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Original Research Article

Co-inoculation of auxin producing PGPR and rhizobia enhanced growth of *Vigna mungo* (L.) under cadmium stress

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Abstract

The main objective of this study was to evaluate the effect of cadmium resistant bacteria to enhance the growth of black gram (*Vigna mungo*) under cadmium ($CdCl_2$) stress. For this purpose, plant growth promoting rhizobacteria (PGPR) and strains of rhizobia were isolated from the rhizosphere of different plants. 16S rRNA gene sequencing of bacteria confirmed the presence of the strains of *Bacillus cereus*, *B. thuringiensis*, *Pseudomonas fluorescens* and *Rhizobium*. L-tryptophan dependent auxin production was determined colorimetrically by growing strains in Nutrient broth. *B. Cereus* HG, *B. thuringiensis* JAF *and Rhizobium sp*. IBA showed highest levels of auxin production that ranges from 140 to 146 µg ml^{-1}. For pot trials, soil was amended with 50, 100 and 200 mg kg^{-1}. In single bacterial inoculations, significant shoot length response was up to 65% and 39% with *P. fluorescens* WN-1 and *B. cereus* CF at 200 mg kg^{-1}, over respective $CdCl_2$ treated control. Similarly, for fresh biomass, 2.2 and 1.3 fold increases were shown by *B. cereus* CF at 100 and 200 mg kg^{-1} $CdCl_2$ stress, respectively. For mixed culture of PGPR, combination MC-3 (CF-WN-1, A-2) significantly promoted shoot length (1 fold) at 50 mg kg^{-1} $CdCl_2$. In case of mixed culture of PGPR and rhizobia, up to 1 fold increases were observed for shoot length, root growth and fresh biomass with MCR-1 (IBA, HG, BS-1) at 100 mg kg^{-1} $CdCl_2$. Finally, it can be concluded that strains isolated from the rhizosphere of different pants showed promising results for plant growth promotion in cadmium amended soils.

Keywords: Bacterial auxin, Cadmium stress, PGPR, Growth promotion, Phytoremediation, Vigna mungo

Introduction

Among all the heavy metals, cadmium (Cd) is wide spread and most toxic pollutant present in the top soil. It is present with Zinc (Zn) and the increased demand of Zn has also increased the amount of Cd in the environment. The use of Cd has also increased in industries as it has many physical and chemical properties that make it useful (Hussain and Islam, 2010). Cadmium releases into environment through mining, smelting, burning of fuel, sewage sludge and application of phosphate fertilizers. In nature volcanic eruption, forest fires and soil erosion are the main sources of cadmium release (Figueira et al., 2005). Cadmium particles travel as dust and fall with rain, there they enter into soil, crops and water supplies. It is very toxic and it is also considered as carcinogenic. It causes certain diseases such as lung cancer, osteomalacia, renal damage by affecting tubular cells, bone demineralization by renal dysfunction (Godt et al., 2006). It also causes damage to liver, reproductive system, emphysema, hypertension, testicular atrophy

and cardiovascular system even at low level of exposures (Bazrafshan et al., 2006). Once it enters in the body it tends to remain there for a longer period of time. It enters in human bodies from diet as many crops like lentils, potato, tobacco, leafy vegetables, sunflowers and root vegetables take up large amount of cadmium (Qing et al., 2007). Cadmium poses risk because it absorbs very easily in the body and its half-life is 10-30 years. Its amount in adults of 45 years of age is about 30-40 mg per total body weight. Its declared lethal dose is 2 mg kg^{-1} body weight that is quit less than other toxic metals. In plants it cause chlorosis, stunted growth of roots, disturbed homeostasis and affect nutrient uptake (Jankiewicz et al., 2000).

In order to relief soil from metal contamination, plant growth promoting rhizobacteria (PGPR) are being used. The plant growth promoting traits of these bacteria are nitrogen fixation, siderophores and phytohormone production (Ladwal et al., 2012; Raheem et al., 2015). Plant resists to heavy metal contamination in the soil by expressing shock absorbent proteins. But when the concentration of such metals increases beyond the limit, plants start to become deficient in certain nutrients. Low iron absorption that leads to under development of chloroplast and hence plants becomes chlorotic exhibiting yellow coloration of leaves. PGPR able to produce siderophores resolves this problem for plants by providing them a large amount of iron to absorb by their roots. (Jing et al., 2007). In addition, it has also been observed that the PGPR also help the seedlings to germinate in the stressed environment. Once the seedling has established, the rhizobacteria help them to grow by providing them with solubilised phosphates and phytohormones (Rajkumar et al., 2010; Raheem et al., 2017).

Bacteria use quorum sensing mechanism for plant growth promotion. They sense the molecular signal, amplify it and attract other microbial species thus forming a biofilm over the roots of plants. Bacteria in return get food and nutrients from the plant root exudates as well as shelter for living (Khan, 2005). Inoculated bacterial strains first share the metal load with plants and after emergence of seedling, these PGPR starts to produce phytohormones like auxin that helps the plant roots to establish for better nutrient uptake (Madhaiyan et al., 2007; Ali, 2015).

