




Integrated role of biochar and PGPR (*Leclercia adecarboxylata* HW04) in enhancing cadmium phytoremediation and stress tolerance in *Glycine max* L

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) and biochar (BC) are recognized as effective biological agents for enhancing stress tolerance and mitigating heavy metal toxicity in crops. Therefore, this study aims to investigate the effects of the cadmium (Cd)-resistant PGPR strain *Leclercia adecarboxylata* HW04 (>4 mM Cd resistance) on soybean plants exposed to 300 μ M Cd. HW04 was observed to possess the innate ability to synthesize indole-3-acetic acid and exopolysaccharides, which facilitated the absorption of Cd in the medium. Scanning electron microscopy (SEM) images revealed that HW04 effectively colonized the porous structure of BC. Their combined treatment significantly enhanced photosynthesis and improved the morphological characteristics of soybean plants. Additionally, the Cd content in soybean shoots significantly increased following both the sole or combined treatments of BC and HW04. However, the combined treatments significantly reduced Cd content in the roots and soil by 31% and 47%, respectively. HW04 inoculation alone increased Cd content in the roots by 43.7% while lowering it in the soil by 7.4%. Additionally, the co-application of HW04 and BC significantly enhanced calcium (Ca) and magnesium (Mg) assimilation while promoting Cd/Fe accumulation in soybean shoots. The higher expression of signaling cascade genes, including *GmCaMK1* and *GmCDPK5* (Ca signaling) and *GmMAPK4a* and *GmMAPK7* (MAP kinase signaling), in the co-applied crops further validate the findings. The increase in abscisic acid levels and the decrease in salicylic acid levels after HW04 and BC application were correlated with enhanced stress tolerance in crops. These findings suggest that the combined application of HW04 and BC could serve as an effective, eco-friendly approach for mitigating heavy metal toxicity in crops and promoting phytoremediation.

1. Introduction

The contemporary food chain is at significant risk of heavy metal contamination (Clemens et al., 2013). The Food and Agriculture Organization classifies heavy metals, such as cadmium (Cd), as nonessential and highly toxic elements. Extensive anthropogenic activities can exacerbate Cd exposure, contributing to biodiversity loss and increased toxicity throughout the food chain (Ali et al., 2019). The persistent nature of heavy metals and their tendency to biomagnify pose significant

threats to biodiversity and the stability of the food chain (Zhang et al., 2021).

Cd exhibits high solubility and mobility within plant organs. After uptake, Cd forms complexes with several organic ligands or is sequestered into intracellular organelles, such as vacuoles, through specific transport proteins (Sterckeman and Thomine, 2020). Cd is absorbed through transporters associated with zinc (Zn) and iron (Fe), including IRT, ZIP, and NRAMP genes. Subsequently, the compound is transported to the xylem via ATP-dependent transporters, such as HMA (He et al.,

Abbreviations: Cd, cadmium; PGPR, plant growth-promoting rhizobacteria; PGP, plant growth-promoting; DAT, day after treatment; ABA, abscisic acid; SA, salicylic acid; CDPKs, calcium-dependent protein kinases; CaMKs, Ca²⁺/calmodulin-dependent protein kinases; MAPKs, mitogen-activated protein kinases; MB, microbes; BC, biochar; MBBC, microbes+biochar; N₂, nitrogen; CAS, chromeazurol S; EPS, exopolysaccharide; PGP, plant growth-promoting traits; IAA, Indole-3-acetic acid; PVK, medium, Pikovskaya medium; TSB, Tryptic Soy Broth; OD600, optical density at 600 nm; ICP-OES, inductively coupled plasma optical emission spectrometer; EtoAc, ethyl acetate; PVP, polyvinylpyrrolidone; HPLC, high-performance liquid chromatography; CRM, Certified Reference Material; ANOVA, analysis of variance; DMRT, Duncan's multiple range test; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; PS II, photosystem II; CaM, calmodulin.

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2017; Takahashi et al., 2012). The physiological and biochemical toxicity of Cd induces ionic imbalances, lipid peroxidation, chromosomal aberrations, inhibition of enzyme activity and cell division, and chlorophyll degradation (Gill et al., 2013). Therefore, plants utilize several protective mechanisms to mitigate the effects of toxic ions, maintain osmotic balance, and facilitate the uptake of essential elements. These strategies involve the modulation of signaling pathways, the enhancement of antioxidant activity, and the regulation of endogenous phytohormones to enhance plant defense mechanisms (Souza et al., 2017; Yang et al., 2023).

Two primary biological strategies for mitigating heavy metal exposure in the food chain are as follows: (i) phytoremediation, which involves the use of heavy metal-tolerant plants to remediate contaminated soil through phytoextraction, and (ii) phytodetoxification, which employs biological mechanisms to stabilize and chelate metals, thereby reducing their uptake by crops.

To enhance phytoremediation or phytodetoxification, the formulation of safe biological tools is essential for mitigating crop toxicity and promoting growth. Plant growth-promoting rhizobacteria (PGPR)—soil microorganisms—and biochar (BC) have been identified as effective, biologically safe, and environmentally sustainable tools for heavy metal detoxification in both crops and soil (Adhikari et al., 2024). The porous structure of biochar offers a large surface area that fosters microbial growth and protects microorganisms from environmental stressors (Fahad et al., 2015).

The effectiveness of leguminous crops, such as soybeans, in phytoremediation has been extensively documented in several studies. For example, wild soybean (*Glycine soja*) has been shown to accumulate 264.57 kg of soluble salts/ha/year in saline soils, demonstrating its potential for soil remediation (Xu et al., 2020). Additionally, Sanderson et al. (2018) identified *Glycine* max as a promising candidate for the phytoremediation of oil-contaminated soils. A study by Illinskiy et al. (2023) suggests that soybean plants possess a significantly high capacity for accumulating pollutants in their biomass. Moreover, they are effective in the remediation of several contaminants, including heavy metals such as Cu, Zn, Pb, and Cd. Hence, this study explored the phytoremediation of Cd using PGPR in cultivating commercially valuable leguminous crops, such as soybeans. However, a significant limitation of using soybean crops for phytoremediation is the potential for crop toxicity. In contrast, the use of leguminous crops, such as soybeans, offers the advantage of nitrogen (N₂) fixation, which reduces the need for synthetic fertilizers. This reduction in fertilizer application helps mitigate heavy metal pollution, eutrophication of water bodies and land, and aquatic ecosystem degradation (Balasuriya et al., 2022; Rong et al., 2020).

Therefore, this study aims to investigate the synergistic combination of Cd-resistant PGPR and BC to limit and manage Cd uptake and deposition in soybean crops under controlled conditions, acknowledging that it may not account for all environmental variables. Additionally, this study seeks to elucidate the underlying mechanisms of the PGPR-Cd-soybean interaction from both physiological and molecular perspectives.

2. Materials and methods

2.1. Microbial bioassay

2.1.1. Collection, isolation, and characterization of plant growth-promoting rhizobacteria

The rhizobacterial isolates used in this study were collected from the rhizospheres of plants located along the beaches of Gyeongju and Pohang, Gyeongsangbuk-do, South Korea. These isolates were subsequently screened for various plant growth-promoting (PGP) traits.

Exopolysaccharide (EPS) production was assessed using Congo red agar medium; siderophore production activity was evaluated on chromeazuroil S (CAS) agar medium. Indole-3-acetic acid (IAA) production

was determined using the Salkowski test, while phosphate solubilization activity was tested in Pikovskaya (PVK) medium. The oxidative stress tolerance of the bacterial strains was evaluated by incubating each isolate in Tryptic Soy Broth (TSB) media supplemented with methyl viologen at 28 °C, with shaking at 150 rpm for 72 h. The optical density at 600 nm (OD₆₀₀) was measured to compare the growth of the treated samples to that of the control.

