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Degradation of heavy metal contaminated soil using plant growth promoting rhizobacteria (PGPR): Assess their remediation potential and growth influence of *Vigna radiata.* L.

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Abstract The contamination of heavy metal is caused by the natural and anthropogenic source which a global environment. The remediation of heavy metal- contaminated soil has faced a critical issue due to toxic effects on living organisms. The heavy metal-tolerant plant growth promoting rhizobacteria (PGPR), *Pseudomonas fluorescens* (PF01), and *Bacillus subtilis* (BS01) revealed to alleviate the heavy metal's toxic effects on the Green gram plant (*Vigna radiata*, L.) and their abilities to promote plant growth in greenhouse. The growth performance, photosynthetic pigments, heavy metal uptake were presented in green gram cultivated in the soil of contaminated with heavy metals under the greenhouse conditions. Results showed that the application of PGPR strains as a biofertilizer to green gram that helped the plant to ignore the toxic effects of heavy metals and enhanced the plant growth characteristics.

Keywords: Heavy metals, Greengram, PGPR, Biofertilizer, Environment

Introduction

Heavy metals in soil are increased as a major pollution in environment nowadays. The most conventional remediation approaches have not provided an appropriate solutions (Cheng, 2003). In recent years, the widespread industrialization and various anthropogenic activities accumulates the large amount of the heavy metals and dust particles to the agriculture soil, which affect to agriculture productivity from year to year (Govindasamy *et al.,* 2011). Heavy metals ions are non degradable and persist in the soil at toxic level to reduce plant growth which hamper to essential plant function and metabolic process (Seneviratne *et al*., 2017).

Plants associated with soil microorganisms, especially plant growth promoting rhizobacteria (PGPR), are accepted to play an important role to promote the plant growth and remediating soils from metal pollutants and organic pollutants by various mechanisms (Rajkumar *et al*., 2012). Application of remediation as phytoremediation appears to be an excellent

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for low cost production. The metal accumulating or tolerant species are used for good energy crops that promising as a renewable energy source, and avoiding the utilization of farmland for the production of non-edible plant biomass (Mlezeck *et al*., 2010). Plant growth-promoting and metal tolerant bacteria have played an important role for plant growth and heavy metal uptake of the plants which decreasing the metal bioavailability in the soils (Chen *et al*., 2006). Beneficial plant-microbe interactions in the rhizosphere influence the plant vigour and soil fertility. PGPR have affected directly and indirectly mechanisms on the plant growth (Dastager *et al*., 2011). The application of biofertilizers to cultivated soil has improved biodegradation of soil organic matter, increased nutrient supply, and enhanced plant tolerance to environmental biotic and abiotic stress. Therefore, biofertilizer are adopted as an efficient soil conditioner to improve soil quality by agriculturists and plant biologists (Bhardwaj *et al*., 2014). The research finding aimed to isolate the rhizobacterial strains from rhizosphere soil and inoculation to *Vigna radiata*. L plants in pot experiment. To estimate their plant growth promoting potential and photosynthetic pigment analysis. PGPR was inoculated to *Vigna radiata*. L plants and analysed the metal accumulation. The regulatory mechanisms of PGPR co-inoculation in counteracting metal toxicity was proved an efficiency strategy for the phytoremediation of metal contaminated soil. The results are expected to provide the theoretical basis for the application and improvement of phytoremediation in *Vigna radiata*. L planted in heavy metal contaminated soil.

Materials and Methods

Soil collection, preparation, and characterization

Soil samples were collected from rhizosphere soil the contaminated site of Salem District, Tamil Nadu, India. The rhizosphere soil samples were collected from Dharmapuri District, Tamil Nadu. All samples were aseptically kept in sterile plastic bags for analysis. The collected soil samples were analysed for physicochemical parameters and heavy metal both in treatment and non-treated control by using the standard procedures at Omega laboratories, Nammakkal , Tamil Nadu.

Isolation and identification of rhizosphere bacteria

Soil samples were collected at the depth of 20 cm and passed through 2 mm sieve to remove the dusts and stones, then placed in plastic bags and stored at 4℃. Each soil sample (10 g) was taken into 250 ml conical flask, added 90 ml of distilled water and performed in a rotary shaker for 15 min. One ml of soil suspension was serially diluted until 10^{-8}

dilutions. A 0.1 ml of sample was spreaded on nutrient agar plates and incubated at 37℃ for 24 hours. Experiment was carried out thrice to get pure cultures.

