Pseudocitrobacter Anthropi Reduces Heavy Metal Uptake and Improves Phytohormones and Antioxidant System in Glycine Max L.

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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 HMs while improving plants' growth is the call of the day. In this study, exposing soybean plants to different concentrations (i.e., 10 and 50 µg/mL) of chromium and arsenic showed a severe reduction in agronomic attributes, higher ROS 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., CAT, APX, POD, and DPPH), higher ROS scavenging, and lower ROS accumulation. Thereby, lowering secondary oxidative stress and subsequent damage. The strain was identified using 16S rDNA sequencing, and was identified as *Pseudocitrobacter anthropi*. Additionally, the strain can endure metals up to 1200 µg/mL and efficient in detoxifying the effect of Cr and As, 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.

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 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 (Gupta and Rastogi 2009; Zhu et al. 2014). Hexavalent chromium is toxic, mutagenic and carcinogenic, exposure to Cr-(VI) increases the risk of lung cancer whereas trivalent chromium is much less toxic (Cheung and Gu 2003; Costa 2003; Viti et al. 2003). In addition to Cr-(VI) toxicity, it is difficult to be absorbed by soil particles and easily enters surface water or groundwater. 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 a low solubility and act as nutrient for various microbes and organisms (Bielicka et al. 2005; Dhal et al. 2013; Xu et al. 2015). On the other hand, arsenic is generally toxic to all lifeforms. 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 in nature and can induce oxidative damage in biological systems (Achour et al. 2007). They are analogs of sulphate and phosphate respectively and therefore, can actively be up taken and accumulated in cells beyond threshold levels, disrupting important physicochemical processes (Shrestha et al. 2008). Bacteria play key role in Cr and As speciation by converting Cr-(VI) to Cr-(III) and As-(V) to As-(III) are of environmental significance due to the formation of uncharged state which has higher mobility than arsenate.

In turn, plants have several detoxification mechanisms to avoid phytotoxicity of these metals. They include phytostabilization 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 induce physiochemical changes, such as impaired water balance, wilting and chlorosis of leaves, and damage to the growth of shoots and roots (Wyszkowski and Radziemska 2010). Metal stress produces reactive oxygen species (ROS) and alters the balance and assimilation of nutrients, metabolism of protein and oxidative phosphorylation in plant tissues (Mittler 2006; Suzuki et al. 2013). HMs also affect the uptake of water, inhibit photosynthesis, and causes lipid peroxidation by altering the cell membrane lipid structure (Flora 2011; Hasanuzzaman et al. 2015; Mirza et al. 2017). To overcome the stress beyond threshold level of the host plants, researchers aim microbial assisted remediation. It is a new, yet novel potential technique to remediate the metal by changing their 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 host's endogenous pool of phytohormones, thus promoting their tolerance toward HMs stresses (Zahoor et al. 2017).

Soybean (*Glycine max* L.) is one of the economically valuable crops and important source of food, protein, and oils in the world. Soybean is one of the cheapest and richest sources of protein and edible oil. It's good for human health and reduces the risk of various health problems like cardiovascular disease, certain cancers, and low sugar blood level. Due to increasing demand and heavy metals pollution the yield and productivity of soybean crops is greatly affected. Therefore, the present work aims to i) isolate chromate and arsenate reducing bacteria from rhizosphere of *Chlorophytum comosum* ii) role of the isolated rhizobacteria in soybean growth promotion and improving plant antioxidant system under metal stress ii) 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 media. After incubation for 24 hours at 28 °C, individual colonies were selected and purified for further studies.

Molecular identification of selected strains

The potent rhizobacterial isolate was subjected to phylogenetic analysis to specie level by standard method of 16S rDNA gene sequencing. The DNA templates were prepared from the selected strain by picking individual colony and amplified by 16S rDNA gene using modern PCR techniques. 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).

Minimum inhibitory concentration

For assessing the ability of potent isolate to tolerate different levels of selected HMs, the isolate was grown in LB broth media having various concentrations (100, 300, 500, 900 and 1200 µg/mL) of chromium and arsenic in the form of potassium chromate and sodium arsenate. Inoculations were made in 50 mL of L.B broth, which was then incubated at 28 °C in the shaking incubator for 24 hours at 150 rpm. After 24 hours, bacterial growth was recorded using a PerkinElmer Lambda 25 double beam spectrophotometer at 600 nm.

Metabolic profiling of culture supernatant

The culture supernatant of the potent isolate was obtained by filtration and centrifuging the LB medium at 4000 rpm for 20 mins. The bacterial culture supernatant was then collected and analyzed for various phytohormones and secondary metabolites.

Determination phytohormones

Salkowski reagent method was carried to estimate the exogenous indole-acetic acid (IAA) in the culture supernatant of the isolate using colorimetric method of (Tsavkelova et al. 2007).

To determine gibberellic acid (GA), 10 healthy embryo-less wheat seeds were sterilized by dipping in ethanol (70%) for 30 seconds. 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 hours. After the incubation, the solution was filtered, and the pH was adjusted to 2.5 with HCl (2N) and the filtered solution was isolated with $NaHCO_3$/ethyl acetate (1:1 v/v). GA was measured with spectrophotometer in the ethyl acetate phase at 254 nm (Cho et al. 1979).

Exogenous salicylic acid (SA) content were estimated using standard protocol (Warrier et al. 2013). To 100 µL of chilled culture supernatant, added freshly prepared ferric chloride (0.1%) and the final volume was adjusted to 3 mL with ddH_2O. The solution mixture turned violet due to formation of complex between Fe^{3+} ions and SA. This change in color to violet was measured spectrophotometrically at 540 nm.

Determination of plant metabolites

Total phenol content

The total phenolic content in samples were determined using Folin-Ciocalteau reagent method slightly modified by (Lee et al. 2015). Colored complex developed in the reaction mixture was quantified by taking

OD at 650 nm, through a spectrophotometer. Total phenol contents were expressed as µg/mL.

Proline

Proline content in samples were determined using acid ninhydrin (Bates et al. 1973). Reaction mixtures consist of equal volume of sample and acid ninhydrin and boiled for 60 minutes 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 was measured at 520 nm.

Total protein content

Total protein estimation was carried out by following (Nićiforović et al. 2010) method with slight modification. Protein extract was prepared by grinding 100 mg of plant material in phosphate buffer (1 mL, pH 7.5). Extract (100 µL) was diluted with distilled water to make 1 mL solution. To the diluted extract, 1 mL of copper reagent (2% sodium carbonate, 0.1N of sodium hydroxide, 1% copper sulfate and 2% sodium potassium tartrate) was added and thoroughly stirred for 10 minutes. Diluted Folin-ciocalteau reagent (100 µL) was added and incubate the mixture in an incubator at room temperature for 30 minutes. The absorbance was read at 650 nm using a spectrophotometer.

Total flavonoids content

Total flavonoid content (TFC) was determined by well-established method (El Far and Taie 2009). The reaction mixtures consisted of 0.5 mL of 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 minutes. The absorbance was measured at 415nm.

Total soluble sugar

Soluble sugar content was determined by slightly modifying phenol-sulfuric acid procedure of (Mohammadkhani and Heidari 2008). To 100 µL of supernatant add 1 mL of 80% phenol and incubated for 10 minutes at room temperature. After incubation, 5 mL of concentrated H_2SO_4 was added to the mixture and the mixture was incubated for 1 hour at room temperature. The absorbance was read at 485 nm using spectrophotometer. The total sugar contents were expressed as mg g^{-1}.

Phosphate solubilization index

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

$$SI = \frac{colony\ diameter\ (cm) + holozone\ diameter\ (cm)}{colony\ diamtere\ (cm)}$$

Hydroponic experiment under heavy metals stress

Soybean (*Glycine max*) seeds were obtained from Agricultural Research Institute Mingora, Swat. Soybean seeds were surface disinfected, using 0.1% mercury chloride ($HgCl_2$), followed by washing three times with double distilled water. Seeds were grown in sterilized sand and seedlings under two fully expanded leaf conditions were shifted to pots containing half strength of Hoagland's solution. The experimental setup consisted of 36 pots with two chromate and arsenic levels, low level (10 µg/mL) and high level (50 µg/mL) along with control (without any treatment). Replicates of each treatment were set in randomized complete block design. Inoculation was made to assess the alleviation potential of the isolate in pot conditions. The pots were transferred to LabTech growth chamber and kept under 25 ^oC temperature, 68% humidity and 13 hours of photoperiod for 14 days. After 14 days, seedlings were harvested, and parameters described below were recorded.

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 minutes at 10000 rpm and the supernatant was collected as enzyme extract. The enzyme extract was added to a 3 mL of reaction mixture. The reaction mixture consists of 2.6 mL of 50 mM potassium phosphate buffer at pH 7.0 and 0.4 mL of 15 mM H_2O_2. The decrease in the absorbance of H_2O_2 (µmol/min) was estimated by 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 H_2O_2 (0.1 mM). The optical density was recorded at 290 nm for every 30 seconds for a period of 5 minutes 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 minutes at 4 °C and 14000 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 H_2O_2 (12.3 mM) and 50 µL

guaiacol solution (20 mM). The POX activity was determined spectrophotometrically by using guaiacol as the substrate. The mixture optical density was recorded at 436 nm and the activity was calculated using the following equation:

$$Enzyme\ activity = (\frac{500}{\Delta t}) \times (\frac{1}{1000}) \times (\frac{TV}{VU}) \times (\frac{1}{f\ wt})$$

Where in equation.

