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Isolation and identification of Rhizospheric and Endophytic Bacteria from Cucumber plants irrigated with wastewater: Exploring their roles in plant growth promotion and disease suppression

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ABSTRACT

Wastewater contains various emerging contaminants, including heavy metals, residues of pesticides, and pharmaceuticals. Therefore, irrigation with wastewater can enhance heavy metal contamination in soil and adversely affect plant growth. To mitigate this problem, plant growth-promoting bacteria (PGPR) can improve plant growth under heavy metal stress. This study aimed to isolate and characterize rhizospheric and endophytic bacteria from the rhizosphere soil and roots of a cucumber plant irrigated with municipal wastewater. A total of 121 morphologically distinct bacterial isolates from the rhizosphere and 90 bacterial isolates from the endophytic region were isolated and tested for heavy metal resistance and *in vitro* plant growth-promoting characteristics, including indole-3-acetic acid (IAA) production, phosphate solubilization, Hydrogen Cyanide (HCN) production, and siderophore production. Most of the bacteria analyzed from the rhizospheric and endophytic regions showed various plant growth-promoting characteristics and were tolerant to different heavy metals at various concentrations. Bacterial strains R1 *(Proteus* sp.*)* and E2 (*Bacillus* sp.*)* were antagonistic to *Fusarium oxysporum* f. sp. *Lycopersici.* Wastewater irrigation increases heavy metal-resistant bacteria in cucumber plants, which can alleviate heavy metal stress. Additionally, *Proteus* sp. and *Bacillus* sp. isolates are potential candidates for removing heavy metal-contaminated soil and could be potential biofertilizer candidates for selected plants and biocontrol agents.

Introduction

Different plant organs are inhabited by various bacteria found in the leaves, stems, fruits, roots, and rhizosphere of nearly all plant species (Ryan et al., 2008). Bacteria harboring rhizospheric and endophytic regions benefit plants diversely (Etesami et al., 2018). Plant activity is directly or indirectly regulated by the biochemical, physiological, and metabolic activities of Plant Growth Promoting Rhizosphere (PGPR). The direct pathways of plant growth include atmospheric nitrogen fixation, mineral solubility, phytohormone production, and plant enzyme production. The indirect types include induction of systemic resistance, plant competition, inhibition, production of siderophores, antibiotics, lytic enzymes, and antibiotic metabolites (Gopalkrishnan et al., 2015). Owing to siderophore production, PGPR protects plants from toxic metals such as Cd, Cu and Pb (Qin et al., 2024). In addition to heavy metal protection, phytohormones produced by plants also regulate root and shoot growth (Zhang et al., 2024). Some PGPRs provide plant micronutrients and solubilize zinc and phosphate that plants can use for growth promotion (Ikiz et al., 2024). Moreover, PGPR is used for phytoremediation and can reduce various pollutants present in soil and

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water (Nedjimi, 2021). Generally, contaminated soil and water are responsible for reducing plant growth and influencing plant diseases (Chen et al., 2023). Therefore, it is crucial to evaluate the structure and diversity of the rhizosphere bacterial community and their relationships and interactions with the soil and plants (Ling et al., 2022).

Currently, water scarcity is a major global problem. Therefore, reusing treated wastewater may be one of the most important solutions for developing water resource management (Silva, 2023). They can also play an important role in the irrigation of agricultural soil and in handling various problems related to the agricultural sector. However, irrigation with reclaimed water can cause toxicity in the soil owing to the high accumulation of heavy metals (Rata et al., 2023). Some metals, such as Cu, Fe, Mn, and Zn, are essential for plant growth, but at low concentrations, above the threshold value, they have a negative impact on plant growth (Arif et al., 2016; Rashid et al., 2023). Toxic heavy metals cause soil salinity, nutrient deficiency, and imbalance by interfering with the uptake and distribution of essential nutrients in plants (Alengebawy et al., 2021). Heavy metals are also transferred into the food chain through plants grown in soils contaminated with heavy metals (Wang et al., 2021). According to previous research, various rhizospheric and endophytic bacteria can control the impact of heavy metal toxicity in plants through various mechanisms, such as biosorption/bioaccumulation and reduced availability (Etesami, 2018). Bacteria associated with roots that can resist the toxicity of heavy metals can be a major technique used in bioremediation (Arantza et al., 2022).

PGPR possess enzymatic capabilities that are very effective in the immobilization or translocation of heavy metals in plants (Khanna et al., 2019). Some PGPR strains, such as *Pseudomonas aeruginosa, Bacillus subtilis*, and *Alcaligenes faecalis*, can tolerate and accumulate heavy metals (Ibrahim et al., 2021; Yadav et al., 2022; Tatung et al., 2024). These bacteria can bind to heavy metal ions, preventing them from entering the environment or affecting plant growth. These characteristics make them useful for the treatment of wastewater contaminated with heavy metals and for establishing symbiotic relationships with plants. PGPR can also enhance nutrient uptake, improve plant health, and increase biomass production, thereby contributing to the overall efficiency of the treatment system (Saeed et al., 2021; Alves et al., 2022). PGPR strains can also be considered biocontrol agents that can suppress the growth of plant pathogens and are a good alternative to chemical pesticides (Mohammed et al., 2020; Mekonnen et al., 2022). However, the effectiveness of biocontrol agents depends on several factors, such as specific bacterial strains, environmental conditions, and other microbial communities (Bonaterra et al., 2022). A major agricultural problem is the pathogenic fungus *Fusarium oxysporum* f. sp. *Lycopersici* strain ITCC 8111 (FOL), which mostly affects economically significant crops, including tomatoes, cucumbers, and chilli peppers. Because of its strong host specificity, aggressive pathogenicity, and abundance of genetic and molecular research tools, this strain was chosen for investigation. FOL has a major global effect, producing Fusarium wilt, which causes severe symptoms such as vascular browning and wilting. Affected areas can have yield losses of up to 50 %. The fungus causes significant financial losses and difficulties for producers due to its persistence in soil and asymptomatic colonisation of plant roots, which complicate treatment. Understanding pathogen development, host-pathogen interactions, and creating integrated pest management (IPM) techniques depend on FOL research. Gained knowledge can aid in the development of crops resistant to disease, lowering the need for chemical fungicides and advancing sustainable farming practices. The creation of quick diagnostic instruments for early disease diagnosis, agronomic and environmental factors-informed preventative measures, and efficient treatment techniques like fungicides and biocontrol agents are only a few of the practical consequences of this study. These developments can lessen crop losses, increase the resilience of agricultural systems, and assist farmers in putting timely solutions into practice. Furthermore, research results can help shape agricultural policies and extension services, offering recommendations for handling Fusarium wilt and assisting decision-makers in creating rules and assistance initiatives.

In the present study, we isolated and characterized rhizospheric and root endophytic bacterial isolates from the rhizospheric and endophytic regions of cucumber plants and screened for their heavy metal resistance and plant growth-promoting traits.

Materials and methods

Sampling sites

Soil samples near the rhizosphere were collected in January 2021 from wastewater-irrigated agricultural fields in the Naini region (25.419406 ° N, 81.857853° E) of Pragaraj district, Uttar Pradesh (Fig. 1). This area has been irrigated with wastewater for the past 25 years. Effluents generated by Naini wastewater treatment plants are enriched with different heavy metals, such as Cr, Cd, Pb, Zn, Cu and Ni (Mani and Mourya, 2013).

Isolation of endophytic and rhizospheric bacteria

Nine soil samples were collected from the wastewater-irrigated agricultural field of Naini, Prayagraj, Uttar Pradesh, India, near the rhizosphere and root endophytic regions of the cucumber plants. Cucumber plants and their associated soil samples were collected during the flowering stage. Cucumber plants with soil samples were collected in sterile plastic bags and transferred to the laboratory in an ice box. Root rhizospheric and endophytic bacteria were isolated using the serial dilution method described by (Estami et al., 2014). Rhizospheric soil was collected by hand-shaking roots for 10 min in one litre of a sterile double distilled water solution to remove the adherent soil and sterilize the surface by following protocol: sample root dipped in 70 % ethanol for 2 min followed by 0.5 % NaOCl for 3 min and again dipped in 70 % ethanol for 30 s. Finally, root tissues were washed with double distilled water. Surface sterilized root materials were cut into small pieces and crushed with 10 mL of sterile phosphate buffer saline, and 10^{-2} dilutions were spread on the Nutrient Agar plate and incubated at 37 °C for 24 h. The rhizospheric soil was serially diluted in 100 mL of distilled water. After serial dilution, 100 μL of each dilution (10^{-4} to 10^{-7}) was spread on Nutrient Agar plates and incubated at 37 °C for 24 h. Rhizospheric and endophytic bacterial colonies on the plate were counted, and morphologically distinct colonies were randomly selected and stored at 4 °C for further analysis. Fresh bacterial inoculum was prepared for each isolate for further tests and experiments.

