Plant growth promoting rhizobacteria that decrease chromium toxicity in *Brassica juncea*

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Abstract

The aim of the present study isto assess the importance of siderophore producing rhizosphere bacteria on the growth of *Brassica juncea*under chromium stress. *Pseudomonas*sp. (A4) produced an iron chelating substance siderophores in iron deficient medium. Under chromium stress condition *Pseudomonas*sp. (A4) markedly increased the root and shoot length and also biomass of *Brassica juncea* as compared to *Pseudomonas* sp. (A3). This plant growth promotion has been related to the microbial production of siderophore.

Introduction

Industrialization has been identified as one of the necessary evils for the economic progress of any region or country. However, Industrialization in the last few decades has neither incorporated environmentally sound strategies nor attempted to balance the ecological equations encountered during the execution of production components. An inevitable outcome of such exercise has been the severe degradation of vital environmental components air, water and soil. One of the common heavy metals affecting the soil quality is chromium, which is introduced into the environment from industries such as leather tanning, electroplating and pigment production. Cr^{6+} compounds are highly water soluble and toxic compared to Cr^{3+} compounds.

Heavy metal contamination of soil causes a variety of environmental problems and the remediation of heavily contaminated soils often involves excavation and removal of soilto secure land fills, a technology that is expensive, and require site restoration. Alternatively,

heavy metal contaminated soil may be dealt with by Rhizoremediation the use of plants and rhizosphere microbes to remove, destroy or sequester hazardous substances from the environment It is a potentially economic and usually unobstructive technology that offers the possibility of biorecovery of resources such as heavy metals. There are number of reports using metal accumulating plants to remove toxic metals from the soil (Cunningham *et al.* 1995). When present at elevated levels in the environment, the heavy metal ions are absorbed by plants leading to impaired metabolism and reduced growth (Burd *et al.* 2000). One of the ways to relieve the toxicity of heavy metals to the plants is to involve the rhizosphere microbes (Glick *et al.* 1998, Glick 1995). Mechanism of plant growth promotion by rhizopsphere bacteriaincludes nitrogen fixation, synthesis of siderophore, production of phytohormones and solubilization of minerals (Kloepper *et al.*1989, Glick 1995). Although several strategies have been successfully applied to generate plants able to accumulate or transfer number of metals (Cunningham and Ow 1996), there are only a few reports on chromium remediation (Cerventes *et al.* 2001) and studies linking the role of rhizobacteriain plants growth under chromium stress. Hence, the present work was undertaken with a view to evaluate the influence of siderophore producing rhizosphere bacteriaon the growth of *Brassica juncea* exposed to chromium stress.

Materials and methods

Isolation of chromium resistant bacteria

Soil samples were collected from the rhizosphere of *Euphorbia hirta* growing in chromium contaminated soil. The samples were serially diluted using 25 mM phosphate buffer and incubated for 24 h at 37 C on nutrient agar amended with 25 µg of Cr^{6+} per mL. From the bacterial colonies different strains were picked and purified on the agar medium containing 25µg of Cr^{6+} per mL employing standard methods (Hasnain and Sabri 1996).Purified colonies were gradually taken to higher concentration of chromium and the procedure was repeated to isolate chromate resistant.

Isolation of plant growth promoting rhizobacteria

Plant growth promoting rhizobactera were isolated from the chromium resistant bacterial culture. The chromium resistant bacterial strains were grown on DF salts minimal medium (Dworkin and Foster 1958) containing 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4$, 1mg $FeSO_4$, 10 µg $MnSO_4$, 70 µg $ZnSO_4$, 50 µg $MnSO_4$, 10 µg MoO_3, 0.2 % gluconic acid, 0.2 % citric acid per L

ofdemineralized water and supplemented with 3 mM of 1-aminocyclopropane 1- carboxylic acid (ACC) to provide a nitrogen source. The bacteria were grown aerobically at 175 rpm for 120 h at 30C. During 120 h study growth was monitored by measuring the optical density of the culture at 600 nm once in 24 h. Dilution of the final culture were plated on to solid DF salts minimal medium with ACC and incubatedfor 48 h at 30C. From the medium, actively growing PGPR were isolated for further studies.

