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Screening of Multiple Metal and Antibiotic Resistant Isolates and Their Plant Growth Promoting Activity

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Abstract: Heavy metal contamination has accelerated due to the rapid industrialization world wide. Accumulation of metals in excess can modify the structure of essential protein or can replace an essential element. *Bradyrhizobium* strains showed tolerance to cadmium, chromium, nickel, lead, zinc and copper. All the isolates showed maximum tolerance towards lead and zinc which was followed by nickel and chromium. These strains also showed tolerance towards most of the antibiotics. *Bradyrhizobium* strains were also tested for their Plant Growth Promoting (PGP) substances, all isolates produced good amount of indole acetic acid and were positive for ammonia but only three strains were positive for HCN and siderophore (RM1, RM2 and RM8), the rest isolates showed negative result. Based on the above intrinsic abilities of *Bradyrhizobium* species, these strains can be used for the growth promotion, as well for the detoxification of the heavy metals in metal polluted soils.

Key words: *Bradyrhizobium*, heavy metal tolerance, antibiotic resistance, PGP activities

INTRODUCTION

Heavy metals and metalloids are major global environmental pollutants and many of them are toxic even at very low concentrations. Such trace elements are released into the biosphere from different industries which uses them frequently for manufacturing various products (Fernandes and Henriques, 1991). The industries using Heavy Metals (HM) include mining, smelting, manufacturing, gas exhaust, energy and fuel production, fertilizer, sewage and pesticide production and municipal waste generation. After discharge, heavy metals are reported to adversely affect about 12% of the world's agricultural land (Moffat, 1999). According to some estimates, metal concentrations in soil range from less than 1 mg kg^{-1} to as high as 100,000 mg kg^{-1}, which could either be geological in origin or may result from different human activities (Blaylock and Huang, 2000). Even though some heavy metals are required by plants to maintain its physiological processes but the excessive accumulation of such metals in plant tissues have shown toxicity symptoms and have often been found lethal as they- (1) can modify structure of some essential proteins (Yruela, 2005; Roy *et al*., 2010) (2) can replace certain elements able to cause chlorosis (Ebbs and Uchil, 2008), growth inhibition, structure damage, browning of roots (Roy *et al*., 2010) and (3) decline in physiological and biochemical activities including inhibition of photosynthesis (Cheng, 2003; Gorhe and Paszkowski, 2006; Wani *et al*., 2007a). The threat by heavy metals to plants and consequently to human and other animals is aggravated by low mobility and their ability to persist in the environment. For instance, lead (Pb), one of the more persistent metals was estimated to have a soil retention time of 150 to 5000 years while the average biological half-life of Cd has been reported as 18 years.

Soils heavily contaminated with several toxic metals, can adversely affect not only beneficial rhizospheric microbes but also plant growth at elevated concentrations (Giller *et al*., 1998; Rajkumar *et al*., 2006). Metal toxicity, however, can be reduced by applying resistant microorganisms (Wani *et al*., 2009). Generally, the Plant Growth Promoting Rhizobacterial (PGPR) strains can promote plant growth and through phosphate solubilization, IAA and synthesis of antimicrobial compounds and siderophores when these strains are applied to seeds or to soils (Wasi *et al*., 2008). Moreover, metal-resistant microbes can detoxify metals either by enzymatically or through the production of metabolites, through accumulation and sequestration of the metal ions

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inside the cell and adsorption/desorption of metal ions (Mamaril *et al*., 1997). Such PGPR strains possessing multiple metal tolerance, antibiotic resistance and plant growth promotion could be of greater importance for both bioremediation and for plant growth. The present study was therefore under taken to (1) Determine the resistance pattern of PGPR to heavy metals and antibiotics (2) Determine the production of plant growth promoting activities.

MATERIALS AND METHODS

Collection of samples: Plant samples for the isolation of bacteria were collected from the soils of the metal contaminated sites of the industrial area of Aligarh, UP, India.

