Original Research

**Rhizobacteria-*Pseudomonas guguanensis* Isolated from Mines Area Assists Green-Remediation of Cadmium by *Brassica juncea*: a Promising Environment Sustainable approach**

Sarita Sharma1,2*, Meenu Saraf 2

**Abstract**
This study investigated how Cd-tolerant rhizobacteria isolated from the mine area and landfill site influence the phytoremediation efficacy of *Brassica juncea* plants in Cd-contaminated soils. Out of four cadmium-tolerant rhizobacteria, isolate SMHMZ4 showed the promising phytoextraction efficacy of *B. juncea*. Isolate SMHMZ4 was identified as *Pseudomonas guguanensis* and submitted to NCBI GenBank under accession number MZ145097. These rhizobia were reported for the first time to support Cd-phytoremediation using *B. juncea*. Compared with the non-inoculated control, SHMMZ4 treatment significantly improved the germination of *B. juncea* seeds and increased soluble Cd in soil by 7.78 times. Growth and health parameters, pigment and Cd accumulation in roots and shoots of isolate SHMMZ4 inoculated *B. juncea* grown in individual soil contaminated with 94.95 μg g⁻¹ CdCl₂ were significantly increased. Pot experiments showed that SHMMZ4 could transfer Cd from soil to roots, from roots to shoots. The translocation, bioconcentration, and bioaccumulation coefficient values were 1.28, 1.22, and 1.72 times higher, respectively, than in the non-inoculated control. The present study demonstrates that the rhizobacteria amendments to *B. juncea* are believed to be a promising method for green remediation of cadmium-polluted areas.

**Keywords**
Rhizobacteria, metal(s), phytoremediation, atomic absorption chromatography, bioconcentration factor, translocation factor

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Rhizobacteria Pseudomonas guguanensis, izolirana iz območja rudnikov, pomaga pri zeleni sanaciji kadmija z Brassica juncea: obetaven okoljski trajnostni pristop

Izvleček
Naša študija je preučevala, kako rizobakterije, tolerantne na kadmij, izolirane iz območja rudnik in odlagališč, vplivajo na učinkovitost fitoremediacije rastlin Brassica juncea v tleh, onesnaženih s kadmijem. Izmed štirih rizobakterij, tolerantnih na kadmij, je izolat SMHMZ4 pokazal obetavno učinkovitost fitoekstrakcije B. juncea. Izolat SMHMZ4 je bil identificiran kot Pseudomonas guguanensis in predložen NCBI GenBank pod pristopno številko MZ145097. Prvič so poročali, da te rizobije podpirajo Cd-fitoremediacijo z B. juncea. V primerjavi z neinokulirano kontrolo je tretma s SHMMZ4 znatno izboljšal kalitev semen B. juncea in povečalo topnost Cd v tleh za 7,78-krat. Rasti parametri, fiziološko stanje, kopičenje pigmenta in kadmija v koreninah in poganjkih B. juncea inokulirane z izolatom SHMMZ4, gojene v posameznih tleh, onesnaženih s 94,95 μg g⁻¹ CdCl₂, so se znatno povečali. Poskusi v loncih so pokazali, da lahko SHMMZ4 vplivana na privzem kadmija iz tal v korenine in iz korenin v poganjke. Vrednosti translokacijskega koeficienta, biokoncentracijskega koeficienta in bioakumulacijskega koeficienta so bile 1,28, 1,22 oziroma 1,72-krat večje, kar je bilo bistveno višje od neinokulirane kontrole. Ta študija dokazuje, da so kombinacija B. juncea z rizobakterijami zelo obetavna metoda za zeleno sanacijo s kadmijem onesnaženih področij.

Ključne besede
Rizobakterije, kovine, fitoremediacija, atomska absorpcijska kromatografija, biokoncentracijski faktor, translokacijski faktor

Introduction
In recent decades, metal(s) contamination of soil has become a serious concern due to anthropogenic activities such as growing industrialization, mining activity, heavy input of various fertilizer applications, weedcides, pesticides, and land irrigation with polluted water sources (Sharma et al., 2021a; Sharma and Saraf, 2023b). Given that metal(s) are quickly incorporated into the food chain through fruits, vegetables, and other edible plant components, metal(s) contamination in agricultural soils is particularly significant (Kaur et al., 2018; Prajapati et al., 2022). Elevated concentrations of potentially hazardous metal(s) in soils are recognized worldwide as a major contributor to environmental deterioration, endangering human health and the ecosystem (Ekobessa et al., 2021). For 25 to 30 years, cadmium (Cd), a dangerous non-essential transition metal, can accumulate in plants and animals. Reactive oxygen species (ROS) were created, photosynthesis and respiration were inhibited, enzyme activities were altered, and plants' capacity to absorb nutrients was diminished when exposed to cadmium (Wang et al., 2022). Sensitive remediation techniques for metal(s)-contaminated soils are desperately needed to protect the environment and reduce the detrimental effects of metal(s) bioaccumulation and biomagnification in biological systems. Many physical, chemical, and/or biological solutions are now available to clean up contaminated environments (Sharma I., 2020). On the other hand, the high cost and labour intensity of physical and chemical procedures are their limitations. Additionally, secondary pollutants such as sludge and piles are produced by chemical operations and will be harsh to the environment (Zainab et al., 2020).

