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Effects of heavy metal contaminated soils using Plant Growth Promoting Rhizobacteria (PGPR)

[1]Santha kumari.R, [2]Lalitha.S

[1,2] Department of Botany,School of Life Sciences, Periyar University,Salem-11, Tamil Nadu.

Abstract: **The environment has been contaminated with organic and inorganic pollutants. Soil contamination was toxic metals, such as Cd, Pb, Cr, Ni, Cu, as result of worldwide industrialization has increased noticeably within past few years. Bioremediation is a biological process for cleanup of pollutants from the environment. In the present study, *Bacillus cereus* and *Pseudomonas aeruginosa* isolated from rhizosphere soil was used for bioremediation. These isolates were characterized for their ability of biochemical tests. *Bacillus cereus* and *Pseudomonas aeruginosa* were inoculated in *Vignamungo*.L plants by pots inoculation method and analyzed for the growth promotion efficacy in heavy metal polluted soil. The plant inoculated with isolates showed Plant height, root length and fresh weight, dry weight, as compared to non-inoculated control.Further analysis of leaves were carried out for the photosynthetic pigment analysis, in *Vignamungo*.L. Results of the study showed positive growth response of *Vignmungo*.L on heavy metal polluted soil in green house condition in plants. The study concluded that the 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. Phytoremediation is the process by which PGPR are stimulated to rapidly degrade the heavy metal contaminated soil to environmentally safe levels in soils. Such metal resistant PGPR, when used as bioinoculant or bio fertilizers, significantly improved the growth of plants in heavy metal contaminated soils. Application of PGPR processing metal detoxifying traits along with plant-beneficial properties is a cost effective and environmental friendly metal bioremediation approach.**

Keywords: **Bioremediation, Biofertilizer, Bioinoculants, Environment, Plant Growth Promoting Rhizobacteria.**

I. INTRODUCTION

Environmental pollution by heavy metals has become a serious problem in the world. Pollution of the biosphere by toxic metals have been accelerated by industrial and technological advancement, it has become one of the most serve environmental problems today.(Hansda et al.,2014) Soil is a complex ecosystem where different microorganisms 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). Heavymetals ions were non degradable and persist in the soil and their presence at toxic level reduce or inhibit plant growth by hampering essential plant function and metabolic process.(Seneviratne et al.,2017).In addition ,the higher concentration of metals not only affects the growth and metabolism but also decreases the biomass of naturally occurring soil microbial communities of beneficial microorganisms around the roots. Aswell, they also exert a negative impact on plant growth (Wani Khan,2010). Phytoremediation is composed of different forms such as, phytoextraction which increase the concentration of metals into the plants, rhizofiltration which is the use of plant roots to absorb heavymetals and phytostabilization that reduce the mobility and bioavailability of heavymetals through absorption in the roots and precipitation by plants.((Hao et al.,2014).The phytoremediation efficiency can be enhanced by increasing the heavy metal solubility and mobilization in the soil and enhanced plant biomass by help of plant growth promoting rhizobacteria(Zhuang et al.,2007).The use of

heavy metal resistant microorganisms for the decontamination of heavymetals from contaminated soil has attracted growing attention because of several problems associated with pollutant removal using conventional methods.(Yang et al.,2015). Beneficial plant-microbe interactions in the rhizosphere can influence plant vigour and soil fertility. These beneficial effects of PGPR have direct and indirect performance on plants(Dastager et al.,2011).Major role of *Baciilus cereus* and *Pseudomonas aeruginosa* secretion in phytoremediation of heavy metals has been reported by (Bakiyaraj et al.,2014). The application of plant growth promoting rhizobacteria (PGPR) as crop inoculants for bio fertilization, phytostimulation, and bio control would be an attractive alternative to decrease the use of chemical fertilizers which also effect environmental pollution (Ali 2010). Aim of this study is the isolation of rhizobacteria from rhizosphere soil and pot experiment inoculation of these isolates in *Vignamungo*.L plants to estimate their plant growth promoting potential .The inoculated plants were also analyzed for accumulation of metals.

