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Heavy Metal Resistant PGPR Bioinoculants as Promising Bioremediating Agents for Modulation in Plant Growth under Chromium Metal Stress

^aNuzhat Jamil*, ^bMuhammad Yasir, ^aAmbreen Ahmed^a Department of Botany, University of the Punjab, Lahore, Pakistan.^b Department of Chemistry, The University of Lahore, Pakistan.

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ABSTRACT

Chromium generally hinders plant growth, but bacterial inoculations by following different mechanisms can enhance tolerance against heavy metal stress in plants. Characterization and identification of the bacterial strains which have capability to improve growth of plants growing under chromium stressed environment by reducing uptake of metal was the main objective of this present study. For this purpose, chromium tolerant bacterial strains were isolated from tannery effluents from industrial area Kasur, Pakistan and finally three competent bacterial isolates showing minimum inhibitory concentration as 300µg/ml were selected for further study. After that 16S rRNA genetic analysis was used for molecular characterization of these bacterial isolates. Seeds of *Helianthus annuus* were inoculated with these bacterial isolates and grown under chromium contaminated soil to determine their effect growth attributes of plants. All the growth attributes of plants, biomass production, stress tolerance index and seedling vigor index was measured. The present study represents that bacterial inoculation of seeds has capability to improve growth attributes of plants growing in heavy metal stressed environment and counteract the damaging effects of toxicity of heavy metal chromium. The objective of this study is to use bacteria as emerging ecofriendly bioremediation approach to grow important crops in heavy metal contaminated areas.

Corresponding Author: Nuzhat Jamil

Email: ashee.jamil@yahoo.com

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INTRODUCTION

From the previous few decades, the content of hexavalent chromium has amplified greatly by human activities which are the major risk for life on earth. So the eradication of this toxic element from the environment has become necessary to save lives (Singh *et al.*, 2021). Physicochemical treatment to remove this carcinogenic material at small concentration is very much costly and inappropriate. Today's agricultural industry rely on usage of chemicals, but increased dependence on chemicals often causes harmful impacts on surroundings including soil along with micro flora, animals and human beings (Ukhurebor *et al.*, 2021). Agricultural products are

greatly affected by chemicals use in terms of quality and quantity and interaction of chemicals and agriculture is an important subject of discussion called as agriculture-environment relationship. Increased world population caused increase in use of fertilizers to enhance production of crops for meeting increasing demands for food (Istudor *et al.*, 2019). As chemical fertilizers have very dangerous effects on environment so it paved the way for the usage of bio-fertilizers (PGPR), because plant growth promoting bacteria (PGPR) can be best substitute for the chemical fertilizers for the attainment of eco-friendly and sustainable agriculture.

They cause plant growth promotion directly by not only

providing nutrients but also indirectly by protecting plants against pathogens and increasing fertility of soil (Singh *et al.*, 2019a). Rhizospheric bacteria directly increase plant growth by producing various enzymes, siderophores production for sequestration of iron, solubilization of phosphate for making available for plants and production of their hormones such as cytokinins, gibberellins and auxins and decreasing ethylene content by ACC deaminase activity (Etesami and Adl, 2020). Plant growth promoting bacteria act as bio-control agent against fungal pathogens and cause indirect improvement in plant growth and rescue them from abiotic stress factors including salts, heavy rains, drought, and heavy metals. Reduction of hexavalent chromium into trivalent form can be used as useful technique to

remove hexavalent chromium from wastewater because it converts highly toxic Cr(VI) into Cr(III) (Nazli *et al.*, 2020). So, present work gives information regarding removal of hexavalent chromium through bacteria. To remove hexavalent chromium efficiently, the conditions using in this technique deciding fate of toxic heavy metals and major machinery, the microbes should be suitable. The elimination of hexavalent Chromium through bacteria and their benefits are described. It is compulsory to comprehend all these aspects to use technological tool from laboratory to at large scale and require focused research in these spheres. This may take towards the execution of this technology on higher level making it most opted on this level. The schematic of proposed study is shown in Figure 1.

