INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0279/2018/20–7–1613–1622
DOI: 10.17957/IJAB/15.0682
http://www.fspublishers.org

Full Length Article

Differential Uptake of Cadmium and Chromium in *Brassica oleraceae* in Response to Application of Plant Growth Promoting Rhizobacteria

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Abstract

Among various toxic heavy metals (HMs), Cadmium (Cd) and Chromium (Cr) have proven to be extremely noxious for environment, hence warranting their remediation. Current study investigated the plant growth promoting rhizobacteria (PGPR) assisted Cd and Cr removal by *Brassica oleraceae* under controlled environment. For this purpose, *B. oleraceae* plants were grown at different concentration of Cd (4, 10 mg kg^{-1}) and Cr (35, 50 mg kg^{-1}) and inoculated with strains evaluated for PGPR characteristics. For heavy metals and growth hormone production experiments, two strains, HRS-C3 and B6 were used. Inoculation with PGPRs significantly increased the fresh and dry biomass of plant shoots as compared to un-inoculated counterparts. Under heavy metal stress conditions, inoculation with HRS-C3 increased the fresh biomass of plants from 535.08 to 657.66 mg and corresponding dry weight from 214.50 to 295.30 mg compared to un-inoculated plants under stressed conditions. Interestingly, PGPRs treatment increased the Cd uptake by plant, while Cr accumulation was significantly reduced in inoculated plants as compared to un-inoculated plants. PGPR strain, HRS-C3 has proved to be more efficient than B6 with reference to growth hormones (IAA and GA) production. Inoculation with mix culture of both strains, HRS-C3 and B6 produced significantly higher concentrations of IAA and GA than singular applications. Overall, PGPRs enhanced Cd availability to plant by reducing stress to tested plants. This phenomenon suggests that microbes played a key role for remediation of medium level contamination of Cd by *B. oleraceae* in contaminated soil. However, the results of same strains indicated that Cr uptake was reduced in *B. oleraceae.* Results suggest that there is need to employ metal specific strains for Cr remediation.

Keywords: PGPRs; Heavy metal stress; Cadmium; Chromium; *Brassica oleraceae*

Introduction

Heavy metals (HMs) have posed serious challenges to both terrestrial and aquatic ecosystems over the last many decades. HMs pave their way in to the environment from both natural and anthropogenic sources including; agrochemicals, industrial waste disposal, vehicle exhausts and mining (Abdullah *et al*., 2015; Bibi *et al*., 2015). Once heavy metals are entered in to the environment and bio-accumulated (Abdullah *et al*., 2015; Zafar *et al*., 2015), because of their non-biodegradable nature, these elements may stay there forever thereafter influencing the surrounding habitat and biota and enter the food chain, threating the food safety and health risks unless remediated. All of the known HMs are not toxic, but some are essential (e.g., Zn, Ni, Cu, Fe) for normal plant growth and development and their absence may jeopardize the biotic life.

Amongst the trace/toxic heavy metals, Cd and Cr are of great concern because of the toxicities induced by these metals in plant and animal kingdom. As of rankings by the Agency for Toxic Substances and Disease Registry (ATSDR) USA, Cd is seventh most toxic heavy metal worldwide (Jaishankar *et al*., 2014), and originates as a byproduct of Zn mining and secondary minerals. For instance, secondary minerals like $ZnCO_3$ contain Cd concentrations of 0.2–0.4% (Alloway, 1995) while Rose *et al*. (1979) reported as high as 50% Cd in secondary minerals. Excessive use of phosphatic fertilizers (e.g., rock phosphate) and many industrial activities including; smelting, mining, batteries and manufacturing of stabilizers have also been reported to be the major sources of Cd in to the environment (Oves *et al*., 2012). Cd bioaccumulation has been reported to cause serious health complications in human beings (Zafar *et al*., 2015) and oxidative stress, disruption of enzymatic cells and nutritional deficiency are well reported abnormalities in plants (Irfan *et al*., 2013). Cr exists in many oxidation states (e.g., Cr^{+2}, Cr^{+3} and Cr^{+6}) and its toxicity very much depends on the oxidation form (Rodríguez *et al*., 2007; Khan *et al*., 2013). Chromium has been reported to be the seventh most

To cite this paper: Asad, S.A., Masood-ur-Rehman, R. Ahmad and M. Umer, 2018. Differential uptake of cadmium and chromium in *Brassica oleraceae* in response to application of plant growth promoting rhizobacteria. *Int. J. Agric. Biol*., 20: 1613–1622

abundant element on earth (Mohanty and Patra, 2013). Fossil fuel burning, tanneries, pigment oxidants, chromium steel production, fertilizers and sewage sludge, electroplating, pulp and paper production are the major sources of Cr release in to the environment (Zayed and Terry, 2003; Ghani, 2011; Khan *et al*., 2013; Jaishankar *et al*., 2014; Kamran *et al*., 2014).