2.1.2. Visualization of plant growth-promoting traits in isolated strains

To identify potential microbes for further investigation, several tests were conducted. EPS production was confirmed by the appearance of a darkened zone around the colony, while siderophore production was confirmed by the formation of an orange halo surrounding the colony. Similarly, a color change in the Salkowski reagent from yellow to pink-red indicated IAA production. Phosphate solubilization activity was observed by the formation of a clear halo zone around the colony.

2.1.3. Cadmium resistance test

The Cd tolerance of each isolate was assessed on TSA agar plates supplemented with Cd concentrations of 0.5, 1, 2, and 4 mM. The isolates were incubated at 28 °C for 72 h, and their growth was compared to that of the control.

2.1.4. Amino and organic acid production tests

To analyze the amino and organic acid contents in bacterial cultures, the isolates were grown in a TSB medium for 3 days, after which the supernatant was collected.

Amino acid content was evaluated using a modified version of the method described by Kazerooni et al. (2021), with analysis performed with an amino acid analyzer (HITACHI L-8900, HITACHI, Japan).

Organic acid content was measured using the method described by Lee et al. (2019), employing a high-performance liquid chromatography (HPLC) system (Prominence, Shimadzu Co., Japan) equipped with a PL Hi-Plex H column (7.7 × 300 mm, Waters Co., Milford, MA, USA) and a refractive index detector (RI, Waters 410, Milford, MA, USA). A pilot experiment was conducted to evaluate the effect of isolate treatment on soybean plants.

Detailed methodologies are available in the Supplementary File. Based on the screening results, strain HW04 was selected for further investigation. HW04 identification was conducted through 16S rRNA gene sequence analysis using Solgent technology (Daejeon, Korea).

Based on the screening results, strain HW04 was selected for further investigation.

2.1.5. Evaluation of cadmium removal efficiency of strain HW04

To evaluate the Cd removal efficiency of strain HW04, the bacterium was cultured in a TSB medium supplemented with Cd (0, 0.5, and 1 mM) for 24 h. Following incubation, the change in Cd concentration in the medium was measured using the method described by Li et al. (2018). The HW04 culture was centrifuged at 6000 rpm for 10 min, with nitric acid (HNO₃) added to the supernatant. Subsequently, the mixture was heated in a heating block at 100 °C for 3 h and filtered. The resulting sample was analyzed using an inductively coupled plasma optical emission spectrometer (ICP-OES; Optima 7900DV, PerkinElmer, Shelton, CT, USA) to quantify the Cd concentration.

2.1.6. Evaluation of biochar porosity and SEM analysis of HW04 colonization visualization

The average pore size and porosity of the BC were measured using a porometer (Autopore V 9620, Micromeritics, Norcross, GA, USA) (Table 1).

Scanning electron microscopy (SEM) was conducted to visualize the colonization of strain HW04 on the BC surface. The method described by Bilal et al. (2018) was followed for BC visualization. Strain HW04 was cultured in TSB medium for 24 h, while sterilized BC was incubated with the bacterial culture for 3 h. Following incubation, the BC was collected

Table 1
Indicators of biochar characteristics used in this study.

Parameter	Measurement
Total pore area	10.3560 m ² /g
Density	Bulk density Apparent density
	0.4027 g/mL 1.1888 g/mL
Average pore diameter	634.2800 nm
Porosity	66.1266%

$$\text{Total chlorophyll (mg/g DW)} = \{[(20.2 \times A645) + (8.02 \times A663)] / 1000 \times W\} \times V$$

by centrifugation at 6000 rpm for 10 min. The sample was freeze-dried and mounted onto aluminum stubs using adhesive carbon tape, followed by platinum coating with a sputter coater. Subsequently, the stub was loaded into the SEM (HITACHI 3500N, HITACHI, Japan) for observation. The average pore size and porosity of the BC were evaluated using a porometer (Autopore V 9620, Micromeritics, USA).

2.2. Plant experiment

2.2.1. Materials

Cd (CdSO₄·8 H₂O; molecular weight: 769.53 g/mol), BC (70% wood char, 10% dolomite, 5% zeolite, 10% molasses, and 5% peat), soybean seeds (cv. Pungsannamul), the rhizobacterium *Leclercia adecarboxylata* HW04, and horticultural bed soil (Shinsung Mineral Co., Ltd., Goesan, South Korea) were utilized in the experiment. The greenhouse conditions were maintained (Temperature 27 ± 2 °C, relative humidity (RH) 60–70%).

2.2.2. Pilot test, experimental conditions, and final plant experiment setup

Supplementary File, Section 1 and Fig. S1 present the details of the pilot test conducted on soybean seedlings. Based on the findings from strain HW04, the final experiment was conducted under conditions similar to those described in the pilot seedling experiment. Table 2 shows the treatment design.

Following the protocol used in the pilot experiments, bacterial inoculum suspension and sterilized distilled water were applied to the pots starting at the V1 growth stage (50 mL/pot). BC was added to each pot at a rate of 1.5 g/pot 1 day before the inoculation with the bacterial suspension. Cd treatment was initiated 4 days after inoculation with HW04 (300 μM, 50 mL/pot). The Cd treatment was administered three times at 2-day intervals. Subsequently, physiological and molecular analyses were conducted on the plants.

2.2.3. Measurement of plant growth parameters and photosynthetic components

Several plant growth parameters were measured 3 days after treatment (3DAT), including shoot length, root length, stem diameter, shoot

Table 2
Experimental design.

Normal condition	Cd-stressed condition
(a) -Cd control (irrigated with distilled water)	(e) + Cd control (treated with Cd)
(b) BC (treated with biochar)	(f) BC + Cd (treated with biochar + Cd)
(c) MB (treated with HW04)	(g) MB + Cd (treated with HW04 + Cd)
(d) MBBC (treated with HW04 + biochar)	(h) MBBC + Cd (treated with HW04 + biochar + Cd)

Note: Each treatment consists of nine replicates.

Abbreviations: BC, biochar; MB, microbe *Leclercia adecarboxylata* HW04 inoculum.

fresh weight, root fresh weight, and leaf number. Additionally, chlorophyll content and leaf fluorescence were measured at 3DAT. Chlorophyll content was evaluated following the method described by Radhakrishnan and Lee (2016). The freeze-dried leaf samples were extracted using 80% acetone and centrifuged at 15,000 rpm for 10 min to isolate the supernatant. Subsequently, absorbance was measured at 663 nm and 645 nm. Chlorophyll content was calculated using the following formula:

In this equation, "W" represents the dry weight of the sample, while "V" denotes the volume of the extract. Chlorophyll fluorescence was measured using a chlorophyll fluorometer (OS5p+, Opti-Sciences, USA). Table S1 describes the specific chlorophyll fluorescence parameters used.

2.2.4. Quantification of endogenous phytohormones: abscisic and salicylic acids

Endogenous abscisic acid (ABA) levels were analyzed using the method described by Shahzad et al. (2016). Briefly, freeze-dried plant samples were treated with an ABA extraction solution (isopropanol/acetic acid, 95/5, v/v). Subsequently, the suspension was spiked with 100 ng of an ABA standard [(±)-3,5,5,7,7,7-d⁶]. The extraction process involved several steps: chlorophyll was removed using dichloromethane (CH₂Cl₂), and ethyl acetate (EtOAc) was employed for extraction. A polyvinylpyrrolidone (PVP) suspension was prepared, followed by pH adjustment and a final extraction with EtOAc. The resulting extract was dried under N₂. Subsequently, the samples were methylated using diazomethane, dissolved in dichloromethane, and injected into a gas chromatography-mass spectrometry/selective ion monitoring (GC-MS/SIM) system (6890N Network GC System and 5973 Network Mass Selective Detector; Agilent Technologies, Santa Clara, CA, USA). The ABA content was calculated by comparing the peak areas of ions 190 and 194.