Heavy metals interfere with the uptake of essential nutrients by roots of plants. Metal resistant bacteria are able to convert the heavy metals from one oxidation state to another thus, lessen the impact of metals on roots and allow them to absorb nutrients. In addition, they also produce phytohormone auxin that increases the surface area of root by enhancing the root hair growth that helps the plants to absorb nutrients far away in the soil. (Belimov et al., *2005*). The main aim of this study was the isolation of cadmium resistant bacteria from the rhizosphere of plants and their application to enhance the growth of black gram i.e. *Vigna mungo* (L.) Hepper in cadmium amended soil. For co-inoculation experiments, rhizobia were also isolated from the root system of *Vicia faba* L. to evaluate their combined effect on the growth of leguminous plant.

Materials and Methods

Isolation and characterization of bacterial strains

For the isolation of plant growth promoting rhizobacteria (PGPR), soil samples were collected from the rhizosphere of *Rosa indica*, *Trifolium alexandrinum*, *Jasminum officinale*, *Solanum tuberosum* and *Brassica campestris*. One gram of soil was dissolved in 99 ml adutoclaved distilled water to prepare 10^{-2} dilution and so on. From each dilution, 50 µl sample was spread on Nutrient agar (N-agar) plates and incubated at 30°C for 24 h. Afterwards, diverse bacterial colonies were selected and purified by quadrant streaking. For rhizobia isolation, roots of *Vicia faba* were collected and sterilized by dipping in 1% solution of bleach for 2-3 min. Afterwards, nodules were washed with distilled autoclaved water three times. The nodules were crushed in 1 ml autoclaved distilled water and loopful inoculum was streaked on Yeast Mannitol Agar (YMA). Finally, different colonies with varied appearance were purified after several rounds of streaking. Gram and spore staining, methyl red Vogus Proskauer test (MR-VP), starch hydrolysis, citrate utilization, nitrogenase activity and Congo red tests were performed.

16S rRNA gene sequencing

Final taxonomic status of a few bacterial strains was determined by 16S rRNA gene sequencing. Genomic DNA was extracted by using Tissue Genomic DNA Extraction Kit (Favorprep™, Favorgen). About 1.5-Kb DNA fragment containing 16S rRNA gene was amplified using forward 27f and reverse 1522r primers as described earlier (Akhtar and Ali, 2011). After PCR amplification, product was purified using FavorPrep™

Gel Purification Kit. Purified fragments were sequenced from First Base Laboratories (Singapore).

Colorimetric analysis of bacterial auxin production

Auxin production in liquid medium was quantified in the presence of L-tryptophan. N-broth was amended with 200 µg ml^{-1} of L-tryptophan. Then, flasks were inoculated with 100 µl of bacterial cell suspension in duplicate. Cultures were incubated around 30ºC for 72 h on shaker at 120 rpm. Then, 1 ml of bacterial culture was centrifuged (at 5000 rpm) and mixed with 2 ml of Salkowski reagent. Test tubes were kept in dark for 25 min and optical density of the samples was recorded at 535 nm with spectrophotometer. Standard curve from different concentrations of authentic auxin was constructed for the estimation of bacterial auxin production.

Analysis of bacterial auxin by HPLC

Auxin was also detected qualitatively by using High Performance Liquid Chromatography (HPLC). For this purpose, bacterial culture supernatants from the above experiment were also used for HPLC analysis. Ten ml of bacterial supernatant was adjusted to pH 3 and mixed with equal volume of ethyl acetate. Afterward, upper aqueous layer was separated in a flask and allowed to dry by placing it in water bath at 50ºC. Then, 5 ml of HPLC grade methanol was added to dissolve the auxin content. Auxin was analyzed qualitatively after injecting 20 µl of each sample in HPLC (Model 203; Sykam, Eresing, Germany) equipped with hypersil-keyston ODS column (5 µm; 4.6 × 250 nm). The mobile phase was methanol/water (90:10) at a flow rate of 1 ml min^{-1}. Elutes were detected at 220 nm and IAA was detected by integrating the areas under the peaks of authentic IAA.

Bacterial cadmium resistance

Bacterial $CdCl_2$ resistance in solid media was determined qualitatively by assessing the abundance of growth appeared on the surface of media. To determine resistance, N-agar was supplemented with different concentrations of metal that included 0.25, 0.5, 1, 1.25 and 1.5 mg ml^{-1}. Bacterial strains were inoculated in duplicate and incubated at 37ºC for 24 h. Bacterial cadmium resistance was determined by evaluating the abundance of growth.

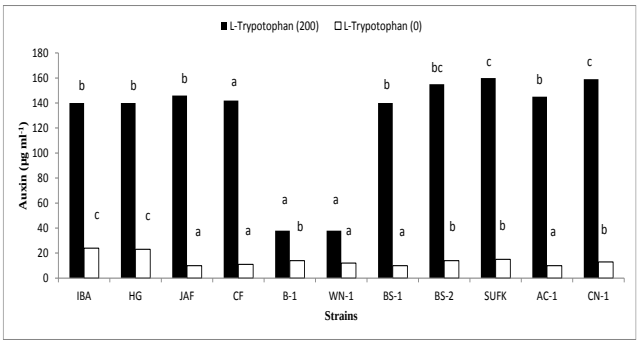

Figure 1. Bacterial auxin production in the presence and absence of L-tryptophan.