Biochemical characterization of bacterial isolates

The bacterial isolates were identified by the morphological, cultural, staining (Vincent, 1970) and biochemical properties. The biochemical characterization was done using Methyl red, VP test, citrate utilization, indole production, catalase oxidase, urease by standard microbiological techniques according the method of Cappuccino and Sherman (2002).

Phosphate solubilisation

The bacterial isolates were screened for phosphate solubilisation according to the method of Chen *et al. (*2006). Pikovsakaya's agar medium amended with inorganic phosphate was prepared and a full loop of bacterial culture was streaked onto the medium plates. The streak plates were incubated at 28 ± 2^0C for 3-4 days. Solubilization of mineral phosphate was observed by a clear halo zone around the bacterial colony.

Siderophore production

Siderophore production was checked on the solid Chromazurol S (CAS) universal blue agar plates (Schwyn and Neilands, 1987). The actively growing cultures were spotted on the CAS agar plates and incubated at 30^0 C for 48 hours. Formation of the yellow-orange halo zone around the colony was indicated to produce and release siderophores on the agar plates.

Antibiotic sensitivity test

The susceptibility to antibacterial agent was tested on antibiotic disks by the method of Bauer *et al* (1966). Bacterial strains were grown in nutrient broth at 28±2℃ for 24 hours. A 0.1 mL of overnight grown culture was spreaded on nutrient agar plates. The antibiotic discs of known potency were placed on the agar surface and incubated at 28±2℃ for 24 h. The inhibition zones around antibiotic discs were measured, and compared to the following antibiotics:- Streptomycin, Chloramphenicol, and Kanamycin.

Tolerance to salt and pH

Salt tolerant of bacterial isolate was investigated by inoculation each bacterial strain to nutrient agar plates with different concentrations of NaCl such as 0.5%, 5%, 10%,15%, 20%, 25% and 30%, respectively and

incubated for 48 hours at 28 ± 2 °C. A pH-tolerant of bacterial strains were investigated in the plate count agar medium with different acetic conditions ranging from 2-12 m, which was adjusted by using 1N NaOH and 4N HCl. After 48 h incubation, Salt and pH-tolerant growth efficiency of bacterial strains were recorded.

Cadmium tolerance test

Cd tolerant efficiency of bacterial strains, the agar dilution method was determined for Cd tolerant of selected bacterial isolates (Cervantes *et al.,* 1986). The freshly growing cultures were streaked on Cd (Cadmium chloride) amended agar plates at different concentration ranging from 25-100 µg/L. Then, the Cd resistance was determined by the viability counting of selected bacteria after the 3 to 4 days of incubation. The minimal inhibitory concentration (MIC) was calculated.

Molecular identification of bacterial isolates

Isolates were molecular phylogenic identified into at species level by PAR Life Sciences, Trichy, Tamilnadu. The molecular identification was used to characterize the bacteria isolates by the gene sequencing of 16S rRNA gene using 27 forward and 1492 reverse primers. The identified bacterial sequences were searched in BLAST and NCBI nucleotide database centre to compare the similarity between sequence (Altschul *et al.,* 1997). The obtained sequencing of 16S rRNA gene compared as a similar with *Pseudomonas fluorescens* (MK478897) and *Bacillus subtilis* (MK483262).

Pot experiments

The collected rhizosphere soils and Cd contaminated agriculture soil were sterilized and placed into plastic pots, and the soil moisture content was maintained approximately 70% of water holding capacity. The seeds of Healthy Green gram (*V. radiata*) were obtained from Tamil Nadu Agriculture University (Coimbatore), and sterilized in 20% sodium hypochlorite for 10 min, then washed three time with deionized water. After the seedling, each pot was inoculated with 2 ml of bacterial suspension (*Pseudomonas fluorescens*-PF01, and *Bacillus subtilis*-BS01). The control was bacterial free suspension. The plants were grown in a greenhouse under natural light conditions. A 1 ml of same bacterial suspension were diluted into 1 ml of distilled water and inoculated into plant growing soil every week. After 45 DAI (day after inoculation), plant biomass (fresh and dry weight, g), shoot and root length (cm) and photosynthetic pigment contents were assessed. The dry weight (g) was dried at 65 ℃ in an hot air oven for 12 hours.