Siderophore assay

The chrome azural S (CAS) assay using mineral salt medium (MSM; 0.36 g KH_2PO_4, 1.4 g K_2 HPO_4,0.25 g $MgSO_4$ 7H2O, 0.2 g NaCl, 0.02 g $CaCl_2$ $2H_2O$, 15 mg EDTA, 0.16 mg $ZnSO_4$, 0.25 mg H_3BO_3, 0.2 mg Na_2MoO_4, 0.2 mg $MnSO_4$ $4H_2O$, 0.02 mg $CuSO_4$ $5H_2O$ per L of demineralized water with 20 mM of mannitol and 10 mM of NH4Cl2) was employed to detect siderophore production (Schwyn and Neilands 1987). Bacterial strains in the log phase were transferred into MSM supplemented with known quantity of filter sterilized FeCl3to get required concentration of Iron. A control series without iron was also prepared. The strains were incubated at 150 rpm for 24 h at 30C. After incubation the cells were separated by centrifugation at 10 000 rpm for 15 min. then 0.5 mL aliquot of supernatant was mixed with 0.5 ml CAS assay solution. Color change in the solution was recorded and the interpretation of the result was done as detailed in Carson *et al.* (2000)

Bacterization of seeds

Bacterization of seeds was performed following Burd *et al.* (2000). *Brassica juncea* seeds were surface sterilized by soaking in 1.5% sodium hypochlorite for 10 min and rinsed thoroughly with sterile distilled water. Sterilized seeds were incubated for 1 h at room temperature either sterile distilled water or in bacterial suspension (10^8 10^9 CFU per ml).

Pot experiments

Experiments on the effects of PGPR on the growth of *Brassica juncea* was performed using plastic pots (top diameter 120 mm, bottom 100 mm and height 90 mm). Seeds, control and inoculated with PGPR were sown in 200 gm of manured and autoclaved soil. The pots were irrigated with 40 mL of either distilled water or potassium dichromate solution containing 50

mg per L, 100 mg per L or 150 mg per L of $Cr^{6+}$and observed for 20 days. After 20 days the plants were carefully removed from the pots and the root surface was cleaned several times with dist water. Growth parameters such as shoot length, root length, fresh weight and dry weight of the plants were measured. The chromium content in shoot and root system was estimated by wet ashing method (Burd *et al.* 2000).

Results

Isolation and identification of bacterial strains

Chromium resistant bacterial strains were isolated from the rhizosphere of *Euphorbia hirta*growing in chromium laden environment. During the initial isolation process sixteen chromium resistant bacteria were isolated, which would tolerate more than 200 mg of Cr^{6+} per L of nutrient agar medium. All the chromate resistants were tested for their ability to grow on DF salts minimal medium with ACC as the sole source of nitrogen. Based on the utilization of ACC in DF salts minimal medium (Fig. 1) two PGPR namely, A3 and A4 were isolated from the sixteen strains and their chromate resistance levels were found to be 550 and 450 mg of Cr^{6+} per L, respectively. From the figure, it is evident that both strains grew well on DF salts minimal medium. However, in the absence of ACC, both strains showed minimal growth. In ACC containing medium, A4 and 32 exhibited growth up to a period of 72 h and the stationary phase reached thereafter. The maximum growth was observed in the strain A4 as compared to strain A3. On the basis of morphological, physiological and biochemical characteristics, the chromate resistant PGPR, A3 and A4, were identified as species belonging to the genus *Pseudomonas*..

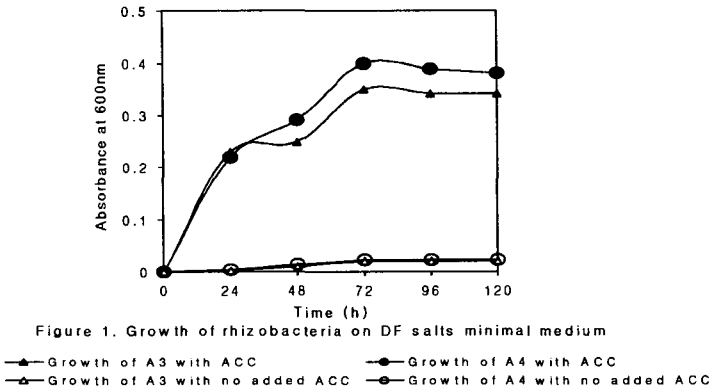

Figure 1. Growth of rhizobacteria on DF salts minimal medium

Siderophore production

The results of siderophore production by PGPR are furnished in table 1. Only the strain A4 gave a positive reaction in CAS assay indicating the production of siderophore, while the strain A3 did not produce the siderophore in iron deficient medium. Further from the table it is evident that higher concentrations of Fe^{3+} in the medium inhibited siderophore production in strain A4. Growth of A3 and A4increased with increases in Fe^{3+} concentration in the medium and the pattern of growth more or less similar at all the concentrations of Fe^{3+}.

