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Research Article

Assessment of Heavy Metals Toxicity on Plant Growth Promoting Rhizobacteria and Seedling Characteristics of *Pseudomonas putida* SFB3 Inoculated Greengram

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Abstract

Heavy metals present a great environmental concern, because of their adverse impact on microflora, plants and humans. The growing environmental awareness necessitates the development of effective and inexpensive methods for metal removal. In the present study, an attempt was made to examine the plant growth promoting abilities of the rhizobacteria isolated from metal contaminated fields. A total of 113 rhizobacterial isolates belonging to genera *Bacillus, Pseudomonas, Azotobacter* and *Rhizobium* were isolated from rhizospheric soils of bajra grown in the fields near Mathura road, U.P., India. The rhizobacterial isolates were characterized biochemically and evaluated for their plant growth promoting traits such as production of indole acetic acid (IAA), ammonia (NH3), hydrogen cyanide (HCN), siderophore and catalase. Also, the metal tolerant ability of the bacterial cultures was determined. All isolates were positive for catalase and NH_3 production. All isolates of *Pseudomonas spp., Bacillus spp.* and *Azotobacter spp*. produced IAA whereas only 57% *Rhizobium spp*. produced IAA. Among the bacterial isolates, *Pseudomonas putida* strain SFB3 (identified using 16S rRNA gene sequence analysis, GenBank accession no. MF284668) showed high level of tolerance to multiple heavy metals and exhibited significant plant growth promoting activities even under metal stress. The strain SFB3 when used as an inoculant enhanced the germination efficiency and seedling vigour of greengram besides increasing the plumula and radicle length both in metal free and metal stress conditions. *Pseudomonas putida* strain SFB3 showing tolerance to multiple heavy metals and exhibiting PGP traits hold promise as effective PGPR for enhancing crop production when applied as biofertilizer under field conditions.

Keywords: Greengram; Metal Tolerance; PGPR; *Pseudomonas*

Introduction

Heavy metals are generally referred to as those metals which possess a specific density greater than 5 g/cm^3 [1]. Rapid industrialization and various anthropogenic activities have been responsible for increased heavy metal release to the environment causing negative impacts on agriculture and human health. Due to non-biodegradable and persistent nature, the excessive accumulation of heavy metals into soils becomes most dangerous to crop plants and affects structure and microbial composition of soils and their activity.

This in turn cause reduction in fertility and concurrently results in yield losses [2,3]. They also pose significant threat to human beings via the food chain [4]. According to the World Health Organization (WHO) Cd, Cr, Co, Cu, Pb, Ni, Hg and Zn are the most hazardous metals [5]. Conventional methods to remediate heavy metals contaminated site are excavation and solidification or stabilization.

Even-though these technologies are suitable to contain contamination but they cannot permanently remove metals from the polluted sites [6]. In addition, these methods are expensive, and generates hazardous by-products. To circumvent such problems, biological methods have been found as inexpensive, easy to operate and they do not produce secondary pollution [7]. Among biological materials used in metal detoxification, microorganisms endowed with metal tolerance ability can be exploited to remove, concentrate and recover metals from contaminated sites [8]. In this context, studies have been conducted to assess the impact of various plant growth promoting bacteria (PGPB) for effective bioremediation of metal contaminated soils. When used as inoculant under metal stressed environment such PGPR stimulates plant growth by- (i) supplying N [9] and P (ii) phytohormone production [10] (iii) enhancing plant resistance to metals and protection of plants from pathogens through release of volatile components (acetoin and 2, 3-butanediol) [11], synthesis of 1-aminocyclo-

propane-1-carboxylate deaminase (ACC) [12], secretion of siderophores and organic acids and (iv) biosorption and accumulation of metals [13,14]. The selection of microorganisms possessing both metal tolerance ability and capability to produce growth regulators could be useful to speed up the recolonization of the plant rhizosphere in the polluted soil. Also, the use of PGPR as inoculant is considered an effective and economical approach to replace/reduce chemical fertilizer [15]. Considering the importance of legumes and PGPR in maintaining soil fertility and the ability of plants to absorb excessively higher amounts of heavy metals, the present study was designed with the following objectives to (i) screen PGP and metal tolerant ability of indigenous microbes isolated from metal contaminated rhizosphere soils and (ii) to evaluate the toxic effect of metals on seedling growth of mungbean [*Vigna radiata* (L) Wilczek] plants inoculated with or without *P. putida* strain SFB3.

