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Phytoremediation of Cadmium Contaminated Soil Using *Sesbania sesban* L. in Association with *Bacillus anthracis* PM21: A Biochemical Analysis

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Abstract: Sustainable food production to feed nine to 10 billion people by 2050 is one of the greatest challenges we face in the 21st century. Due to anthropogenic activities, cadmium (Cd) contamination is ubiquitous with deleterious effects on plant and soil microbiota. In the current study, the phytoremediation potential of *Sesbania sesban* L. was investigated in Cd-spiked soil inoculated with *Bacillus anthracis* PM21. The Cd-spiked soil drastically reduced important plant attributes; however, inoculation of *B. anthracis* PM21 significantly ($p \leq 0.05$) enhanced root length (17.21%), shoot length (15.35%), fresh weight (37.02%), dry weight (28.37%), chlorophyll a (52.79%), chlorophyll b (48.38%), and total chlorophyll contents (17.65%) at the Cd stress level of 200 mg/kg as compared to the respective control. In addition, bacterial inoculation improved superoxide dismutase (11.98%), peroxidase (12.16%), catalase (25.26%), and relative water content (16.66%) whereas it reduced proline content (16.37%), malondialdehyde content (12.67%), and electrolyte leakage (12.5%). Inoculated plants showed significantly ($p \leq 0.05$) higher Cd concentration in the *S. sesban* root (118.6 mg/kg) and shoot (73.4 mg/kg) with a translocation (0.61) and bioconcentration factor (0.36), at 200 mg/kg Cd. Surface characterization of bacteria through Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) predicted the involvement of various functional groups and cell surface morphology in the adsorption of Cd ions. Amplification of the CzcD gene in strain PM21, improved antioxidant activities, and the membrane stability of inoculated *S. sesban* plants conferred Cd tolerance of strain PM21. In addition, the evaluated bacterial strain *B. anthracis* PM21 revealed significant plant growth-promoting potential in *S. sesban*; thus, it can be an effective candidate for phyto-remediation of Cd-polluted soil.

Keywords: antioxidant enzymes; Fourier transform infrared spectroscopy; scanning electron microscopy; CzcD gene; plant growth-promoting rhizobacteria

1. Introduction

Sustainability and food security are the main challenges of the current era. Environmental pollution is drastically increasing due to anthropogenic activities [1]. As a result of anthropogenic activities, the contamination of heavy metals (HMs) causes serious threats to the environment [1]. Being non-degradable and persistent in nature, HMs can easily bio-accumulate in the food chain and eventually induce ill effects on the health of humans.

Contamination of HMs in the soil has become a critical problem in Pakistan, and various HMs have been detected in the soil of Malakand Agency and Vehari [2]. Additionally, HMs are the result of the weathering process [3,4]. According to the Environmental Protection Agency (EPA), cadmium (Cd) is the most widespread in environment [5,6]. High rates of bioaccumulation were observed in plant and animal-based food products [7]. The food contaminated with Cd has negative health consequences, including nephron toxicity, bone deterioration, and neurotoxicity [8,9]. In a previous study, [10] evaluated that daily Cd intake from rice was 2.20 and 1.5 g/kg bw (for adults and children), which was considerably higher than PTMI (daily to monthly intake). Owing to geochemical, industrial, and sewage waste, extremely high amounts of Cd have been found in crabs [11], rice, and vegetables [10]. Furthermore, levels of Cd were 0.001–0.71 in leafy vegetables and 0.38–7.0 mg/kg in rice samples, above the maximum permitted threshold [12].

The level of Cd contamination in some agricultural soils of Pakistan has been detected as 0.29–184 mg/kg, which is above the permissible limits of the WHO [13]. Heavy metal-contaminated soil can be remediated by utilizing various biological and physico-chemical methods. Phytoremediation is a cost-effective method to remediate HM-contaminated soils [14,15]. Soil contaminated with HMs is regarded as a different substrate than air and water. It might be due to the persistent nature of HMs present in soil than other components of the biosphere [16,17].

An excessive amount of Cd in plants activates the production of reactive oxygen species (ROS), which hampers the antioxidant defense machinery and eventually plant growth [18,19]. Antioxidants including superoxide dismutase (SOD), peroxidase (POD), and catalase are enzyme antioxidants that may help plants improve their immunity (CAT). Not only antioxidants, but also other compounds, including malondialdehyde (MDA) contents of plants, were increased. All these factors under stress conditions lead to stunted growth of plants. In this scenario, the Cd tolerance in plants through bacteria has not yet been explored. Various biological agents including bacteria and fungi have been used to ameliorate the heavy metal stress in plants. Among all of these, microorganisms' bacteria have been used most widely. The use of various bacterial genera, including *Bacillus*, *Acidovorax*, *Mycobacterium*, *Paenibacillus*, *Alcaligenes*, *Pseudomonas*, and *Rhodococcus*, in the phytoremediation process has been widely reported [20,21]. Bacteria were found to be quite useful in phytoremediation because they increased metal solubility by producing organic acids, polysaccharides, and plant growth stimulants.

Inoculated bacteria have been reported to promote plant growth under normal and stress conditions through direct and indirect mechanisms [22]. Direct mechanisms include enhanced nutrient availability, phytohormone production, extracellular enzymes (especially ACC deaminase enzyme), exopolysaccharides, and the production of various diffusible and secondary metabolites. Indirect mechanisms displayed by the inoculated bacteria include protection of plants from various pathogenic diseases and abiotic stress conditions [23]. Phytoremediation (by *Bacillus* sp. and *Pseudomonas* sp.) directly affects growth and biomass production of various plant species [24]. The production of various signals and metabolites by diverse groups of microorganisms detoxifies metal-contaminated industrial effluents [25,26]. The bacterial strain *P. aeruginosa* has been identified as the most efficient for dealing with heavy metal emissions. Apart from a diverse group of microbes, the efficacy of *Bacillus, Escherichia*, and *Mycobacterium* during detoxification of Cd and improving soil quality has been well demonstrated.

Phytoremediation has emerged as a promising, ecofriendly, and cheap approach for the extraction, and immobilization of pollutants from groundwater and soil sediments [27]. Methods such as phyto-stabilization, phytoextraction, rhizofiltration, and phytovolatilization can be used for phytoremediation [28]. Hyperaccumulator plants are used for phytoextraction, as they can better tolerate and accumulate various HMs [4,29]. The tolerance of plants to HM toxicity, the interaction between HMs and the native bacterial community, and the uptake of available soil HMs are key factors of phytoremediation [30].

Sesban (*Sesbania sesban* L.) belongs to the Fabaceae or Leguminosae family, which has huge medicinal importance and is widely distributed in tropical regions worldwide [31]. It is also used for fodder and fuel purposes and has high biomass. *S. sesban* is reported to survive in contaminated environments [32]. *S. sesban* could be an effective option for reclamation due to the potential of nitrogen fixation in root nodules [33]. Researchers have also conducted genetic studies to understand the mechanisms adopted by microorganisms for the remediation of heavy metals. For example, the gene cadA, which encodes the cadmium efflux system, was explored and has been commonly observed in Gram-positive bacteria species [34]. In *B. subtilis*, the cadA gene has a role against Cd [35]. Two other genes (TrkA and czcD) in bacteria detoxify metals, and their recombinant boosts the efficiency of *Escherichia coli* [36]. Genes for the biosynthesis of bacilli thiol (BSH) in the genus *Bacillus* are an efficient mechanism for metal decontamination. The lack of BSH in *B. subtilis* increases its sensitivity against Cd [37]. We assessed the biosorption capacity of a plant growth-promoting bacterium, *B. anthracis* PM21, for sorption of Cd under different operational conditions. This research work was mainly focused on highlighting the ability of *B. anthracis* PM21 to tolerate Cd as well as its ability in bacterial-assisted phytoremediation of Cd-spiked soil. Additionally, we evaluated the differential effects of *B. anthracis* PM21 inoculation on important morphological, physiological, and biochemical attributes of *S. sesban* under different Cd concentrations.