Photosynthetic pigment assay

Photosynthetic pigment was assayed the green gram leaf tissue sample of 100 mg by suspended in 10 mL of 80% acetone, mixed and kept at 4℃ overnight in dark condition. Supernatant was withdrawn after centrifugation (5000 rpm), and absorbance was recorded at 663 and 645 nm in Spectrophotometer. The amount of chlorophyll was calculated according to Arnon method (1949).

Heavy metal accumulation of plant samples

Cd uptake in plant was assessed by using Atomic Absorption Spectrophotometer (AAS). The oven dried samples were used to estimate the Cd uptake. The digestion sample was done follow the method of Allen *et al.* (1976). A 0.02 g of oven dried sample was kept in digestion beaker. The samples were digested in sulphuric acid (H_2SO_4), nitric acid (HNO_3) and per-chloric acid ($HCIO_4$) at a ratio of 1:3:1. Every digestion beaker, 5 ml of digestion mixture was added and heated on hot induction plate. The samples were heated until clear. These were filtered after cooling using nylon syringe filters (0.22-µm size). The sample volume was made 50 ml using double distilled water.

Results

Collection and soil sample analysis

The rhizosphere soil and samples were tested in Omega laboratories, Nammakkal. The physicochemical properties of soil areas were found to contaminate the experimental site in Salem District, Tamil Nadu (Table 1).

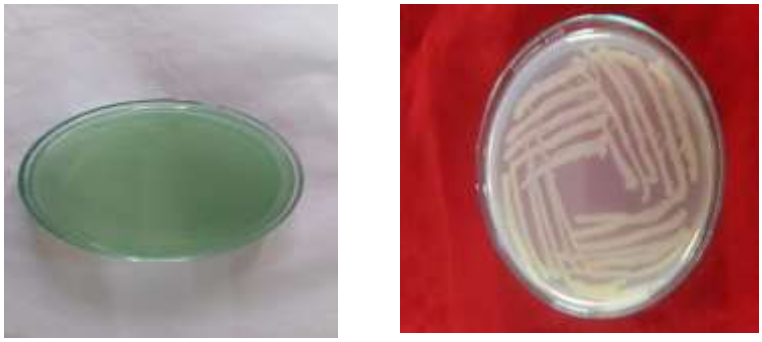
Table 1. Physicochemical analysis of soil samples

Physicochemical Properties	**pH**	**EC**	**N**	P	K	**Cu**	**Mg**	**Ca**	**Cd**	**Zn**
Contaminated soil	8.72	0.88	01.28	0.260	01.06	16	335	298	**12**	310
Normal soil	7.26	0.874	0.95	1.80	00.86	6.4	234	250	1.5	198

Isolation and identification of the bacterial isolates

Result showed that the all bacterial isolates from rhizosphere soil that can be grown in King's B and Nutrient agar media for PGPR screening. The obtained results showed both strains enzyme production activity for citrate, catalase, urease, oxidase and expressed phosphate solubilizing property. Moreover, gram positive nature of *P. fluorescens* (PF01) exhibited siderophore. It was proved by indole, methyl red (MR) and Voges-

Proskauer (VP) tests. But gram negative in *B. subtilis* (BS01) was also exhibited the siderophore. But the PF01 had shown better biochemical properties than the BS01 strain. The presented biochemical parameters of both bacterial strains were seen in Figure 1 and Table 2. Both the bacterial isolates showed the phosphate solubilising activity on Pikovaskaya's agar medium. The phosphate solubilising activity was shown that *P. fluorescens* exhibited higher than *B. subtilis* as it was observed by a clear zone around the inoculated strain after 3 days. The isolates showed siderophore production activity on CAS agar medium. The siderophore production by *P. fluorescens* was higher than *B. subtilis* which observed by a clear zone around the inoculated the bacterial isolates (Table 2).



PF01-*Pseudomonas fluorescens* BS01-*Bacillus subtilis*

Figure 1. Culture characteristics of *Pseudomonas fluorescens* PF01 and *Bacillus subtilis* BS01

Table 2. Biochemical characterization of bacterial isolates

Test	Gram staining	Indole	MR test	VP test	Citrate utilization	Catalase	Oxidase	Urease	Phosphate solubilization	Siderophore production
PF01	+	+	+	+	+	+	+	+	+	+
Bs01	-	-	_	+	+	+	+	+	+	-

Note: (+) – Positive ,(-) Negative

The bacterial isolates found to be much tolerant at lower concentration of 25 µg/ml and less tolerant at concentration of 200 µg/ml. These isolates expressed the tolerance to Cdat concentration of 25-200 µg/ml and tolerant to cadmium at high concentration of 200 µg/ml (Figure. 2). Effect of salt concentration on the growth of bacterial isolates was observed in nutrient agar plates at different salt concentration. *P. fluorescens* can be grown well at salt concentration of 25 %, except unable to grow at 10 %. *B. subtilis* grew well at salt concentration of 30 %, except unable to grow 15% (Table 3).