Different letters on bars indicate significant difference between treatments using Duncan's multiple range test ($P \leq 0.05$).

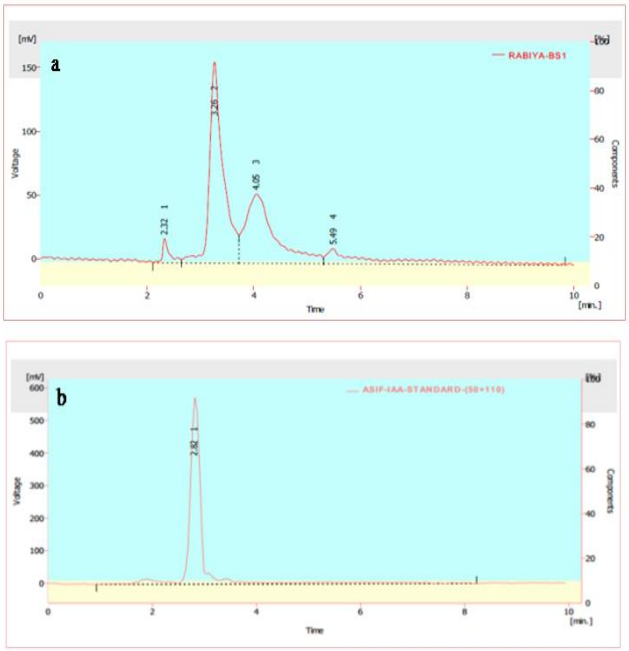

Figure 2. HPLC analysis of bacterial auxin production. (a)-extracts from P. fluorescens WN-1, (b)- standard IAA

Nitrogenase activity of bacterial strains

Bacterial nitrogenase activity was determined in nitrogen free mineral salt medium. Strains were inoculated on the media plate and placed in incubator at 37ºC for 48 h to allow the appearance of growth or hollow zone of growth. Bacterial Congo red absorption was determined in order to indicate the presence of rhizobia by supplementing the yeast mannitol agar with Congo red indicator. *Rhizobium* species took up the colour of Congo red in the presence of $CaCO_3$. Strains were inoculated on the plates and

incubated at 28 ºC for 48 h. After incubation in dark, the plates were incubated in light for 24 h to allow the strains to absorb the Congo red colour. Appearance of pink colouration in colonies upon incubation in light showed the presence of *Rhizobium.*

Pot trails

For pot trials, inoculum was prepared by dissolving the bacterial growth from N-agar to 25 ml of autoclaved distilled water. Optical density of the cultures were adjusted 10^7 CFU ml^{-1}. Sterilized seeds of *Vigna mungo* were inoculated with single bacterial cultures such as HG, WN-1, JAF, B-1, CF and SUFK. Mixed cultures (MC) combinations included MC-1 (WN-1, JAF, A-2), MC-2 (HG, JAF, CF), MC-3 (CF-WN-1, A-2) and MC-4 (CF, HG, JAF). For rhizobia single cultures included Nj-2, Swm-1, Sdm-1, IBA and Wn-2. Mixed cultures of rhizobia with other PGPR strains were also used that included MCR-1 (IBA, HG, BS-1), MCR-2 (IBA, WN-1, AC-1), MCR-3 (IBA, JAF, CN-1) and MCR-4 (IBA, BF-1, SUFK). For bacterization, seeds were treated with bacterial suspensions (single or mixed) for 25 min. For control treatment, seeds were dipped in distilled autoclaved water for the same duration. Soil was prepared by mixing equal ratio of sand and soil (1:1). After mixing, soil was autoclaved to eliminate the growth of previously present microbes. After autoclaving, soil was filled in pots (6×6 cm) and amended with three different concentrations of $CdCl_2$ to a final amount 50, 100 and 200 mg kg^{-1} of soil. Positive controls were inoculated only with bacterial suspensions. Six pots were used for each strain, metal treatments or control. Five seeds were inoculated in each pot at the depth of 1 cm. Pots were moistened with sterilized water immediately after sowing and placed in plant growth chamber at 12 h photoperiod. After 7 days, plants were harvested to record their shoot length, root length and fresh weight. For dry weight, plants were dried at 80ºC in oven for 24 h.

Statistical analysis

Data for auxin production and plant growth parameters was subjected to analysis of variance (ANOVA) and means were separated by using Dnncan's multiple range test ($P \leq 0.05$).

Results

Biochemical characterization

After purification of colonies, 11 strains were selected for further study. Majority of the strains (HG, JAF, B-1, CF) were found to be gram positive, while some were gram negative (WN-1, IBA). Among gram positive strains, majority showed starch hydrolysis and citrate utilization as well as spore formation. Gram negative strains WN-1, AC-1, BS-1 and CN-1 showed positive Congo red test and nitrogenase activity. For MR-VP test HG, SUFK and JAF were found to be MR positive and VP negative. Bacterial strains grown under different concentration of $CdCl_2$ showed varied pattern of resistance. Strains HG, IBA, SUFK and JAF showed high resistance at 0.5 mg ml^{-1}. Whereas, WN-1 showed sensitivity against $CdCl_2$ as metal concentration increased from 0.7 to 5.62 mg ml^{-1}.