Δt = time change in minute

TV = total volume of the extract (mL)

VU = volume used (mL); f wt = weight of the fresh leaf tissue (g)

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 minutes and record the absorbance at 517 nm using the following equation as a percentage of free radical scavenging acidity:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [\frac{(1 - A1)}{A0}] \times 100$$

where in equation, A1 is the absorbance of the extract, and A0 is the absorbance of the blank (DPPH solution without extract).

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 minutes. Boiling decolorized the leaves, except the brown colored spots produced by DAB. After cooling, the tissues were observed under light microscope to visualize the brown spots.

Determination of Cr and As by BCR extraction

Reduction of chromate and arsenate in bacterial culture supernatant and soybean plants were determined using atomic absorption spectroscopy (Perkin Elmer AAnalyst 700). Prior to analysis metal with lower and higher oxidation states were extracted separately by using modified BCR (Community

Bureau of References) sequential extraction procedure (Kazi et al. 2005). Steps of BCR are listed as under.

Step 1: In these step acid soluble and exchangeable components were extracted. To, 0.5 g of air-dried samples, 20 mL of 0.11 M acetic acid (CH_3COOH) was added and shaken for 12-24 hours at 25-30 ^oC in an orbital shaker. This fraction was separated from the matrix through centrifugation (3000 rpm, 15 minutes).

Step 2: In this step, fraction consisted of reducible metal ions were separated. To the residue from step 1, 20 mL of 0.5 M hydroxylamine-hydrochloride ($NH_2OH–HCl$) at pH 1.5 with nitric acid (HNO_3) was added. The samples were stirred for 16 hours at 30 ^oC. This fraction was separated from the matrix through centrifugation (3000 rpm, 15 minutes).

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 (H_2O_2). The mixture was stirred for 1 hour at room temperature. This step was repeated twice. The sample was then heated at 60 ºC to evaporate excess of solvents and treated with 25 mL of 1 M ammonium acetate (CH_3COONH_4). The resulting mixture was stirred for 16 hours. This fraction was separated from the matrix through centrifugation (3000 rpm, 5 minutes). 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 CH_3COOH		12-24 hours at 25-30 °C
2	Reducible	20 mL 0.5 M $NH_2OH–HCl$	1.5	16 hours at 25-30 °C.
3	Oxidizable	5 mL of 30% H_2O_2	2.0	1 hour at 25-30 °C
		Then, 5 mL of 30% H_2O_2	2.0	1 hour at 85 °C
		25 mL of 1 M CH_3COONH_4	2.0	16 hours 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-1. 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:

$$BCF = \frac{Metal\ accumulated\ in\ biomass}{Metal\ add\ to\ media}$$

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 µg/mL of Cr (as K$_2$CrO$_4$) and As (as Na_3AsO_4). Among them, 9 strains were capable to grow in the presence of Cr and As stress upto 1200 µg/mL (Table 1). The isolates that were capable to withstand the Cr-(VI) and As-(V) was selected for further study for plant growth promotion assay.

Table 1
Screening for chromium and arsenic stress tolerance in rhizobacteria

	Cr-(VI)/As-(V) stress					
	Control	100 µg/mL	300 µg/mL	500 µg/mL	900 µg/mL	1200 µg/mL
C1	+	-/+	-/-	-/-	-/-	-/-
C2	+	-/-	-/-	-/-	-/-	-/-
C3	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C4	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C5	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C6	+	-/-	-/-	-/-	-/-	-/-
C7	+	-/-	-/-	-/-	-/-	-/-
C8	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C9	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C10	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C11	+	-/-	-/-	-/-	-/-	-/-
C12	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C13	+	-/-	-/-	-/-	-/-	-/-
C14	+	-/-	-/-	-/-	-/-	-/-
C15	+	-/-	-/-	-/-	-/-	-/-
C16	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
C17	+	-/-	-/-	-/-	-/-	-/-
C18	**+**	**+/+**	**+/+**	**+/+**	**+/+**	**+/+**
(+ Rhizobacteria growth) (- No Rhizobacteria growth)						

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. 1). Based on its performance in soybean growth assay, the isolate C18 was selected for further analysis.

Molecular identification of rhizobacterial isolate C18

Based on the sequence of 16S rDNA, the isolate C18 showed maximum homology (98.01%) with *Pseudocitrobacter anthropi*. For the confirmation of strain identity, sequence of C18 was subjected to phylogenetic analysis. 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.

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 upto 500 µg/mL in the presence of chromate supplements. However, decline phase was recorded in the growth beyond 500 µg/mL in chromate supplementation. Nonetheless, an opposite trend was recorded in case of arsenic supplementation. Arsenic treated bacterial cells tends to decrease with increasing arsenic concentration compared to untreated bacterial cells (Fig 3).

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

Isolate C18 is tolerant to high concentration of Cr-(VI) and As-(V) and have the potential to reduce the concentration of chromium and arsenic in nutrient broth media containing utpo 1200 µg/mL (Fig. 4). In order 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 *P. anthropi* was able to reduce 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 µg/mL, while the level of hexavalent chromium (Cr-VI) was steadily reduced.

Metabolic profiling of *P. anthropi*

Response of the selected rhizobacterial strain *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.

Phytohormones

The ability of rhizobacteria *P. anthropi* (C18) to produce IAA under different concentrations of chromate and arsenate were determined. IAA quantity was increased with increasing levels of metal stress in bacterial culture supernatant. In particular, at 1200 µg/mL isolate produced the highest amount of IAA (59.02 µg/mL), lowest amount was recorded in control (25.91 µg/mL) without any stress (Fig. 5A). 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 500µg/mL. However, higher concentrations of the selected heavy metals in the media significantly reduced the amount of GA, in comparison to control (Fig. 5B). 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 control without any stress. Increasing level of stress showed decline in SA quantity till 900µg/mL. Interestingly, higher concentration (1200 µg/mL) was least toxic for *P. anthropi* to release SA (5C).

Secondary metabolites

Rhizobacteria *P. anthropi* (C18) in the presence of different metal concentrations (0, 100, 200, 300, 500, 900 and 1200 µg/mL) produced flavonoids in LB medium. The content of flavonoids decreases steadily with the gradual increase of Cr-(VI) and As-(V) level in the medium (Fig. 5D). Strain results revealed higher amount (103.43 µg/mL) of flavonoids at 500 µg/mL compared to normal culture media, the content of TFC content is lowest at (57.02µg/mL) at 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. 5E). The phenolic content level was significantly decreased to 823.15 µg/mL in treated culture compared with 1046.15 µg/mL in control untreated. The production of total protein contents was also measured when the strain was exposed to different concentration of the heavy metal (Fig. 5F). 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 µg/mL. The production of proline contents was determined upon exposure of the isolated strain to the selected heavy metal (Fig. 5G). The proline contents showed a slight increase till 300 µg/mL and subsequent decrease in the elevated levels of the metals. The exogenous soluble sugar contents of the *P. anthropi* were also estimated under Cr and As stress conditions (Fig. 3H). The production of endogenous sugar contents was highly influenced by the 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 µg/mL 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. 6). The strain showed a larger halo area in Pikovskaya agar with an average diameter of 26 mm.

Growth parameters

Exposure to aforementioned concentration of chromate and arsenate reduced the host seedling growth by almost by 50% and 49% as compared to the untreated seedlings (Fig. 7A). Consequently, inoculation of *P. anthropi* improves the host seedling growth by 68% and similar trend was recorded in metal treated seedlings as well showing no significant decrease in their shoot length. Contrastingly, 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, major part of which (77.51%) were stored endogenously (Fig. 8A). Exposure to selected levels of metal have an influential impact on the endogenous and exogenous production of IAA. A significant increase in endogenous and decrease in exogenous IAA contents were recorded upon exposure to 10 and 50 µg/mL of selected metal stress. Interestingly, inoculating seedlings with *P. anthropi*, the endogenous levels show a dose dependent decrease in the total IAA contents with the exception of 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 concentration 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 µg/mL however, followed by an abrupt increase in case of chromate supplementation. An opposite trend was recorded in 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, 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.

Metabolites profiling

Soybean seedlings treated with mentioned concentration of chromate and arsenate triggers the host to synthesize higher quantities of total flavonoid contents. Heavy metal stress greatly influences the total flavonoids contents by higher endogenous accumulation of flavonoids however, decline was recorded in the exogenous flavonoid contents (Fig. 8E). Co-cultivating the host seedlings with *P. anthropi*, lower flavonoids accumulation and higher release was 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 µg/mL 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 µg/mL of selected heavy metals (Fig. 8G). Cocultivation of *P. anthropi* with soybean seedling, no significant improvement were 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 µg/mL. An increasing trend was recorded in the proline contents of 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 concentration of both metals. Cocultivation of host seedlings with *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 *P. anthropi* and 50 µg/mL of arsenate. Significant decrease was noted in the endogenous and roots exuded soluble sugar contents was when exposed to 10µg/mL followed by an increase at 50 µg/mL of chromate and arsenate induced stress (Fig. 8K). Inoculating *P. anthropi,* an increase was recorded at 10 µg/mL in production of endogenous and root exuded soluble sugar contents however, decline was recorded at 50 µg/mL. Similar trend was also recorded in the total protein contents of the host treated with mentioned concentration of the selected metals (Fig. 8M). Supplementing medium with chromate and arsenate, 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 *P. anthropi,* no substantial increase was recorded in the endogenous and exogenous protein content however, increase was recorded in the exogenous protein contents of the plants treated with 50 µg/mL of chromate and arsenate.

