

Article

Metal-Tolerant Bioinoculant *Pseudomonas putida* KNP9 Mediated Enhancement of Soybean Growth under Heavy Metal Stress Suitable for Biofuel Production at the Metal-Contaminated Site

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Abstract: The contamination of agricultural land with heavy metals is a global concern. Agricultural products produced in heavy metal-contaminated soil are prone to metal accumulation, and thus, are less fitted for consumption due to food safety issues. The cultivation of biofuel crops in contaminated soil would provide immediate economic benefit to the landholders while simultaneously reclaiming contaminated sites in the long run. The use of edible soybean for biodiesel production is discouraged due to the negative impact on food security. However, soybean produced in metal-contaminated soil would be suitable for biodiesel production. In this study, the tolerance and metal bioaccumulation potential of *Pseudomonas putida* KNP9 for Pb and Cd is investigated, and KNP9 is tested for soybean growth enhancement in cadmium and lead-amended soil. The maximum metal tolerance for the Pb and Cd in KNP9 was 1580 μ M and 546 μ M, respectively. KNP9 was found to be effective in removing both Pb and Cd from the solution. SEM-EDX revealed that KNP9 bioaccumulates both Pb and Cd. In pot trial studies, KNP9 was found to be effective in enhancing soybean growth with respect to untreated control under lead and cadmium stress. Thus, KNP9 inoculation protects soybean plants from the detrimental effects of cadmium and lead stress. Therefore, metal bioaccumulating bacterium *P. putida* KNP9 inoculation in soybean is a promising strategy for soybean growth enhancement, which could be utilized for enhanced biodiesel production from soybean at metal-contaminated sites.

Keywords: biodiesel; heavy metals; *Pseudomonas putida* KNP9; soybean



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1. Introduction

The heavy metal pollution of agricultural land is one of the major environmental problems due to its toxic effects on the environment and tendency to cause adverse health effects on humans. The primary sources of heavy metal pollution in agricultural land include mining, milling, surface finishing industries, and contaminated irrigation water [1,2]. Lead (Pb) and cadmium (Cd) are the two most common toxic heavy metals which can pollute the agricultural produce cultivated on contaminated land. Pb exposure to humans results in learning disabilities, reduced fertility, and cardiovascular diseases [3]. On the other hand, Cd causes various kidney, bone, and lung diseases including cancer.

Agricultural products produced in heavy metal-contaminated soil are prone to metal accumulation, and thus, are less fitted for consumption due to food safety issues [4]. Although metal-contaminated land can be remediated through physical and chemical approaches, these processes are expensive, slow, and cannot remove all heavy metals below the limits. Moreover, these methods are not suitable in places where the heavy metal concentration is low, but still above the permissible limits. On the other hand, the production of biofuels requires substantial areas of agricultural land [5]. Biofuels

produced from renewable organic material are effective in reducing the undesirable impact of fossil fuel production such as greenhouse gas emissions and the dependence on unstable foreign suppliers. Therefore, the cultivation of biofuel crops in contaminated soil would provide immediate economic benefit to the landholders while simultaneously reclaiming contaminated sites in the long run and reducing the undesired effects of fossil fuels on the environment.

Soybean (*Glycine max*) is the potential crop for biofuel production, as soybean oil is used as a major feedstock for biodiesel production [6]. However, soybean is an important crop that is grown for oil and protein, and using this extensively for biodiesel production would have a negative impact on food security. The cultivation of soybean in metal-contaminated sites for biodiesel production would be a suitable strategy for the management of contaminated soil and for enhancing the livelihood of the farmers. Soybean also has a wide adaptability, which makes it ideal for cultivation in metal-contaminated sites for biofuel production with less economic input [7]. Thus, soybean cultivation for biodiesel production in heavy metal-contaminated soil is a sustainable approach for energy production and polluted agricultural soil management.