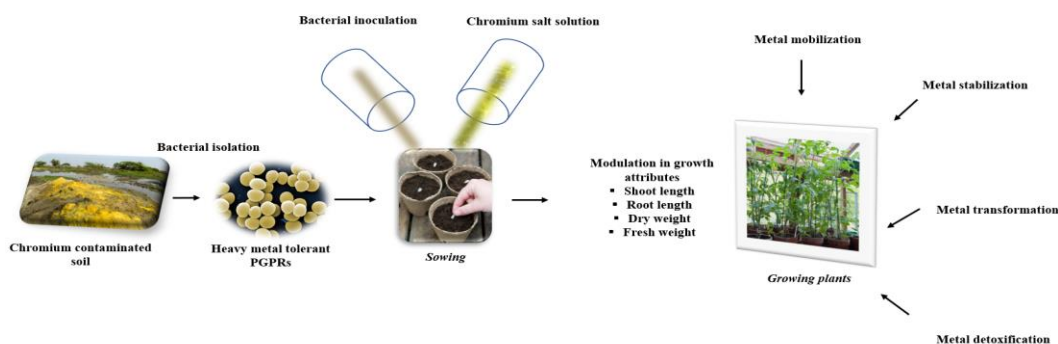


Figure 1. Schematics showing bioremediation of heavy metal toxicity by bacterial inoculations.

MATERIALS AND METHODS

Collection of soil sample

The soil sample was collected from the Botanical Garden, University of the Punjab, Lahore, Pakistan, for this present study. Polythene bags of fine quality were filled with soil sample and wrapped with paper. These polythene bags were placed in autoclave and its temperature and pressure were maintained up to 120 °C and 15 lbs. respectively for 30 minutes. For having 100% sterile soil autoclaving was done repeatedly for 2-3 days.

Seed material

Certified seeds of *Helianthus annus L.* (variety Hysun-33) were bought from Punjab seed corporation, Lahore, Pakistan. Seeds having uniformity in size and weight were selected for growth experiment.

Bacterial isolation, identification and MIC

Bacterial isolates, isolated from industrial effluents of Kasur, Pakistan was purified by Plate streaking methods and single colonies were obtained. PGPR activity and metal stress tolerance of bacterial isolates was estimated

through biochemical tests and MIC respectively (Agarwal *et al.*, 2020). After that further identification of this bacterial isolate was done through 16S rDNA sequencing from Singapore.

Seed sterilization

Healthy seeds of both plants were treated with 0.1% HgCl₂ solution for 3-5 minutes for surface sterilization of Seeds. After that seeds were washed repeatedly 5-6 times by using sterilized distilled water for removing the traces of HgCl₂.

Inoculation of Seed

After surface sterilization seeds were inoculated with 24 hours bacterial cultures that were already adjusted to the same optical density for forty minutes. For control treatment, seeds were treated with autoclaved distilled water for the same time.

Experimental setup

Plant growth experiment was conducted in disposable cups, each containing 300g air dried soil growth chamber at department of botany, university of the Punjab, Lahore.

It was a sandy clay loam textured soil used in experiment. Seven seeds that are already inoculated were seeded in each pot with uniform distance. Experiment was arranged in randomized design with 21 replicates for each bacterial inoculation treatment and without bacterial inoculation was considered as control.

Induction of Cr stress

After germination of seeds, 10 ml solution of 150µg/ml was poured in seven replicates of each bacterial treated pot and in control (without bacterial treatment). Similarly, 10 ml solution of 300µg/ml was poured in other seven replicates for each bacterial treatment and in control also. Seedlings were grown for two weeks. Observations were made daily and general appearance of seedlings was noticed.

Determination of seed germination and growth measurements

After every 24 hours, germination of seeds was noted until it becomes constant. After harvesting seedlings after 30 days of germination, washed carefully with deionized water and traces of soil were removed. Then these seedlings were examined for growth and biochemical parameters. Growth parameters including shoot length, root length, fresh weight and dry weight were studied. After cleaving seedlings at shoot-root junction, the lengths of their shoots and roots were measured in centimeters using metric scale. Fresh weight of seedlings was recorded in grams on an analytical balance, to obtain even dry weight; seedlings were first dried at 60°C for 24 hours in an oven for measuring on analytical balance.