Materials and Methods

Isolation of rhizobacteria

The soil samples were collected from the rhizosphere of *Pennisetum glaucum* L. grown in sewage irrigated fields of Mathura road, Kanpur region, India. The rhizosphere soil samples were kept in plastic bags and stored at 4°C in the laboratory until further use. Soil samples were serially diluted in sterile phosphate-buffered saline (pH- 7.2) and plated onto yeast extract mannitol agar (*Rhizobium*), Ashby agar medium (*Azotobacter*), King's B agar (*Pseudomonas*) and nutrient agar (*Bacillus sp*.) media. Following incubation at 28 ± 2°C, colonies were randomly picked and further purified by streaking. Pure bacterial colonies were maintained as glycerol stocks at -70°C for further use.

Identification and Biochemical Characterization of Rhizobacteria

Rhizobacterial strains were characterized based on their morphological, biochemical and/or physiological characteristics using standard methods [16].

16S rRNA based identification of strain SFB3

"Sequencing of the 16S rRNA of strain SFB3 was done commercially by a DNA sequencing service (Macrogen, Seoul, South Korea) using universal primers". Macrogen (Korea) for 16S full rRNA sequencing. The sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA). Universal primers 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAATTCMTTTRAGTTT) were used for sequencing and another set of universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) were used for the amplification. The sequence (642 bp) so obtained were examined using BLASTn programme at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) to identify and compare the isolate with the nearest neighbour sequence available in the NCBI database. The selected sequences were aligned by using ClustalW, and the aligned data was used for phylogenetic analysis using MEGA7 using neighbour-joining method with 1000 boot strap replicates.

Heavy Metal Tolerance

The selected bacterial strains were tested for their resistance to heavy metals by agar dilution method [17]. Freshly prepared agar plates were amended with various soluble heavy metal salts namely $K_2Cr_2O_7$ [CrVI], $Pb(CH_3COO)_2$ [Pb], $NiCl_2.H_2O$ [Ni], $CdCl_2$ [Cd], $ZnCl_2$ [Zn], and $CuSO_4.5H_2O$ [Cu] at various concentrations ranging from 25 to 2000 µg/ml and metal treated plates were inoculated with overnight grown cultures. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at 37°C for 24 - 48h.

Scanning electron micrograph studies of *P. putida* SFB3

Based on high metal tolerant ability, strain SFB3 was used to assess the cellular distortions employing Scanning Electron microscopy (SEM) by growing bacterial culture under varying concentration (200 µg/ml) of Pb, Ni, Cu, Cd and Cr. Following incubation, the bacterial cells were centrifuged at 10,000 x g for 10 min., cell pellet was re-suspended in PBS and was chemically fixed for a period of 24h at room temperature using a final concentration of 2.5% glutaraldehyde. The samples were then rinsed in PBS thrice to remove traces of glutaraldehyde, and later the samples were dehydrated in grades series of ethanol (30%, 50%, 70%, 90% and 100%) and observed under a JSM 6510 LV scanning electron microscope (JEOL, Japan).

Characterization of Rhizobacteria for PGP Traits

Production of Indole Acetic Acid, Ammonia and Siderophore

Indole acetic acid (IAA) production was detected as described by Brick., *et al.* [18]. *Azotobacter, Pseudomonas, Bacillus* and *Rhizobium* cultures were grown separately on their respective media with 200 µg/ml of L-tryptophan at 30°C for 48h. Fully grown cultures were centrifuged at 8000 rpm for 10 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (concentrated H_2SO_4:150 ml, 0.5M $FeCl_3 \cdot 6H_2O$:7.5 ml, distilled water: 250 ml). Development of pink colour indicates IAA production. Bacterial isolates were also tested for ammonia production in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48 - 72h at 37 ± 2°C.

Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production [19]. Siderophore production was detected by the method of Atkin., *et al.* [20] using 2% $FeCl_3$ solution added to culture supernatant. Formation of red colour was indicative for siderophore production.