Microorganisms are widely implemented for the bioremediation of metal-contaminated soil [8–10]. The ability of the microorganism to grow under high metal concentrations might result from a specific mechanism of resistance. These microorganisms carry out heavy metal detoxification by several mechanisms which mainly include valence transformation, extracellular complexation, volatilization, extracellular chemical precipitation, biosorption, and bioaccumulation [11,12]. The inoculation of metal-resistant microbial inoculants in plants grown in heavy metal-contaminated sites would circumvent the negative effects of metals on plants. Therefore, bioinoculant inoculation in soybean grown in metal-contaminated sites would enhance plant growth, which will ultimately be beneficial for biodiesel production and the management of metal-contaminated agricultural land.

In this study, an indigenous Pb- and Cd-resistant bacterium, *Pseudomonas putida* KNP9 (DQ205427.1), which was previously isolated by Tripathi et al., 2005 from a metal-contaminated site, was evaluated for tolerance in different concentrations of Pb and Cd, and heavy metal bioaccumulation potential [13]. Further, KNP9 was evaluated for plant growth promotion and heavy metal uptake in soybean plants under Pb and Cd stress to assess the bioremediation and plant growth-enhancing potential of KNP9.

2. Materials and Methods

2.1. Heavy Metal Tolerance Experiment

To test the tolerance of KNP9 strain to heavy metals, this bacterium was grown in a nutrient broth medium with varying concentrations of Pb and Cd at 30 °C with 160 rpm. Lead and cadmium were obtained by supplementing the growth medium with lead acetate and cadmium chloride, respectively. Absorbance at 600 nm was recorded at a different time interval to monitor the bacterial growth using a UV-Visible Spectrophotometer (Spectronics-20).

2.2. Heavy Metal Removal Studies

To determine the heavy metal removal efficacy of KNP9, 1 mL of overnight culture was mixed with 99 mL of the nutrient broth medium, supplemented with Pb (100 µg/mL) and Cd (50 µg/mL), and incubated on an incubator shaker for seven days under constant temperature. A total of 5 mL of bacterial culture was removed at required time intervals, followed by centrifugation at 7000 rpm for 7 min at 4 °C. The supernatant was used to analyze the Pb and Cd concentrations. Bacterial cell pellets were dried overnight at 90 °C in the oven. Dried pellets were treated with 5 mL of concentrated HNO₃ and kept overnight. This was followed by the incineration of acid-lysed pellets on a sand bath for 4–6 h under slow heating (45 °C–50 °C) until the white residue was formed. Subsequently, the residue was treated with 1 mL of concentrated HNO₃ and HClO₄ (60%) in a ratio of 6:1, and incinerated on a hot plate for three hours. After the incineration, this white residue

was suspended in 3 mL of deionized water. Cd and Pb concentrations in incinerated cell pellets and the supernatant were then quantified using a flame atomic absorption spectrophotometer (GBC Avanta ΣGBCFS 3000). Estimation of cadmium was performed at 228.8 nm wavelength, with a sensitivity of 0.009 ppm, with the current lamp of 3 mA. For lead estimation, wavelength at 217 nm and the sensitivity of 0.06 ppm with the current lamp of 5 mA were used. The used flame type was air acetylene (oxidizing).

2.3. SEM-EDX Analysis

In order to investigate the effect of heavy metals on bacterial morphology and to confirm the presence of Pb and Cd within the bacterial cell, SEM-EDX analysis was performed. For preparation for scanning electron microscopy, the cell suspension was treated with 1% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 9.0) and kept at 4 °C overnight. This fixed cell suspension was then smeared over the coverslips and coated with a polyline for 30 min. Samples were then dehydrated in graded ethanol series and transferred to the bucket fully immersed in absolute ethanol, followed by drying for 20 min in a critical point dryer (Balzer Union). Coating of cells was performed with 90 Å thick gold palladium in polaron SC7640 sputter coater for 30 min. Cells were viewed at 15 kv using Leo 430 electron microscope. Energy-dispersive X-ray was used for confirming Cd and Pb bioaccumulation.