II. MATERIALS AND METHODS

Soil collection, Preparation, and characterization

Soil collected from site of mining area Dalmia of Salem District,Tamilnadu.Control soil collected from Nallampalli, Dharmapuri,Tamilnadu. soil collected from 20cm temperature 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,Tamilnadu

Isolation and identification of rhizosphere bacteria

Agricultural land soils samples collected from Nallampalli, Dharmapuri district, TamilNadu. Soil samples were placed in plastic bags and stored at4°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. the colonies were identified by performing Gram's staining and biochemical tests like Indole, MR, VP, Citrate. (Cappuccino and Sherman, 2002)

Biochemical Characterization of Bacterial isolates

Gram's staining (Cappuccino and Sherman, 2002)

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 glycerin 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 test (Cappuccino and Sherman, 2002)

The indicator phenyl red solution was prepared by dissolving 0.1gm of bacto methyl –red in 300 ml of 95% alcohol and diluting to 500 ml with the addition of 200 ml of distilled water. 20 the test was performed by inoculating a colony of the test organism in 0.5ml sterile glucose phosphate broth. After overnight incubation of 370^0 C, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of bright red colour, indication of activity. A yellow (or) orange colour was a negative test.

Citrate utilization test (Cappuccino and Sherman, 2002)

This was carried out by inoculating the test organism in test tube containing Simon's citrate medium and this was inoculated for 24 hours to 72 hours. The development of deep blue color after incubation indicates a positive result.

Extra cellular enzyme activity of bacterial isolates

Catalase activity (Ajay Kumar, *et al*., 2012)

48 hrs 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-phenylediamine dihydrochloride). Development of blue or purple coloration was positive to oxidase production.

Urease activity (Mac Faddin, 2000)

The isolates were streaked on christensen's urea agar slants and incubated for 3 days at 28^0C. Observer the slant for a color 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.

Phosphate solubilization tests (Chen, *et al*., 2006)

The bacterial isolates were streaked on pikovsakaya's agar medium. The presence of clearing zones around the bacterial colonies following incubation at 28± 2^0 C for 24 hours indicated positive for phosphate solubilisation.

Qualitative detection of siderophore (plate assay) (Schwyn&Neilands, 1987)

The chrome azurol sulfonate (CAS) assay (universal assay-Schwyn&Neilands, 1987) was used since it is comprehensive, exceptionally responsive, and most convenient. The chrome azurol sulfonate assay agar was used. For the qualitative assay cultures were spot inoculated onto the blue agar and incubated at 37^0C/24-48 hours. The results were interpreted based on the colour change due to transfer of the ferric iron from its intense blue complex to the siderophore. The sizes of yellow-orange haloes around the growth indicated total siderophores activity.

Antibiotic sensitivity test

The susceptibility to antimicrobial agents was tested with antibiotic disks by the method of Bauer *et al* (1966).the commercial 6different antibiotic disks used were Streptomycin, .autoclaved Mueller- Hinton medium at 15 lbs (1210c) 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.

Plant growth test

The experimental plant, the Blackgram *(Vigna mungo.L)* cultivar(vembon)10^{-1}belongs to the family fabaceae is one of the important pulses of the India. Five seeds were sown in 500 ml plastic container containing 150g of steam sterilized soil. Each container was inoculated with 2ml of bacterial suspension (PA01,BS01). The control was uninoculated bacterial suspension. Three replicates for each treatment were made. The plants were incubated in a green house 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 and biomass

Growth parameters of plant height (shoot and root length cm) fresh and dry weight of each plant monitored and measure at 45 days.

Measurement of plant length

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 Doong *et al.,(*1993).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 carotenoid contents.The absorbance of extract-measured at 470, 645, and 663nm in the UV-visiblespectrophotometer. 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

III. RESULTS

Soil collection, Preparation, and characterization

In the present study, bacteria were isolated from rhizosphere soil in legumes plant.The rhizospheric soil samples was tested in a Omega laboratories, Nammakkal. The physicochemical properties of the soil areas were in contaminated site in Dalmia, Salem, Tamilnadu.(Table.1)

Biochemical characterization of *Pseudomonas aeruginosa* and *Bacillus cereus* isolate

Among two isolates were selected on the basis biochemical characterization, in which one was Gram positive.