Evaluation of bacterial strains for metal tolerance: *Bradyrhizobium* strains were isolated from the nodules of greengram (*Vigna radiata* L. Wiclzek) grown in metal contaminated soils of the industrial area of Aligarh, UP, India. *Bradyrhizobium* strains were tested for their resistance to six metals like cadmium, chromium, nickel, lead, zinc and copper not by agar plate dilution method (Holt *et al*., 1994) using YEM agar medium. The freshly prepared agar plates amended with increasing concentration of cadmium (0-400 μg mL^{-1}), chromium (0-500 μg mL^{-1}), nickel (0-500 μg mL^{-1}), lead (0-1500 μg mL^{-1}), zinc (0-1500 μg mL^{-1}) and copper (0-400 μg mL^{-1}) were spot inoculated (100 μL) with 10^8 cells mL. Plates were incubated at 28±2° for 3-5 days. Lowest concentration of metals inhibiting bacterial growth on nutrient agar plate was defined as a minimum inhibitory concentration. Each experiment was replicated three times.

Determination of antibiotic resistance: To determine resistance to antibiotics, the plant growth promoting *Bradyrhizobium* were tested for their sensitivity to ten antibiotics. The reactions to antibiotics were determined by the disc diffusion method (Bauer *et al*., 1966). *Bradyrhizobium* species were grown in YEM broth respectively, at 28±2°C for 24 h. A 0.1 mL of the over night grown culture was spread on the surface of yeast extract mannitol agar. The antibiotic discs of known potency were then placed on the agar surface and the plates were incubated at 28±2°C for 24 h and the zones of inhibition around the antibiotic discs were measured to the following antibiotics (all from Hi-media Mumbai): ampicillin (25 μg), chloramphenicol (25 μg), ciprofloxacin (30 μg), cloxacillin (30 μg), gentamycin (30 μg), methicillin (30 μg), nalidixic acid (30 μg), nitrofurantoin (100 μg), streptomycin (25 μg) and tetracycline (30 μg).

Plant growth promoting activities

Quantitative assay of indole acetic acid: Indole-3-acetic acid was quantitatively assayed by the method of Gordon and Weber (1951), later modified by Bric *et al*. (1991). For the activity of indole acetic acid bacteria were grown in Luria Bertani (LB) broth (g L: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5). Luria Bertani broth (100 mL) supplemented with 0, 20, 40, 60, 80 and 100 μg mL^{-1} of tryptophan was inoculated with 10^8 cells mL^{-1} of one mL culture and was incubated for 24 h at 28±2°C with shaking at 125 rpm. After 24 h, 5 mL of each culture was centrifuged (10,000 rpm) for 15 min. and an aliquot of 2 mL supernatant was mixed with 100 μL of orthophosphoric acid and 4 mL of Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% per-chloric acid) and incubated at 28±2°C in darkness for 1 h. The absorbance of pink color developed was read at 530 nm. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard (Bric *et al*., 1991). The experiments were repeated three times on different time intervals.

Detection and quantification of siderophore: The bacterial strains were further assayed for siderophore production on the Chrome Azurol S (CAS) agar medium by the method of Alexander and Zuberer (1991). Chrome Azurol S agar plates were prepared separately and divided into equal sectors and spot inoculated with 100 μL of 10^8 cells mL^{-1} and incubated at 28±2°C for 72-96 h. Development of yellow orange halo around the growth was considered as positive for siderophore production. Each individual experiment was replicated three times. The siderophore produced by the test strains were further quantitatively assayed using Modi medium (K_2 HPO_4 0.05%; $MgSO_4$ 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1%; NH_4NO_3 0.1%). Modi medium was inoculated with 10^8 cells mL^{-1} of bacterial cultures and incubated at 28±2°C for 5 days. Catechol type phenolates were measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride-ferricyanide reagent of Hathway. Ethyl acetate extracts were prepared by extracting 20 mL of supernatant twice with an equal volume of solvent at pH 2. Hathways reagent was prepared by adding 1 mL of 0.1 M ferric chloride in 0.1 N HCl to 100 mL of distilled water and to this was then added 1 mL 0.1 M potassium ferricyanide (Reeves *et al*., 1983). For the assay, one volume of the reagent was added to one volume of sample and absorbance was determined at 560 nm for salicylates with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2, 3-dihydroxy benzoic acid as standard.

