Pseudocitrobacter anthropi reduces heavy metal uptake and improves phytohormones and antioxidant system in Glycine max L.

Husna1 · Anwar Hussain1 · Mohib Shah1 · Muhammad Hamayun1 · Amjad Iqbal2 · Waheed Murad1 · Muhammad Irshad1 · Muhammad Qadir1 · Ho-Youn Kim3

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Abstract
Heavy metal contamination due to anthropogenic activities is a great threat to modern humanity. A novel and natural technique of bioremediation using microbes for detoxification of heavy metals while improving plants’ growth is the call of the day. In this study, exposing soybean plants to different concentrations (i.e., 10 and 50 ppm) of chromium and arsenic showed a severe reduction in agronomic attributes, higher reactive oxygen species production, and disruption in the antioxidant system. Contrarily, rhizobacterial isolate C18 inoculation not only rescued host growth, but also improved the production of nonenzymatic antioxidants (i.e., flavonoids, phenolic, and proline contents) and enzymatic antioxidants i.e., catalases, ascorbic acid oxidase, peroxidase activity, and 1,1-diphenyl-2-picrylhydrazyl, lower reactive oxygen species accumulation in leaves. Thereby, lowering secondary oxidative stress and subsequent damage. The strain was identified using 16 S rDNA sequencing and was identified as Pseudocitrobacter anthropi. Additionally, the strain can endure metals up to 1200 ppm and efficient in detoxifying the effect of chromium and arsenic by regulating phytohormones (IAA 59.02 µg/mL and GA 101.88 nM/mL) and solubilizing inorganic phosphates, making them excellent phytostimulant, biofertilizers, and heavy metal bio-remediating agent.

Keywords Bioremediation · Arsenic · Hexavalent chromium · PGPR · Bio reduction · Root exudates

Introduction
Heavy metal pollution is an alarming concern for many countries as they have the property of bioaccumulation, making a way to the food chain and resulting in human exposure. Exposure to heavy metals (HMs) results in inducing oncogenicity, carcinogenicity, and organ damage. Among HMs, chromium (Cr) and arsenic (As) are type 1 contaminants, that are mainly released in the natural environment by various industrial sources. Chromium is mostly found in the environment in Cr-VI and Cr-III state, whereas As in inorganic forms, i.e., As-V and As-III (Igboamalu and Chirwa 2017; Jiang et al. 2019; Rahman and Singh 2019). Hexavalent chromium is toxic, mutagenic and carcinogenic, exposure to Cr-(VI) increases the risk of lung cancer whereas trivalent chromium is much less toxic (Chen et al. 2019; DesMarias and Costa 2019; Wang et al. 2017). In addition to Cr-(VI) toxicity, it is readily soluble in water and easily leach out into the ground water, adsorbed by soil particles and easily enters surface water or groundwater (Liang et al. 2021; Wuana and Okieimen 2011). Therefore, it is urgent to take measures to detoxify Cr-(VI) in the environment. Bio-reduction of Cr-(VI) to Cr-(III) is considered to be a feasible method to reduce the toxicity of Cr-(VI), as trivalent chromium has low solubility and acts as a nutrient for various microbes and organisms (Yang et al. 2021). On the other hand, arsenic is generally toxic to all life forms. Like chromium, arsenic mobility in the natural environment is a major concern in arsenic-rich and contaminated areas. Chromium and arsenic both are very toxic and can induce
oxidative damage in biological systems (Pandey et al. 2018; Xiong et al. 2019). They are analogs of sulfate and phosphate respectively and therefore, can actively be up taken and accumulated in cells beyond threshold levels, disrupting important physicochemical processes (Kashyap and Garg 2018). Bacteria plays a key role in Cr and As speciation by converting Cr-(VI) to Cr-(III) and As-(V) to As-(III) and are of environmental significance due to the formation of uncharged state which has higher mobility (Fu et al. 2021).

In turn, plants have several detoxification mechanisms to avoid the phytotoxicity of these metals. They include phyto-stabilization and biotransformation of these metals to their non-toxic form. For instance, beyond threshold levels, these metals induce oxidative stress that disrupts the antioxidant defense system of the host leading to the induced toxicity of the metals. Plant exposure to these heavy metals induces physiochemical changes, such as impaired water balance, wilting and chlorosis of leaves, and damage to the growth of shoots and roots (Ponting et al. 2020). Metal stress produces reactive oxygen species (ROS) and alters the balance and assimilation of nutrients, metabolism of protein, and oxidative phosphorylation in plant tissues (Kapoor et al. 2019). HMs also affect the uptake of water, inhibit photosynthesis, and cause lipid peroxidation by altering the cell membrane lipid structure (Flora 2011; Hasanuzzaman et al. 2015; Mirza et al. 2017). To overcome the stress beyond the threshold level of the host plants, researchers aim for microbial-assisted remediation. It is a new, yet novel potential technique to remediate the metal by changing its valence state and making them biologically less toxic and stable. It is one of the most feasible and reliable techniques to reclaim and reconstruct the natural conditions of the soil that are considered harmful to environmental health (Ayangbenro and Babalola 2017). The root-associated microbes not only reduce HMs toxicity but also improve the host’s endogenous pool of phytohormones, thus promoting their tolerance toward HMs stresses (Zahoor et al. 2017).

Many scholars in various countries have made considerable research on searching plants of high biomass and super-accumulation of HMs. Nowadays ornamental plants are considered as a new source of phytoremediation species because of their use in landscaping and practical applications in air pollution monitoring and control (Cristaldi et al. 2017). Recent studies on ornamental plants to repair of HMs-polluted soils have important practical significance (Khoshru et al. 2020). The associated microbes with such plants enable their hosts to hyperaccumulate metal and efficiently reclaim the contaminated soil (Thjis et al. 2017).

Soybean (Glycine max L.) is one of the economically valuable crops and an important source of food, protein, and edible oil. Soybean is a rich source of protein and the cheapest edible oil (El-Din and Elbana 2021). It’s good for human health and reduces the risk of various health problems like cardiovascular disease, certain cancers, and low blood sugar level (hypoglycemia). Despite the increasing demand, the yield and productivity of soybean crops are greatly affected by heavy metal pollution. Thus decreasing the content of chromium and arsenic in polluted environment is extremely important, by remediating the stress and promoting growth of soybean (Bilal et al. 2020; Cid et al. 2020; Xiao 2008). Therefore, the present work aims to (i) isolate chromate and arsenate reducing bacteria from rhizosphere of Chlorophyllum comosum (ii) role of the isolated rhizobacteria in soybean growth promotion and improving plant antioxidant system under metal stress (iii) The potential of the isolate to cease metal uptake by the host plant, avoiding entry to the food chain ensuring food safety.

**Materials and methods**

**Isolation of heavy metal resistant rhizobacteria**

Rhizospheric soil samples of C. comosum were collected and sent to the Plant-Microbe Interaction (PMI) Lab, Department of Botany, Abdul Wali Khan University Mardan. Heavy metal resistant strains with the ability to promote host plant growth were isolated from rhizospheric soil, using Luria Bertani (LB) agar medium. Simply, the rhizosphere soil was suspended in autoclaved distilled water and serially diluted. Spread plates, containing chromate/arsenic supplemented LB agar, were then prepared from each dilution which were incubated at 28°C for 24 h. Distinctly different bacterial colonies were recovered on fresh media and purified by streaking method. The strains were further screened for their potential to endure elevated levels of the selected metals by supplemented selected levels i.e., 100, 300, 500, 900 and 1200 ppm of chromium and arsenic. Inoculations were made in 25 mL of L.B broth, incubated at shaking incubator for 24 h at 150 rpm and 28°C. Bacterial growth was determined by recording OD of the cultures at 600nm using a UV/Vis spectrophotometer (PerkinElmer Lambda 25 double beam spectrophotometer).