2.4. Pot Trial Experiment

Surface sterilization of soybean seeds was performed via treatment of 0.1% HgCl₂ solution for 2 min, followed by washing three times with sterile distilled water. Seed coating of soybean seeds was performed with the 0.5 optical density (OD) culture of KNP9 and carboxy methyl cellulose, followed by drying at room temperature. Control seeds were treated with an equal volume of sterile water and carboxy methyl cellulose. Soil for conducting the pot trial experiment was collected from the heavy metal contamination-free agricultural field, where both the metal concentrations were below the detection limit. After collection, the soil was sterilized by repeated autoclaving. Seeds were sown in plastic pots filled with sterilized soil. Five seeds were sown per pot, and each treatment had four replicates. Cadmium-treated soil had 110 µM CdCl₂, while lead-treated soil had 660 µM (CH₃COO)₂Pb. The pot trial was conducted in a poly house at 30 °C temperature. Regular irrigation in pots was performed with autoclaved tap water. Plants were harvested after 25 days of cultivation, and plant growth parameters were recorded. Plant shoot length, root length, fresh weight, and dry weight were recorded. Plant chlorophyll was also quantified following the previously described method [14].

2.5. Metal Quantification in Plant and Soil Samples

Shoots and roots were collected and separately dried at 150 °C in the oven. A total of 1 g of dried leaves sample was ground using mortar and pestle. Dried roots were minced with a razor blade, followed by wet ashing [15]. Cd and Pb content in root and shoot samples were analyzed using graphite furnace atomic absorption spectroscopy. For heavy metal quantification in soil, ten grams of dried soil sample was taken in 125 mL conical flasks, and 20 mL of diethylene triamine penta acetic acid (DTPA) extracting solution was added and shaken for 2 h at 120 cycles/min. This soil suspension was filtered and processed for metal quantification. Cd and Pb content in soil samples were analyzed using graphite furnace atomic absorption spectroscopy.

3. Results

3.1. Heavy Metal Tolerance Study

The heavy metal tolerance study revealed that KNP9 resisted very high concentrations of Cd and Pb in the growth medium. The maximum metal tolerance for the lead and cadmium were 1580 µM and 546 µM, respectively. The effect of cadmium and lead on the generation time of KNP9 was also studied to observe the effect of metals on its growth.

It was evident that the generation time was found to be longer in the presence of lead (60 ± 0.86 min) and cadmium (70 ± 1.1 min) in comparison to the control (without metal 50 ± 0.57 min). However, subsequently, the culture achieved a similar biomass in all three conditions. This change in the growth rate could be due to the potentially toxic effects of heavy metals, leading to an extended lag phase, lower cell density, or bacterial cell death [16].

3.2. Heavy Metal Removal Studies

The heavy metal removal study revealed that KNP9 not only resisted high concentrations of Cd and Pb, but also removed the heavy metals from the culture medium containing heavy metals. By quantifying the heavy metals in the pellet and supernatant at different time intervals, it was found that the maximum soluble Cd (85.73%) was removed from the solution after the third day during the stationary phase (Table 1). However, the cadmium concentration increased in the supernatant after that. By the end of the sixth day, 56.94% of cadmium removal from the solution was observed. The cadmium concentration increased in the cell biomass ($111.5 \mu\text{M}$) until the third day, when it decreased, which could be due to the lysis of the cell (Table 1). On the other hand, the lead concentration decreased by 97.6% up to the fourth day from the solution; after that, it increased in the supernatant. By the end of the sixth day, 50.23% of lead removal from the solution was observed. Similarly, the lead concentration increased in the cell biomass up to the fourth day ($792 \mu\text{M}$); after that, it decreased (Table 1).

Table 1. Heavy metal accumulation study of *Pseudomonas putida* KNP9 under Pb and Cd.

Days	Pb in μM		Cd in μM	
	Pellets	Supernatant	Pellets	Supernatant
1st day	615 ± 2.51	185 ± 4.04	106 ± 1.80	44 ± 0.50
3rd day	753 ± 9.64	47 ± 3.60	111.5 ± 3.01	38.9 ± 0.58
4th day	792 ± 4.00	7.21 ± 0.79	103 ± 1.52	47.3 ± 0.90
6th day	647 ± 10.96	153 ± 1.00	32.14 ± 1.43	117.4 ± 0.30

Each value was the mean of three replicates. Values after \pm represent the standard error of the mean.