Screening of heavy metal-tolerant bacteria

For the screening of heavy metal-tolerant rhizospheric and endophytic bacterial isolates, various heavy metals were used, *viz.* Pb, Ni, Cd, Cr, Co, and Cu at different concentrations of 9.0, 8.5, 7.5, 6.5, 5.5, 4.5, 3.5, 2.5, and 1.5 mM. The selected bacterial isolates were grown on a minimal salt medium of Luria Bertani (LB) Agar medium (3 g/L K_2HPO_4, 6 g/L Na_2HPO_4, 6 g/L NaCl, 2 g/L NH_4Cl, 0.1 g/L $MgSO_4$, 8 g/L glucose) consisting of metal salts (Cd as $CdCl_2$, Pb as $PbCl_2$, Cr as $Cr(NO_3)_3$, Co as $CoCl_2$, Ni as $NiCl_2$, and Cu as $CuCl_2$) at different concentrations (pH was adjusted either by NaOH or HCl to 6.8) in a streak method. The cultured bacterial colonies were incubated at 37 °C for 48–72 h. The heavy metal-tolerant bacterial colonies were selected, and their growth was compared to the growth of control plates (without bacterial strains). A culture of 10 μl of growing bacteria (1.0×10^7 CFU/ml) was suspended in 100 μl LB with respective metals incubated for 48 h at 28 °C in a shaking incubator. Bacterial cultures were monitored at an absorbance of 600 nm by a microplate reader (Thermo Scientific multiscanGO) to analyse the heavy metal stress tolerance (Abedinzadeh et al., 2019).

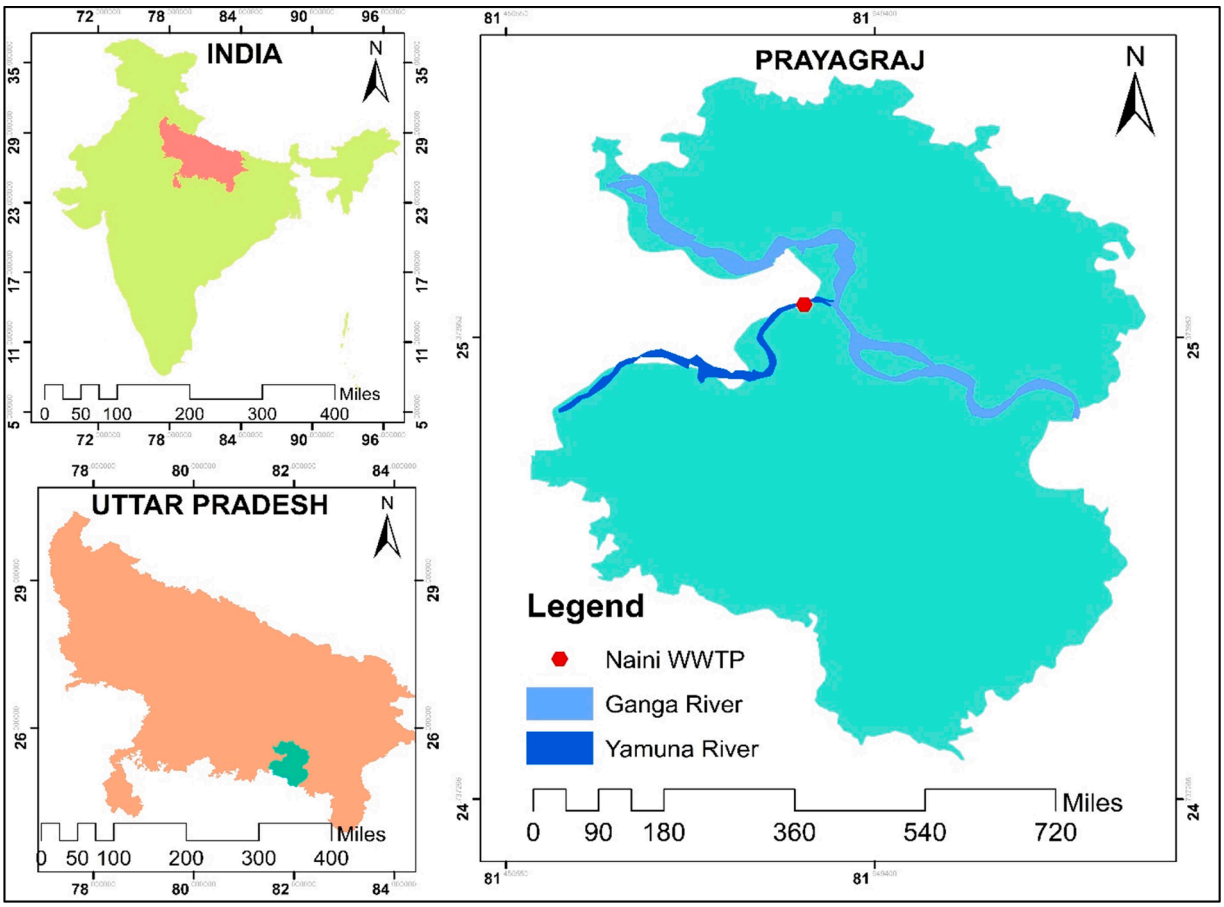


Fig. 1. Sampling location of wastewater irrigated agricultural field near Naini Wastewater Treatment Plants.

Screening of plant growth-promoting (PGP) traits for rhizosphere and endophytic bacteria

IAA (Indole-3-acetic acid) production

The IAA production by these bacterial isolates was evaluated according to the protocol described by Patten and Glick et al. (1996). This method is based on the colorimetric technique with a Salkowski reagent (50 ml, 35 % of perchloric acid, 1 ml 0.5 M $FeCl_3$) solution. IAA production (μg/mL) was observed by the appearance of a pink color after 25 min of incubation, and absorbance was determined by OD at 530 nm using a UV-spectrophotometer (SYSTRONICS-2206, India).

Phosphate solubilization

Phosphate solubilization of the bacterial isolates was evaluated based on their ability to solubilize inorganic phosphate. A Pikovskaya agar medium containing calcium phosphate as the inorganic form of phosphate was used in the assay. Bacterial cultures were streaked onto plates and incubated at 37 °C for 4–5 days. The appearance of a transparent halo around the bacterial colony indicated the phosphate-solubilizing activity of the bacteria (Sperber, 1958).

HCN production

HCN production was detected using a 24-hour grown culture on LA media supplemented with 4.4 g/L glycine. A Whatman filter paper No.1 (soaked in a solution of 2 % sodium carbonate solution in 0.5 % picric acid) was placed in between the base and lid of the culture plate. Production of HCN was indicated by the color change of the filter paper from yellow to orange-brown, while no changes in color indicated a negative result for this experiment (Lukkani et al., 2014).

Ammonia production

To detect the production of ammonia, the rhizospheric and endophytic isolates were grown in a peptone water medium for 48 h. Then, 1 ml of each culture was transferred to 1.5 ml microtubes, and 50 μL of Nessler's reagent (0.5 ml) was added. The development of a faint yellow color indicates a small amount of ammonia production, and a deep yellow to brown color indicates maximum ammonia production (Dye 1962).