**Molecular identification of selected strains**

The potent rhizobacterial isolate was subjected to phylogenetic analysis to specie level by standard method of 16S rRNA gene sequencing. The DNA templates were prepared from the selected strain by picking individual colony. Genomic DNA was used to amplify 16S rRNA gene through PCR techniques. The reaction mixture (25 µL) contained genomic DNA (2.5 µL), rapid mix, universal forward, and reverse primers (518F 5′CCA GAC CAC GCC GGTA ATACG3′ and 800R 5′TACC AGGT ATCTA ATCC3′) purchased from Macrogen Inc., Seoul, South Korea. Initial denaturation was
carried out at 94 °C for 2 min in a thermal-cycler (Thermofisher Veriti 96-Well), followed by 30 cycles consisting of denaturation at 94 °C for 1 min; primer annealing at 52 °C for 1:30 min. and primer extension at 72 °C for 2 min. The final extension was performed at 72 °C for 10 min. The PCR product was run on a 1% agarose gel containing 2 µL of ethidium bromide (20 mg/mL) and using 0.5X TE (Tris-EDTA) buffer. The λ Hind-III ladder was used as a size mark. Purification of amplified PCR products was done by using PCR purification kit (QIAGEN), according to the manufacturer’s recommended standard protocol (Hayat et al. 2013). Purified PCR product samples (1550 bp) were sequenced using the universal 16 S rRNA gene sequencing primers through the DNA sequencing service (http://dna.macrogen.com/eng) of MACROGEN, Korea. The sequence results were blasted through NCBI BLAST to get the exact nomenclature of the isolate. Bioinformatics tool MEGA X was used for phylogenetic analysis (Chun et al. 2007; Tamura et al. 2007).

Profiling of bacterial culture supernatant

Culture supernatant of the selected bacterial isolate was obtained by centrifuging the overnight culture made in LB medium at 4000 rpm for 20 min. The bacterial culture supernatant was then collected and analyzed for various phytohormones and secondary metabolites.

Determination phytohormones

Colorimetric method based on Salkowski reagent was used to estimate the exogenous indole-acetic acid (IAA) in the bacterial culture supernatant (Tsavkelova et al. 2007). Culture supernatant and Salkowski reagent (2 mL of 0.5 FeCl₃ in 49 mL of 35% HClO₄ solution) were mixed in 2:1 ratio and the mixture was then incubated for 30 min in the dark chamber. The colored complex was quantified by taking O.D at 540 nm on a spectrophotometer against Salkowski reagent as blank. Indole-3-acetic acid (10, 20, 30, 40, 60, 80 and 100 µg/mL) was used as a reference to find out concentration of IAA in the sample.

To determine gibberellic acid (GA), 10 healthy embryoless wheat seeds were sterilized by dipping in ethanol (70%) for 30 s. After ethanol treatment, the seeds were washed thrice with double distilled water to remove traces of ethanol. Ethanol-free seeds were placed in petri plates having 10 mL of buffer solution (acetate buffer pH 4.5) and 2 mL of supernatant from bacterial culture. The plates were kept at room temperature for 48 h. After the incubation, the solution of each plate was filtered and 200 µL Benedict’s solution was added and kept in a water bath for 30 min (Coombe et al. 1967). Optical density of the reaction mixture was taken at 254 nm against blank (10 mL acetate buffer with 2 mL LB broth media and 200 µL Benedict’s solution). Standard curve was made by making assaying different concentrations (0.1, 1.10, 100 and 1000 nM/mL) of GA₃ solution.

Exogenous salicylic acid (SA) content was estimated using a standard protocol (Warrier et al. 2013). To 100 µL of culture supernatant, 2.99 mL chilled freshly prepared ferric chloride (0.1%) was added, and the final volume was adjusted to 3 mL. The solution mixture turned violet due to the formation of a complex between Fe³⁺ ions and SA. This change in color to violet was measured spectrophotometrically at 540 nm against ferric blank (0.1% ferric chloride solution). Standard SA (100, 200, 300, 400 and 500 µg/mL) was used as a reference to quantify the amount of SA in the samples.

Determination of metabolites in bacterial culture supernatant

Total flavonoids content

Total flavonoid content (TFC) was determined by a well-established method (El Far and Taie 2009). The reaction mixture consisted of 0.5 mL of culture supernatant, 0.1 mL aluminum chloride (10%), 0.1 mL potassium acetate (10%) and 4.8 mL methanol (80%). The reaction mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance was measured at 415 nm against blank (0.1 mL aluminum chloride, 0.1 mL potassium acetate and 4.8 mL of 80% methanol). Different concentration of quercetin (15, 30, 60, 120, 240 and 480 µg/mL) was used as a reference to quantify the amount of flavonoid in the samples.

Total phenol content

The total phenolic content in bacterial culture supernatant was determined using the Folin-Ciocalteau reagent method with slight modification (Lee et al. 2015). Reaction mixtures consisted of 2 mL culture supernatant mixed with 0.5 mL Folin-Ciocalteau reagent. After 5 min, 2 mL of 20% sodium carbonate (Na₂CO₃) solution was added and the mixture was incubated for 4 min at 25 °C. Heat treatment of the mixture was done by placing the mixture tubes in boiling water. Upon cooling, a colored complex developed in the reaction mixture which was quantified by taking OD at 765 nm against Folin-Ciocalteau reagent as a blank. Different concentration of Gallic acid (100, 200, 300, 500 and 600, 700 and 900 µg/mL) was used as a reference to quantify the amount of phenol in the samples.

Total protein content

Total protein estimation was carried out by following (Lowry et al. 1951) assay. Biuret reagent was prepared by
mixing 0.5 mL of 1% copper sulfate and, 0.5 mL of 2% sodium potassium tartrate, followed by the addition of 50 mL of 2% sodium carbonate in 0.1 N of sodium hydroxide. Bacterial supernatant (100 µL) was diluted with distilled water to make 1 mL solution, equal volume of biuret reagent was added to the sample and thoroughly stirred for 10 min. Followed by addition of 100 µL of diluted Folin-Ciocalteau reagent (1:1) and incubated at room temperature for 30 min. The absorbance was read at 650 nm using a spectrophotometer against Folin-Ciocalteau reagent as a blank. Different concentrations of Bovine serum albumin (20, 40, 60, 80 and 100 µg/mL) was used as reference to quantify total protein content in samples.

Proline

Proline content in bacterial supernatant was determined using acid ninhydrin (Bates et al. 1973). Reaction mixtures consist of an equal volume of sample and acid ninhydrin reagent (1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL phosphoric acid) was boiled for 60 min at 100 °C. After cooling, the mixture was extracted with 4 mL of toluene. The toluene layer was separated with a separating funnel and the absorbance of toluene fraction was measured at 520 nm against using toluene as a blank. Standard proline (2, 4, 6, 8 and 10 mg/mL) was used as a reference to quantify the amount of proline in the samples.

Total soluble sugar

Soluble sugar content was determined by slightly modifying the phenol-sulfuric acid procedure (Dubois et al. 1956). To 100 µL of supernatant, 1 mL of 80% phenol was added and the mixture was incubated for 10 min at room temperature. After incubation, 5 mL of concentrated H₂SO₄ was added to the mixture which was then allowed to stand for 1 h at room temperature. The absorbance was read at 485 nm using a spectrophotometer against blank (1 mL of 80% phenol and 5 mL H₂SO₄). Different concentration of glucose (20, 40, 60, 80 and 100 µg/mL) was used as reference to quantify amount of soluble sugar in the samples.

Phosphate solubilization index

Pikovskaya agar medium was used to determine phosphate-solubilizing index following method of Edi-Premono et al. (1996). The selected isolate was inoculated on Pikovskaya’s media plates in laminar-flow-hood in sterile conditions. Plates were then incubated at 28 °C for 48 h. Halo zone and colony diameters were measured and the capacity of phosphate solubilization activity was found through the following equation:

\[
SI = \frac{\text{colony diameter (cm)} + \text{holozone diameter (cm)}}{\text{colony diamtere (cm)}}
\]

Hydroponic experiment under heavy metals stress

Soybean (G. max) seeds were obtained from Agricultural Research Institute Mingora, Swat. Soybean seeds were surface disinfected, using 0.1% mercury chloride (HgCl₂), followed by washing three times with double distilled water. Seeds were grown in sterilized sand and seedlings with two fully expanded leaves were shifted to pots (500 mL) containing half strength of Hoagland’s solution. The experimental setup consisted of 36 pots with two chromat and arsenic levels, low level (10 ppm) and high level (50 ppm) along with control (without any treatment). Rhizobacterial inoculation (10⁶) was made to assess the alleviation potential of the isolate in pot conditions. Three replicates of each treatment with 4 plants were set in randomized complete block design. The pots were transferred to the LabTech growth chamber and kept under 25 °C temperature, 68% humidity, and 13 h of photoperiod for 14 days. After 14 days, seedlings were harvested, and the parameters described below were recorded.

Root exudates

For exudates sampling 14 days cultivated plants roots from hydroponic system were rinsed and placed in sterile de-ionized water for 6 h (Hao et al. 2010). The root exudates were collected and centrifuged at 3000 rpm for 10 min and various metabolites were studied spectrophotometrically.