3.3. SEM-EDX Analysis

Scanning electron microscopy (SEM) was performed to observe the changes in the bacterial cell morphology in response to cadmium and lead (Figure 1 and Table 2). The control cells (without metal) were rod-shaped and had smooth surfaces. The cell size of KNP9 was found to increase in the presence of Cd and Pb. In the presence of lead, the cell length and breadth increased by 108% and 16.2%, respectively. However, in the presence of cadmium, there was no change in the cell length, while the cell breadth increased by 62%. Furthermore, an analysis via EDX revealed that lead and cadmium were detected inside the cells of KNP9 when it grew in the presence of lead and cadmium (Figure 2). However, no heavy metals were detected inside the bacterial cells in the control (without heavy metals). Therefore, the heavy metal removal studies followed by the SEM-EDX conclude that the increase in cell size in KNP9 under Pb and Cd stress is due to the intracellular accumulation of these heavy metals.

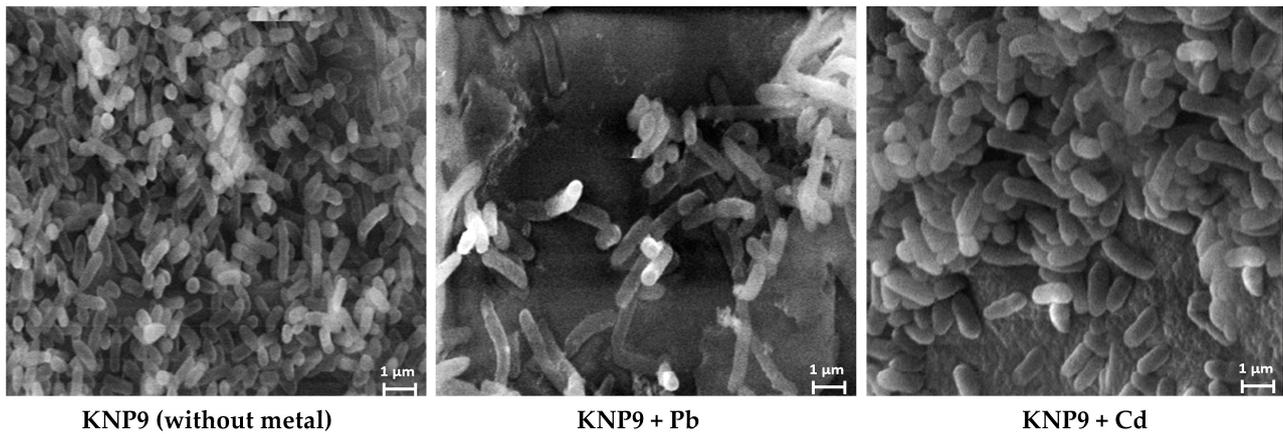


Figure 1. Scanning electron micrographs of KNP9 without the metals, and under Pb and Cd stress at 15.0 kx after 14 h.

Table 2. Scanning electron microscopy-based comparative morphological analysis of *Pseudomonas putida* KNP9 under Pb and Cd.

Treatments	Length (μ)	Breadth (μ)
Without metal (control)	1.086 ± 0.01	0.37 ± 0.03
In the presence of Pb	2.26 ± 0.02	0.43 ± 0.04
In the presence of Cd	1.086 ± 0.01	0.607 ± 0.02

Each value was the mean of three replicates. Values after ± represent the standard error of the mean.

