Research Article

Journal of Environmental Nanotechnology

Effect of *Burkholderia sp.* on Root Nodulation in Black Gram Grown in Cadmium-Contaminated Soil Supplemented with Lignite Humic Acid and Seaweed Biochar

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Received: 02.08.2024 Accepted: 18.11.2024 Published: 30.12.2024
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ABSTRACT

A non-rhizobial, root-nodulating endophytic bacterium, *Burkholderia multivorans* strain Strulens, was isolated from black gram root nodules. Under cadmium (Cd) stress, this bacterium synthesized indole-3-acetic acid, produced exopolysaccharides, and solubilized phosphate. When black gram plants were treated with biochar (2% w/w), humic acid (2% w/w), and *Burkholderia sp.,* they exhibited increased nodule formation and higher leghemoglobin content, even at varying Cd concentrations. Combining humic acid and biochar with *Burkholderia sp.* also reduced Cd translocation from the roots to the shoots, resulting in a translocation factor of 0.17. These findings suggest that incorporating biochar and humic acid into Cd-affected soils, along with *Burkholderia sp.* inoculation, can improve legume growth and reduce Cd uptake in contaminated soils.

Keywords: Biochar; Humic acid; Black gram; *Burkholderia sp.*; Root endophyte; Root nodulation.

1. INTRODUCTION

Currently, many agricultural soils are contaminated with heavy metals such as cadmium, arsenic, lead, mercury, and chromium (Rashid *et al.* 2023). Heavy metal concentrations above a certain level can disrupt various life forms (Briffa *et al.* 2020), affecting soil microbial ecology, reducing crop growth, and posing health risks to humans through the food chain (Alengebawy *et al.* 2021). Among the heavy metals, Cd is particularly toxic to humans, animals, and plants, causing cytotoxic and genotoxic effects (Mitra *et al.* 2022). Due to sewage sludge disposal, excessive fertilizer use (phosphate), and atmospheric deposition, Cd has become prevalent in agricultural lands (Gil *et al.* 2022). Cadmium, even at relatively low concentrations (0.001–0.1 mg L^{-1}) (Alkorta *et al.* 2004), is classified as a human carcinogen (Cui *et al.* 2021), negatively impacting crop growth as well as human health (Vazquez *et al.* 2021). Moreover, Cd in soil affects plant metabolic activity and cell division, as it easily transfers to plants from the soil and through the food chain (Wang *et al.* 2023). It is initially absorbed by the root system from the soil and then moves to the aerial parts, including the edible parts (Jing *et al.* 2023). A thorough investigation is required to prevent the translocation of Cd from soil to edible parts of the plants.

Within the soil, plant growth-promoting bacteria (PGPB) encompass free-living bacteria and rhizobacteria that colonize the root rhizosphere (Ramakrishna *et al.* 2019), with their population being higher than in bulk soil (Vejan *et al.* 2016). The most suitable host plants associated with PGPB belong to the families of Fabaceae, Poaceae, Asteraceae, Brassicaceae, and Solanaceae. The Fabaceae family (legumes) forms a mutualistic relationship with the nitrogen-fixing endophytic bacterium Rhizobium (Muresu *et al.* 2022). Furthermore, many rhizobia belong to a diverse array of Alpha-proteobacteria genera, including *Azorhizobium, Allorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Bradyrhizobium, Sinorhizobium, Divosia, Methylobacterium, Phyllobacterium, Ochrobacterium*, *Aminobacteria*, and *Micrvirga* (Swarnalakshmi *et al.* 2020). However, PGPB associated with legume crops is often related to free-living *Pseudomonas sp.* and *Burkholderia sp.* (Ramakrishna *et al.* 2019). Studies have demonstrated that the genera *Burkholderia* and *Cupriavidus* belong to the Beta-proteobacteria group (Klonowska *et al.* 2018). Endophytes colonizing plant root systems can produce nodules and plant growth-regulating substances even in harsh environmental conditions (Kandel *et al.* 2017). The PGPB enhances crop yield and biofortification and reduces the need for chemical fertilizers. In addition, PGPB offer multiple benefits to agriculture by increasing crop productivity and nutrient content while suppressing the growth of pathogens (Ramakrishna *et al.* 2019).

Soil microorganisms play a role in fixing soluble Cd, thus reducing its bioavailability and toxicity (Meng *et al.* 2019). Cd-tolerant bacteria form a diverse group of bacteria that live in soil, water, and air (Bravo *et al.* 2022). Wang *et al.* (2020) showed that Cd-resistant *Burkholderia sp.* Y4 reduced the accumulation of Cd in rice. *Burkholderia* has been identified as an important

rhizobial genus in South American and Venezuelan soils across diverse climatic and topographical regions, capable of nitrogen fixation and enhancing plant growth under stress conditions (Li *et al.* 2024). Various additives have been developed to mitigate heavy metal pollution in soil, including lime, iron, and ferric salts, phosphate fertilizers, apatite composites, bentonite, zeolite, red mud, silicon, and modified silicon (Gogoi *et al.* 2021). Humic acid (HA) is known for its abundance of oxygen-rich functional groups, particularly carboxyl and phenolic OH groups, which improve soil properties (Meng *et al.* 2017). Adding HA can improve soil microbial activity, support plant growth (Zhang *et al.* 2024), and play a role in the soil nitrogen cycle (Jin *et al.* 2024). Humic acid forms complexes with metal ions, promoting stable metal fractions in soil, thus reducing the bioavailable heavy metals, increasing residual fractions, and enhancing heavy metal stability (Proshad *et al.* 2024). Similarly, biochar application in soil immobilizes heavy metals and reduces their availability to plants (Gogoi *et al.* 2021).

Several studies have shown that the co-application of PGPB with organic amendments improves plant growth-promoting rhizobacteria activity and reduces heavy metal toxicity (Borah *et al.* 2018). Also, prior studies have separately investigated the impact of biochar, humic substances, and microbial inoculants in mitigating heavy metal stress. No studies were conducted to investigate the synergistic effect of *Burkholderia* sp., lignite-derived humic acid, and seaweed biochar on root nodulation in black gram cultivated in heavy metal-contaminated soil. As a result, this study aimed to isolate and identify heavy metal-tolerant PGPBs suited for legume crops and evaluate the influence of heavy metals on plant growth promoting rhizobacteria synthesis by isolated PGPBs. Furthermore, the effect of isolated PGPB on the growth and nodulation of black gram cultivated under Cd stress and supplemented with HA and biochar was investigated. In addition, the impact of isolated PGPB in combination with HA and biochar on the translocation of Cd in black gram was evaluated.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Plant Growth-Promoting Rhizobacteria

The bacterium was isolated from root nodules of black gram plants grown in acidic soil (pH 5.0) (Somasegaran *et al.* 1994). Well-isolated colonies appearing on the culture medium were further purified by streaking them onto the peptone salt yeast extract agar medium. The isolated bacterium was subsequently assessed for its colony morphology, Gram-staining characteristics, and motility.

A single pure colony of the bacterial isolate was cultured overnight in Luria Bertani (LB) broth at 28°C. After centrifuging 2 mL of culture at 5000 rpm, the pellet was suspended in 200 µL TE buffer (pH 8.0). Following the addition of lysis buffer, tubes were incubated at 37°C with periodic shaking. An equal volume of phenol: chloroform: alcohol (25:24:1) was added, mixed, and centrifuged at 14,000 rpm. The upper phase was extracted, treated with chloroform alcohol (24:1), and re-centrifuged. DNA was precipitated with sodium acetate and isopropanol, centrifuged, and washed with ethanol before dissolving in TE buffer. DNAse-free RNAse was added to remove RNA, and the extracted DNA was visualized on 0.8% agarose gel electrophoresis.

The ~1.5 kbp 16S rDNA fragment was amplified using high-fidelity PCR polymerase, with amplicons confirmed via 1% agarose gel electrophoresis. The PCR product was sequenced bidirectionally, and sequence data were aligned to identify the bacterium and its closest relatives. Database matching was conducted using BLASTn (NCBI), and a phylogenetic tree was generated *via* the maximum likelihood method in MEGA X software. The cycling and primer conditions are in Table S1. The 16S rRNA sequence of the isolate was submitted to GenBank with accession number PQ270043.

