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Isolation and characterization of heavy metal resistant PGPR and their role in enhancement of growth of wheat plant under metal (cadmium) stress condition

Chhaya Verma, Pooja Singh and Rajesh Kumar*

Rhizosphere Biology Laboratory, Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar Raibareli Road, Lucknow, India

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ABSTRACT

In present time excessive use of chemical fertilizers in agricultural field causes the environmental hazards and affects the human and animal health. There is an urgent need to reduce the application of chemical fertilizers. Plant growth promoting rhizobacteria (PGPR) is the best alternative of chemical fertilizers, present in root zone of plant and enhanced the plant growth in stress as well as the normal condition. This study was done on heavy metal resistant PGPR, isolated from heavy metal contaminated soil of industrial and agricultural area of Lucknow, Kanpur and Ambedkar Nagar. Out of 27 isolates only 6 were grown on high concentration of cadmium metal. Isolate PBB_1 showed 1000 ppm MIC of cadmium and remaining isolates namely PP_3, PP_2, SNA_3, and SNA_5 were grown on 800 ppm MIC of cadmium. PGPR screening was done for selection of best PGPR to enhance the growth of wheat plant by production of IAA, ammonia and phosphate solubilization. The pot study was done with 100 ppm cadmium amended soil. When cadmium resistant PGPR were applied on seeds of wheat the growth and germination of plants were enhanced. On comparison of finding result with literature, it may be possible that all the isolates may be fluorescent pseudomonads. The result of pot study showed that the Pseudomonas sp. SNA_5 gave the best result in comparison of other isolates.

Keywords: Cadmium, PGPR, *Pseudomonas*, Heavy metal resistant microbes, Pollution

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INTRODUCTION

Heavy metal pollution is increases day by day due to industrialization in all over world. Pollution is generated due to metallic ferrous ores mining and smelting, fossil fuels, sewage, municipal wastes, pesticides and fertilizers [1]. Among all the heavy metal cadmium (Cd) is a very poisonous metal and has 7th rank in all 20 toxin categories because it is highly toxic for cellular enzymes [2]. According to Naidu et al., [3] cadmium shows toxicity against humans, animals, and plants, with long biological life and it alters the cell differentiation, proliferation, apoptosis, and improves activation of oncogene in carcinogenesis mechanisms. In environment sources of cadmium contamination in soil are usage of industrial effluents, phosphatic fertilizers, and municipal sewage sludge and city compost in agricultural field [4, 5]. Alloway [6] said that in human approximately 70% of cadmium intake is occur through vegetable foods and cadmium stay in environment for several years.

In cadmium contaminated soil, microorganisms evolved several mechanisms by which they can survive in stress environment such mechanisms are metal exclusion by barrier of permeability, cellular sequestration that is

intracellular or extracellular [7] active efflux pumps, enzymatic reduction, metal volatilization and bio-precipitation [8, 9]. Wuertz and Mergeay [10] studied about resistance mechanisms and said that the resistance and tolerance mechanisms are controlled by the biochemical and structural qualities, physiological and/or genetic adaptations [11]. In microorganisms different types of resistance mechanisms have been developed by which microorganisms either secrete metal binding protein or accumulate them in your body to develop resistance [12]. In soil many types of cadmium resistant bacteria are found and are also found in rhizosphere.

Rhizosphere is the root surrounding region and have many types of active groups of bacteria [13] termed as "PGPR" (Plant Growth Promoting Rhizobacteria) [14, 15]. Plant-Growth-Promoting Rhizobacteria inhabits in and around the root and useful for plant with the enhancement of growth via two mechanisms first is the direct mechanisms such as nitrogen fixation, growth-regulating agents production, increasing availability of nutrients to the plant, production of plant hormones and vitamins such as Gibberllin, Cytokinin and Oxine and the second is the indirect mechanisms includes antibiotics synthesis, make iron available, competing with root inhabiting species [16, 17], causing systemic resistance in the plant, and promoting plant resistance in stress conditions caused by non-living factors [18].