Siderophore production

The siderophore-producing ability of the selected bacterial strains was assessed using the Chrom azurol S CAS assay described by Schwyn and Neilands (1987). Briefly, 60.5 mg CAS was dissolved in 50 ml sterile distilled water, mixed with 10 ml of 1 mM $FeCl_3$ and 50 ml of hexadecyl trimethyl ammonium bromide (HDTMA) solution, autoclaved, and stored in the dark. The CAS solution (100 mL) was mixed with 900 mL of Nutrient Agar medium and poured into Petri plates for siderophore estimation. Siderophore was performed by taking the supernatant of the bacterial culture grown in LB broth medium. Selected bacterial isolates from the control group were inoculated and incubated at 28 °C for 72 h. The siderophore index (SI) was calculated using the following equation:

Siderophore Index= (total diameter (colony diameter + halo zone diameter/colony diameter))

Cellulase production

Cellulase metabolic activity can be observed by performing it on carboxymethyl cellulose (CMC) medium (0.2 % NaNO3, 0.1 % K2HPO4, 0.05 % MgSO4, 0.05 % KCl, 0.2 % carboxymethyl cellulose salt, 0.02 % peptone, 1.7 % agar). 5μl of the bacterial isolate was inoculated into the CMC media plate and incubated at 28 °C for 48 h. After Subsequently,

each plate was stained with iodine (0.666 % KI; 0.33 % iodine) for 5 min, and positive cellulase-producing strains were shown halo region (Bera et al., 2014).

Protease activity

Protease activity was observed in skim milk agar (SMA) media. The bacterial strain was incubated in LB medium and further streaked on SMA media. Positive activity of the bacterial strain was observed when a halogen region was created (Ghosh et al., 2007).

Exopolysaccharide production

EPS production was determined according to Paulo et al. (2012). Each bacterial strain was inoculated onto a 5-mm diameter paper disc in medium (2 % yeast extract, 1.5 % K_2HPO_4, 0.02 % $MgSO_4$, 0.0015 % $MnSO_4$, 0.0015 % $FeSO_4$, 0.003 % $CaCl_2$, 0.0015 % NaCl, 1.5 % agar). The positive effects of exopolysaccharide production created a halo and slime-like appearance. However, adding a mucoid substance to 2 ml of absolute ethanol confirmed the presence of EPS due to precipitation.

Biosurfactant test

Each bacterial strain was inoculated in a 500 ml Erlenmeyer flask containing LB media. The flask contained sterilized mineral salt medium (1.0 g/L K_2HPO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $FeSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.001 g/L $Na_2MoO_4{\cdot}2H_2O$, 30 g/L NaCl) and crude oil (1.0 %, w/v) and culture flask was incubated for seven days in an incubator shaker at 30 °C and 200 rpm. The biosurfactant oil-spreading test was performed as described by Morikawa et al. (2000). Briefly, 20 ml of distilled water was added to the petri plate simultaneously with 20 μl of crude oil, and then 10 μl of the bacterial culture (1.0×10^7 CFU/mL) was also added to the oil surface. Oil displacement activity should be observed when the oil-free clearing zone and diameter of the clearing zone are formed, indicating that the culture has positive biosurfactant activity.

Molecular identification and DNA sequencing of selected effective isolates

For the molecular charecterization of rhizospheric and endophytic isolates, only one bacterial isolate from endophytes (E2) and one from rhizospheric (R1) region were selected as a representative. The selection of bacterial isolates was based on resistance against heavy metals and having various PGP traits. The selected isolates were shown to have a higher degree of resistance against heavy metals and PGP traits. The molecular identification was based on the 16S rRNA gene amplification using the bacteria-specific primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Hyder et al., 2020). The DNA of each bacterial isolate was extracted using phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol. PCR amplification was performed with (Takara Bio Inc. Japan), 0.02 units μl^{-1} Taq polymerase, and 1.0 μM of each primer. The PCR amplification was achieved in the Bio-Rad thermal cycler consisting of an initial denaturation at 90 °C for 30 s followed by 35 cycles each consisting of initial denaturation at 95 °C for 30 s, primer alignment at 50 °C for 30 s and an extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. Amplified PCR products were sent for Sanger sequencing at Eurofins Pvt., Ltd. Bengaluru after purification. After DNA sequencing, the nucleotide sequences were submitted to the NCBI database and annotated using the NCBI BLAST server. Both the sequences were aligned with MEGA6 software (Tamura et al., 2013) for constructing a phylogenetic tree.

Assay of the growth rate of isolates in the presence of Cd and Pb

In this assay, Pb and Cd heavy metals were selected because they are highly abundant in industrial and municipal wastewater. The viable count method was used to estimate and determine the selected bacterial growth curve in the presence of 4 mM Cd, 4 mM Pb, and 4 mM Cd + 4 mM Pb at different times (0–120 hrs). The experiment was performed by inoculating each bacterial isolate in NB medium supplemented with 4 mM Cd, 4 mM Pb, and 4 mM Cd + 4 mM Pb separately at 28 °C on a rotatory shaker at 120 rpm, and each treatment was performed in triplicate. Similarly, a control was set up without heavy metal supplementation. Finally, after making the dilution series, 0.1 ml of a suspension of each bacterial isolate (1.0×10^7 CFU/mL) was grown in NA plates containing heavy metals (4 mM Cd, 4 mM Pb). After incubating the plates at 28 °C for 24 h, the growth curve was plotted based on the results (Abedinzadeh et al., 2019).

Biocontrol test

(1) A standard culture assay was used to evaluate the *in vitro* antagonistic activity of selected rhizospheric and endophytic bacterial strains against FOL strain. FOL strain was provided by the Division of Plant Pathology, Indian Institute of Agricultural Research, New Delhi. Briefly, PGPR bacterial strains were streaked around the FOL of a 10 cm petri plate containing 50 % LB agar and incubated at 25 °C for 72 h. Simultaneously, mycelial discs of the fungal strains were placed at the centre of the Petri plate. For the negative control plates, only the fungus was cultivated in the plate. The inhibition zone between the bacteria and FOL and the area of fungal mycelial growth was measured after inoculation. Depending on the growth rate, two conditions were observed for *in vitro* antagonistic activity.

Mycelia area: calculate the pathogen-occupied areas based on the following formulae

$$\pi * \left(\frac{D1}{2} + \frac{D2}{2}\right)$$

where D1 is the long diameter, and D2 is the short diameter.

(2) Inhibition Area: The distance between the pathogen and bacteria has been calculated.

Evaluation of the in vitro cell-free supernatant antifungal activity

The Bacterial strain was inoculated in 100 mL of 50 % LB medium in 250 ml of the conical flasks and incubated at 130 rpm for six days in a shaking incubator. Initially, 1 ml of the bacterial inoculum was taken and centrifuged at 10,000 rpm for 5 min. The collected supernatant was filtered through 0.22 μm membranes, and cell-free supernatants (CFS) were stored at 4 °C. Potato dextrose agar (PDA) plates were inoculated with the FOL agar pulp, and 0.5μl of CFS was pipetted into the aseptically created holes placed at a distance of 0.5 cm from the FOL plug. For the negative control plates, LB medium was filled in the holes. The plates were incubated at 25 °C and observed after 1–3 days, depending on the pathogen growth rate. The experiment was conducted in triplicates for statistical analysis (Moshe et al., 2023).