Determination of phytohormones in soybean plant and root exudates

For IAA determination in soybean leaves, 0.2 g fresh leaves were ground in 5 mL of 80% ethanol and centrifuged for 5 min at 3000 rpm. A sample of a different aliquot (10-1000 mg) was extracted in 1 mL of ethanol to determine the solubility of SA from the tissue. SA quantity in root exudates was determined by adding 100 µL of sample to 2.99 mL of chilled 0.1% ferric chloride. Phytohormones (IAA and SA) in all treated plants and exudates were estimated by the method mentioned previously (Tsavkelova et al. 2007; Warrier et al. 2013).

Determination of plant metabolites in soybean plant and root exudates

Primary metabolites and secondary metabolites were determined in plant material and root exudates. 0.5 g of fresh leaves were ground in 5 mL of ethanol (80%). The
homogenate was centrifuged at 3000 rpm for 5 min and supernatant was kept for flavonoids estimation. For extraction of phenolic content, 0.5 g of fresh soybean leaves were homogenized in 10 mL of 80% ethanol. The homogenate was then centrifuged for 20 min at 10,000 rpm. Secondary metabolites were estimated in plant supernatant and root exudates of all treated plants by the methods as mentioned previously (El Far and Taie 2009; Lee et al. 2015).

Protein extract was prepared by grinding 100 mg of plant material in phosphate buffer (1 mL, pH 7.5) and centrifuged for 10 min at 3000 rpm. Proline content was extracted in soybean by grinding 0.1 g fresh leaves of in 4 mL of 3% sulfosalicylic acid solution and incubated at 5 °C for 24 h. The homogenate was then centrifuged at 3000 rpm for 5 min. Soluble sugar content was determined by grinding 0.5 g fresh leaves of soybean in 10 mL of distilled water using mortar and pestle. The homogenate was centrifuged for 5 min at 3000 rpm. Primary metabolites (protein, sugar and proline) in plant material and root exudates were determined spectrophotometrically by the method as mentioned earlier (Bates et al. 1973; Dubois et al. 1956; Lowry et al. 1951).

Antioxidant enzymes

Determination of catalases activity

Catalases activity was assayed as described by Chandlee and Scandalios (1984) with slight modifications. Fresh soybean leaves (100 mg) were homogenized in 1 mL of phosphate buffer (50 mM, pH 7.5). The homogenate was centrifuged for 10 min at 10,000 rpm, and the supernatant was collected as enzyme extract. The enzyme extract was added to 3 mL of the reaction mixture. The reaction mixture consists of 2.6 mL of 50mM potassium phosphate buffer at pH 7.0 and 0.4 mL of 15 mM H₂O₂. The decrease in the absorbance of H₂O₂ (µmol/min) was estimated by a spectrophotometer at 240 nm.

Determination of ascorbic acid oxidase

For the extraction and estimation of ascorbic acid oxidase activity, the method of Oberbacher and Vines (1963) was used with slight modifications. The enzyme extract (200 µL) was mixed with the reaction mixture (800 µL). The reaction mixture consisted of 600 µL of potassium phosphate buffer (50 mM), 100 µL of ascorbic acid (0.5 mM), and 100 µL of H₂O₂ (0.1 mM). The optical density was recorded at 290 nm for every 30 s for 5 min against blank. The enzyme activity was expressed in mg⁻¹ protein.

 Peroxidase's activity

Peroxidase activity was measured by the method of Putter (1974). To isolate the enzyme, fresh samples (0.1 g) were homogenized in 3 mL phosphate buffer (0.1 M) using mortar and pestle. The homogenate was centrifuged for 10 min at 4 °C and 14,000 rpm. The collected supernatant was used as an enzyme extract and mixed with 3 mL phosphate buffer (0.1 M, pH 7.0), 30 µL H₂O₂ (12.3 mM), and 50 µL guaiacol solution (20 mM). The POX activity was determined spectrophotometrically by using guaiacol as the substrate.

Determination of DPPH-radical scavenging activity

Radical scavenging activity 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined spectrophotometrically using Meng et al. (2016) method. The reaction mixture consisted of 2 mL of samples solution and 2 mL of DPPH solution (0.04 mg/mL) in methanol. Leave the reaction mixture in the dark for 30 min and record the absorbance at 517 nm against methanol as blank.

Screening for radical oxygen species (ROS)

For the detection of ROS accumulation in soybean leaves treated with rhizobacteria under chromate and arsenate stress, the method of Scharte et al. (2005) was followed. After 14 days of germination, hydrogen peroxide was detected histochemicaly in leaves by DAB (3,3′-diaminobenzidine) staining. Leaves were dipped in DAB (1 mg/mL, pH 3.8), vacuum infiltrated five times (five minutes each), and incubated for three hours at room temperature. The leaves were then decolorized in boiling ethanol (96% v/v) for 20 min. Boiling decolorized the leaves, except the brown-colored spots produced by DAB. After cooling, the tissues were observed under a light microscope to visualize the brown spots.

Determination of Cr and As by BCR extraction

The reduction of chromate and arsenate in bacterial culture supernatant and soybean plants was determined using atomic absorption spectroscopy (Perkin Elmer AAnalyst 700). Before analysis metal with lower and higher oxidation states were extracted separately by using a modified BCR (Community Bureau of References) sequential extraction procedure (Kazi et al. 2005). Steps of BCR are listed as under.

Step 1: In this step acid-soluble and exchangeable components were extracted. To, 0.5 g of air-dried samples, 20 mL of 0.11 M acetic acid (CH₃COOH) was added and shaken for 12–24 h at 25–30 °C in an orbital shaker. This fraction was separated from the matrix through centrifugation (3000 rpm, 15 min).
Step 2: In this step, fractions consisted of reducible metal ions were separated. To the residue from step 1, 20 mL of 0.5 M hydroxylamine-hydrochloride (NH₂OH·HCl) at pH 1.5 with nitric acid (HNO₃) was added. The samples were stirred for 16 h at 30 °C. This fraction was separated from the matrix through centrifugation (3000 rpm, 15 min).

Step 3: In this step oxidizable metal ions were obtained. The residues from step 2 were mixed in ammonium acetate buffer (pH 2) and then treated with 5 mL of 30% hydrogen peroxide (H₂O₂). The mixture was stirred for 1 h at room temperature. This step was repeated twice. The sample was then heated at 60 °C to evaporate excess of solvents and treated with 25 mL of 1 M ammonium acetate (CH₃COONH₄). The resulting mixture was stirred for 16 h. This fraction was separated from the matrix through centrifugation (3000 rpm, 5 min). The supernatant was carefully poured off to avoid loss of residue.

Table: BCR method for heavy metal speciation.

| Step | Phase                              | Extractants                  | pH      | Shaking time and temperature |
|------|------------------------------------|------------------------------|---------|------------------------------|
| 1    | Acid soluble and exchangeable      | 20 mL of 0.11 M CH₃COOH      | 29-30°C |                              |
| 2    | Reducible                          | 20 mL 0.5 M NH₂OH·HCl        | 1.5     | 16 h at 25-30 °C             |
| 3    | Oxidizable                         | 5 mL of 30% H₂O₂ Then, 5 mL of 30% H₂O₂ | 2.0     | 1 h at 25-30 °C |
|      |                                    | 25 mL of 1 M CH₃COONH₄       | 2.0     | 16 h at 25-30 °C             |

Atomic absorption spectroscopy (AAS)

Total metal concentration and metal ions in different fractions were separately measured using atomic absorption spectroscopy (FAAS). For excitation, an air-acetylene flame was used. The light source was a hollow cathode lamp with a single wavelength. A gas flow rate of 2 L/min. Metal ion analysis of unknown samples was carried out using a known standard. The absorption of standard metal ion solutions was measured and plotted as a standard curve.

Bioconcentration factor (BCF)

Accumulation of heavy metal in the bacterial supernatant and plant biomass of soybean was determined by using the following equation:

\[
BCF = \frac{\text{Metal accumulated in biomass}}{\text{Metal added to media}}
\]

Results

Isolation of metal tolerant rhizobacteria

Metal resistant rhizobacteria were isolated from the rhizosphere of *C. comosum* on Luria Bertani (LB) media. A total of 18 rhizobacterial strains were isolated were cultured in LB broth media supplemented with 100, 300, 500, 900, and 1200 ppm of Cr (as K₂CrO₄) and As (as Na₃AsO₄). Among them, 9 strains were capable to grow in the presence of Cr and As stress up to 1200 ppm (Table 1). The isolates that were capable to withstand the Cr-(VI) and As-(V) were selected for further study for plant growth promotion assay.

