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RESEARCH ARTICLE

Effect of Heavy metals on Growth and PGPR activity of Pseudomonads

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Abstract

Effect of heavy metals such as copper, chromium, nickel and cadmium on growth of *Pseudomonas* as well as PGPR activity was analyzed in this study. Minimum Inhibitory Concentration (MIC) of copper, chromium, nickel and cadmium against *Pseudomonas aeruginosa* strains were 186.9±29.60, 88.0±12.36, 153.81±34.38 and 130.54±28.21 µg/mL respectively. MIC of copper, chromium, nickel, cadmium against *P. putida* strains were 195.45±28.76, 82.72±12.97, 164.54±30.08 and 138.63±33.42 µg/mL. Similarly, MIC of copper, chromium, nickel and cadmium against *P. cepacia* strains were 188.84±27.1, 85.38±14.26, 169.61±28.16 and 137.69±30.00 µg/mL. MIC of copper, chromium, nickel, cadmium against *P. fluorescens* strains were 192.97±28.1, 82.43±13.03, 150.27±37.54 and 144.59±28.92 µg/mL. Present study showed that heavy metals reduced growth as well as Plant growth-promoting rhizobacteria (PGPR) activity of previously characterized *Pseudomonas* strains.

Keywords: Heavy metals, *Pseudomonas*, minimum inhibitory concentration, PGPR, copper, chromium.

Introduction

Pseudomonas sp. is ubiquitous bacteria in agricultural soils and has many traits that make them well suited as Plant growth-promoting rhizobacteria (PGPR). The most effective strains of *Pseudomonas* have been *fluorescent Pseudomonas* spp. Considerable research is underway globally to exploit the potential of one group of bacteria that belong to *fluorescent Pseudomonas*. *Pseudomonas* strains produced indole acetic acid (IAA), siderophore, HCN (hydrogen cyanide) and P-solubilzation (Deshwal and Kumar, 2013). Kannahi and Kowsalya (2013) isolated *Pseudomonas fluorescens* from rhizosphere soil of *vigna mungo* and observed that *Pseudomonas* strains effectively produced PGPR activity. IAA belongs to auxin family which controls many important physiological processes such as cell enlargement and division and tissue differentiation (Teale *et al.,* 2006). *Pseudomonas aeruginosa* showed maximum IAA production among 103 *fluorescent Pseudomonas* (Khare and Arora, 2010). Sasirekha *et al.* (2012) also reported that *P. aeruginosa* strains effectively produced IAA.

Phosphorous is an essential nutrient for plant growth but often limiting its growth due to its low solubility and fixation in the soil. Similarly, Parani and Saha (2012) mentioned that phosphorus deficiency is a major constraint in crop production due to rapid binding of the applied phosphorus into fixed forms not available to the plants. Phosphate solubilizing bacteria improved soluble phosphorous content in soil. Recently, Rajasankar *et al.* (2013) screened phosphate solubilizing *Pseudomonas* from pesticide polluted field soil.

Parani and Saha (2012) reported *P. fluorescens* K-34 solubilized tricalcium phosphate and produced substantial amount of soluble phosphorus (968.5 mg/L) in Pikovskaya's (PVK) broth. Siderophores are low molecular weight, non-ribosomal peptides, secreted under low iron stress conditions and absorb iron from soil (Budzikkiewicz, 1993). *Pseudomonas* is a potentent bio-control agent and produces siderophore that sequester iron in the root environment, making it less available to competing deleterious microflora (Kloepper *et al.*, 1980; Bagnasco *et al.*, 1998; Deshwal *et al.*, 2012). Tailor and Joshi (2012) extensively screened siderophore producing bacteria from sugarcane rhizosphere and isolated *Pseudomonas fluorescens* S-11 as efficient siderophore producer (96% SU).