According to Jeyasundar et al. (2021), "green remediation" refers to a gentle in situ remediation technique that considers the environmental impact of remediation measures at every stage of the process to maximize the overall environmental value of a clean-up. Because they are effective at creating biomass, plants like Brassica juncea are perfect for producing bioenergy (Ying et al., 2021; Sharma and Saraf, 2023a: 2023c; 2023d). According to Amin et al. (2018), plants are believed to be a low-cost way to remove metal(s) with relatively modest environmental management issues. Additionally, rhizobacteria-assisted phytoremediation of metal(s)-contaminated soils, which promotes plant development, is being investigated as an affordable, environmentally friendly option for soil management. Therefore,
to achieve the objectives of "green remediation," plant growth-promoting rhizobacteria (PGPR) from the Zawar mines in Udaipur, Rajasthan, India, and the Pirana garbage dumpsite in Ahmedabad, Gujarat, India, along with the bioenergy crop *B. juncea*, were integrated into this study. The efficacy of phytoremediation may be limited by factors such as plant production, pollutant bioavailability, and plant resistance to stress caused by metal(s) (Antoniadis et al., 2021). The use of various types of organic and inorganic substances as amendments, plant growth promoters, beneficial microorganisms, and genetically engineered microorganisms has been reviewed and suggested by numerous researchers as assisted phytoremediation techniques (Rathore et al., 2019; Saraf et al., 2017; Rostami and Azhdarpoor, 2019). These techniques are thought to help plant stabilization, promoting phytoremediation performance. The remediation potential of these processes is primarily achieved by increasing i) plant biomass, root surface area, and health (Shaikh et al., 2022); ii) metal bioavailability and microbial community composition; and iii) metal translocation within plants via microbe-metal-plant interactions (Sharma and Saraf, 2023a:2023b:2023d). Of these processes, microbial-assisted phytoremediation has recently attracted increased attention due to multiple efficient mechanisms. According to Sullivan and Gadd (2019), microorganisms can interact with metals, food, and toxins. Their exceptional adaptability and metabolic activities also show that they are particularly useful for remediation (Sharma I., 2020; Dabhi et al., 2021).

Determine whether toxic Cd-tolerant rhizobacteria can increase cadmium availability and translocation in *B. juncea* roots and shoots to enhance phytoremediation and make it a viable choice for improving Cd-contaminated locations. This was the main goal of the current investigation. Recent studies (Din et al., 2020; Zhang et al., 2020; Sharma and Saraf, 2023a) focused on single strain-single host plant interactions; nevertheless, microbial populations in the field are always complex mixtures.

### Materials and Methods

#### Metal-tolerant rhizobacteria isolation and selection

Earlier, we recovered 91 multi-metal-tolerant rhizobacteria: 40 from the Pirana dumpsite in Ahmedabad, Gujarat, India, and 51 from the Zawar mines in Udaipur, Rajasthan, India. Because of their strong resistance to Cd, four rhizobacteria were selected for additional study (Sharma et al., 2020; 2021; 2022a; 2023c). We explored how these rhizobacteria affected *B. juncea*’s cadmium accumulation and plant growth.

#### Identification of Cd-tolerant rhizobacteria using 16S rRNA gene sequence

Isolates with greater resistance to cadmium, plant growth-promoting traits, and elevated cadmium accumulation in plants were selected for identification. The pure culture’s DNA was taken out. Using Agarose Gel Electrophoresis, a single band of high molecular weight DNA was discovered, indicating the sample’s purity. Using 16S rRNA primers, a portion of the gene was amplified by PCR. One unique PCR amplicon band was visible when the sample was resolved on an Agarose Gel. SLS’s PCR Purification kit (column-based purification) was used to purify the PCR amplicon to eliminate contaminants. Using the BDT v3.1 Cycle sequencing kit and 16S rRNA Primers, the DNA sequencing reaction of the PCR amplicon was carried out using an ABI 3730xl Genetic Analyzer at SLS Research Pvt. Ltd., Surat, Gujarat, India (Saitou and Nei, 1987). BLAST was run using the gene sequence against the NCBI Genbank database. Various alignment software programs were used to align the first ten sequences, which were selected based on their maximum identity score. Here’s a summary of the primers (Sharma and Saraf, 2023c).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>AGA GTT TGA TCM TGG CTC AG</td>
</tr>
<tr>
<td>1492R</td>
<td>CGG TTA CCT TGT TAC GAC TT</td>
</tr>
</tbody>
</table>

#### Soil sampling and metal spiking

Surface soil (0–15 cm) samples were collected at five separate locations from an agricultural tract near Jagatpur village near Gota, Ahmedabad, Gujarat. The soil samples were bulked up to form a composite sample after being air-dried, crushed, and sieved to a particle size of 2 mm. CdCl₂ was used as a Cd source to spike the soil sample. The soils were spiked, meaning that 1000 g of air-dried parent soil (at a 10:1 solid:liquid ratio) received 100 mL of 1000 mg L⁻¹ metal stock, and the mixture was incubated for four weeks to stabilize the metals in the soil. According to Sharma
and Saraf (2023c), the previously indicated spiking was intended to produce a target concentration of about 100 μg g⁻¹. The atomic absorption spectrophotometer was used to assess the metal concentrations in the contaminated soil after applying the acid digestion method. Following the protocols mentioned above, each spike of soil produced had Cd values of 94.95 μg g⁻¹ after four weeks. These spike soils were then used in pot studies. 