Chromium (VI) and arsenic (V) effect on bacterial growth

From the isolated rhizobacterial strain, C18 was capable to withstand all the metal levels supplemented in the liquid medium. The growth pattern recorded to increase up to 500 ppm in the presence of chromate supplements. However, the decline phase was recorded in the growth beyond 500 ppm in chromate supplementation. Nonetheless, an opposite trend was recorded in the case of arsenic supplementation. Arsenic-treated bacterial cells tend to decrease with increasing arsenic concentration compared to untreated bacterial cells (Fig. 1).

Molecular identification of rhizobacterial isolate C18

Based on the sequence of 16S rDNA, isolate C18 showed maximum homology (98.01%) with *Pseudocitrobacter anthropi*. For the confirmation of strain identity, sequence of C18 was subjected to phylogenetic analysis (GenBank Accession No. MZ567221). The phylogenetic consensus tree was constructed by Maximum Parsimony method using MEGA X software (Fig. 2). The phylogenetic analysis and sequence homology results revealed that the isolate C18 made a clad with *P. anthropi* supported by 98% bootstrap value in the consensus tree.

Cr-(VI) and As-(V) speciation by PGPR isolate C18

Isolate C18 is tolerant to a high concentration of Cr-(VI) and As-(V) and has the potential to reduce the concentration of chromium and arsenic in nutrient broth media containing up to 1200 ppm (Fig. 3). To confirm that the Cr-(VI) and As-(V) supplemented in the medium is bio-transform by the rhizobacteria isolate, we used atomic
absorption spectrophotometry (AAS) to analyze the valence transformation in the medium. The isolate \textit{P. anthropi} was able to reduce a significant amount of Cr-(VI) to Cr-(III) and As-(V) to As-(III). Isolate C18 grew well in Cr-(VI) spiked media till 500 ppm, while the level of hexavalent chromium (Cr-VI) was steadily reduced.

| Cr-(VI)/As-(V) stress | Control | 100 ppm | 300 ppm | 500 ppm | 900 ppm | 1200 ppm |
|-----------------------|---------|---------|---------|---------|---------|----------|
| C1                    | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C2                    | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C3                    | +       | +++     | +++     | +++     | +++     | +++      |
| C4                    | +       | +++     | +++     | +++     | +++     | +++      |
| C5                    | +       | ++/+    | ++/+    | ++/+    | ++/+    | ++/+     |
| C6                    | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C7                    | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C8                    | +       | +++     | +++     | +++     | +++     | +++      |
| C9                    | +       | +++     | +++     | +++     | +++     | +++      |
| C10                   | +       | +++     | +++     | +++     | +++     | +++      |
| C11                   | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C12                   | +       | +++     | +++     | +++     | +++     | +++      |
| C13                   | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C14                   | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C15                   | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C16                   | +       | +++     | +++     | +++     | +++     | +++      |
| C17                   | +       | −/−     | −/−     | −/−     | −/−     | −/−      |
| C18                   | +       | +++     | +++     | +++     | +++     | +++      |

(+Rhizobacteria growth) (− No Rhizobacteria growth)

Metabolic profiling of \textit{P. anthropi}

Response of the selected rhizobacterial strain \textit{P. anthropi} were analyzed in terms of production of phytohormones (IAA, GA, and SA) and stress-related metabolites such as flavonoids, phenols, protein, proline and total sugar content to withstand the stressful environment.

Bacterial culture phytohormones

The ability of rhizobacteria \textit{P. anthropi} (C18) to produce IAA under different concentrations of chromate and arsenate was determined. IAA quantity was increased with increasing levels of metal stress in bacterial culture supernatant. In particular, at 1200 ppm isolate produced the highest amount of IAA (59.02 µg/mL), lowest amount was recorded in the control (25.91 µg/mL) without any stress (Fig. 4A). The release of exogenous gibberellic acid (GA) was also enhanced in media containing salts of As and Cr. Our results report that the maximum GA (101.88 and 95.66 nM/mL) production is observed when the metal concentrations are 300 and 500 ppm. However, higher concentrations of the selected heavy metals in the media significantly reduced the amount of GA, in comparison to control (Fig. 4B). From the spectrophotometric readings, it was observed that the production of salicylic acid gradually decreased with the respective metal concentration. The maximum yield of SA (787.77 µg/mL) was observed in the control without any stress. The increasing level of stress showed a decline in SA quantity till 900
ppm. Interestingly, a higher concentration (1200 ppm) was least toxic for *P. anthropi* to release SA (4 C).

**Bacterial culture metabolites**

Rhizobacteria *P. anthropi* (C18) in the presence of different metal concentrations (0, 100, 200, 300, 500, 900, and 1200 ppm) produced flavonoids in LB medium. The content of flavonoids decreases steadily with the gradual increase of Cr-(VI) and As-(V) levels in the medium (Fig. 4D). Strain results revealed a higher amount (103.43 µg/mL) of flavonoids at 500ppm compared to normal culture media, the content of TFC content is lowest at (57.02 µg/mL) at the maximum level of chromate and arsenate. A decrease in total phenolic content was observed under different metal concentrations for the treated and untreated bacterial culture supernatant (Fig. 4E). The phenolic content level was significantly decreased to 823.15 µg/mL in treated culture compared with 1046.15 µg/mL in the control untreated. The production of total protein contents was also measured when the strain was exposed to different concentrations of the heavy metal (Fig. 4F). Results showed that the cultured filtrate of *P. anthropi* total protein contents tend to increase from 35.25 to 222.22 µg/mL as chromate and arsenate level in the culture media increases 0 µg/mL to 1200 ppm. The production of proline contents was determined upon exposure of the isolated strain to the selected heavy metal (Fig. 4G). The proline contents showed a slight increase to 300 ppm and a subsequent decrease in the elevated levels of the metals. The exogenous soluble sugar contents of *P. anthropi* were also estimated under Cr and As stress conditions (Fig. 4H). The production of endogenous sugar contents was highly influenced by heavy metals. A significant dose-dependent decrease was recorded in the soluble sugar contents from 169.59 to 48.49 µg/mL of the strain from 0 to 1200 ppm of selected heavy metal stress.

**Phosphate solubilization by *P. anthropi***

The potential of *P. anthropi* to convert the inorganic form of phosphorous into the solubilized form was evaluated (Fig. 5). The strain showed a larger halo area in Pikovskaya agar with an average diameter of 26 mm.

**Plant growth promotion assay**

Among the selected isolates, C18 inoculation was the most potent isolate that alleviated chromate and arsenate stress in soybean as reflected by improved growth parameters (Fig. 6). Based on its performance in soybean growth assay, isolate C18 was selected for further analysis.

**Growth parameters**

Exposure to the aforementioned concentration of chromate and arsenate reduced the host seedling growth by almost 50% and 49% as compared to the untreated seedlings (Fig. 7A). Consequently, inoculation of *P. anthropi* improves the host seedling growth by 68%, and a similar trend was recorded in metal-treated seedlings as well showing no significant decrease in their shoot length. Contrastingly, the substantial dose-dependent reduction was recorded in the root growth of seedlings (Fig. 7B). The decline recorded was up to 58% as compared to untreated control seedlings. Co-cultivation of *P.
anthropi with soybean normalizes the root growth showing comparable elongation as control plants however, a significant decrease was recorded in the root length upon exposure to mentioned levels of chromate and arsenate.

**Phytohormones**

Soybean seedlings produce promising quantities of Indole-3-acetic acid, a major part of which (77.51%) were stored endogenously (Fig. 8A). Exposure to selected levels of metal has an influential impact on the endogenous and exogenous production of IAA. A significant increase in endogenous and decrease in exogenous IAA contents was recorded upon exposure to 10 and 50 ppm of selected metal stress. Interestingly, inoculating seedlings with *P. anthropi*, the endogenous levels show a dose dependent decrease in the total IAA contents except for T7 showing higher IAA levels. More fascinating results were recorded in the case of exogenous IAA contents showing a dose-dependent increase at all supplemented concentrations of the selected metals. Similar trends were also recorded in the case of endogenous and root exuded salicylic acid production (Fig. 8C). A catastrophic decrease was recorded in endogenous SA contents at 10 ppm, however, followed by an abrupt increase in the case of chromate supplementation. An opposite trend was recorded in the case of arsenic spiked medium (p < 0.05). For instance, opposite tendencies were recorded in the root-exuded salicylic acid contents. Inoculating the host with *P. anthropi*, comparatively higher quantities of endogenous salicylic acid contents were recorded. Interestingly, the lowest salicylic acid contents were recorded in *P. anthropi* inoculated seedlings however, a significant increase was recorded in the rest of the chromate and arsenate treated seedlings as compared to control.
Plants metabolites profiling

Soybean seedlings treated with a mentioned concentration of chromate and arsenate trigger the host to synthesize higher quantities of total flavonoid contents. Heavy metal stress greatly influences the total flavonoids contents by the higher endogenous accumulation of flavonoids however, the decline was recorded in the exogenous flavonoid contents (Fig. 8E). Co-cultivating the host seedlings with \textit{P. anthropi}, lower flavonoids accumulation and higher release were recorded. The same decline was recorded in endogenous and root exuded flavonoids however, higher accumulation was recorded when the host seedlings were exposed to 10 and 50 ppm of arsenate stress. A decreasing trend was recorded in the total endogenous and exogenous phenolic contents of the host seedling exposed to 10 and 50 ppm of selected heavy metals (Fig. 8G). Cocultivation of \textit{P. anthropi} with soybean seedling, no significant improvement was recorded in the total endogenous and exogenous phenolic contents as compared to control seedlings.