Identification of bacterial strains

16S rRNA gene sequences were compared with online available sequences in GenBank through BLAST. Majority of the strains showed 99% resemblance with their respective genes. Strain HG isolated from *R. indica* showed resemblance with *Bacillus cereus*. WN-1 associated with *T. alexandrinum* showed homology with *Pseudomonas fluorescence*. Similarly, JAF isolated from *J. officinale* showed homology with *Bacillus thuringiensis*. On the other hand, B-1 and CF isolated from *S. tuberosum* and *B. campestris* were similar to *B. cereus*. Strain IBA isolated from *V. faba* showed homology with *Rhizobium* species. The sequences from strains HG, WN-1, JAF, B-1, CF and IBA have been submitted in the GenBank under accession numbers KM206792 to KM206797 (Table 1).

Bacterial auxin production

Bacterial strains were grown in the presence and absence of L-tryptophan. For majority of the strains, auxin production was very low in the absence of L-tryptophan. However, in the presence of substrate higher levels of auxin were recorded. For example *B. Cereus* HG, *B. thuringiensis* JAF *and Rhizobium sp.* IBA showed highest levels of auxin production that ranges from 140 to 146 µg ml^{-1} (Fig. 1). About 4.9 fold increase in auxin production was recorded by *Rhizobium sp.* IBA as compared to un-supplemented medium. *B. cereus* CF and *B. cereus* B-1 also showed 11 and 5 fold increases, respectively. Similarly, 14 fold increases were recorded by *B. thuringiensis* JAF, over respective un-amended control. Bacterial auxin production was further confirmed by analysing the bacterial crude extracts with HPLC. Figure 2 showed the chromatogram of auxin production by *P. fluorescens* WN-1 in comparison with standard.

Pot trials with monocultures

Plants grown under cadmium stress showed a very poor growth as compared to bacterized or water treated treatments. Overall, plant growth enhancement by inoculation with monocultures of PGPR was up to 78% as compared to their respective water treated control (Table 2). For shoot length, single bacterial inoculations in cadmium non-amended soils showed statistically comparable results to water treated controls. However, when plants were subjected to 50, 100 and 200 mg kg^{-1} of $CdCl_2$ stress, significant reduction in shoot length (up to 41%) was observed over water treated control. On the other hand, when plants were grown in $CdCl_2$ amended soils under bacterial inoculations, significant reduction in metal toxicity was recorded in comparison with respective control. For instance, *B.* cereus HG showed up to 60% increase in shoot length over 100 mg kg^{-1} metal treated control. However, maximum shoot length response was up to 65% and 39% with *P. fluorescens* WN-1 and *B. cereus* CF at 200 mg kg^{-1}. Similarly, *B. cereus* B-1 and *B. thuringiensis* JAF showed highest root growth response that was up to 1.2 and 1.8 fold at 50 mg kg^{-1}, respectively, over metal treated control. On the other side, at 100 mg kg^{-1} of stress SUFK showed 1.7 fold increases over respective metal treated control. Similarly, for fresh biomass *B. cereus* CF showed highest response in cadmium non-amended soil as compare to water treated control. However, when plants were exposed to cadmium stress, best recorded plant growth promotion was up to 78% by strain SUFK at 50 mg kg^{-1}. Moreover, 2.2 and 1.3 fold increases were shown by *B. cereus* CF at 100 and 200 mg kg^{-1} cadmium stress respectively. For dry biomass, highly significant increase (4 fold) was shown by *B. cereus* HG at 50 mg kg^{-1}.

Growth response with mixed bacterial culture inoculations

Pot trials were also conducted by using mixed cultures of PGPR and rhizobia. In cadmium non-amended soils, plants showed significant growth under bacterial inoculations as compared to water treated seeds (Table 3). However, under cadmium stress, plants recorded retarded growth response, over control. Nevertheless, mixed culture combination MC-3 significantly performed well at 50 mg kg^{-1} by promoting the shoot length up to 1 fold over respective metal treated control. Similarly, MC-2 and MC--4 enhanced shoot length up to 1.2 fold and 60 % at 100 and 200 mg kg^{-1} of cadmium stress, respectively. As far as root length promotion is concerned, mixed culture induced growth was up to 38% and 26% with combinations MC-3 and MC-1 under 50 and 100 mg kg^{-1} of $CdCl_2$ stress, respectively. The increase in fresh weight was up to 1.3, 2.2 and 1.1 fold with MC-3, MC-4 and MC-2 under 50, 100 and 200 mg kg^{-1}. For dry weight, up to 53% increase was observed with MC-3 at 50 mg kg^{-1} $CdCl_2$ (Table 3).