Plant growth condition in the presence of PGPR and FOL

Cucumber seeds of one Indian cultivar (Heera) were selected to assess their responses to fungal pathogens. Before the experiment, all cucumber seeds were surface sterilized with 2.5 % sodium hypochlorite for 10 min, placed on wet tissue paper inside a Petri plate, and incubated for 3–4 days at 28±2 °C. After the incubation period, uniformly germinated seeds were then transferred to sterile trays for further growth. After two weeks of planting, seedlings were transferred to pots (dimensions: $L \times W \times H$, 3.5 × 3 × 3 inches). All experimental treatments were set up in triplicate with one plant. Seedlings were grouped according to their treatment criteria: (i) plants without any treatment (control), (ii) plants treated with PGPR, (iii) plants infected with FOL (infected control), and (iv) plants treated with PGPR and infected with FOL. The FOL strain ITCC 8111 was used as a fungal pathogen and grown on PDA media for four days at 28 °C. The concentration of the fungal spore suspension was adjusted to 1×10^6 conidia/ml using a hemocytometer. After two weeks of incubation, cucumber plants were

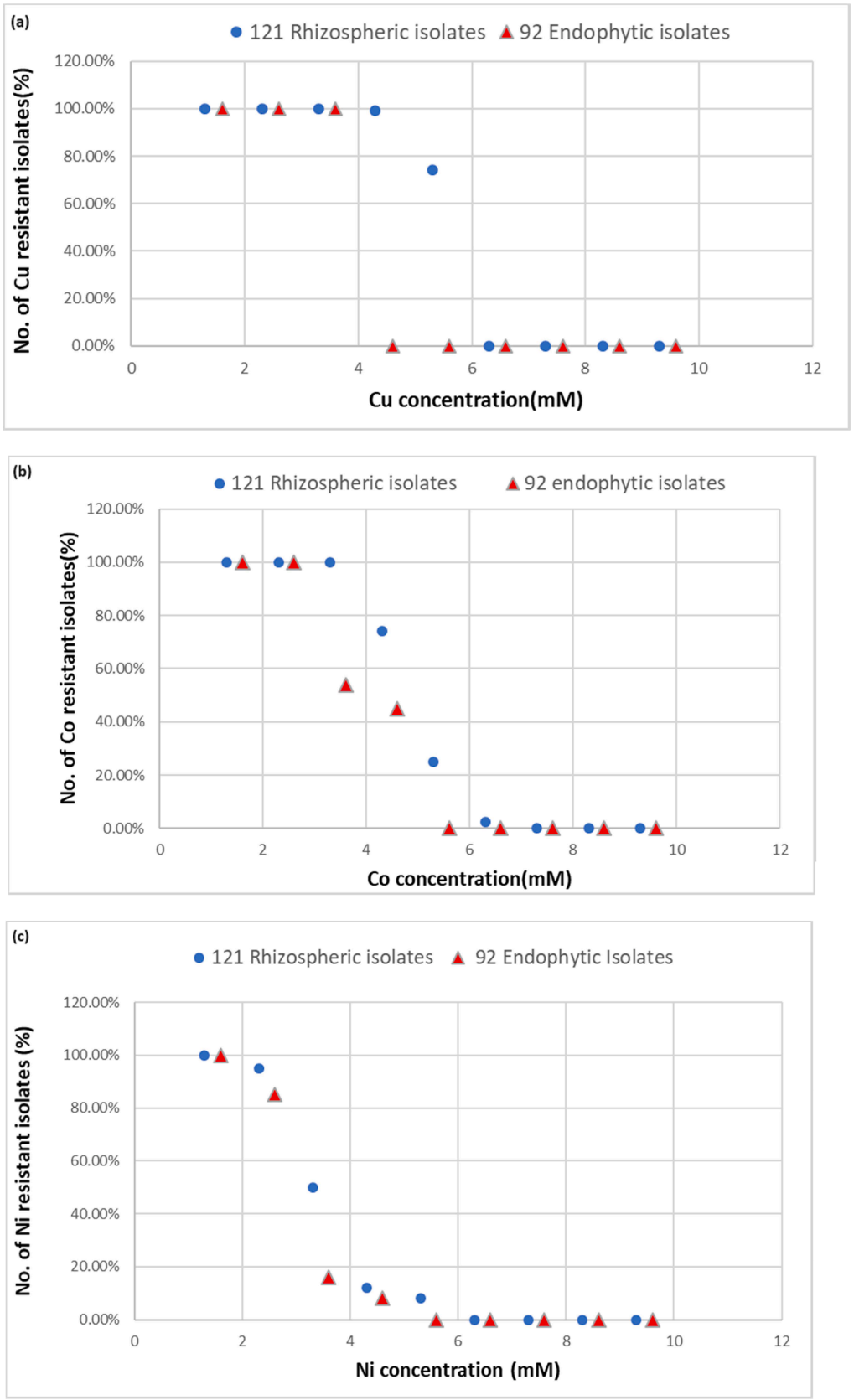
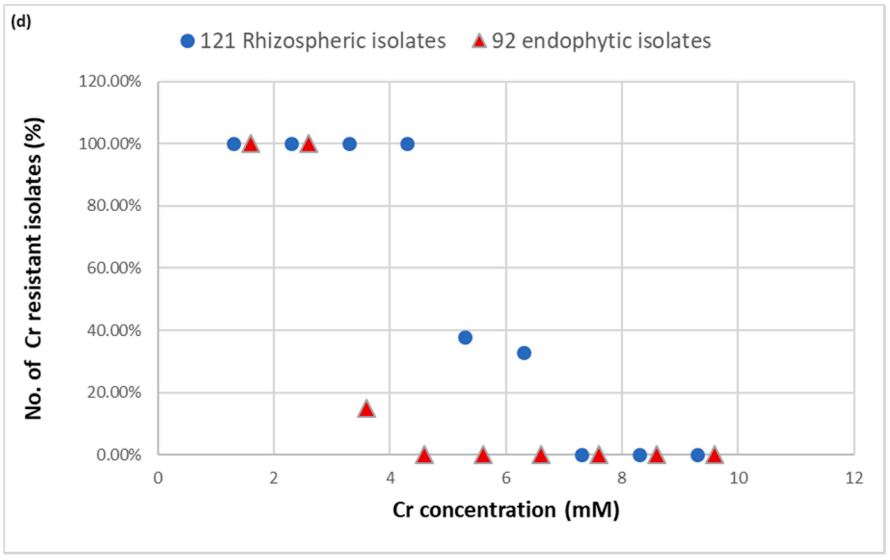
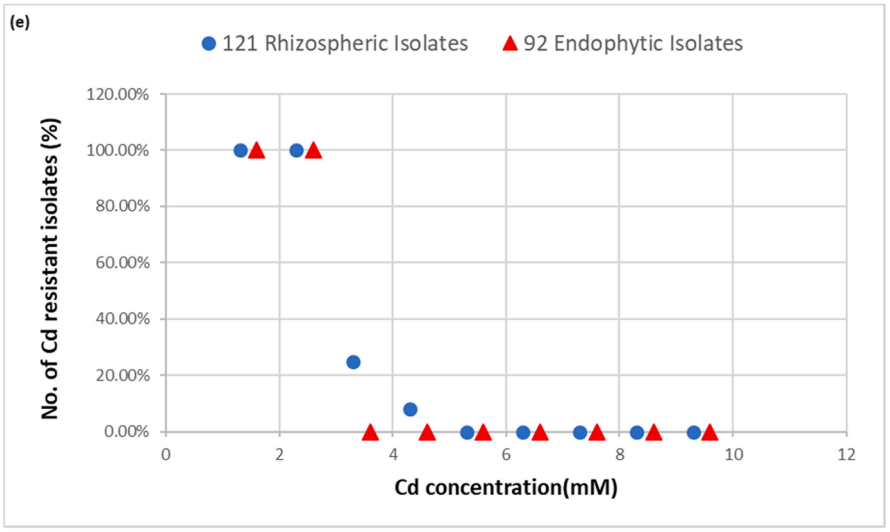


Fig. 2. Abundance of endophytic and rhizosphere bacterial isolates resistant to different concentrations of heavy metals.





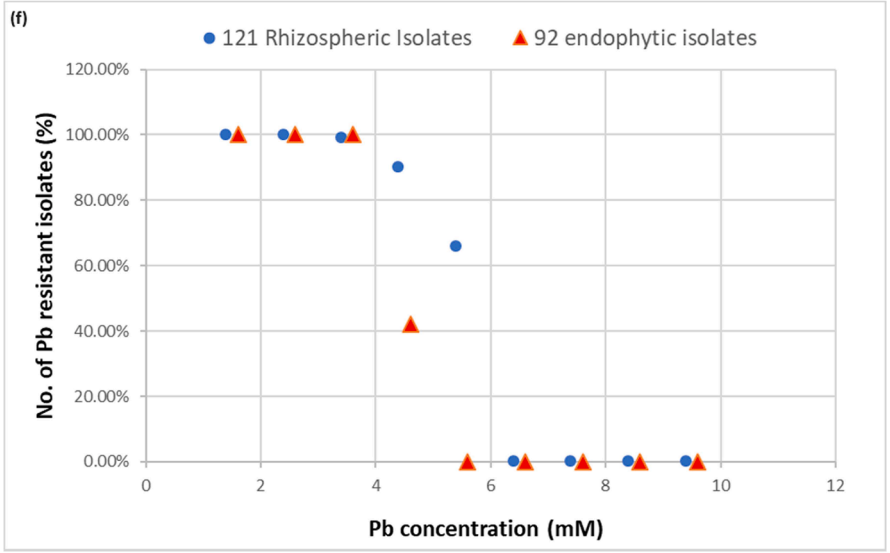


Fig. 2. (*continued*).

treated with the fungal pathogen (1×10^6 conidia/ml), bacterial isolates (2×10^7 CFU/ml), and fungi with bacterial isolates directly to the root surface, and the control plants were treated with sterile distilled water. The soil was maintained at 60 % water-holding capacity. The minimum and maximum temperatures during the experiment were 15 to 29 °C, respectively and humidity was 56 %. After 45 days, the inoculated plants were assessed based on growth parameters, such as plant height (pH), root length (RL), fresh weight (FW), dry weight (DW), and chlorophyll content.