**Rhizobacterial inoculum preparation**

The creation of rhizobacterial inoculum is achieved by growing rhizobacterial isolates in 250 mL Erlenmeyer flasks containing 100 mL of sterilized nutrient broth modified with 100 μg mL⁻¹ of Cd. Flasks were in a shaking incubator set at 37°C ± 2°C and 120 rpm for 48 hours. After centrifuging the bacteria for 15 minutes at 10,000 rpm, they were twice cleaned with sterile distilled water to extract the cells. Cell pellets were resuspended in phosphate buffer (pH 7.0) and adjusted to an absorbance of 1.5 at 600 nm (about = 5.6 × 10⁸ cfu mL⁻¹) using a spectrophotometer (Systronics 166).

**Pot experiment preparation**

The soil was first oven-dried for 72 hours at 70°C. It was then sieved using a 2 mm sieve and autoclaved for 15 minutes at 121°C. After being sterilised, 1000 g of soil were placed into 6-by-7-inch plastic pots and autoclaved for 15 minutes at 121°C. After centrifuging the bacteria for 15 minutes at 120 rpm for 48 hours. After centrifuging the bacteria for 15 minutes at 10,000 rpm, they were twice cleaned with sterile distilled water to extract the cells. Cell pellets were resuspended in phosphate buffer (pH 7.0) and adjusted to an absorbance of 1.5 at 600 nm (about = 5.6 × 10⁸ cfu mL⁻¹) using a spectrophotometer (Systronics 166).

**Rhizobacteria's influence on soil metal mobility**

The rhizobacterial strains were cultivated in 100 mL conical flasks with 50 mL of Nutrient broth and incubated at 37°C ± 2°C and 200 rpm on a Remi, India shaker. After being centrifuged at 8000 rpm for 10 minutes, the bacterial cells were removed from the broth after 24 hours. They were then twice cleaned with phosphate buffer (pH 7.0) and resuspended in 5 mL of sterile double-distilled water. Each bacterial cell suspension’s optical density was adjusted to 1.5 using a UV spectrophotometer. (Systronics 166). 100 g of the soil contaminated with 100 μg g⁻¹ CdCl₂ was placed into 50 ml falcon tubes, and a 1 mL aliquot of SMHMZ2, SMHMZ4, SMHMP4, and SMHMP23 rhizobacterial cell suspension was added (Treatment 2). The tubes were shaken at 200 rpm at room temperature. Treatment 1: (control - Cd spike soil + no rhizobacterial inoculation) received a 1 mL application of sterile distilled water. After seven days, 10 mL of sterile double distilled water was added to each set of falcon tubes to remove water-soluble cadmium. All falcon tubes were centrifuged for 10 minutes at 8000 rpm to get rid of any dirt particles. The resulting solution was then filtered through Whatman filter paper No. 40. The amounts of Cd in the filtrate were measured using an atomic absorption spectrophotometer (ELICO SL 194) (Rajkumar and Freitas, 2008).

**Seed germination test**

To investigate the impact of bacterial inoculation on seed germination in the presence of metal contamination, seed germination tests were conducted using an adapted version of He et al. (2013) methodology. Two folded pieces of filter paper were placed on the bottom of each clean, sterile glass petri plate for this use. 100 μg mL⁻¹ Cd was added to either 10 mL of bacterial solution or sterile tap water (Control). The procedure described by Ndedy Aka et al. (2016) was used to sterilize *B. juncea* seeds. After being incubated at room temperature for two hours in 10 mL of rhizobacterial consortium suspension on a shaker at 150 revolutions per minute, sterile seeds were placed in each petri dish (30 seeds per plate). Every therapy was administered three times. After seven days, the number of seeds germinating in each petri dish was counted. To measure the seedlings’ growth characteristics, three randomly selected seedlings (shoot length and root length) were taken from each plate. The germination percentage and vigour index were computed using the following formula (Rathod et al., 2021).

\[ \text{Germination rate \%} = \frac{n \times 100}{N} \]

Where n is the number of germinated seeds after seven days, and N is the number of total seeds.

\[ \text{Vigour Index} = \% \text{germination} \times \text{Total length of seedling} \]

\[ \text{(Shoot length + Root length)} \]
Cadmium and Cd-tolerant rhizobacteria’s effect on plant growth

The *B. juncea* seeds were sterilised for the pot tests using the procedure described by Ndedy Aka et al. (2016). After being sown for two hours in either sterile water (control) or rhizobacterial cultures, sterilized *B. juncea* seeds were allowed to dry at room temperature. Ten infected and non-inoculated seeds were planted at a depth of 5 cm in each container (6 by 7 inches) containing 1000 g of soil. Eight days after germination, the pots were pruned, each containing seven seedlings. After ten days, bacterial suspensions (10 mL per pot) were added to the soil surrounding the root. When the pots were watered again, the leachate was collected in a plastic tray underneath the treatment pot and reintroduced to the pots. Every day, deionized water was used to irrigate the seedlings. The experiment’s overall plan called for a maximum of six treatments, each with four replicates:

- *B. juncea* + non-contaminated soil (control H2O)
- *B. juncea* + metal-contaminated soil (spiked with-Cd)
- *B. juncea* + metal-contaminated soil (spiked with-Cd) + rhizobacterial isolates (SMHMZ2, SMHMZ4, SMHMP4, and SMHMP23)

After 30 days, each plant was carefully removed from its pot and given a thorough rinse with distilled water to remove any remaining dirt. After measuring each plant’s height and fresh weight, the plants were divided into roots and shoots. Plant tissues, including roots and shoots, were placed inside separate polyethylene bags and dried at 80°C for 72 hours. Plant tissues that had been baked were ground into a powder using a mortar and pestle, and then a Wensar PGB 200 scale was used to weigh each plant individually. Plant samples were appropriately tagged and kept in polyethylene bags for further examination.