Mixed cultures of rhizobia (MCR) with other PGPR also recorded positive results for the growth of *V. mungo* as compare to metal treated controls (Table 4). For shoot length at 100 mg kg^{-1} $CdCl_2$ up to 1 fold increase was observed with MCR-1, over respective control. Similarly, for root length, 1 fold increase was also recorded by MCR-1 combination at 100 mg kg^{-1}. For fresh biomass, significant increase of 1.3 fold was recorded at 50 mg kg^{-1} with MCR-1 combination over metal treated control. In case of dry weight, MCR-1 also performed well at 100 mg kg^{-1} by increasing the weight up to 76% as compare to metal treated control.

Table 1. 16S rRNA gene sequencing of PGPR and Rhizobium isolated from the rhizosphere of plants

S. No.	Source/ Plant	Strain	Identified as	Accessions
1	*Rosa indica* L.	HG	*Bacillus cereus* HG	KM206792
2	*Trifolium alexandrinum* L.	WN-1	*Pseudomonas fluorescens* WN-1	KM206793
3	*Jasminum officinale* L.	JAF	*B. thuringiensis* JAF	KM206794
4	*Solanum tuberosum* L.	B-1	*B. cereus* B-1	KM206795
5	*Brassica Campestris* L.	CF	*B. cereus* CF	KM206796
6	*Vicia faba* L.	IBA	*Rhizobium* sp. IBA	KM206797

Table 2. **Effect of single cultures of PGPR strains on the growth of *V. mungo* in cadmium amended soils**

S. No.	Strains/ Treatmetns	Shoot length (cm)	Root length (cm)	Fresh weight (g)*	Dry weight (g)*
1	Control	19.92 de	11.93 i	2.15 hi	0.233 bc
2	50 mg kg^{-1}	11.61 ab	5.33 ab	0.83 a-e	0.131 b
3	100 mg kg^{-1}	11.10 a	3.94 a	0.63 c	0.12 a
4	200 mg kg^{-1}	11.60 ab	4.10 a	0.80 a-e	0.40 cd
5	HG	19.50 de	9.27 e-i	3.45 j	0.39 a-d
6	HG+50 mg kg^{-1}	16. b-e	7.85 b-f	1.28 b-g	0.67 d
7	HG+100 mg kg^{-1}	17.80 cde	8.70 e-h	1.46 c-h	0.21 abc
8	HG+200 mg kg^{-1}	16.55 cde	3.31 a	1.80 f-i	0.45 abcd
9	WN-1	16.10 b-e	8.0 c-g	1.55 d-h	0.58 cd
10	WN-1+50 mg kg^{-1}	15.65 a-d	10.05 f-i	0.84 a-e	0.20 abc
11	WN-1+100 mg kg^{-1}	16.17 b-e	9.16 e-i	0.75 a-e	0.15 ab
12	WN-1+200 mg kg^{-1}	19.22 de	5.53 abc	1.48 a-h	0.33 ad
13	JAF	16.69 cde	8.31 d-h	0.481 ab	0.21 abc
14	JAF+50 mg kg^{-1}	18.65 cde	11.61 i	0.31 a	0.24 abc
15	JAF+100 mg kg^{-1}	15.56 a-d	9.43 e-i	0.31 a	0.14 ab
16	JAF+200 mg kg^{-1}	13.80 abc	3.18 a	1.04 a-f	0.35 a-d
17	B-1	16.49 cde	10.46 f-i	1.41 c-h	0.33 a-d
18	B-1+ 50 mg kg^{-1}	15.56 a-d	11.83 i	1.49 d-h	0.42 a-d
19	B-1+ 100 mg kg^{-1}	16.89 cde	10.76 ghi	1.57 e-h	0.18 abc
20	B-1+200 mg kg^{-1}	20.70 e	5.71 a-d	1.60 e-h	0.33 a-d
21	CF	15.92 b-e	9.53 e-i	2.53 i	0.34 a-d
22	CF+ 50mg kg^{-1}	16.65 cde	6.95 b-e	1.37 c-h	0.15 ab
23	CF+ 100 mg kg^{-1}	17.81 cde	10.55 f-i	2.05 g-i	0.25 abc
24	CF+ 200 mg kg^{-1}	16.13 b-e	3.16 a	1.85 f-i	0.52 bcd
25	SUFK	16.26 b-e	8.58 e-h	0.71 abcd	0.13 ab
26	SUFK + 50 mg kg^{-1}	18.03 cde	9.40 e-i	0.85 a-e	0.26 abc
27	SUFK + 100 mg kg^{-1}	16.05 b-e	10.86 hi	0.78 a-e	0.20 abc
28	SUFK + 200 mg kg^{-1}	17.44 cde	4.11 a	0.14 c-h	0.32 abcd

Mean of 30 plants. Different letter within same column indicate significant difference between treatments using Duncan's multiple range test ($P \leq 0.05$).

