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Cr(VI) toxicity inhibits microbe enhanced plant growth promotion without affecting bioremediation potential

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ABSTRACT

Hexavalent Chromium [Cr(VI)], a toxic inorganic pollutant of agriculture soil derived from various anthropogenic industrial sources, disturbs vegetation and contaminates the food chain. Chromate microbial toxicity was studied using plant growth-promoting chromate reducing *Pseudomonas aeruginosa* ATCC P15442 (P15). With a minimum inhibitory concentration of 1,250 µg/ml Cr(VI), the isolate is capable of 98% bioreduction of 100 µg/ml Cr(VI) in 24 hours and 83% of 500 µg/ml Cr(VI) in 72 hours. Additionally, P15 shows tolerance to cross heavy metal pollutants (Cd, Pb, and Zn), halotolerance, and the production of plant growth-promoting substance, such as indole acetic acid (IAA), siderophore, and phosphate solubilization in the presence and absence of Cr(VI). This study also reports that 100 and 250 µg/ml Cr(VI) decreases the production of IAA, siderophore, and phosphate solubilization without affecting the growth or Cr(VI) bioreduction ability. In *Vigna mungo* seed bacterization assay, P15 is capable of enhancing root and shoot length in absence of Cr(VI) and reversing toxic effects of 100 µg/ml Cr(VI). No enhancement of plant parameters was observed at higher Cr(VI) concentrations, except reversal of Cr toxicity. These data are indication of the detrimental effect of Cr(VI) pollution on rhizospheric microbial flora associated with plant growth-promoting activities.

1. INTRODUCTION

Hexavalent Cr(VI), a toxic heavy metal pollutant, is derived from industrial waste and spent of electrochemical plating, leather tanning, textile, painting, metallurgical industries, and coal power plants, etc [1]. Cr(VI) toxicity is attributed to its water solubility, mobility, and high oxidizing ability in comparison to Cr(III) and Cr(IV) oxidative species [2]. Cr(VI) is reported to be carcinogenic and mutagenic due to the ability to produce reactive oxygen species [3]. In plants, Cr(VI) toxicity leads to poor germination, affects physiological processes such as photosynthesis, water relation, and nutrient deficiency [4,5]. Microbial toxicity of Cr(VI) has also been reported wherein biodiversity in chromate polluted sites has been severely affected [6,7]. Microbe-assisted phytoremediation of Cr(VI) using a combination of plant growth-promoting (PGP) Cr(VI) reducing bacteria residing in the rhizospheric region of phytoaccumulator plants is considered as an economical, viable, and green alternative to the use of physicochemical methods [8]. Many micro-organisms have been described that can remediate toxic Cr(VI) into non-toxic Cr species using different reduction mechanisms while many organisms are described that can tolerate Cr(VI) concentrations in soil but are unable to reduce into non-toxic variants [9–13]. Such Cr(VI) reducing bacteria have also been reported to produce secondary metabolites that also promote plant growth and immunity [8,14,15]. PGP substances produced by rhizospheric or endophytic bacteria play an important role in plant growth and developments as well as improving stress tolerance to heavy metals aiding phytoremediation in many accumulator plants [8,16]. Among the PGP products found to be positive in the isolates, indole acetic acid is important for improving nutrient absorption and lateral and adventitious root development in plants [17]. Siderophores are particularly important in chelating metals such as Fe for plants and reducing metal toxicity [18]. Organic acid byproducts of phosphate solubilization and mobilization by bacteria make insoluble phosphate available to plants [19]. Hydrogen cyanide (HCN) production act by keeping phytopathogens in control and biofilm exopolysaccharides biosorp metals and make them

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available for reduction while reducing cellular toxicity [20]. The effect of Cr(VI) toxicity on microbial growth and PGP production of rhizospheric bacteria in *Vigna mungo* system was studied to determine the effect of Cr(VI). It is not understood in previous experimental studies if Cr(VI) negatively affects the production of plant growth stimulating products due to inhibited or reduced microbial growth, and hence decreased PGP production or directly affect the PGP production without affecting growth. In this study, we study the effect of different Cr(VI) concentrations on microbial growth as well as indole acetic acid, siderophore, and phosphate solubilizing enzyme production. P15 bacterization of *V. mungo* seeds was performed to understand PGP and Cr(VI) remediation physiology in soil microenvironment.