Response of rhizobacteria inoculated soybean antioxidant enzymes machinery to metal stress

Modulation in the activity of the antioxidant enzymes including CAT, AAO, 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. CAT enzyme level drastically increased with increasing concentration Cr and As. *P. anthropi* inoculated soybean seedlings showed slight decline in CAT activity (Fig. 9A). 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 than that in the control and bacteria inoculated. However, with increasing chromium and arsenic concentration peroxidase activity showed reduction. Plants inoculated with *P. anthropi* isolate showed lowest amount of peroxidases under control conditions, whereas Cr stressed showed an increase in peroxidase activity. An opposite trend was observed in soybean seedlings exposed to As stress (Fig. 9C). DPPH-Radical scavenging activity in the plants got reduced upon exposure to Cr and As stress. However, inoculation of *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). Present study also showed an increased level of H_2O_2 with increasing HMs dose, i.e. from 10 µg/mL to 50 µg/mL. Significant amount of H_2O_2 were accumulated in the leaves of soybean plants when exposed to chromate and arsenate stress. Heavy metal are free radical generators and our study showed higher rate of ROS formation under metal stress compared to control and rhizobacterial inoculation. With the inoculation of *P. anthropi* isolate, the chromate stress was alleviated, hence reducing the H_2O_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 nontoxic form in the surrounding medium was influenced by the plants. As the concentration of the metal in 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 seedling whereas, their biotransformation increases in the host tissue to avoid phytotoxicity. On the other hand, lower arsenic uptake and higher biotransformation was recorded at 10 µg/mL however, an opposite trend was followed at 50 µg/mL showing higher accumulation and biotransformation. Bioconcentration factor of the soybean seedlings treated with different concentration of Cr and As were recorded (Fig. 11B). The bioconcentration increased at 10 µg/mL, whereas declined at 50 µg/mL of the Cr. In contrast, 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 µg/mL and low at 50 µg/mL. In case of As, higher bioaccumulation was recorded at both concentration of As in *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 led to loss in crop yield and effect 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 *Chlorophytum comosum* and were assessed for detoxification effects against chromium and arsenic while improving host plant growth. *P. anthropi* was able to tolerate Cr-(VI) and As-(V) up to 1200 µg/mL. 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 bio-transform 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-García et al. 2020). These enzymes help the bacterium to bio-transform the metal outside of the cell thus avoiding their toxicity. In case of arsenic, bacteria use ArsC-based resistance determinants, whereas, ArsB efflux proteins for protection of 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 for respiration and the most common detoxification mechanism is based on blocking the membrane channels through which toxic substances enter the cell. 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 hours 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 carbohydrate in the culture medium (Ismail et al. 2020b; Ismaila et al. 2018). 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 were observed as an 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 induce microbes to release 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).

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. 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). Increase was recorded in the protein production whereas, 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 condition the bacterial cell produce huge quantities of stress related protein including chaperons, heat shock protein, metalloprotein to cop the heavy metal stress. On the other hand, 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 building block and other used as an energy source to drive the machinery for the production of stress related protein. Among other microbial strategies to cop 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 and safety 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 reduce the growth attributes of productivity and stress related metabolites making the host more susceptible to damage of biotic and abiotic stress factors. Currently, treating *Glycine max* L. with mentioned levels of chromate and arsenate had profound negative impact on the agronomic attribute of the host. Cocultivation of 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). Plant responds at multilevels to counter the stress and these responses are regulated by phytohormones. Hence, higher production of phytohormones by HM stressed soybean seedlings is required to cope with high chromium and arsenic concentration in growth medium. In current scenario, C18 associated seedling exude higher quantities of IAA and SA, aiding the host in attracting microbial partners in the rhizosphere for the establishment of 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 the flavonoids act as nonenzymatic antioxidant and metal quencher. Thus, helping the host to boost antioxidant system by higher accumulation of flavonoid in their tissue while detoxify the metals by chelating them (Pisoschi et al. 2020). An opposite trend was recorded in the production of phenolic contents of the host showing 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 acts as osmolytes, chemical chaperones, direct ROS scavenger 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 plants 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 weigh ($p < 0.05$). C18 had the ability to 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 host to support its protection mechanisms (Sarker and Oba 2018). In plants, oxidative stress can be identified by abnormal activity of antioxidant enzymes under stress situations. As a result of metal stress, excessive ROS generated and seedlings were unable to handle them. Hence, damage was done in seedlings which was 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. Consequences of this was inability of soybean seedlings to scavenge ROS and avoid lipid peroxidation as evident by low amount of lipids in such seedlings. Stressed seedlings were unable to produce optimum amount of IAA, phenol and sugars, which was also an indication of the internal damage to the seedlings. Metal exposure also damaged cell membrane of the seedlings making the membrane porous to electrolytes and depriving seedlings of essential electrolytes (Demidchik 2018). In such 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 host was several folds. Cocultivation of seedlings with C18 interfere 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 case of As, another process, often associated with

detoxification, is arsenic methylation. Some bacteria are able to use As-(III) as a source of electrons for respiration. Current research provides evidence that *P. anthropi* mitigate 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).

Conclusion

Bioremediation by reducing bacteria is highly promising, cost effective and eco-friendly method and shows great potential for future use. The results here indicated that *P. anthropi* has 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 restoration of Cr and As in polluted areas.

Declarations

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Conflicts of interest/Competing interests: We have no competing interests

Availability of data and material: All the data generated and material used during current study are available

Code availability: Not applicable

Authors' contributions: Husna performed the experimental work, AH and MS supervised the study, AH designed the project, MH and WM facilitated the study by discussion and giving valuable suggestions for the refinement of the study. AI finalized the MS, MI and HYK performed statistical analysis and MQ help in methodology.

Ethics 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

1. Achour AR, Bauda P, Billard P (2007) Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. Research in microbiology 158(2):128-137
2. Ayangbenro AS, Babalola OO (2017) A new strategy for heavy metal polluted environments: a review of microbial biosorbents. International journal of environmental research and public health 14(1):94
3. Bates LS, Waldren RP, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant and soil 39(1):205-207
4. 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 doi:10.1016/j.chemosphere.2018.07.197
5. Bielicka A, Bojanowska I, Wisniewski A (2005) Two Faces of Chromium-Pollutant and Bioelement. Polish Journal of Environmental Studies 14(1)
6. Bilal S, Shahzad R, Khan AL, Al-Harrasi A, Kim CK, Lee I-JJJohm (2019) Phytohormones enabled endophytic Penicillium funiculosum LHL06 protects Glycine max L. from synergistic toxicity of heavy metals by hormonal and stress-responsive proteins modulation. 379:120824
7. Bistgani ZE, Hashemi M, DaCosta M, Craker L, Maggi F, Morshedloo MRJIC, Products (2019) Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of Thymus vulgaris L. and Thymus daenensis Celak. 135:311-320
8. Chandlee J, Scandalios J (1984) Analysis of variants affecting the catalase developmental program in maize scutellum. Theoretical and applied genetics 69(1):71-77
9. Chen J, Zhu M, Liu R, Zhang M, Lv Y, Liu Y, Xiao X, Yuan J, Cai HJJoeb (2020) BIOMASS YIELD 1 regulates sorghum biomass and grain yield via the shikimate pathway. 71(18):5506-5520
10. Cheung KH, Gu J-D (2003) Reduction of chromate (CrO42−) by an enrichment consortium and an isolate of marine sulfate-reducing bacteria. Chemosphere 52(9):1523-1529
11. Cho KY, Sakurai A, Kamiya Y, Takahashi N, Tamura S (1979) Effects of the new plant growth retardants of quaternary ammonium iodides on gibberellin biosynthesis in Gibberella fujikuroi. Plant and Cell Physiology 20(1):75-81
12. Chun J, Lee J-H, 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. International journal of systematic and evolutionary microbiology 57(10):2259-2261
13. Conroy BS, Grigg JC, Kolesnikov M, Morales LD, Murphy MEJB (2019) Staphylococcus aureus heme and siderophore-iron acquisition pathways. 32(3):409-424
14. Costa M (2003) Potential hazards of hexavalent chromate in our drinking water. Toxicology and applied pharmacology 188(1):1-5
15. Demidchik VJIjoms (2018) ROS-activated ion channels in plants: biophysical characteristics, physiological functions and molecular nature. 19(4):1263
16. Dhal B, Thatoi H, Das N, Pandey B (2013) Chemical and microbial remediation of hexavalent chromium from contaminated soil and mining/metallurgical solid waste: a review. Journal of