Chlorophyll pigment estimation (chlorophyll a, b and carotenoids)

A 0.5 g leaf was homogenized with acetone at an 80% (v/v) concentration and filtered through filter paper to estimate the amount of chlorophyll pigment. A spectrophotometer was used to test the absorbance of the filter at wavelengths of 663, 645, and 480 nm for carotenoids, chlorophyll a, and b, respectively (Lichtenthaler & Wellburn, 1983).

Proline content

Proline levels were ascertained using the Bates et al. (1973) protocol. We crushed a 500 mg plant sample in 10 mL of 3% sulfosalicylic acid. This was centrifuged at 13,000 rpm for 15 minutes. A 2 mL supernatant sample was heated to 100°C and mixed with 2 mL of ninhydrin and glacial acetic acid. Immediately, the test tubes were submerged in an ice bath to halt the reaction. A 4.0 mL toluene sample was added to the reaction mixture and vortexed for one minute. After removing the aqueous layer, the red toluene layer’s absorbance was measured at 520 nm. The standard curve was made using the L-proline.

Total phenols

The creation of rhizobacterial inoculum is achieved by growing rhizobacterial isolates in 250 mL Erlenmeyer flasks containing 100 mL of sterilized nutrient broth modified with 100 µg/mL of Cd. Flasks were in a shaking incubator set at 37°C ± 2°C and 120 rpm for 48 hours. After centrifuging the bacteria for 15 minutes at 10,000 rpm, they were twice cleaned with sterile distilled water to extract the cells. Cell pellets were resuspended in phosphate buffer (pH 7.0) and adjusted to an absorbance of 1.5 at 600 nm (about = 5.6 × 10^8 cfu mL-1) using a spectrophotometer (Systronics 166).

Flavonoid content

According to Zhishen et al. (1999), the amounts of flavonoids were tested. The dried sample of 100 mg was crushed in 3 mL of 100% alcohol. Four mL of double-distilled water, 3 mL of 5% NaNO2, and 3 mL of 10% AlCl3 were mixed with 1 mL of the extract sample. For 10 minutes, the mixture was incubated with 2 mL of NaOH and 2 mL of distilled water. The absorbance was measured at 510 nm. The standard for determining flavonoid content was quercetin.

Plant metal(s) measurement

Each dried plant sample (roots and shoots) was carefully weighed using a balance before being added to a 100 mL Erlenmeyer flask with 9 mL of HCL, 3 mL of concentrate HNO3 (69 %), and 1 mL of H2O2 for the Cd analysis. The mixture was heated on a hot plate at 100°C in a fume hood until white fumes were released. After digestion, the mixture was allowed to cool before being diluted with...
distilled deionized water to a volume of 50 mL. Before the Cd concentrations in the samples were measured using an Atomic Absorption Spectrophotometer (ELICO SL 194), the resultant solution was twice filtered using filter paper (Ndeddy Aka et al., 2016; Edulamudi et al., 2019).

Evaluating the efficacy of phytoremediation

Phytoextraction indices are a helpful technique for determining the phytoextraction potential of *Brassica juncea* in conjunction with PGPR for removing Cd from polluted soil. The following are the most commonly used phytoextraction indices (Lindsay and Norvell, 1978; Amin et al., 2018):

**Bioconcentration factor (BCF)**

The bioconcentration factor (BCF) was calculated as the metal concentration ratio in plant roots to soil (Lindsay and Norvell, 1978; Amin et al., 2018):

\[ \text{Bioconcentration factor [BCF]} = \frac{[\text{Metal}]_{\text{root}}}{[\text{Metal}]_{\text{soil}}} \]

**Bioaccumulation coefficient (BAC)**

The bioaccumulation coefficient (BAC) was calculated as a ratio of Cd in shoots to Cd in soil (Lindsay and Norvell, 1978; Amin et al., 2018):

\[ \text{Bioconcentration factor [BCF]} = \frac{[\text{Metal}]_{\text{shoot}}}{[\text{Metal}]_{\text{soil}}} \]

**Translocation factor (TF)**

The translocation factor (TF) was calculated as a ratio of Cd in plant shoots to Cd in plant roots (Lindsay and Norvell, 1978; Amin et al., 2018):

\[ \text{Bioconcentration factor [BCF]} = \frac{[\text{Metal}]_{\text{shoot}}}{[\text{Metal}]_{\text{root}}} \]

Statistical Analysis

In this experiment, a randomized block design was utilized. Every experiment was run in triplicate. Each treatment's outcomes were given as an arithmetic mean plus standard error. One-way analysis of variance (ANOVA) was performed on the data using IBM SPSS Statistics version 22 (SPSS Inc. Chicago, USA). The homogeneity of variance was assessed using the Levene test, and differences between the averages of all treatments were tested at p ≤ 0.05 significance using Duncan’s Multiple Range Test (DMRT).

Results

Identification using 16s rRNA sequence

Molecular identification of isolates of rhizobacteria SMHMZ4 was conducted based on the outcomes of phytoextraction efficiency of Cd by *B. juncea*. The partially amplified and sequenced 16S mRNA gene was searched for sequence homology using BLAST. It was discovered that the partial nucleotide sequences of the bacterial strains transcribed by SMHMZ4 and Pseudomonas guguanensis strain CC-G9A were reasonably similar. They were recognized by phylogenetic analysis as gram-negative bacteria (Fig. 1). Partial sequences of rhizobacterial strains tagged with SMHMZ4 were given the following accession codes, which were then added to the NCBI GenBank database under the accession number MZ145097.