Another was Gram negative, and another test was IMVC tests and enzyme activity by *P.aeruginosa* was higher compared to that of *B.cereus* as observed by positive results.**(Table.2**) and the Antibacterial sensitivity by both isolates as observed by positive results.((**Figure. 1).**

Both the isolates showed phosphate solubilising activity on Pikovaskaya's agar medium. The phosphate solubilising activity by *P.aeruginosa* was higher compared to that of *B.cereus* 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.aeruginosa* was higher compared to that of *B.cereus* as observed by a clear zone around the inoculated bacterial isolates**.** (**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 16S rDNA of about 1500pb were obtained for both the isolates as discrete bands in agarose gel.**(Figure. 3)**

In the present study, *Vignamungo.L* plants inoculated with *B.cereus* and *P.aeruginosa*, showed significantly higher plant hight,root length and Fresh weight and dry weigth. Plant inoculated with *B.cereus* showed root, shoot length and fresh,dry weights as compared to non –inoculated control.while *P.aeruginosa* treated plants showed more root,shootlength and fresh,dry weight as compared to non-inoculated control(.**(Figure,4,5)**. Plants inoculated with both *B.cereus and P.aeruginosa* showed plant root,shoot length and fresh,dry weigth as compared to non-inoculated control.**(Figure.6)**

Inoculation with both the isolates showed highest level in photosynthetic pigment analysis in *Vignamungo*.L plants.**(Figure.7)**

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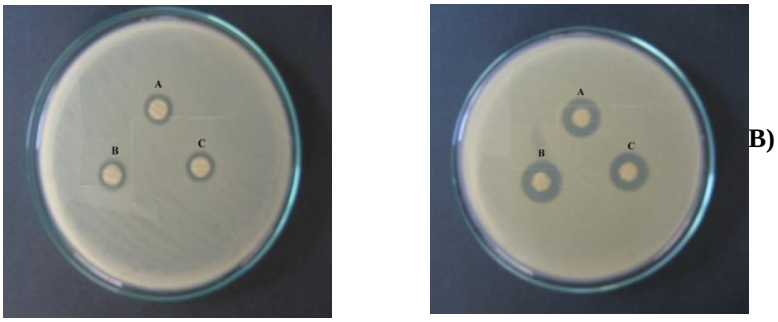
TABLE 1: Physicochemical analysis of soil samples

Physicochemical Properties		
Physicochemical Properties	Contaminated soil	Normal soil
Ph	8.72	7.84
Electrical Conductivity(EC)	0.88	0.2
Available N	01.28	0.45
Available P	02.60	02.25
Available K	01.06	01.02
Copper(mg/kg)	16	7.6
Magnesium (mg/kg)	335	210
Calcium(mg/kg)	298	129
Zinc(mg/kg)	310	260
Cadmium(mg/kg)	12	3.5

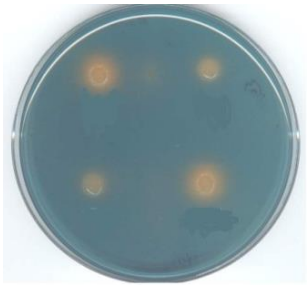
TABLE 2: Biochemical characterization of *P.aeruginosa* spp. and *B.cereus* spp

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**	+	+



A **B)** **B**

Fig 1: Antibacterial activity test, (A) *P.aeruginosa* B) *B.cereus*



A

Fig 2: Siderophore production test- A *)P.aeruginosa* , *B.cereus*

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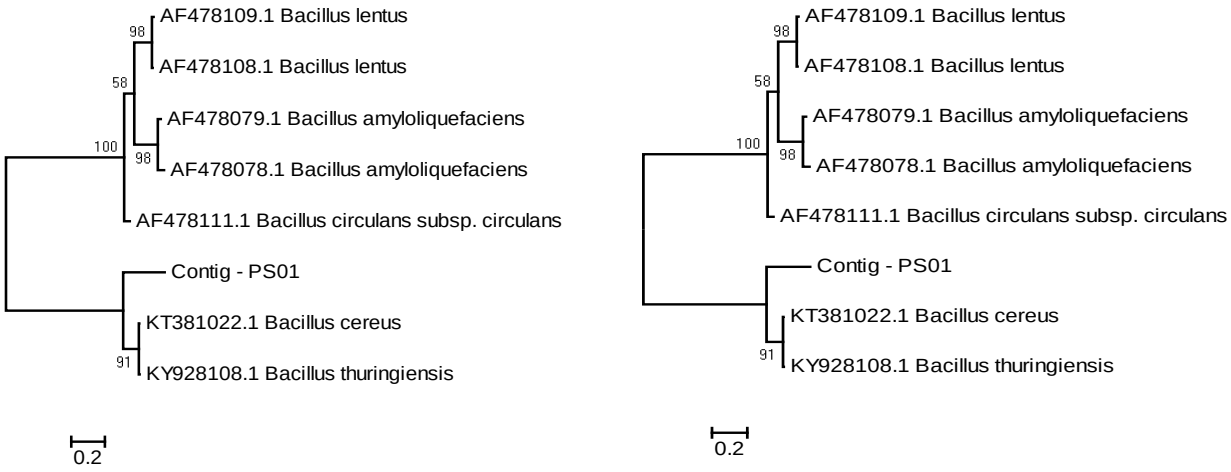

Fig 3: Phylogenetic tree , A)*P.aeruginosa* B)*B.cereus*

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). Bacillus *cereus* and were submitted GenBank under Accession number MH128361.*Pseudomonas aeruginosa* strain Accession number MH128359.