hazardous materials 250:272-291

17. 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. Australian Journal of Basic and Applied Sciences 3(4):3609-3616
18. Etesami HJE, safety e (2018) Bacterial mediated alleviation of heavy metal stress and decreased accumulation of metals in plant tissues: mechanisms and future prospects. 147:175-191
19. Flora SJ (2011) Arsenic-induced oxidative stress and its reversibility. Free radical biology & medicine 2(51):257-281
20. Ghaffari H, Tadayon MR, Nadeem M, Cheema M, Razmjoo JJApp (2019) Proline-mediated changes in antioxidant enzymatic activities and the physiology of sugar beet under drought stress. 41(2):23
21. Gupta V, Rastogi A (2009) Biosorption of hexavalent chromium by raw and acid-treated green alga Oedogonium hatei from aqueous solutions. Journal of Hazardous Materials 163(1):396-402
22. 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. Frontiers in microbiology 8:686
23. Hamayun M, Khan N, Khan MN, Qadir M, Hussain A, Iqbal A, Khan SA, Rehman KU, Lee I-JJB (2021) Antimicrobial and plant growth-promoting activities of bacterial endophytes isolated from Calotropis procera (Ait.) WT Aiton. 45(2):363-369
24. Hasanuzzaman M, Nahar K, Fujita M (2015) Arsenic toxicity in plants and possible remediation. Soil remediation plants: Prospects challenges:433-501
25. Hemmler D, Roullier-Gall C, Marshall JW, Rychlik M, Taylor AJ, Schmitt-Kopplin PJSr (2018) Insights into the chemistry of non-enzymatic browning reactions in different ribose-amino acid model systems. 8(1):1-10
26. Hussain A, Shah ST, Rahman H, Irshad M, Iqbal A (2015) Effect of IAA on in vitro growth and colonization of Nostoc in plant roots. Frontiers in plant science 6:46
27. Ikram M, Ali N, Jan G, Jan FG, Rahman IU, Iqbal A, Hamayun M (2018) IAA producing fungal endophyte *Penicillium roqueforti* Thom., enhances stress tolerance and nutrients uptake in wheat plants grown on heavy metal contaminated soils. PloS One 13(11):e0208150 doi:https://doi.org/10.1371/journal.pone.0208150
28. Ismail, Hamayun M, Anwar H, Sumera Afzal K, Amjad I, In-Jung L (2020a) An endophytic fungus *Aspergillus violaceofuscus* can be used as heat stress adaptive tool for *Glycine max* L. and *Helianthus annuus* L. Journal of Applied Botany and Food Quality 93:112-120
29. Ismail AH, Mehmood A, Qadir M, Husna AI, Hamayun M, Khan NJPJB (2020b) Thermal stress alleviating potential of endophytic fungus rhizopus oryzae inoculated to sunflower (Helianthus annuus L.) and soybean (Glycine max L.). 52(5):1857-1865
30. Ismaila AH, Qadira M, Husnaa MI, Ahmadb A, Hamayuna MJJAEBS (2018) Endophytic Fungi Isolated from Citrullus Colocynthesl. Leaves and Their Potential for Secretion of Indole Acetic Acid and Gibberellin. 8(6):80-84

31. 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. Analytical bioanalytical chemistry
32. 383(2):297-304
33. Khan MA, Sahile AA, Jan R, Asaf S, Hamayun M, Imran M, Adhikari A, Kang S-M, Kim K-M, Lee I-JJBpb (2021) Halotolerant bacteria mitigate the effects of salinity stress on soybean growth by regulating secondary metabolites and molecular responses. 21(1):1-15
34. 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 hydrolase inhibitory activities. Journal of the Science of Food and Agriculture 95(14):2956-2964
35. Machado RA, Theepan V, Robert CA, Züst T, Hu L, Su Q, Schimmel BC, Erb MJPb (2021) The plant metabolome guides fitness-relevant foraging decisions of a specialist herbivore. 19(2):e3001114
36. Mala JGSM, Takeuchi S, Sujatha D, Mani UJAMT, Technology (2020) Microbial Chromate Reductases: Novel and Potent Mediators in Chromium Bioremediation-A Review.32-44
37. Meena VS, Meena SK, Verma JP, Kumar A, Aeron A, Mishra PK, Bisht JK, Pattanayak A, Naveed M, Dotaniya MJEE (2017) Plant beneficial rhizospheric microorganism (PBRM) strategies to improve nutrients use efficiency: a review. 107:8-32
38. 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 Journal of Pharmaceutical Sciences 78(4):436-442
39. Mirza N, Mubarak H, Chai L-Y, Yang Z-H, Mahmood Q, Yong W, Tang C-J, Fahad S, Nasim W (2017) Constitutional tolerance and chlorophyll fluorescence of Boehmeria nivea L in response to the antimony (Sb) and arsenic (As) co-contamination. Toxicological Environmental Chemistry 99(2):265-272
40. Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends in plant science 11(1):15-19
41. Mohammadkhani N, Heidari R (2008) Drought-induced accumulation of soluble sugars and proline in two maize varieties. World Appl Sci J 3(3):448-453
42. Nićiforović N, Mihailović V, Mašković P, Solujić S, Stojković A, Muratspahić DP (2010) Antioxidant activity of selected plant species; potential new sources of natural antioxidants. Food and Chemical Toxicology 48(11):3125-3130
43. Oberbacher M, Vines H (1963) Spectrophotometric assay of ascorbic acid oxidase. Nature 197(4873):1203-1204
44. Padhan D, Rout PP, Kundu R, Adhikary S, Padhi PPJSBAATST (2021) Bioremediation of Heavy Metals and Other Toxic Substances by Microorganisms.285-329
45. Pisoschi AM, Pop A, Iordache F, Stanca L, Predoi G, Serban AIJEJoMC (2020) Oxidative stress mitigation by antioxidants-an overview on their chemistry and influences on health status.112891

46. Premono ME, Moawad A, Vlek P (1996) Effect of phosphate-solubilizing Pseudomonas putida on the growth of maize and its survival in the rhizosphere.
47. Putter J (1974) Peroxidase. In “Methods of Enzymatic Analysis”, ed. Bergmeyer, HU. Academic Press, New York
48. 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 chromate uptake and strengthening antioxidant system. Chemosphere 258:127386 doi:10.1016/j.chemosphere.2020.127386
49. Sarker U, Oba SJSr (2018) Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected Amaranthus tricolor under s alinity stress. 8(1):1-9
50. Scharte J, SCHÖN H, Weis E (2005) Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with Phytophthora nicotianae. Plant, Cell & Environment 28(11):1421-1435
51. Shafiq F, Iqbal M, Ali M, Ashraf MAJE, Safety E (2021) Fullerenol regulates oxidative stress and tissue ionic homeostasis in spring wheat to improve net-primary productivity under salt-stress. 211:111901
52. Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng BJM (2019) Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. 24(13):2452
53. Shrestha RA, Lama B, Joshi J, Sillanpää M (2008) Effects of Mn (II) and Fe (II) on microbial removal of arsenic (III). Environmental Science and Pollution Research-International 15(4):303-307
54. Suzuki N, Miller G, Salazar C, Mondal HA, Shulaev E, Cortes DF, Shuman JL, Luo X, Shah J, Schlauch K (2013) Temporal-spatial interaction between reactive oxygen species and abscisic acid regulates rapid systemic acclimation in plants. The Plant Cell 25(9):3553-3569
55. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular biology and evolution 24(8):1596-1599
56. Tiwari S, Gupta SC, Chauhan PS, Lata CJPCR (2021) An OsNAM gene plays important role in root rhizobacteria interaction in transgenic Arabidopsis through abiotic stress and phytohormone crosstalk. 40(1):143-155
57. 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. Archives of Microbiology 188(6):655-664
58. Valenzuela-García LI, Zapata BL, Ramírez-Ramírez N, Huchin-Mian JP, Robleto EA, Ayala-García VM, Pedraza-Reyes MJA, Microbiology E (2020) Novel Biochemical Properties and Physiological Role of the Flavin Mononucleotide Oxidoreductase YhdA from Bacillus subtilis. 86(20)
59. Viti C, Pace A, Giovannetti L (2003) Characterization of Cr (VI)-resistant bacteria isolated from chromium-contaminated soil by tannery activity. Current Microbiology 46(1):0001-0005
60. Vives-Peris V, Gómez-Cadenas A, Pérez-Clemente RMJPcr (2017) Citrus plants exude proline and phytohormones under abiotic stress conditions. 36(12):1971-1984

61. Warrier R, Paul M, Vineetha MJG (2013) Estimation of salicylic acid in Eucalyptus leaves using spectrophotometric methods. Genetics and plant physiology 3(1-2):90-97
62. Wyszkowski M, Radziemska M (2010) Effects of chromium(III and VI) on spring barley and maize biomass yield and content of nitrogenous compounds. Journal of Toxicology and Environmental Health 73(17-18):1274-82 doi:10.1080/15287394.2010.492016
63. Xu F, Ma T, Zhou L, Hu Z, Shi L (2015) Chromium isotopic fractionation during Cr (VI) reduction by Bacillus sp. under aerobic conditions. Chemosphere 130:46-51
64. 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. Ecotoxicology and environmental safety 142:139-149
65. Zaid A, Mohammad F, Wani SH, Siddique KMJE, safety e (2019) Salicylic acid enhances nickel stress tolerance by up-regulating antioxidant defense and glyoxalase systems in mustard plants. 180:575-587
66. Zhu Y-G, Yoshinaga M, Zhao F-J, Rosen BP (2014) Earth abides arsenic biotransformations. Annual Review of Earth and Planetary Sciences 42:443-467