2. MATERIALS AND METHODS

2.1. Microbial growth

Pseudomonas aeruginosa ATCC 15442 [21] was procured from Hi-Media, Mumbai and maintained on tryptone soyapeptone (TS) media at 37°C. For heavy metal experiments, the isolate was plated on tryptone soyapeptone agar (TSA) supplemented with 100 µg/ml Chromium ($K_2Cr_2O_7$) by standard spread plate method. All media and chemicals used were purchased from HiMedia, India and Merck, India.

2.2. Minimum inhibitory concentration (MIC) and Diphenyl carbazide Assay

P15 was grown in successively increasing Cr(VI) concentrations in TSA medium and growth of plate recorded. Cr (VI) bioreduction ability was estimated using the 1,5 diphenyl carbazide method [15]. Briefly, TS media with varying concentrations of Cr (VI) was inoculated with 24 hours log culture (10% v/v). Aliquots of the sample at different time points were centrifuged and supernatant acidified using 6N H_2SO_4. 25% (w/v) 1, 5 diphenyl carbazide in acetone was added and change in color to dark pink detected by spectrophotometer (Thermo Scientific, Spectronics) at 540 nm.

2.3. Heavy metal and Halotolerance

Heavy metal tolerance was determined for each isolate on TSA supplemented with 100 µg/ml of different heavy metals: $ZnSO_4$, $CdCl_2$, and $Pb(NO_3)_2$ in the absence and presence of 100 µg/ml Cr(VI). Plates were incubated at 37°C for up to 72 hours to measure growth. Halotolerance was similarly determined by incorporating different NaCl concentrations (0.5%–6%) in the absence and presence of 100 µg/ml Cr(VI) in TSA and growth recorded by visual observation.

2.4. Plant growth promontory (PGP) activities

All PGP activity was tested in the presence and absence of varying Cr(VI) concentrations.

2.4.1. Phosphate solubilization

Qualitative phosphate solubilization was checked on Pikovskaya's agar medium by measuring the clearance zone [22]. The quantitative bioassay was carried out using Erlenmeyer flasks (100 ml) containing 10 ml of NBRIP broth medium inoculated with the 10% bacteria cell [23]. The flasks were incubated for 5 days at 28°C on a shaker at 120 rpm. Available phosphorus content in the filter supernatant, as well as control (supernatant obtained from no bacteria inoculation), was estimated using the vanado-molybdate colorimetric method by measuring the absorbance at a wavelength of 450 nm.

2.4.2. Indole acetic acid (IAA) production

Bacterial production of IAA was tested using tryptone broth containing 0.1% tryptophan. The medium was incubated with 10% log culture (1×10^7 cell/ml) for 2 days at 37°C with shaking. After centrifugation at 10,000 g for 10 minutes, 1 ml of supernatant was mixed with 2 ml of Salkowasky's reagent and the optical density was measured at 550 nm [24].

2.4.3. Siderophore production

Siderophore production by the isolates was tested qualitatively using the Chroma Azurol Sulphonate (CAS) Media and quantitatively by CAS-Shuttle assay [25]. Succinate medium inoculated with log phase culture was incubated for 48 hours at 37ºC with constant shaking at 120 rpm. Centrifuged cell-free supernatant was mixed with 0.5 ml of CAS reagent, and the absorbance was measured at 630 nm against reference. Siderophore unit in aliquot was calculated by using following formula: $[(Ar - As)/Ar] \times 100$, where Ar is the absorbance at 630 nm of reference (CAS assay solution+ uninoculated media) and As is the absorbance at 630 nm of the sample (CAS assay solution + supernatant) [26].