Phosphate Solubilization Activity

All isolates were first screened on Pikovskaya's agar plates for solubilization of insoluble phosphate. Bacterial cultures were inoculated on the centre of agar plates under aseptic condition. Inoculated plates were incubated for 4 days at 30°C and clear zone (halo) around the colony was recorded. The solubilization index was determined by measuring the halo (clear zone) diameter and the colony diameter were determined according to Premono., *et al.* [21]. The Solubilisation Efficiency (SE) was determined by Nguyen., *et al.* [22]. The quantitative estimation of phosphate produced in supernatant was done by chloromolybdate method [23].

Catalase and HCN production

Bacterial cultures were grown in nutrient agar medium for 18 - 24h. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of O2. Hydrogen cyanide (HCN) production from glycine was tested by growing the bacteria in Kings B medium supplemented with glycine (4.4 g l^{-1}) and cyanogenesis was revealed using picric acid and Na_2CO_3 (0.5 and 2%, respectively) using the method of Bakker and Schipper [24]. Impregnated filter paper was fixed to the underside of the Petridish lids. Results were recorded after five days of culture growth at 28°C. A change in filter paper colour from yellow to orange-brown indicated production of HCN the degree of reaction was rated as: Yellow (1) - limited cyanide production, orange (2) - moderate cyanide production, light brown (3) - relatively high cyanide production and brown (4) - high cyanide production. Based on efficient metal tolerant ability and PGP potentials, *P. putida* strain SFB3 was assayed further for various PGP activities in the presence of the selected metal salts. The concentration used throughout the *in vitro* studies to assess the effect of metals on plant growth promoting activities of *P. putida* SFB3 were 50, 100 and 200 µg/ml for Cd, Cr and Ni while the dose for Pb and Cu were 200, 400 and 800 µg/ml, respectively. The metal tolerant and efficient PGPR strain SFB3 was further used as inoculant to assay its impact on seedling growth of mungbean grown under metal stress.

Plant Based Experiments

Mungbean germination

Seeds of mungbean were surface sterilized by 3% (w/v) sodium hypochlorite for 3 min. to avoid the fungal contamination, followed by three times washing with distilled water [25]. Sterilized Petri dishes each containing soft agar amended with 200 µg/ml each of Cd, Cr, Pb, Ni and Cu. The seeds were then allowed to germinate at room temperature and growth parameters were measured 4 days after sowing.

Uninoculated and untreated seeds but soaked in water only served as control. Seeds were bacterized by soaking seeds in broth containing 10^8 cells/ml of overnight grown culture of *P. putida* strain SFB3 for 1h.

Finally, the inoculated seeds were placed on petri plates containing appropriate concentration of metals. A- 1-mm radical emergence from seeds was considered as positive seed germination. The total germination percentage was calculated according to Mathivanan., *et al.* [26]. Seedling vigour index was determined according to Abdul-Baki., *et al.* [27].

Results and Discussion

In the present study, the rhizobacterial strains were identified based on morphological, biochemical and molecular characteristics and were tested for their beneficial traits like ability to produce IAA, NH3 and other plant growth promoting substances. Efficient rhizobacterial strain selected based on the above characters were examined for their impact on germination attributes of greengram.

Isolation, Characterization and Identification of Rhizobacteria

Based on cultural, morphological and biochemical characteristics (Table 1), out of total 113 bacteria. *Bacillus* (28), *Pseudomonas* (34), *Azotobacter* (24) and *Rhizobium* (27) were isolated and identified from domestic sewage irrigated rhizospheric soils. Out of 113 isolates, 42 isolates were selected owing to their metal tolerance ability and were screened for the plant growth promoting activities. Most of the isolates were Gram negative except belonging to genus *Bacillus*. Similarly, the rhizospheric bacteria showing multiple plant growth promoting activities and metal tolerance ability were studied by Paredes-Páliz., *et al.* [28] who reported

that the Gram-positive genera to be the most dominant population in contrast to this study where Gram-negative bacteria were the dominating genera. Based on 16S rRNA gene sequence analysis, the isolate SFB3 was identified as *Pseudomonas putida* and the base sequence were deposited in the GenBank sequence database with accession no. MF284668. A phylogenetic tree was constructed based on the aligned sequences retrieved from NCBI of the different bacterial genera using the neighbour-joining algorithm in MEGA7 software (Figure 1).

