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Effect on PGPR (Plant Growth Promoting Rhizobacteria) in phytoremediation of heavy metal contaminated soil using *Vigna radiate*.L

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Abstract
Plant growth promoting rhizobacteria (PGPR) may help reducing the toxicity of heavy metals to plants in polluted soils. In this work the effects of inoculating metal resistant and plant growth promoting bacterial strains on the growth *Vigna radiata* L. grown in cadmium contaminated soils were assessed. The PGPR strains *Bacillus subtilis* and *Pseudomonas fluorescens* isolated from rhizosphere soil was used for bioremediation. These isolates were characterized for their ability of biochemical tests. *Bacillus subtilis* and *Pseudomonas fluorescens* were inoculated in *Vigna radiata* L. plants by pots inoculation method and analysed for the growth promotion efficacy in heavy metal polluted soil. The plant inoculated with isolates showed Plant growth parameters and biomass, as compared to non-inoculated control. Further analyses of leaves were carried out for the photosynthetic pigment analysis, phytochemical analysis. Heavy metal analysis of plants and soil in *Vigna radiata* L. Bacterial strains diversity decreased with increasing levels of metal contamination in the soil, but in rhizosphere soil of plants inoculated with the PGPR strains, a higher bacterial diversity was kept throughout the experimental period. Inoculation of *Vigna radiata* L. particularly *Bacillus subtilis* with *Pseudomonas fluorescens*, appears to be an effective way enhancing the short term rhizoremediation potential of the plant in metal contaminated, lowering losses in plant biomass and degreasing above ground tissue contamination. The study concluded that heavy metal mobilizing PGPR could be used as effective inoculants for improving the phytoremediation in heavy metal contaminated soil as well as reclamation of heavy metal polluted soil.

Keywords: phytoremediation, contamination, bioinoculants, environment, plant growth promoting rhizobacteria, heavy metals

Introduction

Soil is a complex ecosystem where different micro-organisms play important roles in maintaining the soil fertility and plant productivity through the interactions with both biological and physic-chemical components (Kosev and Vasileva,2014) [10].PGPR bacteria can influence plant growth and development by producing and supplying the plant with various types of organic compounds- hormones(Sivasankari *et al.*, 2014) [22]. PGPR nitrogen fixing bacteria have other plant –beneficial properties such as the remediation of soil contaminated with trace elements by increasing plant tolerance to abiotic stresses and limited availability (Parnell *et al*,2016) [19]. Biotechnological approaches that include biostimulation of native microbial populations, bioaccumulation, biosorption, rhizoremediation (plant and microbe interaction), and phytoremediation may offer highly specific alternatives that can be used on a large scale (Sharma, 2012). Phytoremediation is a cheap, energy-efficient, and safer strategy (relative to conventional methods) for accumulating heavy metals from the soil for improved quality of the environment and human health (Khan, 2010).

The microbiological preparation including those of PGPR often called bio fertilizers “a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003) [24]” when applied to seeds and/or soils, have been found to affect the growth of horticultural crops (Esitken, 2011) [6].

The application of bio fertilizers into soil has been shown to improve the production of antibiotics and the biodegradation of soil organic matter, increase nutrient supply, enhance plant tolerance to environmental stress, therefore, biofertilizer has been adopted as a clean and efficient soil conditioner or amendment to improve the quality of soil by agriculturists and plant biologists (Shen *et al.*, 2013) [21]. Biofertilizer are the plant growth promoting microorganisms which by several mechanisms augment plant growth.These potential biological fertilizers would play a key role in productivity and sustainability of soil and also in protecting the environment as eco-friendly and cost effective inputs for the farmers.

Aim of this study is the isolation of rhizobacteria from rhizosphere soil and pot experiment inoculation of these isolates in *Vigna radiata* L. plants to estimate their plant growth promoting potential. The inoculated plants were also analyzed for accumulation of metals. The regulatory mechanisms of PGPR co-inoculation in counteracting metal toxicity and provide an efficiency strategy for the phytoremediation of metal –contaminated soil.

Materials and Methods

Soil collection, Preparation and characterization

Soil collected from contaminated site of mining area of Salem District, Tamil Nadu. Rhizosphere soil collected from Nallampalli, Dharmapuri, Tamil Nadu. Soil collected from 20cm Depth and passed through 2mm sieve to remove dusts and stones. Soil samples were kept aseptically in sterile plastic bags for further analysis. The collected soil (test and control) analysed for physicochemical parameters and heavy metal analysis. These parameters and heavy metals were analysed by (using the standard procedures) Omega laboratories, Nammakkal, Tamil Nadu.