Soybean produce ample quantities of free amino acid ample quantities of which are stored endogenously, and promising quantities are released exogenously (Fig. 8I). Soybean seedlings are treated with 10 and 50 ppm. An increasing trend was recorded in the proline contents of the host however, the increase was comparatively lower as compared to control except for T3 showing higher amounts of endogenous proline. Higher roots exuded proline contents were recorded at all supplemented concentrations of both metals. Cocultivation of host seedlings with \textit{P. anthropi}, an increase was recorded in total endogenous and exogenous proline contents followed by a decreasing trend. The endogenous proline contents were lower as compared to control however, the root exuded proline contents were higher except in plants treated with \textit{P. anthropi} and 50 ppm of arsenate. A significant decrease was noted in the endogenous and roots exuded soluble sugar contents was when exposed to 10ppm followed by an increase at 50 ppm of chromate and arsenate induced stress (Fig. 8K). Inoculating \textit{P. anthropi}, an increase was recorded at 10 ppm in production of endogenous and root exuded soluble sugar contents however, the decline was recorded at 50 ppm. A similar trend was also recorded in the total protein contents of the host treated with a mentioned concentration of the selected metals (Fig. 8M). Supplemented medium with chromate and arsenate, the decline was recorded in the endogenous pool of total protein contents. Nonetheless, the escalation was recorded in the exogenous total protein contents as metal increases in the medium. Inoculating seedlings with \textit{P. anthropi}, no substantial increase was recorded in the endogenous and exogenous protein content however, an increase was recorded in the exogenous protein contents of the plants treated with 50 ppm of chromate and arsenate.

Response of rhizobacteria inoculated soybean antioxidant enzymes machinery to metal stress

Modulation in the activity of the antioxidant enzymes including catalasas (CAT), ascorbic acid oxidase (AAO), peroxidase activity (POD), and DPPD in response to chromate and arsenate stress was studied. The results show that metal stress has different effects on the activities of these antioxidant enzymes (Fig. 9A). CAT enzyme level drastically increased with increasing concentration Cr and As compared to control (0.005 unit enzyme/g/30 s). \textit{P. anthropi} inoculated soybean seedlings showed a slight decline in CAT activity (0.029 unit enzyme/g/30 s). A similar trend was revealed in AAO activity in inoculated and non-inoculated plants under HMs stress (Fig. 9B). In addition, POD activity was higher in metal-treated plants (4.71 and 4.17-unit enzyme/g/30 s) than that in the control (4.01 unit enzyme/g/30 s) and bacteria inoculated. Plants inoculated with \textit{P. anthropi} isolate showed the lowest amount of peroxidases under control conditions 1.096 unit enzyme/g/30 s. However, with increasing chromium and arsenic concentration peroxidase activity showed a reduction from 4.67 to 2.31 unit enzyme/g/30 s in chromate treated plants. An opposite trend was observed in soybean seedlings exposed to As stress from 3.20 to 3.58 unit enzyme/g/30 se (Fig. 9C). DPPH-radical scavenging activity in the plants got reduced upon exposure to Cr and As stress. However, inoculation of \textit{P. anthropi} showed an increase in the radical scavenging activity under metal stress (Fig. 9D).

DAB staining

Exposure of plants to chromium and arsenic stress induced the production of high ROS in plants (Fig. 10). The present study also showed an increased level of H$_2$O$_2$ with increasing HMs dose, i.e. from 10 to 50 ppm. A significant amount of H$_2$O$_2$ was accumulated in the leaves of soybean plants when exposed to chromate and arsenate stress. Heavy metals are free radical generators, and our study showed a higher rate of ROS formation under metal stress compared to control and rhizobacterial inoculation. With the inoculation of \textit{P. anthropi} isolate, the chromate stress was alleviated, hence reducing the H$_2$O$_2$ production with no DAB stains.
Microbial reduction Cr-(VI) and As-(V) in *Glycine max*

The biotransformation of the hexavalent chromium (Cr-VI) and pentavalent arsenic (As-V) form to their non-toxic form in the surrounding medium was influenced by the plants. As the concentration of the metal in the medium increased, the biotransformation of spiked metal also increased (Fig. 11A). Cocultivation of the selected rhizobia with the host plants resulted in higher biotransformation and stabilization. The accumulation of chromium in the plants’ parts was cut by almost 50% in bacterial inoculated seedlings whereas, their biotransformation increases in the host tissue to avoid phytotoxicity. On the other hand, lower arsenic uptake and higher biotransformation were recorded at 10 ppm however, an opposite trend was followed at 50 ppm showing higher
accumulation and biotransformation. The bioconcentration factor of the soybean seedlings treated with different concentrations of Cr and As were recorded (Fig. 11B). The bioconcentration increased at 10 ppm, whereas declined at 50 ppm of the Cr. In contrast, a low bioconcentration factor was recorded at both levels of As. Inoculated plants with the selected rhizobacterial strains showed a high accumulation of Cr at 10 ppm and low at 50 ppm. In the case of As, higher bioaccumulation was recorded at both concentrations of As in \textit{P. anthropi} inoculated soybean seedlings compared to the non-inoculated seedlings.

**Discussion**

Heavy metal contamination in water and soil has become a global problem that can lead to a loss in crop yield and affect human health due to the accumulation in the food chains. Nowadays, microbial assisted remediation is a green technique to reclaim the contaminated environment (Bibi et al. 2018; Hamayun et al. 2017; Ikram et al. 2018; Ismail et al. 2020a; Qadir et al. 2020). In this context, heavy metal resistant strains were isolated from the rhizosphere of \textit{C. comosum} and were assessed for detoxification effects against chromium and arsenic while improving host plant growth. \textit{P. anthropi} was able to tolerate Cr-(VI) and As-(V) up to 1200 ppm. The main role of microbes living in abiotic stress is survival and detoxifying the effect of HMs. To avoid heavy metals, detoxify their toxicity, and induced oxidative damage, microbes tend to actively biotransform the metal by changing their valance state. The biotransformation of chromate in the medium indicates the presence of chromate reductases, quinone reductases, and other enzymes of family oxidoreductases (Mala et al. 2020; Valenzuela-Garcia et al. 2020). These enzymes help the bacterium to bio-transform the metal outside of the cell thus avoiding their toxicity. In the case of arsenic, bacteria use ArsC-based resistance determinants, whereas ArsB efflux proteins for the protection of the cell against arsenate (Mukhopadhyay et al. 2002). Another process, often associated with detoxification, is arsenic methylation. Bacterial cells can use As-(III) as an electron source.
**Fig. 9**  
*P. anthropi* inoculation effect on antioxidant machinery of soybean plants exposed to chromate and arsenate stress.  
**A** CAT activity, **B** AAO activity contents, **C** POX activity, and **D** DPPH assay. Control (without any treatment) T1) Plants exposed to Cr (10 ppm) T2) Plants exposed to Cr (50 ppm) T3) Plants exposed to As (10 ppm) T4) Plants exposed to As (50 ppm) T5) Plants inoculated with *P. anthropi* T6) Plants inoculated with *P. anthropi* exposed to Cr (10 ppm) T7) Plants inoculated with *P. anthropi* exposed to Cr (50 ppm) T8) Plants inoculated with *P. anthropi* exposed to As (10 ppm) T9) Plants inoculated with *P. anthropi* exposed to As (50 ppm)

**Fig. 10** ROS accumulation assay with DAB; localized brown areas on the leaves symbolizing ROS accumulation in  
**a** Control, **b** Cr 10 ppm, **c** Cr ppm, **d** As ppm, **e** As 50 ppm, **f** *P. anthropi*, **g** *P. anthropi* + Cr 10 ppm, **h** *P. anthropi* + Cr 50 ppm, **i** *P. anthropi* + As 10 ppm, and **j** *P. anthropi* + As 50 ppm
for respiration and the most common detoxification mechanism is based on blocking the membrane channels through which toxic substances enter the cell. An additional method for the protection is to use a specific membrane pump to actively reduce metal ions from the cell. In this study, *P. anthropi* (C18) showed a reduction in chromate and arsenate stress within 48 h of incubation. The reduction efficiency of Cr-(VI) and As-(V) was evaluated, showing an increasing tendency with increasing metal levels.