Bacterial strain	Parameters	Fe(μM)				
		0	1	10	25	50
A3	CAS assay	-	-	-	-	-
	Growth(log DFR/mL)	7.74	8.01	8.24	8.18	8.32
A4	CAS assay	+	+	+	-	-
	Growth(log CFU/mL)	7.74	8.25	8.45	8.56	8.59

Table 1. Grwoth and Chrome Azural S (CAS) reaction in A3 and A4 grown in MSM '+' indicates siderophore producers; '-' indicates non siderophore producers.

Plant growth promoting activity of A3 and A4

Effects of PGPR inoculation on the growth of *Brassica junceae*exposed to different concentrations of chromium are presented in table 2. Plants inoculated with PGPR strains (A3 and A4) exhibited significantly higher growth compared to non inoculated plants. The maximum growth of shoot and root system was observed in the plants inoculated with A4. Seeds inoculated with A3 and A4 recorded significant increase in the fresh and dry weight of the plants. In plants exposed to chromium stress, a marked inhibition in growth was observed at all concentration of chromium as compared to control plants. With increase in the concentration of chromium, progressive decrease in growth (root and shoot lenth) and biomass yield (Fresh and dry weight) were noticed in *Brassica juncea.* Seeds inoculated with A3 and A4 exhibited an increase in shoot length and root length under chromium stress. From the table, it is clear that at the lowest concentration of 95.3 μg of Cr^{6+}per g soil, the inoculated plants grew better than the blank. At this concentration, plants inoculated with A4 exhibited a maximum

growth and biomass. The response of *Brassica juncea* to chromium at concentration of 304.4 mg of Cr^{6+} per g of soil, varied marginally with or without PGPR inoculation. However, at this concentration, plants inoculated with A4 exhibited maximum growth and biomass. When grown under chromium stress *B. juncea* exhibited marked increasein fresh and dry weights upon PGPR inoculation. At all the concentration of chromium used, the fresh and dry weight increased significantly when inoculated with A4. Accumulation chromium in the root and shoot systems increased with increase in the initial concentration Cr^{6+} in the soil. From the table it is found that the inoculation with PGPR (both A3 and A4) does not influence the quantity of accumulation of chromium in *B. juncea*. However, the root system exhibited greater accumulation of chromium as compared to the shoot system.

Cr^{6+} in soil (µg/g)	Bacterial strain	Root length (cm)	Shoot Length (cm)	Fresh weight (mg)	Dry weight (mg)	Chromium (µg/g shoot DW)	Chromium (µg/g root DW)
Control	Blank	5.56	7.60	26.10	5.70	-	-
	A3	7.35	8.47	38.56	6.32	-	-
	A4	9.62	9.78	52.67	7.68	-	-
95.3	Blank	4.74	6.22	23.10	4.94	75.52	752.34
	A3	6.06	6.76	30.34	5.74	77.54	753.65
	A4	7.69	8.16	35.00	7.92	73.63	750.22
198.3	Blank	3.88	5.33	18.40	4.07	133.58	895.05
	A3	4.22	5.45	19.34	4.67	135.54	875.43
	A4	4.50	6.79	25.18	5.01	137.79	890.42
304.4	Blank	3.58	4.03	15.55	3.64	192.43	1018.43
	A3	3.89	4.87	17.50	4.42	195.88	1004.76
	A4	4.46	4.95	20.04	4.69	187.68	998.46

Table 2. Effects of chromium stress and rhizosphere bacteria (A3 and A4) on the growth of *Brassica juncea* (values represent average of five samples)

Discussion

The present study helped to evaluate the usefulness of two PGPR strains on the growth of Brassica juncea under chromium stress. A3 and A4 strains were highly effective in countering the inhibitory effects of chromium on *Brassica juncea*. During the study, 16 chromate resistant bacterial strains were isolated from the rhizosphere of *Euphorbia hirta*. The observed poor bacterial diversity can be attributed to the chromium contamination in the soil. It is known that

at higher concentrations, chromium inhibits the growth of most wild type bacteria through carcinogenic and mutagenic effects (Venitt and Levy 1974). Based on the utilization of ACC as a sole source of nitrogen two PGPR namely A3 and A4 were isolated from the chromate resistants.