Heavy metal pollution has been widely reported across many areas in Pakistan. Notably, Cd, Cr, As, Pb and Ni have been reported in varying concentrations in different ecological niches including; water bodies, vegetables, soil, sediments and particulate matter (Waseem *et al*., 2014). For instance, high concentration of arsenic (As) was reported in ground and surface water where respectively 3% and 16% of water resources in Punjab and Sindh contained 50 μg L^{-1} As (Saqib *et al*., 2013) against World Health Organization (WHO) standards of 10 μg L^{-1} for developed countries. Similarly, Cd has been found in water samples collected in Korangi area of Karachi (5.35 mg L^{-1}) and Lahore (0.18 to 0.37 mg L^{-1}) which exceeded the permissible limit of 0.10 mg L^{-1} set by National Environmental Quality Standards Pakistan (NEQs, 2000; Mahmood and Malik, 2014). Soil concentration of Cd varies from 0.02 and 184 mg kg^{-1} (Parveen *et al*., 2012). Cr is usually accumulated in food grown on metal contaminated land or that irrigated with waste water. A study conducted by Parveen *et al*. (2012) observed that vegetables irrigated with waste water in the suburbs of Peshawar accumulated elevated concentrations of Cr ranging from 3.74–3.95 mg kg^{-1} against a minute concentration of 0.004 mg kg^{-1} when same vegetables were irrigated with clean water as noted by Lone *et al*. (2003). All these toxic metals have been reported to cause serious health implications including several types of cancers, lungs and kidney diseases, respiratory and skin diseases, anemia and gastrointestinal problems among the expanding list of health problems.

Hence the scale of heavy metal toxicities and their repercussions in fauna and flora warrant immediate steps to devise cost effective and sustainable ways for remediating these obnoxious compounds (Dell'Amico *et al*., 2008; Bah *et al*., 2010). Many physical and chemical approaches have been in use to remediate metal contaminated environments each with its own merits and demerits. Bioremediation techniques involving plant-microbe ecosystem has attracted the attention of many research groups (Shilev *et al*., 2006) because of its sustainability and cost effectiveness and has yielded valuable results. Suitable plant-microbe combinations may help to revolutionize the metal remediation strategies. Amongst microbes, plant growth promoting rhizobacteria (PGPR) have been widely reported in heavy metal remediation (Hassan *et al*., 2016; Kamran *et al*., 2015; Pramanik *et al*., 2018). PGPRs live in symbiotic relationship with plants, improve plant growth and increase plant' s competitiveness for space and nutrients, hence increasing plant resistance to withstand external stresses (Glick, 2010; Hassan *et al*., 2015, 2016). These novel species of microbes include but not restricted to; *Rhizobium, Pseudomonas, Azospirillum, Azotobacter* genera. These microbes fulfill their nutritional needs from root exudates and in return impart multiple benefits to the plants including; growth hormone production (Miransari and Smith, 2014), antagonism against pathogenicity (Chandra *et al*., 2007; Shilev, 2013), nitrogen fixation (Gaby and Buckley, 2012), plant growth promotion (Arora *et al*., 2013; Miransari and Smith, 2014; Hussain *et al*., 2016), lytic enzymes production (Joshi *et al*., 2012; Nadeem *et al*., 2013) and above all increase the metal solubility in the soil to be easily taken up by plants (Shilev, 2013).

Brassicales are widely researched from phytoremediation perspective, because they easily translocate heavy metals from roots to shoots and withstand elevated soil metal content. They also exhibit fast growing habit and are high biomass producers (Marchiol *et al*., 2004). Empirical evidences indicated an increased amount of heavy metal accumulation in many of the Brassicas (*B. juncea, B. napus and Noccaea caerulescens*) including *B. oleraceae* and proving the high tolerance level against heavy metals stress. The only risk involved in using the brassica vegetables for phytoremediation is that brassica oil may be contaminated because of increased level of metal accumulated in the seed, leading to the toxicity in the food chain and environment. An interesting study was conducted by Park *et al*. (2012) on *B. napus* who concluded that despite plants were exposed to relatively high concentration of combination of heavy metals which there after accumulated exceptionally higher number of elements, but oil contained relatively low content of heavy metals and was safe to use as an energy source. According to their findings, most of the heavy metals were retained in the residue during oil extraction process. A great volume of knowledge exists on heavy metal hyperaccumulator plants (Asad *et al*., 2013, 2015, 2015a), but very limited literature exists on the heavy metal accumulator plants including many brassica vegetables such as *B. oleraceae*. *B. oleraceae* is a metal accumulator plant which accumulates many folds of heavy metals in its body compared with non-accumulators.

B. oleraceae is mainly cultivated for its oil contents or fodder for animals but very rarely for remediating the heavy metals. This is because of risks of contamination because of heavy metal toxicities. However, empirical evidences (e.g., Park *et al*., 2012 and references there in) revealed that heavy metals are not passed in to the oil during extraction process, hence leaving no chance of food chain contamination. Moreover, limited research exists on the involvement of PGPRs to enhance the heavy metal uptake by accumulator plants. Current study was designed to explore the heavy metals, Cd and Cr accumulation potential of *B. oleraceae* under controlled settings. Moreover, study plants growing at varying concentrations of Cd and Cr were inoculated with PGPR strains to observe the impacts of microbial strains on heavy metal uptake by plant. Capability of experimental plants to withstand Cd and Cr toxicity under controlled

conditions and potential role of PGPRs to alleviate the plants against heavy metal stress also formulated the objective of current study. This research would strengthen our knowledge on combined application of PGPR and metal accumulator brassica plants for remediating the medium level Cd and Cr contamination in the soil.