2.4.4. Ammonia production

Log culture of P15 was grown in peptone water at 37ºC for 2 days. After incubation, 1 ml of Nessler's reagent was added into each tube and the development of yellow color indicates ammonia production [27].

2.4.5 Biofilm formation

P15 was streaked on Congo red agar media (0.08% congo red; 5% sucrose) in the absence and presence of 100 µg/ml Cr(VI) and incubated at 37ºC for 1 day. The appearance of the black precipitated colony was indicative of biofilm formation [28].

2.5. Plant bioassay

Experiment was performed to evaluate the toxic effects of Cr(VI) on seed germination and seedling development of *V. mungo* in the presence or absence of isolate [15]. Healthy seeds were firstly surface sterilized in a solution of 0.1% $HgCl_2$ and then washed with sterile water. Log phase culture (10^8 CFU/ml) was used to inoculate sterile seeds while in control treatments, seeds were placed in sterile saline water in autoclaved Petri dishes. About 3 ml of varying $K_2Cr_2O_7$ concentrations (100, 250, and 500 µg/ml) was added in seed containing Petri plates lined with Whatman filter paper. Petri plates were placed in room temperature for germination at 28°C for 7 days and moisture content maintained by adding 2 ml sterile water on alternate days. After 1 week, seedlings were harvested to determine shoot length and root length.

2.6. Statistical analysis

Experiments were performed in triplicates. Student's *t*-test and ANOVA were used wherever applicable. Data were considered significant when $p \leq 0.05$. Statistical analysis was carried out using GraphPad Prism software (version 6.0, USA).

3. RESULTS AND DISCUSSION

3.1. P15 heavy metal and qualitative PGP characterization

Environmental strain *P. aeruginosa* ATCC 15542 (P15) was treated with media containing increasing concentration of Cr(VI). According to Table 1, the minimum inhibitory concentration of Cr(VI) was found to be 1,250 µg/ml on plate assay. The isolate also has the capability to cross tolerate other heavy metals such as $CdCl_2$, $Pb(NO_3)_2$ and $ZnCl_2$, in addition to 100 µg/ml Cr(VI). Tannery effluents are rich in brine and P15 also shows 2% halotolerance (NaCl) in the presence of 100 µg/ml Cr(VI) and 4% halotolerance in the absence of Cr(VI). Qualitative screening for the ability to produce plant growth-promoting products recorded that P15 was positive for ammonia, siderophore, indole acetic acid production biofilm formation even in the presence of 100 µg/ml Cr(VI).

3.2. Cr(VI) reduction and growth kinetics

Figure 1A shows growth kinetic profile of P15 isolate at increasing (100, 250, and 500 µg/ml) Cr(VI) concentration up to day 3 post-inoculation. The isolate shows 23% decrease in growth at 100 and 250 µg/ml Cr(VI), whereas 62% decreased growth is observed at 500 µg/ml Cr(VI) at day 1 post-inoculation compared to Cr(VI) non-amended media ($p \leq 0.001$). By day 3, growth at 100 and 250 µg/ml Cr(VI) was found to be comparable to media with no Cr(VI) likely due to detoxification of Cr(VI) in the media while growth remained inhibited by 39% at 500 µg/ml Cr(VI) concentrations. As shown in Figure 1B at 100 µg/ml Cr(VI), bioreduction was found to be 98% within day 1 of growth. Cr(VI) reduction of 500 µg/ml Cr(VI) was found to be at a slower pace and 42% at day 1 and 83% was reduced at day 3, respectively, post inoculation.

Table 1: Cr(VI) MIC, cross heavy metal, halotolerance and plant growth promoting properties of *Ps. aeruginosa* ATCC 15542 (P15).