2. Materials and Methods

Bacillus anthracis PM21 with NCBI accession number (OK255528) was previously isolated from the rhizosphere of tomato and was found to play a role as plant growth-promoting rhizobacteria. The isolated bacterial strain was tested for growth under different concentrations of Cd for 7 days. According to previous findings, the growth of *Bacillus anthracis* PM21 remained negatively proportional to heavy metal concentrations. The maximum growth for the *B. anthracis* PM21 strain was observed at the sixth day of incubation [27].

2.1. Antibiotic Resistance of Bacillus Anthracis PM21

The disk diffusion method was used for the detection of antibiotic resistance [38]. Bacterial culture was allowed to grow in broth for 24 h. The antibiotic discs were fixed on LB agar plates on 24 h grown culture (100 µL). Incubation of inoculated Petri plates was done overnight at 35 °C. Bacterial resistance to antibiotics was quantified by measuring the diameter of the inhibition zone around the antibiotic disks.

2.2. Inoculum Preparation and Seed Treatment

A 250 mL conical flask containing 100 mL LB broth was used to multiply *B. anthracis* PM21 inoculum. A shaking incubator (DKS-1020, N-Biotek, Bucheon-si, Korea) was set at 30 °C with 120 rpm for 24 h to keep the prepared inoculum. The value of optical density was recorded at 600 nm and 1.00 was obtained that showed bacteria as a 10^9 colony forming unit (CFU/mL). *S. sesban* seeds were initially soaked for 5 min in 75% ethanol and then in HgCl2 (0.1%) for 1 min for sterilization purposes [39]. The seeds were washed 5 to 6 times with double-distilled water (DDW). Seed priming was carried out via immersion in bacterial inoculum for 3–4 h. The control treatment contained seeds that were soaked only in double-distilled water.

2.3. Greenhouse Experiment

Soil was collected from an agricultural and non-contaminated field of the National Agricultural Research Center, Islamabad, Pakistan (33.6701° N, 73.1261° E). The collected soil was autoclaved at 121 °C for 1 h. The nature of the soil was found to be loamy. The properties of the experimental soil prior to the experiment were determined (Table 1).

Table 1. Soil properties of experimental soil prior to experiment.

Soil Properties	Value	References
Soil texture	Loamy	
Clay (%)	15	
Silt (%)	42.5	
Sand (%)	42.5	
pH	7.06	
Electrical conductivity (dS/m)	2.28	
Organic matter (%)	0.7	
Phosphorus (mg/kg)	156	[40]
Potassium (mg/kg)	3.27	
Nitrate-nitrogen (mg/kg)	0.02	
Extractable Cd (mg/kg)	0.4	

Experimental pots each having 23 cm diameter and 19 cm length were filled with autoclaved soil (5 kg). Cadmium was applied in dried form in two levels (100 and 200 mg/kg) in pot treatments. Treatments containing Cd were thoroughly mixed for two weeks for metal solution stabilization to maintain an even metal concentration in the soil before sowing [38]. A completely randomized design with three replications was implemented to manage the experimental treatments. Initially, 10 seeds per pot were sown, and after germination 4–5 plants were left per pot. Moreover, one pot was considered as a replication. Experimental treatments included control (T0), *B. anthracis* PM21 (T1), Cd 100 mg/kg (T2), Cd 100 mg/kg + *B. anthracis* PM21 (T3), Cd 200 mg/kg (T4), and Cd 200 mg/kg + *B. anthracis* PM21 (T5).

2.4. Growth and Photosynthetic Pigments of Sesbania sesban L.

Growth variables were observed in the plants just after harvesting for the experimental treatment. The root and shoot length, fresh (FW), and dry weight (DW) were recorded as plant growth traits. Plant parts were kept in an oven at 70 °C for complete removal of moisture and the dry weight was estimated [27]. The photosynthetic pigments were isolated and estimated by the method used by [41]. Small pieces (0.5 g) of fresh leaves were suspended in falcon tubes containing 5 mL dimethyl-sulfoxide (DMSO) reagent with lids. The combination was incubated in a water bath at 65 oC until the extraction of pigments. Centrifugation (Pro Economy, Centurion Scientific, Chichester UK) of the extract was done and assorted supernatant in 6 mL of C_3H_6O was added followed by centrifugation again. The samples (extracts) were then quantitatively estimated at wavelengths of 663 and 645 nm by means of the formula by [41] for various chlorophyll contents as follows:

$$\text{Chlorophyll a} = (12.7 \times \text{A663}) - (2.49 \times \text{A645})$$

$$\text{Chlorophyll b} = (12.9 \times \text{A645}) - (4.7 \times \text{A663})$$

$$\text{Total Chlorophyll} = (8.2 \times \text{A645}) + (20.2 \times \text{A645})$$

2.5. Electrolyte Leakage (ELL) and Relative Water Content (RWC)

Fresh leaves (0.5 g) from each treatment were kept in water at 4 °C for 4 h to evaluate relative water content. Similarly, plant samples were dried in an oven (80 °C) to obtain the dry weight (DW) [42]. The protocol suggested by [42] was implemented to measure electrolyte leakage (ELL) via the formula given below:

$$\text{electrolyte leakage (ELL)} = \frac{\text{Electrical conductivity 1}}{\text{Electrical conductivity 2}} \times 100 \quad (1)$$

2.6. Estimation of Proline Content, Malondialdehyde (MDA), and Antioxidant Enzyme Activity

The proline content was determined using ninhydrin [38]. A total of 4 mL of sulphosalicylic acid (3%) was used to grind the leaf sample (0.5 g) and placed overnight. Cen-

trifugation (Pro Economy, Centurion Scientific, Chichester UK) of the ground samples was done for 5 min at 3000 rpm and then glacial acetic acid and ninhydrin was added to the suspension. The obtained mixture was heated at 100 °C for 1 h in a water bath. The mixture was transferred to an ice bath to cool it. Toluene was used for extraction of the mixture and its absorbance was noted at 520 nm. A standard curve was used to measure proline concentration and expressed in mmol g^{-1}. A fresh leaf sample of 0.5 g was ground in 10 mL of 0.1% trichloroacetic acid (TCA) to measure MDA [43]. The sample was centrifuged at 15,000× *g* for 15 min. The supernatant was separated and mixed with 4 mL of 0.5% TBA (Thiobarbituric acid) and 20% TCA. The samples were centrifuged (Pro Economy, Centurion Scientific, Chichester UK) at 10,000× *g* for 10 min and absorbance was noted at 440, 532, and 600 nm, respectively. The MDA equivalents were estimated using the formula given below.

$$\text{MDA} = 6.45\ (\text{A532} - \text{A600}) - 0.56 \times \text{A440}$$

Superoxide dismutase (SOD) was recorded as a reduction in superoxide nitro blue tetrazolium complex absorbance through enzymes [44]. The following buffers were prepared: (a) adding 15.6 g monosodium dihydrogen phosphate to 500 mL of distilled water, and (b) adding 53.65 g disodium hydrogen phosphate to 600 mL of distilled water. For the preparation of the buffer solution of pH 7, 183 mL of Na_2HPO_4 disodium monohydrogen phosphate and 117 mL of $NaHPO_4$ monosodium dihydrogen phosphate was allowed to mix to make the last volume of 0.6 L and the pH was adjusted to 7. For the preparation of buffer solution of pH 7.8, both the buffers were mixed by taking 275.5 mL of Na_2HPO_4 disodium monohydrogen phosphate and 25.5 mL of $NaHPO_4$ monosodium dihydrogen phosphate. The last volume was made in a quantity of 0.6 L and the pH was adjusted to 7.8. The superoxide dismutase level was assessed by utilizing the method of [44]. The plant sample (0.2 g) was crushed in a solution (4 mL) formed by adding PVP (1 g) along with disodium EDTA (0.028 g) in 0.1 L of phosphate buffer of pH 7. The mixture was centrifuged for 10 min at 4 °C. The supernatant was collected, and the volume was upgraded to 8 mL using phosphate buffer (pH 7). Na_2EDTA (0.0278 g), methionine (1.5 g), and nitro blue tetrazolium chloride (NBT) (0.04 g) were added in phosphate buffer of pH 7.8 (100 mL). A total of 10 mL was taken in another flask and its volume was increased up to 50 mL. Riboflavin (0.00113 g) was added to the phosphate buffer (100 mL). A total of 20 mL was taken in another flask and its volume was increased up to 50 mL with DH_2O. Then reference, blank, and reaction mixtures were prepared, each containing 2 mL of solution, 0.5 mL of solution two, and 0.5 mL of enzyme extract. The reference treatment was placed in darkness and the reaction mixture was kept in a light chamber. Absorbance was noted as 560 nm. The activity of SOD was presented as unit/100 g of fresh weight.