***In vitro* assay of hydrogen cyanide and ammonia:** Hydrogen cyanide production by bacterial isolates was

detected by the method of Bakker and Schippers, 1987). For HCN production, the bacterial strains were grown on an HCN induction medium (30 g tryptic soy broth, 4.4 g glycine, 15 g agar L^{-1}) at 28±2°C for four days. For each bacterial isolate, 100 µL of 10^8 cells mL^{-1} was placed in the centre of the petri plates. A disk of Whatman filter paper No. 1 dipped in 0.5% picric acid and 2% Na_2CO_3 was placed at the lid of the petri plates. Plates were sealed with parafilm. After four days incubation at 28±2°C, an orange brown colour of the paper indicating HCN production was observed. For ammonia production, the rhizobial strains were grown in peptone water (g L^{-1}: peptone 10 g, NaCl 5 g, pH 7) and incubated at 30±2°C for four days. One mL of Nessler reagent was added to each tube and the development of yellow color indicating ammonia production was recorded (Dye, 1962).

RESULTS

Rhizobacterial tolerance to metals: *Brayrhizobium* strains were tested for their tolerance towards various concentrations of heavy metals like cadmium, chromium, nickel, lead, zinc and copper using agar plate dilution method. Generally, *Bradyrhizobium* strains showed a varied level of tolerance to heavy metals. Among the *Bradyrhizobium* strains, strain RM8 showed highest tolerance to most of the metals (Fig. 1). Strain RM8 tolerated a concentration of 75, 200, 300, 1300, 1500 and 1000 µg mL^{-1} of cadmium, chromium, nickel, lead, zinc and copper, respectively, amended in agar plates.

Resistance of *Bradyrhizobium* to antibiotics: Resistance to antibiotics among metal tolerant rhizobacterial strains differed considerably (Table 1). Among *Bradyrhizobium* spp, 71% of strains were resistant to ampicillin, 35.7% to tetracycline and nitrofurantoin, 28.6% to methicillin while 14.3% were resistant to each cloxacillin and streptomycin. No strain was found to be resistant to nalidixic acid, gentamycin, ciprofloxacin and chloramphenicol.

Bioassay of plant growth promoting activities: The Plant Growth Promoting (PGP) substances like IAA, siderophore, hydrogen cyanide and ammonia synthesized by the metal tolerant PGPR strains were assayed both qualitatively and quantitatively under *in vitro* experiments and are explained as follows.

Indole acetic acid: The production of IAA by the metal tolerant bacterial strains was assayed in LB broth supplemented with different concentrations of tryptophan (Table 2). The *Bradyrhizobium* spp. exhibited a substantial production of IAA after 24 h of incubation. Moreover, the data revealed a concentration dependent increase in IAA, the maximum being 13.3, 10.2, 7.3, 6.2, 4.7 and 3.6 µg of IAA/mL in LB broth supplemented with 100, 80, 60, 40, 20 and 0 (without tryptophan) µg tryptophan/mL, respectively, by strain RM8. This was followed by strain RM2, which produced a maximum amount of 12.5, 9.5, 7.2, 5.8, 3.2 and 2.4 µg IAA/mL in LB broth supplemented with 100, 80, 60, 40, 20 and 0 µg tryptophan/mL, respectively.