Bacterial strains grown on DF salts minimal medium with ACC, possess the enzyme ACC deaminase that would hydrolyse ACC (Jacobson *et al.* 1994). It is known that bacterial strains contain ACC deaminase when present in the soil or bound to seeds promote root elongation (Glick *et al.*1994). Certain heavy metal resistant bacterial strains potentially hydrolyse ACC and promote plant growth. For example, Nickel resistant PGPR, *Kluyvera ascorbata* isolated from soil contaminated with nickel and other heavy metals has been shown to promote plant growth (Burd *et al.* 2000). Hasnain *et al.* (1993) isolated lead resistant Pseudomonads from the rhizosphere of *Cenchrus pennisetiformis* for promoting plant growth. Both PGPR strains showed limited growth on DF salt minimal medium with no added ACC. The limited growth of PGPR may be due to utilization of residual nutrients with in the cells (Jacobson *et al.*1994).

In the present investigation two PGPR isolates (A3 and A4), which showed ACC deaminase activity andsiderophore production, were evaluated for plant growth promotion under chromium stress. In general, chromium contamination in soil is often associated with iron deficiency (Schmidt 1996). Chromium compounds present in the soil interfere the uptake of Fe by the plants (Barcelo *et al.*1985). One of the ways to reduce the iron deficiency in the plant system is to use siderophore producing PGPR. Inoculation with siderophore producing PGPR is known to form siderophore Fe complexes which can be taken up by plants. These serve as an iron source for plants (Bar-Ness *et al.* 1991). In our study, PGPR strain A4 produced siderophore in Fe3+deficient medium. From the present observation, it is evident that higher concentrations of Fe3+ in the medium inhibit siderophore production in strain A4. Inhibition of siderophore production when Fe3+ abundance in the medium has been reported in *Pseudomonas syringea* (Bultreys and Ghaysen 2000).

PGPR inoculation significantly increased the growth of *Brassica juncea*. In every instance, the trends observed indicated that the A3 and A4 helped to promote the growth of *Brassica juncea* under chromium stress. Similar observations on the influence of PGPR belonging to *Pseudomonas sp, Bacillus sp* and other bacterial strains on different plant species have been reported by earlier workers. PGPR having ACC deaminase, IAA and Siderophore producing characters have been found to enhance the plant growth (Pal *et al.* 1999). Strain A4 exhibited maximum fresh and dry weight of *Brassica juncea*under chromium stress as compared to strain A3. It may be due to the siderophore producing isolates which might have helped plant root to

enhance the uptake of soil minerals and nutrients by the host plant (Lambrecht *et al.* 2000).

Inoculation with PGPR strains did not influence the accumulation of chromium in *Brassica juncea*. This result reveal that strain A3 and A4 promotes the growth of plants without any influence on the quantitative accumulation of metal. Burd *et al.*(1998, 2000) have also recorded similar observation upon inoculation with *Kluyvera ascorbata*under Nickel, Lead and Zinc stress. In contrast to the present observations Hasnain and Sabri (1996) have reported that decrease in the accumulation of chromium stimulated the germination and growth of *Triticum aestivum* inoculated with *Pseudomonas sp.* Hoflich and Metz (1997) have reported that some PGPR are able to stimulate Maize growth and metal uptake when the plants were grown in soil polluted with heavy metals. Inoculated and non inoculated root system exhibited greater accumulation as compared to shoot system. This can be attributed to poor translocation of chromium from root to shoot system. Zayad *et al.* (1998) are also reported that the translocation of chromium from root to shoot is extremely limited and accumulation of chromium in roots is 100 fold higher than in the shoots. .

In conclusion, we found that the siderophore producing strain A4 promote the growth of *Brassica juncea* effectively than the non siderophore producing strain A3 underchromium stress. Based on the findings of the present study, siderophore producing PGPR can be used as potential candidate for the remediation of chromium contaminated soil.

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