2.2 Effect of Heavy Metals on the Growth of the Isolated Bacterium (*Burkholderia multivorans* Strain Strulens)

To assess the heavy metal tolerance of the isolated bacterium, cultures at a concentration of 10^8 CFU/mL were inoculated in LB broth with varying concentrations (0–6.0 mM) of Cadmium ($CdCl_2$), Lead ($Pb(NO_3)_2$), and Nickel ($NiCl_2$). Cultures were grown aerobically at 28°C with shaking at 180 rpm. Growth was monitored by measuring OD600 every 6 hours for 36 hours. Viable cell counts were determined by serially diluting the culture to 10^{-8} in sterile water, plating 0.1mL on LB agar with heavy metals, and incubating at 37°C for 2 days. CFU/mL was recorded and converted to $\log_{10}$ values.

2.3 Effect of Cd concentrations on IAA, Exopolysaccharide (EPS) and Soluble Phosphate Production

The impact of Cd on the IAA production of the bacterium was evaluated using a ferric–chloride–perchloric acid reagent ($FeCl_3$–$HClO_4$) (Gordon *et al.* 1951). The EPS produced by the bacterium under varying concentrations of Cd was determined using the method adopted by DuBois *et al.* (1956), and soluble phosphate production was determined using the method employed by Das *et al.* (2003).

2.4 Effect of Cd on Root Nodule Formation Supplemented with Biochar and HA

The modified Leonard jar assembly was used in this investigation, and the study was conducted at the Centre for Applied Research and Development, Neyveli Lignite Corporation Ltd., Neyveli, India (Supplementary material). The study included the following treatments: T1 (control: without Cd and *Burkholderia sp.)*, T2 (*Burkholderia sp.)*, T3 (HA: 2% w/w + *Burkholderia sp.)*, T4 (Biochar 2% w/w + *Burkholderia sp.)*, and T5 (HA 2% w/w + Biochar 2% w/w + *Burkholderia sp.)*. Each treatment involved plants exposed to different $CdCl_2$ concentrations (0–6.0 mg kg^{-1}), with Cd added to the sand before sterilization. The content of Cd^{2+} (mg kg^{-1}) in the growth medium (sand) of each treatment was calculated according to the dry weight of the sand. Cd^{2+} was added to the jars in aliquots of aqueous solution of $CdCl_2$ and thoroughly mixed. Cd content in contaminated soil was 3.99 mg kg^{-1} (Oliva *et al.* 2019). Hence, the concentration in the current study was set at 0–6 mg kg^{-1}. Five replications were maintained for each treatment. For sowing, healthy seeds of black gram (var. VBN 4) were surface sterilized (Somasegaran *et al.* 1994), treated with *Burkholderia sp.* ($\times 10^8$ CFU mL^{-1}) for 20 min, and pre-germinated in sterile sand. Then, five seedlings were planted per apparatus and later thinned to three. After 45 days, the plants were carefully removed without disturbing nodules, and the number of nodules produced per plant was examined.

2.4.1 Effect of Cd on Leghemoglobin Content

The leghemoglobin content of the nodules was measured using the method established by Schiffmann *et al.* (1973). The nodules were washed and crushed in a Tris-acetic acid solution. The resulting extract was centrifuged at 3000 rpm for 20 min (Remi RM - 02 Plus Mini Centrifuge, India. An aliquot of 0.1–1.0 mL of the supernatant was then collected and adjusted to obtain an absorbance value in the range of 0.2–0.4. This aliquot was subsequently diluted to a final volume of 4.0 mL with Tris–acetic buffer.

Subsequently, 2.0 mL of freshly prepared benzidine reagent was added, and the color formation intensity was measured at 540 nm using the Spectronic 20+ (LABINDIA Ltd., India) spectrophotometer. A standard graph was developed by plotting the absorbance after 30 s against varied levels of ox-blood hemoglobin (0.8–1.5 µm mL^{-1}). The leghemoglobin concentration of the samples was determined using the standard graph and presented in mg g^{-1} nodule on a fresh weight basis.

2.5 Microscopic Analysis

2.5.1 Scanning Electron Microscopic (SEM) Analysis of Black Gram Roots and Nodules

The samples (1 cm) were fixed with glutaraldehyde (2.5%) in phosphate buffer and dehydrated twice with 20%, 40%, 60%, 70%, and anhydrous ethanol (Sigma) for 4 minutes each (Balasubramaniyam *et al.* 2014). The samples were then dried and coated with gold-palladium in a sputter coater before being examined using Scanning Electron Microscopy (Tescan Vega3 paired with Oxford XMax N, Rupgong Scientifics, Bangladesh).

2.5.2 Epifluorescent Confocal Microscopic Analysis of Black Gram Root Nodules

For confocal microscopic analysis, nodules were coated with wax, and slices of 50 µm were made using a Leica VT1000 S vibrating blade microtome (Leica Biosystems, USA). The slices were examined with a Nikon AXR—NSPARC Confocal laser scanning microscope (Nikon Instruments, Japan) at 430 nm.

2.6 Cd Uptake by Black Gram Plants

The plant samples (root system and aerial part) were prepared according to the method employed by Li *et al.* (2021). The digested mixture was transferred to a 10 mL standard flask and used for analyzing Cd using an Atomic Absorption Spectrophotometer Varian Model: Spectra AA 220 (Labindia Analytical Instruments Pvt. Ltd., India). After determining the Cd content in the aerial part and root system, the translocation factor (TF) was calculated using the formula:

TF = (Concentration in aerial parts) / (Concentration in root system)

2.7 Statistical Analysis

The normality of the data was evaluated using the Shapiro-Wilk test and SPSS version 22 was used for the statistical analysis of the data. To determine whether there was a statistically significant difference in the mean values between the various treatments, a one-way analysis of variance (ANOVA) was carried out. Duncan's Multiple Range Test, a post-hoc test, was performed following ANOVA to compare significant differences among treatments at a significance level of $P = 0.05$.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterization of PGPB

A bacterial species associated with the root nodules of black gram grown in acid soil was isolated, and colonies showing different morphologies were subjected to further characterization. Further

characterization was performed by 16S rRNA sequencing, revealing the bacterium to be *Burkholderia multivorans* strain *Strulens*. The electrophoresis of PCR amplified DNA isolated from *Burkholderia sp.* and the phylogenetic tree of *Burkholderia sp.* reconstructed is presented in Fig 1.

The sequence obtained from the isolated strain was compared against the GenBank nucleotide database. The analysis placed the strain close to various *Burkholderia* species, such as *B. multivorans* strain *Strulens* 16s ribosomal RNA (99.62%, NR-029358.1), *B. multivorans* ATCC BAA-247 strain CIP105 49516s (99.62%, NR-116152.1), *B. multivorans* ATCC BAA-247 strain LMG 1301016s (99.62%, NR-114523.1), *B. latent* strain R-5630 16s ribosomal RNA (99.25%, NR-042632.1), *B. vietnamiensis* strain TVV75 16S ribosomal RNA (99.17%, NR-118872.1), *B. cenocepacia* strain LMG 16656 16S ribosomal RNA (99.10%, NR_025013.1), *B. vietnamiensis* strain CIP 105875 16S ribosomal RNA (99.08%, NR_116154.1), *B. vietnamiensis* strain LMG 10929 16S ribosomal RNA (99.02%, NR_041720.1), *B. pseudomultivorans* strain LMG 26883 16S ribosomal RNA (98.95%, NR_117661.1), and *B. dolosa* strain LMG 18943 16S ribosomal RNA (98.95%, NR_104973.1).