The following formula was used to determine SOD activity.

R^1, R^2, and R^3 represent the OD of the reference, OD of the blank, and OD of the sample, respectively.

$R^4 = R^3 - R^2$ and Final = R^4/A

In a similar way, peroxidase (POD) activity was observed by following [44]. A total of 4.45 g Na_2HPO_4 disodium monohydrogen phosphate and 3.9 g $NaHPO_4$ monosodium dihydrogen phosphate was taken in 0.5 L distilled water for the preparation of 0.1 M phosphate buffer of 6.5 pH. One-percent hydrogen peroxide was made in phosphate buffer (0.1 M) of pH 6.5. By using 10 mL of phosphate buffer of pH 6.5, a fresh shoot (1 g) sample was crushed, then spun, and the supernatant was obtained. A spectrophotometer was adapted to zero and values were recorded at 430 nm. H_2O_2 (0.5 mL) was added and mixed for each sample in the test cuvette. The absorbance of the supernatant was recorded at 430 nm for a consecutive 3 min to observe changes in the absorbance values. The absorbance change per minute at 430 nm was taken as one unit of peroxides. The protocol suggested by [45] was implemented to determine catalase activity by H_2O_2 decomposition. Disodium monohydrogen phosphate (5.963 g) and monosodium dihydrogen phosphate

(5.226 g) were added to 500 mL distilled water to make 0.067 M phosphate buffer of pH 7.0. Hydrogen peroxide (12.6 µL) was added to 100 mL of phosphate buffer to make 2 mM hydrogen peroxide solution. Plant tissues (0.5 g) were measured and crushed in 8 mL of phosphate buffer. The extract was spun, and the supernatant obtained was used for the enzyme assay. A total of 40 µL supernatant was mixed completely in 3 mL of 2 mM hydrogen peroxide solution already prepared by using 0.067 M phosphate buffer (100 mL phosphate buffer + 26.5 µL H_2O_2). The absorbance of the samples was recorded at 240 nm while taking phosphate buffer as blank. The optical density was measured at 240 nm using a spectrophotometer (752N UV-VIS, Beijing, China).

2.7. Metal Analysis of Plants by Wet Acid Digestion

Cadmium (Cd) uptake was achieved by employing the method of wet acid digestion [46]. Plant material (1 g) was finely ground via pestle and mortar and transferred to a 0.1 L conical flask. About 10 mL nitric acid and perchloric acid (HNO_3-$HClO_4$ in a 3:1 ratio) were kept in the flask and kept overnight in a fume hood. Then the flasks were kept in a fume hood for 60 min, maintaining the temperature of 70 °C. During this process, brown fumes turned white. The flasks containing mixture were permitted for a few seconds to cool. Distilled water was added to dilute the mixture. The obtained extract was filtered through Whatman No. 42 and double-distilled water was added to raise the total volume up to 50 mL. These samples were utilized to quantify Cd concentration with the help of the flame atomic absorption spectrometry method (Varian FAAS-240, Triad Scientific, New York, NJ, USA). To assure quality, the FAAS instrument was calibrated with standards (R^2 = 0.9999) given by the firm (SRM 3108 for cadmium). Periodic calibration of the FAAS instrument using metal standards at intervals of 10 samples ensured the accuracy of the Cd readings. The FAAS instrument has a detection limit of 0.002 µg/L for Cd.

2.8. Bioconcentration Factor (BCF) and Translocation Factor (TF)

The bioconcentration factor (BCF) was determined to evaluate the metal uptake capacity that occurred from the soil to plant tissues. The BCF was calculated for individual plant parts i.e., roots and shoots. The translocation factor (TF) determined the ability of a given plant species as a phytoremediator. The translocation factor is the ratio between the concentration (Conc.) of Cd present in the plant shoot to the root. The formulae proposed by [47] were used to calculate BCF and TF.

$$\text{BCF} = \frac{\text{Conc. of metal in root}}{\text{Conc. of metals in shoot}} \quad (2)$$

$$\text{TF} = \frac{\text{Conc. of metal in shoot}}{\text{Conc. of metals in root}} \quad (3)$$

2.9. Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

The functional groups involved in the adsorption of Cd ions on the surface of bacterial cells were identified through Fourier transform infrared spectrophotometric (FTIR) analysis. Initially, bacterial culture was inoculated in LB broth with and without the amendment of Cd (100–200 mg/L). After an incubation of 24 h, *B. anthracis* PM21 culture was centrifuged for 10 min at 8000 rpm by maintaining a temperature of 4 °C. Supernatant was discarded and pelleted bacterial cells were separated. Bacterial cells were washed three times with sodium chloride solution (0.85%) followed by de-ionized (DDI) H_2O and then oven dried at 50 °C [48]. Furthermore, 1 mg of crushed dried bacterial cells were mixed with 400 mg of KBr (potassium bromide). Thoroughly mixed cells were ground into fine powder and a manual hydraulic press at 100 kg/cm2 pressure was applied for 10 min to obtain transparent sample disks. Fixation of the disks was done in FTIR (Nicolet TM, Thermo Scientific, Waltham, MA, USA) to observe specific functional groups at a wavelength ranging from 400 to 4000 cm [49].

2.10. Scanning Electron Microscopy (SEM)

SEM was executed to examine the impact of Cd on bacterial cell morphology. Bacteria were grown at 30 °C for 24 h at 120 rpm in LB broth in the presence (200 mg/L) and absence of Cd. The LB broth that did not contain Cd was considered a control treatment. Centrifugation of the bacterial cells was performed at 4 °C for 10 min at 8000 rpm after 24 h of incubation. Phosphate buffer saline (PBS) was used to wash pelleted bacterial cells thrice. Glutaraldehyde (2.5%) was used to pre-fix the washed bacterial cells for 4–6 h at 4 °C [50]. The PBS (pH 7.2) was again used to wash the pre-fixed bacterial cells twice. Osmium tetroxide (1%) was used for post-fixation purposes for 1 h followed by PBS washing and dehydration with acetone (*v/v*) at 20%, 40%, 60%, 80%, and 100%. Bacterial cells were dried with the help of a critical point dryer (CPD) and platinum-coated ion sputter coater (JFC 1600 Auto Fine Coater, JEOL, Tokyo, Japan) and analyzed under SEM (JSM-6490LV, JEOL) to investigate the morphological changes in bacterial cells treated with or without Cd.

2.11. Amplification of Heavy Metal Resistance CzcD Gene

The CzcD gene responsible for cadmium resistance was amplified in *B. anthracis* PM21. The sequence of the CzcD primer set is F-CAGGTCACTGACACGACCAT, R-CATGCTGATGAGATTGATGATC, with an amplicon size of about 398 base pairs [51]. Reactions were performed in the sequence of denaturation at 95 °C for 5 min, followed by 34 cycles at 94 °C for 90 s, 52 °C for 90 s, 72 °C for 2 min, and a final extension step of 72 °C for 7 min [52].