A. Minimum inhibitory concentration (MIC)	**24h**	**48h**
250 µg/ml	+++	++++
500 µg/ml	+++	++++
1,000 µg/ml	++	+++
1,250 µg/ml	-	-
1,500 µg/ml	-	-
	No Cr	**Cr(VI)** (100 µg/ml)
B. Cross heavy metal tolerance		
100 µg/ml $CdCl_2$	+	+
100 µg/ml $ZnCl_2$	+	+
100 µg/ml $Pb(NO_3)_2$	+	+
C. Halo tolerance		
NaCl	4%	2%
D. Plant Growth promoting activity		
Biofilm formation	+	+
Ammonia	+++	+++
Phosphate solubilization	++	-
Siderophore	+++	+
Indole acetic acid	++	+

A. P15 was grown in increasing concentration of Cr(VI) in TSA at 24 and 48h until no further growth was obtained. + indicative of amount of visual microbial growth on TSA.
B. P15 was grown on TSA containing 100 µg/ml heavy metals in absence and presence of 100 µg/ml Cr(VI).
C. Maximum concentration at which growth was recorded in absence and presence of 100 µg/ml Cr(VI).
D. Qualitative screening for plant growth promoting properties as described in materials and methods in absence and presence of 100 µg/ml Cr(VI).

3.3. PGP production and biotoxic effect of Cr(VI)

Pseudomonas aeruginosa P15 was found to be positive for qualitative production of IAA, siderophore, ammonia production, and phosphate solubilization (Table 1). In order to study the effect of increasing Cr(VI) toxicity on growth and PGP production, P15 isolate was treated with 100 and 250 µg/ml Cr(VI) and the quantitative production of IAA, siderophore production, and phosphate solubilization was measured. Figure 2A shows no effect of 100 µg/ml Cr(VI) on growth or IAA production ($p \geq 0.05$) with negative effect on growth ($p \leq 0.05$) and IAA production ($p = 0.01$) at 250 µg/ml Cr(VI). Siderophore production was negatively affected in a concentration-dependent manner [Cr(VI) 100 µg/ml, $p = 0.002$; 250 µg/ml, $p = 0.0009$] even as growth was unaffected [Cr(VI) 100 µg/ml, $p = 0.22$; 250 µg/ml, $p = 0.06$] (Figure 2B). Phosphate solubilization was completely inhibited even at 100 µg/ml Cr(VI) ($p \leq 0.001$) with no effect on growth (Figure 2C). Hence, plant growth-promoting activity of P15 was negatively affected by the presence of increasing Cr(VI) concentrations even though the MIC was recorded to be 1,250 µg/ml Cr(VI) and the isolate was capable of reducing Cr(VI) within few days.

3.4. Plant bioassay

In order to study if the decrease in PGP production of indole acetic acid, siderophore, and phosphate solubilization had an effect on plant growth in the presence and absence of Cr(VI), p15 inoculated *V. mungo* seed germination was studied. As shown in Figure 3, bacterization of seeds with P15 in the absence of Cr(VI) showed a significant increase in the shoot (22.4%) and root length (64.58%) hence showing that PGP effect was evident upon p15 seed treatment ($p < 0.05$). Concentration-dependent decrease in shoot and root length, respectively, was observed at 100 µg/ml (11.9%, 8.33%), 250 µg/ml (22.3%, 23.5%), and at 500 µg/ml (31.13%, 61.33%, $p < 0.05$) Cr(VI) concentrations, respectively. In P15 treated seeds at 100 µg/ml treatment, reversal of Cr(VI) toxicity in addition to enhanced growth in the shoot (13.11%) and root (8.3%) length was observed ($p < 0.05$). However, at both 250 and 500 µg/ml treatment, Cr(VI) toxicity reversal that is similar growth to control untreated seeds is seen without any increase in plant growth parameters. However, in comparison to untreated seeds at 250 and 500 µg/ml Cr(VI), an increase in shoot length but not root length was observed for P15 bacterized seeds ($p < 0.05$). Bioreduction ability of P15 led to decreased Cr(VI) toxicity to plants with no positive effects of PGP production at higher concentrations. It is likely that Cr(VI) induced repression of rooting hormone IAA production in P15 led to decreased root length. Shoot length increase was likely observed due to decreased

Cr(VI) toxicity due to bioremediation potential of P15. This experiment is also indicative that PGP activity without Cr(VI) reduction and heavy metal tolerance abilities will be ineffective in microbe-assisted phytoremediation strategies.