PGPR can participate in bio-control by competing or destroying other microbes or pathogens in case of plants [19, 20]. For example fluorescent pseudomonads can causes root diseases in crop via the competition, antibiotics production, siderophores or HCN production [21]. In soil many types of bacteria have tolerant property against heavy metals through mobilization or immobilization processes [22]. Not only soil bacteria various types of PGPR have ability to solubilize ''unavailable" forms of heavy metal-binding minerals with the help of organic acids [23]. Therefore, interaction of plants and PGPR can be helpful for microbes assisted phytoremediation and they may accumulate or adsorb the heavy metal with improvement of the phytoremediation technology [24, 25].

Study of metal ion resistance gives us an idea about insight environmental processes and provides an understanding of basic living processes. Mostly the heavy metal resistant genes are found on plasmid but many studied proved that genes for resistance to inorganic salts of soft metals are found both on plasmids and in chromosomes. Genes that are present on plasmid generally causes resistance while those genes that are chromosomally encoded may provide metal ion homeostasis [10, 26].

MATERIALS AND METHODS

Plant growth experiment were conducted under standard controlled conditions (temperature 28 ± 2^0C; 65 % relative humidity) with sterile soil as a substrate, plants were watered with sterile de-ionized water regularly whenever required. Standard controlled condition is required for the experiment of plant growth in sterile soil and plants were continuously watered with distilled water. Experiment was completed in small disposable pots of 5x4 cm with a 50 gm of soil as a substrate.

1.1. Bacterial cultures:

Twenty Seven fluorescent bacteria were isolated from rhizosphere region of mustard and maize. Soil samples were collected from Lucknow region of Uttar Pradesh, India in polythene bag and stored at 4^oC for further analysis. Diluted soil samples were spread on King's B agar plates [27]. Pure cultures of all isolates were maintained on nutrient agar plates and in slant at 4^oC for further studies.

1.2. Cadmium Tolerance Test:

All the selected fluorescent bacteria were analyzed for their tolerance to cadmium by following agar dilution method [28]. In this method freshly growing cultures were streaked on cadmium (Cadmium nitrate) amended agar plates at different concentration ranging from 50 to 1000 mg/l. Cadmium resistance was determined by the appearance of growth of bacteria after the 3 to 4 days of incubation. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of metal ion that completely inhibited growth.

1.3. Biochemical Characterization:

The selected bacterial isolates PP3, PBB1, SNA3, SNA5 and PP2 were characterized by following standard methods of Cappuccino and Sherman, 1992 [29] and Aneja, 2003 [30]. The isolate were characterized by Gram staining, Methyl Red, Voges Proskauer, Citrate, oxidase test, catalase test, starch hydrolysis, dextrose metabolism, urease test, rhamnolipid production test, indole test.

1.4. Screening of plant growth promoting activities of *Pseudomonas* sp.
Qualitative characterization of all the isolates were required for the best result of pot experiment and all the thirty isolates were qualitatively characterized by following standard protocols.

1.4.1.Production of Indole acetic acid:
Brick et al., [31] described the production of Indole acetic acid in culture medium. Bacterial cultures were inoculated in mineral salt media amended with 1% typtophan and incubated for 48 hrs at 28±2^{o}C. After incubation cultures were centrifuged at 5000 rpm for 15 min. After this the 2 ml of supernatant was added with few drops of Orthophosphoric acid and 2 ml of Salkowaski's reagent. Development of pink colour indicates the presence of IAA in the tubes.

1.4.2.Ammonia Production: (Cappuccino and Sherman, 1992 [29])
Peptone water broth was used for testing of ammonia production and each isolates were inoculated with freshly grown culture and incubated for 48 to 72 hrs at ±28^{0}C. When broth becomes turbid 0.5 ml of Nesselers's reagent was added in each tube and observed for appearance of yellow to brown. A faint yellow (small amount of ammonia) and brown color (high production of ammonia) indicates the accumulation of ammonia in medium.

1.4.3.Production of HCN:
All the isolates were tested for the HCN production with the help of methodology of Castric [32]. A Whatman no.1 filter paper placed on lid of Petri plates and then autoclaved. Selected isolates were streaked on nutrient agar plates amended with 4.4 g per liter of glycine. Soaked the filter paper in 2% sodium bicarbonate and 0.5% picric acid solution and put inside the lid of the plates and sealed properly with parafilm and incubated for 3-4 days at ±30^{o}C. HCN production is indicated by the appearance of color of filter paper from light brown to dark brown.

