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Toxicity, PGP Activity, Bioaccumulation of Cadmium, Copper and Chromium (VI) in Nitrogen-fixing Rhizobacteria

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ABSTRACT

Heavy metal pollution of soil is a significant environmental problem and has its negative impact on human health and agriculture. Rhizobial isolates present in rhizospheric region can play a key role in remediation of polluted sites, in which, microbial populations are known to affect heavy metal mobility and availability to the plant through release of indole acetic acid, siderophores, ammonia, phosphate solubilizing agents and other Plant Growth Promoting (PGP) substances and therefore, have potential to enhance bioremediation processes. Bioremediation strategies with suitable heavy metal-adapted rhizobacteria have received a lot of attention. This study tells the effect of heavy metals (Cd^{2+}, Cu^{2+} and Cr^{6+}) on the growth of different rhizobial cultures, their PGP producing ability and significance of bioaccumulation of toxic heavy metals on rhizobium.

Key words: Rhizobium, rhizobacteria, plant growth promoting activity, heavy metals, bioaccumulation

INTRODUCTION

With the rapid industrialization and development of the agricultural practices, heavy metal pollution in soil has increasingly becomes a serious threat to the environment. Giller *et al.* (1998) reported that metal-polluted environment poses a damaging effect to soil microbial diversity and microbial activities (indices of microbial metabolism and of soil fertility). Their accumulation on the culturable layer of soil changes the trace element profile and thereby causing physiological and genetic changes to various lives (Mudakavi and Narayana, 1997; Chhonkar *et al.*, 2006). Different techniques developed so far for the metal removal, are quite expensive and requires use of contaminating product for desorption of metals and for cleaning up of inorganic matrix. Removal of toxic heavy metal from the soils is of great importance not only because of the decontamination effect but also because this removal protect plants from the effect of toxic metal and ensures the functioning of plants. Metal accumulation is an alternative mechanism for metal detoxification in bacteria (Gadd, 1990). Bacteria have evolved several types of mechanisms to tolerate the heavy metal ions. These include reduction of the heavy metal ions to a less toxic state (Nies, 1999), the

efflux of metal ions outside the cell, accumulation and formation of complexes and synthesis of binding proteins like MTs and PCs (Rajendran *et al*., 2003).

Heavy metals, when present in concentration greater than the normal in soils, are known to affect both the beneficial rhizospheric microflora and crops. As a common rhizospheric microorganism, *Rhizobium*, exists everywhere in the soil but is largely found symbiotic partner of leguminous plants. Rhizobia facing heavy metal contaminations also develop resistance and show effective nodulations (Smith and Giller, 1992). Besides the plant growth promoting activity, several reports have shown the response of rhizobium against salts and heavy metals on the metabolic properties and stress tolerance of leguminous plants (Abbas and Kamel, 2004). Elevated resistance against Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} in *Rhizobium leguminosarum* has shown the presence of high intracellular carbohydrates and larger cell inclusions (Purchase *et al*., 1997).

With these views, the aim of this study was to determine Minimum Inhibitory Concentration (MIC) for different heavy metals and their binary combinations, to screen out the most resistant bacteria by observing growth rate experiment under heavy metal stress, to verify Plant Growth Promoting (PGP) activity in normal and heavy metal stress conditions from the selected resistant isolates and to observe the bioaccumulation potential of the most resistant isolates.

MATERIALS AND METHODS

Isalation of rhizobial strains: Nodules of chick-pea (*Cicer aurentium*) and pea (*Pisum sativum*) grown at the experimental fields were collected. The nodules, after surface sterilization, crushed and serially diluted into normal saline solution. The nodular suspension was plated on solid Yeast Extract Mannitol (YEM) agar plates with Congo red. The plates were incubated at 28±2°C for 3 days. Red, mucoid, gummy and glistening colonies were again streaked thrice on solid YEM agar plates for checking purity. The mucoid and gummy colonies were maintained on YEM agar slants at 4°C.

All the morphological, physiological and biochemical tests of the cultures were performed as per standard procedures.