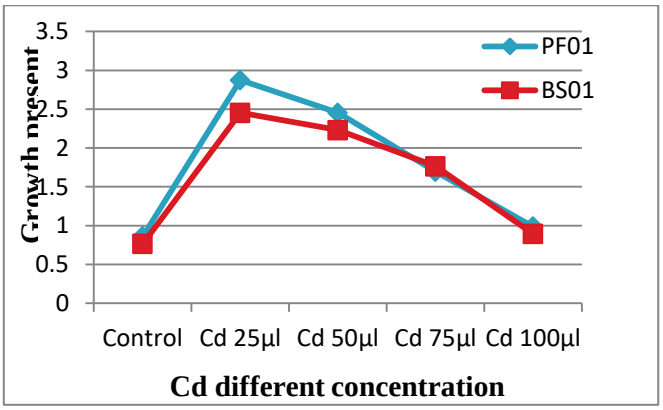


Figure 2. Quantitative analysis of heavy metal tolerance

Table 3. Effect of different salt concentrations on the growth of bacterial isolates

	Salt concentration						
No	0.5%	5%	10%	15%	20%	25%	30%
PF01	+++	+++	++	+	-	-	-
BS01	+++	+++	++	++	+	-	-

Note: "+" or "-" sign indicate the growth of the bacteria in particular condition

Effect of pH on growth of bacterial isolates in nutrient agar plates showed that *P. fluorescens* grew well at pH 10 except, but unable to grow at pH 2. *B. subtilis* grew well at pH 12, except unable to grow at pH 4 (Table 4). The antibacterial sensitivity was positively shown in both isolates (Table 5).

Table 4. Effect of various pH on the growth of bacterial isolates

	p H values					
	2	4	6	8	10	12
PF01	-	-	++	+	+	-
BS01	-	-	++	++	+	-

Note: "+" or "-" sign indicate the growth of the bacteria in particular condition

Table 5. Antibiotic sensitivity test of PF01 and BS01 isolates

No.	Commercial antibiotic disc	Inhibition zone diameter (mm)	
		PF01	BS01
1.	Streptomycin[10]	13 mm	16 mm
2.	Chloromphecol[25]	21 mm	24mm
3.	Kanamycin[10]	22 mm	26mm

The molecular identification was confirmed species by total genomic DNA and amplified by 16S rDNA specific primers. PCR amplicons of 16S

rDNA of about 1500 pb were obtained for both the isolates to discrete bands in agarose gel electrophororsis (Figure 3). Phylogenetic tree of 16S r RNA gene sequences showed the realationships among the isolates of bacteria isolated from the rhizoshere soils. The related species were retrived from GenBank database. *Pseudomonas fluorescens* PF01 and *Bacillus subtilis* BS01 are deposited as accession number MK478897 and -MK483262, respectively in GenBank database.

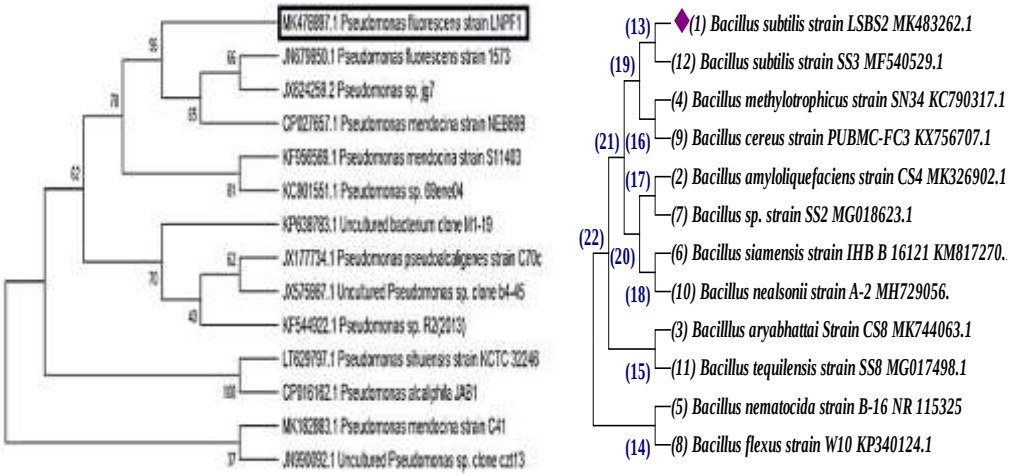


Figure 3. Phylogenic tree of *Pseudomonas fluorescens* PF01 and *Bacillus subtilis* BS01

