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Research Article
Effect of Chromium (Vi) Reducing *Bacillus* species PZ3 on the Growth of Pea Plants in Chromium Amended Soil

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Abstract
Use of Cr (VI) in various industries like leather, tanning, metallurgy, electroplating, textile and pigment manufacturing may lead to the contamination of the environment with Cr (VI). Aim of this study is to check the bacterial species for their tolerance towards multiple metals, antibiotics, plant growth promoting activity, Cr (VI) reducing activity and to further check its effect on pea growth in Cr (VI) contaminated soil. Bacterial strains were isolated from metal contaminated soils of Abeokuta, Ogun State, Nigeria. All of the isolates showed tolerance to lead, zinc and chromium (VI). All bacterial species were positive to ammonia, Bacillus PZ3 and *Treptococcus* spp. PZ4 were positive to HCN. Bacterial strains also showed reduction of chromium (VI) under varying pH and chromium concentration. Maximum reduction (87.5%) of chromium (VI) was observed at pH 7 by Bacillus PZ3. Chromium (VI) was also reduced significantly at pH 5, 6, 8 and 9. The PGPR strain PZ3 reduced chromium (VI) at a concentration of 50 µg Cr/mL (47.5%), 100 µg Cr/mL (87.5%) and 150 µg Cr/mL (133.33%). Chromium reducing Bacillus PZ3 when inoculated in chromium amended soil increased the growth, nodulation, leghaemoglobin, chlorophyll and protein content of pea plant in comparison to control plants.

Key words: *Bacillus* species, metal tolerance, chromium (VI) reduction, plant growth promoting activities, pea growth

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Release of chromium waste in the environment is due to the use of toxic chromium in various industries like leather tanning, metallurgy, electroplating, textile and pigment manufacturing (Sultan and Hasnain, 2007). Chromium occurs either in trivalent or hexavalent form which can cause negative effect on the population of various types of microbes present in the soil (Ortegel *et al*., 2002). Hexavalent chromium is more toxic and carcinogenic than trivalent due to its permeability through biological membranes. When Cr (VI) is taken up by the bacterial cell, interacts with proteins and nucleic acids, leads to the damage to these molecules inside the bacterial cell (Ackerley *et al*., 2006). Reduction of Cr (VI) results in the formation of stables, less soluble and less toxic Cr (III) and is thus an important process for the transformation of Cr (VI) contaminated soil environments (Jeyasingh and Phillip, 2005). The microbial transformation of Cr (VI) to Cr (III) is a cheap and environment friendly technique which protects the soil system from metal toxicity. The microbial detoxification of Cr (VI) to Cr (III) has been studied in Bacillus (Elangovan *et al*., 2006; Chaturvedi, 2011), *Pseudomonas* sp. (Wani and Ayoola, 2015; Rahman *et al*., 2007), *Escherichia coli* (Bae *et al*., 2005), Microbacterium, *Ochrobactrum intermedium* (Faisal and Hasnain, 2005) and Micrococcus (Sultan and Hasnain, 2005).