Figures

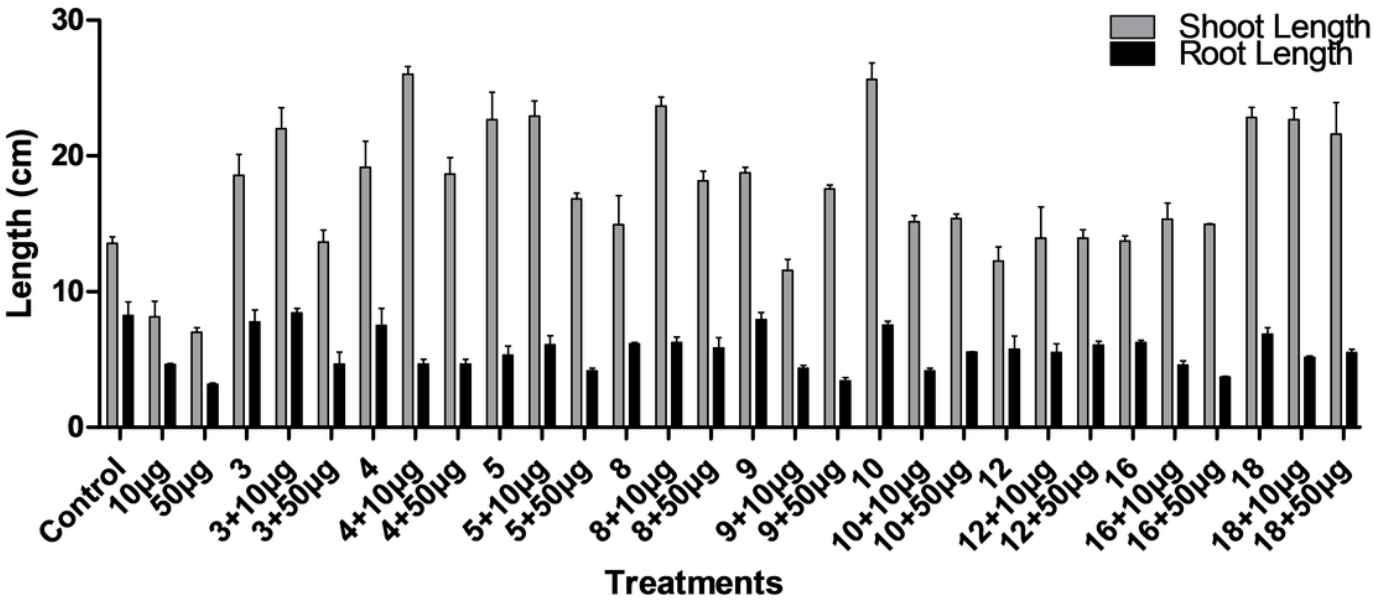

Figure 1

Effect of rhizobacterial isolates on plant growth under heavy metal stress. Values are mean of replicates with ± SE (Duncan test; $p < 0.05$).

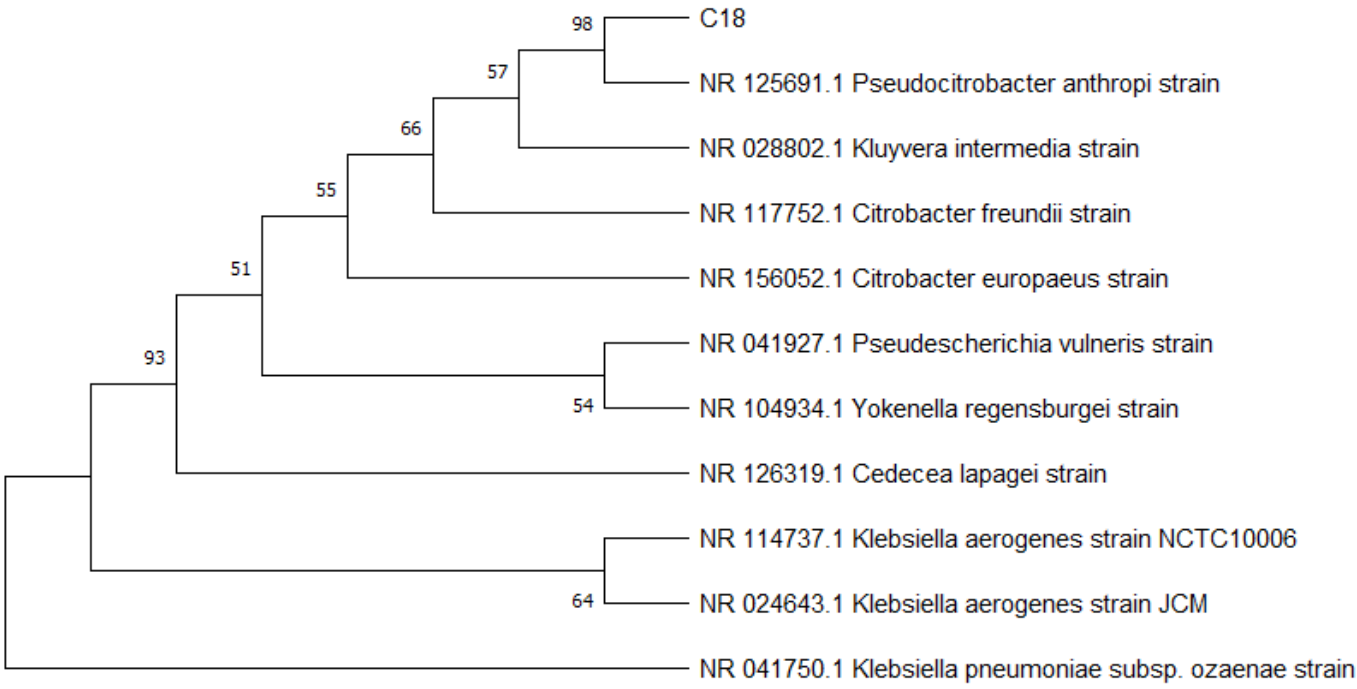

Figure 2

Phylogenetic consensus tree construction for the identification of bacterial isolate C18 using Maximum Parsimony method. 98% bootstrap value confirmed isolate C18 as Pseudocitrobacter anthropi.

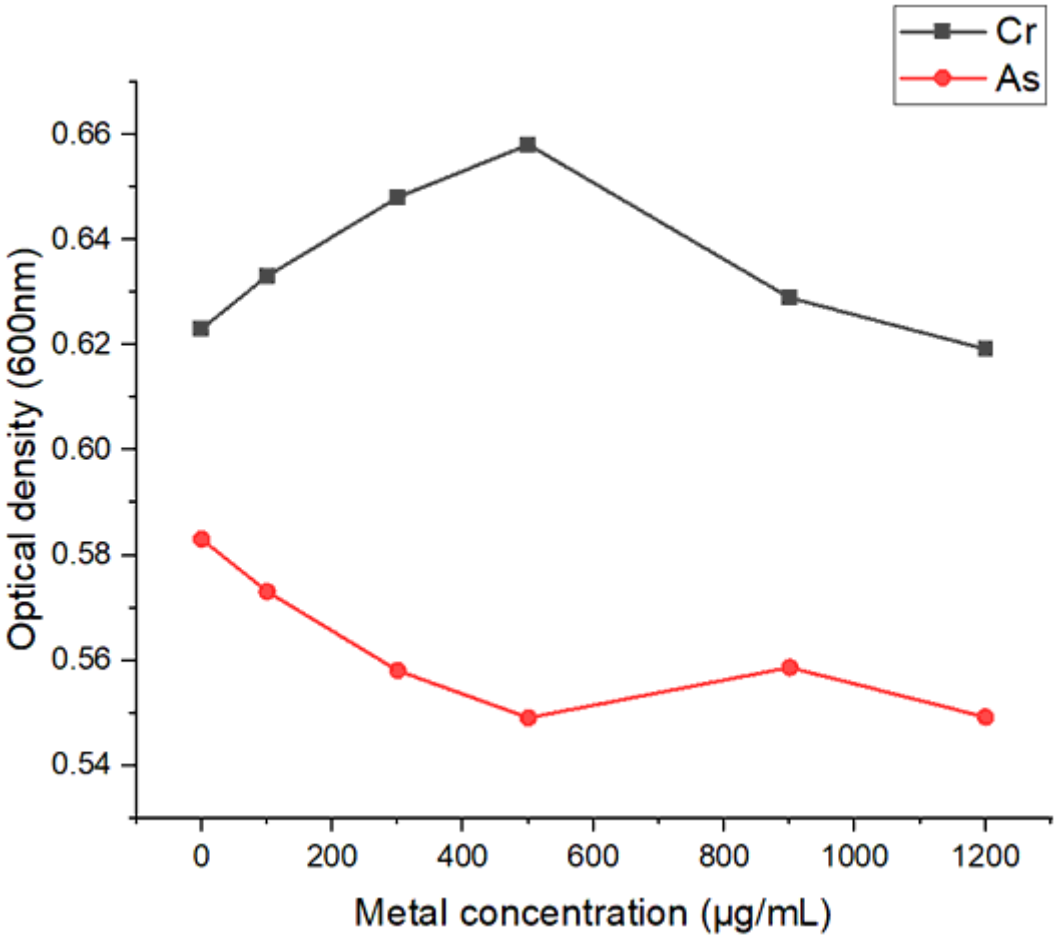

Figure 3

Growth curve of C18 in the presence and absence of heavy metal. Black indicates chromate treated bacterial cells; Red indicates arsenic treated bacterial cells.