Other strategies include the production of higher quantities of phytohormones including IAA and GA and stress-related metabolites including flavonoids, phenolics, proline, metalloprotein, lower molecular weight carbohydrates in the culture medium (Ismail et al. 2018, 2020b). The strain was also able to effectively solubilize the inorganic phosphate. Exposing strains to elevated levels of selected metals modulate the phytohormones and metabolites production. Release of IAA and GA were positively regulated whereas, SA was negatively correlated under metal stress (Tiwari et al. 2021; Zaid et al. 2019). The positive correlation of IAA and GA was observed as a persuasive constituent of defense responses via regulation of several genes and negotiation of crosstalk between abiotic and biotic stress responses by overexpression of IAA30 gene of NTM2 and TaMYB73 gene respectively. This means that at higher concentrations of Cr and As, *P. anthropi* cells started to release IAA and GA in excess to protect themselves from environmental stress by regulated gene expression profile (Hamayun et al. 2021; Hussain et al. 2015). The negative correlation of SA has been suggested that exposure to heavy metal induces microbes to release an excess of SA, which is re-absorbed by the culture and converted to SA based metabolites, such as siderophores. The production of SA based siderophores can be used to acquire Fe and detoxify heavy metals by chelating them (Conroy et al. 2019; Forchetti et al. 2010; Hudson and Bentley 1970; Marshall and Ratledge 1972).

Primary and secondary metabolites produced by the strains relieving metal and secondary oxidative stress in the microbes and are also responsible for mitigating heavy metal stress in their host by fostering growth, improving the antioxidant and metabolic systems. A decrease in the production of flavonoids and phenolic contents occurs due to the alteration of phenylalanine and shikimate pathway respectively (Chen et al. 2020; Sharma et al. 2019). An increase was recorded in protein production whereas, a decline in the proline and sugar contents were recorded (Ghaffari et al. 2019; Shafiq et al. 2021). The increase in protein content was possibly due to the reason that under stress conditions the bacterial cell produces huge quantities of stress-related protein including chaperons, heat shock protein, metalloprotein to cope with the heavy metal stress. On the other hand, a decrease was recorded in the production of free amino acid and sugar because they are actively used for the production of protein (Hemmler et al. 2018). One acts as a building block and the other is used as an energy source to drive the machinery for the production of stress-related protein. Among other microbial strategies to cope with the harsh stressful condition are bioreduction and biotransformation of metal to their non-toxic and immobile form thereby relieving stress. Elevated levels of the metals stimulate the microbes to release defense molecules for relieving stress thereby showing increasing trends as metal increases while growing normally (Etesami 2018). These strategies of the rhizobacteria not only help to assist their growth but also assist their macro-symbiont to withstand harsh environmental conditions making the strain best fit for symbiotic association with their host.

Phytotoxicity of chromate and arsenate catastrophically reduces the growth attributes of productivity and stress-related metabolites making the host more susceptible to

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**Fig. 11** Bio-reduction and bioconcentration factor of soybean plants inoculated with isolate *P. anthropi* under chromium and arsenic stress
the damage of biotic and abiotic stress factors. Currently, treating G. max L. with mentioned levels of chromate and arsenate had a profound negative impact on the agronomic attribute of the host. Cocultivation of the host with rhizobacterial isolate C18, stimulate the growth of stressed seedlings by the enhanced production of phytohormones and stress-related metabolites (Khan et al. 2021). The plant responds at multi-levels to counter the stress and these responses are regulated by phytohormones. Hence, higher production of phytohormones by HM stressed soybean seedlings are required to cope with high chromium and arsenic concentration in the growth medium. In the current scenario, C18 associated seedlings exude higher quantities of IAA and SA, aiding the host in attracting microbial partners in the rhizosphere for the establishment of the beneficial association and enhance stress tolerance (Meena et al. 2017).

On the other hand, in C18 associated seedlings, higher flavonoid accumulation was recorded in the host tissue and lower quantities were exuded in the rhizosphere (Machado et al. 2021). This is possibly due to the reason that flavonoids act as a nonenzymatic antioxidant and metal quencher. Thus, helping the host to boost the antioxidant system by a higher accumulation of flavonoids in their tissue while detoxifying the metals by chelating them (Pisoschi et al. 2020). An opposite trend was recorded in the production of phenolic contents of the host showing a decline in the endogenous and root exuded phenolic content (Bistgani et al. 2019) however, higher endogenous and lower root exuded proline and sugar contents were recorded in the plant parts. This is possibly due to the reason that proline and sugar act as osmolytes, chemical chaperones, direct ROS scavengers, and as well as regulate intracellular redox homeostasis (e.g., ratio of NADP+/NADPH and GSH/GSSG by proline) (Vives-Peris et al. 2017). Thus, tends to maintain the integrity of the photosynthetic machinery under stress indicated from the higher sugar production and their accumulation in plant parts. During the study, it was found that exposing soybean to Cr-(VI) and As-(V) reduced the growth of the host significantly in terms of roots shoot length and fresh/dry weight \( p < 0.05 \). C18 could stimulate the development of Cr and As stressed soybean seedlings showed that detoxifying Cr and As and subsequently decreased absorption were not the sole mechanism for phyto-stimulation rather it also strengthens the antioxidant system of the host. Due to secondary oxidative damage, abnormal production was noted in the enzymatic antioxidant of the host plants. However, the deposition of antioxidants (APX, CAT, POD, DPPH, and SOD) modulated by selected strains may have more efficiently scavenged stored ROS in the host to support its protection mechanisms (Sarker and Oba 2018). In plants, oxidative stress can be identified by the abnormal activity of antioxidant enzymes under stress situations. As a result of metal stress, excessive ROS was generated, and seedlings were unable to handle them. Hence, the damage was done in seedlings that were phenotypically recognized as retarded growth. Cr and As exposed seedlings tried to control the excess of ROS by producing abnormally higher quantities of CAT, AAO, peroxidase, and SOD. The consequences of this were the inability of soybean seedlings to scavenge ROS and avoid lipid peroxidation as evident by a low amount of lipids in such seedlings. Stressed seedlings were unable to produce the optimum amount of IAA, phenol, and sugars, which was also an indication of the internal damage to the seedlings. Metal exposure also damaged the cell membrane of the seedlings making the membrane porous to electrolytes and depriving seedlings of essential electrolytes (Demidchik 2018). In such a situation, the isolate C18 has aided the soybean in producing substantial quantities of enzymatic antioxidants under HMs stress. Production of nonenzymatic (flavonoids and proline) and enzymatic antioxidant (i.e., AAO, CAT, POD, and DPPH) has scavenged the ROS, allowing the plant to grow normally under severe HMs toxic environment.

Apart from phytostimulation, the strain P. anthropi was also able to bio-transform the toxic heavy metals from their high toxic to least toxic and immobile form. The uptake of metal by the host was several folds. Cocultivation of seedlings with C18 interferes with the uptake capability of the host by reducing metal uptake by almost 50% (Padhan et al. 2021). This is possibly due to the reason that C18 might downregulate heavy metal ATPase genes (GmHMA13, GmHMA14, GmHMA19) and GmMATE1 compared to non-inoculated plants (Bilal et al. 2019). In the case of As, another process, often associated with detoxification, is arsenic methylation. Some bacteria can use As-(III) as a source of electrons for respiration. Current research provides evidence that P. anthropi mitigates chromate and arsenate stress through biotransformation and bio-reduction rendering them unavailable to root. The enhanced microbial colonization in roots stressed with high levels of heavy metal exposure has provided empirical evidence of symbiotic association (Qadir et al. 2020).

