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ORIGINAL ARTICLE

Comparative in situ remediation potential of *Pseudomonas putida* 710A and *Commamonas aquatica* 710B using plant (*Vigna radiata* (L.) wilczek) assay

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Abstract A greenhouse study was conducted to determine the effect of cadmium (Cd)-resistant *Pseudomonas putida* strain 710A and Commamonas aquatica strain 710B, used either alone or as a binary mixture on the toxicity of cadmium to mungbean [*Vigna radiata* (L.) wilczek] plants. The bacterial strains 710A and 710B, isolated from the Semra mines in Ranchi, India, were resistant to 0.5 mM and 1 mM, $CdCl_2$ respectively. Moreover, both strains grew well at 10 °C and 30 °C. Of the two bacterial strains, strain 710B showed 129.6 and 83.5 times higher P solubilizing activity than strain 710A, when grown in liquid broth supplemented with cadmium at 10 °C and 30 °C, respectively. Furthermore, 16S ribosomal DNA (rDNA) sequencing identified 710A as a *Pseudomonas putida* strain and 710B as a *Commamonas aquatica* strain. The strain 710A significantly ($p<0.05$) stimulated the growth of roots (35.2 %) and shoots (30 %) of mungbean plants grown in soil treated with 110 μM $CdCl_2$. However, the increase in the case of 710B was found to be 15.4 % and 5.17 %, respectively. The loss of chlorophyll content was replenished by up to 80 %, 66 %, and 77.3 % by inoculation of 710A, 710B and binary mixture, respectively. Moreover the strains were able to reduce Cd accumulation in roots and shoots. Most significantly, residual Cd concentration in soil was reduced in the presence of bioinoculants. However, in terms of efficiency, the strains were found to be in the following order: 710A>710A +710B>710B. The results suggest that P. *putida* 710A strain is a potentially effective candidate metal to be used for sequestering and as a growth-promoting bioinoculant in Cd polluted soil.

Keywords Bioremediation · Phosphate solubilization · Cadmium · Commamonas · Transmission electron microscopy

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Introduction

Many metal ions are essential as trace elements, but at higher concentrations they become toxic. Heavy metals are difficult to remove from the environment and unlike many other pollutants, they cannot be chemically or biologically degraded. The main sources of heavy metal soil contamination are of both natural and anthopogenic origin (Ahmed et al. 2005). At present, however, the release of heavy metals into the environment is mainly due to human activities (Cortez et al. 2010) that include agriculture and metallurgical activities.

Metals and metalloids can pose a threat when they build up in soil due to many forms of anthropogenic activities. However, the nonessential metals such as lead (Pb), Cd, arsenic (As) and mercury (Hg) tend to build up in soils and when their bioavailability becomes high, toxicity can result. These negative effects can occur in soil microbes, soil fauna, higher animals, plants and humans homogenously. Accumulation of toxic metals in agricultural soils is an issue of health concern (Giller et al. 1998; Singh and Kalamdhad 2011).

Among the different heavy metals, Cd has received special attention due to its strength and persistence in accumulating in ecosystems, where it causes damage by moving up the food chain to finally accrue in human beings, who are at

the top of this chain (Machado et al. 2008). Cadmium is known to bind with essential respiratory enzymes (Nies 2003), causing oxidative stress and cancer (Banjerdkji et al. 2005). Cadmium is nonessential and highly toxic (Vassilev et al. 1998) to most organisms, having moderate toxicity (2–20 times toxicity higher than other heavy metals). It is readily taken up by many crops including cereals, potatoes, vegetables and fruits. Total Cd levels exceeding 0.001 $mgkg^{-1}$ are considered toxic to plants (Kabata-Pendius and Pendius 1992). Because of toxicity and persistence in nature, the levels of heavy metals in the environment need to be controlled. Physical, chemical and biological strategies have been considered in the remediation of contaminated soil. The biological option known as natural attenuation, bioattenuation or intrinsic bioremediation refers to the use of (micr-)organisms to reduce or remove the effect of harmful contaminants in the environment. The incessant exposure of soil microorganisms to sublethal doses of heavy metals/agrochemicals develops the enhanced detoxification potential and acquisition of new traits (Kavamura and Esposito 2010).