Chlorophyll content

The chlorophyll content (a) and (b) of the plant leaf was observed by the Tahri et al. (1998) method. The total chlorophyll content ($a + b$) was calculated according to Wellburn and Lichtenthaler (1984) formula:

$$Chlorophyll\text{‘}a\text{’}\,(mg/gF.W.) = 12.7 * (A663) - -2.69 * (A645) * \frac{V}{1000 * W * a}$$

$$Chlorophyll\text{‘}b\text{’}\,(mg/gF.W.) = 22.9 * (A663) - -4.68 * (A645) * \frac{V}{1000 * W * a}$$

$$Total\,Chlorophyll\,(mg/gF.W.) = 20.2 * (A663) + 8.02 * (A645) * \frac{V}{1000 * W * a}$$

where,

A645 = Absorbance of the extract at 645 nm
A663 = Absorbance of the extract at 663 nm
a = Path length of cuvette (1 cm)
V = final volume of the chlorophyll extract (10 ml)
W = Fresh weight of the sample (0.10 g)

Statistical analysis

The differences between treatments were statistically analyzed using analysis of variance (ANOVA) and subsequently by Tukey's multiple range test at $p < 0.05$. Experimental data obtained from this study were statistically analysed (calculation of average value, standard deviation through Microsoft Excel 2016 software (Microsoft, Redmond, WA, USA).

Results

Isolation of endophytic and rhizospheric bacteria

A total of 211 bacterial isolates with different morphologies, colors, and sizes were isolated from the rhizospheric and endophytic regions of cucumber plants irrigated with industrial wastewater. Of these, 121 (57.35 %) isolates were rhizospheric, and 90 (42.65 %) were endophytic isolates.

Heavy metals–resistant rhizosphere and endophytic bacteria

Rhizospheric and endophytic bacterial isolates showed heavy metal resistance, as demonstrated in Fig. 2 (a-f). Based on this assay, it was observed that increasing the heavy metal concentration in the culture medium decreased bacterial tolerance. The MIC of these Rhizospheric bacterial strains reached 6.5 mM Pb, 5.5 mM Cd, 6.5 mM Cu, 7.5 mM Co, 6.5 mM Ni, and 7.5 mM Cr, respectively. Similarly, MIC of endophytic isolates reached 5.5 mM Pb, 3.5 mM Cd, 4.5 mM Cu, 5.5 mM Co, 5.5 mM Ni, and 4.5 mM Cr, respectively. This assay showed Cd was toxic to the bacterial strain even at lower concentrations. The results showed that 100 % of rhizospheric and endophytic bacterial strains tolerated 0.5 to 2.5 mM for Cr, Ni, and Cd, whereas 0.5 to 4.5 mM for Co and Pb, and 0.5 to 3.5 mM for Cu heavy metal. Rhizospheric and endophytic isolates showed simultaneous resistance to several heavy metals. The general sequential pattern of endophytic bacteria reducing their capacity to tolerate heavy metal concentrations were Cr, Pb, Cu, Co, Cd and Ni. However, rhizospheric bacteria showed a sequential pattern of reduced capacity to tolerate heavy metal concentrations of Co, Pb, Cr, Co, Cd, and Ni.

PGP traits of rhizospheric and endophytic bacteria

A total of 210 bacterial isolates were selected to analyse their PGP characteristics. A Total of 83.4 % rhizospheric isolates and 80.4 % endophytic isolates produced IAA. Approximately 63 % of the endophytic isolates produced siderophores, 66 % produced HCN, 67 % of these isolates were able to solubilize mineral phosphate, and 89 % of isolates showed the highest ammonia production, 11 % of these bacteria were able to produce halo region for producing cellulose activity, 5 % were able to produce oil displacement effect, *i.e.,* having biosurfactant behavior, 11 % were able to produce the mucoid substance in the endeavor to show exopolysaccharide test. However, 77.68 % of the rhizosphere isolates produced siderophores, 89.25% showed the activity of Ammonia Production, 83.47 % produced HCN, 61 % of these isolates were capable of phosphate solubilizing, 8 % of these bacteria were able to produce halo region for producing cellulose activity, 6 % were able to produce oil displacement effect, *i.e.*, having biosurfactant behavior, 8 % were able to produce the mucoid substance in the endeavor to show exopolysaccharide test. Besides these tests, 11 % of rhizospheric and 8 % of endophytic bacteria produced a halo region in skim milk agar, which can show protease activity. As per the trend, we observed that most of the rhizospheric isolates were highly IAA-producing isolates compared to endophytes. Phosphate-solubilizing ability was observed more in rhizospheric bacteria than in endophytic isolates. Based on the PGP traits, rhizospheric and endophytic isolates were selected for molecular characterization and identification.

Table 1
Morphology, Physiology, biochemical test, PGP traits and MIC of Rhizospheric and Endophytic bacterial isolates isolated from rhizosphere and endophytic region of wastewater-treated cucumber plants.

SN	Isolate Code	Bacterial Name	Morphology, physiology and biochemical tests	PGP Traits	MIC value of the Heavy metals (mM)
1	R1	*Proteus* sp. (accession number: PP665705)	Rod, motile, G-, Endospore formation (-), Catalase (+), Citrate (+), Oxidase (-), Urease (+), Starch hydrolysis (-), Glucose (+), Lactose (+), Manitol (-), Arabinose (-)	IAA (82.25ug/mL), HCN (++), Siderophore (++), Phosphate solubilizing (++), Ammonia production (+++), Protease k (++), cellulose activity (++), Exopolysaccharide (++), Biosurfactant (+)	Cd (4.5), Pb (6.5), Cr (7.6), Co (6.0), Cu (6.2), Ni (7.5)
2	E2	*Bacillus* sp. (accession number: PP665709)	Rod, motile, *G+*, Endospore formation (+), Catalase (+), Citrate (+), Oxidase (+), Urease (+), Starch hydrolysis (+), Glucose (+), Lactose (-), Manitol (+), Arabinose (+)	IAA (62.31ug/mL), HCN (++), Siderophore (++), Phosphate solubilizing (+), Ammonia production (++), Protease k (++), cellulose activity (++), Exopolysaccharide (++), Biosurfactant (++)	Cd (2.6), Pb (3.5), Cr (2.5), Co (2.0), Cu (3), Ni (1.5)

Biochemical and molecular characterization of bacterial isolates

Molecular identification and characterization of rhizospheric and endophytic bacteria were based on morphological, physiological, biochemical, and PCR amplification of the 16S rRNA gene. The morphological, physiological, biochemical, and 16S rRNA PCR amplification results are summarized in Table 1. These bacterial strains were selected based on their potential heavy metal tolerance and exhibited all PGPR traits. The 16S rRNA gene was successfully amplified by PCR, and approximately 1465 bp gene products were sent for Sanger sequencing. The BLAST-n program of the NCBI database was used for the similarity search for sequences, revealing that the rhizospheric isolate was *Proteus* sp. (accession number: PP665705). However, the endophytic bacterial isolate was similar to *Bacillus* sp. (accession number: PP665709). The identified nucleotide sequences were submitted to the GenBank database. A phylogenetic tree, constructed based on the 16S rRNA gene