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Fig 4: Growth characterization *Vigna mungo* L. at 45 DAI (cm/plant)

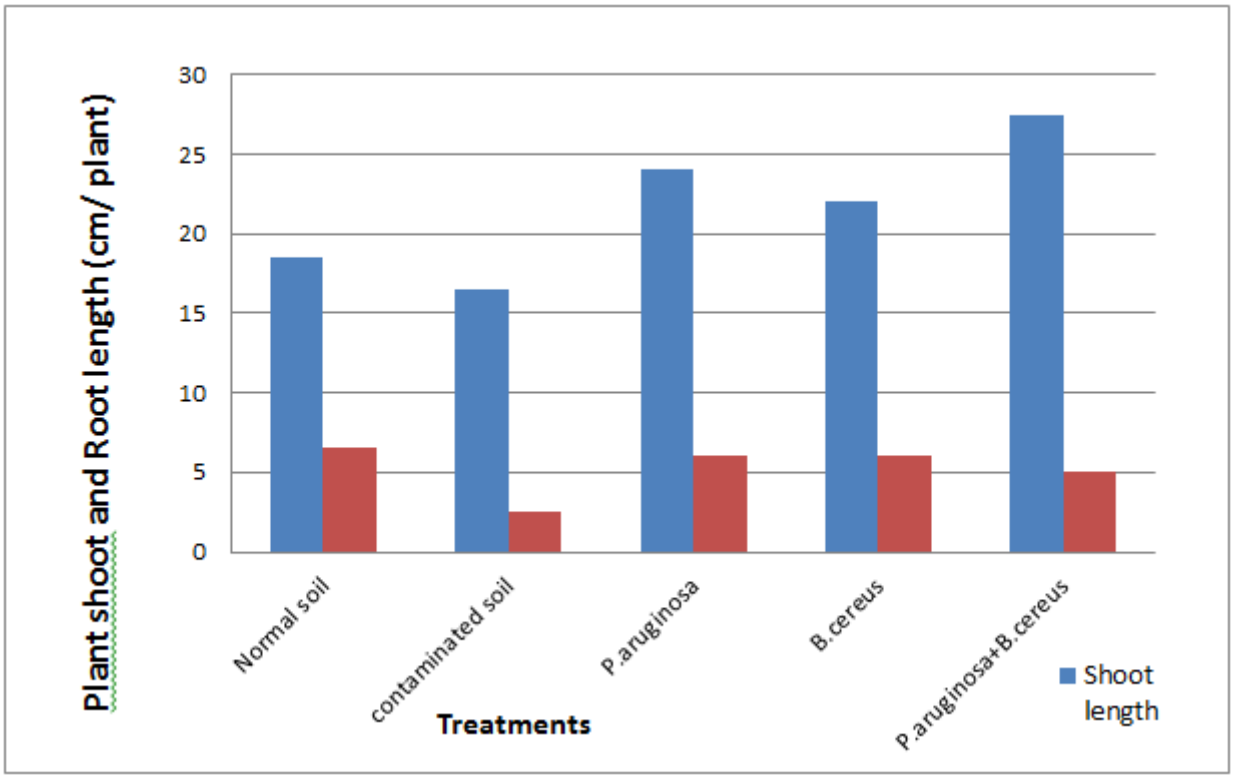

Fig 5: Growth characteristic of Vigna mungo L. Shoot /root length at 45 DAI (cm/plant)