Salicylic acid (SA) was measured using a modified version of the method described by Seskar et al. (1998). First, the freeze-dried sample was extracted using 90% methanol and subsequently dried using a Savant SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the residue was sonicated with 5% trichloroacetic acid and partitioned using an SA extraction solution (C₄H₈O₂/C₅H₁₀/C₃H₈O, 49.5/49.5/1, v/v/v). The organic solvent layer was concentrated using a Savant SpeedVac concentrator. The concentrated sample was dissolved in 100% methanol and subsequently injected into an HPLC system equipped with a fluorescence detector (Shimadzu RF-10AXL, Columbia, MD, USA).

2.2.5. Quantification of cadmium and mineral nutrient content in soybean plants and soil

The mineral nutrients and Cd content were quantified following the method described by Khan et al. (2014). A 0.1 g portion of the freeze-dried sample was digested in nitric acid (HNO₃) for 3 h at 100 °C using a heating block. After digestion, the solution was diluted with distilled water, before being analyzed using ICP-OES. Certified Reference Material (CRM), specifically the ICP Multi-Element Standard Solution IV Certipur® (Matrix: 6% HNO₃; Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany), was used as the standard for quantification.

2.2.6. Quantification of gene expression using Real-Time PCR

Gene expression analysis was conducted on plant samples collected at 3DAT. Total RNA was extracted using the HiGene™ Total RNA Prep Kit (Biofact Co., Ltd., Daejeon, South Korea) following the instructions of the manufacturer. Subsequently, complementary DNA (cDNA) was synthesized using the BioFACT™ RT-Kit (Biofact Co., Ltd., Daejeon, South Korea), following the instructions of the manufacturer. The synthesized cDNA was used for quantitative reverse transcription PCR (qRT-PCR) to analyze gene expression, following the method described by Kazerooni et al. (2021). This analysis was conducted using the Illumina platform (Illumina, Inc., San Diego, CA, USA). Table S2 presents the genes analyzed and their corresponding primers.

2.3. Statistical analysis

Comparison of media components before and after culture was performed using a paired *t*-test ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$). Other results were analyzed using analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) ($p < 0.05$). Statistical analyses were conducted using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC, USA) and IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY, USA). Control groups, which received no treatment, served as the baseline for comparison with treated groups.

3. Result

3.1. Screening and identification of bacterial isolates

The rhizobacterial isolates were collected and evaluated for their PGP traits and resistance to heavy metals. The evaluation included tests for EPS production, siderophore production, IAA production, phosphate solubilization, and oxidative stress tolerance. Based on the PGP trait evaluations, seven isolates were selected (Fig. S2). Subsequently, these isolates were exposed to several Cd concentrations (0.5, 1, 2, and 4 mM) to assess their Cd tolerance. Among these, HW02, HW04, HW06, and HW07 demonstrated the highest tolerance to Cd (Table S3) and they

were subsequently selected for further investigation.

3.2. Bioanalysis of organic and amino acids in pure cultures of bacterial isolates

Cluster heatmap analysis was conducted to evaluate the amino and organic acids produced by bacterial cultures. The analysis revealed three distinct clusters: one containing isolates HW02, HW06, and HW07 and two separate clusters for HW04 and the control group. HW04 demonstrated a significant production of amino acids, including isoleucine (141%), leucine (118%), tyrosine (107%), phenylalanine (94%), and lysine (57%). Additionally, HW04 produced a significant amount of propionic acid, with a concentration of $2314 \mu\text{g mL}^{-1}$ (Fig. 1).

3.3. Pilot experiments for evaluating plant growth-promoting effects

The pilot experiment results demonstrated that inoculation with HW02, HW06, and HW07 did not induce significant changes compared to that of the broth treatment. However, HW04 significantly enhanced overall soybean growth compared to that of the broth treatment, leading to increases in shoot length (11%), shoot fresh weight (23%), and leaf number (27%). Additionally, root length and root fresh weight increased by 17% and 22%, respectively; however, these increases were not statistically significant between treatments (Fig. S1). Therefore, HW04 was selected for further experiments, as it demonstrated the highest seedling growth rate compared to those of the other treatments and the control.

3.4. HW04 identification, biochar colonization, and cadmium mobility testing

The 16S rRNA sequence of HW04 was compared against the NCBI database, revealing a 99% similarity to *Leclercia adecarboxylata*. Phylogenetic analysis, conducted using MEGA11 software, was used to construct a tree illustrating its relatedness to other strains (Fig. 2A). The HW04 sequence has been deposited in the NCBI database under accession number OR294030.

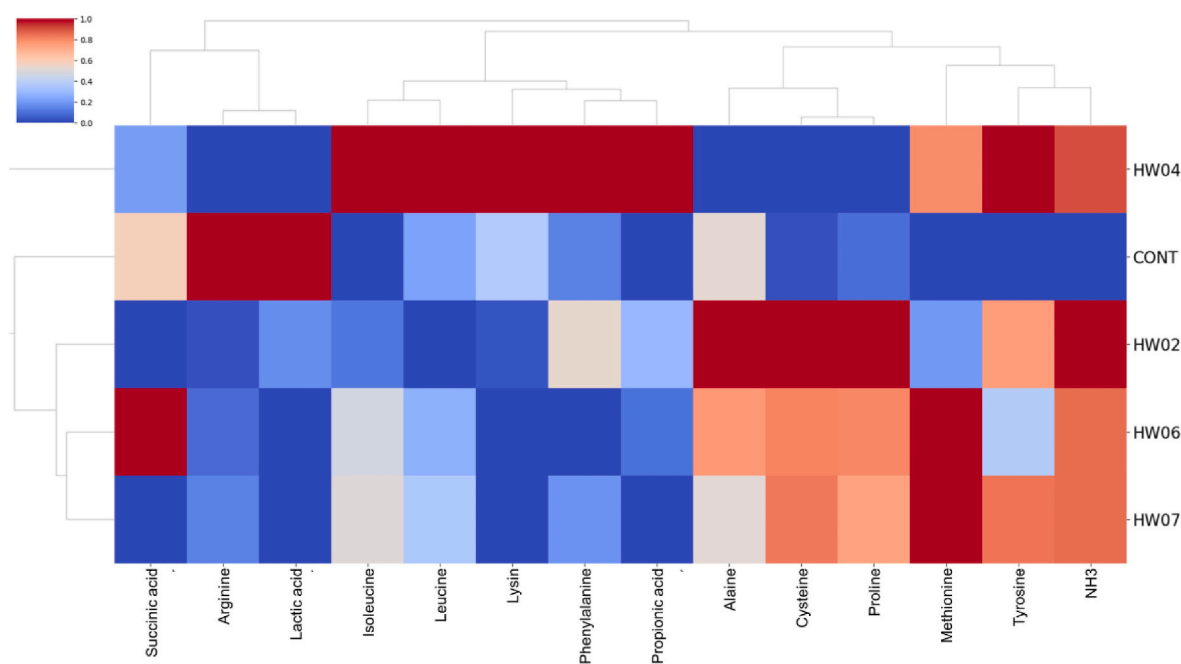


Fig. 1. Cluster analysis and heatmap of amino and organic acid content in bacterial isolates. The heatmap illustrates the normalized concentrations of amino and organic acids, where red indicates higher concentrations, and blue represents lower concentrations. The intensity of each color represents the magnitude of the value, with darker shades indicating higher concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