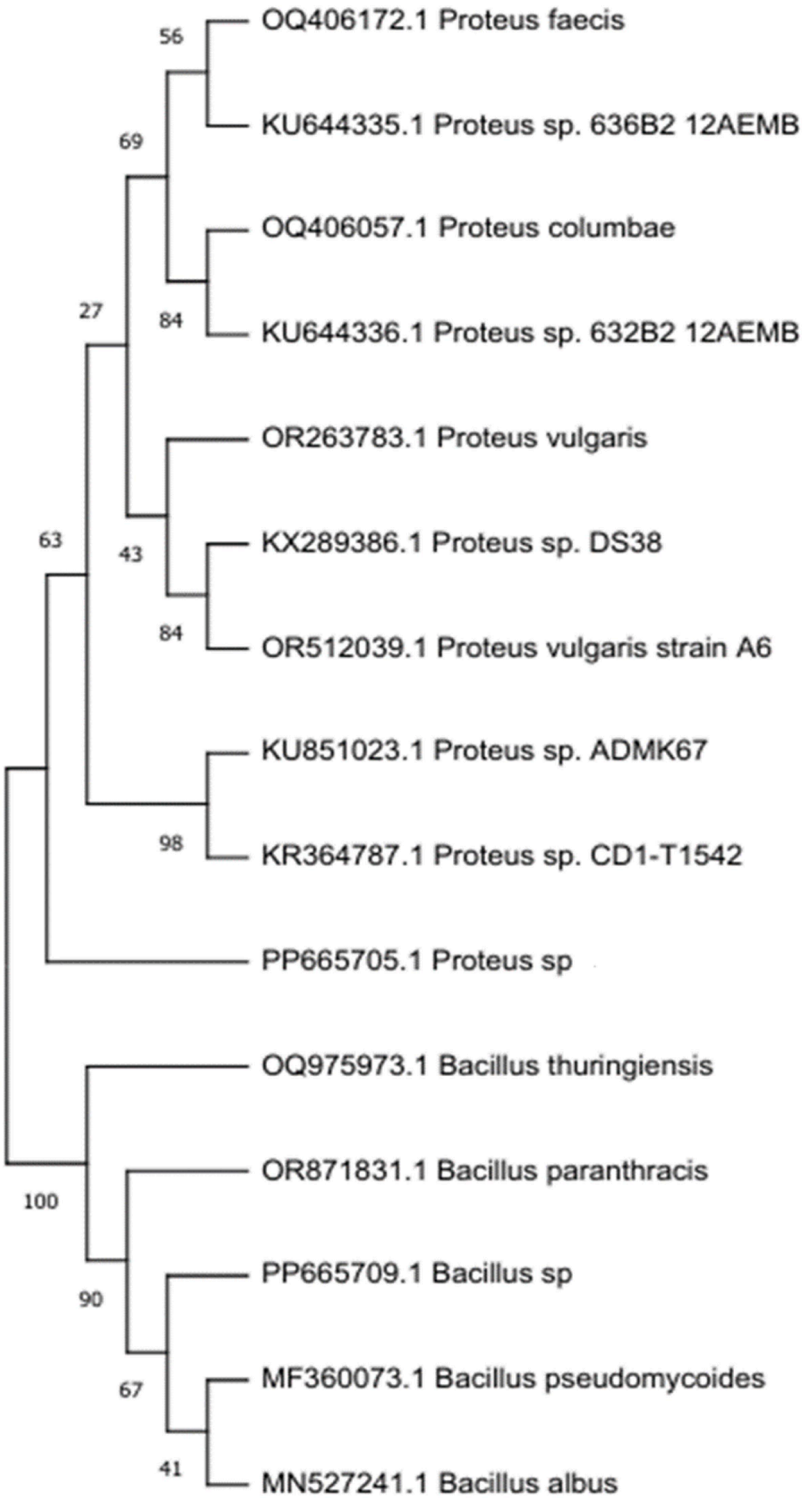
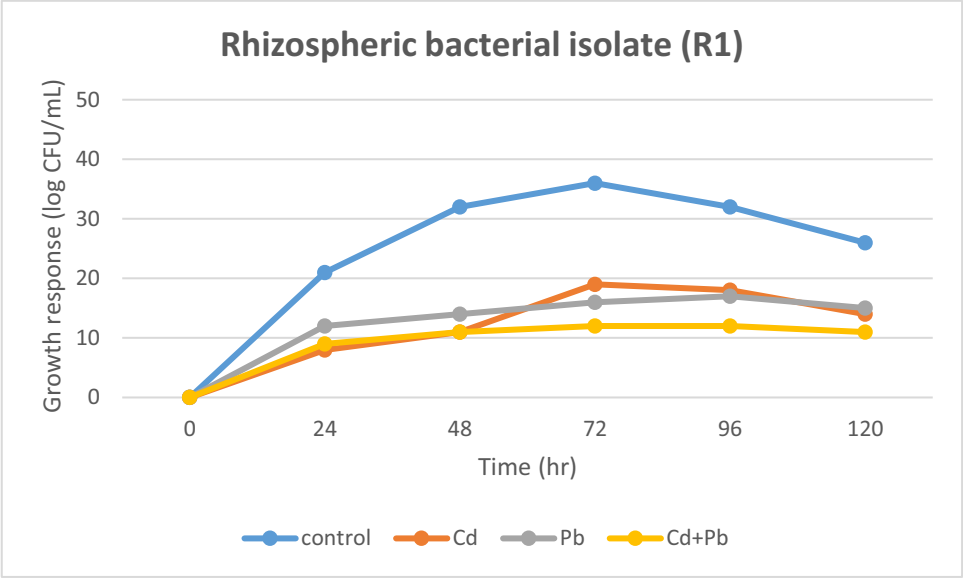


Fig. 3. Phylogenetic analysis of *Proteus* sp. (R1) and *Bacillus* sp. (E2) based on 16S rRNA gene sequences. The phylogenetic tree was constructed through the Neighbor-Joining algorithm with a Bootstrap value of 500. The tree is drawn to a scale of 0.05 substitutions per nucleotide position.



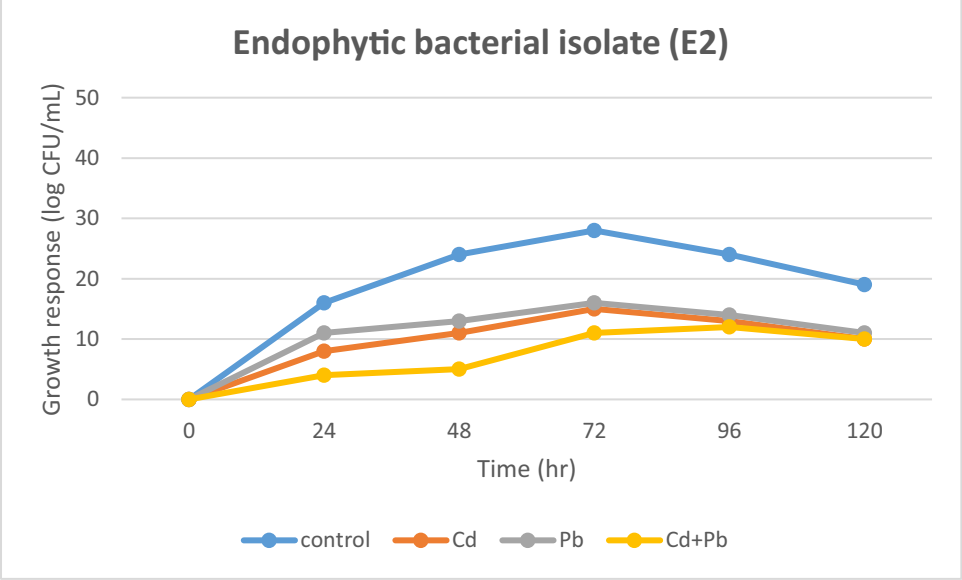


Fig. 4. Comparative growth response of rhizosphere (R1, *Proteus* sp.) and endophytic (E2, *Bacillus* sp.) strain grown in a nutrient (NB broth) medium with or without (control) Cd (4Mm) Pb (4Mm) and Pb+Cd.

sequences, indicated that the R1 strain was closely related to the Proteus vulgaris and E2 isolates were lie Bacillus species, mainly *Bacillus thuringiensis, Bacillus paranthracis* and *Bacillus pseudomycoides* isolates (Fig. 3).