Germination percentage

Germination percentage is the ratio of number of seeds that are germinated to the total number of seeds that were planted. To get the percentage germination following formula was used (Chakma *et al.*, 2019). % Germination = (Number of germinated seeds/Total number of planted seeds) × 100

Seedling vigor index

Seedling vigor index describes performance and activity level of seeds during germinating period and emergence of seedlings. Seedling vigor index was determined using following formula: (Itrotwar *et al.*, 2020). Seedling vigor index (SVI)=Germination percentage x Seedling length

Stress tolerance index

Stress tolerance index determines the yield and genotypic potential to tolerate stress. By using the following formulas stress tolerance indices were calculated for different growth attributes. (Khayatnezhad and Gholamin

2020).

Root length stress tolerance index (RLSTI) = (Root length of stress plant/Root length of control plant) × 100

Shoot length stress tolerance index (SLSTI) = (Shoot length of stress plant/Shoot length of control plant) × 100

Root fresh weight (RFSTI) = (Root fresh weight of stress plant/Root fresh weight of control plant) × 100

Shoot fresh weight stress tolerance index (SFSTI) = (Shoot fresh weight of stress plant/Shoot fresh weight of control plant) × 100

Root dry weight stress tolerance index (RDSTI) = (Root dry weight of stress plant/Root dry weight of control plant) × 100

Shoot dry weight stress tolerance index (SDSTI) = (Shoot dry weight of stress plant/Shoot dry weight of control plant) × 100

Statistical analysis

Data was analyzed statistically by using SPSS software.

RESULTS

Tolerance of bacterial strain was evaluated through minimum inhibitory concentration to chromium; results expressed that chromium stress greater than 300µg/ml led to inhibition of bacterial growth. So, this chromium concentration was considered as MIC and efficient bacterial strain (AR, ALA, BL2) was selected for further studies. The bacterial isolates were molecularly characterized through 16S rDNA sequencing. After matching partial sequences of 16S rDNA with sequences in GenBank through the BLAST suggested that AR, ALA, and BL2 expressed highest similarity with *Bacillus cereus*, *Bacillus pumilus* and *Bacillus atrophaeus*. These sequences were submitted in the GenBank database under accession number KT321456, MG988291 and MG988292 respectively.

Chromium metal is somewhat important biologically, but it becomes toxic when crosses certain level. Increased concentration of chromium in environment by anthropogenic activities is a major human concern. So, it is very important to remove chromium metal from our surroundings and making plants able to uptake less chromium from soil. Present study focused on screening of bacterial isolates which have capacity to stimulate plant growth under metal stressed environment. Three bacillus species were used to demonstrate the stimulated growth including enhancements in growth parameters and matter production under heavy metal stress treatments of different chromium concentration. At the end BL2 was designated as the best biological tool for growth stimulation in *Helianthus annuus* under chromium concentration of 0, 150 and

300µg/ml.

Effect of Cr stress on seed germination

The current study shows that germination of *Helianthus annuus* was greatly influenced by increasing chromium stress up to 300µg/ml.

Effect of Cr stress on seedling vigor index

The highest Cr concentration greatly affect the SVI of *Helianthus annuus*. SVI of *Helianthus annuus* slowly declined with enhanced chromium metal concentration at 0, 150

and 300µg/ml (Table 1). The average seedling vigor index of *Helianthus annuus* reduced from 1192.17, 686.7 and 508.79 respectively due to enhanced chromium concentration of 0, 150 and 300µg/ml. But inoculations of seeds with bacterial isolates AR, Ala and BL2 increased the seedling vigor index up to 1495.11, 1659.24 and 1748.38 without chromium stress. Inoculation with isolate BL2 increased the SVI up to 1115.8 and 836.85 under chromium stress of 150 and 300µg/ml respectively.

Table 1. Effects of various Cr Concentrations on Seed Germination and SVI of *Helianthus annuus*.