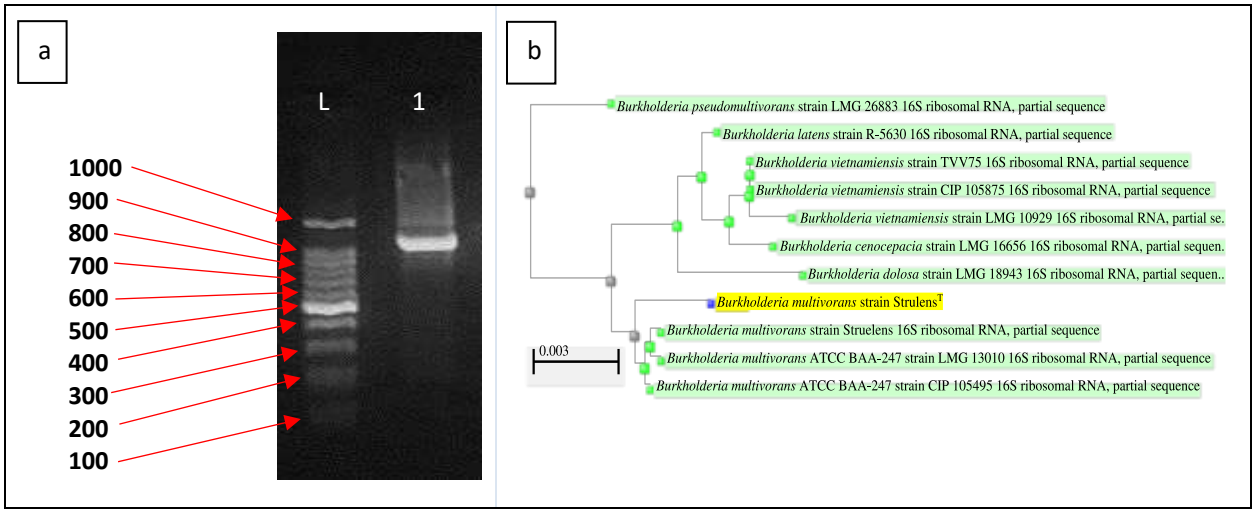


Fig. 1: (a) Agarose gel electrophoresis of PCR amplified DNA isolated from *Burkholderia sp.* (L- 100 base pair DNA ladder (1) Sample DNA (*Burkholderia sp.*) (b) Phylogenetic tree of endophytic bacterium *Burkholderia sp.* reconstructed from neighbor-joining analysis and related sequences from identified bacteria in the database

The genetic relatedness of the isolated species with reported nodulating *Burkholderia sp.* is depicted in the phylogenetic tree constructed using the neighbor-joining method (Saitou *et al.* 1987), based on 1400 nucleotides of the 16S ribosomal gene sequences according to the distance matrix developed. The phylogenetic tree based on 16S rRNA gene sequences illustrates the relationships between *B. multivorans* strain Strulens and the related species.

B. multivorans is a member of the *B. cepacia* complex (Bcc) and is considered a human pathogen. However, studies have shown that Bcc comprises several closely related *Burkholderia* species known for possessing PGP properties (Pal *et al.* 2022). Members of the *Burkholderia* genus are frequent soil inhabitants, with their biological distribution significantly affected by soil pH (Pal *et al.* 2022). In this study, the bacterium was isolated from black gram nodules grown in soil with pH 5.0, showing inherent acid tolerance and thriving in acidic environments. α-proteobacteria genera such as *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium,* and *Azorhizobium* are traditionally associated with nodulation and symbiotic nitrogen fixation in legume crops (Chen *et al.* 2016), and β-proteobacteria, including *Burkholderia*, from root nodules of legume crops, indicating a multifaceted role (Pal *et al.* 2022). The *Burkholderia sp.* isolated from the nodules are considered endophytes and promote plant growth (Rana *et al.* 2020). Additionally, previous studies highlighted the association between *B. cepacia* and various leguminous and non-leguminous plant species (Janaki *et al.* 2024).

3.2 Effect of Heavy Metals on the Growth of *Burkholderia sp.*

Among the heavy metals tested, no viable cells were formed when the bacterium was grown in a medium with Ni and Pb. The bacterium was tolerant to Cd, and growth was observed up to a 6.0 mM concentration of Cd. Therefore, Cd was considered for further study. The cells were grown up to 36 hour, OD values were recorded at 6-hour intervals, and the viable cell count was subsequently enumerated. The maximum population

was attained at 24-hour after inoculation, after which there was a decline in growth. The highest $\log_{10}$ CFU value of 9.7±0.6 was recorded at the 30th hour when the cells were grown at 0- mM Cd concentration (Fig. 2). The bacterium exhibited better growth up to 3.0 mM Cd concentrations, and lower $\log_{10}$ CFU values were noted at 4.0 and 5.0 mM, indicating the toxic effect of Cd at higher concentrations. The bacterium experiences oxidative stress due to Cd exposure, leading to cell damage. High Cd concentrations affect bacterial growth through mechanisms such as inhibition of transcription, protein denaturation, enzyme inhibition, and membrane collapse (Jiang *et al.* 2020). Cd binding to cell wall components such as metallothionein, exopolysaccharides, or other anionic groups, and intercellular detoxification via functional proteins and efflux with cation pumps contribute to cell damage (Zeng *et al.* 2020). This study's findings align with previous reports, as *Burkholderia sp.* isolated from mining soil exhibited Cd resistance and promoted crop growth in Cd-contaminated soil (Liu *et al.* 2023).

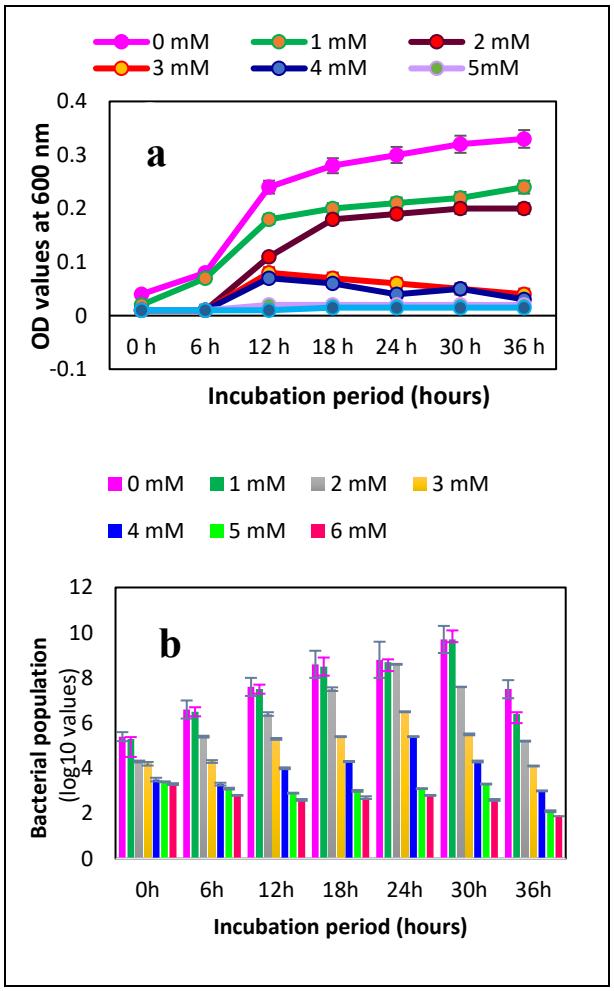


Fig. 2: Effect of Cd on the growth of *Burkholderia* sp. (a) Growth of *Burkholderia* sp. on different concentrations of Cd (b) Log10 transformed values of colony forming units (Error bars represent ± Standard Deviation; n= 5)

3.3 Effect of Cd on IAA Production by the Isolated Bacterium

IAA production by the isolated *Burkholderia sp.* grown under different Cd concentrations (0–6.0 mM) was examined with and without L-tryptophan supplementation, and the results are presented in Fig 3(a). IAA production by the bacterium decreased slightly to a Cd concentration of 3.0 mM under in vitro conditions. However, when the concentration was increased above 3.0 mM, there was a significant reduction in IAA production. Moreover, IAA production was the highest when the bacterium was grown without Cd. IAA production was found to be 4.82±0.8 µg mL^{-1} without tryptophan supplementation and 25.3±0.4 µg mL^{-1} with tryptophan, which is consistent with the findings of a previous study (Goud *et al.* 2024). When the Cd concentration was 6.0 mM, the IAA produced was least compared with other treatments, with values of 1.85±0.4 (without tryptophan) and 18.9±0.2 µg mL^{-1} (with tryptophan). Microbes produce secondary metabolites, including amino acids, carbohydrates, and organic acids, which aid in plants' physiology. Additionally, rhizospheric bacteria produce a significant amount of IAA due to the availability of substrates for secondary metabolite synthesis. Plant growth regulators play a considerable role in controlling plant growth and development, and inoculation of IAA-producing bacteria increases root development, improving water absorption and nutrient uptake (Lobo *et al.* 2023). Production of IAA by the bacterium increased with the addition of L-tryptophan, consistent with the findings of Saowapar Khianngam *et al.* (2023). Vejan *et al.* (2016) reported that *B. cepacia* isolated from oil palm roots produced 50.88 µg mL^{-1} of IAA when the growth medium was supplemented with 4 mg L^{-1} of L-tryptophan. The results of the current experiment revealed that the presence of Cd in the culture medium reduced IAA production, aligning with the findings of Khiangte *et al.* (2021).