Assessment of removal of Cd and Pb by biomass of strain

In this experiment, *Proteus* sp. (R1) and *Bacillus* sp. (E2) bacterial isolates were grown in NB medium containing 4 mM Cd and 4 mM Pb heavy metal separately and in combination (Fig. 4). As shown in Fig 2, both rhizospheric and endophytic bacterial strains grew in the presence of Pb, Cd, and Pb+Cd media and showed similar trends to the control. This experiment revealed that Cd and Pb were toxic to both strains.

Biocontrol test

The antagonistic activity of *Proteus* sp. (R1) and *Bacillus* sp. (E2) against pathogenic *FOL* was further assessed using a dual culture assay (Fig. 5). The result revealed that both isolates showed antagonistic activity and significantly inhibited mycelial growth of the pathogen, with a clear inhibition zone. However, according to the inhibition rate of analysis, R1 (72 %) showed the highest antagonistic activity compared to the E2 (69 %) bacterial strain.

Evaluation of the in vitro cell-free supernatant antifungal activity

The antagonistic effect against the FOL pathogen was also tested with cell-free supernatants of *Proteus* sp. (R1) and *Bacillus* sp. (E2) bacterial strains. The culture filtrates of both strains also inhibited the growth of the FOL pathogen and showed a clear inhibitory effect (Fig. 6). The culture filtrate of *Proteus* sp. (R1) showed an inhibition rate of 22 %, and *Bacillus* sp. (E2) strain of 16 % against fungal pathogens compared to the control. These results suggest that both strains have broad-spectrum antagonistic activities against FOL.

Plant growth promotion and biocontrol activities

The plantlets were grown with *Proteus* sp. (R1) and *Bacillus* sp. (E2)

(a) (b)

Fig. 5. The antagonistic effect of (a) *Proteus* sp. (R1) and (b) *Bacillus* sp. (E2) bacterial strains against *Fusarium oxysporum f.* sp. *Lycopersici* strains. Images of biocontrol experiments (a) and inhibition zone measurements of 72 hrs old *Proteus* sp. (R1) strain (b) *Bacillus* sp. strain on 50 % LB medium at 25 °C.

Fig. 6. Antagonist effect of cell-free supernatant (CFS) of two bacterial strains, *Proteus* sp. (R1) and *Bacillus* sp. (E2), against the FOL. *Proteus* sp. (R1) and *Bacillus* sp. (E2) clearly showed an antagonistic effect on the mycelial growth of FOL inoculated in the centre of the Petri plate, mediated by CFS of five days old bacterial strains grown on 50 % LB.

to analyse the plant growth promotion activities and with spore suspension of FOL to determine the response of cucumber cultivars against fungal pathogens. The few cucumber plantlets were also grown with *Proteus* sp. (R1) + FOL and *Bacillus* sp. (E2) + FOL to analyse the biocontrol activities of both rhizospheric and endophytic bacterial strains. These strains were applied near the root surfaces, and the plants were incubated under higher humid conditions for successful infection. After 14 days of inoculation, the *Proteus* sp. (R1) and *Bacillus* sp. (E2) strains had a significant plant-growth-promoting and antagonistic effect against the FOL pathogen compared with the untreated control in pots. The *Proteus* sp. (R1) and *Bacillus* sp. (E2) strains were able to reduce the effect of FOL and showed a significant increase in plant weight from 400 %, shoot length from 66.6 %, root length from 75 %, and chlorophyll content from 50 % relative to the pathogen and control (Fig. 8). However, Cucumber plants inoculated with FOL exhibited clear symptoms of wilting diseases and severe growth hindrance within two weeks as compared to tolerant plants (Fig. 7). Therefore, cucumber plants treated with *Proteus* sp. (R1) and *Bacillus* sp. (E2) isolates significantly recovered the growth promotion and reduced the wilt disease symptoms. In particular, the *Bacillus* sp. (E2) bacterial isolate showed significantly higher shoot (52.06 %) and root lengths (53.5 %), fresh weight (173.34 %), dry weights (124.13 %) and chlorophyll content (54.53 %) compared to control. However, *Proteus* sp. (R1) strains showed shoot (39.56 %) and root lengths (35.5 %), fresh weight (50.79 %), dry weights (79.31 %) and chlorophyll content (54.6 %) compared to the control plant.

Discussion

Rhizospheric and endophytic strains possess various mechanisms that increase the survival of plants in heavy metal-contaminated soil. Rhizosheric isolates, due to their PGPR traits, can remediate heavy metals in contaminated soil. In this research, heavy metal-resistant rhizospheric and endophytic bacterial isolates were isolated from wastewater-irrigated rhizospheric and endophytic regions of cucumber plants. Recent advances in plant-pathogen interactions and abiotic stress research have revealed the fascinating ability of plants to interact with the rhizosphere and endophytic microbiota effectively. Environmental stress conditions can influence the characteristics of bacterial communities, which can be useful for plant growth. Traditionally, rhizosphere and endophytic microbiome composition has been determined primarily by soil microbial communities and their interactions with roots. However, emerging research has suggested that plants are important for the establishment and maintenance of microbial communities. The results obtained from this study demonstrated that the rhizospheric and root endophytic isolates of cucumber plants irrigated with wastewater showed heavy metal resistance with several PGP traits. Previous studies have also reported the presence of various heavy metal-resistant rhizospheric and endophytic bacterial isolates in plants grown in heavy metal-contaminated soils (Fan et al., 2018; Oubohssaine et al., 2022). Heavy metal tolerance results showed the ability of rhizospheric and

Fig. 7. *In vivo*, Plant growth promoting characteristics of Rhizospheric and endophytic isolates and antagonistic activity against FOL in cucumber plants. The scale bar represents 1 cm.

root endophytic bacterial isolates to tolerate and survive the stress of heavy metals without any toxicity reported in the sampled cucumber plants. The MIC value represents the rhizospheric isolates showed higher tolerance against each heavy metal compared to endophytic isolates. The presence of culturable rhizospheric isolates has also been reported to be higher in roots than in endophytic isolates (Abid et al., 2022). Riseh et al. (2023) reported that rhizosphere organisms are exposed to heavy metals in the soil environment and have developed mechanisms to escape and evade their presence. Root endophytic bacteria in tissues may be less exposed to heavy metals, leading to a lower tolerance level.

In this study, nearly all rhizospheric and root endophytic isolates showed resistance to all heavy metals at different concentrations. The results indicated that rhizospheric and root endophytic bacterial isolates showed resistance to cadmium, which is toxic even at low concentrations. Results from assessing PGP factors, particularly IAA production by rhizosphere and root endophyte isolates, revealed interesting patterns. The data show that Most of the rhizosphere and root endophytic isolates produced IAA, which enhanced plant growth and development. The percentage of IAA-producing bacteria in the rhizosphere (83.47 %, 101/121) was slightly higher than that of IAA-producing root endophytic bacteria (80.43 %, 74/92). These findings indicated that a significant portion of the bacterial community associated with the rhizosphere and root endosphere of cucumber plants can produce IAA and affect plant growth. Mohite (2013) suggested that bacterial IAA production may positively affect plant growth through various mechanisms. IAA can promote root growth, improve nutrient absorption and water utilization, and support overall plant growth (*Etesami and Glick, 2024*). The abundance of IAA-producing bacteria in the rhizosphere and endosphere indicates their ability to support plant growth and development. Chandra et al. (2018) showed that the relatively small number of IAA-producing rhizosphere isolates might be due to the direct effect of root exudates and the proximity of these organisms to the roots. The rhizosphere is a nutrient-rich region where root exudates provide carbon for microbial growth and promote colonization by IAA-producing bacteria. Greater availability of nutrients and organic compounds in the rhizosphere increases the number of IAA producers in this region. Other PGP factors, including siderophore production, ammonia production, hydrogen cyanide (HCN) production, and phosphate solubility, help to understand the properties of cucumber-associated rhizosphere and endophytic bacterial isolates. Most rhizosphere isolates showed good PGP quality. The results showed that 77.68 % of the rhizosphere was isolated as siderophores, metal-chelating compounds that increase plant iron utilization. Siderophores produced by these isolates have demonstrated their ability to increase iron absorption in plants, which is important for many physiological processes (Singh et al., 2022). Additionally, 89.25 % of the rhizosphere isolates showed ammonia activity, indicating they could provide nitrogen for plant growth. Ammonia production is associated with the nitrogen cycle, which can improve nutrition and promote plant growth (Nonthakaew et al., 2022). HCN production was found in 81.81 % of rhizosphere isolates. This is interesting because HCN can act as a biocontrol agent for many diseases. The ability of these isolates to produce HCN demonstrates their potential role in combating diseases and improving plant health (Sehrawat et al., 2022). Moreover, siderophores have the ability to bind with various metals and solubilise heavy metals from soil to facilitate their bioremediation. Regarding phosphate solubility, 61 % of rhizosphere isolates could solubilize phosphate. This property is important because it allows bacteria to release phosphate in an incomplete form, making it easier for plants to use microbial tools to control the bacteria. It has gained in popularity in recent years. In particular, PGPR and its interactions with plants under biotic or abiotic stress have gained importance in improving crop and agricultural yields. This biological control system is environmentally friendly and has become popular, reliable, and durable.