Sr. #	Treatment	Percentage germination	Percentage reduction in germination	SVI
1	Control	73.5±6.1	26.5	1192.17
2	AR	81.7±4.1	18.3	1495.11
3	Ala	83.8±3.7	16.2	1659.24
4	BL2	81.7±4.1	18.3	1748.38
5	C+ Cr(150µg/ml)	65.4±6.9	35.5	686.7
6	AR+ Cr(150µg/ml)	83.8±3.7	16.2	997.22
7	Ala+ Cr(150µg/ml)	79.7±4.3	20.3	1020.16
8	BL2+ Cr(150µg/ml)	79.7±5.2	20.3	1115.8
9	C+ Cr(300µg/ml)	61.3±6.8	38.7	508.79
10	AR+ Cr(300µg/ml)	73.6±7.8	26.4	677.12
11	Ala+ Cr(300µg/ml)	75.5±2.6	24.5	755
12	BL2+ Cr(300µg/ml)	79.7±6.8	20.3	836.85

Effect of Cr stress on root and shoot elongation

After 30 days of induction of Cr stress, *Helianthus annuus* expressed significant reduction in shoot and root length of seedlings. (Table 2). The average root elongation was considerably reduced from 7.02±0.2 cm to 5.4±0.3 cm and 4.4±0.2 cm against Cr stress of 150 and 300µg/ml without bacterial inoculations. But bacterial isolate BL2 caused an increment in root length up to 7.0±0.2 cm and 5.5±0.3 cm under chromium stress treatment of 150 and 300µg/ml respectively over inoculated control treatment without stress.

Similarly, the seedlings expressed prominent enhancements in average shoot length after inoculation with bacterial isolates without chromium stress. But chromium stress treatment of 150 and 300µg/ml caused reduction in shoot length from 9.2±0.3 cm to 5.1±0.1 cm and 3.9±0.2 cm respectively. Inoculation with bacterial isolate BL2 caused enhancements in shoot length of seedlings to 7.0±0.1cm and 5.0±0.2 cm against Cr stress of 150 and 300µg/ml respectively as compared to non-inoculated without Cr stress control treatment.

Effect of Cr stress on seedling fresh weight

The seedling fresh weight of *Helianthus annuus* was

rigorously affected by increasing chromium content in soil (Table 2). Results reveals that chromium stress of 150 and 300µg/ml significantly reduced seedling fresh weight from 0.5±0.03 gm to 0.4±0.01 gm and 0.3±0.01 gm respectively without bacterial treatment. After bacterial inoculations, seedlings expressed enhanced fresh weight. Under Cr stress of 150 and 300µg/ml, bacterial inoculations with bacterial isolate BL2 increased fresh weight up to 0.6±0.02 gm and 0.4±0.02 gm respectively over non inoculated control with respective stress treatment.

Effect of Cr stress on seedling dry weight

The seedling dry weight of *Helianthus annuus* was reduced by increasing chromium concentration (Table 2). Chromium concentration of 150 and 300 µg/ml appreciably enhanced seedling dry weight up to 0.026±0.003 gm and 0.03±0.005 gm respectively over non inoculated without stress control showing seedling dry weight up to 0.01±0.003 gm. Bacterial treatment expressed significant effects by increasing dry weight of seedlings. Bacterial treatment of isolate BL2 caused considerable increase in fresh weight up to 0.025±0.002 gm and 0.023±0.002 gm against Cr stress of 150 and 300

µg/ml respectively as compared to non-inoculated with respective stress control.

Table 2. Effects of various Cr Concentrations on Seedling Growth of *Helianthus annuus*.

Sr. #	Treatment	SL (cm)	RL (cm)	FW (gm)	DW (gm)
1	Control	9.2+0.3	7.02+0.2	0.5+0.03	0.01+0.003
2	AR	10.6+0.2	7.7+0.3	0.6+0.04	0.021+0.0008
3	Ala	11.2+0.2	8.6+0.3	0.7+0.04	0.023+0.003
4	Bl2	12.2+0.2	9.2+0.1	0.8+0.04	0.026+0.003
5	C+ Cr(150µg/ml)	5.1+0.1	5.4+0.3	0.4+0.01	0.026+0.003
6	AR+ Cr(150µg/ml)	5.8+0.2	6.1+0.3	0.4+0.04	0.020+0.0003
7	Ala+ Cr(150µg/ml)	6.0+0.3	6.8+0.2	0.5+0.01	0.022+0.0008
8	Bl2+ Cr(150µg/ml)	7.0+0.1	7.0+0.2	0.6+0.02	0.025+0.002
9	C+ Cr(300µg/ml)	3.9+0.2	4.4+0.2	0.3+0.01	0.03+0.005
10	AR+ Cr(300µg/ml)	4.2+0.2	5.0+0.2	0.39+0.01	0.019+0.0006
11	Ala+ Cr(300µg/ml)	4.7+0.2	5.3+0.2	0.4+0.01	0.020+0.0003
12	Bl2+ Cr(300µg/ml)	5.0+0.2	5.5+0.3	0.4+0.02	0.023+0.002