Materials and Methods

Description of Microbial Culture used in the Experimentation

Five strains of plant growth promoting rhizobacteria (PGPR) were obtained from the soil biology and biochemistry laboratory of National Agricultural Research Centre (NARC), Islamabad. These microbial isolates named as; HRS-C3, IRS-C5, GRP-C3, ERS-C7 and GRS-C4 were previously isolated from the canola roots and maintained in the culture bank of NARC. In addition to these strains, five microbial strains (B2, B3, B4, B6 and H3) were isolated from the rhizosphere soils of *B. oleracea* plants at three different locations in Abbottabad district and maintained at Environmental Sciences Laboratory at COMSATS institute of information technology, Abbottabad. Pakistan.

Isolation and Characterization of Microbial Strains

Microbes were isolated by mixing soil through serial dilutions (10^{-1}–10^{-9}) and inoculating on the Lysogenic Broth (LB) media as described by Sambrook and Russell (2001). From each serial dilution, 0.1 mL was poured in petri plates by spreading the solution evenly on the surface of solidified media. Inoculated plates were placed in the incubator at 28°C for bacterial growth. After 72 h, single isolated colony from each replicate plate was transferred to fresh media and stored at 28°C in the incubator. Isolates were characterized morphologically (Goenadi *et al*., 2000) and biochemically through following tests.

Phosphorus Solubilizing Ability

Phosphorus solubilizing ability of the isolates was determined by inoculating the bacterial cultures on agar medium containing $Ca_3(PO_4)_2$. Solubilization index was measured with Eq. 1 (Edi-Premono *et al*., 1996) below:

$$\text{Solubilization Index} = \frac{\text{Colony diameter+holozone diameter}}{\text{Colony diameter}} \quad \text{(Eq. 1)}$$

Enzyme Activity Determinations

Catalase, oxidase, and amylase enzyme activities of microbial strains were determined as; for catalase, fresh bacterial colony (16–24 h old) was placed on a clean dry glass slide and mixed with a drop of H_2O_2 (3%). Appearance of bubbles indicated that strains were positive in catalase production. To determine the oxidase activity of isolates, 1% kovacs oxidizing reagent was applied to the bacterial cultures. Appearance of blue or purple color indicated oxidase activity of strains. Amylase activity (starch hydrolysis ability of isolates) was carried out by using the method of Deepthi *et al*. (2012). For this purpose, bacterial strains were grown on starch-agar media prepared by dissolving peptone (10 g), NaCl (5 g), agar (12 g) starch (1%) in 1000 mL distilled water. After 4 days, the prepared media was flooded with Gram's Iodine. Appearance of clear zone around the colonies was the indication of amylase production by isolates. These clear zones were measured.

Anti-pathogenic Activity of Bacterial Isolates

Antagonistic potential of bacterial strains against bacterial (*Staphylococcus aurcus*) and fungal (*Epideimophyton floccosum*) pathogens was determined. For this purpose, agar media containing peptone (10 g), and dextrose (40 g) was prepared and pH was adjusted to 5.6 before sterilization and inoculated with one-day old bacterial colonies. Afterwards, sterile cotton buds containing pathogenic bacterial and fungal cultures were swabbed over separate plates and incubated at 37°C for 24 h and the zone of inhibition was measured. Similar procedure was adopted for estimating the antagonistic potential of isolated strains against fungal pathogen *E. floccosum.*

Soil Chemistry

Soil used for pot experiments was tested for the physicochemical properties at two stages i.e., pre-sowing and post-harvest. Soil pH was measured by mixing soil with distilled water in the ratio of 1:5 and putting the solution on shaker to ensure the homogeneity of solution. Pre and post-harvest pH was recorded to be 7.32 and 7.61 respectively. Organic matter contents of soil samples were determined by drying the soil at 105°C in the muffle furnace. Afterwards, 50 g of sample was taken and heated at 360°C for two hours. Difference in mass of sample before and after heating was recorded as the mass of organic matter. Organic matter contents were recorded as 3.86 and 3.94 g for pre-sowing and post-harvest soil samples respectively.

Sowing and Germination Conditions

B. oleraceae seeds were obtained from the oil seed program of National Agricultural Research Center (NARC), Islamabad. Before sowing, seeds were surface sterilized with 95% ethanol followed by dipping in 40% sodium hypo chloride (NaClO) for 3–6 min as described by Abd-Alla *et al*. (2012). After sterilization, dried seeds were inoculated with respective PGPR strains and sown in sterilized experimental pots (16×18 cm) already filled with 1 kg of sterilized soil and stored under axenic conditions. Experimental plants were maintained in the glass house at

the experimental site of Department of Environmental Sciences, COMSATS institute of information technology, Abbottabad. Glass house was fitted with supplementary lights to have 16/8 h' light/dark period and temperatures of 30/18°C for day and night, respectively. One week after germination, pots were spiked with Cd and Cr in solution form to obtain the required concentration of respective heavy metal. Heavy metal concentration and growth hormone production from shoot samples were determined after eight weeks of heavy metal application. Eleven weeks after germination, all experimental plants were destructively harvested, washed with tap water, blotted dried and processed for fresh and dry biomass of plant.