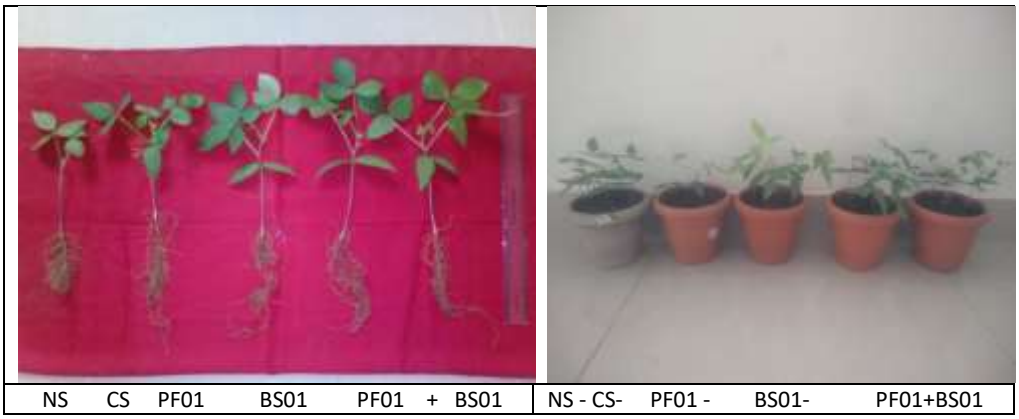


Figure 4. Growth characterization *Vignaradiata* L. at 45 DAI

Vigna radiata inoculated with *B. subtilis* and *P. fluorescens* showed significantly higher shoot, root length, fresh and dry weight than the control.

The plant inoculated with *B. subtilis* showed better root, shoot length and fresh, dry weights than the non–inoculated control (Figure 4 and 5). The plant inoculated with both the bacterial isolates showed highest level in photosynthetic pigment analysis (Table 6).

Heavy metal analysis for accumulation in plants in PF01, BS01, PF01+BS01 resulted to be 1.5 µg/g-^{1}, 1.6 µg/g-1, and 1.3 µg/g-1, respectively (Table 7). The inoculated bacterial isolates decreased heavy metals level when compared to uninoculated control. PGPR minimized the limiting factors in associated with the phytoremediation method for soil chemistry, intensity of soil contamination and metal solubility.

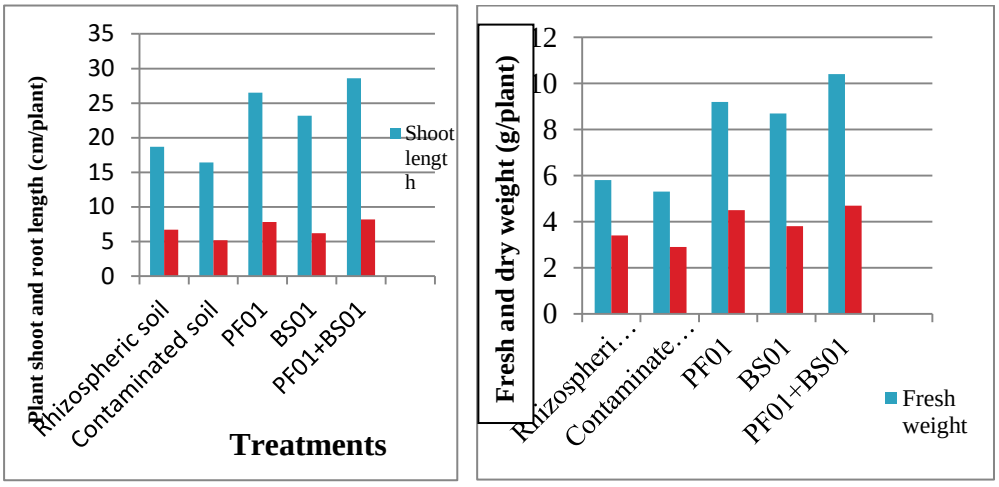


Figure 5. Growth characteristic and biomass of *Vigna radiata* at 45 DAI

Table 6. Averaged data of total chlorophyll and carotenoids for *Vigna radiata* L.