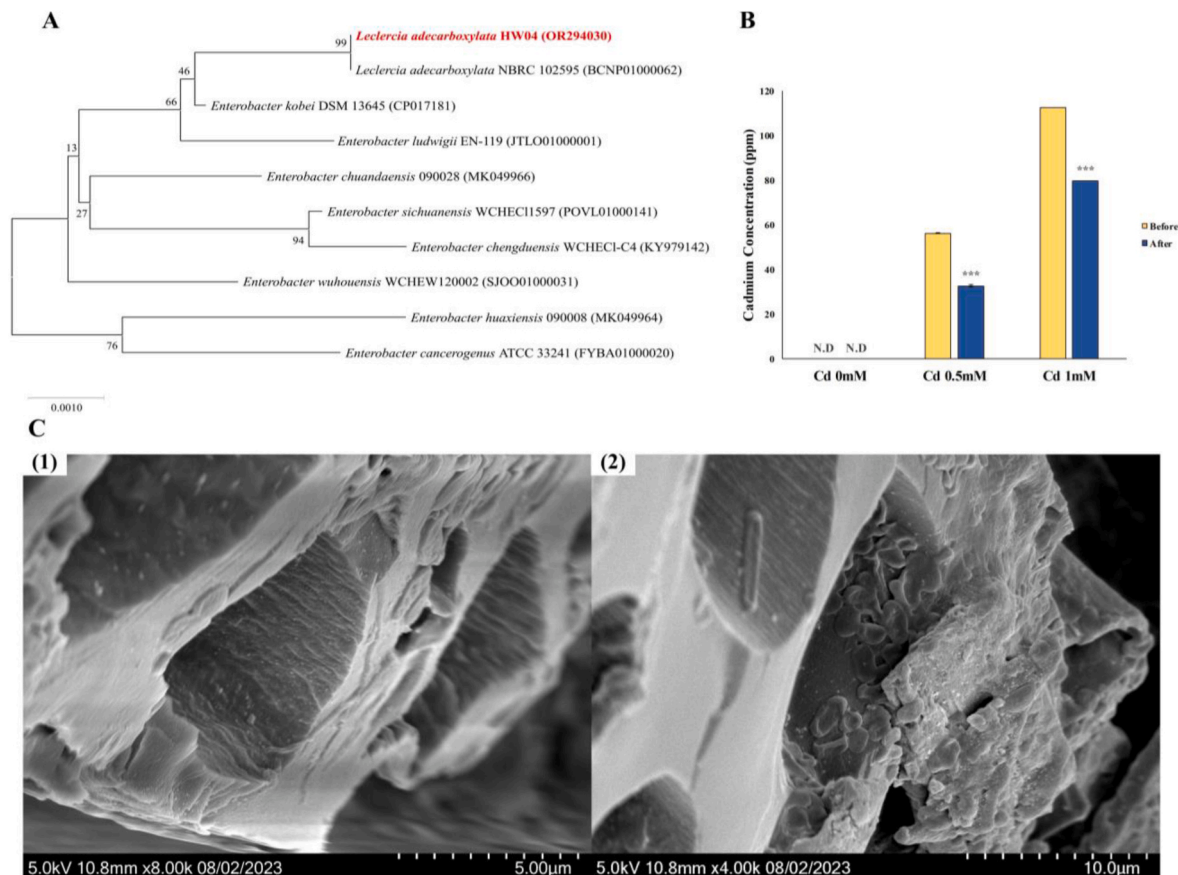


Fig. 2. (A) Neighbor-joining phylogenetic tree of *Leclercia adecarboxylata* HW04 based on partial 16S rRNA sequences. The phylogram illustrates the evolutionary relationships between HW04 and closely related taxa. Bootstrap values, calculated from 1000 replications, are indicated at the branch points. (B) Variation in Cd content in HW04 cultures grown in Cd-supplemented TSB media (0.5 mM and 1 mM). Data points represent the mean of three independent replicates, with error bars indicating the standard error of the mean (SEM). Statistical significance was determined using a paired *t*-test ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$). (C) SEM images showing (1) BC and (2) BC colonized by HW04.

Abbreviations: Cd, cadmium; SEM, scanning electron microscope; BC, biochar.

To evaluate the Cd removal efficiency of HW04, the amount of Cd removed over 24 h was measured. The findings suggest a 40% reduction in Cd concentration in the 0.5 mM Cd medium and a 29% reduction in the 1 mM Cd medium (Fig. 2B).

Fig. 2C illustrates the colonization of BC by HW04, showing that the bacterial strain effectively inhibited the porous structure of the BC.

3.5. Response of soybean seedlings to inoculation with plant growth-promoting rhizobacteria under cadmium stress

3.5.1. Plant growth characteristics

The soybean seedlings were treated based on the experimental design, which included individual and combined applications of HW04 and BC under Cd stress. Under control conditions, the biomass of the seedlings significantly increased by 31.6%, 7.8%, and 44.1% following treatments with MB, BC, and MBBC combination, respectively. Visual observations revealed red coloration on the venation and stems of seedlings exposed to Cd. However, these symptoms were alleviated with co-application treatments. Furthermore, the co-application mitigated the Cd-induced reduction in growth, particularly in shoot growth. The shoot fresh weight of Cd-exposed seedlings increased by 74% with co-application (Fig. 3). Both BC and HW04 significantly increased all growth parameters under both normal and Cd-stressed conditions.

3.5.2. Measurement of chlorophyll content and photosynthetic parameters using OJIP curves

The assessment of photosynthetic parameters through OJIP curves is

crucial for evaluating quantum yield and stress levels in crops. Chlorophyll content showed no significant differences under normal conditions but significantly increased in MB and BC-treated groups under Cd exposure (Fig. 4A). Under normal conditions, co-application enhanced electron transport flux and activated the PSII reaction center in seedlings. Similar trends were observed in Cd-exposed soybean plants, though with subtle variations. The sole application of HW04 slightly increased the dissipation energy flux, an effect mitigated when combined with that of BC. Furthermore, the co-application significantly increased the performance index (PI), indicating enhanced electron flow through PSII (Fig. 4B and C). The parameters TR_o/ABS , ET_o/TR_o , ET_o/CS , RC/CS_o , RC/CS_m , P_t , and DIO/CS increased with both individual and combined treatments of BC and HW04, with the highest PI value observed. Table S1 lists the abbreviations used in the OJIP curves.

3.5.3. Phytohormone regulation in soybean plants

Cd exposure significantly increased ABA accumulation by 29%, which was further enhanced by HW04 inoculation (Fig. 5A). In contrast, HW04 inoculation significantly downregulated *GmNCED3* expression, which encodes the enzyme NCED3 involved in ABA biosynthesis, compared to that in the control (Fig. 5B).

The SA analysis revealed a similar trend of increase by 191% under Cd exposure, then gradually declined with BC and HW04 treatments. Under normal conditions, treatments slightly reduced SA levels. However, under Cd-stress, BC and HW04 alone reduced SA levels by 25% and 42%, respectively, while co-application achieved a 50% reduction (Fig. 5C). These findings indicate an antagonistic interaction between