Isolation and identification of rhizosphere bacteria
Agricultural land soils samples collected from Nallampalli, Dharmapuri district, Tamil Nadu. Soil samples were placed in plastic bags and stored at 4 °C. Soil samples (10g) were taken into 250ml conical flask, to that 90ml of distilled water was added and kept in a rotary shaker for 15 min.1ml of soil suspension was serially diluted up to 10^{-8} dillutions.0.1 ml of sample was spread on nutrient agar plates and incubated at 37 °C for 24 hours. Experiment was carried out thrice to get a pure culture.

Biochemical Characterization of Bacterial isolates
The isolated bacterial strains were characterized by the morphological, cultural, staining and biochemical properties. The biochemical characterization (Methyl red, VP test, Citrate utilization, Indole production, Catalase, Oxidase, Urease) of the isolates were performed by standard microbiological techniques.

Gram staining (Vincent 1970)
A thin smear of test culture was prepared on the clean slide and heat fixed. Few drops of crystal violet were poured on the smear for about 1 min. washed the slide with running tap water. Flooded the smear with Gram's iodine and kept for 2 min. decolorized the stain with Ethyl alcohol (95%) drop wise. Poured few drops of safranine for 2-4 min. washed the slide with tap and mounted in glycerine or oil emulsion and examined under microscope.

Indole test (Cappuccino and Sherman, 2002)
Two ml of peptone water was inoculated with 5 ml of bacteria culture and incubated for 48 hours. 0.5 ml of Kovac's reagent was added; shacked well and examined after 1 minute. A red colour in the reagent layer indicated indole. In negative case there is no development of red colour.

Methyl red and Voges –Proskaur test (Cappuccino and Sherman, 2002)
This test is used to determine two things. The MR portion is used to determine conversion of glucose to acid products like lactate, acetate and formate. The VP portion is used to determine conversion of glucose to acetone. Two set of glucose peptone broth was inoculated with the isolates and incubated at 30C for 72 hours. One set was used for methyl red test, in which development of colour was checked after adding 5 drops of methyl red indicator. Second set was used for the Voges Proskauer test, in which development of colour was checked after adding 0.6ml of 2 –napthol and 0.2 ml of KOH solution in 1ml culture broth.

Citrate utilization test (Cappuccino and Sherman, 2002)
This was carried out by inoculating the test organism in test tube containing Simmon's citrate medium and this was inoculated for 24 hours to 72 hours. The development of deep blue colour after incubation indicates a positive result.

Extra cellular enzyme activity of bacterial isolates
Catalase activity (Ajay Kumar, *et al*., 2012) [1]
48hrs old test bacterial cultures were placed on a clean glass slide and 3% of H_2O_2 was dropped and mixed with tooth pick. Observation of bubble formation indicates the positive test for catalase.

Oxidase production
The isolates were streaked on year extract mannitol agar plates and incubated for 3 days at 28^0 C. after incubation a loopful of isolates was placed over oxidase disc (N, N-Tertra methyl –para-phenylediaminedihydrochloride). Development of blue or purple coloration was positive to oxidase production.

Urease activity (Mac Faddin, 2000) [12].
The isolates were streaked on Christensen's urea agar slants and incubated for 3 days at 28 ^{0}C.Observer the slant for a colour change at 6 hours, 24 hours, and every day for up to 6 days. Urease production is indicated by a bright pink colour on the slant that may extend into the butt.

Quantitative of Phosphate solubilization
The bacterial isolates were screened for inorganic phosphate solubilisation according to (Chen, 2006) [5]. Pikovsakaya's agar medium amended with inorganic phosphate was prepared and loopful of fresh bacterial culture was streaked on to the plates. Plates were incubated at 28± 2^0C for 3-4 days. Solubilisation of mineral phosphate was observed by a clear halo around the bacterial colony.

Quantitative of Siderophore production
Siderophore production was checked on thesolid CAS (Chromazurol S) universal blue agar plates (Schwyn and Neilands 1987) [20]. Actively growing cultures were spot inoculated on the C blue agar plate and incubated at 30^0 C for48h.Formation of yellow-orange halo around the colony indicated production and release of the siderophores on the agar plates.

Molecular identification of bacterial isolates
Isolates were identified at species level for this the pure cultures of potential isolates were sent to PAR Life sciences, Trichy, Tamil Nadu, for identification of bacteria at molecular level by sequencing of 16S rRNA gene using Forward and Revers primer. After obtaining the sequencing that were subjected to the BLASTn search program to look for nucleotide sequencing homology (Altchul *et al.*, 1997) [2] with NCBI, *Bacillus cereus* and were submittedGenBank under Accession numberMH 128361**.**.*Pseudomonas aeruginosa* strain Accession number MH128359.