3.4 Effect of Cd on EPS Production

Exopolysaccharide production ranged from 110.2±10 to 360.1±8.0 µg mL^{-1}. The highest EPS production of 360.1±8.0 µg mL^{-1} was observed in the control treatment (0 mM Cd), followed by the 1.0 mM concentration. The results indicated that EPS production was not significantly reduced up to 1.0 mM Cd concentration compared to the control. To successfully colonize the interior parts of plants, bacteria need effective endophytic colonization, which includes entry through openings and proliferation in host tissues (Kandel *et al.* 2017). Bacterial flagella, adhesion, survival on plant surfaces, and effective endophytic colonization are essential for surface attachment and epiphytic survival (Afzal *et al.* 2019). Many bacteria produce and secrete EPSs, high-molecular-weight sugar-based polymers critical for bacterial adaptability to diverse stress situations, establishing symbiotic and

pathogenic relationships with hosts, and forming mature biofilm formations. In addition to improving soil structure and permeability, biofilm protects bacteria from adverse conditions, stimulates the synthesis of phytohormones, inhibits pathogens from entering through roots, and bioremediates metals (Carezzano *et al*. 2023).

Burkholderia sp. can produce several types of EPSs (dos Santos *et al.* 2022). Exopolysaccharides can serve as major organic carbon reservoirs, providing critical nutrition sources for microorganisms, and can function in metal toxicity and nutrient starvation (Borah *et al.* 2018). In this study, EPS production was not significantly reduced to a Cd concentration of 1.0 mM compared with the control. This behavior, as also noted by Naveed *et al.* (2020), indicates a microbial stress response to Cd, attempting to mitigate the detrimental effects of toxic chemicals. Because Cd binds to essential bacterial proteins, high concentrations of Cd prevent bacteria from producing EPS (Nazli et al. 2021; Gupta *et al*. 2017).

Furthermore, *Burkholderia sp.* may secrete less EPS at greater concentrations of Cd due to toxicity, which is in line with other research that has demonstrated decreased EPS synthesis under heavy metal stress (Naveed *et al.* 2020). Thomas and Benov (2018) reported that higher levels of Cd have an impact on the growth of bacteria by causing DNA damage, denaturing proteins, and inhibiting bacterial cell division. Beneficial bacteria in the soil produce IAA, which is essential for root proliferation and nutrient uptake in plants (Seneviratne *et al*. 2016). Fig. 3 (a) and (b) show the effect of Cd on IAA and EPS production, respectively.

Soluble phosphate produced by the isolated bacterium was evaluated at different Cd concentrations, and the results are presented in Fig. 3(c). Soluble phosphate production ranged from 370.25±10.2 to 510.46±10.4 µg mL^{-1}. Notably, the highest concentration of soluble phosphate was produced at 0 mM Cd concentration, followed by 1.0 mM Cd concentration. A significant reduction in phosphate solubilization was observed when Cd concentration exceeded 2.0 mM. Phosphorus is an essential nutrient for crop growth and productivity in intensive agricultural production (Zhang *et al.* 2022).

The main sources of phosphorus are rocks and other deposits such as primary apatites and other primary minerals formed during the geological age (El Bamiki *et al.* 2021). However, the availability of soluble forms of phosphorus for plants in the soil is limited due to its fixation as insoluble phosphates or iron, aluminium, and calcium in the soil (Kalayu *et al.* 2019). Nonetheless, phosphate-solubilizing bacteria present in the soil can dissolve insoluble phosphate by secreting organic acids, making it available to plants (Khourchi *et al.* 2022). In soil, bacteria belonging to the genera *Pseudomonas, Azospirillum, Azotobacter, Rhizobium, Enterobacter, Bacillus, Flavobacterium,* and *Burkholderia* can dissolve insoluble phosphate and improve plant uptake (Afzal *et al.* 2023). One of the main factors for inorganic phosphate solubilization is the acidification of the medium by PSB, resulting in the secretion of organic acids such as citric, oxalic, and gluconic acid (Teng *et al.* 2019). In the present study, soluble phosphate produced by *Burkholderia sp.* was higher in the absence of Cd in the medium. However, the bacterium could produce soluble phosphate even in the Cd-supplemented condition. Plant growth-promoting bacteria can reduce the toxic effect of Cd (Li *et al.* 2020) by synthesizing siderophores (Chlebek *et al.* 2021) and phenolics (Noor *et al.* 203).

3.5 Effect on Nodule Number and Leghemoglobin Content

A study was conducted to evaluate the effect of Cd on nodulation in black gram plants by *Burkholderia sp.*, adding biochar and HA (Fig. 4). In all the Cd-contaminated treatments, without *Burkholderia sp.* inoculation, the black gram plants produced no nodules because the sand used as a growth medium was sterilized and the seeds were surface sterilized. So, statistical analysis of the treatment without *Burkholderia* was not considered. When the Cd concentration was 0 mg kg^{-1}, the number of nodules produced per plant ranged from 2.8±4.2 to 48.0±4.2. The plants grown with the application of HA (2% w/w), Biochar (2% w/w), and treatment of *Burkholderia sp.* recorded the highest number of 48.0±4.2 nodules per plant. At 1.0 mg kg^{-1} Cd concentration, the number of nodules per plant formed ranged from 26±3.4 to 42.0±3.8 nodules per plant. When the plants were grown at 2.0 mg kg^{-1} Cd concentration, the number of nodules produced ranged from 24±3.2 to 36.0±2.8 nodules per plant. The same trend was observed at concentrations of 3.0, 4.0, 5.0-, and 6.0- mg kg^{-1} Cd. However, the number of nodules produced by the plants was comparatively less at higher Cd concentrations.

In the current investigation, root nodule development was found in black gram roots after inoculation with *Burkholderia sp.* Burkholderia strains belonging to the Bcc possess the nodC gene, indicating them as possible new nodulating bacteria (Fig. 5). When Cd was added, the number of nodules produced was fewer. At the cellular level, Cd inhibits the activities of several groups of enzymes involved in antioxidant activity, the Calvin cycle, and carbohydrate, protein, and nitrogen metabolism (Zulfiqar *et al.* 2022).