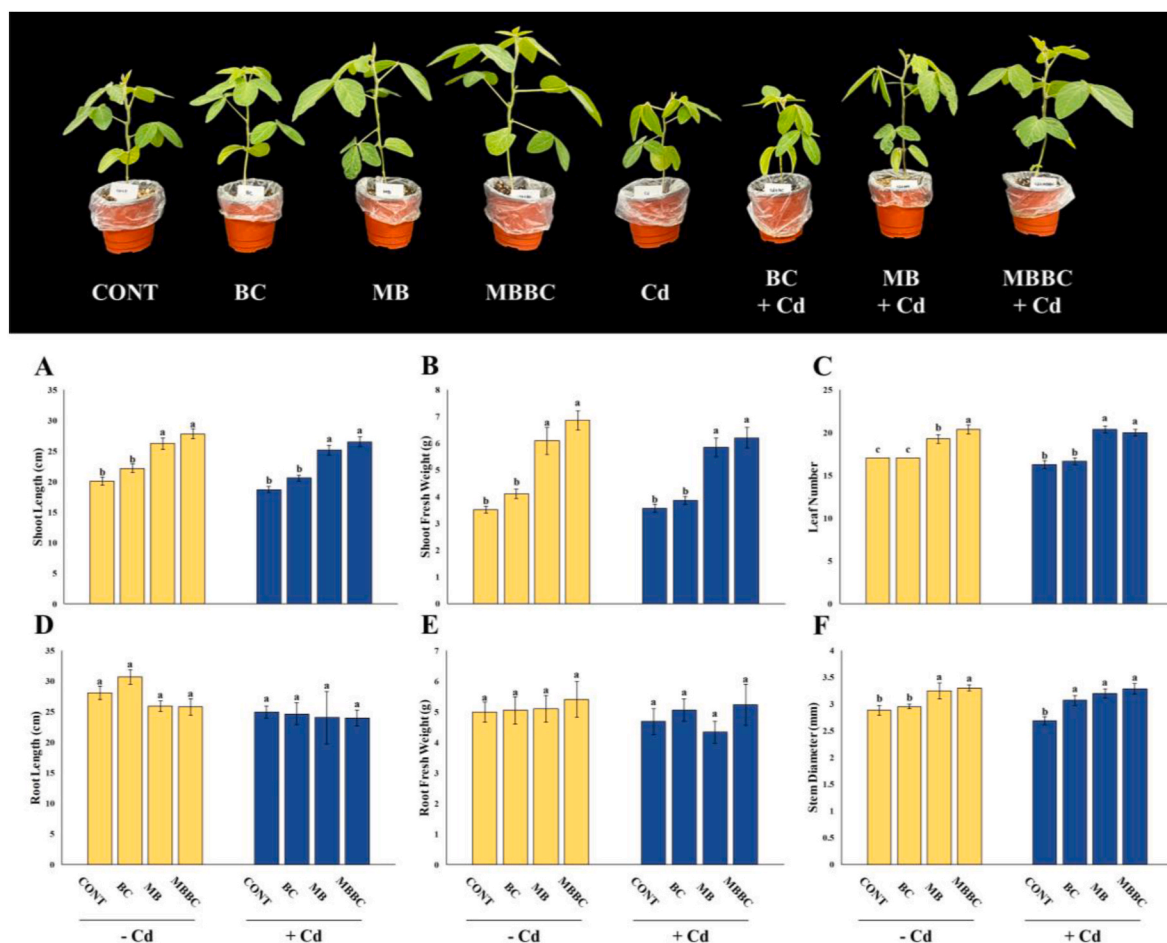


Fig. 3. Effects of HW04 and biochar on soybean growth under cadmium stress conditions. (A) Shoot length, (B) Shoot fresh weight, (C) Leaf number, (D) Root length, (E) Root fresh weight, and (F) Stem diameter of soybean plants. Data are presented as the standard error of the mean (SEM) from five experimental replicates. Statistically significant differences ($p < 0.05$) are indicated by different letters above the bars, as determined using Duncan's Multiple Range Test (DMRT). Table 2 presents the abbreviations used in the treatments.

ABA and SA under Cd-exposed conditions.

3.5.4. Quantification of mineral nutrients (calcium, magnesium, and iron) and cadmium content

In Plants: The ICP quantification analysis revealed that Cd exposure significantly reduced calcium (Ca) translocation compared to that of the control. In contrast, the sole or co-application of HW04/BC significantly increased Ca translocation from root to shoot (Fig. 6A–C). Root Cd accumulation (+Cd control) was measured at 39.67 mg kg^{-1} and significantly reduced by 28% with HW04 and BC co-application (Fig. 6G). The sole application of HW04 or BC had no significant effect on Cd content; however, the co-application increased shoot Cd content by 18%. HW04 treatment alone significantly increased root Cd content, while co-application with BC significantly reduced it. These findings suggest that HW04 potentially enhances phytoremediation, while BC application could facilitate greater Cd accumulation in the shoots and reduce root Cd levels.

In soil: Under normal conditions, soil samples were free of Cd content, and nutrient bioavailability for elements—Ca, magnesium (Mg), and iron (Fe)—was maximized (Fig. 6D–F). Under Cd exposure, the MBBC treatment significantly reduced soil Cd content by 47.3%, followed by a 47.3% reduction with BC. The control exhibited the highest Cd levels, with a 7.4% reduction in the MB treatment (Fig. 6H).

3.5.5. Evaluation of plant signaling: calcium and MAPK signaling

In this study, the expression levels of Ca^{2+} sensor-related genes

(*GmCDPK1*, *GmCDPK5*, and *GmCaMK1*) and mitogen-activated protein kinase (MAPK) cascade-related genes (*GmMAPK4a* and *GmMAPK7*) were analyzed to investigate Ca signaling and biochemical crosstalk. Cd exposure significantly downregulated *GmCDPK1* (66%), *GmCDPK5* (55%), and *GmCaMK1* (57%) expression in Cd-stressed control plants compared to those in the normal control. However, co-application of HW04 and BC significantly upregulated these genes by 52%, 178%, and 158%, respectively, compared to those in the Cd + control (Fig. 7A–C). Similarly, while Cd exposure reduced MAPK signaling, co-application restored it, increasing *GmMAPK4a* and *GmMAPK7* expression by 133% and 271%, respectively, compared to those in the +Cd control (Fig. 7D and E).

4. Discussion

Detoxifying pollutants from crops in agricultural land poses a significant challenge for sustainable agriculture. Although various technological methods have been developed to address these challenges, many are energy-intensive, costly, and may have potential long-term side effects. Therefore, biological methods, such as PGPR and BC, offer a more efficient alternative despite certain inherent challenges.