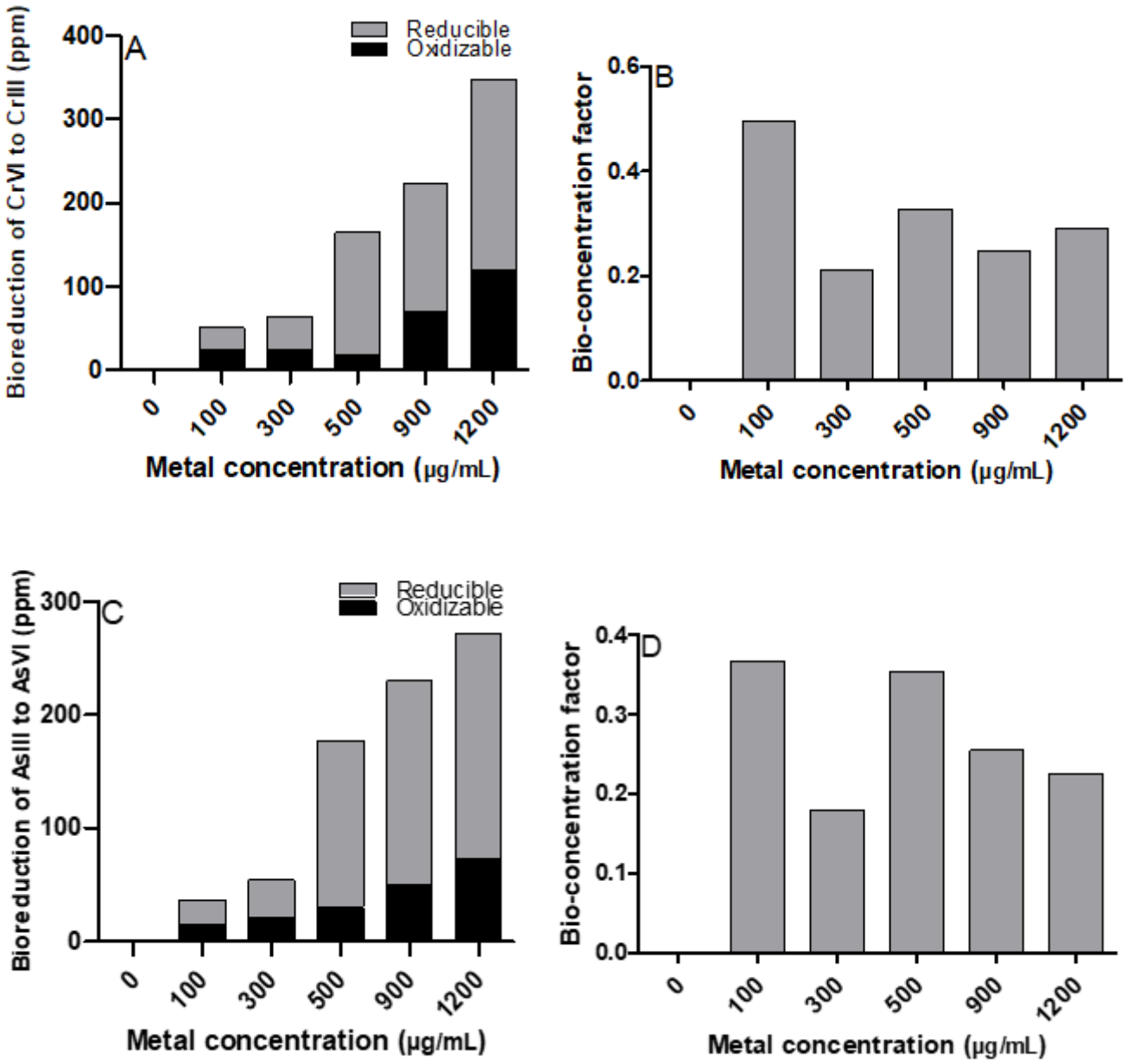

Figure 4

The capability of Cr-(VI) and As-(V) reduction by isolate C18 (P. anthropi) at 0, 100, 300, 500, 900 and 1200 µg/mL of Cr-(VI)/As-(V).

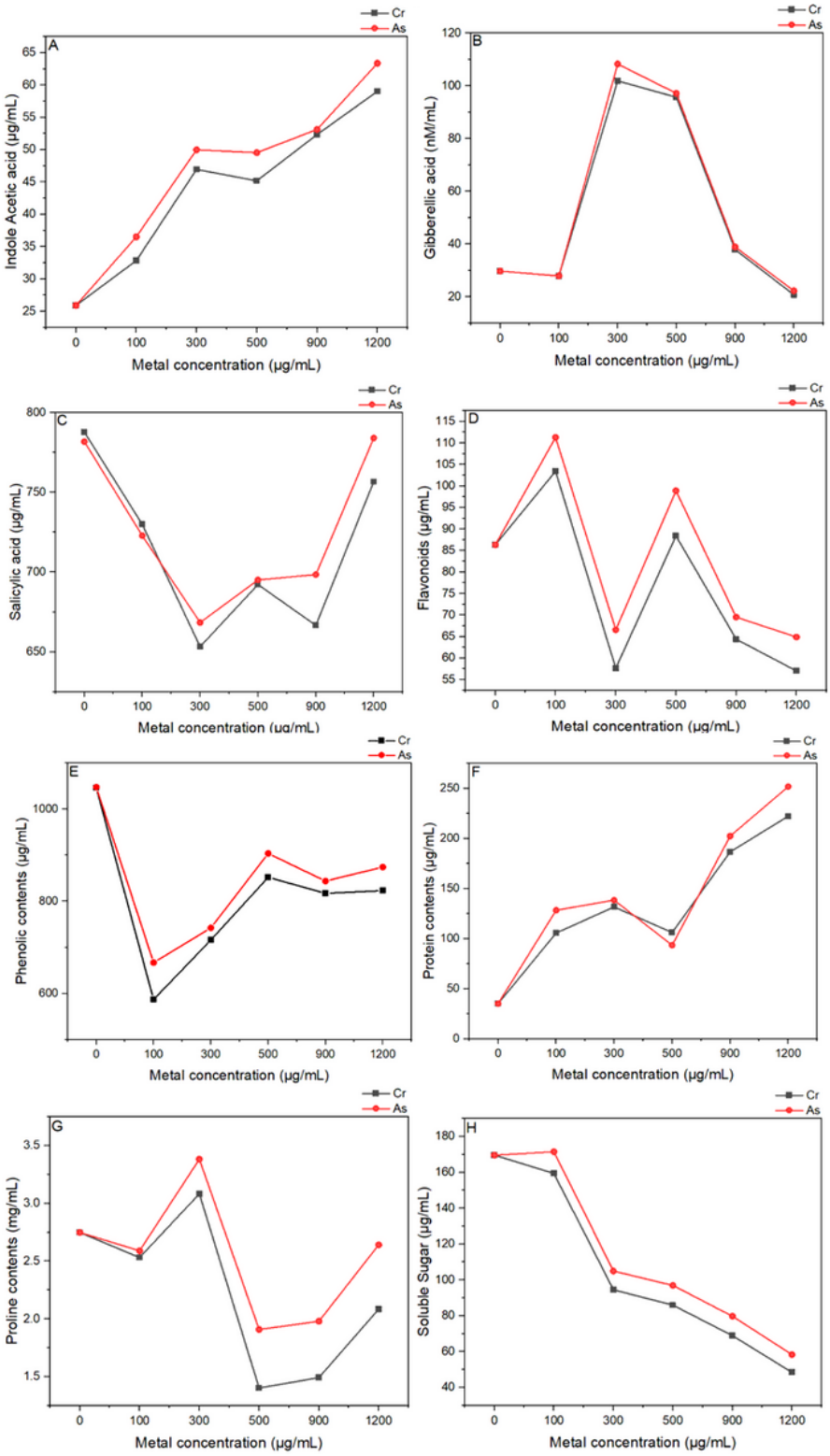

Figure 5

Response of phytohormones and plants metabolites in Pseudocitrobacter anthropi bacterial supernatant to various concentration of chromium and arsenic A) IAA B) GA C) SA D) Flavonoids E) Phenolic content, F) Protein content G) Proline H) Soluble sugar content. Bars followed by different letters are significantly different at p=0.05, using Duncan multiple range test. Each bar represents mean of replicated data with ± SE.