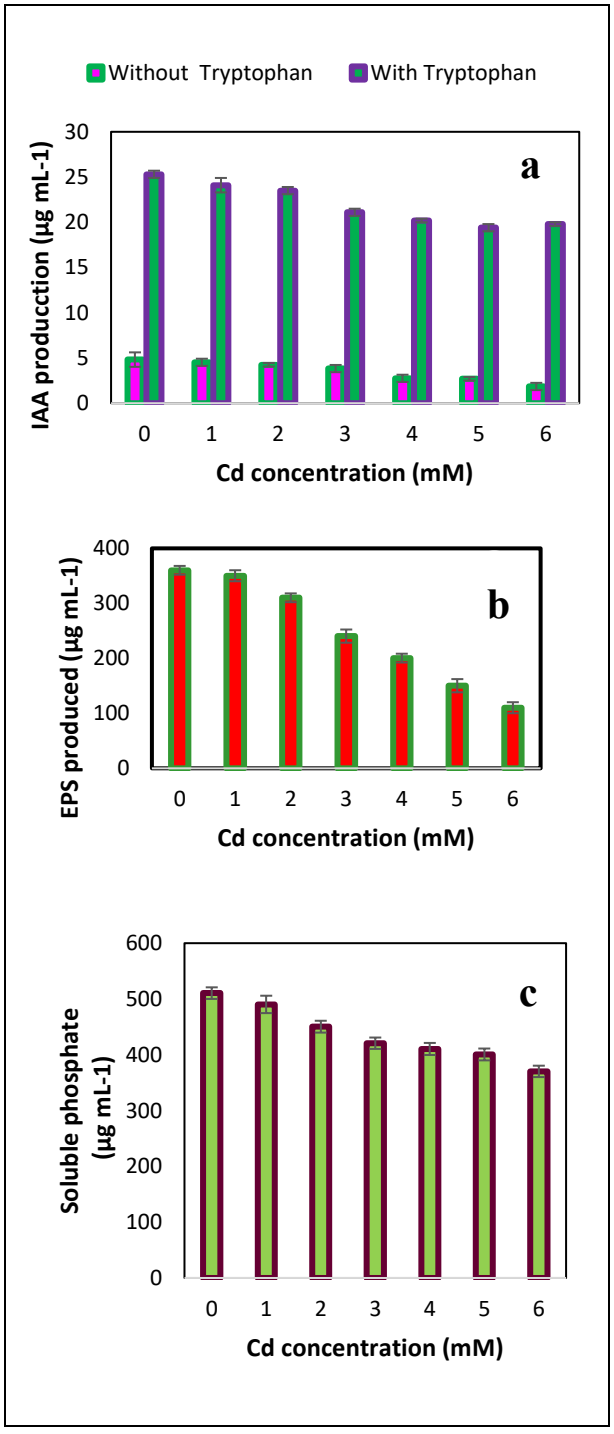


Fig. 3: Effect of Cd on (a) IAA production, (b) EPS production, and (c) Soluble phosphate production by *Burkholderia* sp. (Bars represent ± S.E; n = 5); Mean values sharing common letters are not significant at P = 5% level according to duncan's multiple range test

The leghemoglobin content of fresh nodules collected from black gram plants was determined, and the results are shown in Fig. 6. When the plants were grown in the absence of Cd (0 mg kg^{-1}), the nodules collected from the plants grown with HA 2% w/w, Biochar 2% w/w, and *Burkholderia sp.* recorded the highest leghemoglobin content of 380.6±14.8 µg g^{-1} nodule, followed by the treatment wherein Biochar 2% w/w and *Burkholderia sp.* was added (350.2±12.4 µg g^{-1}). The leghemoglobin content of the nodules collected from the plants grown with HA (2% w/w) and *Burkholderia sp.* was 345.5±10.2 µg g^{-1} nodule. When plants were inoculated with *Burkholderia sp.* without HA or biochar, the leghemoglobin content was 340.8±10.8 µg g^{-1} nodule. Similarly, when 1.0 mg kg^{-1} Cd concentration was added, the highest leghemoglobin content was 376.8±12.6 µg g^{-1} nodule. The leghemoglobin content of the nodules collected from the plants grown with biochar (2% w/w) + *Burkholderia sp.*, and HA (2% w/w) + *Burkholderia sp.;* and *Burkholderia sp.* was 326.2±10.8, 320.4±14.2, and 310.2±12.2 µg g^{-1} nodules, respectively. Likewise, at 2.0 mg kg^{-1} Cd concentration, the highest leghemoglobin content of 355.4±10.8 µg g^{-1} nodule was recorded for HA (2% w/w) + BC (2% w/w) + *Burkholderia sp.* Likewise, the leghemoglobin content of the nodules collected from the plants grown with HA (2% w/w) + BC (2% w/w) + *Burkholderia sp.* was 332.8±14.4 µg g^{-1} nodule, 290.2±14.2 µg g^{-1} nodule, 286.2±11.2 µg g^{-1} nodule, and 264.2±10 µg g^{-1} nodule for 3.0 mg kg^{-1}, 4.0 mg kg^{-1}, 5.0 mg kg^{-1}, and 6.0 mg kg^{-1} Cd concentrations respectively. Additionally, in all the treatments, with increasing Cd concentration, there was a reduction in the leghemoglobin content of the nodules. Certain endophytes are bacteria that aid in the growth of plants and are beneficial in harsh environments (Muresu *et al.* 2022). Previously, it was believed that legumes are nodulated by rhizobia and the associative genera belonging to the alpha subclass of Proteobacteria such as *Bradyrhizobium* sp. and *Sinorhizobium* sp. However, at present, a new group of bacteria belonging to genera other than rhizobia, namely, *Methylobacterium, Devosia, Ochrobacterium, Shinella, Burkholderia,* and *Cupriavidus* capable of root nodulation, was identified. Among them, *Burkholderia* and *Cupriavidus* fall into the subclass of beta-proteobacteria (AL-Nussairawi *et al.* 2020).

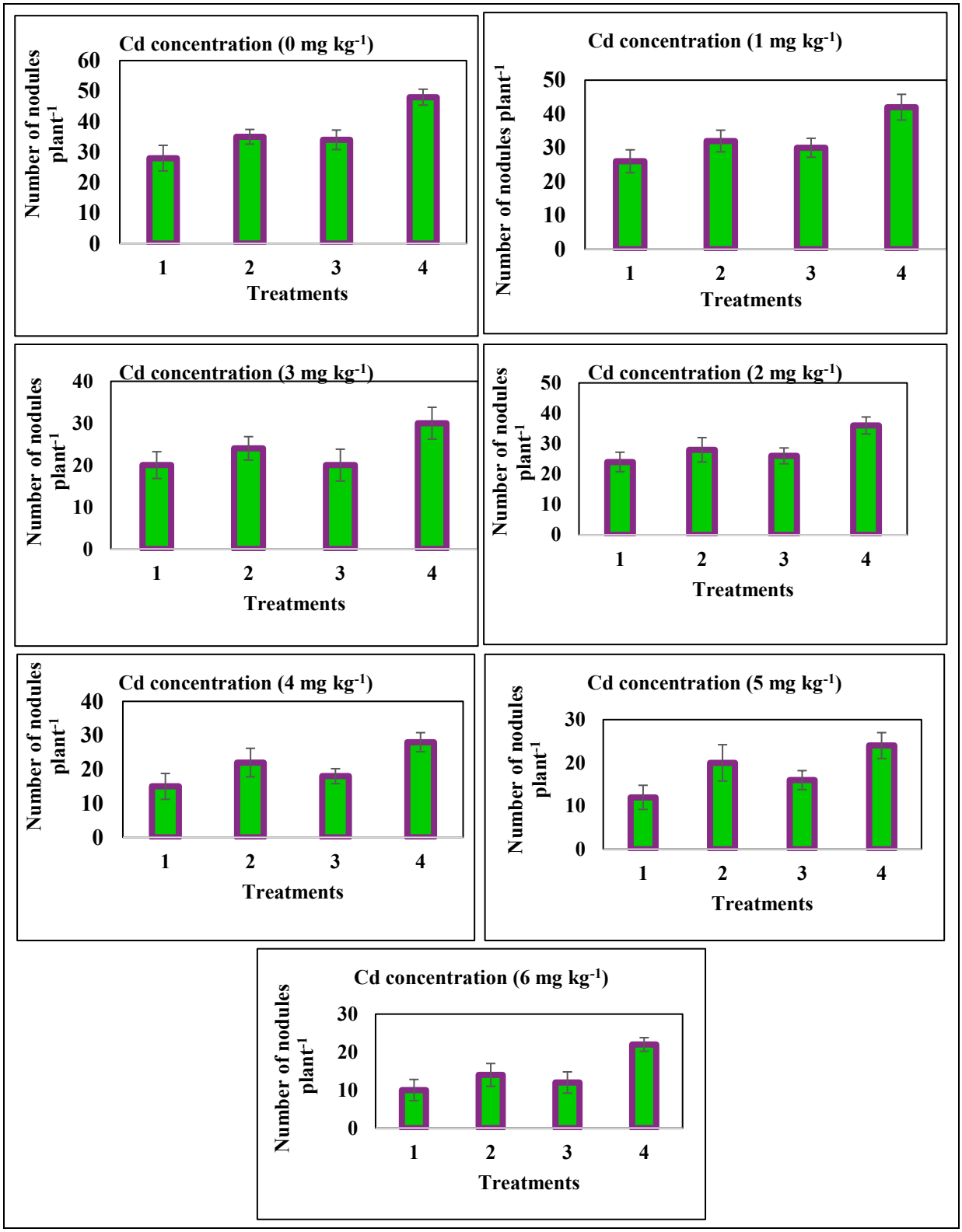


Fig. 4: Effect of humic acid and biochar on nodule produced by black gram inoculated with *Burkholderia sp.* under Cd stress; Error bars indicate ± S.E; n= 5); Mean values sharing common letters are not significant at P = 5% level according to Duncan's multiple range test