Antibiotic sensitivity test
The susceptibility to antimicrobial agents was tested with antibiotic disks by the method of Bauer *et al.*, (1966). The commercial different antibiotic disks used were Streptomycin, Chloromphenicol, Kanamycin. Autoclaved Mueller- Hinton medium at 15 lbs (121^0c) for 15 min dipped a sterilized swab into the 24hours old culture broth and expressed any excess moisture by pressing the swab against the side of the tube. Swabbed the surface of the agar completely allowed the surface to dry for about 5 minutes before placing antibiotic disks on the agar with the help of sterilized forceps.
Then it was incubated at 300c for 24 hours or until bacterial growth was observed. at the end of incubation, the diameter (mm) of each zone (including the diameter of the disk) was measured and recorded.

Heavy metal tolerance

The selected bacterial isolates were tested for their resistance to heavy metals by agar dilution method. Freshly prepared agar plates were amended with different various soluble heavy metal like Cadmium at various concentration ranging from 25- 200 µg/ml-1 were inoculated with overnight grown cultures. Heavy metal tolerance was observed by the appearance of the bacterial growth on the plates after the incubation at room temperature for 24 to 48 hours (Yogendra *et al,* 2013).

Tolerance to salt and p^H by isolated bacteria

Bacterial isolates were separately inoculated in the nutrient agar medium with different concentration of NaCl such as 0.5%,5%,10%,15%,20%,25%,30%.was added to the medium for maintaining salt concentration and incubated for 48 hours at 28 ± 2^0C.It was also studied that each of the bacterial isolates inoculated into the plat count agar medium of different (2,4,6,8,10,12),which was adjusted by using 1N NaOH and 4N HCl. After incubation, the growth of the isolates in different pH medium was recorded.

Mass production of bacterial isolates

Bacillus subtilis

Nutrient broth is prepared and sterilized at 1.1kg/CM2 pressure for 20 minutes. One loopful of *Bacillus subtilis* is inoculated for 24 hours. This serves as mother culture.100ml of mother culture is transferred to 1 litres of sterilized nutrient broth in a fermenter bacterial growth is harvested after 72 hours. Then it is mixed with 2kg of sterilized peat soil amended with 250g calcium carbonate, dried in shade and packed in polythene bags.

Pseudomonas fluorescens

Pseudomonas fluorescens multiplied in sterilized King's broth for 48 hours. The pH of the substrate (talc) is adjusted to 7 by adding calcium carbonate 150g/kg. The substrate is then sterilized at 1.1kg/cm2 pressure for 30 minutes for two days.400ml of *Pseudomonas fluorescens* suspension is added it 1kg of substrate containing 5g of carboxymethyl cellulose and mixed well. The formation is packed in polythene covers and can be stored for one month.

Pot experiments

The collected soil was packed into plastic pots, and the moisture content was maintained at -70%of the maximum water holding capacity. Green gram *(Vigna radiata.L)* cultivar (vembon) seeds (TNAU) were sterilized in 20% Sodium hypo chloride for 10min, washed three times with tap water, and rinsed with deionised water. Each container was inoculated with 2ml of bacterial biofertilzer (*Pseudomonas fluorescens* PF01, *Bacillus subtilis*-BS01). The control was uninoculated bacterial suspension. Each treatment had three replicates. The plants were incubated in a greenhouse under natural light conditions. Up to 1ml of bacterial suspension were inoculated into each cup every other week, along with 1ml of distilled water. After 45 days, plat biomass (fresh and dry weight, shoot and root length and chlorophyll contents were determined.

Measurement of Growth parameters

Plants from each treatment after 45days of seed sowing, were collected carefully with plant shoot, root length were measured.

Estimation of Fresh weight and Dry weight

The fresh and dry weight were recorded on 45 DAI and expressed in gram per plant (g/plant).The fresh weight of the plants was determined by weighing the individual plants immediately after harvesting. The dry weight was estimated after drying the plants at 65 °C in an oven for 12 hours.

Photosynthetic pigment assay

Photosynthetic pigment assay of plant samples were carryout by the modified method of Arnon (1949) [3]. Fresh leaves were collected from the green house and washed with tap water to remove the soil particles. Leaf samples (100mg) crushed with mortar and pestle (ice cold condition) with 1ml of 80% acetone (v/v).Homogenized leaves samples centrifuged at 5000rpm for 10 minutes. After centrifugation, two or three times reducing in plant pigment colour, the supernatant tested for the chlorophyll a/b and carotenoids contents. The absorbance of extract-measured at 470, 645, and 663nm in the UV-visible spectrophotometer. The contents of chlorophyll a, chlorophyll b and carotenoid estimated according to the standard formula.