Reduction of Cr (VI) is either direct or indirect mechanism and is affected by chromium concentration, pH of the medium, incubation period and type of microorganisms. In the direct mechanism, chromium reductases reduce chromium (VI) (Losi *et al*., 1994), whereas in case of indirect mode, it is either reductants or oxidants eg., H_2S which detoxify chromium (VI) into chromium (III) (De Filippi and Lupton, 1992). Reduction of chromium (VI) to chromium (III) is either aerobic or anaerobic but not both when electron donors are added to the culture medium. Reduction of Cr (VI) to Cr (III) by chromium reductase is anaerobic (Lovley and Phillips, 1994), aerobic (Cervantes *et al*., 2001) and both anaerobic and aerobic (Marsh and McInerney, 2001). Chromium reductases are found in the membrane of the cells, as studied in *Pseudomonas fluorescens* and *Enterobacter cloacae* (Wang *et al*., 1990). Reduction of Cr (VI) to Cr (III) forms insoluble precipitate [$Cr(OH)_3$], which is easily removed from wastewater (Jeyasingh and Philip, 2005). The enzyme chromium reductase found in *P. ambigua* and *Bacillus* sp. (Wang *et al*., 1991) were purified and characterized (Puzon *et al*., 2002). Precipitation of Cr (III) following the reduction of Cr (VI) by H_2S produced by bacteria, is an important mechanism in sulfate-rich soil environment under anaerobic conditions (Losi *et al*., 1994). Thus these bacteria can be used in chromium contaminated soils for the removal of chromium toxicity (Wani *et al*., 2007; Wani and Khan, 2010) and consequently enhance the growth and yield of plants (Wani and Khan, 2013). These chromium reducing bacteria can also produce some growth promoting substances which can enhance the yield of the crop (Wani and Khan, 2013). Therefore, the use of chromium tolerant bacteria is a cost effective bioremediation process for detoxification of chromium. The present study was therefore under taken (1) To determine the resistance pattern of bacteria to heavy metals and antibiotics, (2) To check Cr (VI) reduction under varying pH and chromium concentration and (3) To check the effect of *Bacillus* sp. PZ3 on pea growth in the absence and presence of chromium.

MATERIALS AND METHODS

Isolation of bacteria: Bacteria were isolated from the contaminated soils of Abeokuta on nutrient agar medium by spread plate technique. One gram of soil sample was added to 10 mL of normal saline solution and diluted serially. Ten microliters of the suspension was spread plated on the surface of the solid nutrient agar plates and incubated at 28±2°C for 24 h. Isolated bacterial cultures were then purified and preserved on nutrient agar slants for further experiments.

Evaluation of bacterial strains for metal tolerance: Bacterial strains were tested for their resistance to three heavy metals viz., chromium, zinc and copper by agar plate dilution method (Holt *et al*., 1994). Agar plates were then amended with increasing concentration of Cr (0-700 μg mL^{-1}), Zn (0-700 μg mL^{-1}) and Cu (0-700 μg mL^{-1}) which were then spot inoculated (10 μL) with 10^8 cells mL^{-1}. Plates were incubated at 28±2°C for 72 h and highest concentration of heavy metals supporting growth was defined as Maximum Resistance Level (MRL). Each experiment was replicated three times.

Evaluation of bacterial strains for antibiotic sensitivity: Bacterial strains were tested for their sensitivity to ten antibiotics and their reactions to antibiotics were determined by the disc diffusion method (Bauer *et al*., 1966).

***In vitro* assay of hydrogen cyanide and ammonia:** Hydrogen cyanide and ammonia production by bacterial isolates was detected by the method of Bakker and Schippers (1987) and Dye (1962), respectively. 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) 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 bacterial strains were grown in peptone water (g L^{-1}: Peptone10 g, $NaCl_5$ g, pH 7) and incubated at 30±2°C for four days. One milliliter of Nessler reagent was added to each tube and the development of yellow color indicating ammonia production was recorded (Dye, 1962).

Chromium (VI) reduction: In order to check the effect of pH on hexavalent chromium [Cr (VI)] reduction *in vitro*, the nutrient broth (NB) was amended with 100 µg mL^{-1} of Cr (VI), adjusted to pH 5, 6, 7, 8 and 9 with 1M HCL or 1M NaOH and incubated at 28±2°C for 120 h. Further, to study the effect of different concentrations (0, 50,100 and 150 µg mL^{-1}) of Cr (VI), the $K_2Cr_2O_7$ were amended in nutrient broth and incubated at 28±2°C for 120 h. For Cr (VI) reduction, 1 mL culture from each flask was centrifuged (6000 rpm) for 10 min at 10°C and Cr (VI) in the supernatant was determined by 1,5-diphenyl carbazide method (Eaton *et al*., 1992) upto 120 h. Briefly, the test samples were acidified (pH 1-2) and 1,5 diphenyl carbazide (50 µg mL^{-1}) was added and Cr (VI) concentration was detected by UV-VIS spectrophotometer (752N Lamfield medical England) at 540 nm.