Fig. 5: Effect of Cd (2 mg kg^{-1} concentration) on root nodule development in black gram by *Burkholderia sp.* (a) Leonard Jar Assembly, (b) Control (without adding biochar, humic acid, and inoculation of *Burkholderia sp.)*, (c) Plants inoculated with *Burkholderia sp.,* (d) Plants inoculated with *Burkholderia sp.* and added with biochar (2% w/w), (d) Plants inoculated with *Burkholderia sp.* and added with biochar (2% w/w) and humic acid (2% w/w)

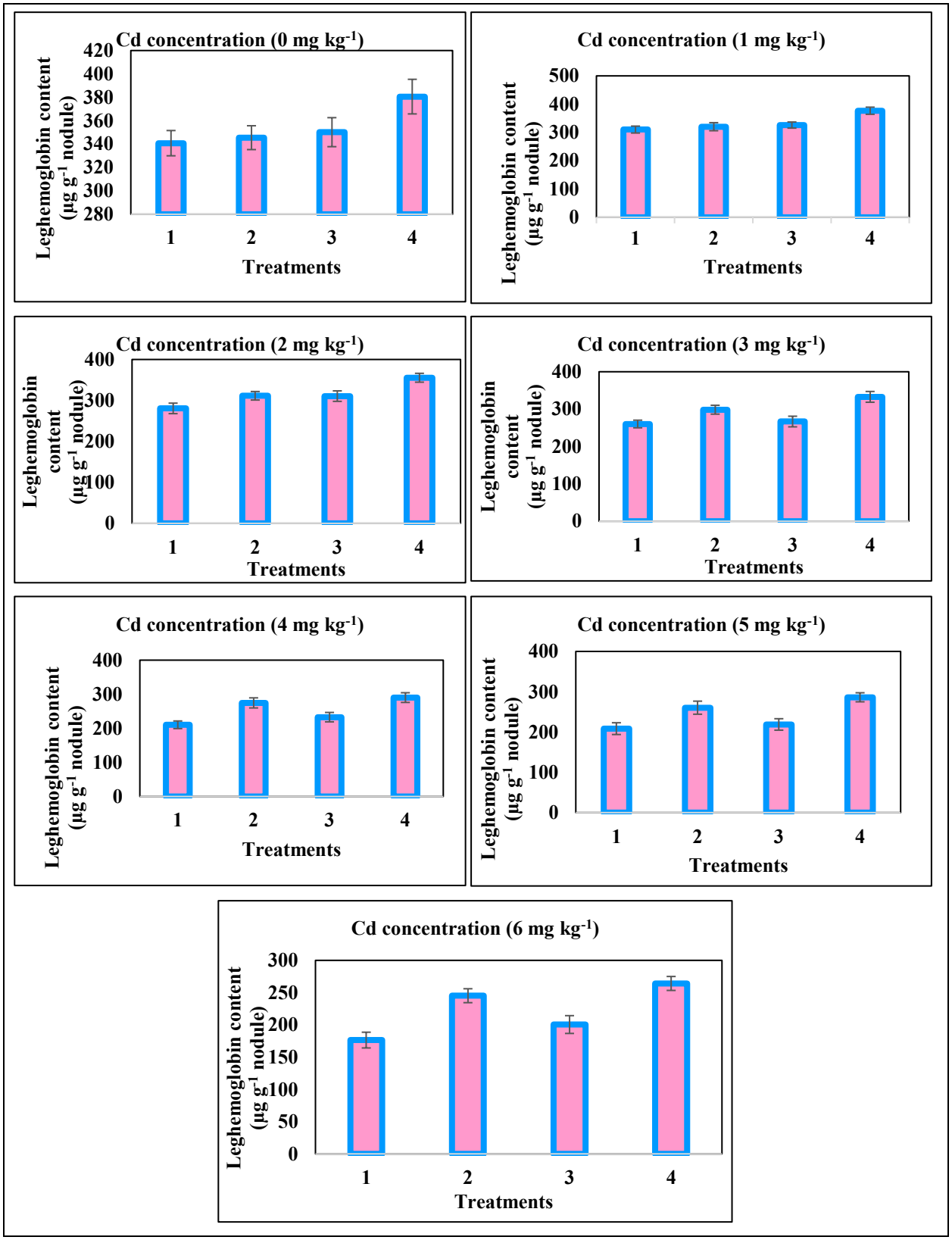


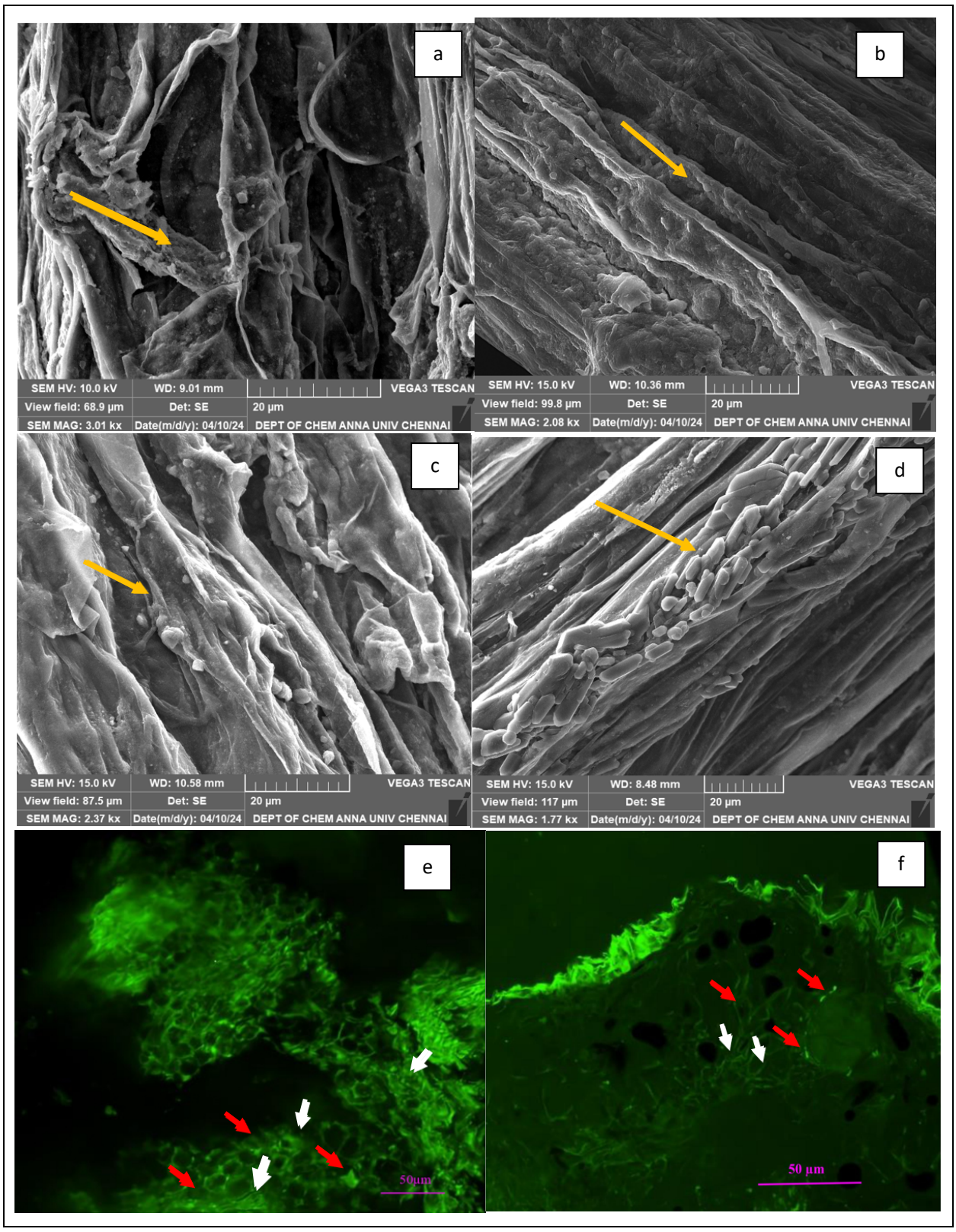
Fig. 6: Effect of humic acid and biochar on leghemoglobin content of black gram nodule inoculated with *Burkholderia sp.* under Cd stress; Error bars indicate ± S.E; n= 5; Mean values sharing common letters are not significant at P = 5% level according to Duncan's Multiple Range Test