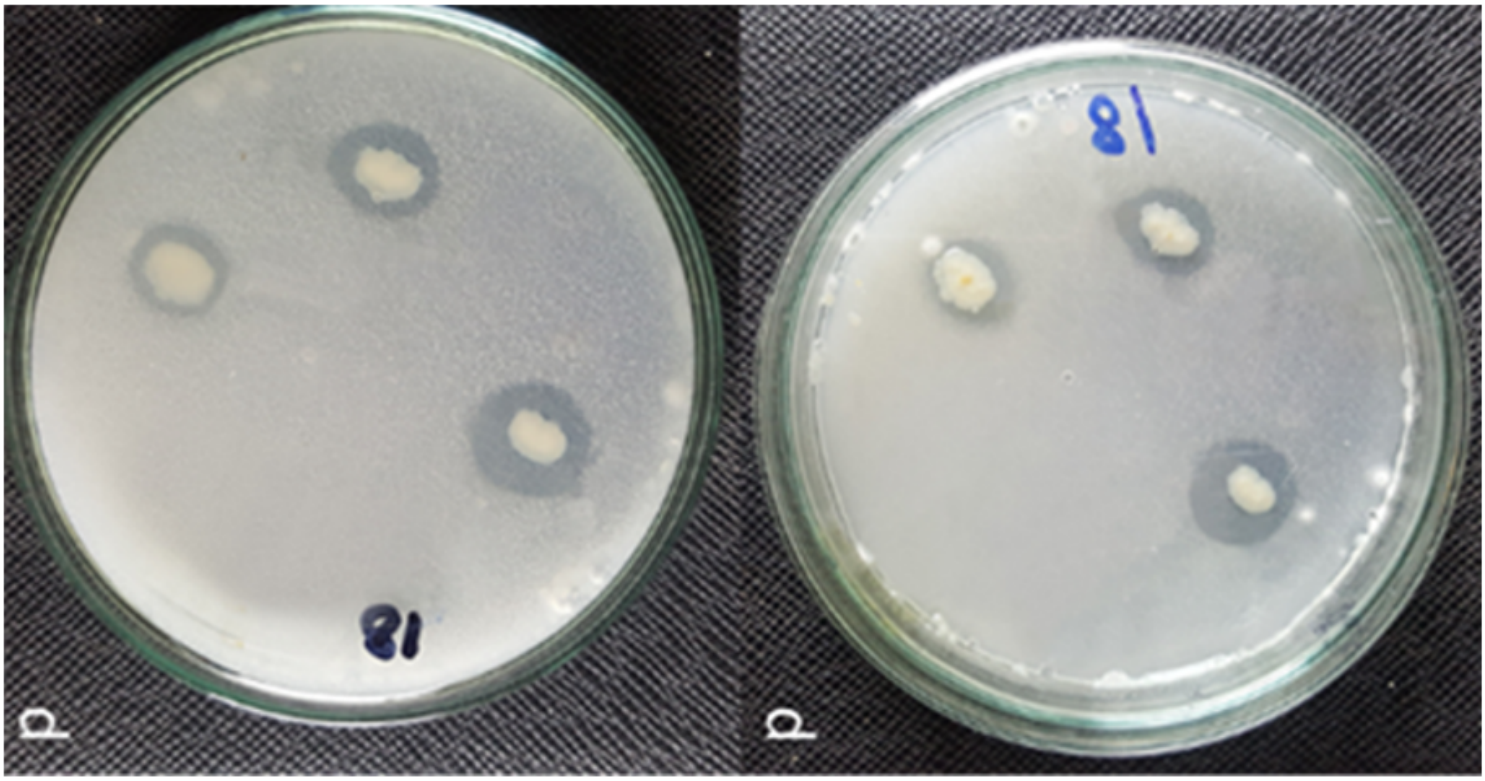

Figure 6

Rhizobacteria P. anthropi show positive phosphate solubilizing activity on Pikovskaya's agar plates by after 24 hours of incubation.

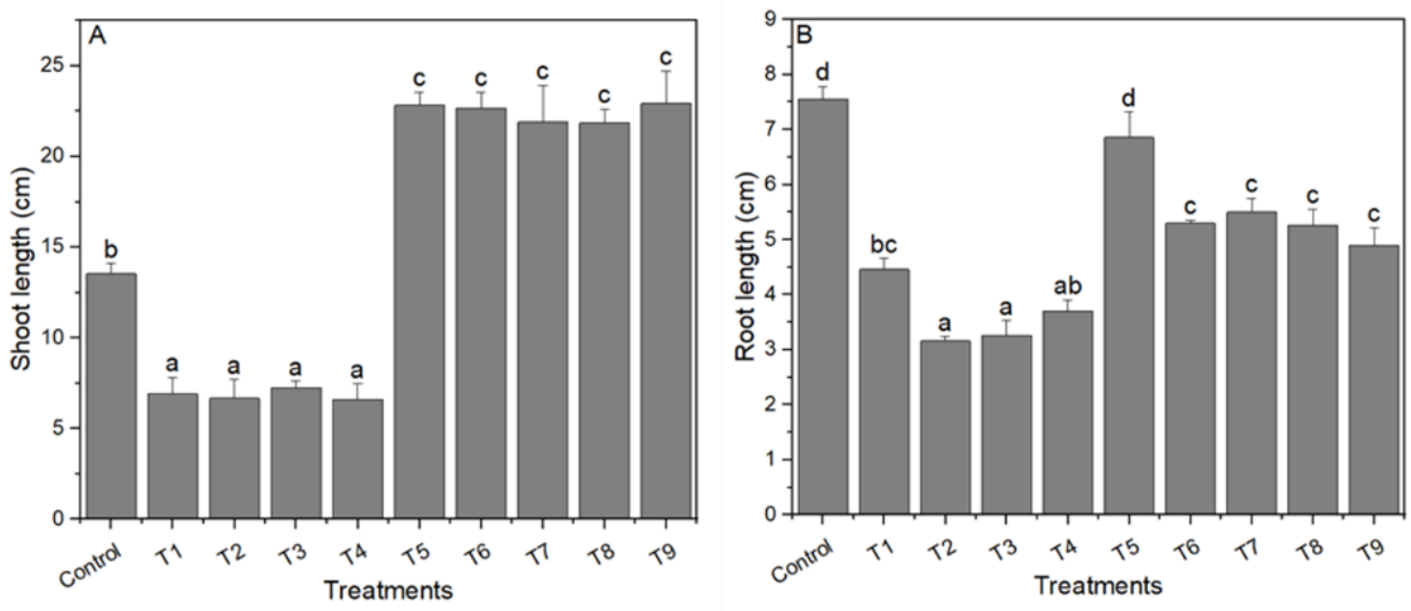

Figure 7

Rhizobacteria isolate P. anthropi effect on A) shoot B) root length of Glycine max under chromium and arsenic stress. Bars followed by different letters are significantly different at p=0.05, using Duncan multiple range test. Each bar represents mean of replicated data with ± SE.

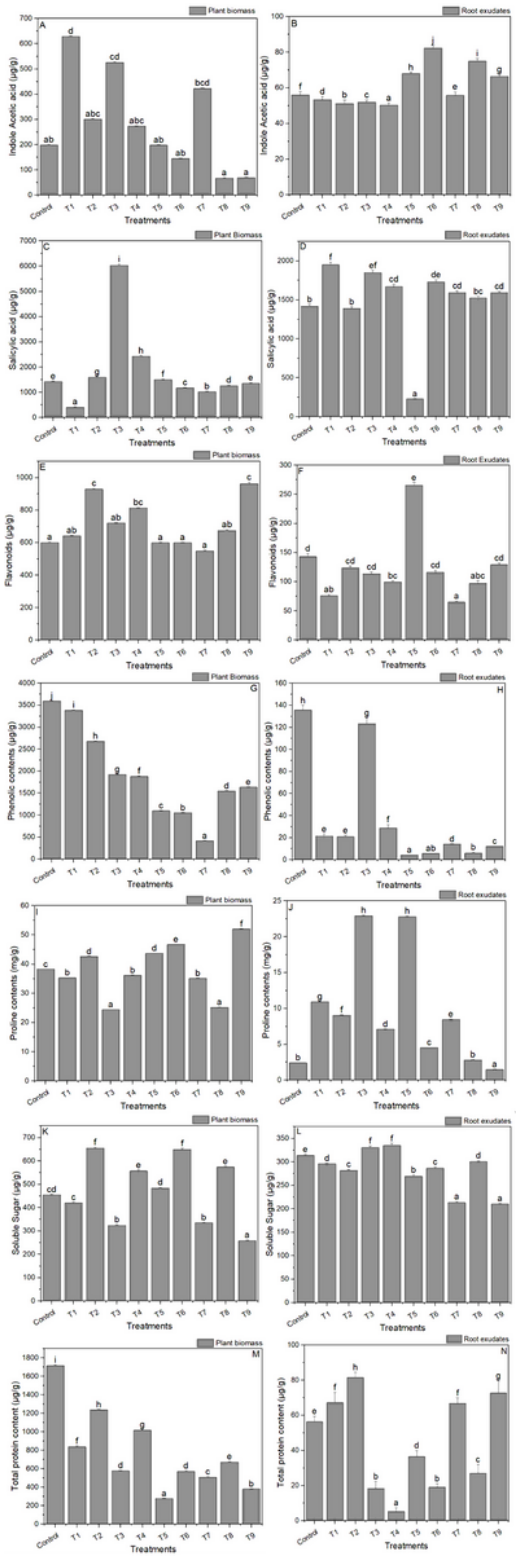

Figure 8

Response of phytohormones and plants metabolites in 14-days old soybean seedling inoculated with P. anthropi to various concentration of chromium and arsenic Control) without any treatment T1) Plants exposed to Cr (10µg/mL) T2) Plants exposed to Cr (50µg/mL) T3) Plants exposed to As (10µg/mL) T4) Plants exposed to As (50µg/mL) T5) Plants inoculated with P. anthropi T6) Plants inoculated with P. anthropi exposed to Cr (10µg/mL) T7) Plants inoculated with P. anthropi exposed to Cr (50µg/mL) T8)

Plants inoculated with P. anthropi exposed to As (10µg/mL) T9) Plants inoculated with P. anthropi exposed to As (50µg/mL). Bars followed by different letters are significantly different at p=0.05, using Duncan multiple range test. Each bar represents mean of replicated data with ± SE.