Effect of chromium reducing *Bacillus* species on pea in chromium amended soil: The soil which is used in this study was sandy clay loam (organic carbon 0.33%, Kjeldahl N 0.61 g kg^{-1}, Olsen P 18 mg kg^{-1}, pH 7.1 and WHC 0.35 mL g^{-1}). Seeds which were used in this experiment were sterilized with the help of 70% ethanol, 3 min; 3% sodium hypochlorite, 3 min, after sterilization of the seeds, these seeds were then rinsed six times with sterile water and ultimately these seeds were dried in the shade. The sterilized seeds were coated with *Bacillus* sp. PZ3 after growing them in nutrient broth, for two hours using 10% gum Arabic as adhesive to deliver approximately 10^8 cells $seed^{-1}$. The control seeds were soaked in sterile water after sterilizing the seeds. The clay pots (25 cm high, 22 cm internal diameter) having three kg sterilized soil with control (with out chromium) and three treatments having 25, 50 and 100 mg Cr/kg soil) were sown with the seeds (10 seeds per pot) with and with out *Bacillus* species PZ3. The normal concentration of Cr (50 mg Cr/kg) used in this study was comparable to those found in sewage soil used for pea production. Three pots were arranged in a complete randomized design. After one week of growth of these seeds in the pots, the pea plants were thinned to three and were watered as per the requirement and were maintained in an open field condition. All plants were removed at 120 Days After Seeding (DAS) and were observed for plant growth and nodulation. Plants harvested after 120 DAS were oven-dried at 80°C and dry matter was measured. Total chlorophyll and leghaemglobin contents in fresh foliage were measured after 120 days of harvesting by the method of Arnon (1949) and Sadasivam and Manickam (1992), respectively. Grain protein was estimated by the method of Lowry *et al*. (1951). Data of the measured parameters were subjected to analysis of variance (ANOVA) for two factor pot culture experiment and significant partial difference (LSD) was calculated at 5% probability level.

RESULTS AND DISCUSSION

Evaluation of bacterial strains for metal tolerance: The selected bacterial strains were tested for their tolerate to chromium (VI) and other metals (Fig. 1). Among the bacterial strains, *Bacillus* spp., PZ3 and *Streptococcus* spp., PZ4 showed highest tolerance to chromium (VI) (700 µg mL^{-1}), whereas *Pseudomonas* spp. PZ1 and *Streptococcus* spp., PZ2 (700 µg mL^{-1} each) to copper and zinc. There are reports which showed sufficiently high tolerance to heavy metals by the bacterial isolates (Wani *et al*., 2007; Wani and Khan, 2013). There are conflicting reports available in many studies for the level of tolerance exhibited by many bacteria, which may be due to the variation in the tolerance level of bacteria and their growth conditions (Rajkumar *et al*., 2005). For example, *Rhizobium leguminosarum* isolated from metal contaminated soil tolerated 92.9 µM of zinc (Delorme *et al*., 2003), while as *Rhizobium* species tolerated 300 mg kg^{-1} nickel and showed an effective symbiosis with *Trifolium repense*, when the legume was grown in nickel amended

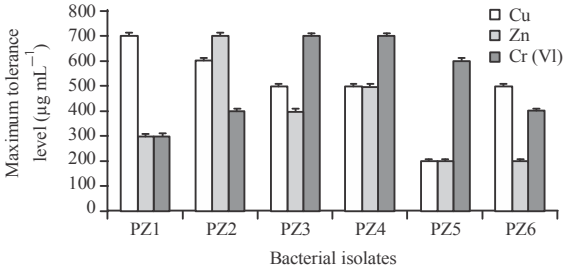

Fig. 1: Maximum tolerance level shown by different bacterial isolates against different metals

Table 1: Resistance pattern of different bacterial species to various antibiotics