PLANT GROWTH PROMOTING ACTIVITY EVALUATION

- **Indole Acetic Acid (IAA) production:** IAA production of the rhizobial isolates was quantitatively assayed using Luria Bertani (LB) by the method of Bric *et al*. (1991) after 48, 72 and 96 h. Nine milliliter of LB broth amended with tryptophan (100, 500 mg 100 mL^{-1}) was inoculated with a loopful culture (10^8 cells mL^{-1}) and incubated at 28±2°C for 48, 72 and 96 h. After incubation, 3 mL of each culture was agitated at 10,000 rpm for 15 min. To the 2 mL of supernatant, 2 mL of Salkowasky reagent was added along with few drops of orthophosphoric acid and re-incubated for 1 h under dark. The concentration of IAA was measured using spectrophotometer (λ = 540 nm) against the standard IAA curve
- **Siderophore production:** Siderophore production was determined by the $FeCl_3$ test (Jalal and Vander Helm, 1991). Overnight grown cultures of each isolate was inoculated with 10 mL of nutrient broth and grown for 4 days at 28±2°C. The cultures were then centrifuged at 3000 rpm for 20 min. To 1 mL of supernatant, 1 mL of 2% aqueous $FeCl_3$ solution was added to each tube. Formation of reddish brown to red color indicated the siderophore production

- **Ammonia production:** Production of ammonia was checked by addition of Nesseler's reagent in incubated rhizobial isolates under peptone water. A 10 mL peptone water was incorporated with isolates and overgrown for 4 days at 28±2°C. After incubation, 1 mL of Nesseler's reagent was added to each tube and the color was observed. The development of yellow color indicated the positive results

Rhizobial strains and tolerance of heavy metals: MIC of cadmium, chromium (VI) and copper was determined by Summers and Sliver (1972) method. The Cd^{2+}, Cr^{6+}, Cu^{2+} and their combinations were taken as $CdCl_2$, $K_2Cr_2O_7$ and $CuCl_2$ in varying concentration from 6.25 to 3200 μg mL^{-1} and were amended in sterilized YEM agar plates. The plates were then spot inoculated with a loopful of test cultures. Plates were incubated at 28±2°C for 24 h. The concentration at which no growth observed was regarded as MIC of the isolate against that metal.

Growth test of rhizobial isolates: Growth rate of rhizobial isolates resistant to these metals were determined by growing them in 50 mL of YEM broth (YEMB) medium with and without heavy metal(s) using subMIC value of each metal at 28±2°C for 60 h. After incubation, OD was taken at λ = 650 nm for the observation of growth.

Evaluation of bioaccumulation property of resistant rhizobial strains: To test the bioaccumulation of these isolates, we used Da Costa and Duta (2001) method with a slight modification. Resistant cultures were grown in Erlenmeyer flasks containing 50 mL of sterilized YEMB and YEMB with glucose (0.5%) at subMIC level of the respective metal for 60 h on rotatory shaker incubator. Whole broth was centrifuged for pellet and then digested in nitric acid solution (98%). The digested solution was used for quantification of the heavy metal by atomic absorption spectrophotometer (GBC, Australia).

RESULTS AND DISCUSSION

With the development, many of the metals after their discharge accumulate into the soil and in turn affect the equilibrium of the soil ecosystem. The strains of *Mesorhizobium ciceri* (chickpea) and *Rhizobium leguminosarum* (pea) formed white, gummy, glistening and translucent colonies on YEM agar plates. Generally, they were short rods, semi translucent, convex, raised and Gram negative. All the mesorhizobial and rhizobial isolates were positive for catalase, but negative for starch and gelatin hydrolysis activity. Interestingly, 20 and 40% of the isolates obtained from chickpea and pea were negative for citrate utilization (data not shown).