4.1. Challenges and potential of biological methods in cadmium detoxification

Detoxifying pollutants from crops in agrarian land presents a

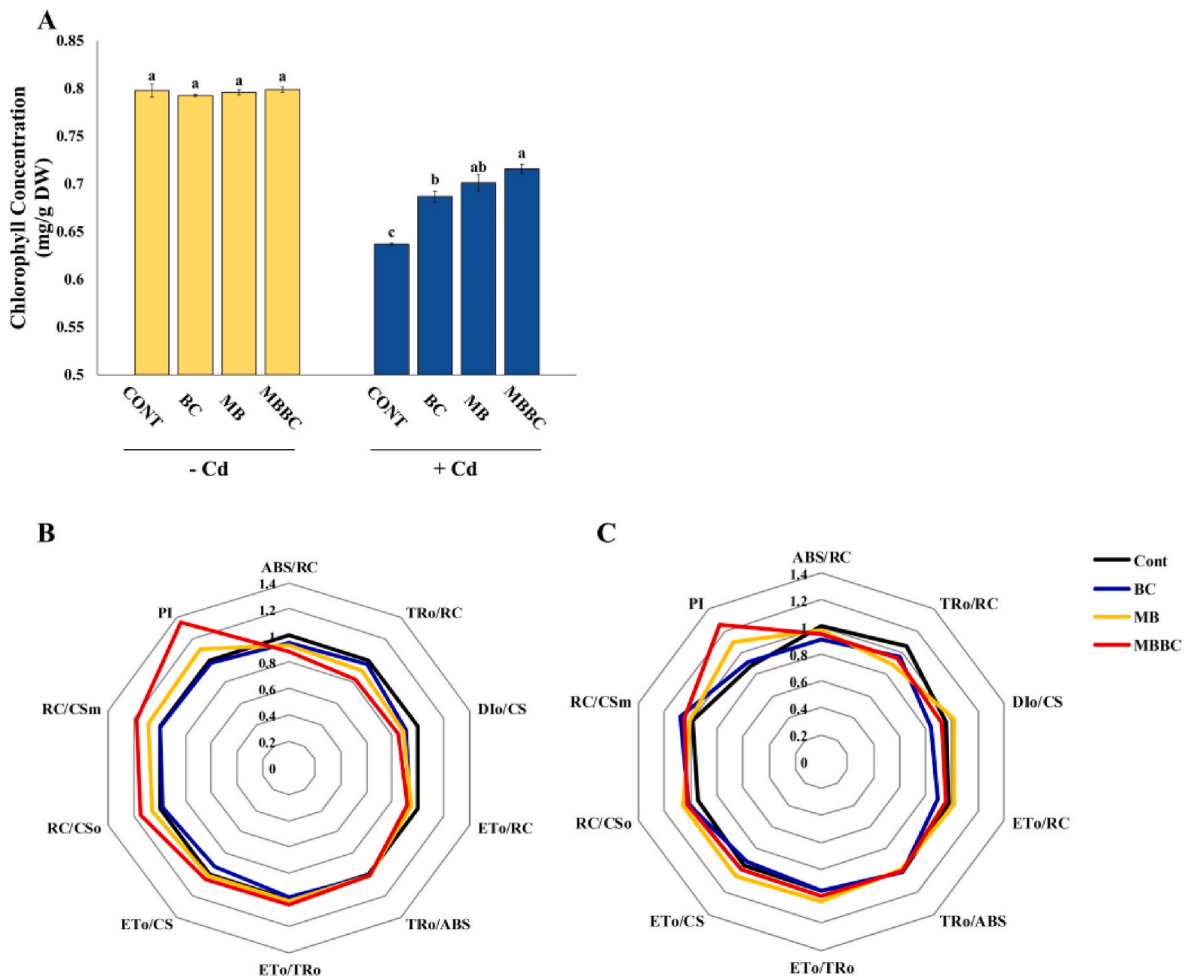


Fig. 4. Effects of HW04 and biochar on chlorophyll content and fluorescence in soybean under cadmium stress. (A) Chlorophyll concentration. Photosynthetic parameters measured using the OJIP curve under (B) normal and (C) cadmium-stressed conditions. Data are represented as the mean of five experimental replicates, with error bars indicating the standard error of the mean. Different letters above the bars indicate statistically significant differences at $p < 0.05$, as determined using Duncan's Multiple Range Test (DMRT).

significant challenge for sustainable agriculture. While various technological methods have been developed to address these challenges, many are energy-intensive, costly, and may have potential long-term side effects. Consequently, employing biological methods, such as PGPR and BC, represents a more efficient alternative despite some inherent challenges.

4.2. Role of *Leclercia adecarboxylata* HW04 in stress mitigation

In this study, the role of co-applying BC and *Leclercia adecarboxylata* HW04 in alleviating stress was evaluated, exploring the underlying mechanisms from physiological and molecular perspectives. The bacterial strain *Leclercia adecarboxylata* HW04 exhibited several PGP traits, including metabolite production, mineral solubilization, and resistance to Cd (>4 mM). Additionally, HW04 demonstrated the innate ability to absorb Cd, reducing its levels by 40% in a Cd-supplemented pure culture medium. These findings are consistent with those of previous studies, showing that several microorganisms can reduce Cd bioavailability through mechanisms such as biosorption, bioaccumulation, and complexation (Hetzer et al., 2006; Wu et al., 2009).

4.3. Role of biochar in enhancing microbial efficiency

Optimal conditions for microbial growth are crucial for maximizing efficiency (Chompa et al., 2023). In this study, the combination of

microbes and BC enabled effective microbial colonization of the porous matrix of BC, as confirmed by SEM. This finding suggests that the porous structure of BC provides an ideal environment for HW04 activity and, in combination with these tools, promotes synergistic effects on soil health and plant metabolism.

4.4. Enhancement of soybean growth and photosynthetic attribute

Pilot experiments show that HW04 significantly promotes soybean growth, necessitating further investigation. This enhancement is potentially owing to the ability of HW04 to improve the bioavailability of essential minerals, produce siderophore, and synthesize other beneficial biochemicals.

Further experimental results demonstrated that co-application significantly enhanced vegetative growth and photosynthetic parameters compared to those of the control. Photosynthetic components that determine chlorophyll fluorescence indices are key indicators for assessing metal-induced stress levels. In this study, HW04 and BC applications improved several PS II-regulating components, including the performance index, electron transfer, heat dissipation, and energy dissipation. These components enhance quantum energy absorption, alleviate photo-oxidative damage, and increase the net photosynthetic rate (Zhang et al., 2020).

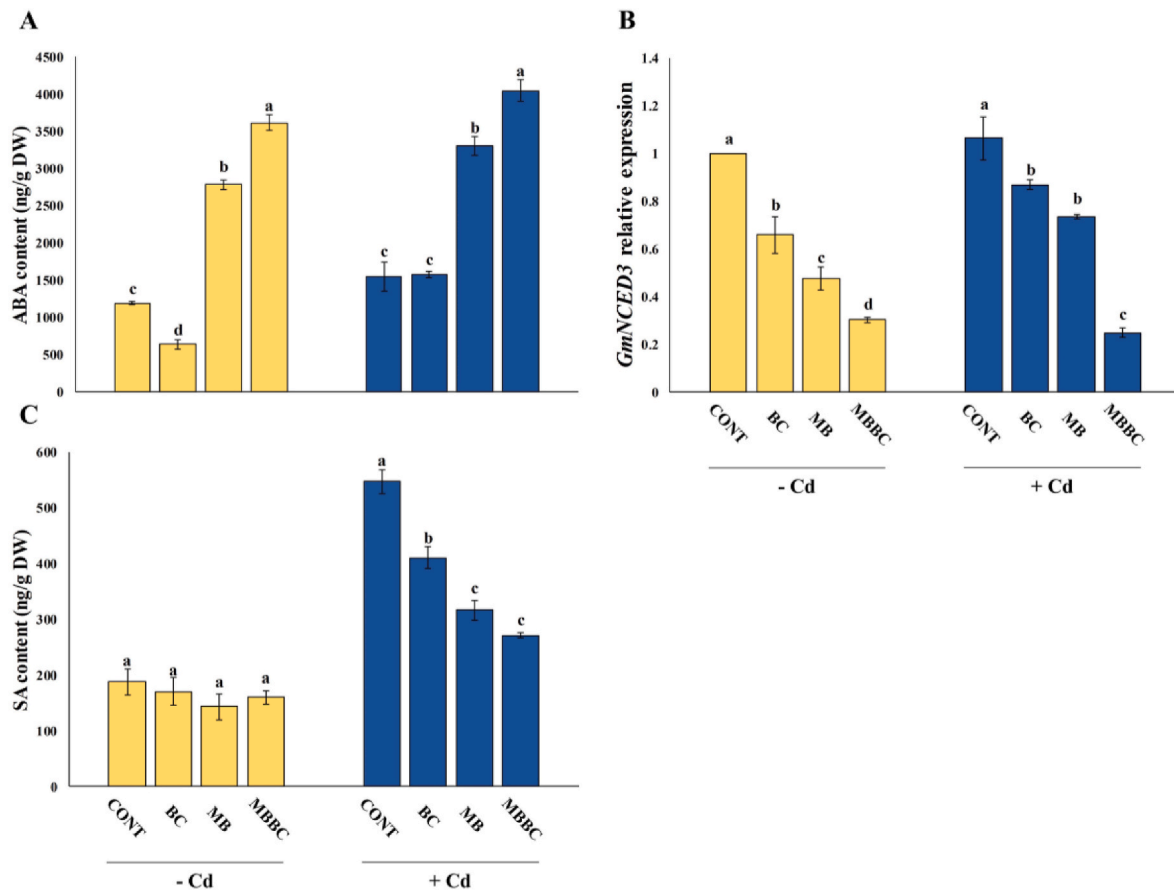


Fig. 5. Regulation of phytohormones by HW04 and biochar under cadmium stress. (A) ABA content, (B) Expression levels of *GmNCED3*, (C) SA content. Each bar represents the mean of five experimental replicates, with error bars indicating the standard error of the mean. Different letters above the bars indicate significant differences between treatments at $p < 0.05$, as determined using Duncan's Multiple Range Test (DMRT). Table 2 provides the abbreviations used in the treatments.