Antibiotics used	Concentrations of antibiotic (μg mL^{-1})	Number of resistant isolate (%)
Septrin	30	6 (100)
Chloramphenicol	30	6 (100)
Sparfloxacin	10	6 (100)
Ciprofloxacin	10	2 (33.33)
Amoxacillin	30	6 (100)
Augumentin	30	6 (100)
Gentamycin	10	5 (83.33)
Pefloxacin	30	5 (83.33)
Tarivid	10	6 (100)
Streptomycin	30	6 (100)

soils (Smith and Giller, 1992). In the present studies, *Bacillus* spp., PZ3 and *Streptococcus* spp., PZ4 showed highest tolerance to chromium (VI), *Pseudomonas* spp., PZ1 and *Streptococcus* spp. PZ2 showed highest tolerance to copper and zinc, respectively. The metal tolerant strains were characterized by physiological, morphological and biochemical characteristics. The strain PZ1 and PZ6 was characterized as *Pseudomonas* spp., PZ2 and PZ4 was characterized as *Streptococcus* spp., PZ3 was characterized as *Bacillus* spp., while PZ5 was characterized as *Micrococcus* spp.

Evaluation of bacterial strains for antibiotics resistance: Resistance of bacterial strains to different antibiotics varied considerably (Table 1). Among the bacterial species, 100% of were resistant to septrin, chloramphenicol, sparfloxacin, amoxacillin, augumentin, tarivid and streptomycin. Resistance to antibiotics may be due to the genetic mutation or by transfer of antibiotic resistant genes between organisms (Spain and Alm, 2003). Multiple antibiotic resistances shown by the bacterial species in this study (e.g. *Bacillus* spp., PZ3 and *Pseudomonas* spp., PZ6) could be due to a high degree of tolerance to metals. In many studies, metal tolerance and antibiotic resistance have been reported (Wani *et al*., 2009; Yilmaz, 2003).

***In vitro* assay of HCN and ammonia:** All the isolated bacterial strains were found to be positive for ammonia while as strain *Bacillus* spp.PZ3 and *Streptococcus* spp., PZ4 were only found positive for HCN. The ammonia synthesized by these bacterial strains plays a signalling role in the interaction between plant growth promoting bacteria and plants (Becker *et al*., 2002). Ammonia is known to increase the glutamine synthetase activity (Sood *et al*., 2002). In addition, ammonia transporters found in bacterial species may 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).

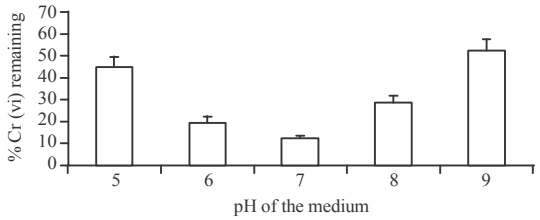

Fig. 2: Effect of pH on Cr (VI) reduction ability by *Bacillus* spp. PZ3 after 120 h of growth

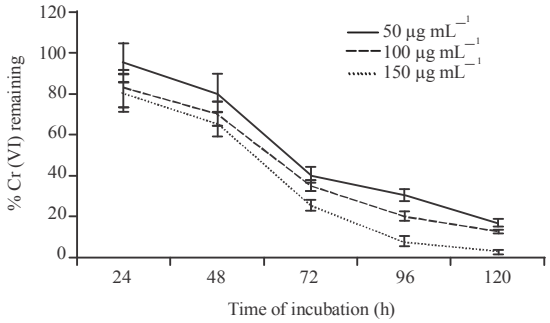

Fig. 3: Effect of Cr (VI) concentrations on Cr (VI) reduction ability of *Bacillus* spp. PZ3 after 120 h of incubation

Chromium (VI) reduction: Chromium, an environmental pollutant is released from various industries. Among the two forms of chromium, Cr (VI) is the most toxic and carcinogenic (Kamaludeen *et al*., 2003). It's toxicity is due to high solubility in water, rapid permeability and its interaction with intracellular proteins and nucleic acids. Reduction of Cr (VI) results in the formation of stable, less soluble and less toxic Cr (III) and is thus an important bioremediation process in chromium affected environments (Thacker *et al*., 2007). Therefore, the present study was designed to determine the Cr (VI) reducing ability of the metal tolerant bacterial strain.