* Fresh and dry weights represented by three replicates of 5 plants

Table 3. **Effect of mixed cultures of PGPR on growth of *V. mungo* in cadmium amended soils**

S. No.	Strains/ Treatments	Shoot length (cm)	Root length (cm)	Fresh weight (g)*	Dry weight (g)*
1	Control	19.92 cdef	11.93 e	2.15 ab	0.233 a-e
2	50 mg kg^{-1}	11.61 ab	5.33 ab	0.83 a	0.131 ab
3	100 mg kg^{-1}	11.10 a	3.94 a	0.63 a	0.12 ab
4	200 mg kg^{-1}	11.60 ab	4.10 a	0.80 a	0.4 cde
5	MC-1**	15.03 abc	7.97 d	0.98 a	0.36 b-e
6	MC-1+50 mg kg^{-1}	14.86 abc	4.86 ab	1.75 ab	0.17 a-d
7	MC-1+100 mg kg^{-1}	14.29 abc	4.99 ab	1.11 ab	0.09 a
8	MC-1+200 mg kg^{-1}	18.59 cdef	3.91 a	1.48 ab	0.14 ab
9	MC-2**	17.79 b-e	9.90 de	2.51 b	0.44 e
10	MC-2 +50 mg kg^{-1}	18.16 c-f	5.90 abc	1.41 ab	0.12 ab
11	MC-2 +100 mg kg^{-1}	24.20 f	4.72 a	1.5 ab	0.11 ab
12	MC-2 +200 mg kg^{-1}	18.54 c-f	4.14 a	1.7 ab	0.24 a-e
13	MC-3**	15.76 abc	9.21 d	1.22 ab	0.42 de
14	MC-3+50 mg kg^{-1}	23.23 ef	7.39 bcd	1.95 c ab	0.2 a-d
15	MC-3+100 mg kg^{-1}	19.95 c-f	3.94 a	1.45 ab	0.15 abc

16	MC-3+200 mg kg^{-1}	17.01 a-e	3.51 a	1.45 ab	0.14 ab
17	MC-4**	15.57 abc	7.95 cd	2.51 b	0.44 e
18	MC-4+50 mg kg^{-1}	16.77 a-d	3.53 a	1.35 ab	0.12 ab
19	MC-4+100 mg kg^{-1}	23.06 def	3.95 a	2.05 ab	0.18 a-d
20	MC-4+200 mg kg^{-1}	18.67 c-f	3.1 a	1.4 ab	0.121 ab

Mean of 30 plants. Different letter within same column indicate significant difference between treatments using Duncan's multiple range test ($P \leq 0.05$).

* Fresh and dry weights represented by three replicates of 5 plants

** Mixed culture combinations: MC-1 (WN-1, JAF, A-2), MC-2 (HG, JAF, CF), MC-3 (CF, WN-1, A-2), MC-4 (CF, HG, JAF)

Table 4. Effect of mixed culture of PGPR and rhizobia on the growth of *V. mungo* in cadmium amended soils

S. No.	Strains	Shoot length (cm)	Root length (cm)	Fresh weight (g)*	Dry weight (g)*
1	Control	19.92 cd	11.93 f	2.15 d	0.233 ab
2	50 mg kg^{-1}	11.61 b	5.33 bcd	0.83 abc	0.131 ab
3	100 mg kg^{-1}	11.10 b	3.94 abc	0.63 ab	0.116 ab
4	200 mg kg^{-1}	11.60 b	4.10 abc	0.80 abc	0.40 ab
5	MCR- 1**	23.40 d	11.30 f	1.57 bcd	0.36 ab
6	MCR-1+50 mg kg^{-1}	19.88 cd	5.34 bcd	1.95 cd	0.13 ab
7	MCR-1 +100 mg kg^{-1}	22.11 d	7.94 de	1.08 abcd	0.14 ab
8	MCR-1 +200 mg kg^{-1}	6.4 a	2.16 a	0.09 a	0.24 ab
9	MCR -2**	22.20 d	10.84 f	0.78 abc	1.21 c
10	MCR -2 +50 mg kg^{-1}	22.31 d	6.31 cd	1.08 abcd	0.23 ab
11	MCR-2 +100 mg kg^{-1}	19.70 cd	5.74 bcd	1.12 abcd	0.11 ab
12	MCR-2 +200 mg kg^{-1}	15.91 b	4.08 abc	0.53 ab	0.05 a
13	MCR -3**	23.68 d	10.02 ef	1.59 bcd	0.25 ab
14	MCR-3 + 50 mg kg^{-1}	22.05 d	5.22 bcd	1.36 abcd	0.16 ab
15	MCR-3 + 100 mg kg^{-1}	19.16 cd	5.56 bcd	0.9 abcd	0.1 ab
16	MCR-3 + 200 mg kg^{-1}	13.66 b	2.85 ab	1 abcd	0.11 ab
17	MCR -4**	22.0 d	10.74 f	1.3a-d	0.82 bc
18	MCR -4 + 50 mg kg^{-1}	22.88 d	7.1 d	1.28 abcd	0.2 ab
19	MCR -4 + 100 mg kg^{-1}	22.00 d	5.71 bcd	0.8 abc	0.12 ab
20	MCR-4 + 200 mg kg^{-1}	14.45 b	2.36 a	1 abcd	0.15 ab

Mean of 30 plants. Different letter within same column indicate significant difference between treatments using Duncan's multiple range test ($P \leq 0.05$).