Chlorophyll (a) =12.7(ab663)-2.69(ab645) × v/1000×w

Chlorophyll (b) =22.9(ab645)-4.68(ab663) ×v/1000×w

Total chlorophyll (a) and (b) =20.2(ab645) + 8.02(ab663) ×v/1000×w

Carotenoid = (ab480)-chlorophyll (a)-(b)/245×v/1000×w

Phytochemical Composition of Plant Samples

Determination of Flavonoid Content

Plant samples were determined according to the method described by (Harborne 1973) [8]. Weigh accurately 5 g of plant sample in a round bottom flask and then boiled and refluxed in 50 mL of 2 M HCl solution for 35 minutes. The mixture was allowed to cool followed by filtration using what man filter paper. The filtrate was treated with equal volume of ethyl acetate drop wise until precipitation was completed. The precipitate was recovered by filtration using a previously weighed filter paper. The weight of the precipitate was measured and recorded.

Determination of Alkaloids

This was performed by gravimetric method described by (Harborne 1973) [8]. 5g of plant sample was dispersed in 10% acetic acid solution in ethanol (1:10). The mixture was allowed to stand for 4 hours at 28 °C, filtered, washed with 1% ammonia solution and dried in an oven at 80 °C. Alkaloid content was then calculated and expressed as a percentage of the weight of plant sample analyzed.

Determination of Tannin

500 mg of the plant sample was weighed into 100 mL plastic bottle followed by addition of 50 ml of distilled and then shaken for 1 h using a mechanical shaker. The mixture was filtered into a 50 ml volumetric flask and diluted up to the mark. 5 ml of the filtrate was then pipette into a cuvette followed by addition of 3 ml of 0.1 M $FeCl_3$ in 0.1 N HCl And 0.008 M potassium Ferro cyanide. The content of the cuvette was thoroughly mixed and the absorbance was taken at 120 nm wavelength using a spectrophotometer. The blank sample was prepared and measurement made at the same wavelength. Tannin acid was used in the preparation of 100 ppm standard solution and absorbance measured (Okwu 2005) [17].

Determination of Saponins
20 g of plant sample was dispersed in 200 ml of 20% ethanol. The mixture was heated in a hot water bath for 4 h with vigorous stirring at 55 °C followed by filtration. The residue was re-extracted with another 200 ml of 20% ethanol.
The combined extracts were concentrated to 40 ml by evaporation in a water bath at 90 °C. 20 ml of diethyl ether was added to the concentrate in a 250 ml separating funnel with vigorous shaking. The ether layer was discarded while the aqueous layer was recovered followed repetition of the purification process with 60 ml of n-butanol. The combined n-butanol extracts were washed with 10 ml of 5% aqueous NaCl then evaporated in water bath, dried in an oven to a constant weight. The % saponincontent was then calculated (Obadoni 2001) [15].

Determination of Anthocyanin
5 g of each plant sample was boiled in 100ml of 2M HCl solution for 35 minutes followed by filtration of the hydrolysate using whatman No 42 filter paper. Ethyl acetate was poured into a previously washed separation funnel which contains the filtrate. The content of the separating funnel was thoroughly mixed and then allowed to separate into two distinct layers.
The extract (ethyl acetate layer) noted and evaporated to dryness in a crucible over a steam bath. Concentrated amyl alcohol was added to the dried extract in other to release the anthocyanins followed by filtration. The combined filtrate and alcohol extract were transferred to an evaporating dish and evaporated to dryness, dried in the oven at about 30 °C for 30 min and cooled.
Then the % anthocyanin content was calculated and adequately recorded (Onyeka 2007) [18].

Determination of Steroid Content
10 g of plant sample was dissolve in 100 ml distilled water with vigorous stirring and blended by the aid of a laboratory blender. The supernatant solution was filtered using whatman No 42 filter paper and then ammonium hydroxide solution was used to elute the filtrate at pH 9.3ml of chloroform was mixed with 3 ml of the eluate in a previously washed test tube. 5ml of acetic anhydride (ice-cold) were poured into the mixture of chloroform and eluate in a clean round bottom flask and 3 drops of concentrated tetraoxosulphate acid were as well added. Similarly a standard solution of sterol was prepared and treated as described previously.
The absorbance of both prepared sample and the standard solution was measured at 420 nm using a spectrophotometer (Okeke 2003) [16].

Test for phenols (Mayr *et al.*, 1995) [14]
Three drops of methanol extract was taken in a spot Appearance of purple colour showed the presence of phenolic compounds.

Heavy metal accumulation of plant samples
Plant leaf samples were washed thoroughly with deionised water to remove surface dust and soil, dried at 80 °c unit completely dry, weighed. and ground to <0.5 mm size. Plant samples (0.5g) of finely ground tissue were digested with concentrated hno3 and hclo4 in 5:1 ration metal concentration in plants were determined by flame atomic absorption spectrophotometry. Two measurements of heavy metals were performed for each sample.