Additionally, Cd toxicity hinders water and nutrient uptake by leguminous plants, which in turn inhibits root development and nodule formation (Ahmad *et al.* 2015). Furthermore, Cd toxicity leads to tissue expansion, inflammation, and cell wall thickening, which eventually causes tissue damage (Das *et al.* 2019). In the present study, *Burkholderia sp.* inoculation enhanced nodule production in the plants grown with the addition of HA and biochar, although Cd was added. The application of biochar modifies the physicochemical properties of soil (Mansoor *et al.* 2021), and inhibits the mobility of heavy metals (Meng *et al.* 2024). In the present study, the co-application of biochar with HA increased the root nodule production in Cd-contaminated soil. Humic substances (HS) have the potential to stimulate crop growth and improve the metabolism of microorganisms in the soil, which is in connection with their general buffering capacity (Pukalchik *et al.* 2019). Furthermore, HS can reduce the toxicity of organic pollutants and heavy metals and have a positive and indirect effect on the growth of microorganisms (Rong *et al.* 2020). Under Cd stress conditions, a decrease in biochemical compounds is a prevailing problem (Shahbaz *et al.* 2018). The addition of HS can reduce the toxic effect of heavy metals such as Cd by adsorption onto the surface of humate complexes, thus improving the biochemical compounds (Klučáková *et al.* 2017). Additionally, siderophore-producing bacteria such as *Pseudomonas sp.* and *Burkholderia sp.* redistribute different forms and availability of Cd by forming stable metal-humate complexes (Li *et al.* 2023). The induction of antioxidant systems plays a crucial role in mitigating the severe effects of abiotic stress in legume plants. In legumes, oxygen availability is regulated by factors such as the O_2-binding protein leghemoglobin or the variable oxygen diffusion barrier in the nodule endodermis, which maintains a microaerobic environment within the infected zone of the nodules (Rutten *et al.* 2021). In plants, Cd toxicity is associated with the overproduction of reactive oxygen species (Cuypers *et al.* 2023). However, under Cd stress, cowpea (*Pisum sativum* (L.)) exhibited histological damage, while without Cd treatment, it formed indeterminate nodules with typical histological organization (Tsyganov *et al.* 2020). Studies have also shown that amendment with 40 µM $CdCl_2$ produced a minimal effect, indicating the plant's defense mechanisms could protect it at this level. These defense mechanisms may involve the production of phytochelatins and metallothioneins, among other detoxifying strategies (Cobbett *et al.* 2000). In this study, nodules and leghemoglobin content formation in plants treated with T5 under Cd stress were comparatively higher than in other treatments, suggesting that biochar, HA, and *Burkholderia sp.* could immobilize Cd and protect plants from toxicity.

3.6 Colonization of *Burkholderia sp.* on Roots and Nodules of Black Gram

The roots and nodules collected from black gram plants inoculated with *Burkholderia sp.* grown under Cd stress conditions (2 mg kg^{-1}) were examined under SEM and fluorescence confocal microscopy after 45 days of sowing, and the results are illustrated in Fig. 7. (a) *Burkholderia* sp.; (b) HA (2% w/w+*Burkholderia* sp.); (c) Biochar (2% w/w) + *Burkholderia* sp.; (d) HA (2% w/w) + Biochar (2% w/w) + *Burkholderia* sp., (e & f) Fluorescence confocal microscopy images showing root nodule section (e) Nodule section colonized with *Burkholderia* sp. (black gram grown with 2 mg kg^{-1} Cd and added with biochar (2% w/w); (f) Nodule section colonized with *Burkholderia* sp. (black gram grown with 2 mg kg^{-1} Cd and added with biochar (2% w/w) and HA 2% w/w); Red arrowheads indicating colonization of *Burkholderia* sp. within root nodule tissues shown by intense autofluorescence, white arrowheads showing infection threads by intense autofluorescence.

The micrographs revealed that the roots are colonized with the inoculated *Burkholderia sp.* However, the combined application of HA (2% w/w) and biochar (2% w/w) facilitated more colonization of *Burkholderia sp.* than that of individual application of HA and biochar. Furthermore, while examining thin slices of root nodules using a fluorescence confocal microscope and SEM, it was revealed that the utilization of biochar (2% w/w) and HA (2% w/w) together resulted in an increased formation of infection threads, leading to the colonization of nodules by *Burkholderia sp.* (Fig. 7 g and h). The first physical step of the attachment of bacteria on the root surface is to secure a prime location for the subsequent development of intimate associations (Wheatley *et al.* 2020). Further, the colonization of bacteria on the root surface is associated with the synthesis of adhesive substances such as exopolysaccharides and lipopolysaccharides (Bhagat *et al.* 2021). The colonization of roots with important agricultural bacterial species namely *Rhizobium, Pseudomonas, Azospirillum, Agrobacterium,* and *Burkholderia sp.* has been studied (Maqsood *et al.* 2021). In the present investigation, adding HA and biochar enhanced the colonization of *Burkholderia sp.* under Cd stress. Microorganisms specifically colonize root surfaces by secreting EPS to enhance crop growth and provide stress tolerance (Sun *et al.* 2022). Also, the porous structure of biochar acts as a microhabitat for beneficial microorganisms and protects against stress (Zhang *et al.* 2023). Also, HA addition enhanced the production of growth-promoting substances like auxins by beneficial organisms and the elevated auxin concentration increased root branching, providing a greater surface for colonization.


a
SEM HV: 10.0 kV
WD: 9.01 mm
VEGA3 TESCAN
View field: 68.9 µm
Det: SE
20 µm
SEM MAG: 3.01 kx
Date(m/d/y): 04/10/24
DEPT OF CHEM ANNA UNIV CHENNAI
b
SEM HV: 15.0 kV
WD: 10.36 mm
VEGA3 TESCAN
View field: 99.8 µm
Det: SE
20 µm
SEM MAG: 2.08 kx
Date(m/d/y): 04/10/24
DEPT OF CHEM ANNA UNIV CHENNAI
c
SEM HV: 15.0 kV
WD: 10.58 mm
VEGA3 TESCAN
View field: 87.5 µm
Det: SE
20 µm
SEM MAG: 2.37 kx
Date(m/d/y): 04/10/24
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d
SEM HV: 15.0 kV
WD: 8.48 mm
VEGA3 TESCAN
View field: 117 µm
Det: SE
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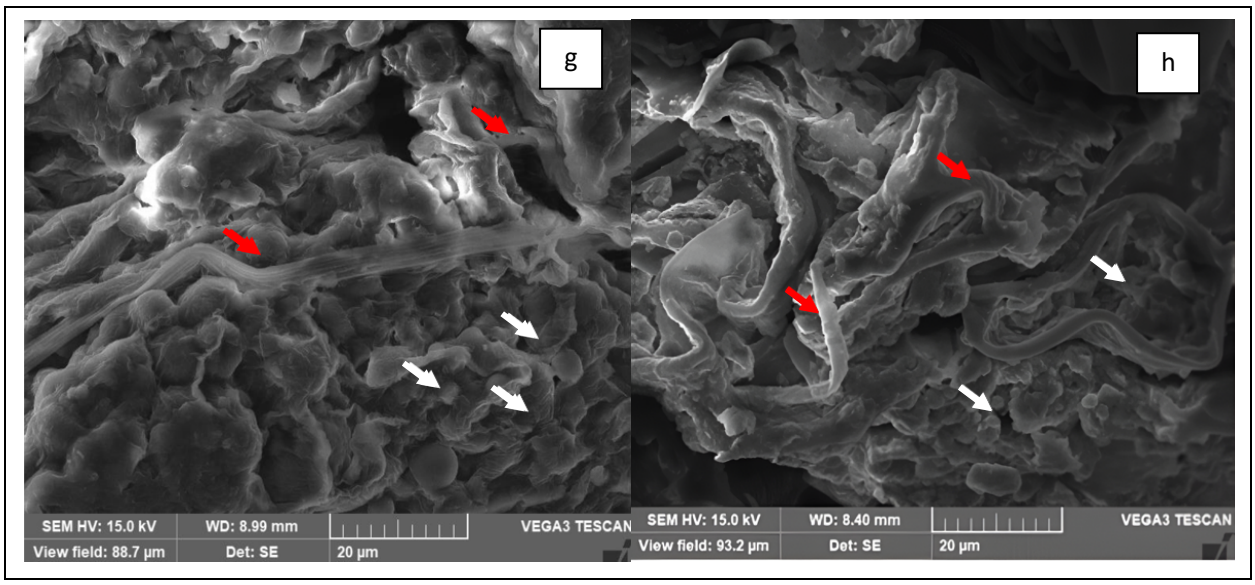