* Fresh and dry weights represented by three replicates of 5 plants

** Mixed culture combinations: MCR-1 (IBA, HG, BS-1), MCR-2 (IBA, WN-1, AC-1), MCR-3 (IBA, JAF, CN-1), MCR-4 (IBA, BF-1, SUFK)

Discussion

This study was conducted to demonstrate the cadmium stress alleviation of *Vigna mungo* after inoculation with rhizobacteria. The metal especially cadmium contamination of soil is hazardous for animals as well as humans and plants. In the present study, cadmium resistant strains that have the ability to produce auxin were isolated. Finally, selected strains were used to determine their potential of plant growth promotion under cadmium stress. Highest auxin production by rhizobacteria was ranged from 140-160 µg ml^{-1}. Auxin is a phytohormone that is involved in increasing root growth of plants. Increase in adventitious roots causes an increased level of nutrient absorption from the soil and thus enhance the plant growth parameters like shoot growth and leaf development (Iqbal and Hasnain, 2013). Bacteria have a number of mechanisms to resist the presence of metal either by converting it into less toxic compound or by up taking it in combined form with minerals. Bacteria associated with the rhizosphere have the ability to resist the cadmium and other metal stress. They do so either by immobilizing, pumping, blocking or binding the metal

to certain specific parts of the cells (Balestrazzi et al., 2009).

After screening, strains were used to observe their growth promoting ability in the presence of metal stress. Experiments demonstrated that the plants recorded suppression in growth when sown solely in metal amended soils. However, plants showed statistically good growth response under metal stress after bacterial inoculations. But, when these seeds were sown along with the bacterial co-inoculation, plants showed better growth. It was recorded that increase in plant root and shoot lengths were up to 1.8 fold and 65% under metal stress conditions. Cadmium metal inhabits the root and shoots growth by imposing damages to the plants normal physiological actions (Rathour et al., 2013). Cadmium can cause oxidative damage in *Vigna mungo* that can be mitigated by eliciting certain antioxidant defence mechanisms (Molina et al., 2008). Recorded increment in fresh and dry biomass of plants with single culture inoculation was 2.2 fold. Ali et al. (2013) has reported that co-inoculation of seeds with auxin producing PGPR increase the biomass of plants by promoting the nutrient availability. Depending upon the applied concentration of metal stress, PGPR immobilize the metal ions in the rhizosphere, thus inhabiting their uptake by plants. In addition these bacteria produce auxin hormone which cause the root enlargement and hence promoting the mineral uptake by the roots (Belimov et al., 2004).

When the bacterial strains were used as mixed culture, plant growth promotion was recorded that was up to 1 fold in terms of plant shoot and root length under cadmium stress. Similarly, fresh weight enhancement was up to 2.2 fold and dry biomass increase was up to 76% in cadmium amended soil. Bacteria associated with the plant roots enhance plant growth by various mechanisms like nitrogen fixation, nutrients conversion and phosphorous availability. PGPR also resist cadmium and can enhance plant growth in the presence of metal stress (Belimov et al., 2005). Co-inoculation with *Bacillus* and *Rhizobium sp.* can enhance the plant growth by enhancing root nodulation or biological nitrogen fixation. In addition, *Bacillus sp.* cause change in composition of rhizosphere leading to enhanced yield of plants in terms of height and weight (Venkatesan et al., 2011; Raheem et al., 2017). Sajid et al. (2017) also reported the plant growth enhancement by using single or mixed bacterial inoculations of *Bacillus* strains under stress conditions. Use of mixed culture of different PGPR either belonging to different species or from same species enhance the performance of these bacterial strains. One property of a strain complements the other property of the strain. As *Rhizobium* fix the nitrogen in the roots of plants, and the other bacteria with the auxin producing ability will enhance their work and plant will be benefited as compared to single strain inoculation (Yu et al., 2012).

Conclusion

In conclusion, strains isolated in this study showed promising results for plant growth promotion under axenic conditions in cadmium amended soils. Majority of the strains showed resistance to cadmium stress. Highest auxin production was ranged from 140- 160 µg ml^{-1} by *B. cereus* HG and *B. thuringiensis* JAF. In pot trials, *B. cereus* HG, *B. cereus* CG and *P. fluorescens* WN-1 showed significant growth response in the presence of cadmium stress. In mixed culture of PGPR, combinations of MC-2 and MC-3 performed to enhance vegetative growth parameters. In co-inoculation experiments of PGPR and rhizobia, mixed culture combination MCR-1 was the most promising as compared to other combinations. Overall, strains showed good potential to facilitate crop growth under cadmium contaminated environment.

References

Ali B, 2015. Bacterial auxin signaling: Comparative study of growth induction in *Arabidopsis thaliana* and *Triticum aestivum*. Turk. J. Bot. 39: 1-9.

Ali T, Mahmood S, Khan MY, Aslam A, Hussain MB, Asghar HN and Akhtar JA, 2013. Phytoremediation of cadmium contaminated soil by auxin assisted bacterial co-inoculation. Asian J. Agri. Biol. 1: 79-84.

Akhtar S and Ali B, 2011. Evaluation of rhizobacteria as non-rhizobial inoculants for mung beans. Aust. J. Crop Sci. 5: 1723-1729.