Heavy Metal Analysis of the Soil Samples
Total concentration of some heavy metals of interest was determined according to the method of Lokeshwari and Chandrappa (2006) [11]. Weigh accurately 2g each of soil samples in a 250 ml glass beaker followed by digestion with 8 mL of aqua-regia on a sand bath for 120 minutes. The sample was evaporated to dryness and then dissolved with 10 mL of 2% nitric acid, filtered and diluted to 50 ml with deionized water.
The filtrate was measured of total concentrations of the heavy metals of interest using atomic absorption spectrophotometer (FS240) and conditions as described above.

Soil microbial population (CFU/g.soil)
Microbial population in the soil samples was determined by the dilution plate's techniques.
The results were expressed as colony forming units per gram soil. Dry weight basis, Nutrient Agar (NA), each culture medium was also used in the liquid form without added for dilution preparation.

Results
Soil Collection, Preparation, and Characterization
The rhizospheric soil collected from Dharmapuri district and contaminated site in Salem, Tamil Nadu. Soil samples were tested in a Omega laboratories, Nammakkal. The physicochemical properties of the soil areas were in (Table.1)

Biochemical Characterization of *Pseudomonas Aeruginosa* and *Bacillus Cereus* Isolate
Among two isolates were selected on the basis biochemical characterization, (Figure.1) in which one *B.subtilis* was Gram positive. Another *P. fluorescens* was Gram negative, and another test was IMVC tests and enzyme activity by *P. fluorescens* was higher compared to that of *B.subtilis* as observed by positive results (Table.2) and the Antibacterial sensitivity by both isolates as observed by positive results (Table.5)
Both the isolates showed phosphate solubilising activity on Pikovaskaya's agar medium. The phosphate solubilising activity by *P. fluorescens* was higher compared to that of *B.subtilis* as 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 activity by *P. fluorescens* was higher compared to that of *B. fluorescens* as observed by a clear zone around the inoculated bacterial isolates. (Table.2) (Figure. 2).
The bacterial isolates were molecular biologically identified by the isolation of total genomic DNA and amplified by 16S rDNA specific primers. PCR amplicons of 16SrDNA of about 1500pb were obtained for both the isolates as discrete bands in agarose gel. (Figure. 3)

Heavy metal tolerance, Tolerance to salt and p^H by bacterial isolates
The bacterial isolates are to be found to be more tolerant at lower concentration of 25µg/ml and less tolerant at

concentration of 200µg/ml. Bacterial isolates showed tolerance to Cd, Cr, at concentration 25-200 µg/ml. Bacterial isolates were tolerant to cadmium even high concentration of 200 µg/ml.(Figure. 4).

Effect of salt concentration on growth of isolates was observed that the growth on nutrient agar plates varied with salt concentration.

The growth of *P. fluorescens* good salt concentration range of 25% except unable to grow 10%.The growth of *B.subtilis* good salt concentration range of 30 %except unable to grow 15%(Table.3).

Effect of p^H on growth of isolates was observed that the growth on nutrient agar plates varied with p^H level. The growth of *P. fluorescens* good p^H range of 10 except unable to grow 2.The growth of *B.subtilis* good p^H range of 12 except unable to grow 4 (Table.4).

In the present study, *Vigna radiata L.* plants inoculated with mass production of *B.cereus*and *P. fluorescens*, showed significantly higher plant height, root length and Fresh weight and dry weight (Figure.5). Plant Inoculated with *B.subtilis* showed root, shoot length and fresh, dry weights as compared to non –inoculated control. While *P.aeruginosa* treated plants showed more root, shoot length and fresh, dry weight as compared to non-inoculated control(.(Figure.6). Plants inoculated with both *B.cereus and P.aeruginosa* showed plant root, shoot length and fresh, dry weight as compared to non-inoculated control (Figure.7).

Inoculation with both the isolates showed highest level in photosynthetic pigment analysis in *Vignaradiata*.L plants (Table.6).

Inoculation of bacterial isolates of *Vignaradiata*.Lplant in phytochemical analysis results ofPhenolsand Flavonoids arepositive. Tannin,Saponin,Anthocyanin and Steroid content are negative in plant samples.Phoenols in increased in Pa01(Table.7).

Bacterial isolates Pa01and Bs01 were inoculation of plants after the analysis of heavy metals soil samples. Inoculation of bacterial isolates decreasing of heavy metals level compared to uninoculated control. PGPR sustainability minimize the limiting factors associated with phytoremediation entailing soil chemistry, intensity of contamination, and metal solubility.(Table.8).