4.5. Role of phytohormones in cadmium stress response

Phytohormones, including ABA and SA, play crucial roles in plant stress responses through intricate crosstalk mechanisms under stress conditions (Verma et al., 2016). ABA is recognized as a stress indicator, and its endogenous content is significantly induced by Cd through various pathways (Labidi et al., 2021; Liu et al., 2023). In this study, the role of *GmNCED3*—a key enzyme coding ABA biosynthesis—was investigated. *GmNCED3* catalyzes 9-cis-violaxanthin and 9-cis-neoxanthin cleavage to produce xanthoxin, the initial C15 intermediate in the ABA biosynthesis pathway (Wang et al., 2016). The findings revealed that Cd-exposed plants exhibited significantly higher ABA levels than those of unexposed plants. Furthermore, compared to non-inoculated plants, HW04 significantly increased ABA content, with a more pronounced effect when combined with BC. However, this contrasts with *GmNCED3* expression, encoding enzyme 9-cis-epoxycarotenoid dioxygenase (NCED3), crucial for ABA biosynthesis. The elevated ABA levels indicate heightened stress, influenced by various environmental and biochemical factors.

SA is a crucial signaling molecule, triggering plant defense responses and leading to systemic acquired resistance (SAR) activation (Horváth et al., 2007). SA activates antioxidant mechanisms to maintain the redox balance within plants (Sharma et al., 1996). However, excessive SA accumulation can induce programmed cell death (Radojčić et al., 2018). SA also reduces Cd absorption and chelates or sequesters absorbed Cd (Bai et al., 2015; Noriega et al., 2012). These findings indicate that Cd exposure increases SA levels, which was reduced with HW04 and BC application, either individually or combined. The findings suggest that the co-application of HW04 and BC may alleviate Cd-induced stress,

subsequently reducing SA levels. This finding is consistent with that of previous studies linking increased Cd tolerance to decreased endogenous SA (Zawoznik et al., 2007). Additionally, Koo et al. (2020) reported that elevated SA levels inhibit plant growth and development. The reduced SA content in HW04-inoculated plants potentially contributed to decreased growth inhibition compared to that in non-inoculated plants. Further research is needed to explore these tolerance mechanisms, particularly the interaction between metal-regulating genes and mineral ions dynamics.

4.6. Calcium-dependent signaling and stress response

The influx and efflux rate of mineral elements are crucial for maintaining osmotic balance and regulating plant metabolic rates (Sarwar et al., 2010). Cd, a nonessential element, is competitively absorbed by plants through channel proteins shared with essential divalent cations such as Ca^{2+} , Mg^{2+} , and Fe^{2+} , inhibiting their absorption (Lux et al., 2011). This study shows that co-application significantly enhanced the translocation of essential mineral nutrients— Ca^{2+} , Mg^{2+} , and Fe^{2+} . Deficiencies in mineral elements impair key physiological processes in plants, such as growth, photosynthesis, cellular respiration, and signal transduction (Pandey, 2018). In this study, co-application increased plant Cd accumulation while simultaneously enhancing their nutritional status, resulting in photosynthesis recovery and enhanced Cd stress tolerance. The elevated Cd accumulation correlated with higher Fe, Ca, and Mg levels, suggesting a shared transporter pathway for these elements.

These findings were further examined by analyzing calcium-dependent protein kinases (CDPKs), Ca^{2+} /calmodulin-dependent

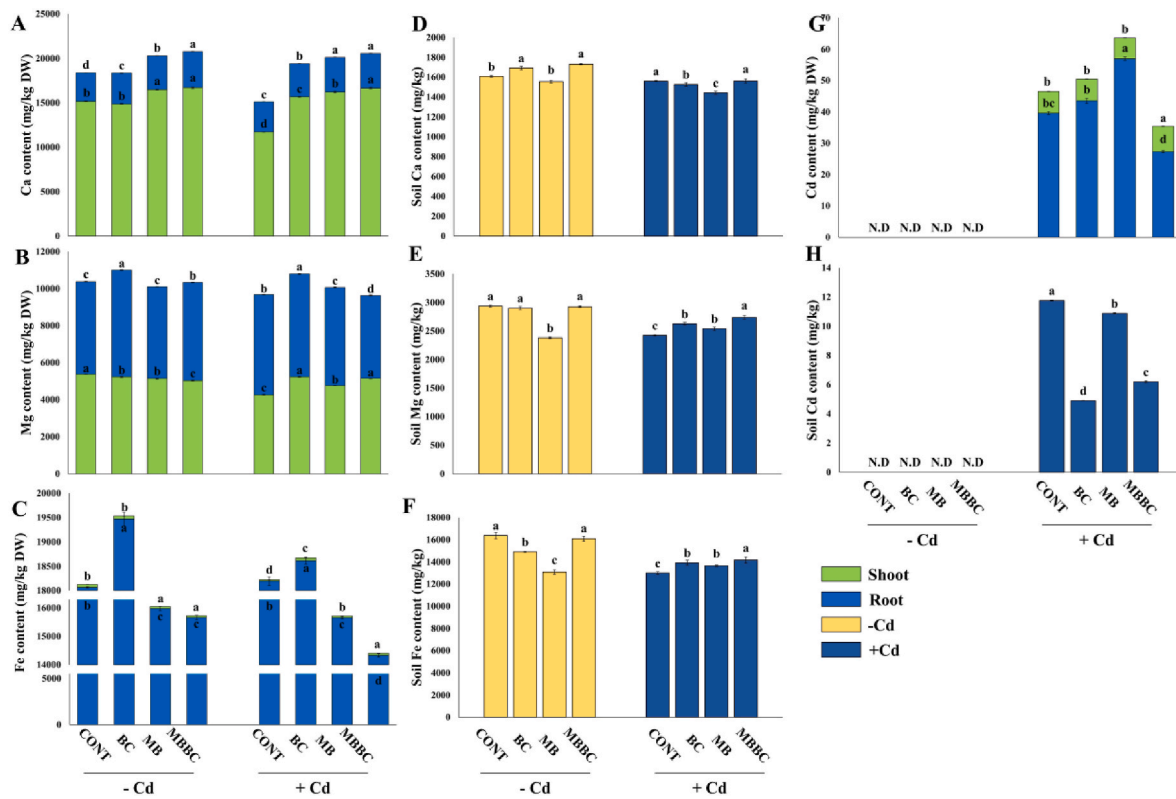


Fig. 6. Measurement of inorganic elements and cadmium contents in soybean roots, shoots, and soils under normal and cadmium-stressed conditions. (A) Calcium content in soybean roots and shoots, (B) Magnesium content in soybean roots and shoots, (C) Iron content in soybean roots and shoots, (D) Calcium content in the soil, (E) Magnesium content in the soil, (F) Iron content in the soil, (G) Cadmium content in soybean roots and shoots, (H) Cadmium content in the soil. Each bar represents the mean of five experimental replicates, with error bars indicating the standard error of the mean (SEM). Different letters above the bars represent statistically significant differences ($p < 0.05$), as determined using Duncan's Multiple Range Test (DMRT). Table 2 presents the abbreviations used in the treatment.