Table 1: Morphological and Biochemical based on Identification of the Bacteria Isolated from Bajra Rhizosphere Grown in Metal Contaminated Soil.

Characteristics				
Morphological				
– Gram reaction	Gram negative	Gram negative	Gram negative	Gram positive
– Cell shape	Short rods	Short rods	Short rods	Rods
– Pigments	Brown	Fluorescent	Translucent	White
Biochemical				
– Indole	-	+	+	-
– Methyl Red	-	+	+	+
– Voges Proskauer	+	-	+	+
– Simmon Citrate Utilisation	+	+	+	-
– Nitrate reduction	+	+	-	+
– Catalase	+	+	+	+
– Oxidase	+	+	-	+
– Starch hydrolysis	+	+	-	+
– Gelatin hydrolysis	-	-	+	-
– Lipid hydrolysis	+	-	+	+
– Carbohydrate Utilisation				
– Sucrose	+	+	+	+
– Glucose	+	+	+	+
– Mannitol	+	-	+	+
– Fructose	+	+		+
– Lactose	-	-	-	+
– Presumptive identification	*Azotobacter* spp	*Pseudomonas* spp.	*Rhizobium* spp	*Bacillus* spp

'+' and '-' Indicate Positive and Negative Reaction Respectively.

Figure 1: The Evolutionary History of Strain P. Putida SFB3 was Inferred using the Neighbour-Joining Method. The Bootstrap Consensus Tree Inferred from 1000 Replicates is taken to Represent the Evolutionary History of the Taxa Analysed. The Evolutionary Distances were Computed using the Maximum Composite Likelihood method and are in the Units of the Number of Base Substitutions Per Site.

Heavy Metal Tolerance of bacterial isolates associated with rhizosphere of *P. glaucum*

A total of 42 bacterial isolates were also checked for their tolerance ability against Cd, Cr, Cu, Pb, Ni and Zn and data on few selected isolates is presented in Table 2. In this study, *Pseudomonas, Azotobacter* and *Rhizobium* showed maximum tolerance to multiple heavy metals while *Bacillus spp*. was found tolerant to a lesser extent (Table 2 and 3). Among all bacterial genera, *Pseudomonas* demonstrated highest tolerance to all the metals tested except for Ni for which the maximum tolerance was displayed by *Rhizobium* spp.

The metal-microbe interaction in natural environment is influenced by pH and organic matter content [29]. In the present study, selected strains showed different heavy metal tolerance with the highest tolerance observed for Cd (400 µg/ml), Cr (400 µg/ml), Cu (1000 µg/ml), Pb (1200 µg/ml), Ni (1000 µg/ml) and Zn (800 µg/ml). Among all bacterial isolates, strain *P. putida* SFB3 survived under all metals stress significantly and tolerated maximally Cd at 400, Cr at 400, Cu at 1000, Pb at 1200, Ni at 800 and Zn at 800 µg/ml, respectively. This high level of tolerance among microorganisms owes to specific genetic mechanisms that impart resistance to heavy metals and may exhibit tolerance by immobilizing metal on cell surfaces or transforming them into less toxic forms [30].

Table 2: Metal Tolerable Concentration of Bacterial Isolates.
Values indicate mean of three independent replicates.

Bacteria	Strain	Metals					
		Cd	Cr	Cu	Ni	Pb	Zn
Pseudomonas	SFB1	100	200	800	200	400	400
Pseudomonas	SFB3	400	400	1000	800	1200	800
Pseudomonas	SFB2	100	200	1000	200	1200	400
Pseudomonas	SFB4	200	100	1000	400	1000	800
	Mean	200	225	950	400	950	600
Azotobacter	SFA3	200	100	800	200	1000	400
Azotobacter	SFA5	100	200	800	200	800	400
Azotobacter	SFA7	200	100	1000	400	800	400
	Mean	166.7	133.3	866.7	266.7	866.7	400
Rhizobium	RZ1	200	200	1000	1000	1000	800
Rhizobium	RZ3	100	100	800	400	1200	400
Rhizobium	RZ5	100	100	800	400	1000	400
	Mean	133.3	133.3	866.7	600	1066.6	533.3
Bacillus	BC7	100	100	800	200	800	400
Bacillus	BC5	100	100	800	400	800	800
	Mean	100	100	800	300	800	600

Table 3: Plant Growth Promoting activities of the Isolated Strains.

