of phosphate. The maximum solubilization was achieved after 168 h of incubation (89.6 µg of P mL^{-1}). Further incubation up to 192 h,

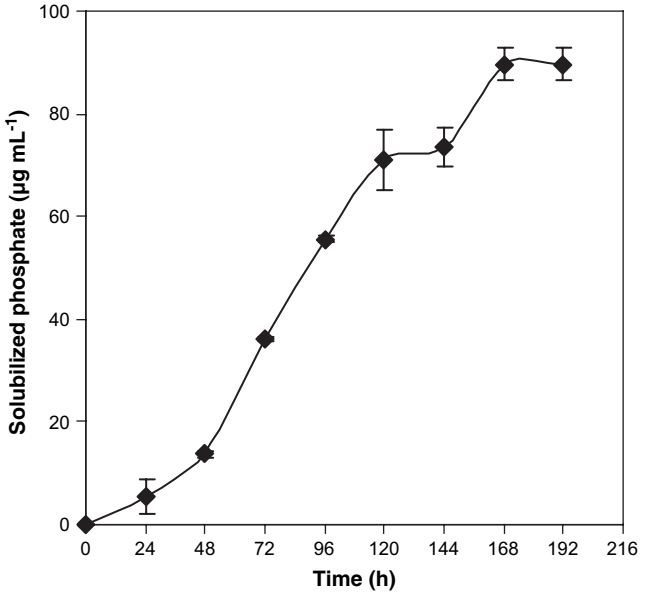

Fig. 4. Phosphate solubilization by *A. xylosoxidans* Ax10. The amount of soluble phosphates released was determined from the absorbance data using the calibration curve of KH_2PO_4 at 880 nm. Each value is the mean of triplicates. Error bars represent standard deviation.

did not improve the extent of solubilization. The solubilization of insoluble P by the rhizosphere microorganism was observed by several authors (Cunningham and Kuiack, 1992; Bar-Yosef et al., 1999) and reported to be due to the excretion of organic acids.

Earlier studies have reported the potential of soil microorganisms to assist plant establishment on contaminated soils through mediating nutrient mineralization and uptake by plants (Gupta et al., 2002), production of plant growth hormones (Patten and Glick, 1996) and utilization of ACC as a sole N source (Belimov et al., 2005; Burd et al., 2000). In this study, the increase in plant growth caused by Ax10 may be attributed to the production of IAA and solubilization of phosphate. In general the elevated levels of heavy metals in soil interfere with uptake of nutrients such as P and lead to plant growth retardation (Halstead et al., 1969). It is likely that the phosphate solubilizing isolates might have helped plant root proliferation and enhanced the uptake of soil minerals such as P by the host plant (Gupta et al., 2002; Zaidi et al., 2006). Further, the IAA produced by PGPB promotes root growth by directly stimulating plant cell elongation or cell division (Glick et al., 1998).

3.3. Accumulation of copper in *B. juncea*

The metal concentration in plant tissues and total metal removal by shoots are important indicators of whether the addition of a bacterial inoculation affects metal uptake by *B. juncea*. Microbes possibly affect trace metal availability in the rhizosphere and subsequently to the plant through the release of chelating substances, acidification of the microenvironment, and by influencing changes in redox potential (Smith and Read, 1997). Fig. 5A and B show the Cu distribution profile in shoot and root systems of *B. juncea* grown at varying concentrations of Cu in the soil. Accumulation of copper in the root and shoot systems increased with increase in the initial concentration of Cu in soil. Further, the data exhibit significantly higher Cu accumulation in tissues of inoculated plants compared with non-inoculated plants ($P < 0.05$; Fig. 5). Similar observations have also reported by Whiting et al. (2001), who found that the addition of a mixed inoculum of *Microbacterium saperdae*, *Pseudomonas monteilii*, and *Enterobacter cancerogenus* to surface sterilized seeds of *Thalaspi caerulescens* sown in autoclaved soil increased the Zn concentration in shoots 2-fold compared with non-inoculated controls; the total accumulation of Zn was enhanced 4-fold. In our study, the maximum accumulation of Cu in the root and shoot were determined to be 180 and 8 mg kg^{-1}, respectively, in the presence of *A. xylosoxidans* Ax10, at the highest concentration of 150 mg Cu kg^{-1} soil. However, in non-inoculated plants, the Cu accumulation in the root and shoot tissues were assessed to be 42 and 4 mg kg^{-1}, respectively. The increased accumulation of Cu in presence of *A. xylosoxidans* Ax10 might be due to more Cu uptake under acidic soil conditions, which develops as a result of activity of phosphate solubilization in soil. Sheng and Xia (2006) reported that the addition of Cd-resistant bacterial strains to *Brassica napus* grown in metal