Treatments and Layout of Experiments

All of the 10 isolates were tested for their morphological, biochemical and anti-pathogenic characteristics. However, during growth hormone production and investigating the microbes-heavy metals-plant interactions experiments, two isolates; HRS-C3 and B6 were used because these two strains exhibited the maximum traits of PGPR. Moreover, due to logistic constraints it was not possible to proceed with all 10 isolates. Different concentrations of Cd and Cr were obtained from $CdCl_2$ and $Cr_2(SO_4)_3.6H_2O$ salts, respectively. These salts were dissolved in deionized water and applied to experimental pots in solution form. Solution volume was used to obtain the final concentrations of 4 and 10 mg Cd and 35 and 50 mg Cr kg^{-1} of soil. PGPR were applied to the sterilized seeds of *B. oleraceae* before sowing. Throughout the experiments, plants were irrigated with distilled sterilized water. The experiments were laid out in completely randomized design to make sure that all treatments were equally exposed to different environmental conditions inside the glass house. There were three replicates for each treatment.

Plant Analysis

Fresh and dry weights of plants were recorded to observe the effects of different treatments on plant health.

Plant Metal Determinations

Approximately, 50 mg of dried plant material was taken and digested in a mixture of HNO_3, H_2O_2 and H_2O mixed in the ratio of 3:2:3 respectively. The samples were then heated on hot plate at 150°C until the disappearance of green color of sample solution. After digestion, the volume of digested plant material was made up to 10 mL by adding distilled water.

Extraction, Purification and Analysis of Growth Hormones from Bacterial Culture and Plant Tissues

Bacterial culture: Growth hormones (indole acetic acid (IAA) and gibberellic acid (GA) production ability of bacterial strains was determined by growing the microbes in lysogenic broth (without agar) for 6 days and extraction of hormones from this bacterial culture. For this purpose, freshly prepared broth was inoculated with 5 days old bacterial colonies and maintained at a temperature of 27±1°C for 48 h in the water bath revolving at 180 rpm. Six days old bacterial culture was harvested by centrifugation at 10,000 rpm for 20 min. Palette was discarded and pH of the supernatant was adjusted to 2.5–3.0 with 1 N HCl. Supernatant collected was mixed with 1/3 volume of ethyl acetate followed by collecting the liquid phase and discarding the palette. This step was repeated three times and supernatant collected during three steps was amalgamated. The supernatant was dried at 30–40°C and residue was collected by dissolving in 1.5 mL of HPLC grade methanol. Afterwards, the sample was filtered through Millipore syringe filter (0.45 μm) and run on HPLC against the respective standards of hormones as described by Wurst *et al.* (1984).

Plant Material

One gram of freshly harvested biomass of experimental plants was washed with tap water to remove the dirt from leaf surface and dried with paper towel. Clean and dried plant materials were dipped in liquid nitrogen for 10 sec to stop the metabolic processes. Afterwards, these samples were freeze dried and ground with pestle and mortal. Samples were treated with 6 mL of methanol (80%) and allowed to remain for 72 h. After 72 h, samples were centrifuged for 10 min at a speed of 2500 rpm followed by evaporation of supernatant with rotary evaporator. The residue in the rotary flask was collected by dissolving in distilled water and pH of the solution was adjusted to 2.5 to 3.0 with HCl (1 N). Collected solution was partitioned with 1/3 volume of ethyl acetate. This process was repeated three times whereby three liquid phases were amalgamated and dried afterwards. Final residue was collected by dissolving in 1.5 mL of HPLC grade methanol and samples were run on HPLC against the respective standards of hormones as described by Wurst *et al.* (1984).

Statistical Analyses

Three replicates were used for each treatment. The data collected was analyzed using data analysis tool pack of Microsoft excel 2016 and conducting one-way analysis of variance. The means were compared by LSD (least significant difference) test at $p \leq 0.05$ (Steel *et al.*, 1997).

Results

Efficacy of PGPRs to influence the heavy metal uptake by *B. oleraceae* plants exposed to varying concentrations of Cd and Cr was observed under glass house conditions. For this

Table 1: Morphological characteristics of bacterial isolates used in the experimentation

Microbial strain	Colony morphology				
	Form	Margin	Elevation	Opacity	Colour
HRS-C3	Circular	Entire	Raised	Opaque	White
IRS-C5	Circular	Entire	Convex	Opaque	Yellow
GRP-C3	Circular	Entire	Pulvinate	Opaque	Yellow
ERS-C7	Circular	Entire	Convex	Opaque	White
GRS-C4	Circular	Entire	Raised	Opaque	White
B2	Irregular	Undulate	Flat	Opaque	White
B3	Circular	Curled	Raised	Opaque	White
B4	Circular	Entire	Pulvinate	Translucent	White
B6	Circular	Entire	Convex	Opaque	White
H3	Circular	Entire	Raised	Opaque	White

Table 2: Biochemical tests performed on bacterial strains collected from NARC and those isolated from the rhizosphere soil of *Brassica oleraceae*. Positive sign (+) indicates that respective isolate exhibited that ability while (-) demonstrate that isolates lacked that characteristic