Treatments	***Vigna radiata L***	
	Chlorophyll (mg/g)	Carotenoids (mg/g)
Rhizospheric soil	0.064±0.067	0.035±0.02
Contaminated soil	0.061±0.023	0.026±0.034
PF01	1.124±0.06	0.054±0.01
BS01	1.105±0.24	0.038±0.04
PF01+ BS01	1.165±0.18	0.041±0.012

Table 7. Averaged data of heavy metal analysis of plant samples in *Vigna radiata*

No	Treatments	Cd level in plant (mg/g)
1.	Normal soil	0.8
2.	Contaminated soil	2.1
3.	PF01	1.3
4.	BS01	1.5
5.	PF01+BS01	1.7

Discussion

The pootent for phytoremediation depends upon the interactions of among the soil, heavy metals, bacteria and plants. The roots of plants interact with a large number of different soil microorganisms that are major of determinants of the extent of phytoremediation. *Bacillus, Pseudomonas* and *Bravibacillus* are well known to promote the plant growth characters and yield in different non-leguminous plants (Jha *et al.,* 2016). In the study, bacterial isolates were screened for their plant growth promoting activities viz. indole production, MR-VP, citrate utilization, phosphate solubilization, lytic enzymes eg catalase, urease, and oxidase The selected rhizobacterial isolates were chracteriszed by using standard methods, morphological and cultural characteristics on agar plate, growth on broth media and NaCl, which also described in Bergy's Manual of Systematic Bacteriology (Tein *et al.,* 1979). Plant growth in agricultural soil is influenced by the several environmental factors. Beneficial soil microorganisms can be achieved significantly for yield. In the present study, *Vigna radiata* L inoculated with *B. subtilis* and *P. fluorescens* showed significantly higher plant growth parameter and plant biomass than the control. The effect of these isolated rhizobacteria in phytoremediation of contaminated soil, and rhizobacterium for plant growth and protection are approprioated strategy and clean environment as stated by Sowmya *et al.* (2014). The related experiment which inoculated *Burkholderia spp*. to sorghum and maize resulted in increasing plant root and shoot biomass (Govindarajan *et al*., 2006). There is a direct correlation of the plant growth and biomass with plant growth promoting rhizobacteria (PGPR) which was found to be increased after augmentation of PGPR under Cd toxicity (Kamran *et al*., 2015). Our studies were in the agreement with the previous studies which conducted by (Treesubsuntorn *et al.,* 2018 who found that *B. subtilis* and *B. cereus* when inoculated to Cd exposed *Oryza sativa* resulted in higher root and shoot biomass. Inoculation of *Klebsella pneumonia* in *Vigna mungo* enhanced the level of chlorophyll under Cd stress (Dutta *et al.,* 2018). The results of the present study showed that Cd content was more in roots than in shoots. Similarly result stated by Han *et al*. (2018) that enhanced metal accumulation of seed under Cd presence (Han *et al*., 2018).

The use of PGPR in phytoremediation and crop production are shown some limitation, the most important of the difficulty to achieve that similar results under other fields, soil types, and plant conditions as suggested by Parnell *et al.* (2016). Plants inoculated with PGPR resulted to increase the plant biomass, photosynthetic pigment and developed the tolerance to heavy metal contaminated soil, where the metal exceeded to plant tolerance. Rhizosphere microorganisms (PGPR) with growth properties and heavy metal resistance would be the best choices in sustainable agriculture.

The study was found that microorganisms are helpful to reduce the leaching of heavy metals in environment. Hence, these isolates can be reached for minimization of toxicity of cadmium and enhancement of plant growth under the metal stress condition. This study is also helpful for bioremediation due to high level of MIC of isolates. PGPR may help to reduce the toxicity of heavy metals to plants in polluted environments. This study is identified specific PGPR with multiple plant growth promoting characteristics as novel PGPR strain for bio-inoculant and applied to soil in various agro-climatic conditions and crop varieties. However, phytoremediation is a slow process, removal of heavy metal from the polluted soil is done by increasing the amount of plant biomass and increasing the metal accumulation ability of the plants. The contribution of PGPR in decreasing Cd toxicity and accumulation implicated their roles to achieve the goal of lower Cd concentration in green gram plants.

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