2.12. Re-Isolation of Inoculated Strain

Re-isolation was performed as reported by [53]. The re-isolation experiment was performed for the confirmation of inoculated strain PM21.

2.13. Statistical Analysis

Normality of the data was calculated through the Shapiro–Wilk test using SPSS software (IBM SPSS Statistics 21) (Table S1) [54]. The experiment was organized as a completely randomized design (CRD) in triplicate. Statistix version 8.1 was used to perform the analysis of variance (ANOVA) (Table S2). Data are presented as means ± standard error; statistical significance between the treatments was calculated using a least significant difference (LSD) value of $p \leq 0.05$.

3. Results

3.1. Antibiotic Resistance of Bacillus Anthracis PM21

The bacterial strain was tested using the disk method for antibiotic resistance. The strain was resistant against the maximum number of antibiotics applied. A total of 20 antibiotics were tested and the appearance of a halo zone around the disk was considered a sign of susceptibility to bacterial strain. The antibiotic resistance ability of the strain is shown in (Table S3). The *B. anthracis* PM21 was highly resistant and showed resistant against 15 antibiotics.

3.2. Growth and Photosynthetic Pigments of Sesbania sesban L.

A significant increase in the root length of *S. sesban* L. was observed in Cd-stressed and non-stressed treatments with the inoculation of *B. anthracis* PM21 (Table 2). Both Cd contamination levels, 100 and 200 mg/kg (T2), significantly decreased root length (47.68%) and shoot length (33.29%) compared to the control. Inoculation of strain PM21 increased shoot and root length in both non-stress and Cd-stress treatments (Table 2). Comparing the level of Cd, the maximum root length (38.39%) and shoot length (32.67%) was noted with 200 mg/kg Cd (T5) under strain PM21 inoculation compared to control. Fresh weight was decreased under contaminated treatments compared to uninoculated control. The Cd contamination at the level of 200 mg/kg reduced fresh weight (47.95%) and dry weight (62.48%) compared to control treatment. However, *S. sesban* L. inoculated

with strain PM21 showed fresh weight (43.48%) and dry weight (50.84%) at 200 mg/kg of Cd (Table 2, Figure 1). A significant reduction in photosynthetic pigments was noted in Cd-contaminated treatments of *S. sesban* compared to control treatment (Table 2). Chlorophyll a and b and total chlorophyll content were reduced by 27.34, 62.79, and to 43.71%, respectively, at a Cd stress level of 200 mg/kg compared to control treatment. Chlorophyll a and b and total chlorophyll contents were considerably improved due to the application of strain PM21 compared to the respective control. Enhanced chlorophyll a (60.08) and b (48.38%) and total chlorophyll contents (17.65%) were noted with the application of strain PM21 at the level of Cd stress of 200 mg/kg.

Table 2. Influence of bacterium inoculum PM21 on important agronomic traits and photosynthetic pigments of *Sesbania sesban* L. in Cd-spiked soil. T0 = Control, T1 = *Bacillus anthracis* PM21, T2 = Cd 100 mg/kg, T3 = Cd 100 mg/kg + *Bacillus anthracis* PM21, T4 = Cd 200 mg/kg, and T5 = Cd 200 mg/kg + *Bacillus anthracis* PM21 (n = 3). Data in each column show mean ± SD and treatment means sharing different letter(s) in each column are significantly different at $p < 0.05$ level.

Treatments	Root Length (cm)	Shoot Length (cm)	Fresh Weight (g)	Dry Weight (g)	Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Total Chlorophyll (mg/g)
T0	25.67 ± 0.33 ^{b}	63.01 ± 0.10 ^{b}	20.02 ± 0.01 ^{ab}	7.73 ± 0.08 ^{b}	1.28 ± 0.03 ^{e}	0.43 ± 0.03 ^{c}	12.10 ± 0.05 ^{b}
T1	30.33 ± 0.33 ^{a}	66.50 ± 0.73 ^{a}	25.06 ± 0.02 ^{a}	9.55 ± 0.01 ^{a}	2.75 ± 0.08 ^{a}	0.65 ± 0.03 ^{a}	15.16 ± 0.03 ^{a}
T2	15.16 ± 0.29 ^{d}	46.5 ± 0.17 ^{d}	13.04 ± 0.01 ^{c}	3.04 ± 0.08 ^{e}	1.47 ± 0.03 ^{d}	0.35 ± 0.05 ^{d}	9.35 ± 0.01 ^{d}
T3	24.2 ± 0.12 ^{c}	64.67 ± 0.36 ^{c}	20.03 ± 3.33 ^{bc}	6.51 ± 0.01 ^{c}	2.55 ± 0.01 ^{b}	0.52 ± 0.01 ^{b}	10.50 ± 0.05 ^{c}
T4	13.43 ± 0.05 ^{e}	42.03 ± 0.29 ^{f}	11.02 ± 0.08 ^{c}	2.90 ± 0.08 ^{ef}	0.93 ± 0.01 ^{f}	0.16 ± 0.08 ^{f}	6.81 ± 0.01 ^{f}
T5	22.01 ± 0.11 ^{d}	62.43 ± 0.06 ^{e}	19.50 ± 0.08 ^{b}	5.9 ± 0.26 ^{cd}	2.33 ± 0.01 ^{c}	0.31 ± 0.01 ^{e}	8.27 ± 0.01 ^{e}

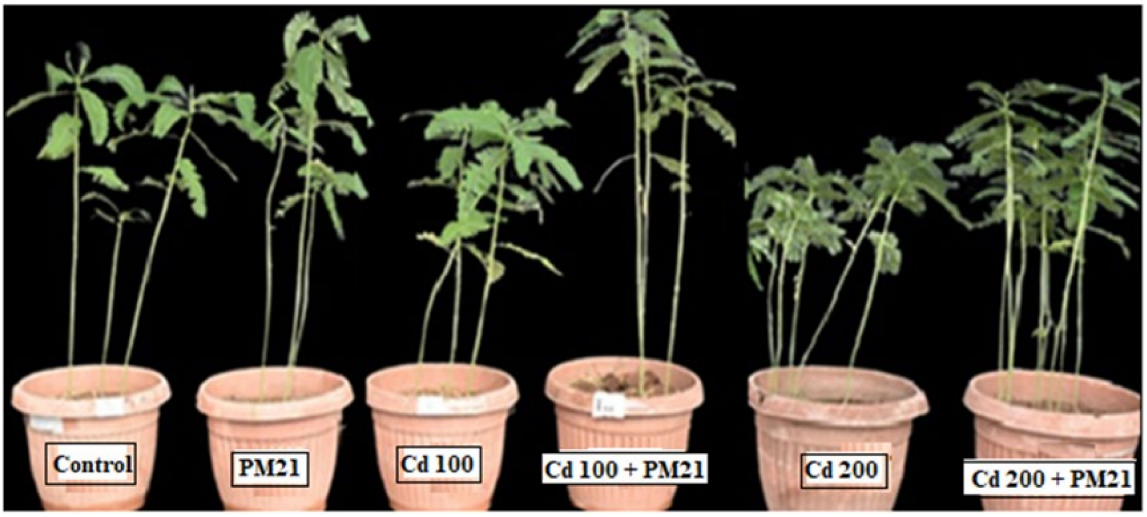


Figure 1. Showing the effect of metal-tolerant strain PM21 on the growth of *Sesbania sesban* under normal and Cd-stress conditions.