**Rhizobacteria's influence on soil metal mobility**

In this work, the levels of water-soluble Cd in soil were examined to assess the effectiveness of individual rhizobacteria in promoting soil Cd solubilization. When compared to the control treatment, soil inoculation with SMHMZ4 increased the quantity of soluble Cd in the soil by a factor of 7.78 (Fig. 2).

**Cadmium-tolerant rhizobacteria's effect on germination of *B. Juncea* seedlings under Cd stress environment**

Table 1 shows that rhizobacterial strain inoculation of seeds significantly increased plant height, root length, seed germination, and vigour index (p<0.05) compared to control. For seedlings treated with SMHMZ4 and SMHP23, maximum germination% of 80% and 76.66%, respectively, were registered. With Cd cultivation, uninoculated seedlings exhibited the lowest germination rate (43.33 %), considerably (p<0.05). The results showed that seeds treated with SMHMZ4 and SMHP23 had considerably (p<0.05) higher seedling vigour indexes, 768.0 and 691.77, respectively. The seeds treated with Cd yielded the lowest value (326.4).
Figure 1. The Phylogenetic relationship of rhizobacterial strain coded with SMHMZ4 closely related sequences based on partial 16S rRNA gene sequence.

Slika 1. Filogenetsko razmerje seva rizobakterij, kodiranega s tesno povezanimi sekvencami SMHMZ4 na podlagi delnega zaporedja gena 16S rRNA.

Figure 2. Effect of Cd-tolerant rhizobacterial isolates on the concentration of water-soluble Cd in soil. Treatment 1 Control (metal contaminated soils (100 μg g⁻¹ CdCl₂) with no bacteria inoculation); Treatment 2 (metal-contaminated soils + individual rhizobacteria). According to Duncan's Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant. Data are means (n = 3±SD), with an superscript indicating considerably higher values, and later alphabets indicating significantly lower values.

Slika 2. Vpliv izolatov rizobakterij, tolerantnih na Cd, na koncentracijo vodotopnega kadmija v tleh. Obdelava 1 Kontrola (s kovinami onesnažena tla (100 μg g⁻¹ CdCl₂) brez inokulacije bakterij); Obdelava 2 (s kovinami onesnažena tla + posamezne rizobakterije). Po Dunca novem Multiple Range Testu (p<0.05) so podobne črke v istem stolpcu statistično neznačilne. Podatki so povprečja (n = 3±SD), z nadnapisom, ki označuje znatno višje vrednosti, kasnejše črke pa označujejo bistveno nižje vrednosti.
Table 1. Effect of Cd-tolerant rhizobacteria and Cd on shoot length, root length, vigour index, and germination of *B. juncea* seedlings.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot length (cm)</th>
<th>Root length (cm)</th>
<th>Vigor index</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H₂O)</td>
<td>3.80±0.06</td>
<td>4.40±0.06</td>
<td>519.3±6.3</td>
<td>63.33</td>
</tr>
<tr>
<td>Control (M)</td>
<td>3.76±0.09</td>
<td>3.77±0.09</td>
<td>326.4±7.2</td>
<td>43.33</td>
</tr>
<tr>
<td>SMHMZ2 (M)</td>
<td>3.70±0.09</td>
<td>4.50±0.06</td>
<td>555.5±2.2</td>
<td>66.67</td>
</tr>
<tr>
<td>SMHMZ4 (M)</td>
<td>3.83±0.07</td>
<td>5.90±0.06</td>
<td>768.0±9.2</td>
<td>80</td>
</tr>
<tr>
<td>SMHPM4 (M)</td>
<td>3.43±0.12</td>
<td>5.37±0.03</td>
<td>586.6±10.2</td>
<td>66.67</td>
</tr>
<tr>
<td>SMHMMP23 (M)</td>
<td>3.60±0.06</td>
<td>5.87±0.09</td>
<td>691.7±6.47</td>
<td>76.66</td>
</tr>
</tbody>
</table>

Effect of Cd-tolerant rhizobacteria on *B. juncea* growth under Cd stress condition

In comparison to the same level of Cd stress without inoculation, the results showed that rhizobacteria SMHMZ4 significantly (p≤0.05) improved the root length (1.33-fold), shoot length (1.94-fold), fresh weight of shoot and root (1.15-fold and 1.80-fold), and dry weight of shoot and root (1.46-fold and 2.22-fold) of *B. juncea* at 94.95 μg g⁻¹ of Cd contaminated soil (Fig.3 and Table 2).

Table 2. Effect of Cd-tolerant rhizobacteria and Cd stress on the growth of *B. juncea*.