Microbes can affect heavy metal transport in the subsurface environment. Bacterial surfaces are often negatively charged in natural environments and their surfaces display functional groups that effectively bind dissolved cationic metals (Pang et al. 2005). Such microorganisms could be exploited for bioaugmentation of contaminated soils in a cost-effective manner for bioremediation and soil health restoration.

One way to relieve the toxicity of heavy metals to plants might involve the use of plant-growth promoting bacteria that exert some beneficial effect on plant development when they are either applied to seeds or incorporated into the soil (Rodriguez et al. 2008; Khan et al. 2009). Mechanisms of plant growth promotion include N_2 fixation, synthesis of siderophores, production of phytohormones and solubilization of minerals such as phosphorus.

The purpose of this study was to evaluate comparative potential of *Pseudomonas putida* 710A and *Commamonas aquatica* 710B in remediation of soil contaminated with Cd and therefore reducing Cd toxicity to the mung bean plant at low temperature (10±5 °C). Moreover, the combined effect of binary inoculants (710A+710B) was compared. In the present study, we also investigated the change in microbial cells in the presence of Cd using transmission electron microscopy (TEM).

Materials and methods

Strains and culture conditions

Strain 710A and strain 710B, originally isolated from soil samples from the Semra mines in Palamau, Jharkhand, were obtained from a departmental culture collection. Both the strains were maintained on nutrient agar medium (Hi Media) supplement with 1 mM $CdCl_2$ and 0.5 mM $CdCl_2$ concentration, respectively. The plates were incubated at 10 °C.

P- solubilization

The strains were screened for P-solubilization on Pikovskaya's agar plates (Das et al. 2003). Further quantitative estimation of P-solubilization was done in National Botanical Research Institute's phosphate growth medium (NBRIP) broth at 10 °C and 30 °C, in the presence and absence of Cd. The flasks were incubated for 3 days.

16S rDNA sequencing

The 16S rDNA sequencing was done at the National Centre of Cell Science, Pune University Campus, Gomeshkhind, Pune. Polymerase chain reaction (PCR) amplification of an almost full length 16S rRNA gene was carried out with the primer set 16 F27 (5′-CCAGAGTTTGATCMTGGCTCAG-3′) and 16 R15 25xP (5′- TTCTGCAGTCTAGAAGGAGG TGWTCCAGGC-3′). PCR was performed in an automated Gene amplification PCR system 9700 thermal cycler (Applied Biosystem, Foster City, USA), and PCR product was sequenced using a Big Dye terminator cycle sequencing kit (V 3.1) in an AB1 Prism 3730 Genetic Analyzer (Applied Biosystem, USA) using primer 16 F27 to yield a 700- base 5′ end sequence. The sequences were then analyzed at the RDII and NCBI database.

In situ characterization

A greenhouse study was conducted using strains 710A and 710B. The seeds of mung bean were bacterized (10^8 cells/seed) using carboxymethyl cellulose [Katiyar and Goel 2003] with respective strains. Nonbacterized seeds served as a control. To detect the remediation ability of the strains and the subsequent effect to the mung bean, cadmium (110 μM $CdCl_2$) was added to the sterilized soil. In brief, the soil was loam in texture, soil pH was 7.7, organic carbon was 0.82 % and cation exchange capacity (CEC) 7 cmol (+) kg^{-1} soil. Pots were kept in a green house (10±5 °C) for 90 days. Three replicates for each treatment were taken. Agronomical parameters of plants were measured after harvesting.

Chlorophyll assay

The chlorophyll content of the plant leaves was measured using the method described by Tripathi et al (2005).

Heavy metal analysis

After the plants were harvested, the roots and shoots were washed in a solution containing 5 mM Tris HCl (pH 6.0) and 5 mM EDTA, and then in distilled water, followed by drying at 60 °C for 24 h followed by 70 °C for 3 h. After grinding, aliquots (1 g) of root and shoot samples were analyzed by modified methods of wet ashing (Gupta et al. 2005).