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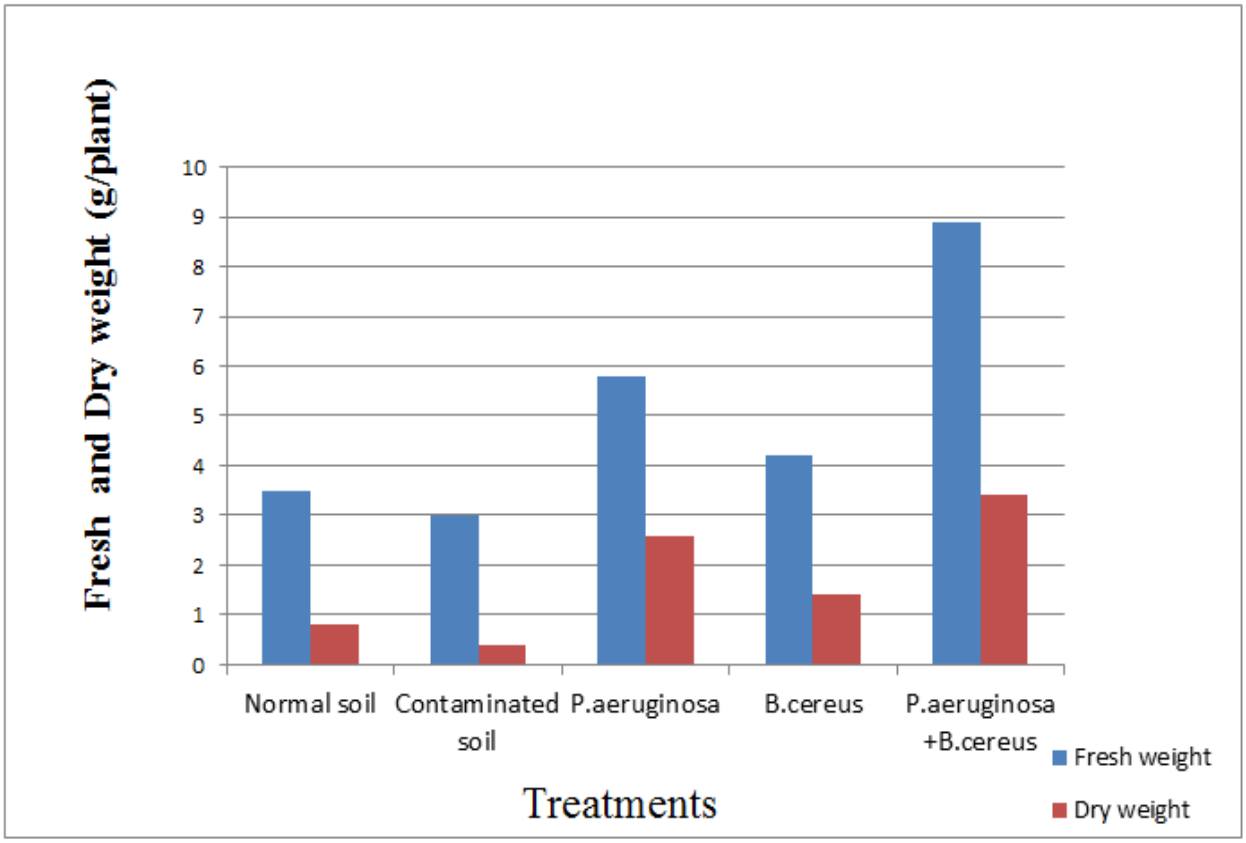

Fig 6: Growth characteristic of *Vigna mungo* L. fresh weight and dry weight at 45 DAI (g/plant)