Organism	Strain	IAA*	Phosphate solubilization		Catalase	HCN	Ammonia	Siderophore
			Zone size (mm)	Liquid media (µg/ml)				
Pseudomonas	SFB1	53	21	117	+	+	+++	++
Pseudomonas	SFB3	70	29	181	+	++++	++++	+++
Pseudomonas	SFB2	69	15	56	+	++	+++	++
Pseudomonas	SFB4	55	23	108	+	+	++	++
Azotobacter	SFA3	78	24	118	+	+	+++	+
Azotobacter	SFA5	38	20	69	+	-	+++	+
Azotobacter	SFA7	52	-	-	+	-	+++	+
Rhizobium	RZ1	65	-	-	+	+	++	-
Rhizobium	RZ3	48	27	110	+	-	+++	+
Rhizobium	RZ5	53	16	78	+	-	++	-
Bacillus	BC7	32	14	59	+	-	+++	+
Bacillus	BC5	34	-	-	+	-	+	+

'+' and '-' indicate positive and negative reaction respectively. Values are mean of three independent replicates.
*Tryptophan concentration used was 200 µg/ml.

SEM studies of *P. putida* SFB3 under metal stress

The scanning electron micrograph of *P. putida* SFB3 grown under metal stress demonstrated distinct changes in cell size and surface features (Figure 2). The SEM image shows shrinkage of cells when grown under Pb stress. This alteration in surface features may be due to uptake of metal by strain SFB3. Also, the cells displayed irregular shapes as well as cell aggregation in the presence of Ni and Cu while cells were elongated under Cr stress. These results are in collaboration with earlier reports which have shown a clear cellular deformation while growing under different metal stress [31,32].

Figure 2: SEM Micrographs of P. putida SFB3 (A) Control and Treated with (B) Cr(VI), (C) Cd (D) Cu (E) Ni and (F) Pb.

Plant Growth Promoting Characteristics of Test Isolates

The PGP traits of *Bacillus, Pseudomonas, Azotobacter* and *Rhizobium* rhizobacteria are presented in Table 3. The order of IAA produced by rhizobacteria was: *Pseudomonas>Azotobacter>Rhizobium>Bacillus*. It is well documented that IAA is the most important auxin (phytohormone) produced by plants and many soil bacteria and plays an important role in variety of physiological activities, including embryo development, root initiation and development, apical dominance, leaf formation and fruit development. IAA is derived mainly from tryptophan through multiple enzymatic pathways by many different genera of PGPR like *Rhizobium, Bacillus, Pseudomonas, Azotobacter,* Enterobacter, *BradyRhizobium, Xanthomonas* and *Alcaligenes* [33,34]. However, the selected rhizobacterial isolates showed variable P solubilisation activity.

The most significant P solubilisation activity was exhibited by *Pseudomonas* followed by *Rhizobium* and *Azotobacter* while only one *Bacillus* sp. showed P solubilisation. All rhizobacterial isolates showed positive catalase reaction. Catalase activity in the bacterial strains may be very advantageous and bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. HCN production was detected higher in *Pseudomonas* spp. as compared to the *Bacillus* spp., *Azotobacter* spp. and *Rhizobium* spp. isolates. Higher HCN production by *Pseudomonas fluorescens* and *P. aeruginosa* has also been reported by other researchers [35,36]. Ammonia production was detected in *Bacillus, Pseudomonas, Azotobacter* and *Rhizobium* which is an important attribute of PGPR that influences plant growth indirectly.

This accumulation of ammonia in soil may increase in pH creating alkaline condition of soil at pH 9-9.5. It suppresses the growth of certain fungi and nitrobacteria due to it potent inhibition effect besides inhibiting germination of spores of many fungi [37]. Also, the siderophore production was significantly higher in *Pseudomonas* spp. and *Azotobacter* but the activity was not profound in *Bacillus* and *Rhizobium*. The activity has the growth promoting attribute as siderophores are low molecular- weight molecules that are secreted by many microorganisms and act as solubilising agent for Fe from minerals under Fe deficient condition.