A high tolerant strain was thus chosen for chromium (VI) reduction under *in vitro* conditions. This study was carried out to access (i) effect of different pH on reduction of Cr (VI) and (ii) effect of chromate concentration on Cr (VI) reduction.

The effect of pH on chromium (VI) reduction is shown in Fig. 2. Maximum reduction (87.5%) was observed at pH 7 by *Bacillus* spp., PZ3. Similarly, *Bacillus* spp., PZ3 significantly reduced the chromium (VI) at pH 5 (55%), pH 6 (80%), pH 8 (71.25%) and pH 9 (47.5%), respectively, at 100 μg Cr/mL after 120 h of incubation.

In second experiment, the chromium reduction was observed in the chosen bacterial strain in nutrient broth amended with 50, 100 and 150 μg mL^{-1} of $K_2Cr_2O_7$ under *in vitro* conditions. The time for total reduction of chromium

Table 2: Effect of Cr (VI) concentration on growth and nodulation of pea plants at 120 DAS grown in the presence and absence of *Bacillus* sp. PZ3

		Length/plant (cm)		Nodulation		
Treatment	Dose (mg kg^{-1} soil)	Root	Shoot	No. of plant	Dry weight (g/plant)	Total dry weight (g/plant)
Uninoculated	Control	22	28	75	0.213	3.7
	25	20	26	72	0.211	3.6
	50	17	24	63	0.219	3.5
	100	14	21	55	0.215	2.4
Inoculated	Control	27	33	82	0.235	4.2
	25	29	35	86	0.315	4.5
	50	31	38	88	0.332	4.6
	100	28	32	74	0.249	4.3
LSD		1.3	1.5	0.22	0.06	0.47
F-value	Inoculation (df = 1)	925*	679*	1.44	18.4*	30.5*
	Cr (df = 3)	31.8*	46.6*	12.4*	2.6	3.2
	Interaction (df = 3)	52.4*	36.7*	20.2*	2.4	1.9

Each value is a mean of three replicates where each replicate constituted three plants/pot; Mean values are significant at $p \geq 0.05$

Table 3: Effect of Cr (VI) concentrations on chlorophyll, leghaemoglobin and seed protein of pea plants at 90 DAS grown in soil inoculated with and with out *Bacillus* sp., PZ3

Treatment	Dose (mg kg^{-1} soil)	Chlorophyll content (mg g^{-1})	Leghaemoglobin content [mmol g^{-1} f.m]	Seed protein (mg g^{-1})
Uninoculated	Control	0.84	0.13	230
	25	0.82	0.12	229
	50	0.79	0.09	224
	100	0.75	0.07	220
Inoculated	Control	0.88	0.16	240
	25	0.91	0.18	244
	50	0.94	0.20	249
	100	0.92	0.17	242
LSD		0.05	0.02	1.6
F-value	Inoculation (df = 1)	107.2*	299.3*	2258*
	Cr (VI) (df = 3)	1.7	9.5*	46.2*
	Interaction (df = 3)	7.3*	17.5*	84.5*

(VI) increased with increase in the concentration of chromium (VI). During this study, almost complete reduction of chromium (VI) occurred after 120 h by *Bacillus* spp., PZ3 (Fig. 3) at 50 µg mL^{-1} of chromium. *Bacillus* spp., PZ3 reduced chromium (VI) at concentration of 50 µg Cr/ mL (97.5%), 100 µg Cr/mL (87.5%) and 150 µg Cr/mL (66.7 %), respectively. Our study is in correlation with the study of Yang *et al*. (2009) who observed significant reduction of chromium (VI). In this study, *Intrasporangium* species Q5-1 reduced chromium (VI) by 98% after 84 h of incubation at 2,3 or 4 mM of Cr (VI) whereas at 5mM Cr (VI), the same bacterial species reduced Cr (VI) by 70% after 72 h of incubation. In another study, *Bacillus* species isolated from tannery effluent reduced 71.4% of Cr (VI) at 1100 mg L^{-1} of Cr (VI) after 24 h of incubation (Chaturvedi, 2011). Ibrahim *et al*. (2011) found that the *Bacillus* species KSUCr5 reduced 100% of Cr (VI) concentration of 40 mg L^{-1} within 24 h whereas 100% chromium reduction was achieved for 60-80 and 100 mg L^{-1} of Cr (VI) after 48 and 72 h, respectively. It was also observed that *Bacillus* species KSUCr5 reduced only 78.2 and 44.2% of Cr (VI) at 150 and 200 mg L^{-1}, respectively after 72 h of incubation. The strain KSUCr5 could also reduce Cr (VI) between a pH range of 7-12 with a maximum reduction at pH 10.