Chick-pea (100%) and pea rhizobia (84%) were positive for ammonia production, whereas 50 and 46% of the same isolates showed siderophore production (Table 1). The PGP activity of the tested strains differed considerably in 100 and 500 μg 100 mL^{-1} of tryptophan supplementation. Generally, the maximum amount of IAA was produced at 500 μg 100 mL^{-1} of tryptophan by all the mesorhizobial and rhizobial strains. Among chickpea rhizobia, *M. ciceri* RC5 produced a maximum amount of 39.3 μg mL^{-1} of IAA at 100 μg 100 mL^{-1} of tryptophan at 72 h of incubation at 28±2°C. In comparison, RC8 showed a greater production of 216.7 μg mL^{-1} at 500 μg 100 mL^{-1} of tryptophan with same conditions. In the *R. leguminosarum* strains, among the two concentrations of tryptophan, the maximum production of IAA was observed at 500 μg 100 mL^{-1}, where RP12

Table 1: Plant growth promoting (PGP) activity of *Mesorhizobium ciceri* and *Rhizobium leguminosarum* bv. *viciae*

	Name of isolate											
PGP activity of												
Mesorhizobium ciceri	RC1	RC2	RC3	RC4	RC5	RC6	RC7	RC8	RC9	RC10	RC11	RC12
Ammonia production	+++	+++	++	+	+++	+++	++++	+++	+++	+++	+++	++++
Siderophore production	++++	-	+	-	++	-	-	-	++	-	+++	++
IAA production at 100 µg 100 mL^{-1}												
48 (h)	0.8	8.7	5.9	8.	19.0	23.4	4.1	22.5	14.4	20.0	5.5	10.5
72 (h)	8.7	19.8	14.8	19.6	39.3	34.3	14.1	41.2	26.4	30.6	19.6	21.6
96 (h)	2.9	13.4	10.0	12.3	23.4	23.4	5.3	27.8	18.2	27.4	12.3	15.9
IAA production at 500 µg 100 mL^{-1}												
48 (h)	11.7	44.3	62.7	80.8	120.0	141.7	85.0	128.7	67.7	94.3	28.3	39.3
72 (h)	59.3	100.8	123.3	141.7	194.2	214.3	134.3	216.7	106.7	132.7	71.7	70.5
96 (h)	31.0	64.2	92.7	113.3	140.0	182.5	116.7	184.3	100.0	126.0	107.0	57.5
	Name of isolate											
PGP activity of												
Rhizobium leguminosarum	RP1	RP2	RP3	RP4	RP5	RP6	RP7	RP8	RP9	RP10	RP11	RP12	RP13
Ammonia production	+	-	+	+++	+	-	+	++	++	++	++	+++	+++
Siderophore production	+	+++	-	-	-	+	-	++	-	++	-	-	+
IAA production at 100 µg 100 mL^{-1}												
48 (h)	3.7	13.30	10.0	8.7	11.7	6.0	18.9	26.4	9.3	2.1	3.3	16.2	15.30
72 (h)	12.6	19.58	24.1	24.1	24.4	9.3	37.4	39.7	17.7	6.0	5.9	24.4	22.20
96 (h)	8.0	12.00	12.8	19.0	16.9	5.3	28.7	33.0	7.7	3.3	3.2	18.1	10.00
IAA production at 500 µg 100 mL^{-1}												
48 (h)	61.7	79.17	45.8	25.8	35.0	11.7	71.8	92.7	68.3	67.5	62.5	111.7	70.70
72 (h)	145.3	152.50	140.0	87.3	136.7	49.2	128.3	177.5	136.7	128.3	133.3	201.6	128.3
96 (h)	100.8	113.30	85.0	64.2	84.2	25.0	89.0	115.0	97.5	98.3	95.0	131.7	99.20

RC: *Mesorhizobium ciceri*, RP: *Rhizobium leguminosarum*, -: No production, +: Low production, ++: Moderate production, +++: Higher production, ++++: Highest production

Table 2: Minimum inhibitory concentration (MIC) of rhizobial isolates towards heavy metals (HMs)

	Rhizobial isolates											
Heavy metal and its combination	RC3	RC4	RC5	RC6	RC7	RC8	RC9	RC10	RC11	RP2	RP5	Mean*
Cd^{2+}	25.00	25.00	50.00	25	50.00	25.00	12.50	6.25	6.25	6.25	6.25	21.6
Cu^{2+}	12.50	50.00	6.25	50	12.50	50.00	50.00	50.00	50.00	25.00	12.50	33.5
Cr^{6+}	50.00	25.00	12.50	25	6.25	6.25	50.00	50.00	12.50	25.00	25.00	26.1
Cd^{2+}+Cu^{2+}	25.00	50.00	12.50	100	50.00	12.50	25.00	100.00	25.00	25.00	25.00	40.9
Cu^{2+}+Cr^{6+}	12.50	25.00	6.25	50	12.50	25.00	25.00	12.50	50.00	100.00	100.00	38.1
Cr^{6+}+Cd^{2+}	50.00	12.50	12.50	25	12.50	12.50	12.50	25.00	12.50	50.00	6.25	21.0