Table 1: Resistant pattern of *Bradyrhizobium* to various antibiotics

Antibiotics used	Concentrations (µg $disc^{-1}$)	Number of resistant isolates (%)
Nalidixic acid	30	ND
Ampicillin	25	10 (71)
Gentamycin	30	ND
Tetracycline	30	5 (35.7)
Nitrofurantoin	100	5 (35.7)
Cloxacillin	30	2(14.3)
Streptomycin	25	2 (14.3)
Methicillin	30	4 (28.6)
Ciprofloxacin	30	ND
Chloramphenicol	25	ND

ND: Not detected, Total No. of isolates = 14

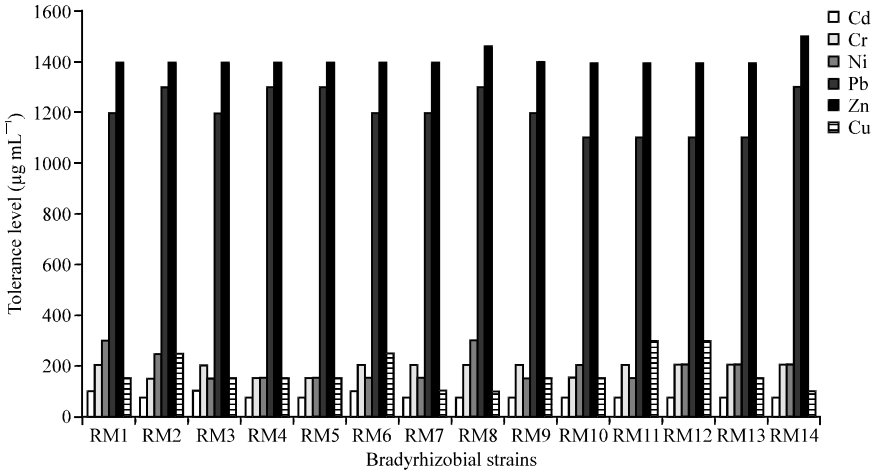

Fig. 1: Maximum tolerance level of *Bradyrhizobium* strains to different heavy metals

Table 2: Plant growth promoting activities of *Bradyrhizobium* species (N = 14) isolated from greengram nodules

	Plant growth promoting activity										
			Siderophores			Indole acetic acid (μg mL^{-1})					
Rhizobial strains	Ammonia	HCN[a]	Zone on CAS agar[b] (mm)	SA[c] (mg L^{-1})	2,3 DHBA[d] (mg L^{-1})	0 T[e]	20 T	40 T	60 T	80 T	100 T
RM1	+	+	8±1	15.5±0.8	14.1±1.1	2.2±0.4	2.5±0.5	6.0±0.9	7.2±1.0	9.7±1.3	12.2±1.2
RM2	+	+	9±1	15.8±0.9	15.0±1.3	2.4±0.5	3.2±0.7	5.8±0.7	7.2±1.1	9.5±1.0	12.5±1.4
RM3	+	-	-	-	-	2.2±0.3	2.6±0.6	5.5±0.5	7.0±0.9	9.0±1.2	11.1±1.1
RM4	+	-	-	-	-	2.0±0.3	2.5±0.5	4.8±0.4	6.2±0.8	8.0±1.3	10.8±0.9
RM5	+	-	-	-	-	2.0±0.5	2.3±0.6	4.3±0.5	5.9±0.5	7.9±1.0	10.3±0.8
RM6	+	-	-	-	-	2.1±0.4	2.6±0.7	4.7±0.6	6.5±0.6	8.6±1.0	10.6±0.9
RM7	+	-	-	-	-	2.0±0.3	2.5±0.4	5.0±0.7	7.0±0.7	8.1±1.1	10.7±1.2
RM8	+	+	12±2	17.4±0.6	16.3±1.2	3.6±0.6	4.7±0.8	6.2±0.4	7.3±0.6	10.2±0.8	13.3±1.4
RM9	+	-	-	-	-	2.4±0.4	2.8±0.3	3.9±0.3	5.7±0.5	7.1±0.7	9.1±0.9
RM10	+	-	-	-	-	2.2±0.5	2.8±0.4	5.1±0.6	6.9±0.7	8.6±0.6	10.5±1.0
RM11	+	-				2.2±0.4	2.7±0.5	3.6±0.4	4.6±0.5	5.8±0.5	7.4±0.6
RM12	+	-				1.3±0.3	2.1±0.3	4.2±0.6	5.5±0.6	6.6±0.4	8.3±0.8
RM13	+	-				1.4±0.3	2.3±0.5	5.8±0.6	7.0±0.7	8.6±0.8	13.1±1.0
RM14	+	-				1.5±0.4	2.1±0.3	4.3±0.5	5.8±0.5	7.3±0.7	9.1±0.7