3.3. Relative Water Content, Electrolyte Leakage, MDA, and Proline

Reduced RWC and enhanced electrolyte leakage was noted in Cd-contaminated treatments compared to control (Figure 2). The Cd stress at 200 mg/kg significantly reduced RWC (16.66%) and enhanced membrane electrolyte leakage (14.28%) compared to control treatment (Figure 2a,b). Inoculation of *B. anthracis* PM21 under Cd stress at 200 mg/kg enhanced RWC (16.66%) and reduced ELL (12.5%). The Cd contamination enhanced the proline and MDA content of *S. sesban* (Figure 2c,d). The Cd stress at 200 mg/kg recorded maximum proline (37.75%) and MDA content (41.04%) compared to control. Bacterium inoculation under Cd stress at 200 mg/kg observed maximum reduction in proline (16.37%) and MDA content (12.65%).

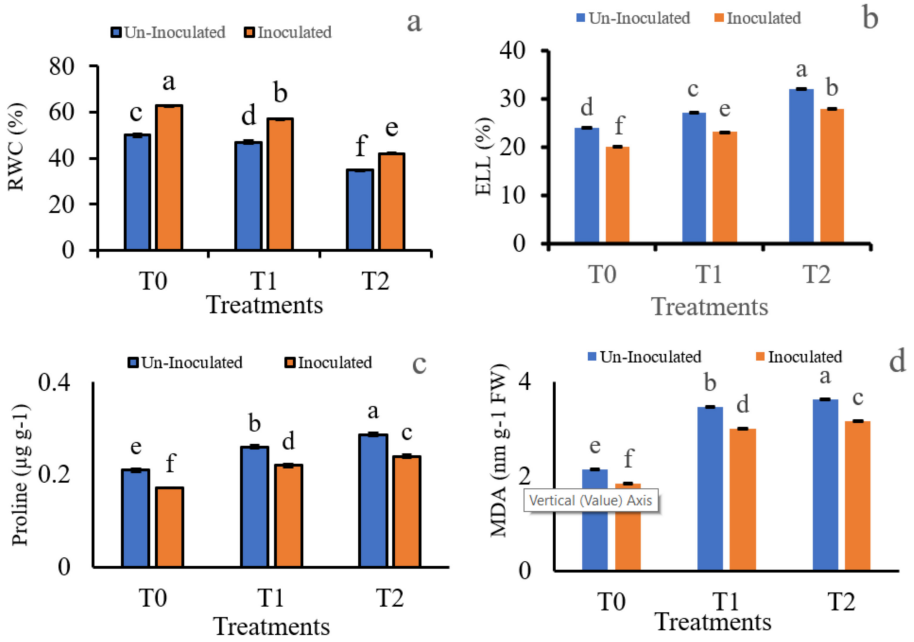


Figure 2. Influence of bacterium inoculum PM21 on (**a**) RWC, (**b**) membrane electrolyte leakage, (**c**) proline, and (**d**) MDA of *Sesbania sesban* in Cd-spiked soil. T0: control, *B. anthracis* PM21; T1: Cd 100 mg/kg, *B. anthracis* PM21; T2: Cd 200 mg/kg, *B. anthracis* PM21.

3.4. Antioxidant Activity

Important antioxidant activities were significantly enhanced with the application of *B. anthracis* PM21 under control and Cd-stress treatments (Figure 3). The *B. anthracis* PM21 inoculation significantly ($p < 0.001$) promoted antioxidant activities, i.e., SOD (11.98%), POD (12.16%), and CAT (4.46%), at 200 mg/kg Cd stress.

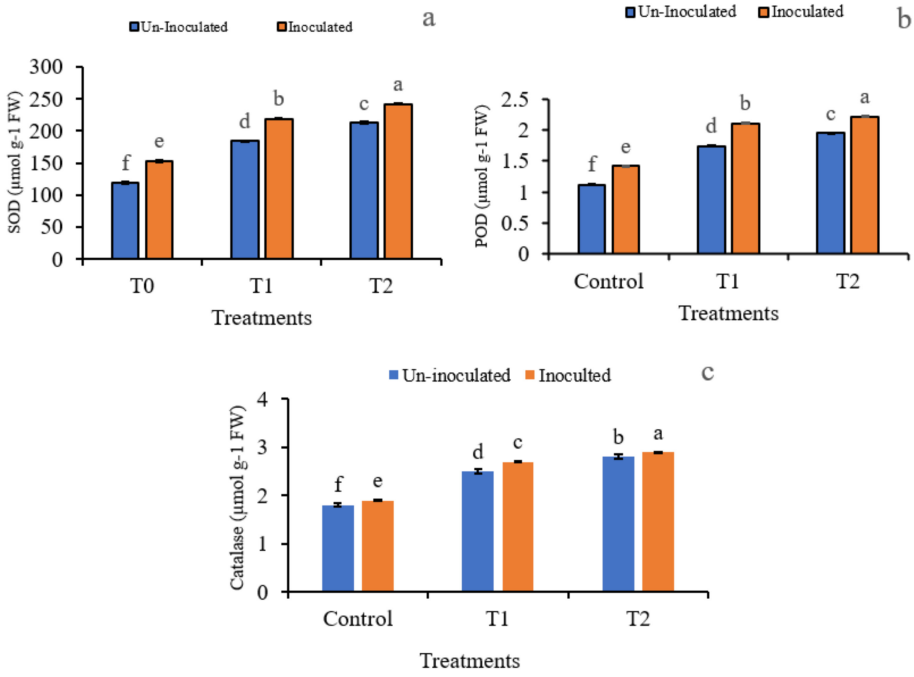


Figure 3. Influence of bacterium inoculum PM21 on (**a**) SOD, (**b**) POD, and (**c**) Catalase important antioxidant enzymes of *S. sesban* in Cd-spiked soil. T0: control, *B. anthracis* PM21; T1: Cd 100 mg/kg, *B. anthracis* PM21; T2: Cd 200 mg/kg, *B. anthracis* PM21.

3.5. Tolerance Index, Translocation Factor (TF), and Bioconcentration Factor (BCF)

The enhanced Cd tolerance and high biomass values were noted in plants inoculated with strain PM21 compared to control (Table 3). *S. sesban* L. plants shown a high Cd absorption upon exposure to increased levels of Cd (Table 3). The application of strain PM21 showed high Cd absorption, resulting in the best cadmium contents in inoculated Cd 200 plants. In all treatments, plant roots had higher Cd than shoots. In comparison to roots of un-inoculated *S. sesban* L., strain PM21-inoculated plants had 12.42 and 11.38% more Cd content in roots for Cd 100 and Cd 200 mg/kg, respectively. Inoculated plants had 24.58 and 18.39% more Cd in their shoots under Cd 100, and Cd 200 mg/kg, respectively, compared to the respective control. The current findings show that strain PM21-inoculated plants exposed to Cd 100 and Cd 200 mg/kg have high TF and BCF compared to the respective control. When compared to un-inoculated Cd 100 plants, Cd 100 mg/kg with strain PM21 had 13.9% and 23.44% high TF and BCF values, respectively. Cadmium 200 + strain PM21 plants showed an 8.19 and 19.44% increase in TF and BCF, respectively, compared to the respective control (Table 3). The value of Cd accumulated in whole plants (mg/plant) was calculated by multiplying Cd content in plant tissues by the dry weight of plants. A high level of Cd was exhibited by plants inoculated with strain PM21 in both treatments (Table 3).

Table 3. Cadmium (Cd) uptake of *Sesbania sesban* L. T0: control, T1: *Bacillus anthracis* PM21; T2: Cd 100 mg/kg, T3: Cd 100 mg/kg + *B. anthracis* PM21; T4: Cd 200 mg/kg; T5: Cd 200 mg/kg + *B. anthracis* PM21, TI: tolerance index; TF: translocation factor; BCF: bioconcentration factor (n = 3). Treatment means sharing different letter(s) are significantly different at $p < 0.05$ level.