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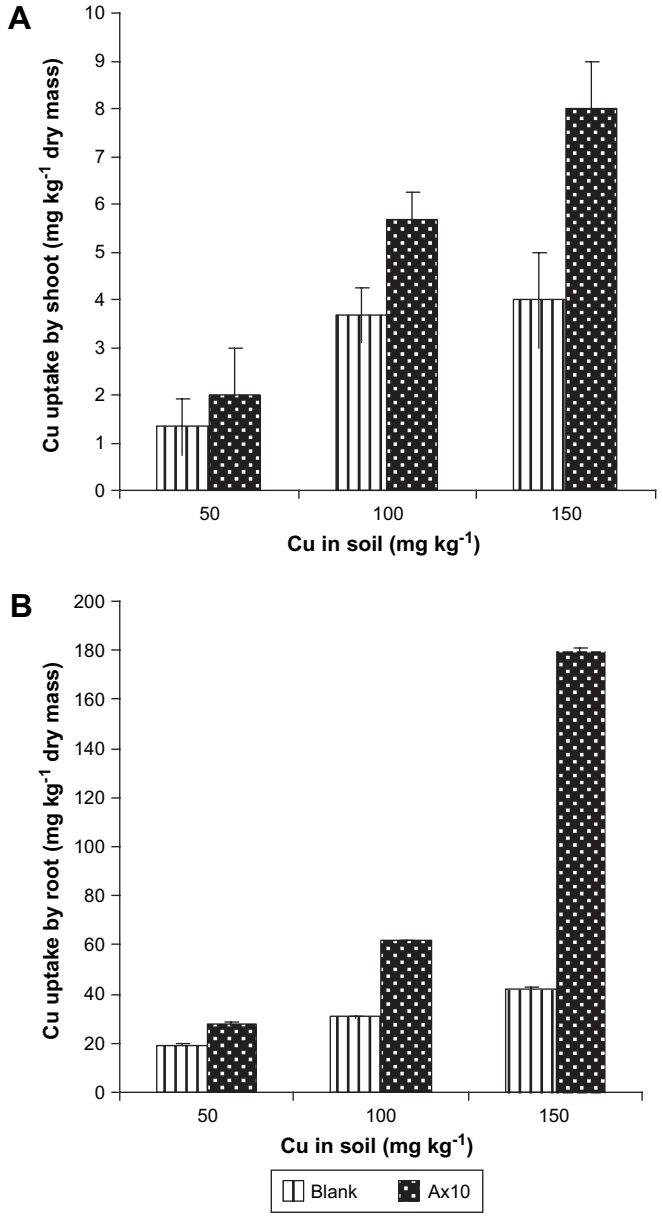

Fig. 5. Cu concentration (mg kg^{-1}) in the shoot (A) and root (B) of *Brassica juncea*. Each value is the mean of triplicates. Error bars represent standard deviation.

contaminated soil significantly increased the plant uptake of Cd when compared with the non-inoculated controls as a result of pH reduction. Similarly, Delorme et al. (2001) hypothesized that soil acidification in the rhizosphere of *Thalaspi caerulescens* facilitates metal ion uptake by increasing metal ion mobility around the roots.

To the best of our knowledge, this is the first report elucidating the role of a Cu-resistant *A. xylosoxidans* in Cu accumulation by *B. juncea* with concurrent reduction of Cu phytotoxicity and promotion of plant growth. Rhizosphere bacteria play a crucial role not only in improving plant growth and nutrition but also in protecting the host plant against biotic and abiotic stresses. A number of studies have demonstrated the importance of bacterial inoculation for plant survival and development in heavy-metal-polluted environments (Abou-Shanab et al., 2003; Sheng and Xia, 2006; Whiting et al., 2001). In the present study, the Cu-resistant *A. xylosoxidans* Ax10 could stimulate plant growth by utilizing ACC, producing IAA and solubilizing the phosphate. Further, studies have evidenced that the rhizosphere bacteria can enhance metal uptake by hyperaccumulator plants (Abou-Shanab et al., 2003; Sheng and Xia, 2006; Zaidi et al., 2006) as was shown in our experiment that the Cu uptake by *B. juncea* was enhanced by the Cu-resistant *A. xylosoxidans* Ax10. Since, the Cu uptake is also influenced by several possible mechanisms. The mechanisms include the release of plant root exudates which can directly solubilize and sequester metal ion from the soil (Rengel et al., 1998); and solubilization of hardly soluble metal bound phosphorous by phosphate solubilizing active substances of root cell wall (Ae and Shen, 2002). Hence, further research, including the interactive effects of rhizosphere bacteria and plant root exudates on the solubilization, uptake and translocation of Cu, is required to elucidate the mechanisms underlying bacterial assisted phytoremediation.

4. Conclusions

In conclusion, the present study has provided a new insight into the phytoremediation of Cu-contaminated soil. The data revealed that inoculation of *A. xylosoxidans* Ax10 not only protects plant from Cu toxicity but also enhances the Cu accumulation in plant tissue with concurrent stimulation of plant growth. Owing to its wide action spectrum, the Cu-resistant *Achromobacter xylosoxidans* Ax10 could serve as a proficient bioinoculant for the remediation of Cu-contaminated soil. Further research will be aimed to assess the suitability of *A. xylosoxidans* Ax10 for efficient bioremediation of heavy metals in natural ecosystem.

Acknowledgement

M. Rajkumar is grateful to Portuguese Foundation for Science and Technology (FCT) for providing financial assistance in terms of fellowship (SFRH/BPD/21309/2005).

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Please cite this article in press as: Ma, Y. et al., Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*, Journal of Environmental Management (2008), doi:10.1016/j.jenvman.2008.01.014