1.4.4.Determination of Phosphate solubilization:
For estimation of phosphate solubilization all isolates were inoculated on the Pikovskaya's agar medium [33]. After 3 to 6 days of incubation at ±28^{0}C, when bacteria solubilised the phosphate clear zone appear around the spot inoculums. Halo zone around the growth was measured for the obtaining the phosphate solubilization index (SI) according to Premono et al., [34].

1.5. Seed germination test
Germination test was done by the paper towel method. For seed germination test firstly seeds were surface sterilized. Then seeds were mixed with gum acacia and treated with selected bacterial inoculums. Surface sterilized and uniform size treated seeds were placed at wet filter paper in sterilized Petri dishes cadmium is also amended in this. After this all the plates were incubated in dark for three days at favorable temperature. Untreated seeds were analyzed as a control. After three days of incubation number of germinated seeds was counted for germination percentage. The seedlings were taken out after 7 days of incubation for several studies also like vigor index, root and shoot length and the data were recorded.

The total germination percentage was calculated by using the following formula [35]

$$\text{Germination} = \frac{\text{Total number of seeds germinated} \times 100\%}{\text{Total number of seed sown}}$$

Vigor index was calculated by following formula [36]:

Vigor index = (Mean Root Length + Mean shoot length) × % germination

1.6. Preparation of metal contaminated soil:
The soil for the pot experiment was collected from green house of BBA University, Lucknow, India. Before use the soil was sieved and autoclaved for 1 hr at 100^{o}C then 100 ppm of cadmium is amended in soil. For the pot experiment this soil is kept in the environment for one month to stabilize metal [37].

1.7. Pot trials:
The experimental setup consisted of 20 treatments of selected isolates with and without cadmium amendment. Firstly the seeds of wheat were surface sterilized by using ethanol and H_2O_2 for 10 min then washed the seeds with

sterilized de-ionized water for 5 times [37]. The inocula for the pot experiment were prepared in nutrient broth at 28±2°C. The sterile soil is treated with the inoculums suspension that has cfu 10^8–10^9 ml^{-1}. For treatments in inoculated soil 10% sugar solution was added and the un-inoculated seeds worked as control. Three replicates of all the treatment were randomly designed in a laboratory under favorable environment. Five inoculated seeds of wheat were sown in each pots having 50 gm soil. All pots were irrigated continuously with de-ionized water and the fertilizer was applied in it. Pots were irrigated when needed [38]. After ten days of sowing whole plants were carefully removed with root and washed with EDTA and de-ionized water. Root length and shoot length were measured and plants were dried for dry weight measurement.

RESULTS AND DISCUSSION

1.8. Isolation and Characterization of cadmium resistant PGPR:

Twenty seven bacteria were isolated from all the eight soil samples and the details are given in Table.1 and Fig.1. Five bacterial strains were isolated from rhizosphere soils on King's B media amended with cadmium (100 ppm). For PGPR screening all the isolates were qualitatively analyzed. Here four isolates namely SNA_5, SNA_3, PP_3 and PBB_1 shows all the PGPR properties (Table.2) and selected for pot study. HCN was not produced by any isolates. These four selected strains were characterized as *Pseudomonas sp*. on the basis of morphological and physiological characterization. Isolate PBB1 have high level of MIC (1000) for cadmium metal and remaining strains shows 800 ppm MIC illustrated in Fig.2.

1.9. Effects of PGPR on growth of wheat:

In this experiment the seeds are used have 50 % germination rate. Four best PGPR were selected for growth treatment of seeds of wheat plants for their growth enhancement analysis (Fig.3). In this experiment in cadmium amended treatment PP3 strain shows maximum VI in germination test other than four isolates, but SNA5 and PBB1 isolates enhance the growth of plant positively very early with 100 % germination shown in Table.3 and in Fig.4. Noel et al., [39] also reported this. In the present study we have found that fluorescent pseudomonads were mostly inhabited in metal contaminated rhizosphere soil. Rhizosphere bacteria of heavy metal tolerant plants have important roles in plant growth enhancement and remediation of heavy metal contaminated soils [40]. Several researchers worked in this field from many years and very few strain were characterized but more research is required to find best metal remediating PGPR strain. According to Pal et al., [41] bacteria developed various resistance mechanisms to adopt the metal contaminated environment.