	Cadmium Uptake				
Treatments	**Root (cm)**	**Shoot (cm)**	**TI**	**TF**	**BCF**
T0	0.1 ± 0.01e	0.01 ± 0.01e	-	0.05 ± 0.01d	0.02 ± 0.02e
T1	0.2 ± 0.05e	0.05 ± 0.05e	-	0.1 ± 0.01d	0.12 ±0.01d
T2	46.5 ± 0.11d	31.9 ± 0.11d	72.36 ± 0.02c	0.68 ± 0.01b	0.32 ±0.01c
T3	53.1 ± 0.02c	42.3 ± 0.12c	100.05 ± 0.01a	0.79 ± 0.02a	0.42 ±0.02b
T4	105.1 ± 0.03b	59.9 ± 0.30b	61.15 ± 0.01d	0.56 ± 0.01c	0.34 ±0.01c
T5	118.6 ± 0.11a	73.4 ± 0.05a	97.11 ± 0.02b	0.61 ± 0.01c	0.36 ± 0.01a

3.6. Determination of Different Functional Groups through Fourier Transform Infrared Spectroscopy (FT-IR)

Bacterial biomass exposed to Cd resulted in different absorption peaks in FTIR analysis and proposed various functional groups such as O-H stretching (3280.89 cm^{-1}) strong bonding with alcohol, C-H stretching (2922.60 cm^{-1}) medium bonding with alkene, C = C stretching (1633.93 cm^{-1}) medium bonding with alkene, N-O stretching (1530.09 cm^{-1}) strong bonding with nitro compound, C-H bending (1451.62 cm^{-1}) medium bonding with alkane, S = O stretching (1393.40 cm^{-1}) strong bonding with sulfonyl chloride, C-O stretching (1230.41 cm^{-1}) strong bonding with alkyl aryl ether, and C-O stretching (1057.56 cm^{-1}) strong bonding with primary alcohol of *Bacillus anthracis* PM21 at different wavelengths (Figure 4a). However, the absorption peaks and functional groups showed at O-H stretching (3287.54 cm^{-1}) strong bonding with alcohol, C-H stretching (2929.53 cm^{-1}) medium bonding with alkene, C = C stretching (1637.79 cm^{-1}) medium bonding with alkene, N-O stretching (1535.31 cm^{-1}) strong bonding with nitro compound, O-H bending (1319.67 cm^{-1}) medium bonding with phenol, C-O stretching (1229.99 cm^{-1}) strong bonding with alkyl aryl ether, and C-O stretching (1055.04 cm^{-1}) strong bonding with primary alcohol on bacterial cell surface grown under Cd 200 mg/L stress at different wavelengths (Figure 4b).

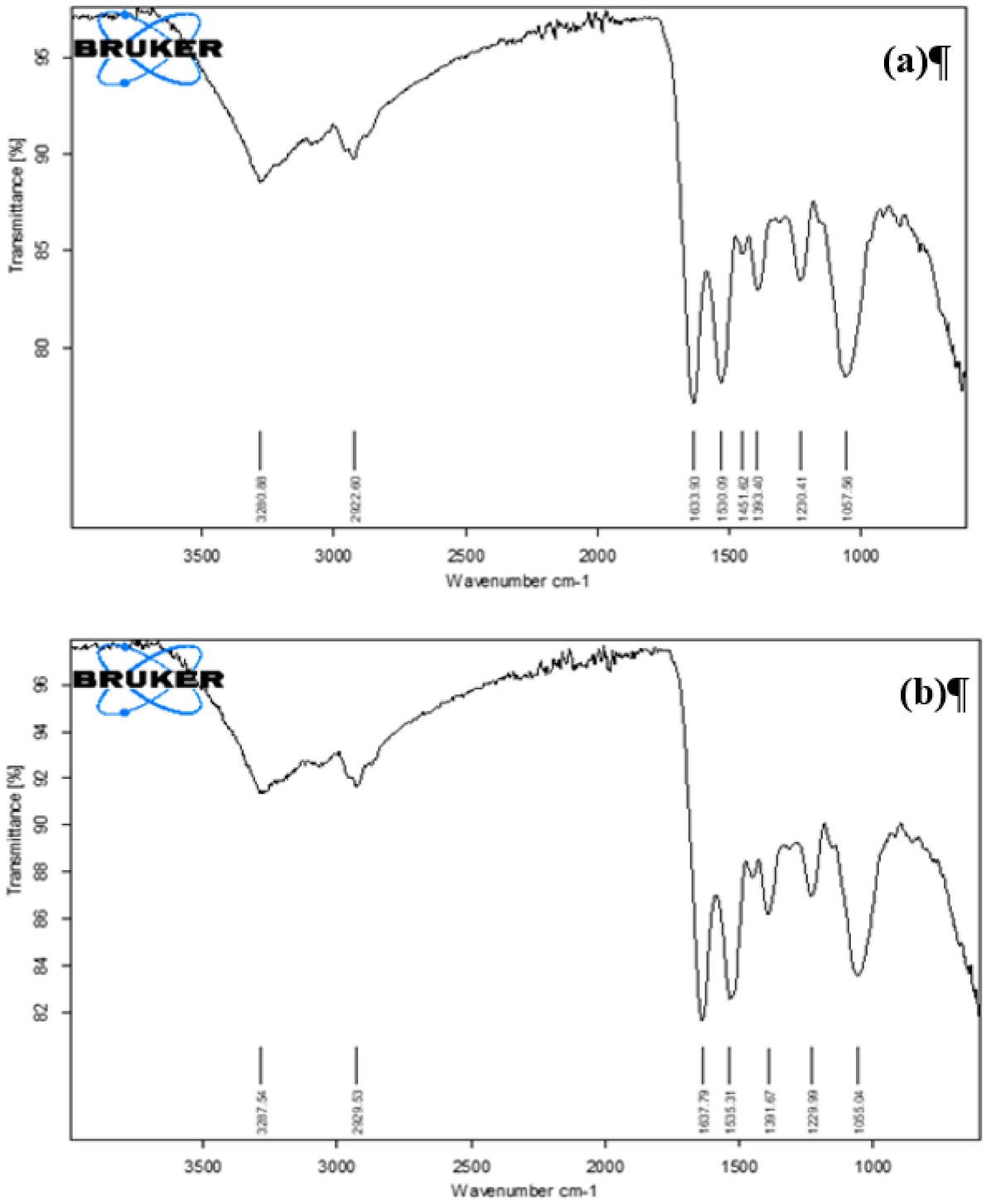


Figure 4. (**a**) FTIR analysis of *Bacillus anthracis* PM21 (**b**) and Cd stress at the level of 200 mg/L and cells of *Bacillus anthracis* PM21.

3.7. Scanning Electron Microscope (SEM)

Compared to unexposed cells having a smooth cell surface, bacterial cells subjected to Cd stress became rough, exhibited surface depression, and showed increased cell size (Figure 5a,b).

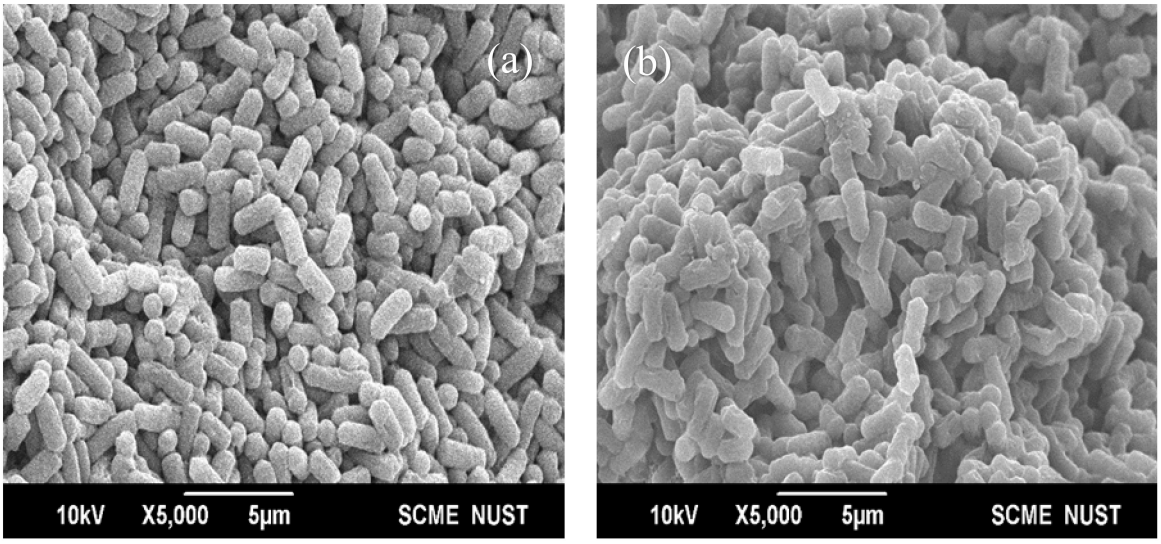


Figure 5. (**a**) SEM analysis of *Bacillus anthracis* PM21 and (**b**) Cd stress at the level of 200 mg/L and cells of *Bacillus anthracis* PM21.