Effect of Cr stress on tolerance index

Results expressed that increased Cr concentration reduced tolerance percentage in *Helianthus annuus* (Table 3). Shoot length of *Helianthus annuus* seedlings showed tolerance index up to 55.43 and 42.39 against Cr stress of 150 and 300µg/ml respectively. Inoculation of seeds with bacterial isolates expressed significant results by increasing chromium stress tolerance index. Bacterial treatment of Bl2 increased tolerance index up to 76.08 and 54.34 under both chromium stress treatment respectively. Similarly, tolerance level of *Helianthus annuus* root length decreased up to 76.92 and 62.67 respectively by increasing chromium concentration to 150 and 300µg/ml. But inoculation with bacterial isolate

increased the tolerance percentage. The seedling fresh weight stress tolerance index reduced as 80 and 60 folds with increasing chromium content up to 150 and 300µg/ml respectively. Bacterial treatment with Bl2 increased FSTI up to 120 and 80 folds under both stress treatments. Cr metal stress badly affects the DSTI of *Helianthus annuus*. DSTI of *Helianthus annuus* was lowered as 70 and 60 folds with increasing chromium concentration up to 150 and 300µg/ml respectively as compared to non-inoculated without chromium stress control. Bacterial inoculations of seeds caused increase in DSTI of seedling. Maximum increase in DSTI of *Helianthus annuus* was as 88 and 75 folds under chromium stress treatment of 150 and 300µg/ml respectively after inoculation with Bl2.

Table 3. STI (%) of *Helianthus annuus* Growing in various Treatments of Cr Stress.

Treatment	SLSTI	RLSTI	FSTI	DSTI
Control	0.00	0.00	0.00	0.00
C+ Cr(150µg/ml)	55.43	76.92	80.0	70.0
AR+ Cr(150µg/ml)	63.04	86.89	80.0	78.0
Ala+ Cr(150µg/ml)	65.2	96.89	100.0	82.0
Bl2+ Cr(150µg/ml)	76.08	99.71	120.0	88.0
C+ Cr(300µg/ml)	42.39	62.67	60.0	60.0
AR+ Cr(300µg/ml)	45.6	71.22	78.0	66.0
Ala+ Cr(300µg/ml)	51.08	75.49	80.0	72.0
Bl2+ Cr(300µg/ml)	54.34	78.34	80.0	75.0

DISCUSSION

Chromium metal is somewhat important biologically, but it becomes toxic when crosses certain level. Increased concentration of chromium in environment by anthropogenic activities is a major human concern. So, it is very important to remove chromium metal from our

surroundings and making plants able to uptake less chromium from soil. Present study focused on screening of bacterial isolates which have capacity to stimulate plant growth under metal stressed environment. Three bacillus species were used to demonstrate the stimulated growth including enhancements in growth parameters

and matter production under heavy metal stress treatments of different chromium concentration. At the end BI2 was designated as the best biological tool for growth stimulation in *Helianthus annuus* under chromium concentration of 0, 150 and 300µg/ml.