Pseudomonads produce HCN which controls the root rot pathogen. Thomshow and Weller (1995) observed beneficial effect on plants by the production of diverse microbial metabolites like HCN. Kaur and Sharma (2013) isolated HCN producing *Pseudomonas* from 25 soil samples from healthy chickpea rhizosphere. Similarly, Deshwal and Kumar (2013) isolated HCN producing *Pseudomonas aeruginosa*, *P. putida*, *P. cepacia* and *P. fluorescens*. Heavy metals like copper (Cu), iron (Fe), and zinc (Zn) are required in trace amounts for microbial growth but it is toxic if present in excess. Chromium is an essential micronutrient required for the growth of many organisms. But high concentration is toxic, carcinogenic and teratogenic (Poornima *et al.*, 2010). Nickel is one of the most common metal contaminants in environment and high concentration of nickel is harmful.

Nickel toxicity is generally a consequence of nickel binding to sulf-hydryl groups of sensitive enzymes or displacing essential metal ions in variety of biological processes (Valko *et al.*, 2005; Cheng *et al.*, 2009). Cadmium is introduced into the bodies of water from smelting, metal plating, cadmium-nickel batteries, phosphate fertilizer, mining, pigments, stabilizers, alloy industries and sewage sludge (Nanganuru and Korrapati, 2012). Such type of toxicity in biological systems occurs through a variety of mechanism. These heavy metals disrupt protein structure or its function (Teitzel *et al.*, 2006). Scientists are gaining considerable attention to few heavy metals which are non-degradable such as mercury, lead, chromium, nickel, copper, cadmium and zinc (Raghuraman *et al.*, 2013). These metals disrupt the soil fertility as well as reduce the microbial load. Available literature suggests that few microorganisms can survive in presence of high concentration of metal. Cazorla *et al.* (2002) collected *P. syringae* pv. *syringae* isolates for copper resistance showed that 59% were resistant to cupric sulfate. Rahman *et al.* (2007) reported a strain of *Pseudomonas* sp. C-171 capable of tolerating hexavalent chromium (Cr^{+6}) up to 2000 ppm as potassium dichromate. Poornima *et al.* (2010) studied chromium degrading *Pseudomonas* SP8 showed 90% chromium degradation. *Pseudomonas aeruginosa* PDMZnCd2003 is a plant growth promoting metal resistant bacteria which showed different type of mechanisms respond in Cd as compared to Zn and Zn+Cd (Meesungnoen *et al.*, 2012). Few researches indicated about genes responsible for metal resistance in bacterial populations and czc resistance determinant is responsible for cadmium, zinc, and cobalt resistance systems in gram negative bacteria (Nies *et al.*, 1989). Against these backdrops, this study was carried out to evaluate the effect of heavy metals on the growth and PGPR activity of previously characterized *Pseudomonas* strains.

Materials and methods

Pseudomonas strains: Previously characterized strains namely 55 *Pseudomonas aeruginosa*, 22 *P. putida*, 26 *P. cepacia* and 37 *P. fluorescens* strains were selected for the present study (Deshwal *et al.*, 2013; Deshwal and Kumar, 2013).

Effect of heavy metals on growth of Pseudomonas strains: Copper, chromium, nickel and cadmium were selected for the present study and individual metal effect was analyzed. Heavy metal incorporated into trypticase soy agar medium at concentrations of 10-300 µg/mL. Four cultures were streaked on agar medium.

Effect of heavy metals on PGPR of Pseudomonas strains: Various concentrations of copper, chromium, nickel and cadmium were added as mentioned above and the following tests were conducted.

Indole production test: Heavy metal was added to Tryptone broth and transferred into test tubes. Control tube did not contain heavy metal. After sterilization, these test tubes were then inoculated with the culture and one tube was kept uninoculated as control. These inoculated tubes were incubated at 28°C for 24 h. After 24h of incubation, 1 mL of kovac's reagent was added to each tube including control, shaked the tubes gently after intervals for 10-15 min and allowed tubes in standing position. Development of cherry red color in the top layer of the tube indicated a positive result.