3.8. Amplification of Heavy Metal Resistance CzcD Gene

The bacterial strain PM21 was screened for the Cd resistance gene (CzcD) by performing PCR amplification of genomic DNA. *Bacillus anthracis* PM21 possessed the resistant gene for Cd (CzcD) (Figure 6).

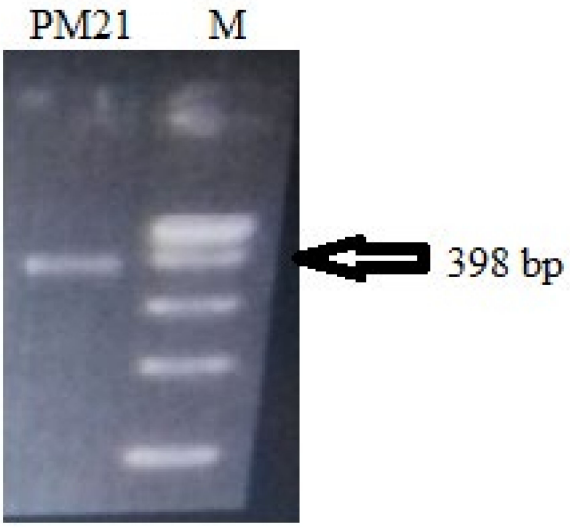


Figure 6. Photograph of the agarose gel showing CzcD gene amplification in *Bacillus anthracis* PM21.

4. Discussion

Agricultural soil contaminated with Cd poses major risks to crop productivity [55]. Microbial-assisted phytoremediation is considered an effective approach for the reclamation of polluted soils [56,57]. The inoculation of stress-tolerant PGPR can reduce the detrimental impact of heavy metals (HMs) in sustainable agriculture [27]. Bacteria can tolerate HMs and can enhance plant growth by producing antioxidants [57]. The current investigation explained *S. sesban* phytoremediation potential for Cd with and without bacterial inoculation (*B. anthracis* PM21) to analyze the potential regulation of physiological parameters, biochemical parameters, and antioxidant enzymes. The application of HM-tolerant bacteria can be used as a promising technique to induce a Cd stress-tolerance mechanism in plants [27,42].

Antibiotic-resistant PGPR can be utilized in the form of inoculum to reduce competition. In the current study, *B. anthracis* PM21 showed resistance against 15 antibiotics. Ref. [58] reported that bacteria can benefit from antibiotic resistance to enhance their colonization and niche with plants compared to others. Genes responsible for antibiotic resistance are considered to provide a key role in the mechanism proposed for antibiotic resistance. The genes are located either in chromosomes or in plasmid. The presence of multifunctional proteins involved in functions such as efflux of metals or molecules are considered for antibiotic resistance [59].

The application of strain PM21 increased agro-morphological traits, including fresh weight (FW), dry weight (DW), root length (RL), and shoot length (SL), of the *S. sesban* plant in the presence and absence of Cd stress. The RL (47.68%), SL (33.29%), FW (44.95%), and DW (62.48%) of *S. sesban* were significantly decreased in Cd-spiked soil compared to control treatment. Previous results reported that plants at 150 µM Cd reduced root length (27.56%), shoot length (30.95%), fresh weight (28.46%), and dry weight (32.24%) compared to control [60]. Several studies have shown that Cd hampers various biological processes in plants, including impregnation through ammonia and other compounds. Cadmium stress also brings negative physiological, biochemical, and genetic changes in plants, which might be a reason for the decreased growth parameters of *S. sesban* L. under Cd stress [42]. Furthermore, Cd levels above the permissible limit may cause the inhibition of enzymatic functions, leaf chlorosis, reduced plant fresh and dry weight, and sometimes death [61].

The increased growth parameters of *S. sesban* was observed through the inoculation of strain PM21 at the concentration of 200 mg/kg Cd. Inoculation of strain PM21 significantly increased RL, SL, FW, and DW up to 38.39, 32.67, 43.48, and 50.84%, respectively, under 200 mg/kg of Cd compared to control (Table 2). This could be due to the modulation of antioxidant mechanisms and photosynthetic pigments of the studied plant with the application of strain PM21 [62]. It has been well documented that inoculating Cd-stressed *Serratia marcescens* BM1 to soyabean enhanced root (14.58%) and shoot length (21.51%) and fresh (11.11%) and dry weight (5.11%) [63]. Usually, applied bacteria benefit plants in phytoremediation under abiotic stress conditions by increasing metal solubility with the synthesis of various organic acids, ACC deaminase enzyme, and exopolysaccharides produced by bacteria [26]. Moreover, a reduction in plant growth and biomass of *Solanum nigrum* was examined when plants were under stress with Cd contamination only, but the application of *Serratia* sp. RSC-14 significantly reduced Cd stress and facilitated plant growth [64].

Chlorophyll a (27.34%) and b (62.79%) and total chlorophyll content (43.71%) of *S. sesban* was significantly decreased in Cd-spiked soil compared to control treatment. In the same way, in an investigation conducted by [65], chlorophyll a (29.25%) and b (43.22%), and total chlorophyll content (61.73%) were decreased under Cd stress. Low synthesis of photosynthetic pigments could be due to a change in structural compounds and gas exchange factors that are blocked under stress conditions [46]. This reduction in chlorophyll content of the *S. sesban* plant could be due to the activation of chlorophyll and membrane damage [26,66]. On the other hand, the chlorophyll a (60.08%), chlorophyll b (48.38%), and total chlorophyll content (17.65%) were positively improved with the application of strain PM21 (Table 2). The chlorophyll contents, including chlorophyll a (28.26%), chlorophyll b (30.54%), and total chlorophyll (58.01%), of the maize plant were increased by bacterial inoculation under 30 µM of Cd [36,67]. Plant growth-promoting bacteria could enhance chlorophyll that upsurges nutrient uptake in plants through PSB and exudates essential substances that have a role in the synthesis of photosynthetic pigments required for photo assimilation [68,69]. In comparison with the reported literature, our results showed significant improvement in the following growth parameters: root length, shoot length, fresh weight, dry weight, and chlorophyll a, chlorophyll b, and total chlorophyll.