In addition, siderophores form stable complexes with heavy metals and increases the soluble metal concentration [38]. Thus, it helps to alleviate the stresses imposed on plants by heavy metals in soil. Based on the PGP activities tested, the isolate *P. putida* SFB3 showed potential PGP activities as compared to other isolates.

Plant growth promoting activity of *P. putida* strain SFB3 under metal stress

The toxicity of different metals on metabolic activities has been reported [39]. In this study *P. putida* strain SFB3 was found to possess efficient PGP potential besides tolerating multiple metals significantly. Hence, the effect of metals on PGP activities of metal tolerant *P. putida* strain SFB3 was studied. IAA production was significantly reduced at different metal concentrations and decreased in the order: Cd > Cr > Pb > Cu > Ni. *Pseudomonas* sp. SFB3 was most efficient P solubilizer expressing SE value of 368.9 after four days of growth. In general, the SE of *Pseudomonas* sp. SFB3 ranged from 174.3 (Ni stress) to 368.9 (without metal). The

SI was found maximum (4.7) in metal free condition whereas Ni showed highest toxicity on SI (2.7). However, copper among metals showed least toxic impact on SI (4.7). It was clear from the data that with increasing concentration of metals, the colony size of bacterium decreased which concurrently adversely affected the SE and SI of *Pseudomonas* sp. SFB3 when grown under metal stress. Similarly, the P solubilisation was reduced by more than 50% in the presence of Ni, Cd, Cr and Pb.

The maximum toxicity to P solubilisation was observed in case of Ni at 200 µg/ml, followed by Cr (200 µg/ml), Cd (200 µg/ml), Pb (800 µg/ml) and Cu (800 µg/ml). The HCN and catalase production was severely affected by Cd, Cr and Ni but the activity was shown even at higher concentrations of Cu and Pb. In contrast, the strain showed significant production of NH3 and siderophore even in the presence of different metals. These results are in close agreement with the findings of Oves., *et al.* [40] where Cr at higher concentrations didn't have significant impact on HCN and NH3 production. Similar results of reduction in various PGP activities due to metal toxicity were observed in *Pseudomonas* sp [39] and *Rhizobium* sp [40].

Growth enhancement in Vigna radiata seedlings

The major effects of heavy metals on seeds are revealed by overall abnormalities and decrease in germination, reduced root and shoot elongation, dry weight, total soluble protein level, oxidative damage, membrane alteration, altered sugar and protein metabolisms, nutrient loss resulting in productivity loss [41]. Considering these, the impact of different on inoculated and uninoculated greengram was determined in plate assay. Mungbean seeds inoculated with metal tolerant PGPR had significantly enhanced germination and seedling vigour. *Pseudomonas putida* SFB3 increased seed germination over metal treated seedlings (Table 4). The highest enhancement of seedling vigor indexes was obtained from bacterial treatment with *P. putida* strain SFB3, which recorded 1610 vigor index over uninoculated control. Based on the results of *in vitro* plant growth promotion and stress tolerance the best selected strain was evaluated on early establishment of mungbean seedlings. Analytical results of germination percentage, Plumula and radicle length of 7 days mungbean seedlings treated with metal tolerant PGPR *P. putida* SFB3 displayed significantly higher values over control treatment (Table 5). It was observed that shoot length was significantly decreased by 89.5, 84, 63, 77 and 39% in Cd, Cr, Cu, Ni and Pb, respectively as compared to untreated control. Similarly, significant reduction was observed in root length by 59, 60, 34, 58 and 42% at 200 µg/ml of Cd, Cr, Cu, Ni and Pb, respectively in comparison to control. The non-significant reduction was recorded at 200 µg/ml of Cu application in almost all the parameters observed.