Phytotoxicity of chromium metal cause inhibition of germination of seeds. With increasing chromium stress inhibitory effects on seed germination increases as compared to less chromium stress which show less damage (Zaheer *et al.*, 2020a). Plants grown in chromium stress become hard in texture, observe hypertrophy in cells and destruction in root systems (Wakeel and Xu 2020). Important mechanisms including photosynthetic activity, reduction of nitrates and synthesis of proteins are seriously disturbed (Zaheer *et al.*, 2020a). It is reported that toxicity of chromium metal is somewhat function of membrane permeability because biological membranes are permeable for hexavalent chromium and impermeable for trivalent form. Once hexavalent form of chromium enters cytoplasm by crossing membranes it changes into trivalent form after reduction. In this form it damages DNA, inhibits the enzyme functions and causes cellular reduction (Taufikurahman *et al.*, 2022). The effect of hexavalent and trivalent form can be observed by relating with their solubility. Hexavalent chromium is soluble in water and produce dichromate and chromate ions. Hexavalent chromium is more toxic because of its bioavailability and having ability to cross biological membranes easily (Alvarez *et al.*, 2021).

Bacterial inoculation of seeds has capacity to stimulate seed germination under chromium and without chromium stress. Significant enhancement was observed in growth attributes and matter production (Oleńska *et al.*, 2020). Bacterial isolates produce certain hormones which promote growth and improve general morphology of the plants. Bacterial inoculation expressed considerable enhancements in length of roots and shoots, lateral roots, fresh and dry matter production, and caused enlargements in cortex area by increasing cell division in meristems. Bacterial inoculation not only stimulated germination and growth in chromium stressed environment but the damages of chromium toxicity were also reduced including improvement in texture, reduction in spots on leaves and root system development. Bacterial treatment also improved the water level in plants by decreasing chromium uptake which results in increased fresh weight of seedlings. Bacterial isolates also decrease the chromium

concentration in plants by reduction of hexavalent into trivalent form and alleviate the heavy metal stress. Many authors proposed that heavy metal stress interferes with division of cells, causes unstable mitosis and chromosomal aberrations which result in decreased root growth and overall seedling growth (Goyal *et al.*, 2020). It is proposed that seedlings are more sensitive to metal toxicity than seeds at germinating stage (Seneviratne *et al.*, 2019). The reduction in fresh weight of roots and shoots of *Helianthus annuus* with increased chromium concentration was also reported in literature (Zaheer *et al.*, 2020b). By treating *Brassica oleracea* (L.) with different chromium treatments researchers determined the toxicity of chromium on the fresh weight of shoots and roots after 8 days of seedling growth (Ahmad *et al.*, 2020). Chromium concentration of 5 - 200 mgL⁻¹ caused decrease in seedling vigor index in four different varieties of soybean in comparison to control treatment (Basit *et al.*, 2022). Significant decrease in dry weight and growth of four different soybean varieties was observed by (Basit *et al.*, 2022) with chromium stress up to 5 - 200 mg·L⁻¹ as compared to control treatment. It was reported in literature that chromium stress of 2.5 m·L⁻¹Ag had very adverse effects on dry matter of seedlings while working on *Vallisneria spiralis* for the evaluation of relationship between chromium toxicity and its build up in shoots (Sharma *et al.*, 2020). A study regarding heavy metal stress tolerance expressed that tolerance mechanisms aid plants to sustain growth under heavy metal stressed environment. Shoot and root growth was considered as important factor to classify metal tolerance. As biological membranes are of prime importance for heavy metal injury, there is reduction in fresh weight of seedlings due to little uptake of water (Singh *et al.*, 2019b). Heavy metal tolerant bacterial isolates are potential candidates as bioremediating agents to modulate the growth attributes of plants growing in heavy metal stressed environment.

CONCLUSION

The extent of toxicity on growth of plants depends upon the metal uptake from specific environment. The wide use of chromium in industries has contaminated the environment. Due to presence of increased concentration of chromium in environment its toxicity has become major concern now days. High concentration of chromium considerably affects germination of seeds, growth, and biochemical parameters of *Helianthus annuus*.

By and large the tolerating capacity of seedlings against metal toxicity was prominent in bacterial treated *Helianthus annuus* seedlings which was expressed as tolerance index. This helps to evaluate the tolerating capacity of *Helianthus annuus* under various chromium concentrations. The result of this research helps in the successful growth in chromium affected areas. Further study is required to find out the effects of various metal concentration on different plant parts in to grow plants in highly metal contaminated areas.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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