Balestrazii A, Bonadei M, Quarttrini E and Carbonera D, 2009. Occurrence of multiple metal-resistance in bacterial isolates associated with transgenic white poplars (*Populous alba* L.). Ann. Microbiol. 59: 17-23

Bazrafshan E, Mahvi AH, Nasseri S, Mesdaghinia AR, Vaezi F and Nazmara S, 2006. Removal of cadmium from industrial effluents by electrocoagulation process using iron electrodes, Iran. J. Environ. Health Sci. Eng. 3: 261-266.

Belimov AA, Hontzeas N, Safronova VI, Demchinsakaya SV, Piluzza G, Bullitta S and Glick BR, 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea L. Czern.*). Soil Biol. Biochem. 37: 241-250.

Belimov AA, Kunakova AM, Safronova VI, Stepanok VV, Yudkin LY, Alekseev VY and Kozhemyakov AP, 2004. Employment of rhizobacteria for the inoculation of barley plants cultivated in soil contamination with lead and cadmium. Microbiol. 73: 99-106.

Figueira PAME, Lima GIA and Rereira SIA, 2005. Cadmium tolerance plasticity in *Rhizobium leguminosarum* bv. viciae: glutathione as a detoxifying agent. Can. J. Microbiol. 51: 7-14.

Godt J, Scheidig F, Grosse-Siestrup C, Esche V, Brandenburg P, Reich A and Groneberg DA, 2006. The toxicity of cadmium and resulting hazards for human health. J. Occup. Med. Toxicol. 1: 22. doi: 10.1186/1745-6673-1-22.

Hussain Z and Islam M, 2010. Leaching of heavy metals from contaminated soils using inductively coupled plasma optical emission spectrometer (ICP-OES) and atomic adsorption spectrometer (ASS). J. Sci. Res. 2: 47-53.

Iqbal A and Hasnain S, 2013. Auxin producing bacilli: Potential candidates in growth stimulation of *Vigna radiata* (L.). Int. Proc. Soc. Behav. Sci. 1: 145-151.

Jankiewicz B, Ptaszynski B and Wieczorek M, 2000. Spectrophotometric determination of cadmium (II) in soil of allotment gardens in lodz. Pol. J. Environ. Stud. 9: 83-86.

Jing YD, He ZL and Yang XE, 2007. Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soil. J. Zhejiang Univ. Sci. *B.* 8: 192-207.

Khan GA, 2005. Role of soil microbes in the rhizosphere of plants growing in trace metal contaminated soils in phytoremediation. J. Trace Elem. Med. Biol. 18: 355-364.

Ladwal A, Bhatia D and Malik KD, 2012. Effect of co-inoculation of *Mesorhizobium cicer* with PGPR on *Cicer arietinum.* Aust. J. Basic Appl. Sci. 6: 183-187.

Madhaiyan M, Poonguzhali S and Sa T, 2007. Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). Chemosphere. 69: 220-228.

Molina SA, Nievas C, Chaca PVM, Garibotto F, Gonza U, Marsa MS, Luna C, Gimenez SM and Zirulnik F, 2008. Cadmium-induced oxidative damage and antioxidative defense mechanisms in *Vigna mungo* (L.). Plant Growth Reg. 56(3): 285-295.

Rahim A, Shaposhnikov A, Belimov AA, Dodd IC and Ali B, 2017. Auxin production by rhizobacteria was associated with improved yield of wheat (*Triticum aestivum* L.) under drought stress. Arch. Agron. Soil Sci. doi: 10.1080/03650340.2017.1362105 (in press).

Raheem A and Ali B, 2015. Halotolerant rhizobacteria: Beneficial plant metabolites and growth enhancement of *Triticum aestivum* L. in salt amended soils. Arch. Agron. Soil Sci. 61: 1691-1705.

Rajkumar M, Ae N, Prasad MNV and Freitas H, 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol. 28: 142-149.

Rathour P, Ramteka WP, Raja W and John AS, 2013. Isolation and characterization of nickel and cadmium tolerant plant growth promoting rhizobacteria from rhizosphere of *Withania somnifera*. J. Biodivers. Environ. Sci. 6: 253-261.

Sajid S, Raheem A and Ali B, 2017. Phylogenetic diversity of drought tolerant *Bacillus* spp. and their growth stimulation of *Zea mays* L. under different water regimes. Res. J. Biotechnol. 12: 38-46.

Qing HU, Dou MN, Qi HY, Zie XM, Zhuang GQ and Yang M, 2007. Detection, isolation and identification of cadmium resistant bacteria based on PCR-DGGE, J. Environ. Sci. 19: 1114-1119.

Venkatesan S, Kirithika M, Rajapriya R, Ganesan R and Muthuchelian K, 2011. Improvement of economic phytoremediation with heavy metal tolerant rhizosphere bacteria. Int. J. Environ. Sci. 1: 1864-1873.

Yu X, Liu X, Zhu H-T, Liu GH and Mao C, 2012. Co-inoculation with phosphate-solubilizing and nitrogen-fixing bacteria on solubilization of rock phosphate and their effect on growth promotion and nutrient uptake by walnut. Eur. J. Soil Microbiol. 50: 112-117.