The correlation between incubation days and colony forming units of Pa01 and Bs01.Essential to establish the role of bacterial isolates for bio fertilizers tobe used in large scale level for the benefit of agricultural crop productivity(Table.9)

Table 1: Physicochemical analysis of soil samples

Physicochemical Properties		
Physicochemical Properties	**Contaminated soil**	**Normal soil**
pH	7.9	7.64
(EC)Electrical Conductivity	0.10	0.88
Available N (mg/kg)	56	128
Available P (mg/kg)	8	2.60
Available K (mg/kg)	79	106
Copper(mg/kg)	114.3	128
Lead(mg/kg)	126.8	275
Zinc(mg/kg)	106	310
Cadmium(mg/kg)	12	8.22

Table 2: Biochemical characterization of *P.aeruginosa*spp. and *B.cereus*spp.

S.no	Test	*Pseudomonas aeruginosa*	*Bacillus cereus*
1	Gram staining	-	+
2	IMVic test		
	Indole production	+	-
	Methyl red test	+	-
	Vogas-Proskaur	+	+
	Citrate utilization	+	+
3	Extra-cellular enzymes		
	Catalase activity	+	+
	Oxidase production	+	+
	Urease activity	+	+
4	Phosphate solubilization	+	+
5	Siderosphore production	+	+

(+) – Positive, (-) Negative

Table 3: Effect of various salt concentrations on the growth of bacterial isolates

	Salt concentration						
S.No	**0.5%**	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**
Pa01	+++	+++	++	+	-	-	-
Bc01	+++	+++	++	++	+	-	-

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

Table 4: Effect of Various p H on the growth of bacterial isolates

	p H values					
	2	**4**	**6**	**8**	**10**	**12**
Pa01	-	-	++	+	+	-
Bc01	-	-	++	++	+	-

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

Pa01-*Pseudomonas aeruginosa* Bc01-*Bacillus cereus*

Fig 1: Isolates Pa01 and Bc01

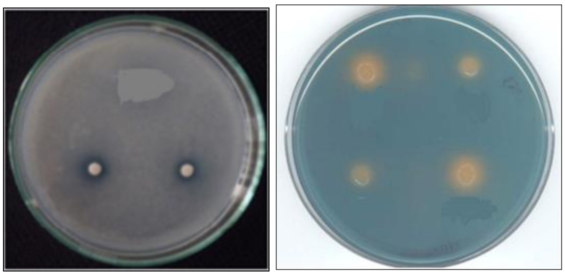
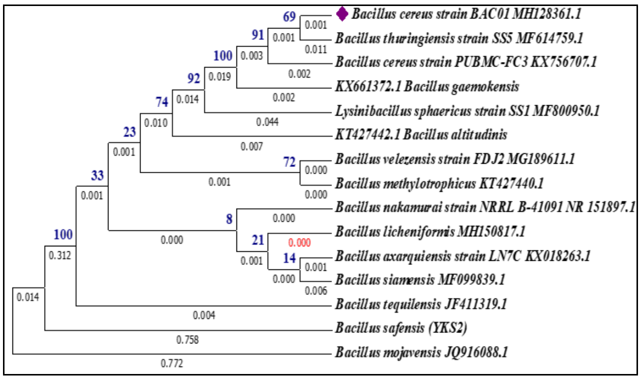
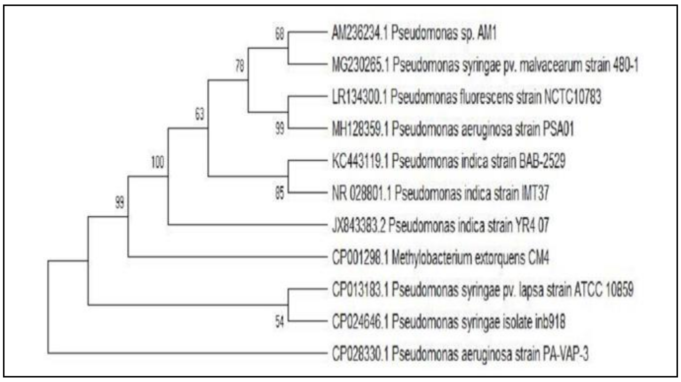


Fig 2: Isolates showing Phosphate solubilizationand Siderophore production

Table 5: Antibiotic Sensitivity test of (Pa01 & Bc01)

S.No.	Commercial antibiotic disc	Inhibition zone diameter (mm)	
		Pa01	**Bc01**
1.	Streptomycin	12mm	17 mm
2.	Chloramphenicol	19 mm	21mm
3.	Kanamycin	25 mm	23mm





B-*B.cereus* **A-*P.aeruginosa***

Fig 3: Phylogenic Tree of Bacterial isolates

Phylogenetic tree of 16S r RNA gene sequences showing the realationships among the isolates isolated from the soils of plant rhizosphere region Salem district, Tamil Nadu. The data of type strains of related species were from GenBank database (the accession numbers are given in parentheses). *Bacillussubtilis* and were submittedGenBank under Accession number*Bacillus cereus*-MK483261.*Pseudomonas aeruginosa* strain Accession number MH128359.