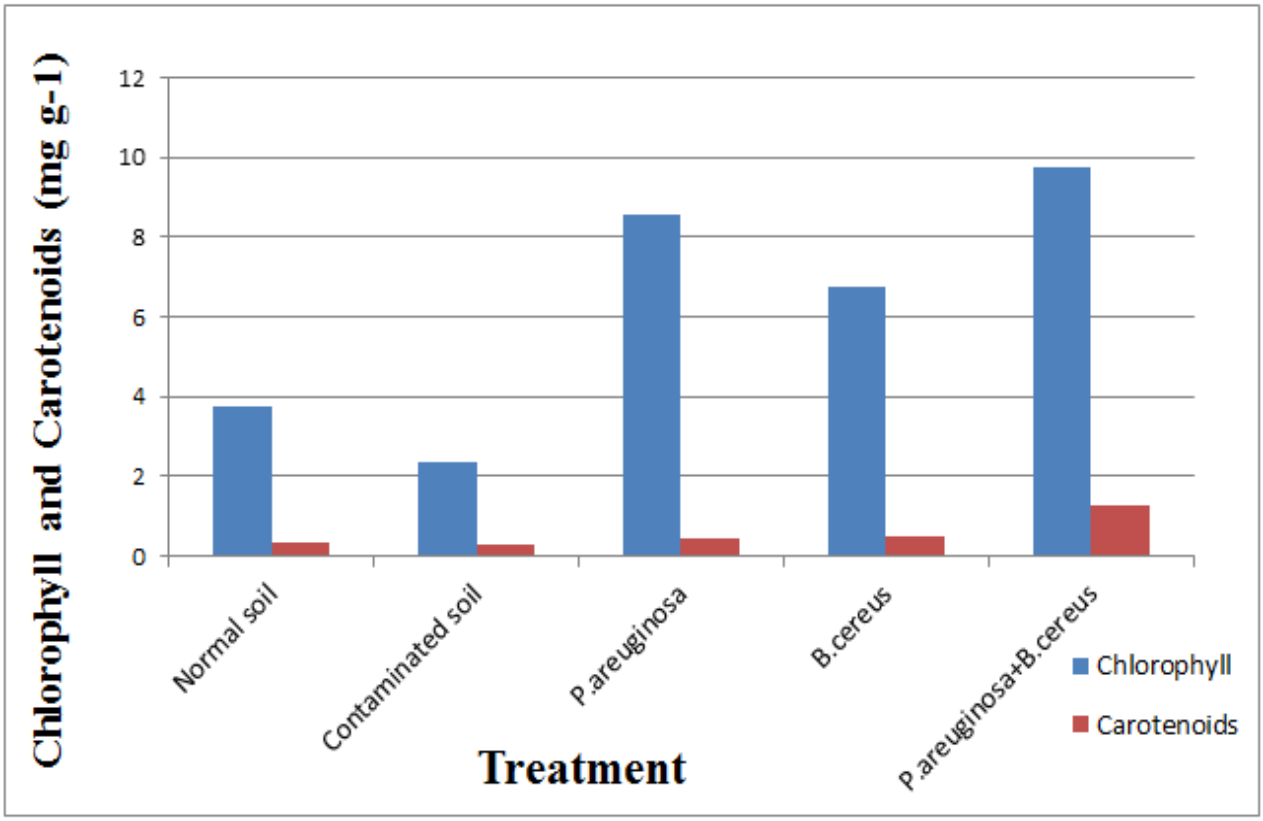

Fig 7: Estimation of Total chlorophyll & carotenoids for *Vigna mungo* L

IV. DISCUSSION

Slow growth, low biomass, and even failure of growth are the major issues encountered in phytoremediation around mining wastelands due to poor soil fertility and heavy metal toxicity (Wong 2003).Potential for phytoremediation depends upon the intractions among the soil, heavymetals,rhizobacteriaand plants. The roots of plants interact with a large number of different microorganisms that are major determinants of the extent of phytoremediation. Different bacterial genera are vital components of soils, involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production(Glick 2012).

PGPR isolates with varied characteristics. The isolates were screened for their plant growth promoting activities viz., Indole production,MR-VP, Citrate , Phosphate solubilization, other lytic enzymes like Catalase, urease, oxidase, Characteration of selected rhizobacterial isolates by using conventional 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).

Bacterial genera such as *Bacillus*, *Pseudomonas* and *Brevibacillus* are well known to promote growth and yield in different non-leguminous plants (Jha,2016) Bacterial isolates isolated from rhizosphere soils have adapted to multiple heavymetal stress by developing various mechanisms.

Plant growth in agricultural soil is influences by several environmental factors. Beneficial microorganisms can be a significant component for achieve the yield.in the present study, *Vignamungo*.L inoculated *B.cereus* and *P.areuginosa*, showed significantally higher plant root, shootlength and fresh, dry weight.

The effect of these isolated rhizobacteria in phytoremediation of contaminated soil, effects used by bacterium in plant growth and protection is a good strategy and clean environment(Sowmya 2014).When considering approaches to alter heavy metal mobilization, there are several advantages to the use of beneficial microbes rather than chemical amendments because the microbial metabolites are biodegradable, less toxic, and it may be possible to produce them in situ at rhizosphere soils. Phytoremediation is an effective method used to remediate heavymetal-polluted sites.Therefore plant-microbe partnership are an effective way utilized to improve biomass production and remediation((Ma et al.,2016) Plants inoculated with plant growth promoting rhizobacteria have increased plant biomass and the plants to develop tolearence in heavymetal contaminated soil,where the metal content exceeds limit of plant tolearence.

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