Microbial strain	Phosphate solubilizing	Catalase test	Oxidase test	Amylase (starch hydrolysing ability)
HRS-C3	+	+	+	+
IRS-C5	+	+	+	-
GRP-C3	+	+	+	+
ERS-C7	-	+	+	-
GRS-C4	-	+	+	-
B2	-	-	+	+
B3	+	-	+	+
B4	-	+	+	-
B6	+	+	+	+
H3	-	-	+	+

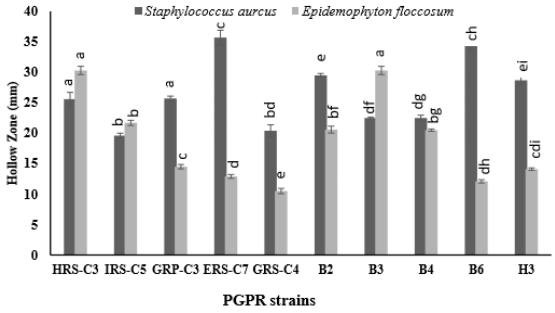

Fig. 1: Efficacy of bacterial isolates to antagonize fungal (*E. floccosum*) and bacterial (*S. aurcus*) pathogens. The data are means of three replicates± SE. LSD; *S. aurcus* =2.18, *E. floccosum* =1.31

purpose, selected isolates were morphologically and biochemically characterized to evaluate their PGPR like traits. Moreover, the influence of tested isolates on plant growth parameters was determined as detailed in the paragraphs below.

Colony Morphology

The outlook of bacterial colonies was shown in Table 1. The data indicated mixed types of margins, forms, color and opacity of bacterial colonies. It was observed that most of the bacterial strains regardless of their source formed circular and opaque colonies with entire margins and white color. Only one strain (B2) isolated from local soil exhibited irregular colony and undulate margins, while B4 isolate exhibited translucent opacity. Contrary to almost similar outlook attributes by majority of the isolates, colony elevation ranged from raised to flat and pulvinate to convex, varying from one isolate to another.

Biochemical Tests

Biochemical tests performed on bacterial isolates are presented in Table 2. Data revealed that 50% of the microbial isolates were capable to solubilize phosphates. Majority of the phosphate solubilizers were those from NARC collection while only 40% of locally isolated strains were able to solubilize phosphate. Further, 7 out of 10 isolates were positive for catalase production test where all the 5 strains from NARC and 2 locally isolated strains exhibited the property of being catalase positive. The most interesting results were observed in oxidase test where all the tested isolates produced cytochrome C oxidase. Contrary to the trend observed in all other biochemical tests, 60% of local and 40% of NARC isolates expressed the characteristics to hydrolyze the starch (Amylase +).

Antagonistic Potential of Bacterial Isolates against Bacterial and Fungal Pathogen

Bacterial isolates were tested on bacterial (*S. aurcus*) and fungal (*E. floccosum*) pathogens to determine their efficacy for antagonizing the host pathogens. Data in Fig. 1 indicated that antagonistic potential of PGPR strains was more prominent against bacterial pathogens as compared with that against fungal pathogens. PGPR strain ERS-C7 showed 35 mm inhibition zone against bacterial pathogen which was reported to be the maximum hollow zone among all strains under study. Same PGPR isolate produced a hollow zone of 12 mm when tested against fungal pathogen, *E. floccosum.*

This trend was observed in majority of the PGPR strains where inhibition zones against bacterial pathogens were higher than those reported against fungal pathogens. Contrary to the general trend of enhanced efficacy of bacterial isolates against bacterial pathogens, two strains; HRS-C3 and B3 exhibited more pronounced effects against fungal compared with bacterial pathogen. Minimum antagonistic effects (10 mm inhibition zone) was exhibited by strain GRS-C4 followed by ERS-C7 and B6 strains against the same fungal pathogen.

Effect of PGPRs on Fresh and Dry Weights (mg) of Plant Shoots

Fresh and dry weights of experimental plants exposed to different combinations of two PGPR isolates; HRS-C3, B6

Table 3: Effect of PGPR on fresh and dry biomass of plants treated with Cd and Cr

Treatments	Shoot fresh weight (mg)		Shoot dry weight (mg)	
Control	1218	a	487.6	a
Cd 4*	483.5	b	197	b
Cr 35**	564.3	c	231.8	c
Cd 4+Cr 35	535.0	d	214.5	bcd
HRS-C3	1443	e	642.6	e
HRS-C3+Cd 4	571	cf	255.6	f
HRS-C3+Cr 35	779.9	g	371.1	g
HRS-C3+Cd 4+Cr 35	657.6	h	295.3	h
B6	1405	i	619.6	i
B6+Cd 4	1045.3	j	450.5	j
B6+Cr 35	1004.6	k	398.3	k
B6+Cd 4+Cr 35	922.9	l	412.2	kl
LSD	22.85		20.42	