Soil samples were collected periodically (0, 7, 15, 30, 45, 60 and 90 days, respectively) and analyzed for residual Cd concentration using a graphite atomic absorption spectrophotometer (GBC Avanta Ver 1.33, Australia). In each case, negative controls (natural soil and plant) were also analyzed for initial Cd content (Rani et al. 2008).

Transmission electron microscopy

Cultures were grown for 24 h in the presence of subtoxic concentration (0.5 mM) of Cd and transmission electron microscopy of the cultures was done as described by Rani et al. (2009).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA). Mean difference of the treatments was considered to be significant at the 5 % level.

Result and discussion

Selection of strains

Strains 710A and 710B were resistant up to 1 mM $CdCl_2$ and 0.5 mM $CdCl_2$, respectively. Both the strains were screened for P-solubilization potential at 10 °C and 30 °C, respectively. The results showed that the strains 710 A and 710 B solubilized 'P' (240 μg/m) and 1.76 μg/ml respectively at 10 °C, while 'P' solubilization at 30 °C was 297.5 μg/ml and 2.59 μg/ml respectively (Table 1). Furthermore, 16S rDNA sequencing identified 710A as a strain of *Pseudomonas putida* (Accession no.-EF207715) and 710B as a strain of *Commamonas aquatica* (Accession no.-EF207716), respectively.

In soils contaminated with metal, the natural role of metal-tolerant, plant growth promoting strains in maintaining soil fertility is more important in metal stressed soil. Besides their role in metal detoxification, strains also promote the growth of plants through the production of plant growth promoting substances, siderophores and phosphate solubilization (Khan et al. 2009). The microorganism possessing a phosphate solubilizing ability can convert the insoluble phosphatic compounds into soluble forms in soil and make them available to the crops (Kang et al. 2002; Pradhan and sukla 2005).

Effect of bioinoculants on plant growth and metal toxicity

Cadmium toxicity is also correlated with disturbances in uptake and distribution of macro and micronutrients in plants. Since there is tremendous difference in "P" solubilzation potential of both the strains,we assessed the comparative remediation properties in plant growth promotion in the presence of Cd.

In the absence of cadmium

A persistent and statistically significant increase in all the variables used to measure growth (root length, 7.4 %; shoot length, 12.4 %; wet weight, 46.9 %; dry weight, 45.4 %; chlorophyll content, 7.1 %) is evident in the plants whose seeds were bacterized with *Commamonas aquatica* 710B (Table 2). The increase was also observed in the plants whose seeds were treated with strain *Pseudomonas putida* 710A (root length, 2.1 %; shoot length, 10.5 %; wet weight, 9.6 %; dry weight, 6 %; chlorophyll content, 3.5 %), but it was less in comparison to the 710B strain. When seeds were bacterized with binary mixture (710A+710B), the increases in all the parameters were found to be less than in the 710B-treated seeds but more than in the 710A-treated seeds (Table 2). This increase was relative to the "P" solubilization potential and in each case, *Commamonas aquatica* 710B performed better than *Pseudomonas putida* 710A.

However, when binary mixture was used as bioinoculant, no synergistic effect of the strains was observed. Further, the

Table 1 Comparative "P" solubilization potential of *Pseudomonas putida* 710A and *Commamonas aquatica* 710B at 10 °C and 30 °C in the absence and presence of cadmium, respectively

	P- solubilization (μg/ml)			
	Without Cd (10 °C)	With Cd (10 °C)	Without Cd (30 °C)	With Cd (30 °C)
Pseudomonas putida 710A	1.76	0.52	2.59	1.02
Commamonas aquatica 710B	240	67.4	297.5	85.2

Table 2 Two-way ANOVA depicting the effect of *Pseudomonas putida* 710A and *Commamonas aquatica* 710B on mung bean under greenhouse conditions (10±5 °C) after 90 days of germination,respectively