Fig. 7: Colonization of *Burkholderia sp.* on the surface of black gram root grown in Cd (2.0 mg kg^{-1}) stress condition supplemented with Humic acid and Biochar (a) *Burkholderia sp.* ; (b) HA (2% w/w+*Burkholderia sp.*); (c) Biochar (2% w/w) + *Burkholderia sp.* ; (d) HA (2% w/w) + Biochar (2% w/w) + *Burkholderia* sp. ; (e & f) Fluorescence confocal microscopy images showing (e) Nodule section colonized with Burkholderia sp. (black gram grown with 2 mg kg-1 Cd and added with biochar (2% w/w); (f) Nodule section colonized with Burkholderia sp. (black gram grown with 2 mg kg-1 Cd and added with biochar (2% w/w) and HA 2% w/w);(g & h) SEM micrographs of roots colonized with the inoculated *Burkholderia sp.* and combined application of HA (2% w/w) and biochar (2% w/w)

3.7 Cd Accumulation by Plants

The Cd content of black gram plants grown under 2 mg kg^{-1} Cd conditions was analyzed, and the results are presented in Table 1. In all treatments, the root system consistently exhibited higher Cd concentrations than the aerial parts of the plant. Among the treatments, plants in treatment T5 (humic acid (2% w/w) + biochar (2% w/w) + *Burkholderia sp.*) accumulated the least Cd, with values of 0.11 mg kg^{-1} in the aerial system and 0.66 mg kg^{-1} in the root system. In contrast, the control group (T1) recorded the highest Cd accumulation, with 0.60 mg kg^{-1} in the aerial system and 1.00 mg kg^{-1} in the root system.

Cd is a non-biodegradable heavy metal that, once introduced into the environment, can bioaccumulate in the food chain, posing significant risks to plant growth and overall ecological health (Angon *et al.* 2024). Absorption and translocation of Cd in plants depend significantly on its concentration in the soil (Huang *et al.* 2020). Organic amendments have been shown to stabilize Cd, thereby reducing its uptake and translocation into plant tissues (Taeprayoon *et al.* 2022). These amendments enhance soil properties, including pH buffering capacity, base saturation, enzymatic activity, and nutrient availability (Ng *et al.* 2022). Among organic amendments, biochar is particularly effective at immobilizing Cd in the soil, limiting its translocation to the shoot system (Ketaubon *et al.* 2024). Furthermore, a 95% reduction in Cd uptake by plants was observed when humic acid and fulvic acid were applied to the soil, demonstrating their potential for mitigating Cd contamination (Yildirim *et al.* 2021). Moreover, HA + FA changes the solubility and bioavailability of toxic heavy metals by forming compounds with them through strong bonds, reducing the heavy metal stress on plant growth (Wang *et al.* 2020). These strong bonds formed with heavy metals are a result of the cation exchange capacities of humic and fulvic acids owing to the high number of carboxyl and phenolic hydroxyl groups in their structure (Deng *et al.* 2021).

In addition, some microorganisms namely *Rhizobium pusense* KG2 and *Burkholderia sp.* QY14 can immobilize Cd from the soil by intracellular accumulation (Feria-Cáceres *et al.* 2022). In the present investigation, the co-application of *Burkholderia sp.* with biochar and humic acid reduced the accumulation of Cd in the root and aerial systems, which is in agreement with the previous study (Xu *et al.* 2024). Adding biochar significantly reduced acid-soluble soil Cd by 22 – 25% (Zhang *et al.* 2023). Biochar treatments significantly increased the soil pH, leading to the precipitation of Cd as the less soluble form, for example: Cd $(OH)_2$ which is less bioavailable (Zang *et al.* 2023). Also, the surface functional groups namely hydroxyl, and carboxyl on biochar can bind with Cd ions, reducing their mobility (Xu *et al.* 2024). Also, it has been demonstrated that higher humic contents are associated with higher Cd immobilization (Qu *et al.* 2022). Humus is rich in functional groups like carboxyl and hydroxyl groups that can form complexes with cations, leading to greater

stability due to internal hydrogen bonds within the humus (Piccolo *et al.* 2019). Humic acid can interact with metal ions, thereby decreasing their speciation and mobility in soils (Lasota *et al.* 2020). In addition, *Burkholderia sp.* can secrete EPS, which can trap cadmium ions, reducing their mobility. *Burkholderia sp.* can enhance the effectiveness of biochar and humic acid by altering the soil environment, such as producing biosurfactants that increase the bioavailability of nutrients or change the soil pH, which can further immobilize cadmium (Manikandan *et al.* 2023). Thus, combining biochar, humic acid, and *Burkholderia sp.* can create a synergistic effect. In the present study, when compared to the aerial part, the root system accumulated more Cd in all treatments as explained by (Liu *et al.* 2023). Translocation factor of heavy metals measures a plant's ability to move heavy metals from its roots to its shoots and leaves. In the present study, the translocation factor was found to be 0.17 (minimum) to 0.60 (maximum) and comparable with previous research (Rezapour *et al.* 2019).

Many environmental conditions can influence the effectiveness of HA and biochar (Wang *et al*. 2021). Additional research is needed to examine the influence of biotic and abiotic factors on the persistence of biochar and the effects of HA under field conditions.

Table 1. Cd accumulation in plants (Incorporated with 2 mg kg^{-1} Cd)

Treatments	Concentration of Cd in the Aerial Part (mg kg^{-1})	Concentration of Cd in the Root (mg kg^{-1})	Translocation Factor
T1	0.60±0.40 (30%)	1.00±0.04 (50%)	0.60
T2	0.50±0.20 (25%)	0.90±0.02 (45%)	0.56
T3	0.32±0.20 (15%)	0.84±0.04 (42%)	0.38
T4	0.28±0.08 (14%)	0.82±0.04 (41%)	0.34
T5	0.11±0.04 (5.5%)	0.66±0.02 (33%)	0.17

4. CONCLUSION

The current study identified *B. multivorans* strain Strulens as a non-rhizobial endophytic bacterium isolated from black gram root nodules using phylogeny and 16S rRNA sequencing. This bacterium demonstrated the ability to thrive in different Cd concentrations (0–6.0 mM). However, there was a reduction in viable cell count at higher Cd concentrations. Nevertheless, the bacterium could produce IAA and EPS under Cd stress conditions. Inoculating black gram plants with *Burkholderia sp.* in combination with biochar and HA resulted in a higher number of nodules at various Cd concentrations. The number of nodules per plant was 48±2.6, 42±3.8, 36±2.8, 30±3.8, 28±2.8, 24±3.0, and 22±1.8 when the Cd concentration was 0, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg kg^{-1}, respectively. Furthermore, adding HA (2% w/w), biochar (2% w/w), and *Burkholderia sp.* enhanced the leghemoglobin content of the nodules and the phyllosphere bacterial population under Cd stress. The translocation of Cd in the aerial part was the lowest when the plants were inoculated with *Burkholderia sp.* and supplemented with HA (2% w/w) and biochar (2% w/w), which was indicated by the least translocation factor (0.17). Applying biochar and HA helped establish *Burkholderia sp.* on the root surface of black gram, developing infection threads and producing a lot of nodules. Furthermore, effective colonization of the bacteria within the nodule was achieved. From this study, it can be concluded that the endophyte *Burkholderia sp.* holds promise for improving the growth of legume crops. Additionally, further enhancements in plant growth could be achieved through the combined application of HA and biochar along with the inoculation of *Burkholderia sp.* in Cd-contaminated soils.

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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