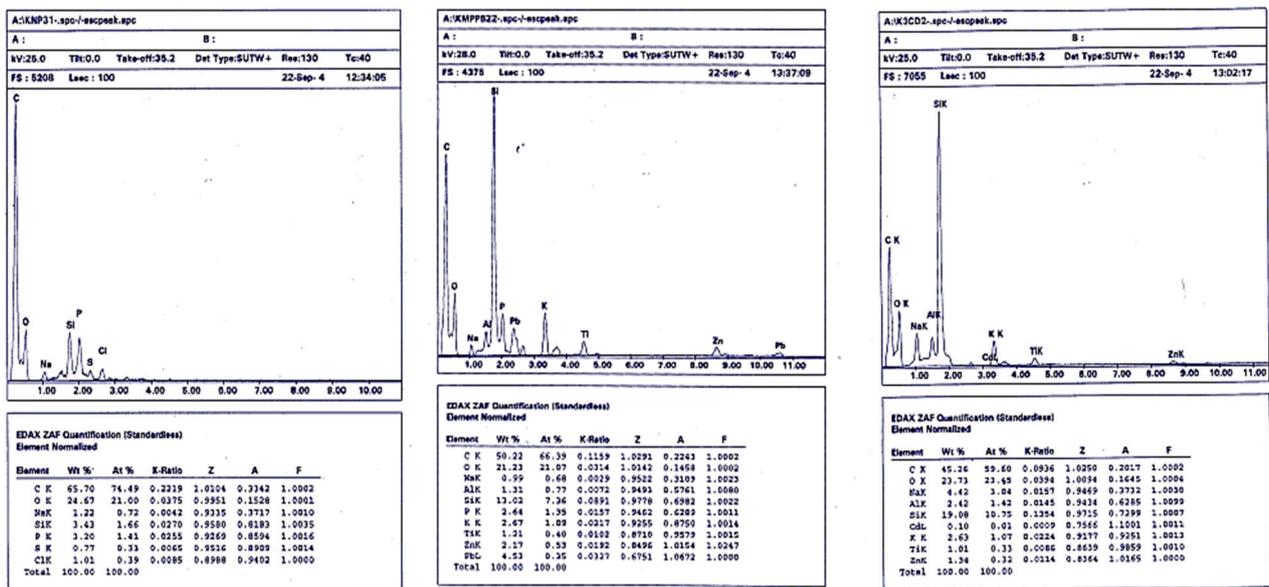


Figure 2. EDX analysis of KNP9 without the metals and under Pb and Cd stress after 14 h.

3.4. In Situ Pot Trial Experiment on Soybean

To assess the ability of KNP9 to promote plant growth in soybean, a pot experiment was conducted using sterilized soil (Figures S1 and S2). The amendment of lead in the soil causes a significant reduction in the shoot length, root length, fresh weight, dry weight, and total chlorophyll content of soybean with 20%, 9.7%, 33.7%, 43%, and 20%, respectively, in comparison to the control (without lead). Similarly, cadmium treatment resulted in

a significant reduction in the soybean shoot length, root length, fresh weight, and dry weight with a reduction of 6.8%, 21.5%, 15.84%, and 26.6% in comparison to the control, respectively. A significant enhancement in the plant growth parameters of soybean was evident in the bioinoculant treatment. KNP9 treatment resulted in an increase in the shoot length, root length, fresh weight, and dry weight in comparison to the uninoculated one by 70.6%, 28.57%, 50.72%, and 76.4%, respectively (Table 3). The soybean shoot and root lead content were reduced to the extent of 48.1% and 41.83%, respectively. The total soil lead content (50.4 μM) in the absence of bioinoculant was greater than in the presence of KNP9 (13.8 μM).

Table 3. Impact of KNP9 on Cd and Pb toxicity of soybean in autoclaved soil under a greenhouse at 30 °C after 25 days.

		Shoot Length ^b (cm)	Root Length ^b (cm)	Fresh Weight ^a (g)	Dry Weight ^a (g)	Chlorophyll ^c (mg g ⁻¹)
Without Metal	Mean (control)	26	9.3	1.01	0.3	4.79
	Mean (treated)	33.5 (28.8)	12 (29.0)	1.34 (32.6)	0.32 (6.66)	5.15 (7.5)
With Lead	Mean (control)	21.0	8.4	0.67	0.17	3.8
	Mean (treated)	36.0 (70.6)	10.8 (28.57)	1.01 (50.74)	0.30 (76.47)	4.9 (28.94)
Critical difference at 5%		6.02	4.29	0.29	0.29	1.05
With Cadmium	Mean (control)	24.60	6.4	0.85	0.22	2.27
	Mean (treated)	35.70 (45.12)	10.90 (70.3)	1.24 (45.8)	0.32 (45.0)	4.5 (98.25)
Critical difference at 5%		4.91	2.16	0.23	0.24	1.34

^a Mean of three replicates of 10 plants. ^b Mean of ten replicates. ^c Mean of four replicates. Value in the parentheses () indicates % increase over the respective control.