HCN production: Pseudomonas strains were streaked on Metal-TSM medium and the plates were supplemented with 4.4 g/L glycine with simultaneously supplemented filter paper soaked in a 0.5% picric acid in 1% Na_2CO_3 in the upper lid of petri plate. The plates were sealed with paraffin and control plates did not receive any *Pseudomonas* inoculum. Plates were incubated at 28°C for 1-2 d. Change in color of the filter paper from yellow to brown indicated a positive result.

Siderophore production: Pseudomonas strains were spread over metal-tryptic soya agar medium and tryptic soya agar. Plates ware incubated at 28±1°C for 24 h. Thereafter, a thin layer of CAS reagent in 0.7% agar was spread over the colonies of *Pseudomonas* and plates were reincubated at 28±1°C for 24-48 h. Observe formation of yellow-orange halo around the colony shows siderophore production.

P-solubilization test: Characterized *Pseudomonas* strains were transferred on metal-Pikovskya's agar medium and Pikovskya's agar medium. Plates were inoculated at 28±1°C for 3-5 d and clear zone around the colony showed P-solubilization.

Results

Heavy metals such as Cu, Cr, Ni and Cd showed effects on growth as well as plant growth activity of isolated *Pseudomonas* strains. Minimum inhibitory concentrations of Cu, Cr, Ni and Cd against *Pseudomonas aeruginosa* strains ranged from 120-250, 60-120, 70-210, 80-180 µg/mL respectively (Table 1). MIC of Cu, Cr, Ni and Cd against *P. putida*, *P. cepacia* and *P. fluorescens* ranged from 150-250, 60-110, 80-210, 80-180 µg/mL (Table 2, 3 and 4). Further, results suggested that heavy metal reduced indole acetic acid, hydrogen cyanide, siderophore production and P-solubilization (Table 5). *Pseudomonas aeruginosa* with copper showed 32 IAA, 29 HCN, 28 siderophore productions and 29 P-solubilization. Chromium and cadmium reduced more PGPR activity of *P. aeruginosa* as compared to copper and nickel. Copper reduced more IAA and P-solubilization of *P. putida* as compared to other metals. Comparatively, Cd reduced more HCN and siderophore production of *P. putida*.

Table 1. Effect of heavy metals on growth of *P. aeruginosa*.

Strains	MIC (μg/mL)			
	Cu	Cr	Ni	Cd
PW-1	150	100	190	160
PW-4	180	90	200	150
PW-6	250	110	210	180
PW-9	160	90	120	110
PW-12	160	100	130	150
PW-13	180	60	90	80
PW-14	160	90	120	110
PW-20	180	60	180	120
PW-21	150	70	100	80
PW-24	160	80	110	130
PW-27	250	100	190	170
PW-29	200	80	140	120
PW-31	160	110	150	90
PW-32	180	70	90	90
PW-33	180	60	90	80
PW-36	110	100	170	100
PW-42	220	70	160	170
PW-47	170	110	190	110
PW-48	120	80	90	160
PW-52	210	100	180	170
PW-54	250	110	190	150
PW-57	120	80	120	180
PW-58	150	80	90	120
PW-59	180	90	100	150
PW-63	130	70	190	140
PW-64	240	80	90	130
PW-70	180	100	120	140
PW-72	150	90	100	120
PW-74	220	90	190	170
PW-77	160	80	200	130
PW-79	230	100	180	80
PW-81	150	120	100	120
PW-84	160	110	170	90
PW-85	240	90	180	130
PW-86	190	80	140	150
PW-90	170	110	130	170
PW-91	240	100	130	120
PW-95	160	80	190	80
PW-96	220	90	210	90
PW-97	180	80	140	90
PW-98	200	80	160	150
PW-99	240	100	160	170
PW-100	190	70	190	90
PW-105	210	70	200	180
PW-108	240	90	170	150
PW-111	160	80	160	160
PW-115	180	100	170	160
PW-118	220	90	160	90
PW-123	170	80	200	150
PW-125	190	90	140	170
PW-128	250	70	150	80
PW-131	180	80	210	180
PW-135	190	70	120	120
PW-136	220	100	210	140
PW-140	190	110	200	110
5^{th} percentile	127	67	90	80
90^{th} percentile	240	110	200	170
Mean value	186.90	88.00	153.81	130.54
SD	29.60	12.36	34.38	28.21

Table 2. Effect of heavy metals on growth of *P. putida*.