4. DISCUSSION

This study highlights the role of chromate toxicity on micro-organisms by affecting the plant growth-promoting potential of environmental strains. Environmental strain *P. aeruginosa* ATCC 15542 can produce PGP products, reduce toxic Cr(VI), and help in plant growth restoration of *V. mungo* in Cr(VI) amended soils only at low Cr(VI) concentrations. This isolate showed minimum growth inhibitory concentration (MIC^{G}) of 1,250 µg/ml Cr(VI), halotolerance (2%–4%) as well as cross tolerance to different heavy metals but minimum PGP inhibitory concentration (MIC^{PGP}) of 100 µg/ml Cr(VI).

Microbial-derived PGP indirectly augments the bioremediation of pollutants as well as help plant growth in nutrient limiting and

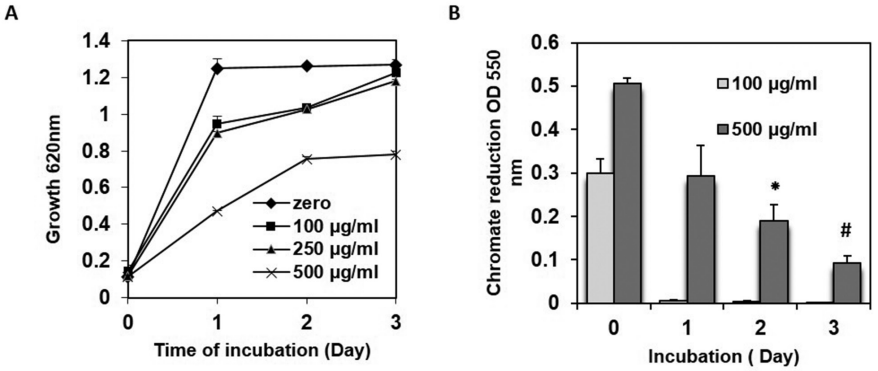

Figure 1: (A) Growth kinetics of *P. aeruginosa* ATCC 15442 (P15) at different Cr(VI) concentrations 100, 250, 500 µg/ml) in TSB media up to 3 days post-inoculation (B) Chromium reducing ability at 100 and 500 µg/ml concentration of Cr(VI) in TSB media up to 3 days post-inoculation. Data are mean of three replicates with standard deviation. *$p \leq 0.05$, #$p \leq 0.001$