Bioremediation by reducing bacteria is a highly promising, cost-effective, and eco-friendly method and shows great potential for future use. The results here indicated that P. anthropi has a relatively high tolerance to Cr-(VI) and As-(V) and reduction rate. Our findings imply that P. anthropi have sufficient potential to detoxify Cr-(VI) and As-(V), especially in alkaline soils polluted by Cr and As. The natural ability of the microorganisms to produce phytohormones can be applied to reduce the adverse effects of stress and to improve the health and development of plants under different environmental conditions. Primary metal stress and secondary oxidative stress cause damage to the host plants by altering their biochemistry, however, P. anthropi minimize the toxic effects of chromium and arsenic.
in host plants by producing phytohormones and changing the exudates secretion. Therefore, such rhizobacteria may not only be used as biofertilizers, but also for the restoration of Cr and As in polluted areas.

**Author contributions** Husna performed the experimental work, AH and MS supervised the study, AH designed the project, MH and WM facilitated the study by discussing and giving valuable suggestions for the refinement of the study. AI finalized the MS, MI and HYK performed the statistical analysis, and MQ helped in methodology.

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**Availability of data and material** All the data generated, and material used during the current study are available.

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** All the co-authors have read and approved the final version of MS.

**References**

Ayangbenro AS, Babalola OO (2017) A new strategy for heavy metal polluted environments: a review of microbial biosorbents. Int J Environ Res Public Health 14:94

Bates LS, Waldren RP, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant soil 39:205–207

Bibi S, Hussain A, Hamayun M, Rahman H, Iqbal A, Shah M, Irshad M, Qasim M, Islam B (2018) Bioremediation of hexavalent chromium by endophytic fungi; safe and improved production of *Lactuca sativa* L. Chemosphere 211:653–663. https://doi.org/10.1016/j.chemosphere.2018.07.197

Bilal S, Shahzad R, Khan AL, Al-Harrasi A, Kim CK, Lee I-J (2019) Phytohormones enabled endophytic *Penicillium funiculosum* LHL06 protects *Glycine max* L. from synergistic toxicity of heavy metals by hormonal and stress-responsive proteins modulation. J Hazard Mater 379:120824

Bilal S, Shahzad R, Imran M, Jan R, Kim KM, Lee I-J (2020) Synergistic association of endophytic fungi enhances *Glycine max* L. resilience to combined abiotic stresses: heavy metals, high temperature and drought stress. Ind Crops Prod 143:111931

Bistgani ZE, Hashemi M, DaCosta M, Craker L, Maggi F, Morshedloo MR (2019) Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of *Thymus vulgaris* L. and *Thymus daenensis* Celak. Ind Crops Prod 135:311–320

Chandlee J, Scandalios J (1984) Analysis of variants affecting the catalase developmental program in maize scutellum. Theor Appl Genet 69:71–77

Chen QY, Murphy A, Sun H, Costa M (2019) Molecular and epigenetic mechanisms of Cr (VI)-induced carcinogenesis. Toxicol Appl Pharmacol 377:114636

Chen J, Zhu M, Liu R, Zhang M, Lv Y, Liu Y, Xiao X, Yuan J, Cai H (2020) BIOMASS YIELD 1 regulates sorghum biomass and grain yield via the shikimate pathway. J Exp Bot 71:5506–5520

Chun J, Lee H-J, Jung Y, Kim M, Kim S, Kim BK, Lim Y-W (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol MicroBiol 57:2259–2261

Cid CV, Pignata ML, Rodriguez JH (2020) Effects of co-cropping on soybean growth and stress response in lead-polluted soils. Chemosphere 246:125833

Conroy BS, Grigg JC, Kolesnikov M, Morales LD, Murphy ME (2019) *Staphylococcus aureus* heme and siderophore-iron acquisition pathways. Biometals 32:409–424

Coombe B, Cohen D, Paleg L (1967) Barley endosperm bioassay for gibberellins. I. Parameters of the response system. Plant Physiol 42:105–112

Cristaldi A, Conti GO, Jho EH, Zuccarello P, Grasso A, Copat C, Ferrante M (2017) Phyto remediation of contaminated soils by heavy metals and PAHs. A brief review. Environ Technol Innov 8:309–326

Demidchik V (2018) ROS-activated ion channels in plants: biochemical characteristics, physiological functions and molecular nature. Int J Mol Sci 19:1263

DesMarais TL, Costa M (2019) Mechanisms of chromium-induced toxicity. Curr Opin Toxicol 14:1–7

Dubois M, Gilles KA, Hamilton JK, Rebers P, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Edi-Premono M, Moawad A, Vlek P (1996) Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indonesian J Crop Sci 11:13–23

El-Din AZ, Abdalla, Bana N (2021) Soybean oil production line design project method. https://doi.org/10.13140/RG.2.2.13019.90409

El Far M, Taie HA (2009) Antioxidant activities, total anthocyanins, phenolics and flavonoids contents of some sweetpotato genotypes under stress of different concentrations of sucrose and sorbitol. Aust J Basic Appl Sci 3:3609–3616

Etesami H (2018) Bacterial mediated alleviation of heavy metal stress and decreased accumulation of metals in plant tissues: mechanisms and future prospects. Ecotoxicol Environ Saf 147:175–191

Flora SJ (2011) Arsenic-induced oxidative stress and its reversibility. Free Radic Biol Med 2:257–281

Forchetti G, Masiarelli O, Izaguirre MJ, Allemano S, Alvarez D, Abdala G (2010) Endophytic bacteria improve seedling growth and soybean yield via the shikimate pathway. J Exp Bot 71:5506–5520

Fu Y, Wang L, Peng W, Fan Q, Li Q, Dong Y, Liu Y, Boczkaj G, Wang Z (2021) Enabling simultaneous redox transformation of toxic chromium (VI) and arsenic (III) in aqueous media—a review. J Hazard Mater. https://doi.org/10.1016/j.jhazmat.2021.126041

Ghaffari H, Tadayon MR, Nadeem M, Cheema M, Razmjoo J (2019) Proline-mediated changes in antioxidant enzymatic activities and its survival in the rhizosphere. Indonesian J Crop Sci 11:13–23