Strains	MIC (μg/mL)			
	Cu	Cr	Ni	Cd
PW-2	250	100	210	160
PW-10	250	90	160	80
PW-11	160	80	180	120
PW-15	180	90	200	180
PW-16	150	60	200	80
PW-26	190	60	80	110
PW-41	160	110	160	120
PW-49	250	80	180	180
PW-50	180	70	170	160
PW-55	160	90	190	170
PW-56	190	60	200	80
PW-62	170	80	120	160
PW-82	180	110	200	160
PW-83	200	80	180	180
PW-93	240	70	80	110
PW-101	190	90	160	100
PW-109	220	110	200	80
PW-110	150	80	170	150
PW-120	240	90	80	160
PW-124	170	70	190	160
PW-134	190	90	150	170
PW-137	230	60	160	180
5^{th} percentile	150.5	60	80	80
90^{th} percentile	249	109	200	180
Mean value	195.45	82.72	164.54	138.63
SD	28.76	12.97	30.08	33.42

Table 3. Effect of heavy metals on growth of *P. cepacia*.

Strains	MIC (μg/mL)			
	Cu	Cr	Ni	Cd
PW-3	160	110	150	180
PW-8	180	80	80	150
PW-18	250	90	210	160
PW-23	250	110	210	170
PW-34	220	90	190	180
PW-38	190	60	160	180
PW-39	170	70	170	110
PW-40	220	80	210	180
PW-43	180	80	160	120
PW-44	190	110	80	80
PW-51	150	90	180	130
PW-60	160	60	180	110
PW-66	230	70	200	100
PW-69	150	80	210	180
PW-76	170	110	190	130
PW-80	180	100	170	80
PW-89	180	80	150	120
PW-103	230	70	80	130
PW-106	230	60	160	180
PW-112	220	90	170	150
PW-113	200	80	210	80
PW-119	160	110	180	150
PW-121	150	110	210	140
PW-122	160	60	190	130
PW-129	180	80	150	180
PW-133	150	90	160	80
5^{th} percentile	150	60	80	80
90^{th} percentile	230	110	210	180
Mean value	188.84	85.38	169.61	137.69
SD	27.13	14.26	28.16	30.00

Table 4. Effect of heavy metals on growth of *P. fluorescens.*

Strains	MIC (µg/mL)			
	Cu	Cr	Ni	Cd
PW-5	230	100	110	160
PW-7	150	80	100	170
PW-17	170	90	150	80
PW-19	180	60	160	180
PW-22	250	110	210	180
PW-25	230	80	160	150
PW-28	230	110	170	120
PW-30	160	100	200	130
PW-35	170	80	100	170
PW-37	190	60	150	80
PW-45	250	90	200	170
PW-46	180	80	160	80
PW-53	150	70	110	180
PW-61	250	60	210	150
PW-65	190	80	150	80
PW-67	180	60	170	160
PW-68	180	70	100	170
PW-71	220	90	90	80
PW-73	150	100	210	180
PW-75	150	90	80	90
PW-78	220	110	210	180
PW-87	190	60	80	150
PW-88	250	100	150	180
PW-92	170	90	180	160
PW-94	180	100	80	110
PW-102	180	80	160	100
PW-104	150	70	170	140
PW-107	200	70	180	150
PW-114	190	90	180	160
PW-116	180	80	90	110
PW-117	250	100	200	180
PW-126	200	60	150	150
PW-127	190	80	180	160
PW-130	150	70	80	170
PW-132	250	90	90	180
PW-138	180	80	190	150
PW-139	150	60	200	160
5th percentile	150	60	80	80
90th percentile	250	100	204	180
Mean value	192.97	82.43	150.27	144.59
SD	28.18	13.03	37.54	28.92

Copper reduced more HCN and P-solubilization of *P. cepacia* as compared to other metals. Cadmium reduced more IAA and siderophore production of *P. putida*. Cadmium reduced more IAA, siderophore and P-solubilization of *P. fluorescens* as compared to other metals. Copper reduced more HCN production of *P. putida* (Table 5).