In this experiment the bacterial isolates SNA5 and PBB1 shows high degree of tolerance to cadmium metal isolated from rhizosphere region of hyperaccumulated plant *Brassica juncea.* Various researchers reported that the metal resistant PGPRs are helpful in growth enhancement of both hyperaccumulator and non-hyperaccumulator plants in metal polluted soil [42, 43]. In this study Strain SNA5 and PBB1 improves the growth and germination of wheat plant in metal amended soil in comparison to control with the production of NH_3, IAA and phosphate solubilization. All the tested isolates show three or four traits of PGPR. IAA is helpful in stimulating plant growth through cell elongation or cell division [18]. All the tested PGPR produce high levels of IAA and other researcher also reported such type of study [44]. In the present study we have found that fluorescent pseudomonads were mostly inhabited in metal contaminated rhizosphere soil and significant increase in shoot and root length were reported in comparison of control, Klopper [46] also worked on same type of study. On the basis of this study we can concluded that the strain SNA5 relatively more helpful in enhancement of growth parameters of wheat in the pot with PGPR properties.

Table.1.Details of sampling sites and isolates

Sampling site	Microbial load (cfu/ml)	Types of colonies
Sarojani nagar industrial area A, Lucknow (SNA)	73×10^5	5
Panki, Kanpur (PP)	106×10^5	2
Mohanlalganj, Lucknow (ML)	39×10^5	2
Bijnaur, Lucknow (BL)	32×10^5	4
Sarojani Nagar Industrial area B, Lucknow (SNB)	11×10^5	3
Kankha, Lucknow (KL)	85×10^5	4
Tanda thermal power plant, Ambedkarnagar (TP)	137×10^5	2
BBA University, Lucknow (PBB)	58×10^5	5

Table No.2.Showing the result of PGPR test

Test/strain	SNA3	SNA5	PP2	PP3	PBB1
Ammonia production test	+++	++	+++	++	+++
IAA production test	+++	+++	++	+++	+++
Phosphate solubilization test	+	+	+	+	+
HCN production test	-	-	-	-	-

Table No.3.Result of Pot Experiments

Treatments	Germination %	Germination type	Growth type	Plant length (cm)	Biomass (g)(dry weight)
Control	40	Late	Medium	12.25	0.031
PBB1	100	Late	Fast	28.8	0.198
PP3	40	Medium	Medium	26.25	0.180
SNA3	80	Medium	Medium	23.90	0.137
SNA5	100	Early	Fast	31.50	0.187