		Root length[a] (cm)	Shoot length[a] (cm)	Wet weight[b] (g)	Dry weight[b] (g)	Chlorophyl[c](mg/g)
Without cadmium	Mean (control)	9.4 (0.52[d])	16.1(0.81)	8.3 (0.17)	3.3 (0.25)	2.8 (0.14)
	Mean (710A strain treated)	9.6 (0.65) 2.1%[e]	17.8 (0.9) 10.5 %	9.1(0.66) 9.6 %	3.5 (0.11) 6.0 %	2.9(0.3) 3.5 %
	Mean (710B strain treated)	10.1(0.4) 7.4 %	18.1(0.75) 12.4 %	12.2(0.23) 46.9 %	4.8 (0.17) 45.4 %	3.0 (0.14) 7.1 %
	Mean (710A+710B strains treated)	9.4 (0.49) 0 %	16.6(0.56) 3.1 %	9.4 (0.23) 13.2 %	4.2 (0.20) 27.2 %	3.2 (0.2) 14.2 %
With cadmium	Mean (control)	7.1 (0.56)	11.6 (0.36)	3.5 (0.20)	1.5 (0.14)	1.5 (0.16)
	Mean (710A strain treated)	9.6 (0.39) 35.2 %	15.1 (0.88) 30.1 %	6.3 (0.24) 80 %	2.4 (0.15) 60 %	2.7 (0.10) 80 %
	Mean (710B strain treated)	8.2 (0.82) 15.4 %	12.2 (0.56) 5.17 %	4.2 (0.14) 20 %	2.0 (0.88) 33 %	2.5 (0.16) 66 %
	Mean (710A+710B strains treated)	8.9 (0.56) 25.3 %	12.9 (0.71) 11.2 %	4.5 (0.17) 28.5 %	3.2 (0.17) 113 %	2.6 (0.79) 75.3 %
Critical difference at 5 %		1.60	2.02	0.57	0.51	0.51

[a] Means of ten replicates

[b] Means of ten replicates

[c] means of four replicates

[d] Value in parentheses () are±SEM

[e] Values indicate the % increase over the respective control

binary mixture was found inferior to *Commamonas aquatica* 710B and superior to *Pseudomonas putida* 710A.

In the presence of cadmium

Cadmium is a nonessential element that negatively affects plant growth and development (Kloepper et al. 1989; Das et al. 1997). Studies carried out in different plant species have revealed that Cd is strongly phytotoxic and causes growth inhibition and eventually plant death. With a higher level of Cd (110 mM), a reduction in mung bean root length, shoot length, wet weight and dry weight was found to be 1.3, 1.4, 2.3, and 2.2-fold, respectively. However, the addition of *Pseudomonas putida* 710A, *Commamonas aquatica* 710B and binary mixture (710A+710B) all improved the health of mung bean plants in the presence of Cd, with inoculation with the *Pseudomonas putida* 710A strain demonstrating the maximum increase for all of the growth parameters

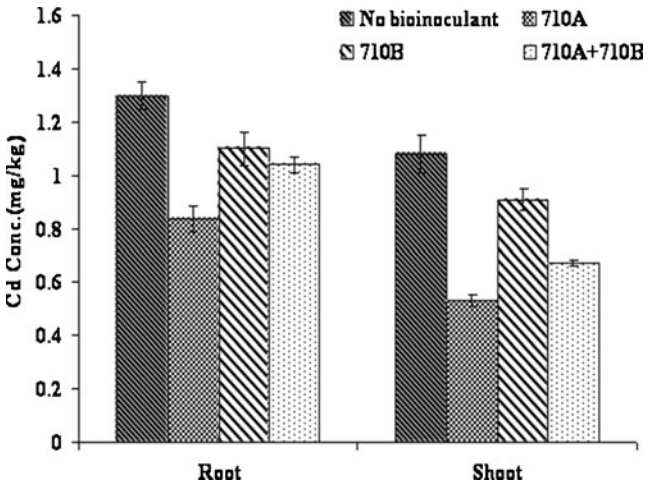

Fig. 1 Effect of bioinoculants *Pseudomonas putida* 710A, *Commamonas aquatica* 710B and their binary mixture (710A+710B) on bioaccumulation of cadmium in roots and shoots of Mung bean plants (SD±7, *n*=7)