[a]Hydrogen cyanide, [b]Chrome azurol S agar, [c]Salicylic acid, [d]2,3 Dihydroxy benzoic acid, [e]Tryptophan concentration (mg mL^{-1}), +: Indicates positive reaction, -: Indicates no reaction. The values indicate Mean±SD of three replicates

Bioassay of siderophore: Another important trait of plant growth promoting rhizobacteria is the production of siderophores that may indirectly affect the growth of plants. In the present investigation, the metal tolerant PGPR strains were tested for both qualitative and quantitative production of siderophores using CAS agar and ethyl acetate extraction method (Table 2). Among all the strains, RM1, RM2 and RM8 displayed 8, 9 and 12 mm colored zone on CAS plates after four days of incubation. Further, the ethyl acetate extraction from culture supernatant of *Bradyrhizobium* strain RM1 yielded 15.5 and 14.1 mg mL^{-1} salicylate (SA) and 2,3-dihydroxy benzoic acid (DHBA), strain RM2 produced 15.8 and 15 mg mL^{-1} of SA and DHBA and strain RM8 yielded 17.4 and 16.3 mg mL^{-1} SA and DHBA, respectively (Table 2).

***In vitro* assay of ammonia and HCN:** The metal tolerant plant growth promoting rhizobacterial strains were tested further for the synthesis of ammonia and hydrogen cyanide using peptone water and HCN induction medium, respectively. Generally, all PGPR strains were found positive for ammonia while as only strain RM1, RM2 and RM8 were positive for HCN (Table 2).

DISCUSSION

Higher concentration of metal in the soil is due the long deposition of these metals which adversely affects the microflora of the soil (Matsuda *et al*., 2002). Heavy metals show toxic impact on the microbes either by blocking their functional groups or they modify the active sites of the biological molecules. Many plant growth promoting rhizobacteria including symbiotic nitrogen fixing bacteria can growth at higher concentrations of heavy metals (Lakzian *et al*., 2002) and is the result of either intrinsic or induced mechanism (Giller *et al*., 1998). There are many reports which has shown a high level tolerance to heavy metals by rhizobacteria (Wasi *et al*., 2008; Wani and Khan, 2010). Many reports are available on the tolerance level of rhizobia, which could possibly be due to the variation in the tolerance level of rhizobia and growth conditions employed (Rajkumar *et al*., 2005). For instance, *Rhizobium leguminosarum* isolated from metal contaminated soil tolerated 92.9 μM of zinc (Delorme *et al*., 2003), Similarly Wasi *et al*. (2008) isolated *Rhizobium* RL9 from metal contaminated soil was resistant to the toxic effects of multiple heavy metals.

In the present study, *Bradyrhizobium* showed high resistance towards all the metals, maximum being towards lead and zinc, which was followed by copper and nickel, whereas cadmium was found to be less toxic than the other heavy metals. Bradyrhizobium strains were characterized by physiological and biochemical methods. Many studies have also shown tolerance towards these heavy metals. For instance, *Protobacteria, Actinobacteria* and *Bacteroidetes* were resistant to Zn and Cadmium (Kuffner *et al*., 2010). *Rhizobium* RL9 showed resistance towards multiple metals which were isolated from metal contaminated sites of Aligarh region. Nickel and zinc tolerance by *Rhizobium leguminosarum* biovar *trifolii* isolated from sewage sludge treated soil was also reported by Purchase and Miles (2001), who observed a metal tolerance of 0.24-0.26 mM Ni^{2+} and 6.0-8.0 mM Zn^{2+}. Similarly, metal tolerance by *Rhizobium*, *Bradyrhizobium* and *Azotobacter* (Pajuelo *et al*., 2008) and varying level of resistance among other PGPR (*Bacillus* and *Pseudomonas*) have also been reported (Yilmaz, 2003; Thacker *et al*., 2007; Wasi *et al*., 2008).