*Mean value is calculated by adding MIC of all rhizobial isolates and dividing them by No. of isolates

increased the IAA by 7 fold, while RP8 had increased the IAA by 300 folds above the 100 µg 100 mL^{-1}. Generally, the production of IAA by all the isolates at different incubation periods decreased in the order 72<96<48 h (Table 1). This finding is in close agreement to those referred by the Tank and Saraf (2003).

Generally, the MIC value of all the rhizobial stains towards heavy metal varied considerably (Table 2). Among the mesorhizobial isolates, the highest MIC value of chromium (VI), copper and cadmium was 50 after 24 h of growth in nutrient broth medium. However, in dual metal treatment,

the MIC value decreased for pea rhizobial strains against single and dual metal treatments. While comparing the sum of the mean values of MIC of each metal used singly, the toxicity decreased in the order: Cadmium<chromium<copper. The variation in the toxicities of heavy metals (HMs) towards rhizobial populations could be due to the differences in their metabolism, e.g., by thick polysaccharide capsule (Ehrlich, 1997) that might have prevented the uptake of specific metal into the cell; alternatively, low toxicity to heavy metals in the rhizobial species could be due to the generation of alkaline environment by the rhizobia which in turn helps to reduce metal solubilization and thereby activity of HMs. Cadmium was found as most toxic metal for *Mesorhizobium* and *Rhizobium* strains. The toxicity of Cd^{2+} could be due to the results of inactivation or inhibition of certain metal enzymes of the nodule bacteria (Ehrlich, 1997; Table 2).

It is evident from the study that when two metals were combined, the toxicity on tested cultures, in general, was increased. The levels of resistance that substantially increased against all the isolates could possibly be due to the synergistic effect of one metal on another metal ion. When Cu^{2+} was combined with Cr^{6+}, resistance level increased upto less than 100 μg mL^{-1} from less than 25 μg mL^{-1} of Cu^{2+} for RP2. Likewise, Cd^{2+} when combined with Cr^{6+}, MIC increased to greater than twice for *Mesorhizobium ciceri* RC3 compared to the single application of Cd^{2+}. However, the toxicity of the Cd^{2+} was increased in comparison with other combinations (on the basis of mean value calculated). The increase in resistance by the cultures towards the pairing of metals could possibly be due to the antagonistic effects between two HM ions (Table 2).

The cultures resistant to cadmium, copper and chromium (VI) were tested to evaluate their growth pattern in YEMB supplemented with HMs at their subMIC level. It was generally found that under HM stress, the growth rate were almost reduced by a factor of 2 in case of Cr^{6+}, Cu^{2+}, Cr^{6+}+Cu^{2+} and Cd^{2+}+Cu^{2+}. In case of Cd^{2+}, it decreases by 4. However, in Cd^{2+}+Cr^{6+} combination, growth rate of RC3 decreased 8 times as compared with 3 times reduction of RP2 populations after 48 h growth in YEMB medium. Thus, among single and dual combinations, the Cd^{2+}+Cr^{6+} combination had the greatest toxic effects on RC3 and RP2 populations compared to other metal treatments (Fig. 1).

The rhizobial strains showing resistance to Cr^{6+}, Cu^{2+} and Cd^{2+} were tested to determine their PGP activity under HM stress condition. The selected cultures showed production of ammonia and siderophore in both single and dual treatments (Table 3). RC9 (resistant to Cu^{2+}) showed the least decline in IAA production having Cu^{2+} alone (50 μg mL^{-1}) compared to the medium free of Cu^{2+}. In comparison, RC6 (resistant to Cd^{2+}+Cr^{6+}) showed maximum decrease (15.4%) in IAA production after 72 h of incubation compared to the medium with no HMs (Table 4a, b).