KNP9 treatment in cadmium-containing soil increased the shoot length, root length, fresh weight, and dry weight by 45.12%, 70.3%, 45.8%, and 45%, respectively, over the uninoculated control (Table 3). Moreover, the Cd content in the soybean shoots and roots was found to decrease by 54.83% and 92.82%, respectively when treated with KNP9. The soil Cd content was reduced to 0.392 μM in the KNP9-treated soil compared to 0.920 μM in the uninoculated cadmium-treated soil.

4. Discussion

The heavy metal contamination of agricultural land is a global concern affecting both crop productivity and food security. Agricultural products produced in heavy metal-contaminated soil are prone to metal contamination, and thus, are less fitted for consumption. Metal-tolerant microorganisms can tolerate wide concentrations of heavy metals, and thus, are suitable for addressing problems associated with heavy metal pollution. The ineffectiveness of conventional heavy metal soil remediation techniques leads to the search for environmentally friendly and cost-effective methods for metal removal from polluted soil. The use of metal-tolerant microorganisms for the bioremediation of heavy metals from contaminated environments is a sustainable approach to tackle this problem [2,12]. In recent years, extensive efforts were made to develop management strategies and remediation strategies for the heavy metal-contaminated soil. In this study, the in vitro bioremediation potential of heavy metal-resistant *P. putida* KNP9 is studied. Further, the impact of KNP9

inoculation on soybean growth promotion and heavy metal uptake for biodiesel production is also studied in Pb- and Cd-contaminated soil.

The maximum metal tolerance in *P. putida* KNP9 for lead and cadmium was 1580 μM and 546 μM , respectively. However, generation time was found to be longer in the presence of lead (60 ± 0.86 min) and cadmium (70 ± 1.1 min) in comparison to the control (without metal 50 ± 0.57 min). Slow doubling time in the presence of both metals suggests that both Cd and Pb reduce the KNP9 growth to a little extent when compared to the growth without heavy metals. Further, quantification of heavy metals in the cell pellet and supernatant at different time intervals revealed that this bacterium effectively bioaccumulates both Cd and Pb. Bioaccumulation of heavy metals by bacteria is a well known metal remediation mechanism where bacteria reduce the effective metal concentration in the vicinity of plant roots thus protecting plants from the negative effects of heavy metals and metalloids [11].

Morphological variations in bacteria are the primary stress combating mechanism under heavy metal stress [17]. Enlargement of cell size in KNP9 was observed while growing in the presence of Cd and Pb. Heavy metal removal studies followed by SEM-EDX conclude that the increase in cell size in KNP9 under Pb and Cd stress is due to the intracellular accumulation of these heavy metals. In the presence of the lead, cell length and breadth increased by 108% and 16.2%, respectively. However, in the presence of cadmium, no change in cell length was observed but cell breadth was found to be increased by 62%. Similar morphological changes in the cell size under heavy metal stress were reported in a previous study where cell size in manganese-oxidizing bacteria was found to increase under cobalt stress [17]. Morphological changes in cells were observed in the presence of Ni and Cr when compared to the control while showing the accumulation of metal inside the cells which was evident in the TEM micrographs of *Lactobacillus plantarum* MF042018 [18]. Related results were obtained in the previous study where biosorption of Pb^{+2} in *Gelidium amansii* was found to reach the maximum when Pb^{2+} concentration reached up to 200 mg/L [19]. In the acidophilic heterotrophic bacterium, *Acidiphilium symbioticum* H8 the changes in size from loosely packed coccobacillus-type (normal cells) to chains of coccoidal lenticular shape with constrictions at the junctions between them were found in the presence of Cd [20].