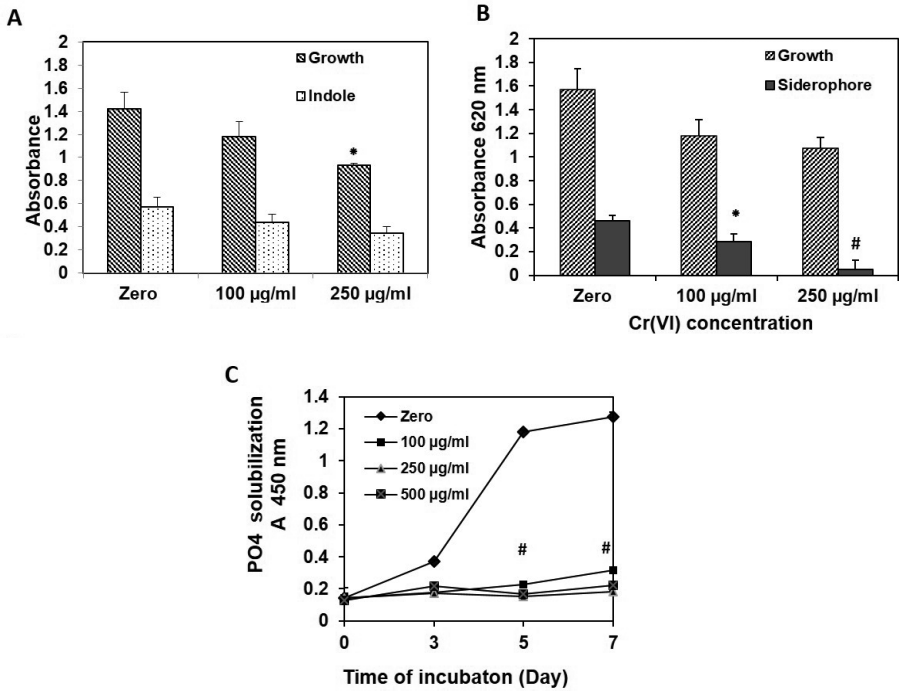

Figure 2: Plant growth promotion by *P. aeruginosa* (P15) at different concentrations of Cr(VI) (0, 100, 250 µg/ml. (A) IAA production in tryptone broth with 0.1%tryptophan at 2 days post-inoculation. (B) Siderophore production in CAS media at 2 days post-inoculation. (C) Phosphate solubilization in NBRIP media. Data are mean of three replicates with standard deviation. * $p \leq 0.05$, # $p \leq 0.001$.

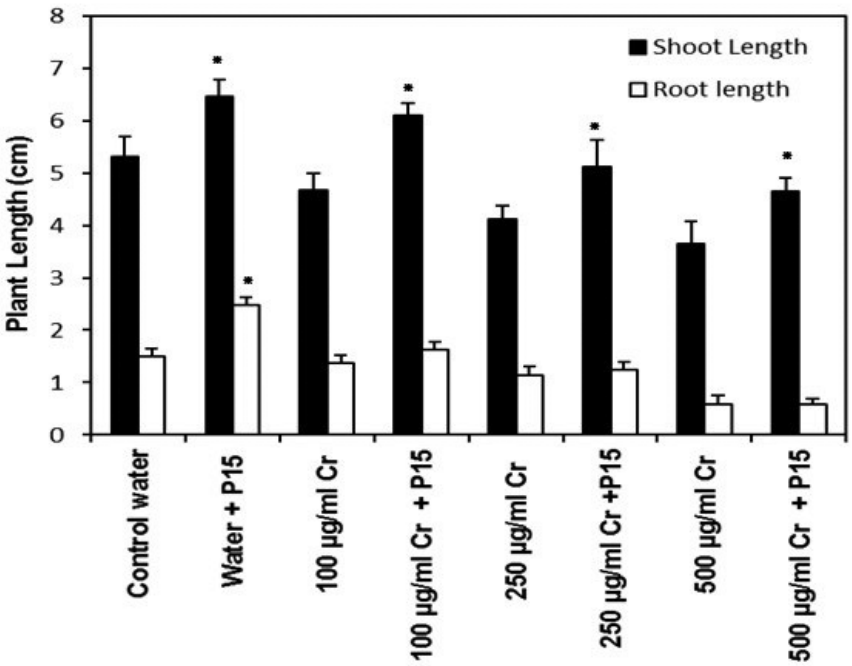

Figure 3: Seed bacterization of *V. mungo* with *P. aeruginosa* (P15) affects shoot and root length development at day 7 in the absence (control) and presence of different Cr(VI) concentrations (0, 100, 250, and 500 µg/ml). Multiple comparisons among treatments were made using ANOVA with Tukey's multiple comparison test. Data are mean of three replicates with standard error of mean (SEM) . *$p \leq 0.05$ of P(15) inoculated in comparison to uninoculated seeds.