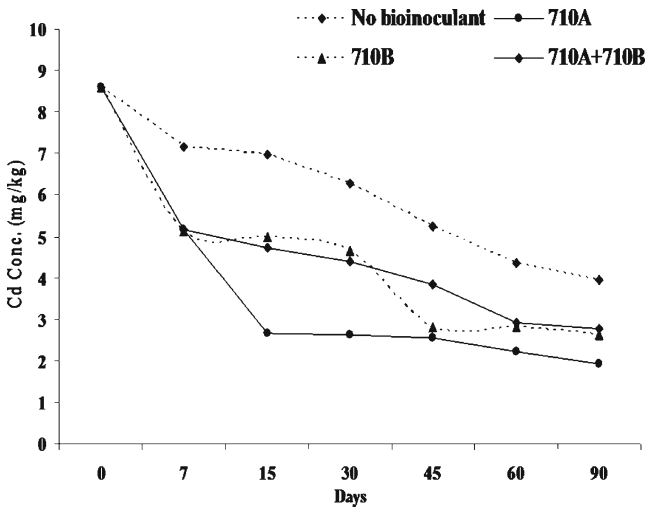

Fig. 2 Comparative residual cadmium concentration in soil (SD±7, *n*=7) the in presence and absence of bioinoculant, respectively. Each figure is a mean of seven replicates

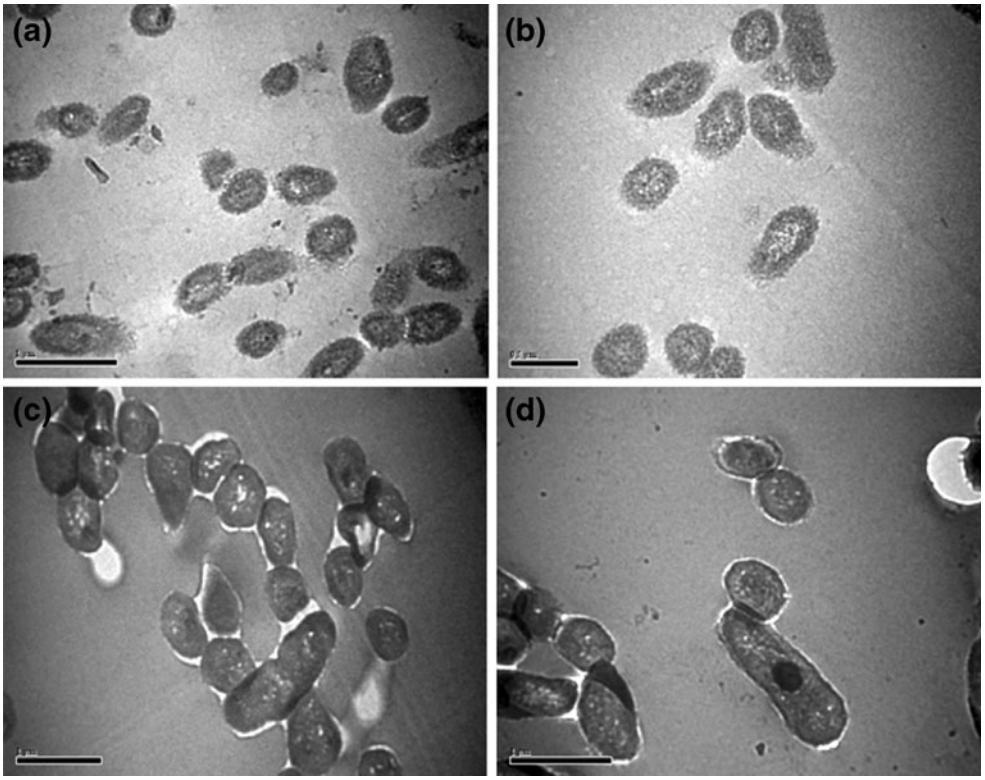

Fig. 3 Transmission electron micrograph of *P. putida* 710A (**a**, **b**) and *C. aquatica* 710B (**c**, **d**) in the absence and presence of cadmium, respectively

(Table 2). Similar observations were also made by Tripathi et al (2005).