*4 mg Cd kg^{-1}; ** 35 mg Cr kg^{-1}

Data are means of three replicates. LSD; fresh weight = 22.85, dry weight = 20.42. Values followed by different letters are statistically different from each other (P=0.05)

and heavy metals; Cd and Cr was depicted in Table 3. Data revealed that plants treated with PGPR have gained significant higher fresh and dry weights of foliage as compared with control and those which were treated with only Cd and/or Cr. Plants inoculated with PGPR strains, HRS-C3 produced two folds higher fresh and dry biomass (1443 and 642 mg, respectively) compared with plants receiving Cd treatment only. The lowest biomass (483 and 197 mg fresh and dry weights respectively) was reported in plants treated with Cd only followed by those receiving both Cd and Cr. Inoculation with PGPR strain, HRS-C3 significantly increased the fresh and dry biomass of plants (657.66 and 295.3 mg), respectively as compared with uninoculated control plants (535.08 and 214.50 mg, fresh and dry biomass, respectively) receiving the same concentration of Cd and Cr. Under dual metal (Cd+Cr) application trial, B6 isolate performed better than HRS-C3 in terms of plant biomass increase, where fresh biomass of plants treated with B6+Cd+Cr was almost double compared to the biomass of plants treated with HRS-C3+Cd+Cr. Inter metal comparison revealed that Cd treated plants were more stressed, eventually resulting in less foliage production compared with their counterparts exposed to Cr.

Effect of PGPR Inoculation on Cd uptake by Plants

Data presented in Fig. 2, revealed the positive correlation between metal application in the soil and that accumulated by plants where increasing metal concentration in the soil resulted in higher metal uptake by plants and vice versa. PGPR exerted varying effects on Cd uptake by *B. oleraceae* plants exposed to different concentrations of Cd. Cd uptake was significantly higher in the B6 inoculated plants compared with un-inoculated counter parts. Percent increase of Cd uptake in the PGPR inoculated plants ranged from 4–46% compared with un-inoculated plants treated with Cd only. Locally isolated B6 increased Cd uptake by plants considerably higher than HRS-C3 strain. Interestingly, B6 inoculated plants accumulated Cd in their tissues almost twice compared with that taken up by plants inoculated by PGPR strain, HRS-C3 regardless of any metal concentration applied in the soil.

Chromium uptake by *B. oleraceae* with and without PGPR Application

Influence of PGPR on Cr uptake was investigated in *B. oleraceae* plants (Fig. 3) growing at two metal concentrations (35 and 50 mg kg^{-1}) with and without inoculation with PGPR strains. Like that in Cd experiment, positive correlation was found between Cr applied in the soil and that accumulated by *Brassica* plants. However, Cr concentration taken up by plants was approximately half of that applied in soil in all Cr treatments without PGPR inoculation. Contrary to the findings in case of Cd treatment, where inoculated plants accumulated significantly higher amount of Cd compared with control, Cr uptake was considerably less in PGPR inoculated plants in comparison to the un-inoculated counterparts growing at same Cr concentration. Comparison of PGPR strains revealed that plants inoculated with, HRS-C3 accumulated approximately 19% and 15% less Cr compared with B6 inoculated plants exposed to 35 and 50 mg kg^{-1} Cr, respectively.

Concentrations of IAA, GA in broth Culture

Plant growth hormones; indole acetic acid (IAA) and gibberellic Acid (GA) were assayed in five days old liquid broth cultures inoculated with individual as well as mixed PGPR strains; HRS-C3, B6. Data in Fig. 4 revealed that PGPR strain, HRS-C3 proved to be more efficient in producing growth hormones compared with B6. Concentration of IAA and GA in the broth culture inoculated with HRS-C3 was, respectively, one and two folds higher than that of B6 inoculated culture. As expected, synergistic effect was observed whilst during combined inoculation of HRS-C3 and B6. Mixed cultures of microbial strains produced 6.11 and 11.86 (mg L^{-1}) of IAA and GA, respectively. The concentrations of IAA and GA produced by combined inoculation of PGPR were significantly higher than hormones concentrations measured in case of singular inoculations of HRS-C3 (Fig. 4).

Growth Hormone Production Ability of PGPR in *B. oleraceae* Exposed to Heavy Metals

Efficacy of PGPR to produce growth hormones; IAA and GA in *B. oleraceae* plants exposed to varying concentrations of Cd and Cr was investigated. Results depicted in Table 4 indicate that plants treated only with

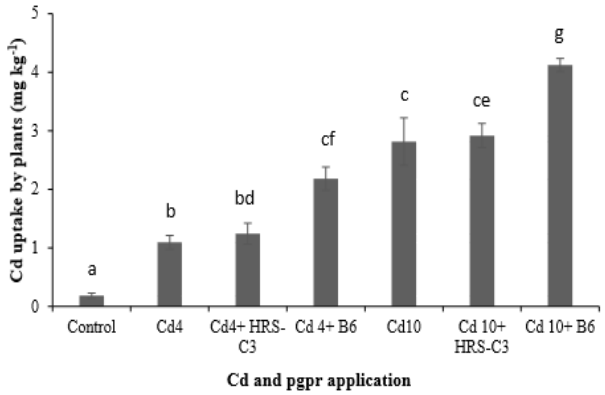

Fig. 2: Cd uptake by *B. oleraceae* plants exposed to varying concentrations of Cd and inoculated with PGPR strains (HRS-C3 and B6). Cd4 and Cd10 indicate the concentrations i.e., 4 and 10 mg Cd kg^{-1} of soil. Data are means of three replicates± SE. LSD=0.634