However, significant improvement was recorded in Plumula length as the reduction observed was comparatively lesser in Cd (43%), Cr (23%), Cu (14%), Ni (18%) and Pb (14%) as compared to treated plants. On the other hand, radicle length was increased by 50, 66.6, 39, 74 and 28 % in case of Cd, Cr, Cu, Ni and Pb, respectively as compared to metal treated seedlings. It can be concluded that to survive under metal stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal outside the cell, accumulation and complexation of metal ions outside the cell and reduction of heavy metal ions to less toxic state [42]. The seedling vigour index calculated for different metal treatments were highly influenced by the toxic impact of metals and were considerably reduced as compared to control. However, bacterial treatment significantly enhanced seedling vigour index even under metal stress by several folds which might be attributed to the plant growth promoting activities of *P. putida* SFB3 observed even under different concentrations of metals.

Table 4: Effect of different metals on Plant Growth Promoting activities of *P. putida* SFB3 isolated from metal polluted soil.

Bacterial strain	Treatment	Plant growth promoting activities								
		IAA production	Phosphate solubilisation on/in				NH_3	HCN	Catalase	Siderophores production
		200 T[f]	Solid Pikovskaya medium	Liquid Pikovskaya medium (µg/ml)	SI	SE				
			Zone of solubilisation (mm)							
SFB3	Control	64	27.3	181	4.7	368.9	++++	++++	+	++++
Cd	50	31	19	127	4.5	345.5	+++	-	+	+++
	100	23.6	10.8	87.5	3.8	276.9	++	-	-	++
	200	11.8	10.5	68.2	3.6	262.5	+	-	-	+
% reduction (control vs 3X)		81.6	61.5	62.3	23.4	28.8				
Cr	50	35.7	20.1	137	4.1	314.1	++	-	-	+++
	100	20.8	14.3	94.5	3.5	246.6	++	-	-	+
	200	12.2	9	37.2	3.3	225.0	+	-	-	-
% reduction (control vs 3X)		65.8	67.0	40.3	29.7	39.0				
Cu	200	54.5	25.3	140	4.7	366.7	+++	++	+	+++
	400	41.9	19.5	133	4.3	325.0	++	++	+	+++
	800	30.0	15.5	90	4.4	344.4	+	+	+	+
% reduction (control vs 3X)		45.0	43.2	35.7	6.3	6.6				
Ni	50	27.4	21.5	106	4.4	335.9	+++	-	+	+++
	100	26.3	12.7	90	2.7	174.3	+++	-	-	++
	200	24.3	ND	-	-	-	+	-	-	+
% reduction (control vs 3X)		11.3	ND	-	-	-				
Pb	200	33.4	21.6	124	4.6	360.0	++	++	+	+++
	400	21.4	17.5	78.6	4.6	357.1	+	++	+	++
	800	10.3	10.6	67.4	3.5	252.4	+	+	-	++
% reduction (control vs 3X)		69.2	61.2	45.6	25.5	31.5				

'+' & '-' indicates degree of positive and negative reaction respectively. Values represent mean of three independent replicates.
ND: Not Detected.

Table 5: Effect of Heavy Metals and PGP Activity of Strain SFB3 on Germination Attributes of Mungbean after 4 Days of Incubation. Values indicate mean of three independent replicates. Metals concentration used: 200 µg/ml.

Treatment	Germination percentage	Mean Plumula length(cm)	Mean Radicle length(cm)	Seedling Vigor Index
Uninoculated Control	100	5.7	7.4	1310
Inoculated Control	100	6.4	9.7	1610
Cd	64	0.6	2.8	217.6
Cd+SFB3	78	3.4	4.2	592.8
Cr	75	0.9	3.0	292.5
Cr+SFB3	89	4.4	5.0	836.6
Cu	100	2.1	4.9	679
Cu+SFB3	100	4.9	6.8	1146.6
Ni	88	1.3	3.1	387.2
Ni+SFB3	94	4.7	5.4	949.4
Pb	78	3.5	4.3	608.4
Pb+SFB3	85	4.9	5.5	968.2

Conclusion

Microbial diversity and metal tolerance and plant growth promoting potential of *P. putida* SFB3 varied considerably. *Pseudomonas putida* strain SFB3 significantly enhanced the biological characteristics of mungbean even under metal stress establishing the potential of metal tolerant *P. putida* strain SFB3. Based on these, *P. putida* SFB3 could be developed as a bioinoculant for application in fields to enhance the production of compatible crops in metals contaminated soil.

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Conflict of Interest

None declared.

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