Discussion

Pseudomonas strains are beneficial plant growth promoting bacteria. Heavy metals in soil disrupt the soil microbial population. Few soil microorganisms are resistant against heavy metals. Low concentrations of heavy metals are beneficial for growth of microorganisms but higher concentration of heavy metal is harmful. Present study showed that heavy metal reduced growth as well as PGPR activity of previously characterized *Pseudomonas* strains. MIC of Cu against *P. aeruginosa* was lowest i.e. 120 µg/mL but MIC of Cr was highest (120 µg/mL) as compared to other *Pseudomonas* strains. Similarly, previous research reports suggested that *Pseudomonas* strains were resistant against Cu (Cazorla *et al.*, 2002). MIC of Ni against *Pseudomonas putida*, *P. cepacia* and *P. fluorescens* ranged from 80-210 µg/mL but *P. aeruginosa* showed MIC of same from 70-210 µg/mL. Similarly, Cardon *et al.* (2010) evaluated the effect of metals on growth of rhizobacteria and reported that Cd was the most toxic metal at its lowest concentration of 0.1 mM, followed by Ni with a relatively toxic effect at concentrations between 0.1-1.0 mM. Metal resistant plant growth promoting bacteria improved plant growth and productivity. Research reports suggested that diverse group of free-living soil bacteria can improve host plant growth and alleviate toxic effects of heavy metals on the plants (Belimov *et al.*, 2004; Wani *et al.*, 2008). Osborne *et al.* (2010) isolated plant growth promoting rhizobacteria and evaluated metal tolerance concentration (MTC) of PGPR strains which were able to survive till 300 mg/L on Cd amended minimal medium.

Table 5. Effect of heavy metals on plant growth activity of Pseudomonads.

Pseudomonas + metal	IAA	HCN	Siderophore production	P-solubilization
P. aeruginosa + copper	32	29	28	39
P. aeruginosa + Chromium	26	27	27	35
P. aeruginosa + Nickel	24	28	27	36
P. aeruginosa + Cadmium	22	29	25	41
P. putida + copper	9	12	12	11
P. putida + Chromium	10	11	12	14
P. putida + Nickel	9	12	12	13
P. putida + Cadmium	11	11	10	*12*
P. cepacia + copper	18	15	14	13
P. cepacia + Chromium	18	15	14	15
P. cepacia + Nickel	18	16	15	14
P. cepacia + Cadmium	16	16	14	13
P. fluorescens + copper	22	18	20	23
P. fluorescens + Chromium	21	19	21	23
P. fluorescens + Nickel	21	19	20	23
P. fluorescens + Cadmium	21	19	20	22
P. aeruginosa (control)*	44	34	35	43
P. putida (control)*	14	12	14	14
P. cepacia (control)*	19	16	15	15
P. fluorescens (control)*	22	19	21	23

* Published data (Deshwal and Kumar, 2013). 55 *P. aeruginosa*, 22 *P. putida*, 26 *P. cepacia*, 37 *P. fluorescens.*

Conclusion

Minimum inhibitory concentration of Cu, Cr, Ni and Cd ranged from 120-250, 60-110, 70-210 and 80-180 µg/mL and showed PGPR strains can multiply less than MIC. Major isolated *Pseudomonas* showed PGPR activity in metal containing medium which clearly indicated that our plant growth promoting *Pseudomonas* may improve plant growth in metal containing soil.

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