Hamayun M, Hussain A, Khan SA, Kim H-Y, Khan AL, Waqas M, Irshad M, Iqbal A, Rehman G, Jan S (2017) Gibberellins producing endophytic fungus *Porostereum spadiceum* AGH786 rescues growth of salt affected soybean. Front Microbiol 8:686
Hamayun M, Khan N, Khan MN, Qadir M, Hussain A, Iqbal A, Khan SA, Rehman KU, Lee I-J (2021) Antimicrobial and plant growth-promoting activities of bacterial endophytes isolated from Calotropis procera (Ait.) WT Aiton. Biocell 45:363–369
Hao W-y, Ren L-x, Ran W, Shen Q-r (2015) Allelopathic effects of root exudates from watermelon and rice plants on Fusarium oxysporum f. sp. niveum. Plant Soil 336:485–497
Ismail AH, Qadir M, Husna MI, Ahmad A, Hamayun M (2018) Endophytic fungi isolated from Citrullus colocynthis. Leaves and Their potential for secretion of indole acetic acid and gibberellin. J Appl Environ Biol Sci 8:80–84
Ismael HA, Mehmood A, Qadir M, Husna, Iqbal A, Hamayun M, Khan N (2020) Thermal stress alleviating potential of endophytic fungus rhizopus oryzae inoculated to sunflower (Helianthus annuus L.) and soybean (Glycine max L.). Pak J Bot 52:1857–1865
Ismail, Hamayun M, Anwar H, Sumera Afzal K, Amjad I, In-Jung L (2020) An endophytic fungus Aspergillus violaceofuscus can be used as heat stress adaptive tool for Glycine max L. and Helianthus annuus L. J Appl Bot Food Qual 93:112–120
Jiang B, Gong Y, Gao J, Sun T, Liu Y, Oturan N, Oturan MA (2018) Insights into the chemistry of non-enzymatic browning reactions in different ribose-amine acid model systems. Sci Rep 8:1–10
Hudson AT, Bentley R (1970) Utilization of shikimic acid for the for-
Hao W-y, Ren L-x, Ran W, Shen Q-r (2010) Allelopathic effects of root exudates from watermelon and rice plants on Fusarium oxysporum f. sp. niveum. Plant Soil 336:485–497
Hasanuzzaman M, Nahar K, Hakeem K, Ozturk M, Fujita M (2015) Arsenic toxicity in plants and possible remediation. Soil Remediation and Plants: Prospects and Challenges: Academic Press, pp 433–501
Hayat R, Khalid R, Ehsan M, Ahmed I, Yokota A, Ali S (2013) Molec-
Hao W-y, Ren L-x, Ran W, Shen Q-r (2010) Allelopathic effects of root exudates from watermelon and rice plants on Fusarium oxysporum f. sp. niveum. Plant Soil 336:485–497
Hasanuzzaman M, Nahar K, Hakeem K, Ozturk M, Fujita M (2015) Arsenic toxicity in plants and possible remediation. Soil Remediation and Plants: Prospects and Challenges: Academic Press, pp 433–501
Hayat R, Khalid R, Ehsan M, Ahmed I, Yokota A, Ali S (2013) Molec-
Kapoor D, Singh S, Kumar V, Romero R, Prasad R, Singh J (2019) Antioxidant enzymes regulation in plants in reference to reactive oxygen species (ROS) and reactive nitrogen species (RNS). Plant Gene 19:100182
Kashyap L, Garg N (2018) Arsenic toxicity in crop plants: responses and remediation strategies. Mechanisms of arsenic toxicity and tolerance in plants. Springer, New York
Kazi T, Jamali M, Kazi G, Arain M, Afridi H, Siddiqui A (2005) Evaluating the mobility of toxic metals in untreated industrial wastewater sludge using a BCR sequential extraction procedure and a leaching test. Analy Biosanal Chem 383:297–304
Khoshrou B, Mitra D, Khoshmanzar E, Myo EM, Uniyal N, Mahakur B, Mohapatra PKD, Panneerselvam P, Boutaj H, Alizadeh M (2020) Current scenario and future prospects of plant growth-promoting rhizobacteria: An economic valuable resource for the agriculture renewal under stressful conditions. J Plant Nutr 43:3062–3092
Lee YH, Choo C, Watawana MI, Jayawardena N, Waisundara VY (2015) An appraisal of eighteen commonly consumed edible plants as functional food based on their antioxidant and starch hydrolyase inhibitory activities. J Sci Food Agric 95:2936–2964
Liang J, Huang X, Yan J, Li Y, Zhao Z, Liu Y, Ye J, Wei Y (2021) A review of the formation of Cr (VI) via Cr (III) oxidation in soils and groundwater. Sci Total Environ. https://doi.org/10.1016/j.scitotenv.2021.145762
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
Machado RA, Theepvan P, Robert CA, Züst T, Hu L, Su Q, Schimmel BC, Erb M (2021) The plant metabolome guides fitness-relevant foraging decisions of a specialist herbivore. PLoS Biol 19:e3001114
Mala JGSM, Takeuchi S, Sujatha D, Mani U (2020) Microbial chro-
Marshall BJ, Ralledge C (1972) Salicylic acid biosynthesis and its control in Mycobacterium smegmatis. Biochimica et Biophysica Acta 264:106–116
Meena VS, Meena SK, Verma JP, Kumar A, Aeron A, Mishra PK, Bishh JK, Pattanayak A, Naveed M, Dotaniya M (2017) Plant benefi-
cial rhizospheric microorganisms (PBRM) strategies to improve nutrients use efficiency: a review. Ecol Eng 107:8–32
Meng G, Tian Y, Yang Y, Shi J (2016) Evaluation of DPPH free radical scavenging activity of various extracts of Ligularia fischeri in vitro: a case study of Shaanxi region. Indian J Pharm Sci 78:436–442
Mirza N, Mubarak H, Chai L-Y, Yang Z-H, Mahmood Q, Yong W, Wang C-J, Fahad S, Nasim W (2017) Constitutional tolerance and chlorophyll fluorescence of Boehmeria nivea L in response to the antimody (Sb) and arsenic (As) co-contamination. Toxicol Environ Chem 99:265–272
Mukhopadhyay R, Rosen BP, Phung LT, Silver S (2002) Microbial arsenic: from geocycles to genes and enzymes. FEMS Microbiol Rev 26:311–325
Oberbacher M, Vines H (1963) Spectrophotometric assay of ascorbic acid oxidase. Nature 197:1203–1204
Padhan D, Rout PP, Kundu R, Adhikary S, Padhi PP (2021) Biore-
media in heavy metals and other toxic substances by micro-
organisms. Soil Bioremediat: Approach Towards Sustain Technol. https://doi.org/10.1002/9788119547976.ch12
Pandey N, Chandrakar V, Keshavkant S (2018) Mitigating arsenic tox-
icity in plants: role of microbiota. Mechanisms of arsenic toxicity and tolerance in plants. Springer, Singapore
Pisoschi AM, Pop A, Iordache F, Stanca L, Predoi G, Serban AI (2020) The impact of increased flooding occurrence on the mobility of potentially toxic elements in floodplain soil—a review. HU. Academic Press, New York
Putter J (1974) Peroxidase. In: Bergmeyer (ed) Methods of enzymatic analysis. HU. Academic Press, New York
Qadir M, Hussain A, Hamayun M, Shah M, Iqbal A, Husna, Murad W (2020) Phytohormones producing rhizobacterium alleviates chromium toxicity in Helianthus annuus L. by reducing chrome
uptake and strengthening antioxidant system. Chemosphere 258:127386. https://doi.org/10.1016/j.chemosphere.2020.127386

Rahman Z, Singh VP (2019) The relative impact of toxic heavy metals (THMs)(arsenic (As), cadmium (Cd), chromium (Cr)(VI), mercury (Hg), and lead (Pb)) on the total environment: an overview. Environ Monit Assess 191:1–21

Sarker U, Oba S (2018) Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected Amaranthus tricolor under s alinity stress. Sci Rep 8:1–9

Scharte J, SCHÖN H, Weis E (2005) Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with Phytophthora nicotianae. Plant Cell Environ 28:1421–1435

Shafiq F, Iqbal M, Ali M, Ashraf MA (2021) Fullerol regulates oxidative stress and tissue ionic homeostasis in spring wheat to improve net-primary productivity under salt-stress. Ecotoxicol Environ Saf 211:111901

Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng B (2019) Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules 24:2452

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thijs S, Langill T, Vangronsveld J (2017) The bacterial and fungal microbiota of hyperaccumulator plants: small organisms, large influence. Adv Bot Res 83:43–86

Tiwari S, Gupta SC, Chauhan PS, Lata C (2021) An OsNAM gene plays important role in root rhizobacteria interaction in transgenic Arabidopsis through abiotic stress and phytohormone crosstalk. Plant Cell Rep 40:143–155

Tsavkelova EA, Cherdyntseva TA, Klimova SY, Shestakov AI, Botina SG, Netrusov AI (2007) Orchid-associated bacteria produce indole-3-acetic acid, promote seed germination, and increase their microbial yield in response to exogenous auxin. Arch Microbiol 188:655–664

Valenzuela-García LI, Zapata BL, Ramírez-Ramírez N, Huchín-Mian JP, Robleto EA, Ayala-García VM, Pedraza-Reyes M (2020) Novel biochemical properties and physiological role of the flavin mononucleotide oxidoreductase YhdA from Bacillus subtilis. Appl Environ Microbiol 86:e01688–e01620

Vives-Peris V, Gómez-Cadenas A, Pérez-Clemente RM (2017) Citrus plants exude proline and phytohormones under abiotic stress conditions. Plant cell Rep 36:1971–1984

Wang Y, Su H, Gu Y, Song X, Zhao J (2017) Carcinogenicity of chromium and chemoprevention: a brief update. OncoTargets Ther 10:4065

Warrier R, Paul M, Vineetha M (2013) Estimation of salicylic acid in Eucalyptus leaves using spectrophotometric methods. Genet Plant Physiol 3:90–97

Wuana RA, Okieimen FE (2011) Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. International Scholarly Research Notices 2011

Xiao CW (2008) Health effects of soy protein and isoflavones in humans. J Nutr 138:1244S–1249S

Xiong X, Liu X, Iris K, Wang L, Zhou J, Sun X, Rinklebe J, Shaheen SM, Ok YS, Lin Z (2019) Potentially toxic elements in solid waste streams: fate and management approaches. Environ pollut 253:680–707

Yang Z, Zhang X, Li Q, Huang P, Zheng C, Liao Q, Yang W (2021) Reductive materials for remediation of hexavalent chromium contaminated soil—a review. Sci Total Environ. https://doi.org/10.1016/j.scitotenv.2021.145654

Zahoor M, Irshad M, Rahman H, Qasim M, Afridi SG, Qadir M, Hussain A (2017) Alleviation of heavy metal toxicity and phytostimulation of Brassica campestris L. by endophytic Mucor sp. MHR-7. Ecotoxicol Environ Saf 142:139–149

Zaid A, Mohammad F, Wani SH, Siddique KM (2019) Salicylic acid enhances nickel stress tolerance by up-regulating antioxidant defense and glyoxalase systems in mustard plants. Ecotoxicol Environ Saf 180:575–587

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