Fig.1.Isolation of Fluorescent Pseudomonads on King's B Plates

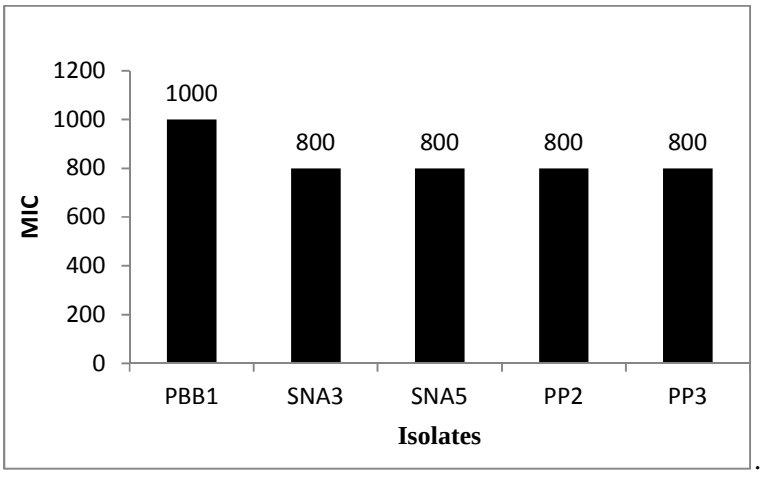

Fig.2.MIC of selected isolates

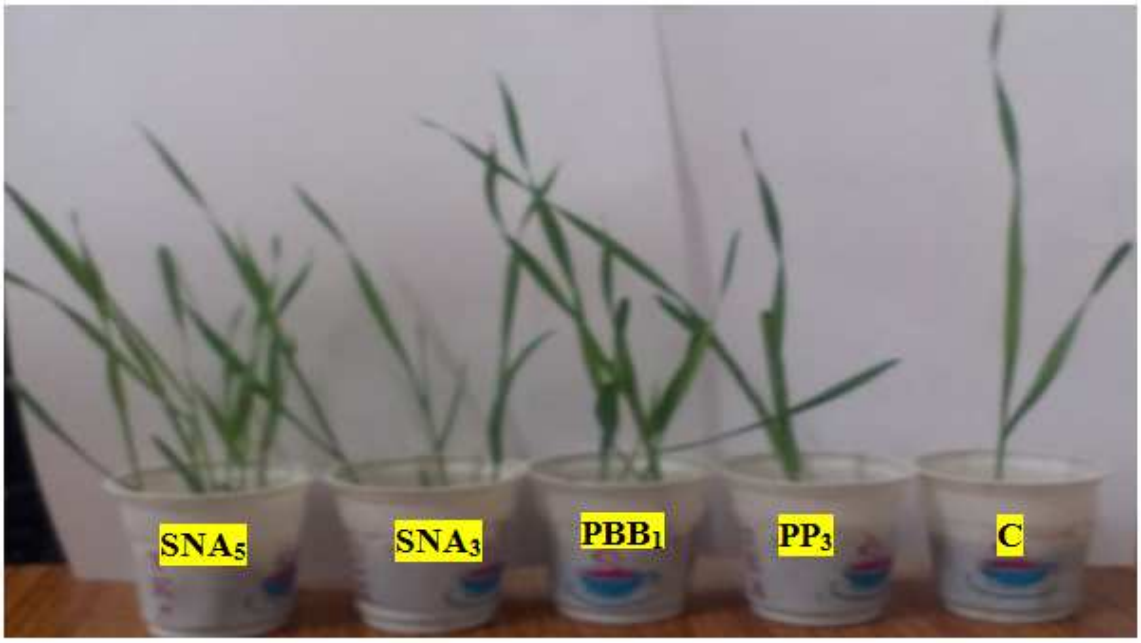

Fig.3.Showing the effects of selected PGPR on growth of wheat plant under cadmium stress condition

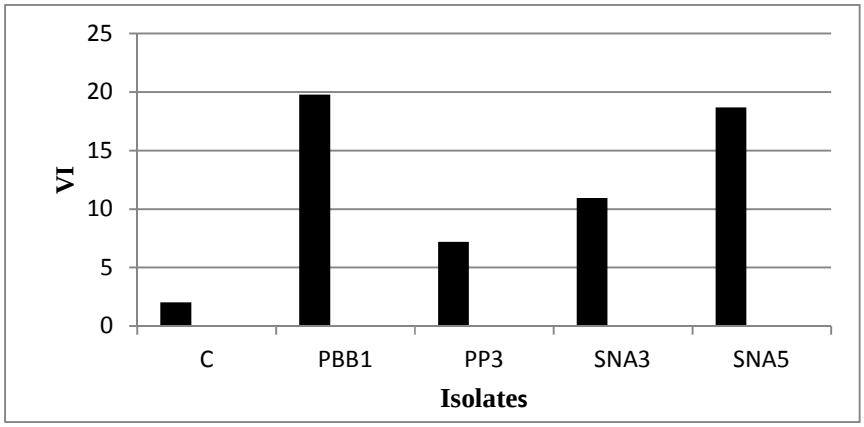

Fig.4. Vigour index of selected PGPR isolates

CONCLUSION

The study has been done is demonstrated that microorganism are helpful for reduction of leaching of heavy metals in environment. Hence, we can use these isolates for minimization of toxicity of cadmium and enhancement of plant growth under metal stress condition. This study is also helpful for bioremediation because of high level of MIC of isolates. All the tested bacteria isolated from rhizosphere and non rhizosphere region. Such heavy metal resistant bacteria are shown best PGPR activity and useful for enhancement of plant growth and germination with re-colonization of plant's rhizosphere region in metal polluted soil.

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