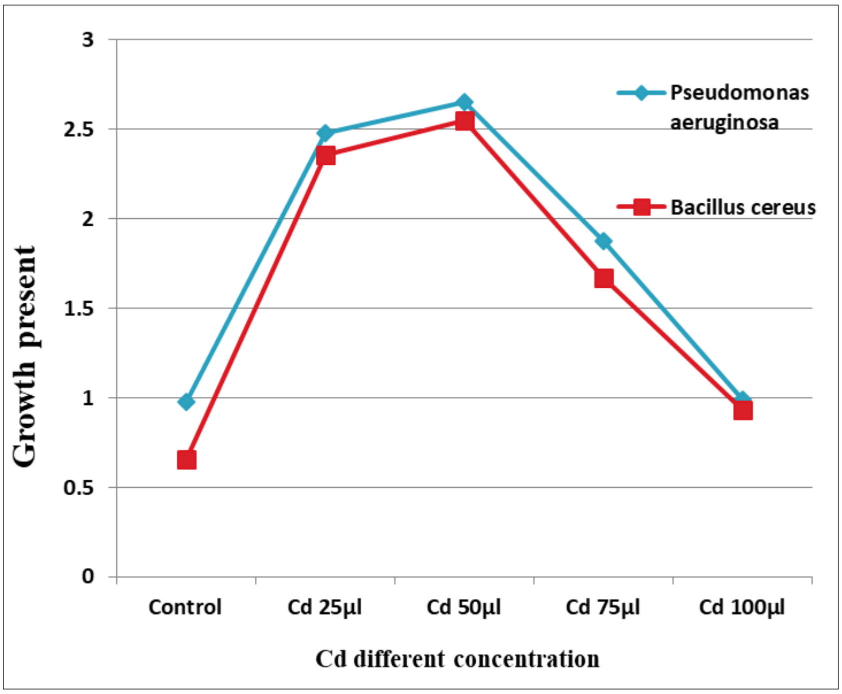


Fig 4: Heavy metal tolerance of bacterial isolates

Fig 5: Mass production of *Bacillus cereus* and *Pseudomonas aeruginosa*

Fig 6: Growth characterization *Vigna radiata* L. at 45 DAI (cm/plant)

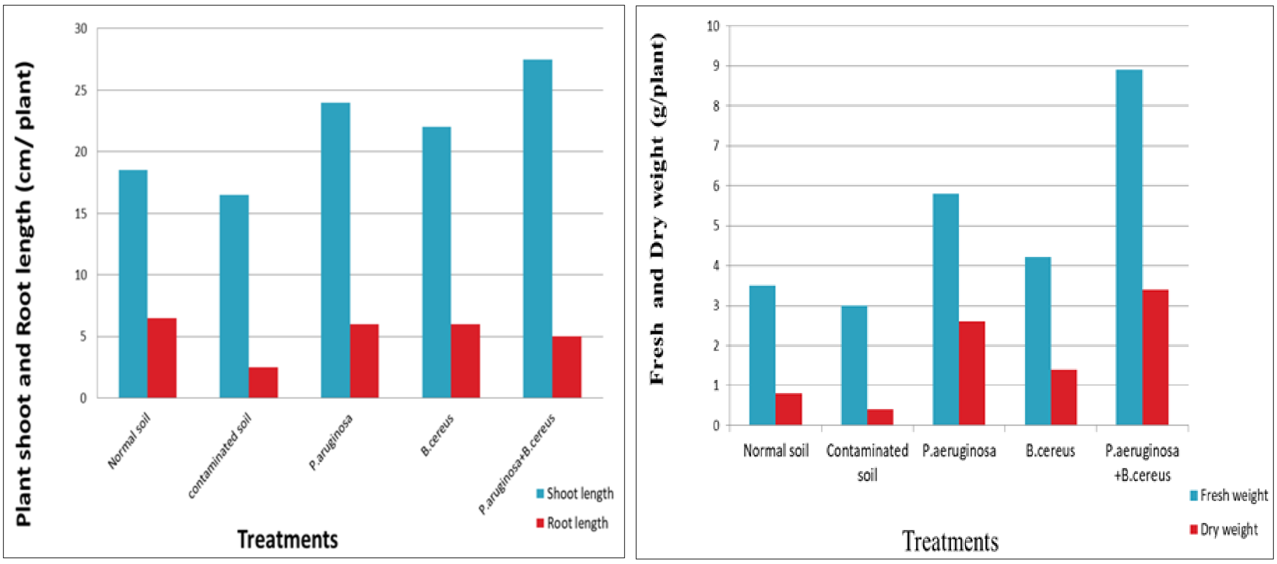


Fig 7: Growth characteristic and plant biomass of *Vigna radiata*L.