<table>
<thead>
<tr>
<th>Cd stress</th>
<th>Shoot length (cm)</th>
<th>Root length (cm)</th>
<th>Fresh weight of Shoot (g Pot⁻¹)</th>
<th>Dry weight of Shoot (g Pot⁻¹)</th>
<th>Fresh weight of Root (g Pot⁻¹)</th>
<th>Dry weight of Root (g Pot⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H₂O)</td>
<td>13.25±0.06</td>
<td>6.15±0.09</td>
<td>35.36±0.16</td>
<td>2.19±0.03</td>
<td>1.84±0.12</td>
<td>0.168±0.01</td>
</tr>
<tr>
<td>Control (M)</td>
<td>10.63±0.09</td>
<td>4.68±0.18</td>
<td>30.71±0.05</td>
<td>1.48±0.01</td>
<td>1.47±0.03</td>
<td>0.139±0.04</td>
</tr>
<tr>
<td>SMHMZ2</td>
<td>14.28±0.26</td>
<td>6.75±0.06</td>
<td>36.46±0.04</td>
<td>2.27±0.01</td>
<td>2.00±0.04</td>
<td>0.188±0.02</td>
</tr>
<tr>
<td>SMHMZ4</td>
<td>25.75±0.06</td>
<td>8.16±0.01</td>
<td>40.88±0.12</td>
<td>3.21±0.03</td>
<td>3.33±0.04</td>
<td>0.373±0.02</td>
</tr>
<tr>
<td>SMHPM4</td>
<td>15.45±0.06</td>
<td>7.55±0.06</td>
<td>37.11±0.03</td>
<td>2.30±0.01</td>
<td>2.15±0.01</td>
<td>0.205±0.01</td>
</tr>
<tr>
<td>SMHMMP23</td>
<td>24.38±0.13</td>
<td>7.96±0.05</td>
<td>40.05±0.08</td>
<td>3.08±0.03</td>
<td>3.2±0.01</td>
<td>0.353±0.05</td>
</tr>
</tbody>
</table>

Note: According to Duncan’s Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant. Data are means (n = 4±SD), with a superscript indicating considerably higher values and later alphabets indicating significantly lower values.
Effect of Cd and Cd-tolerant rhizobacteria on pigments, total phenolic content, flavonoid content, and proline of *Brassica juncea*

Rhizobacteria SMHMZ4 considerably raised the carotenoid and chlorophyll a and b content of the plant as compared to the non-inoculated control plant (Fig. 4). *B. juncea* treated with hazardous metal (Cd) showed higher amounts of total phenols, flavonoids, and proline; these levels increased even more following inoculation with Cd-tolerant rhizobacteria. After a month of exposure to Cd toxicity, the total phenols, flavonoids, and proline content level in a *B. juncea* plant (187.67, 61.32, and 7.86%, respectively). But under Cd-stressed conditions, supplementation with SMHMZ4 further raised total phenols, flavonoids, and proline levels by 124.46, 73.09, and 66.11%, respectively (Table 3).

![Figure 4.](image)

**Figure 4.** Effect of Cd and Cd-tolerant rhizobacteria on pigments (Chlorophyll a and b, carotenoid content) of *B. juncea* under pot experiments. Note: According to Duncan’s Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant. Data are means (n = 4±SD), with a in superscript indicating considerably higher values, and later alphabets indicating significantly lower values.

**Table 3.** Effect of Cd-tolerant rhizobacteria and Cd stress on the level of total phenols, flavonoids, and proline content in *B. juncea*.

<table>
<thead>
<tr>
<th>Cd stress</th>
<th>Total phenol content mg g⁻¹ DW</th>
<th>Flavonoid content mg g⁻¹ DW</th>
<th>Proline µmole g⁻¹ DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H₂O)</td>
<td>5.03±0.15e</td>
<td>1.06±0.03f</td>
<td>3.94±0.0e</td>
</tr>
<tr>
<td>Control</td>
<td>14.47±0.09d</td>
<td>1.71±0.03e</td>
<td>4.25±0.0d</td>
</tr>
<tr>
<td>SMHMZ2</td>
<td>17.21±0.10c</td>
<td>2.44±0.05c</td>
<td>4.49±0.09c</td>
</tr>
<tr>
<td>SMHMZ4</td>
<td>32.48±0.14a</td>
<td>2.96±0.03a</td>
<td>7.06±0.10a</td>
</tr>
<tr>
<td>SMHMP4</td>
<td>15.20±0.10d</td>
<td>2.26±0.03d</td>
<td>4.33±0.0cd</td>
</tr>
<tr>
<td>SMHMP23</td>
<td>31.23±0.06b</td>
<td>2.80±0.03b</td>
<td>6.60±0.0b</td>
</tr>
</tbody>
</table>

Note: According to Duncan’s Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant. Data are means (n = 4±SD), with a in superscript indicating considerably higher values and later alphabets indicating significantly lower values.
Effect of Cd-tolerant rhizobacteria and Cd on the growth tolerance indices (TIs) of *B. Juncea*

Our study examined *B. juncea*’s tolerance indices (TIs) in the presence of Cd and Cd-tolerant rhizobacteria. The TIs for root and shoot lengths, fresh and dried weights of the roots and shoots, and weights during Cd treatment are displayed in Table 4. Other treatments include metal stress without bacterial inoculation and treatment with bacterial inoculation plus individual metal.

Table 4. Effect of Cd-tolerant rhizobacteria and Cd stress on the growth of *B. juncea*.

<table>
<thead>
<tr>
<th>Cd stress</th>
<th>Tolerance Index (TIs)</th>
<th>Tolerance Index % (TIs)</th>
<th>Tolerance Index (TIs)</th>
<th>Tolerance Index % (TIs)</th>
<th>Tolerance Index (TIs)</th>
<th>Tolerance Index % (TIs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.19±0.71e</td>
<td>76.02±2.85i</td>
<td>86.84±1.65c</td>
<td>6716±0.34d</td>
<td>79.62±1.62e</td>
<td>82.35±0.0e</td>
</tr>
<tr>
<td>SMHMZ2</td>
<td>107.74±2.0d</td>
<td>109.76±1.055</td>
<td>103.10±0.123</td>
<td>103.07±0.221</td>
<td>108.97±2.272</td>
<td>111.76±2.45b</td>
</tr>
<tr>
<td>SMHMZ4</td>
<td>194.34±0.49a</td>
<td>132.64±0.244</td>
<td>115.61±0.33a</td>
<td>146.36±1.166</td>
<td>181.11±2.35a</td>
<td>220.59±12.82a</td>
</tr>
<tr>
<td>SMHMP4</td>
<td>116.60±0.49c</td>
<td>122.76±1.055</td>
<td>104.95±0.09c</td>
<td>104.55±0.411</td>
<td>116.71±0.26c</td>
<td>122.06±1.47b</td>
</tr>
<tr>
<td>SMHMMP23</td>
<td>183.96±0.94a</td>
<td>129.43±0.84a</td>
<td>113.24±0.24a</td>
<td>140.34±1.36b</td>
<td>173.91±0.22a</td>
<td>210.30±4.41b</td>
</tr>
</tbody>
</table>