Resistance shown by many bacteria towards antibiotics is an emerging problem and is acquired by a their change in the genetic make up, can occur either by gene mutation or by transfer of antibiotic resistant genes between organisms in the environment (Spain and Alm, 2003). The use of antibiotics in health care as well as in agriculture, is also responsible for the resistance of bacteria towards antibiotics. Heavy metals as well as antibiotics which are increasingly used has created selective pressure in the environment which leads to the mutation in organism thus allowing the microbes to survive better and thus multiply. Genes on a plasmid, are important for the survival of the organism and are most likely transferred together in the event of conjugation. Thus, in an environment with multiple stresses, for example antibiotics and heavy metals, it would be more ecologically favorable in terms of survival for a bacterium to acquire resistance to both stresses. If the resistance is plasmid mediated, bacteria harbouring clustered genes are more likely to pass on those genes to other neighbouring bacteria which would then have a better chance of survival. With these considerations, the antibiotic resistance among PGPR was studied which differed from antibiotic to antibiotic for all the PGPR strains. Microorganisms showing resistance to many antibiotics which can be a factor for a high degree of tolerance to metals. In many studies, metal tolerance and antibiotic resistance have been reported (Wasi *et al*., 2008; Wani *et al*., 2009; Yilmaz, 2003; Verma *et al*., 2001). It is suggested that under metal stress, metal and antibiotic resistant microbes will adapt faster by R-factors than mutation and natural selection (Silver and Misra, 1988). Similar observations on antibiotics resistance by PGPR strains have been reported (Thacker *et al*., 2007). The variation shown by many microbes towards the resistance of antibacterial drugs (antibiotics) may possibly be due to the differences in growth conditions and stress conditions or toxic material as well as presence or absence of resistance mechanisms that could be encoded either by chromosome and/or R-plasmid (Spain and Alm, 2003).

In the present study Bradyrhizobium strains were positive for plant growth promoting activities and produced substantial amount of IAA, siderophore, HCN and ammonia. Bacterial strains produce ammonia; this ammonia plays a signalling role when rhizobacteria and plants interact with each other (Becker *et al*., 2002). Moreover, the ammonia released by the bacterial strains are known to increase the glutamine synthetase activity (Chitra *et al*., 2002). In addition, ammonium transporters found in several plant growth promoting rhizobacteria are thought to be involved in the reabsorption of NH_4+ released as a consequence of NH_3 diffusion through the bacterial membrane (Van Dommelen *et al*., 1997). Similarly phytohormone production (Wasi *et al*., 2008; Rajkumar *et al*., 2006; Ahmad *et al*., 2008) and IAA by rhizobia is reported (Antoun *et al*., 1998). Siderophores is also known to bind to the available form of iron Fe^{3+} in the rhizosphere thus making it unavailable to the phytopathogens and consequently protects the plant health. In other study, the heavy metal resistant *Bacillus* species are also known to produce considerable amount of plant growth promoting substances (Wani *et al*., 2007a, b).

CONCLUSION

This study concluded that the *Bradyrhizobial* strains were tolerant not only to heavy metals but also to antibiotics. They also produced substantial amount of plant growth promoting substances. Due to multifarious properties shown by *Brazyrhizobial* strains, these strains could therefore, be used as bioinoculant to remediate metals in metal contaminated soils and also increase the performance of crops in soils contaminated with heavy metals.

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