Decreased plant growth is one of the indicators of heavy metal stress in contaminated soils compared to growth in normal soil. Inoculation of PGPR strains in metal-contaminated soil promotes plant growth. In this study, Cd and Pb were found to reduce soybean plant growth which was evident by the decreased shoot length, root length, fresh weight, and dry weight. However, inoculation of KNP9 resulted in enhanced shoot length, root length, fresh weight, and dry weight over the uninoculated control which shows the plant growth protecting attribute of KNP9 under Cd and Pb stress. KNP9 had increased the soybean shoot length, root length, fresh weight, and dry weight under lead treatment in comparison to the uninoculated one by 70.6%, 28.57%, 50.72%, and 76.4% percent, respectively. On the other hand, bioinoculation with KNP9 in cadmium treated soil increased the soybean shoot length, root length, fresh weight, and dry weight by 45.12%, 70.3%, 45.8%, and 45%, respectively, over the uninoculated control (Table 3). Thus, KNP9 is effective to enhance soybean plant growth in Cd- and Pb-contaminated soil.

Plant growth-promoting rhizobacteria *Acinetobacter beijerinckii* (C5) and *Raoultella planticola* (C9) were reported to enhance the physiological and metabolic responses of soybean under chromate and arsenic stress [21]. Similarly, the novel *Bacillus cereus* strain, ALT1, enhances the growth of soybean under cadmium stress [22]. Moreover, the establishment of a symbiotic association between soybean and *Bradyrhizobium japonicum* is also affected by heavy metals [23]. If plant and symbiotic bacterial partner tolerance to heavy metals is improved under heavy metal stress, it is possible to have an effective nitrogen-fixing symbiosis under metal stress. The plant growth-enhancing properties of PGPR were also reported in several other plants under heavy metal and metalloid stresses [2,15,21]. These studies reveal that PGPR not only mitigates heavy metal stress in plants, but also promotes plant growth through their plant growth promotory actions.

Considering the decline of world oil reserves, biodiesel has been considered as a potential alternative fuel over the last few years. Biodiesel is obtained from biomass feedstock and is therefore considered a renewable energy source. Biodiesel production from soybean is a sustainable way to enhance energy production. The utilization of heavy metal-contaminated agriculture land for soybean production intended for biodiesel is a good strategy for the management of metal-contaminated agricultural land. However, the heavy metal contamination of biofuel feedstock has the risk of a higher heavy metal content in biodiesel. The removal of heavy metals from biodiesel is expensive and less feasible. KNP9 treatment in soybean under lead treatment also reduced lead content in shoots and roots by 48.1% and 41.83%, respectively. Similarly, KNP9 reduced the Cd concentrations in soybean shoots and roots by 54.83% and 92.82%, respectively. This demonstrates that KNP9 effectively decreases the heavy metal content in soybean plants while simultaneously promoting plant growth. The inoculation of KNP9 has the potential for enhancing the growth of soybean grown in Pb- and Cd-contaminated soil while lowering the content of both heavy metals. Therefore, the bioinoculant application is a potential solution for the management of heavy metal-contaminated soil by growing soybean for biodiesel production.

5. Conclusions

The ability of soil bacteria to bioaccumulate heavy metals is one of the crucial strategies in microorganisms to carry out metal bioremediation. In this study, *Pseudomonas putida* KNP9 was found to be effective in tolerating high Cd and Pb concentrations and removing both the heavy metals (lead and cadmium) from the solution. The SEM-EDX study revealed that KNP9 bioaccumulated both the Cd and Pb inside the cell. The heavy metal removal studies, followed by SEM-EDX, also conclude that the increase in cell size in KNP9 under Pb and Cd stress is due to the intracellular accumulation of these heavy metals. The pot trial experiment revealed that KNP9 inoculation leads to enhanced plant growth and less heavy metal accumulation in soybean plants. Therefore, *P. putida* KNP9 can be a promising bacterial species for promoting soybean growth, which could be used for biodiesel production. However, further research is necessary for understanding the bioremediation mechanisms of *P. putida* KNP9 in detail.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/en16114508/s1>, Figure S1: Pictures of pot trial experiment. Where (a) represents negative control without bioinoculant and (b) represents KNP9 treatment; Figure S2: Comparative soybean plant growth images of the pot trial experiment.

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Conflicts of Interest: The authors declare no conflict of interest.

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