Table 6: Estimation of Total chlorophyll & carotenoids for*Vigna radiata.* L

Treatments	Chlorophyll (mg/g)	Carotenoids (mg/g)
Normal soil	0.127±0.08	0.147±0.001
Contaminated soil	0.061±0.047	0.143±0.0070
Pa01	1.162±0.013	0.834±0.468
Bc01	1.182±0.0029	1.078±0.037
Pa01+Bc01	1.467±0.385	1.69±0.221

Table 7: Quantitative Phytochemical analysis of plant samples

S.No	Treatments	Phenols	Flavonoids
1.	Normal soil	43.1±1.12	59.5±0.71
2.	Contaminated soil	38.7±0.46	56.4±0.66
3.	Pa01	73.6±0.74	95.4±0.61
4.	Bc01	67.2±1.12	85.4±0.87
5.	Pa01+Bc01	77.5±0.57	76.6±1.21

Table 8: Heavy Metal Analysis of the Soil Samples

Physicochemical properties	
Heavy metal analysis	Contaminated soil (mg/g)
Normal soil	3.5
Contaminated soil	12
Pa01	10.4
Bco1	11.3
Pa01+Bc01	9.2

Table 9: Impact of after inoculated soil Counting of Microbial population at 45 DAI

S.No	Treatments	Total bacterial population in soil (CFU/g soil)
1	Control	1.4 X10^7
2	Pa01	4.3 X10^7
3	Bc01	6.1 X10^7
4	Pa01+BcO1	5.4 X10^7

CFU – Colony forming unit

Discussion

Potential for phytoremediation depends upon the interactions among the soil, heavy metals, bacteria and plants. The roots of plants interact with a large number of different microorganisms that are major determinants of the extent of phytoremediation. Bacterial genera such as *Bacillus*, *Pseudomonas and Bravibacillus* are well known to promote growth and yield in different non-leguminous plants (Jha *et al*., 2016) [9] PGPR isolates with varied characteristics. The isolates were screened for their plant growth promoting activities viz., Indoleproduction, MR-VP, Citrate. Phosphate solubilization, other lytic enzymes like Catalase, urease, oxidase, Characterationof selected rhizobacterial isolates by using standard methods like morphological characters, cultural characteristics on agar plate, growth on broth media, growth on NaCl, was done as

Described in Bergy's Manual of Systematic Bacteriology (Tein *et al*., 1979) [23]. Plant growth in agricultural soil is influences by several environmental factors. Beneficial microorganisms canbea significant component for achieve the yield. However, inoculated plants showed more chlorophyll contents than those Cd-treated and noninoculated plants. These results are in agreement with the finding that rhizobacteria positively influence the chlorophyll under Cd stress (Zhang *et al*., 2011) [27].

The plant growth promoting rhizobacteria could be developed as inoculants to increase plant biomass and thereby to stabilize and remediate metal polluted soils. Such as can be made nutrient rich by applying metal-tolerant microorganisms, especially the plant growth promoting rhizobacteria. which would provide not only the essential nutrients to the plants growing in the contaminant sites but would also play a major role in detoxifying heavy metals (mayak *et al*.,2004) [13] and thus help plants capable of remediating heavy metals (Glick,2003) [7].

Conclusion

Plant growing on heavymetal polluted soil shows a reduction in growth due to changes in their physiological and biochemical activities. Bioremediation canbe effectively used for the treatmentof heavy metal pollute soil. Microorganisms and plants employ different mechanisms in the remediation of heavy metal soil. This study was identify specific PGPR with multiple plant growth regulating characteristics, characterize molecularly to identify a suitable and novel PGPR strain for inoculant production and select and apply suitable bioinoculants with respect to soil nature, agro climatic conditions and crop variety. The application of bio fertilizer was effective in alleviating the phytotoxicity of contaminated soils by changing the composition of the rhizosphere bacterial communityPGPR (rhizospheric and endophytic microorganisms) are one of the best suited choices, in the development of phytoremediation techniques and has to be elucidating to speed up the mitigation processes. Therefore, the present observations demonstrated that plant growth promoting rhizobacteria were valuable microorganism resource which can be exploited to improve the efficiency of phytoremediation and plant growth.

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