The growth inhibition produced by cadmium could be mainly due to the effect of this heavy metal on the photosynthetic rate. This reduction could be due in part to the decrease in chlorophyll content, which was reduced 1.8-fold in the presence of Cd (110 mM). When bioinoculants 710A, 710B and 710A+710B were used, this loss was overcome; however, the only significant effect was observed in the case of strain 710A. Chlorosis, leaf rolls and stunting are the primary and easily visible symptoms of Cd toxicity in plants. Chlorosis may appear due to Fe deficiency. The inhibition of root Fe (III) reductase induced by Cd led to Fe (II) deficiency and it seriously affected photosynthesis (Alcantara et al. 1994).

Impact of bioinoculants on cadmium accumulation in plants and soil

a) In plants

The Cd concentration in roots and shoots decreased in the presence of bioinoculants. The *P. putida* 710A, *C. aquatica* 710B and binary mixture of these strains reduced cadmium accumulation in roots (39.6 %, 20.8 % and 25.1 %, respectively) and shoots (50.9 %, 15.7 % and 41 %, respectively) significantly (Fig. 1).

b) In soil

Moreover, the Cd concentration in soil was checked periodically at 0, 7, 15, 30, 45, 60 and 90 days, respectively. The Cd concentration in the control treatment was determined as a basal concentration which was deducted from each sample. The results showed a 54.1 % decrease in the control (untreated) treatment at 90 days. But in the presence of a bioinoculant, *P. putida* 710A, it showed a sharp decline (77.5 %) in Cd concentration in comparison to *C. aquatica* 710B (67.6 %). The decrease in the case of the binary mixture (710A+710B) was found to be 69.4 % per day (Fig. 2).

Transmission electron microscopy

Transmission electron microscopy (Fig. 3) of *Pseudomonas putida* 710A and *Commamonas aquatica* 710B showed that neither of the bacterial strains biosorbed or accumulated Cd, as no change in the cells' morphology was observed in the presence of Cd (Fig. 3). The unchanged cells of *Pseudomonas putida* 710A and *Commamonas aquatica* 710B indicated that cells might be using efflux or some other mechanism of resistance which needs to be documented. The resistance mechanism of bacteria to heavy metals include: (1) metal exclusion by permeability barrier; (2) active transport of the metal away from the cell/organism; (3) intracellular sequestration of the metal by protein binding; (4) extracellular sequestration; (5) enzymatic detoxification of the metal to a less toxic form; and (6) reduction in metal sensitivity of cellular

targets (Bruins et al. 2000). Cadmium resistance occurs though all of the biochemical resistance mechanisms except through enzymatic detoxification.

Conclusion

High concentration of heavy metals in the soil negatively impacts plant growth. Heavy metals are not mobile, thus some remediative measures are necessary to overcome the negative effects of heavy metals on plant growth. In this study, the remediation potential of two Cd-resistant strains, *Pseudomonas putida* 710A and *Commamonas aquatica* 710B, and their binary mixture was investigated using soil and the mung bean system. This study clearly suggested a superior impact of *Pseudomonas putida* 710A over the *Commamonas aquatica* 710B strain, considering their respective demonstration of remediation potential in both plants and soil. Further, in each case the binary mixture (710A+710B) was not found to be effective, as it did not show synergistic/additive effects either in the presence or absence of cadmium. The strains were able to reduce the heavy metal accumulation in plants as well as soil at low temperature, while the same strains showed greater P-solubilization at 30 °C. Therefore, we propose further exploration of these bioinoculants for Cd remediation, as such microbial populations are well adapted to survive at different temperatures.

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