In YEMB medium having 25 μg of Cu^{2+}, *M. ciceri* RC9 accumulated 0.76 μg 50 mL^{-1}. However, the accumulation increased to 1.13 μg 50 mL^{-1}, when glucose was supplemented. Moreover, the bioaccumulation of Cu^{2+} and Cd^{2+} by the resistant rhizobial isolates increased considerably when cultures were grown in YEMB along with glucose than medium devoid of glucose. These findings suggest that glucose aguments the bioaccumulation process of Cu^{2+} and Cd^{2+}. In contrast, a greater accumulation of chromium was observed with cultures, when they were grown only in YEMB medium as compared to glucose supplemented YEMB (Fig. 2). However, no significant difference in bioaccumulation of HMs was observed with *R. leguminosarum* strain RP2 in YEMB with or without glucose.

Table 3: Comparison of plant growth promoting activity by resistant isolates of Rhizobium under normal and heavy metal strains

Rhizobial isolate	Heavy metal	Ammonia production under normal condition	Ammonia production under metal stress	Siderophore production under normal condition	Siderophore production in metal stress
RC3	Cr^{6+}	++	+	+	-
RC9	Cu^{2+}	+++	-	++	+
RC7	Cd^{2+}	++++	+	-	-
RC6	Cd^{2+}+Cr^{6+}	+++	-	-	-
RP2	Cu^{2+}+Cr^{6+}	-	-	+++	+
RP2	Cd^{2+}+Cu^{2+}	-	-	+++	+

RC: *Mesorhizobium ciceri*, RP: *Rhizobium leguminosarum*, -: No production, +: Low production, ++: Moderate production, +++: Higher production, ++++: Highest production

Table 4: Comparative production of IAA in normal and metal amended LB broth

Rhizobial isolate	Heavy metal	Isolates grown in LB (h)			Isolates grown in LB with metal (h)			Percentage decrease in IAA production (%) (h)		
		48	72	96	48	72	96	48	72	96
(a) IAA production by resistant isolates 100 µg 100 mL^{-1}										
RC7	Cd^{2+}	4.10	14.10	5.30	ND	ND	ND	ND	ND	ND
RC9	Cu^{2+}	14.40	26.40	18.20	8.67	17.67	14.08	5.75	8.75	4.39
RC3	Cr^{6+}	5.90	14.80	10.00	ND	ND	ND	ND	ND	ND
RP2	Cd^{2+}+Cu^{2+}	13.30	19.58	12.00	ND	ND	ND	ND	ND	ND
RP2	Cu^{2+}+Cr^{6+}	13.30	19.58	12.00	3.51	8.64	5.01	10.61	10.94	7.75
RC6	Cd^{2+}+Cr^{6+}	23.40	34.30	23.40	10.00	18.92	14.08	13.42	15.41	14.34
(b) IAA production by resistant isolates 500 µg 100 mL^{-1}										
RC5	Cd^{2+}	120.0	194.2	140.0	34.78	68.51	42.92	71.02	64.72	69.34
RC7		85.0	134.3	116.7	ND	38.43	25.84	ND	71.83	77.86
RC6	Cu^{2+}	141.7	214.3	182.5	76.84	134.25	117.36	45.77	37.35	35.69
RC9		67.7	106.7	100.0	41.36	72.54	68.08	38.91	32.01	38.92
RC3	Cr^{6+}	62.7	123.3	92.7	ND	ND	ND	ND	ND	ND
RP2	Cd^{2+}+Cu^{2+}	79.2	152.5	113.3	ND	48.86	37.25	ND	67.96	67.12
RP2	Cu^{2+}+Cr^{6+}	79.2	152.5	113.3	27.81	52.48	38.94	64.89	65.59	65.63
RC6	Cd^{2+}+Cr^{6+}	141.7	214.3	182.5	40.87	64.56	52.67	71.16	69.87	71.14

RC: *Mesorhizobium ciceri*, RP: *Rhizobium leguminosarum*, ND: Not detected, -: No production, +: Low production, ++: Moderate production, +++: Higher production, ++++: Highest production