Note: According to Duncan’s Multiple Range Test (*p*<0.05), similar letters in the same column are statistically non-significant. Data are means (*n* = 4±SD), with ain superscript indicating considerably higher values and later alphabets indicating significantly lower values.

Cadmium accumulation in *B. juncea* plant

When compared to non-inoculated controls in this study, there was a significant (*p*≤0.05) increase in Cd accumulation in the root and shoot tissues of *B. juncea* due to the increase in plant biomass and metal availability brought about by inoculation with toxic Cd-tolerant rhizobacteria treatments (Table 5). For example, the rhizobacteria SMHMZ4 significantly increased the Cd level in *B. juncea* roots and shoots tissues by 24.03 and 71.71%, respectively (*p*≤0.05).

Table 5. Effect of Cd and Cd-tolerant rhizobacteria on accumulation of Cd in root and shoot of *B. juncea*.

<table>
<thead>
<tr>
<th></th>
<th>Root Concentration (µg g⁻¹ DW)</th>
<th>Shoot Concentration (µg g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H2O)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control</td>
<td>95.32±0.05d</td>
<td>95.05±0.05e</td>
</tr>
<tr>
<td>SMHMZ2</td>
<td>114.25±0.03c</td>
<td>134.12±0.01d</td>
</tr>
<tr>
<td>SMHMZ4</td>
<td>118.23±0.14a</td>
<td>163.21±0.13a</td>
</tr>
<tr>
<td>SMHMP4</td>
<td>114.03±0.01c</td>
<td>138.11±0.04c</td>
</tr>
<tr>
<td>SMHMMP23</td>
<td>117.22±0.14b</td>
<td>156.29±0.10b</td>
</tr>
</tbody>
</table>

Note: According to Duncan’s Multiple Range Test (*p*<0.05), similar letters in the same column are statistically non-significant. Data are means (*n* = 4±SD), with ain superscript indicating considerably higher values and later alphabets indicating significantly lower values.
Phytoremediation potential of *Brassica juncea*

Inoculation with Cd-tolerant rhizobacteria led to a considerable increase in Cd accumulation by the roots and shoots of *B. juncea* compared to the non-inoculated control. The *B. juncea* root and shoot exhibit a significant (*p ≤0.05*)

Discussion

Based on the outcomes of phytoextraction efficiency of *B. Juncea*, molecular identification of rhizobacteria SMHMZ4 was carried out and identified as *P. guguanensis* SMHMZ4 and submitted to NCBI Genebank with accession number MZ145097 (Fig. 1).

The low availability of metal(s) in soils is another factor restricting phytoextraction, in addition to the constrained biomass of plants. Rhizobacteria can transform dangerous metal(s) into less toxic forms that plants can absorb, hence increasing the availability of metal(s) in soils (He et al., 2013). Water-soluble Cd levels demonstrated low levels of Cd availability in soils lacking a bacterial inoculum (control set). All of the rhizobacteria utilized in this investigation are capable of both insoluble Cd solubilization and Cd tolerance (Fig. 2). The synthesis of organic acids like indole-3-acetic acid, phosphate solubilization, and oxidation-reduction reactions may be connected to the increase in water-soluble metal concentrations brought on by rhizobacteria (Sharma and Saraf, 2023a).

Germination parameters were investigated to learn more about plant tolerance to cadmium, the quality of seeds, and the ability of rhizobacteria to promote plant growth. The health of the seedlings produced, including their capacity to withstand a range of stressful conditions,
is assessed by the Vigor Index. As compared to the non-inoculated control, the results displayed in Table 1 demonstrate that inoculating B. juncea seeds with SMHMZ4 raised the shoot length, root length, seed germination, and vigour index by 1.02-fold, 1.56-fold, 1.85-fold, and 2.35-fold, respectively. Increased synthesis of many metabolites, including GA, cytokinins, alpha-amylase, and IAA, was linked to the better health of seedlings (Sharma et al., 2023a: 2023c).

Compared to uninoculated plants growing in non-contaminated soil, cadmium poisoning considerably shortened the length of the roots and shoots (Table 2). Also, metal(s) can prevent plants from absorbing nutrients, leading to stunting, chlorosis, and even plant death (Sadiq et al., 2017). Rhizobacteria provide essential nutrients to their host plants, eliminate toxins that hinder their growth, and support one another through biochemical and physical processes. This enhances the intake of minerals and nutrients and promotes the growth of plant roots and shoots. Plants inoculated with SMHMZ4 exhibited more chlorophyll content than non-inoculated control plants, with SMHMP23 following closely behind (Fig. 4). Similar results in Fe-contaminated soil were reported by Jinal et al. (2019).