In the current study, the electrolyte leakage (ELL) (14.28%) and relative water content (RWC) (16.66%) were significantly decreased upon exposure to Cd compared to control. Similar findings were observed with decreased ELL (20.14%) and RWC (29.44%) under

Cr 200 mg/kg [38]. A positive correlation among enhanced ELL and a decrease in RWC represented membrane damage in *S. sesban* upon exposure to Cd [26]. The present investigation demonstrated that the inoculation of strain PM21 reduced ELL (12.5%) and enhanced RWC (16.66%). In comparison, inoculation of *B. xiamenensis* PM14 to *S. sesban* minimized ELL (2.73%) and improved RWC (25.79%) [38]. Bacterium inoculation enhanced water absorption due to improved root surface area, which resulted in improved physiological parameters of the host plant [70]. Under Cd exposure, proline (37.75%) and MDA content (41.01%) were significantly decreased in *S. sesban*. In the same way, physiological parameters proline (37.75%) and MDA contents (34.84%) were decreased in *S. sesban* when exposed to Cd [38]. Cadmium stress produced ROS that led to plant oxidative stress with increased ELL and lipid peroxidation [71]. Malondialdehyde content is an indication of cell membrane damage due to its reaction with protein amino groups [25]. Improved physiological mechanisms like malondialdehyde (MDA) and proline content have been documented in bacterial-inoculated plants [38]. Increased proline content (16.37%) could be due exopolysaccharide synthesis by strain PM21 under Cd stress condition [38]. Bacterial-inoculated *S. sesban* plants showed a significant decrease in MDA content (12.65%), which could have been due to the mitigation of Cd stress by the application of ACC deaminase, producing microbial solution. The results presented by [38] are in line with current results, in which proline (12.33%) was improved and MDA (29.53%) was decreased with *B. xiamenensis* PM14 in *S. sesban*. Plants can tolerate Cd stress with the production of antioxidant enzymes that scavenge produced reactive oxygen species (ROS). Maximum oxidative stress in *S. sesban* was triggered with Cd stress applied at the level of 200 mg/kg, which led to the enhanced production of important antioxidant activities (SOD, POD, and CAT) (Figure 3) [72]. However, inoculation with strain PM21 significantly improved the antioxidants activities of SOD (11.98%), POD (12.16%), and CAT (4.46%) at 200 mg/kg of Cd (Figure 3). It was previously reported that SOD (24.54%), POD (26.03%), and CAT (30.54%) at a 30 µM Cd stress level increased by bacterial inoculation [72]. The *acinetobacter* controls the activities of antioxidant enzymes, as reported in a previous research study, due to the initiation of antioxidant enzyme mRNA expression [73]. As one of the mitigation strategies to deal with the drastic effects of metal stress, particularly in susceptible ones, improved antioxidant enzymes under abiotic stress using PGPR could be predicted [74]. Enhanced activities of SOD, POD, and CAT in inoculated chickpea improved plant growth through the protection of chloroplasts and other organelles in which important metabolic mechanisms occur [60].

Both bioconcentration factor (BCF) and translocation factor (TF) can be used as possible tools to identify the ability of plants to absorb metal ions. The TF and BCF of the strain PM21-inoculated *S. sesban* plants are shown in Table 3. Compared to the control plants, inoculated *S. sesban* showed increased root (118.6 mg/kg), shoot (73.4 mg/kg), TF (0.61), and BCF (0.36), respectively. It was previously reported that accumulation of Cd increased in root (57.2 mg/kg) and shoot (9.02 mg/kg), resulting in higher TF (0.18) and BAF (0.06) values of *Sesbania sesban* [75]. Because of their growth-promoting biochemicals and boosting nutrient uptake capabilities, PGPR can co-currently enhance phytoremediation and plant growth [76]. It was observed that the tolerance index (TI) of strain PM21-inoculated plants was 97.11% with 200 mg/kg of Cd. The tolerance index was 49.6% and 48.14%, respectively, as reported by [77]. The growth and phytoextraction of *S. sesban* were improved by inoculating *B. anthracis* PM21. Moreover, plant growth-promoting rhizobacteria boosted bioaccumulation, bioavailability, and plant biomass in inoculated plants [78,79]. Bacteria can produce chelating biochemicals, siderophores, and phosphate solubilization, which have a role in improved Cd uptake and alleviation of stress-inoculated plants [80]. The current study suggests that strain PM21 is involved in bioavailability in Cd, due to which accumulation and uptake of Cd is enhanced in *S. sesban* [79]. In accordance with the reported literature, our results revealed a considerable improvement in Cd uptake of the root, shoot, TF, BCF, and TI of *S. sesban*. The FTIR spectral results of strain *B. anthracis* PM21 demonstrated that treatments showed a clear shift in the peaks of the hydroxyl functional

group present in alcohol, alkene, nitro compounds, alkane, sulfonyl chloride, alkyl aryl ether, and primary alcohol groups of bacterial biomasses. These functional groups are supposed to relate to polysaccharides and proteins found on the cell surface during their interaction with Cd, as demonstrated by [81]. The peaks observed at 3280.89 cm^{-1} and 2922.60 cm^{-1} resulted from the O-H stretching form of alcohol. The overlap of alcohol and hydroxyl stretching on the surface of cells has been clearly observed [81]. However, in Cd stress-exposed cells, an unchanged absorption peak was observed at 2929.53 cm^{-1}, suggesting the O-H stretching of lipids, nucleic acid, proteins, and polysaccharides in the cell wall. A small difference in the peak of absorption from lower (1633.93 cm^{-1}) to higher (1637.79 cm^{-1}) was also observed, suggesting C = C stretching primarily associated with the mode of deformation of C = C [82]. Scanning electron microscopy of strain *B. anthracis* PM21 revealed clear adsorption of Cd. The SEM results revealed the changes in cell surface morphology, which was Cd concentration dependent. It might be due to different surface responses against Cd stress applied at 200 mgL^{-1}. Extracellular adsorption, including surface complexation, could affect its structure, increasing in cell surface roughness [82]. However, these changes help with adaptation to stressful conditions [83]. Previously, it was also noted that a relative decrease in bacterial cell surface area could reduce the toxic effects of pollutants [83,84]. The CzcD operon that is responsible for Cd resistance is owned by the bacterial strain PM21. The CzcD operon is used to detoxify Cd by exporting it into the extracellular medium from the cytoplasm and/or periplasm [84].

5. Conclusions

Bacterial-assisted phytoremediation is considered an effective approach to reclaim polluted soils for sustainable food production. Phytoremediation in combination with inoculation of PGPR can be used efficiently to manage soil polluted under metal stress. Our findings highlighted remarkable improvements after inoculation of plants with the *B. anthracis* PM21 strain as a plant growth-promoting rhizobacteria. Application of *B. anthracis* PM21 significantly ($p \leq 0.05$) enhanced root length (26.77%), shoot length (12.65%), fresh weight (37.02%), dry weight (28.37%), chlorophyll a (52.79%), chlorophyll b (48.38%), and total chlorophyll contents (17.65%) compared to uninoculated ones. Application of *B. anthracis* PM21 significantly ($p \leq 0.05$) enhanced Cd concentration in root (118.6 mg/kg) and shoot (73.4 mg/kg), and the translocation (0.61) and bioconcentration factor (0.36). The PGPR, like *B. anthracis* PM21, has bioremediation potential and can be utilized to decontaminate HM-contaminated soil. *B. anthracis* PM21 was also applied for the first time in bacterial-assisted phytoremediation that enhanced plant growth of *S. sesban* under Cd stress. It is evident that *B. anthracis* PM21 showed Cd tolerance through the amplification of the CzcD gene. SEM and FTIR analysis conferred surface moieties for the adsorption of Cd on cell surface, growth, and antioxidant improvement.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su132413529/s1, Table S1: Shapiro–Wilk test showed normal distribution for all the studied parameters, Table S2: Analysis of variance revealed highly significant difference for the studied parameters, Table S3: Antibiotic resistance of *Bacillus anthracis* PM21.

Author Contributions: H.J.C. conceived the presented idea and supervised during the study. J.A. performed the experiment and wrote the manuscript. X.W., funding acquisition. M.R. and I.A. carried out the analytical and statistical analysis and revised the article. S.F., data curation. M.F.H.M. carried out the analysis of the experiment. All authors discussed the results and contributed to the final manuscript under the supervision of H.J.C. All authors have read and agreed to the published version of the manuscript.

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