Effect of chromium reducing *Bacillus* species on pea in chromium amended soil: In the present study, effect of chromium on plant growth was studied both under unicoculated and inoculated conditions (Table 2 and 3). Pea plants showed severe toxic effects of chromium when grown in the absence of the bacterial strain. But when these pea plants were grown in sandy clay loam soil amended with chromium in the presence of chromium reducing *Bacillus* sp., PZ3 increased the measured parameters (Table 2). The Bacillus PZ3 when grown in the presence of 50 mg kg^{-1} Cr (VI), increased the root length, shoot length, nodule numbers, nodule dry weight and total dry weight by 15, 15, 7, 41 and 10%, at 120 DAS, respectively, compared to control. While comparing the effects of Bacillus PZ3 on pea applied with different concentrations of chromium, a maximum increase of

82, 58, 40, 52 and 31% at 120 DAS, in root length, shoot length, nodule numbers, dry nodule mass and total dry mass, respectively was observed at 50 mg Cr/kg soil compared to non-inoculated but having same concentration of chromium. The two way ANOVA revealed that the individual effects of inoculation, chromium and interaction was significant ($p \geq 0.05$) for the measured parameters except the individual effects of inoculation on nodule number and chromium and interaction on nodule dry weight and total dry weight at 120 DAS.

Symbiotic association between chromium reducing strains and legumes are very important because of their potential for detoxification of chromium in chromium contaminated soils (Wani and Khan, 2010, 2013). In the present study, strain PZ3 increased plant growth and nodulation compared to uninoculated plants. Increase in plant growth and nodulation is due to chromium reduction and phytohormone production by strain PZ3 (Wani *et al*., 2007). There may be another reason for the increase in pea growth and nodulation that is mineral uptake and phytohormone production by the host plant which will ultimately protect the plants against the toxicity of chromium (Wani *et al*., 2008b). Similar results of increase in plant growth by the chromium reducing bacteria have been reported (Wani *et al*., 2008a; Wani and Khan, 2010).

Chlorophyll, leghaemoglobin and protein content at 120 DAS decreased significantly with increase in the concentration of Cr (VI) (Table 3) without the inoculation of strain PZ3. Chromium at 100 mg kg^{-1} decreased chlorophyll, leghaemoglobin and protein compared to un-inoculated control. The bio-inoculant with 50 mg Cr (VI)/kg, increased chlorophyll, leghaemoglobin in fresh nodules and protein in seeds, compared to un-inoculated but 50 mg Cr (VI)/kg amended soil. Two factors ANOVA revealed that the individual effects of inoculation and Cr (VI) and their interaction (inoculation x Cr (VI) were significant ($p \geq 0.05$)) for the measured parameters except Cr (VI) for chlorophyll. Similar increase in chlorophyll, leghaemoglobin and protein has also been reported (Wani *et al*., 2008a; Wani and Khan, 2013). Burd *et al*. (2000) also observed an increase in protein of tomato, canola and Indian mustard when plants were grown in the presence of Kluyvera ascorbata SUD 165 in the presence of high concentration of Ni, Pb and Zn.

CONCLUSIONS

This study concludes that the bacterial strains not only produced PGPR substances, reduced chromium (VI) but also increased plant growth under metal stress. Due to multifarious properties, these strains could therefore be used as bioremediators of metals in soils contaminated with heavy metals.

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