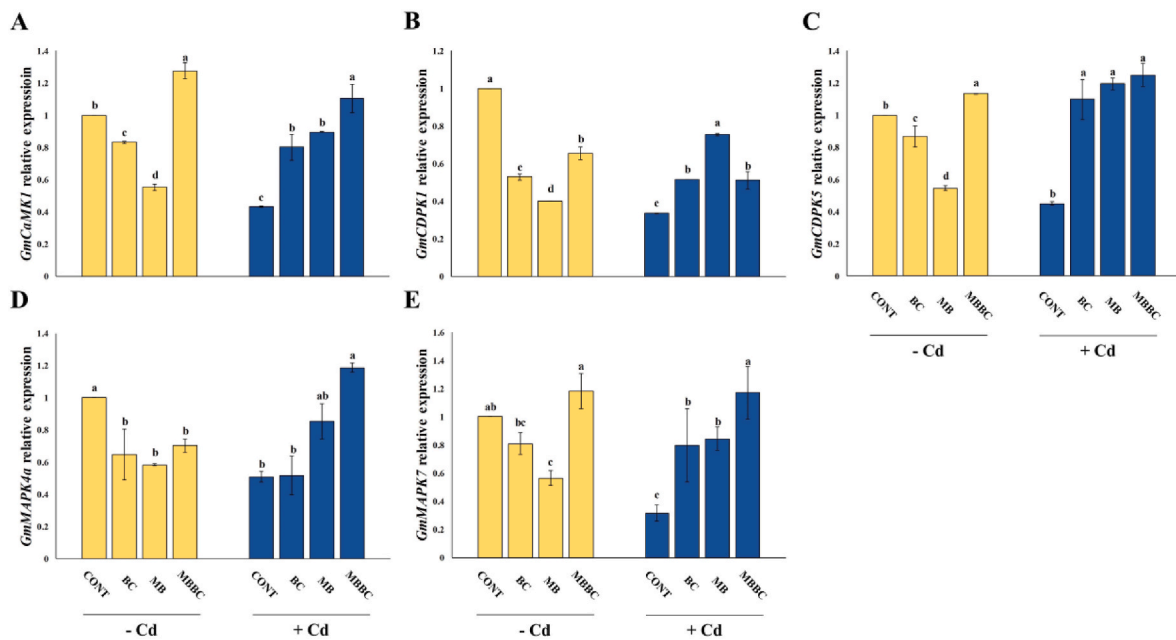


Fig. 7. 2 Relative expression levels of stress-response genes in soybean plants subjected to different treatments. (A) GmCDPK1, (B) GmCDPK5, (C) GmCaMK1, (D) GmMAPK4a, (E) GmMAPK7, and (F) GmNCED3. Each bar represents the mean expression levels from five experimental replicates, with error bars indicating the standard error of the mean. Different letters above the bars represent statistically significant differences between treatments ($p < 0.05$), as determined using Duncan's Multiple Range Test (DMRT).

protein kinases (CaMKs), and MAPKs. These calcium sensor proteins are involved in plant signaling, regulating various physiological processes, including plant development and stress responses (Harper et al., 2004; Bigeard and Hirt, 2018). In rice and maize, ZmCCAMK and OsMPK interact to phosphorylate and activate ABA-induced antioxidant defense responses (Shi et al., 2014; Zhu et al., 2016). These signaling cascades also influence auxin signaling and root development (Yang et al., 2021). The increased ABA levels, despite reduced NCED3 expression, may result from differential expression patterns regulated by CAMK and MAPK pathways.

Exposure to Cd significantly downregulated CDPKs, CaMKs, and MAPKs expressions compared to that of the control, thereby suppressing the related signaling pathways. However, HW04 inoculation upregulated these signal transduction proteins. This finding is consistent with that of previous studies reporting an upregulation of calmodulin (CaM) in response to elevated Ca levels in plants (Xie et al., 2021; Yang et al., 2022). The increased Ca levels in Cd-exposed plants following co-application may correspond to subsequent Ca-CaM binding, thereby enhancing CDPKs and CaMKs expression. However, while the specific mechanism remains unclear, studies report PGPR-induced MAPK upregulation under stress conditions (Fan and Smith, 2021; Kim et al., 2014). Furthermore, Wurzinger et al. (2011) demonstrated that MAPKs and CDPKs function within comprehensive, cooperative networks rather than isolated pathways. The findings of this study suggest that co-application activates signaling in Cd-exposed plants, enhancing the influx rate of essential elements.

Energy dissipation and light intensity are crucial in regulating the photosystem and electron transport. External applications of bio-stimulants and mineral treatments enhance defense mechanisms and maintain ionic balance, preventing programmed cell death. HW04 and BC preserved chlorophyll and improved photosynthetic components by normalizing electron transport flux through the activation of the reaction center, light absorption, heat dissipation, and quantum yield. In contrast, Cd-exposed untreated plants exhibited reaction center deactivation, reducing absorption and trapping flux.

4.7. Biochemical pathways in heavy metal detoxification

Organic and amino acids, intermediates in the Krebs cycle and interconnected with lipid and sugar metabolism, are crucial components in gluconeogenesis—the conversion of metabolites into glucose in plants (Walker et al., 2021; Kocaman, 2023) reported that these acids function synergistically to bind heavy metals through phytochelation in crops. Organic acids inhibit the movement of free heavy metal ions in the cytoplasm, while amino acids facilitate its chelation in cells and the xylem. This process reduces metal bioavailability and forms strong bonds in root exudates, aiding detoxification. Additionally, organic and amino acid exudates from the roots are crucial for heavy metal binding and nutrient bioavailability alongside several rhizospheric processes (Montiel-Rozas et al., 2016; Sharma and Dietz, 2006). The release of amino acids—isoleucine, leucine, tyrosine, phenylalanine, and lysine—and organic acids, particularly propionic acid, by HW04 potentially facilitates nutrient absorption in crops. Moreover, the PGP traits of HW04—EPS formation, phosphorus solubilization, IAA production, and oxidative stress tolerance—may contribute to stress signaling and enhance crop tolerance.

4.8. Limitations and future prospects

A key limitation of this study is the sustainability of PGPR application under harsh environmental conditions. Using suitable carrier materials can create favorable conditions for PGPR, enhancing its survival and maintaining its soil activity. Therefore, BC offers a cost-effective, non-toxic, chemically stable, easily accessible, and user-friendly option for sustaining microbial activity and ensuring long-lasting effects in agricultural processes.

Crops respond to pollutants in dual ways; for instance, while contaminant hyperaccumulation could cause toxicity in the food chain, the detoxification process may increase persistence and bio-magnification in the soil. Therefore, strategies must be content-specific. Another limitation is that while soybean crops may be effective in contaminant uptake, they could pose risks if consumed. Despite these limitations, phytoremediation remains crucial for long-term effects in highly polluted areas, particularly when implemented from the initial stage. Although soybeans used in phytoremediation may not be suitable for dietary consumption, their role as a legume offers additional benefits, such as atmospheric N₂ fixation, potentially reducing the need for synthetic fertilizers.

5. Conclusions

This study explores the scope and challenges of phytodetoxification and phytoremediation, highlighting their limitations. These findings offer valuable insights for developing targeted strategies to address contaminants in agricultural land tailored to specific objectives and situational contexts. Overall, the results demonstrate that HW04 and BC alleviate Cd stress in soybeans, showing their potential as biofertilizers for stable crop production in Cd-contaminated soils. However, further validation is needed to assess the effect of environmental variables and the feasibility of soybean usage in this process.

CRedit authorship contribution statement

Ji-In Woo: Conceptualization, Investigation, Writing – original draft. Arjun Adhikari: Writing – review & editing, Data Curation. Ho-Jun Gam: Methodology, Investigation. Jin Ryeol Jeon, Eun-Hae Kwon, Da-Sol Lee: Investigation. Sang-Mo Kang: Project administration. Byung-Wook Yun: Supervision. In-Jung Lee: Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2025.109489>.

Data availability

Data will be made available on request.

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