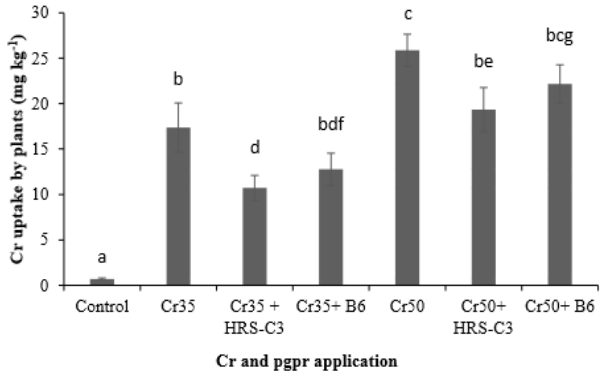

Fig. 3: Cr uptake by *Brassica oleraceae* plants exposed to varying concentrations of Cr and inoculated with PGPR strains (HRS-C3 and B6). Cr35 and Cr50 indicate the concentrations i.e., 35 and 50 mg Cr kg^{-1} soil. Data are means of three replicates ± SE. LSD=5.83

heavy metals produced less amounts of growth hormones compared with PGPR inoculated plants at same metal stress level. Moreover, the control plants produced significantly higher concentration of IAA and GA in reference to those exposed to heavy metals Cd or Cr. The concentration of growth hormones in plant shoot continued to decrease with increasing metal concentrations and reached to the lowest i.e., 0.22 and 2.85 mg L^{-1} of IAA and GA respectively in plants exposed to 10 mg Cd kg^{-1}. The highest concentration of IAA and GA (6.01 and 10.75 mg L^{-1}, respectively) were measured in plants growing at 4 mg kg^{-1} Cd and inoculated with HRS-C3.

Discussion

Results revealed that microbial strains used in the current

Table 4: Effect of PGPR on IAA and GA production in plants treated with different concentrations of Cd and Cr

Treatments	IAA (mg L^{-1})	GA (mg L^{-1})
Control	1.25 a	4.92 a
Cd4	0.44 b	2.98 b
Cd10	0.22 c	2.85 bc
Cr35	2.07 d	3.12 bd
Cr50	1.64 e	3.53 e
Cd4+HRS-C3	6.01 f	10.75 f
Cd4+B6	3.26 g	2.64 bg
Cd10+HRS-C3	4.21 h	10.09 h
Cd10+B6	5.11 I	7.63 i
Cr35+HRS-C3	3.17 Jg	9.68 hj
Cr35+B6	4.42 k	2.63 bk
Cr50+HRS-C3	1.28 AI	9.17 I
Cr50+B6	1.52 Em	4.71 am
LSD	0.167	0.409

The data are means of three replicates. LSD; IAA= 0.167, GA= 0.409. Values followed by different letters are statistically different from each other (P=0.05)

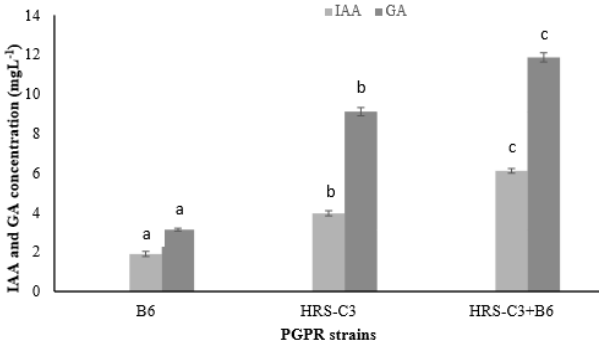

Fig. 4: Growth hormones (IAA, GA) production ability of PGPR strains, HRS-C3 and B6 in 5 days old broth culture with singular and joint inoculation. Data are means of three replicates± SE. LSD; IAA= 0.354, GA= 0.521

investigation exhibited the characteristics of plant growth promoting rhizobacteria. The antagonistic potential of PGPRs yielded interesting results which revealed their potential to antagonize both microbial and bacterial pathogens (Fig. 1). PGPR have been widely reported to impart strong inhibitory effect against various phytopathogens including; *Fusarium oxysporum*, *Rhizoctonia solani* and *Macrophomia phaseolina* under controlled environment (Kumar *et al*., 2012). This inhibitory effect could be attributed to cyanogen production by PGPR which inhibited fungal growth (Datta *et al*., 2011). Antimicrobial enzymes including; cellulase, chitinolytic, and competition for nutrients could also be involved in reducing the radial growth of target pathogens by PGPR (Chung *et al*., 2008; Singh *et al*., 2008). This characteristic of PGPR warrants investigating the biological control activity of these microbes. Perhaps antagonistic potential of PGPR is the most important characteristic of the microbes, the pre-requisite for plant

growth promotion. PGPR significantly increased the fresh and dry weights of plants compared with control plants and those treated with Cd and Cr (Table 3). Interestingly, plants treated with only Cd showed significantly less biomass as compared to the counter parts that received same Cd concentration but inoculated with PGPR. This observation highlights the stress alleviating potential of PGPR. Both Cd and Cr are non-essential heavy metals, not involved in any physiological functions and once taken up by plants disrupt the plant metabolism obviously resulting in stress of the whole plant. PGPR employ numerous strategies to impart health benefits to plants growing under heavy metal stress including; accumulation, transformation or detoxification of heavy metals (Mishra *et al*., 2017). These microbes alleviate the heavy metal stress through; induced systemic resistance (ISR), synthesis of antioxidant enzymes like catalase, superoxide dismutase and peroxidase (Sarma *et al*., 2012; Islam *et al*., 2014). Stress alleviation in plants growing in extreme environment, has been reported in all the major genera of PGPR i.e. from actinobacteria to proteobacteria, pseudomonas, bacillus and rhizobia (Pires *et al*., 2017). Nitrogen fixing nodule formation and nitrogenase are very sensitive to heavy metal toxicity, but they have been reported to induce nodulation from contaminated sites which indicates that rhizobia effectively detoxify heavy metal toxicity during nodulation process (Checcucci *et al*., 2017).