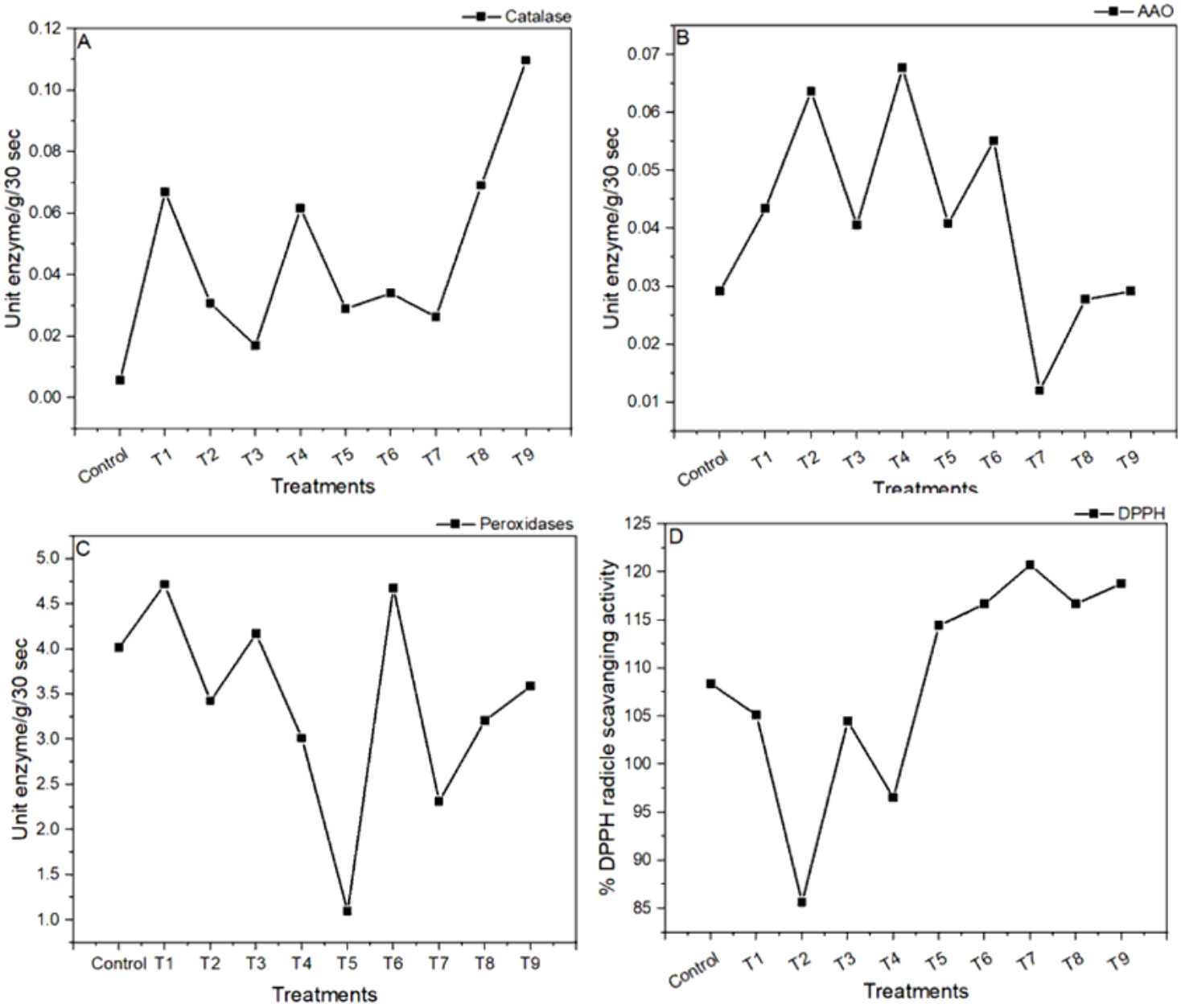

Figure 9

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 D) DPPH assay. Control) without any treatment T1) Plants exposed to Cr (10µg/mL) T2) Plants exposed to Cr (50µg/mL) T3) Plants exposed to As (10µg/mL) T4) Plants exposed to As (50µg/mL) T5) Plants inoculated with P. anthropi T6) Plants inoculated with P. anthropi exposed to Cr (10µg/mL) T7) Plants inoculated with P. anthropi exposed to Cr (50µg/mL) T8) Plants inoculated with P. anthropi exposed to As (10µg/mL) T9) Plants inoculated with P. anthropi exposed to As (50µg/mL).

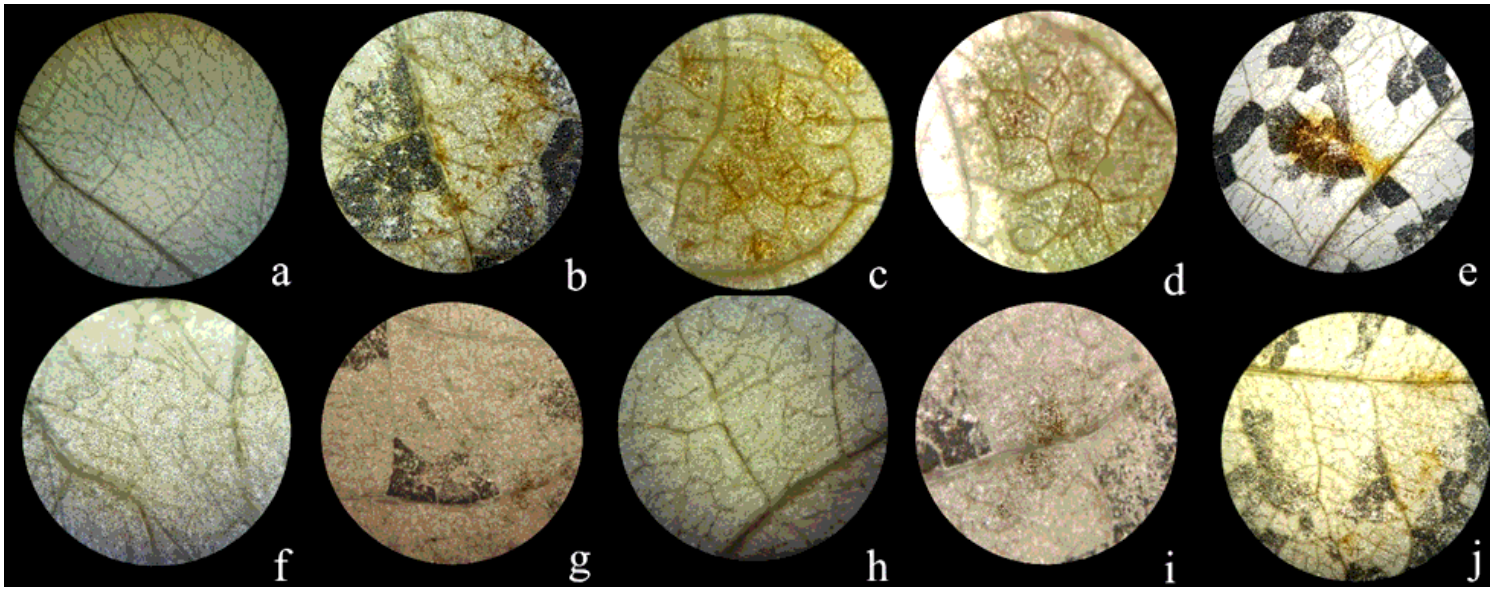

Figure 10

ROS accumulation assay with DAB; localized brown areas on the leaves symbolizing ROS accumulation in a) Control b) Cr 10µg/mL c) Cr 50µg/mL d) As 10µg/mL e) As 50µg/mL f) P. anthropi g) P. anthropi + Cr 10µg/mL h) P. anthropi +Cr 50µg/mL i) P. anthropi +As 10µg/mL j) P. anthropi +As 50µg/mL.

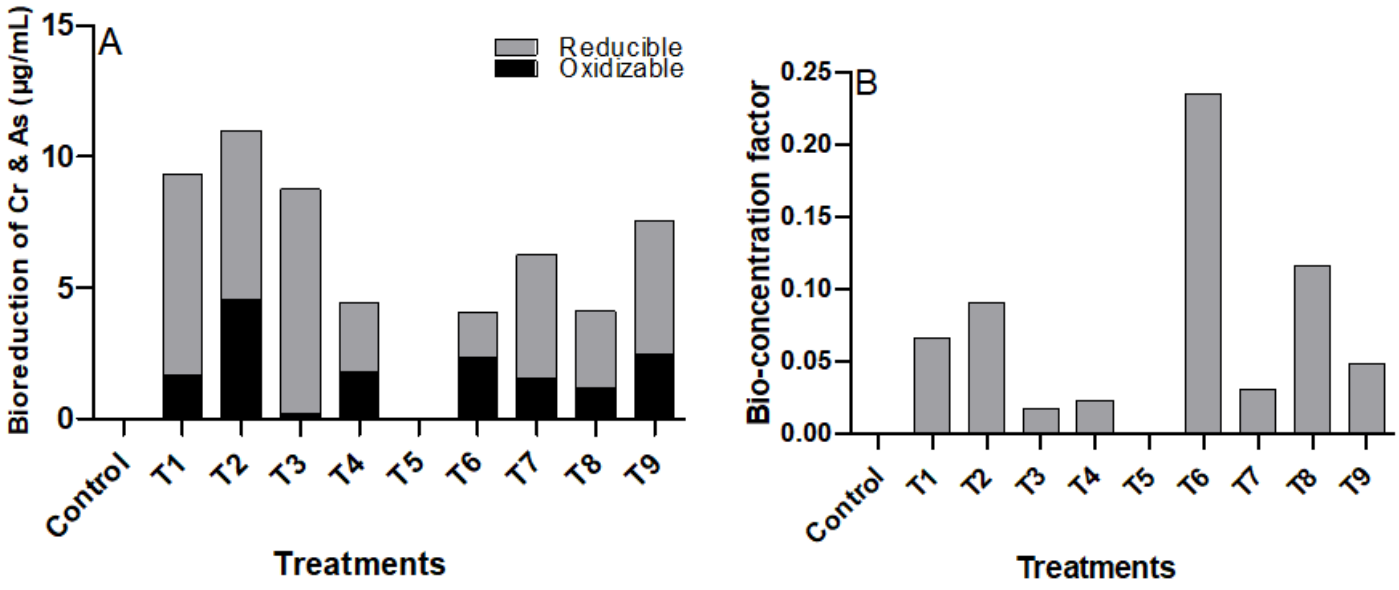

Figure 11

Bio-reduction and bioconcentration factor of soybean plants inoculated with isolate P. anthropi under chromium and arsenic stress.