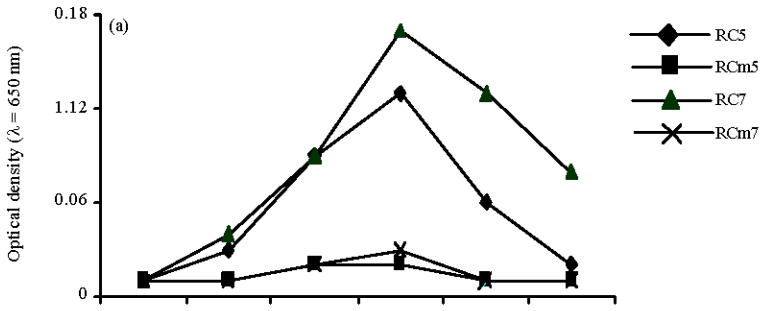

Fig. 1(a-f): Countinue

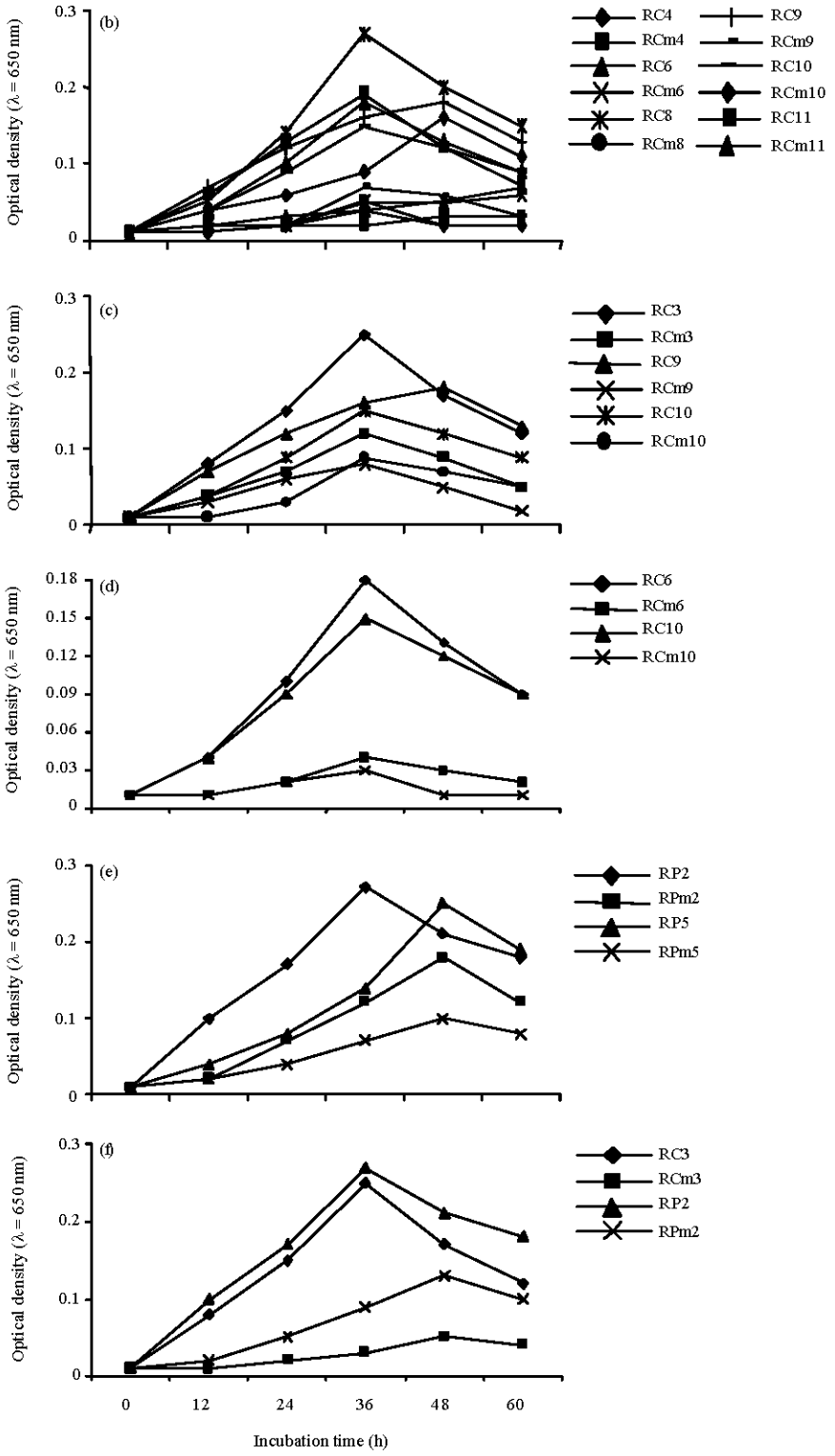

Fig. 1(a-f): Growth rate of rhizobial isolates under normal (a) Cd^{2+}, (b) Cu^{2+}, (c) Cr^{6+}, (d) Cd^{2+}+Cu^{2+}, (e) Cu^{2+} and Cr^{6+} and (f) Cr^{6+} and Cd^{2+} added medium

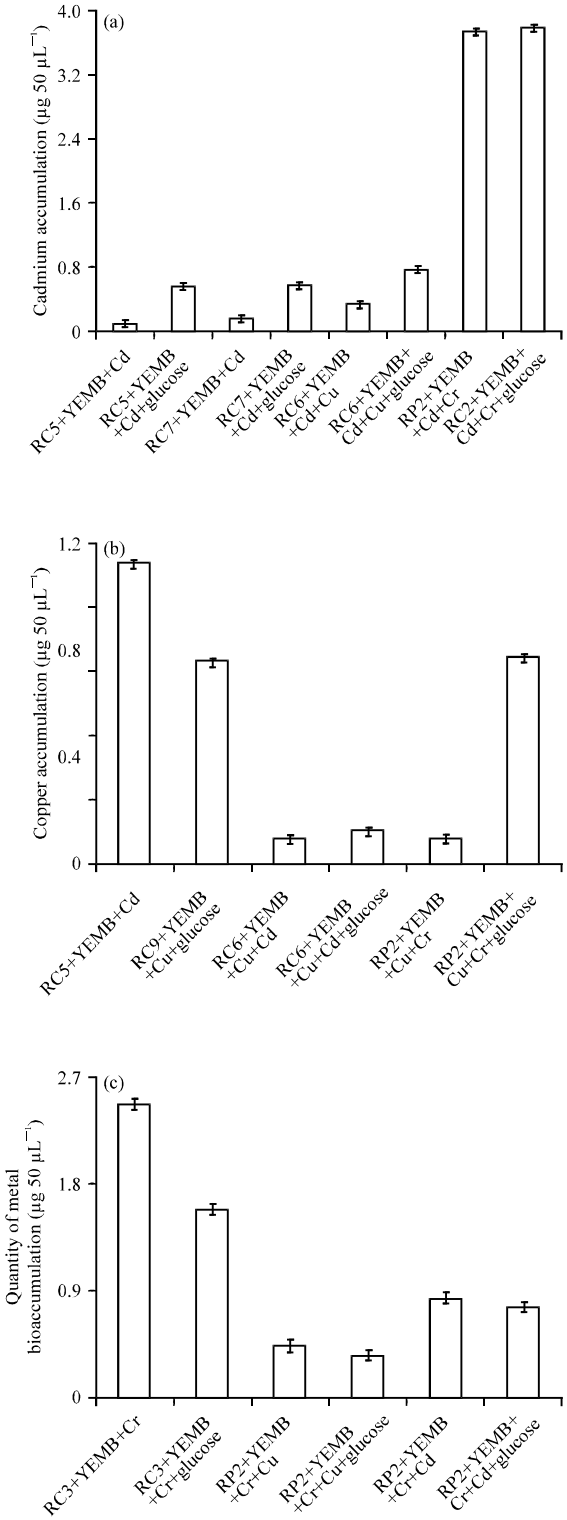

Fig. 2(a-c): Bioaccumulation of Cd^{2+} heavy metal (a) Yeast extract mannitol broth (YEMB) medium, (b) Different isolates and (c) Rhizobial isolate

CONCLUSION

The symbiotic nitrogen-fixing organisms, in addition to providing nitrogen to the legume crops, can also significantly affect the distribution of metals in the environment. This study further suggests that the symbiotic N_2-fixer can be used in effective, economical and eco-friendly metal bioremediation technologies. Conclusively, root nodule bacteria can be used for cleaning up of the soils contaminated with heavy metals.

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