Cd uptake by brassica plants was significantly higher in PGPR inoculated plants (Fig. 2) compared with uninoculated controls. Overall, Cd accumulation in PGPR treated plants increased from 4–47% in line with previous researches where PGPR inoculation lead to significant increases in Cd uptake in brassica plants (Asad *et al*., 2013; Kamran *et al*., 2015). Increased Cd accumulation by *Eruca sativa* was attributed to PGPR (*Pseudomonas putida*) inoculation which also positively influenced growth parameters including, root/shoot length, fresh/dry biomass and chlorophyll contents (Kamran *et al*., 2015). PGPRs are known to enhance plant growth under stressed conditions and perhaps ACC-deaminase could possibly be involved in growth enhancement. This can be ascertained by the fact that ACC deaminase, an enzyme secreted by PGPR actively blocks the synthesis of ethylene production (Cheng *et al*., 2007; Sun *et al*., 2009). Enhanced level of Cd accumulation in the PGPR inoculated plants may be because of greater biomass and subsequently increased concentration of phytohormones (IAA, GA) induced in plants after PGPR inoculation (Asad *et al*., 2004; Baharlouei *et al*., 2011). This puts some credence on our synthesis that single drawback of heavy metal hyperaccumulator is less biomass and slow growth habit. Once plants are inoculated with PGPR, their growth is promoted which ultimately results in enhanced level of metal uptake with increasing biomass. In current study, fresh and dry weights of inoculated plants were significantly higher than uninoculated counter parts, thus it seems logical to conclude that more biomass would have accumulated more metal and vice versa. Contrary to the Cd uptake, Cr accumulation was reduced in PGPR inoculated plants compared with uninoculated control growing at same metal concentration which contradicts previous researches (Ma *et al*., 2011; Kamran *et al*., 2017 and references therein) who noticed significantly enhanced uptake of Cr after brassica plant, *Eruca sativa* was inoculated with PGPR strains. This difference might be because, *E. sativa* is heavy metal hyperaccumulator while, *B. oleracea* is accumulator and both inherit different physiology to uptake and accumulate heavy metals where formers take up excessive amounts to their above ground parts and vice versa (Rascio and Navari-Izzo, 2011). Hence, reduced Cr uptake in current study may be because of poor translocation mechanism from root to shoot (Zayad *et al*., 1998). Metal uptake is facilitated by the secretion of different enzymes and increased uptake of Cr in Kamran *et al*. (2017) study may be because of various enzymes produced by *P. putida* in the contaminated soil (Ma *et al*., 2011). PGPR inoculation is known to increase the solubility and bioavailability of heavy metals, as was observed in case of increased Cd uptake (Fig. 2). ACC-deaminase activity was not determined, so it could be possible that PGPR strains used (HRS-C3 and B6) did not exhibit ACCD activity which was essential for enhancing plants growth and hence metal uptake. Moreover, it might be because specific activity of PGPR to reduce soil pH was not expressed at specific time which hampered PGPR ability to increase nutrient and metal uptake by plant. It is worth noting, that growth hormones; IAA and GA were increased in response to PGPR inoculation (Fig. 4 and Table 4) both in pure culture as well as in plant tissues regardless of any metal concentrations in the soil. These phytohormones play a key role in enhancing the plant biomass (Dell'Amico *et al*., 2008; Rajkumar and Freitas, 2008; Baharlouei *et al*., 2011) thereby reducing the metal toxicity and/or increasing the uptake of essential nutrients. Despite, significant increases in IAA and GA, fresh and dry biomass of plants, Cr uptake by inoculated plants was reduced. Although Cr concentration in the inoculated plant roots was not calculated, but there is possibility that immobilization of Cr in the vacuoles of root cells resulted in less uptake of Cr, as a toxicity response of plants (Shanker *et al*., 2004; Khan *et al*. 2013). However, lack of information on translocation mechanism of Cr from roots to shoot limits our understanding on reduced uptake of this heavy metal after inoculation with PGPR.

Conclusion

PGPR inoculation influenced Cd and Cr uptake differently by *B. oleraceae* plants, where Cd uptake was significantly increased in response to PGPR inoculation while Cr uptake

was decreased. Combined application of PGPR strains, HRS-C3 and B6 resulted in more pronounced increase in plant growth parameters (e.g., shoot fresh and dry weights, growth hormone production) than singular applications of same strains. Suitable combinations of metal specific PGPRs could enhance the heavy metal solubility and uptake by *B. oleraceae*. This technique could be highly effective for remediating the medium-level contaminated soil, promoting the plant growth without causing any toxicity in the food chain.

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(Received 20 February 2018; Accepted 24 March 2018)