The tolerance index values of plants inoculated with PGPR were higher than those of the non-inoculated controls (Table 4). Compared to the control, SMHMZ4 dramatically increased the Cd concentration in the root and shoot in the current investigation (Table 5). A similar observation was made by Din et al. (2020), who reported that the synthesis of plant growth substances like IAA, siderophore production, and the solubilization of minerals like phosphorus caused the improved mineral and nutrient uptake resulting from the bacterial inoculation. These processes promote plant root elongation and shoot growth. Ammonia and HCN generation are crucial factors that have been proven to significantly affect biocontrol and may also indirectly impact root and plant growth (Rochlani et al., 2022). The capacity of a metal(s) to restrict root and/or shoot growth in a medium is used to determine a plant's tolerance to heavy metal stress. If the TI value is less than one, it indicates that metal contamination caused stress to the plant, which led to a net reduction in biomass. On the other hand, plants that have evolved tolerance and a net increase in biomass (hyperaccumulator) are indicated by TI values greater than one (or > 100%). In contrast to the control treatments, the plant is not impacted by metal pollution if the TI value is 1 (Amin et al., 2018).

Comparison to non-inoculated controls, the results demonstrated that treatments containing rhizobacteria under Cd stress had tolerance index values of more than one, indicating that plants developed tolerance significantly and with a notable net increase in biomass. Plants that were injected with PGP rhizobacteria experienced a reduction in metal stress. Additionally, the microbial inoculations resulted in a significant (p≤0.05) increase in root-to-shoot translocation. These findings align with previous research (Mendoza-Hernandez et al., 2019; Jeyasunda et al., 2021) that found that Brassica plants inoculated with different microbial strains had higher Cd concentrations than the corresponding controls.

According to Wang and colleagues (2022), inoculants derived from Plant growth promoting bacteria (PGPB) consortia, both rhizospheric and endophytic, enhanced plant growth (6.9%–22.1%), facilitated B. juncea's Cd uptake (230.0%–350.0%), increased Cd phytoextraction efficiency (343.0%–441.0%), and improved soil Cd removal rates (92.0%–144.0%). According to Ali et al. (2021), the utilization of toxic metal-resistant polymer-grafting resin (PGPR) for metal(s) alleviation is not only cost-effective and environmentally friendly, but it also fosters plant growth by reducing the stress caused by metal(s) and generating compounds that stimulate plant growth. A comparable study on the synergistic effects of Pseudomonas aeruginosa, Burkholderia gladioli, and plant growth-promoting rhizobacteria on several physiological and biochemical activities of 10-day-old Solanum lycopersicum seedlings under Cd stress was published by Khanna et al. in 2019. In seedlings treated with Cd toxicity, total phenols, flavonoids, and proline levels increased by 30.2%, 92.72%, and 60.11 per cent, respectively (Table 3). In contrast, P. aeruginosa (M1) supplementation raised proline, flavonoids, and total phenols levels in Cd-stressed seedlings by 51.3%, 28.33%, and 59.51%, respectively (Table 3). In contrast, P. aeruginosam (M1) supplementation raised proline, flavonoids, and total phenols levels in Cd-stressed seedlings by 51.3%, 28.33%, and 59.51%, respectively (Table 3).

By characterizing metal accumulation and translocation behaviours in plants, the bioconcentration factor (BCF), bioaccumulation coefficient (BAC), and translocation factor (TF) values (Fig. 6) help determine plant suitability for phytoextraction. According to Amin et al. (2018), plants that have BAC, TF, and BCF values greater than one are considered potential phytoextractors and acceptable for phytoextraction, while those that have TF and BCF...
values less than one are not suitable for phytoextraction or phytostabilization. In our investigation, *B. juncea*'s capacity to absorb additional Cd from the soil with a translocation factor value >1 was enhanced by the rhizobacteria SMHMZ4 (Fig. 5).

**Conclusions**

The current study concluded that Cd negatively affects *Brassica juncea* plant development and health indices (total phenol, flavonoid, and proline). Nevertheless, inoculating plants with *Pseudomonos guguanensis* SMHMZ4 multi-metal tolerant rhizobacterial strains not only shields them from Cd-induced growth inhibition but also promotes plant growth, biomass production, and metal bioavailability in the soil, all while simultaneously increasing Cd accumulation in the aerial part of the plant caused by *B. juncea*. *P. guguanensis* SMHMZ4 rhizobacteria are essential for boosting the values of the translocation factor, bioconcentration factor, and bioaccumulation coefficient; these factors improve the effectiveness of phyto remediation and reduce the amount of Cd in polluted soil. The findings showed that rhizobacteria-rich *B. juncea* amendments were more successful at cadmium green remediation. Additionally, because *B. juncea* plant species may supply valuable biomass that can be used to create income and remediate places contaminated with metals, they have ecological and economic significance. After harvesting, the biomass could be burned and disposed of, or the metals could be extracted and used to make biofuels again.

**Author Contributions**

Conceptualization: S.S., Methodology: S.S. & M.S., Software: S.S., Validation: S.S., Formal Analysis: S.S., Investigation: B.O.K., S.S., Resources: S.S., Data Curation: S.S., Writing – Original Draft: S.A.S., Writing – Review & Editing: S.S., M.S., Supervision: S.S. All authors have read and agreed to the published version of the manuscript.”

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**Conflicts of Interest**

The authors declare that they have no known conflicts of interest associated with this study.

**References**


Sharma S., Saraf M., (2022a). Isolation, Screening and Biochemical characterizations with multiple traits of Heavy Metal Tolerant Rhizobacteria from Mining Area and Landfill site. Advances in Bioressearch, 13 (1), 147-156.