environmentally unsuitable conditions [8]. IAA, an intercellular communication signaling molecule, also functions as a root elongation hormone [29]. Siderophore production augments bioremediation processes by chelating iron and enhancing tolerance of micro-organisms to abiotic stresses such as pollutants [30]. Phosphate solubilizing organic acids produced by microbes make unavailable complex form of tricalcium phosphorus into products easily utilized by bacteria as well as plants and enhance their nutrition and immunity to environmental pollutants [19]. Yet high concentrations of environmental pollutant such as Cr(VI) also show microbial toxicity [6,7]. We observed that at >100 µg/ml concentrations of Cr(VI), PGP production of indole acetic acid, siderophore, and phosphate solubilization was negatively affected even as the isolate could reduce up to 500 µg/ml Cr(VI) levels to negligible measurable concentrations and grow in the presence 1,250 µg/ml Cr(VI). Hence, it is likely that Cr(VI) and its byproducts may be transcriptionally inhibiting the production of PGP in the presence of stress. Transcriptional regulation of genes in the presence of Cr(VI) stress has been studied to downregulate gene expression [31]. Hence, Cr(VI) concentration even as sub MIC levels shows biotoxic effect on the plant growth-promoting abilities of environmental micro-organisms without affecting their growth. Biotoxic effects of Cr(VI) on plant growth promotion have been reported for *Cellulosimicrobium funkei* (KM263188) isolated from *Phaseolus vulgaris* rhizosphere [32]. Heavy metals, such as copper (Cu), chromium (Cr), nickel (Ni), and cadmium, (Cd) were shown to reduce growth and PGP properties of *P. aeruginosa* and *P. fluorescens* [33]. Cr and Cd significantly reduced PGP activities, such as IAA, Hydrogen cyanide (HCN), siderophore, and P-solubilization compared to Cu and Ni.

Pseudomonas aeruginosa has until recently been considered a medically important opportunistic pathogen until reports of the rhizospheric and environmental *P. aeruginosa* as pathogenic or beneficial strains were published [34,35]. 75% Cr(VI) bioreduction at 400 mg/l and tolerance to several heavy metals have been reported for *P. aeruginosa* CCTCC AB93066 strain [36]. Recently, the role of *Pseudomonas* sp. (strain CPSB21, capable of Cr^{6+} bioreduction), in phytoremediation of Cr stress in sunflower plants (but not tomato plants) showed enhanced growth parameters, nutrient uptake, and increase in oxidative stress tolerance [37].

The use of Cr reducing and PGP producing *P. aeruginosa* strain OSG41 isolated from rhizospheric soil of mustard plant showed enhanced dry matter accumulation, nodule formation, grain yield, and protein in chickpea compared to non-inoculated plants while decreasing Cr uptake by the plant [38]. The effect of plant growth-promoting *P. aeruginosa* inhibiting germination of seeds of *Zea mays* and *Triticum aestivum* has also been reported [39]. Hence, PGP producing heavy metal stress-tolerant and Cr(VI) reducing bacteria which can enhance growth and reverse Cr toxicity in *V. mungo* can be used as potentially useful bioinoculant for soil remediation as well as biofertilizers (remedifertilization) for microbe-assisted phytoremediation.

5. CONCLUSION

Plant growth-producing *P. aeruginosa* ATCC15442 (P15) showed reduction in indole acetic acid, siderophore, and phosphate

solubilizing enzymes at sub-inhibitory concentrations of Cr(VI). Hence, the presence of heavy metals such as Cr(VI) affect the beneficial properties of rhizospheric microflora. The Cr(VI) reducing ability of P15 however showed a complete reversal of Cr(VI) toxicity in *V. mungo* plants even when PGP activity was hindered. Selection and subsequent use of concurrently heavy metal remediating and plant growth-promoting substance producing bacteria are promising isolates to be used for sustainable enhancement of soil productivity.

AUTHOR CONTRIBUTION

VK, RAO, and PDU contributed to design, experimentation, and data acquisition. VK and SDK contributed to the concept, design, data analysis, interpretation, and manuscript preparation**.**

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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