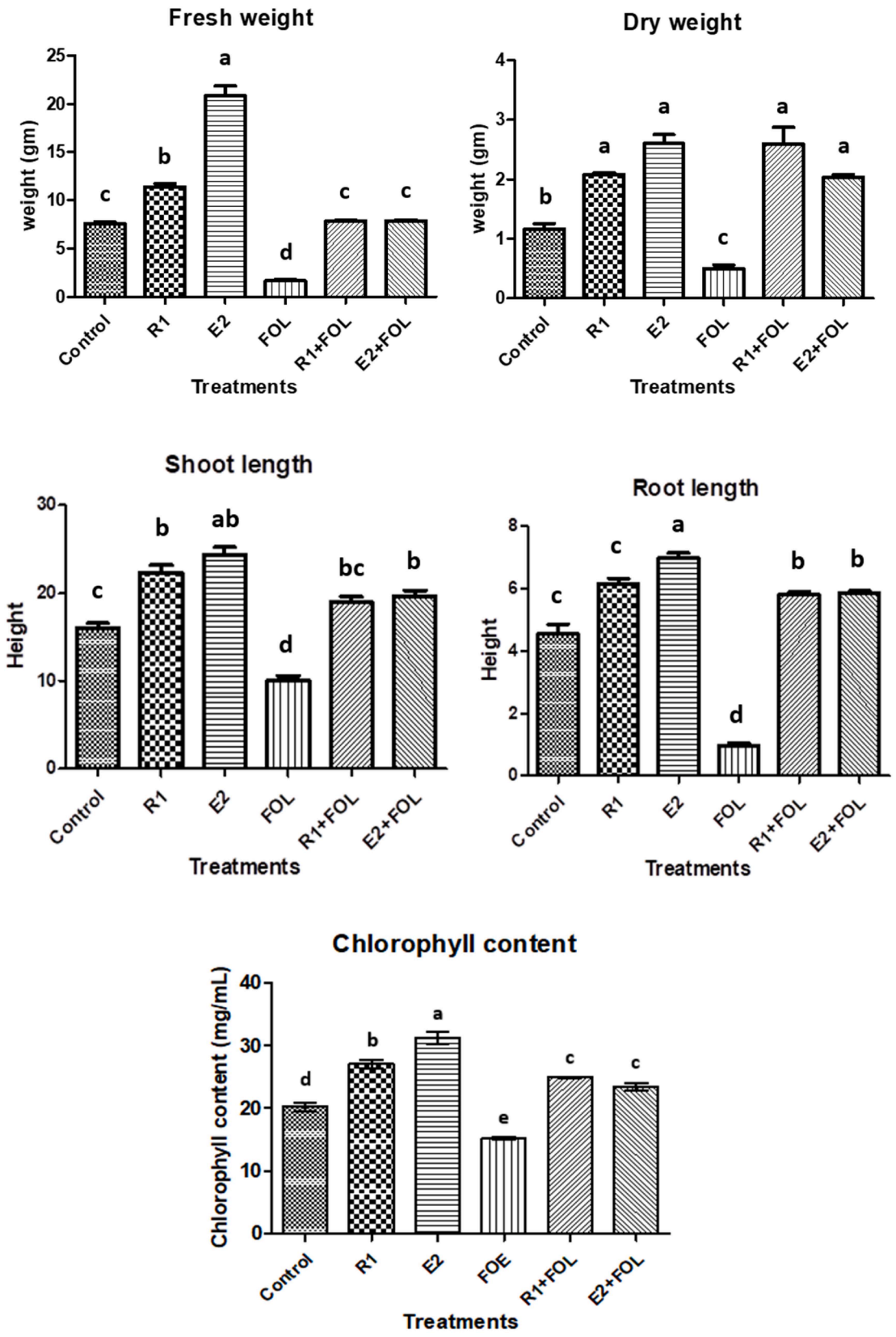


Fig. 8. *In vivo* test of Rhizospheric and Endophytic isolates for plant growth promotion and antagonistic effect against FOL. Data shown are mean ± standard deviation of fresh weight, dry weight, shoot length, root length, and Chlorophyll content. Lowercase letters indicate a significant difference ($P < 0.05$).

PGPR can improve plant growth and disease management directly or indirectly by interacting with host plants (Vejan et al., 2016). There has been much research in recent years, and many studies have shown *Bacillus* and *Proteus* species are widely recognized as important sources for promoting plant growth and reducing disease symptoms (Sundaramoorthy and Balabaskar, 2013; Chaves-López et al., 2015). Fungal diseases can decrease crop yield and production losses, thereby threatening food security. FOL is a fungal disease affecting the vascular system and causing serious damage to tomatoes and cucumbers worldwide. Shahzad et al. (2016) reported that endophyte *i.e. Bacillus amyloliquefaciens* promoted plant growth and resistance to FOL in cucumbers. It has also been reported that *B. aryabhatta*i SRB02 plays a role in oxidative and nitrosative stress tolerance and promotes the growth of bean and rice plants by regulating phytohormone production (Park et al., 2017a). However, it is not yet clear whether *Proteus* sp. (R1) and *Bacillus* sp. (E2) can protect plants from biotic stress.

This study showed that PGPR in pathogenic bacteria protects plants from pathogens and improves plant growth and biomass. These benefits may be achieved by inhibiting fungal pathogens, increasing nutrient absorption, producing phosphorus-solubilizing compounds, or stimulating hormone biosynthesis. Previous studies have also strengthened the present findings and the role of *Bacillus* and *Proteus* species as PGPR and biocontrol agents against many diseases in different plant species, such as root wilt, damping off, Fusarium wilt, ring rot, and charcoal rot in soybean, banana, and apple (Yu et al., 2002; Vitullo et al., 2012; Sun et al., 2022; Han et al., 2021; Li et al., 2024). The present study also confirmed that *Bacillus* sp. and *Proteus* sp. can help to protect cucumber plants from Fusarium wilt diseases, similar to previous reports.

Conclusions

This study revealed that the rhizospheric and root endophytic regions of cucumber plants irrigated with wastewater were enriched with heavy metal-resistant bacterial strains with various PGP traits and antagonistic effects against the FOL pathogen. Most bacterial isolates isolated from the rhizospheric region showed higher resistance against heavy metals and various plant growth-promoting characteristics. The use of heavy metal-resistant bacteria in the agriculture sector can reduce the accumulation of heavy metals and relieve stress originating from the use of treated wastewater in the irrigation of cucumber plants. In this study, *Proteus* sp. and *Bacillus* sp. strains isolated from the rhizospheric and root endophytic region showed higher resistance against various heavy metals and multiple PGP traits, which should be an excellent economical and eco-friendly bio-absorbant for heavy metal contaminated sites. The present study also revealed that both strains can enhance plant growth and biomass under fungal infection. Also, it proved the role of *Proteus* and *Bacillus* species as PGPR and biocontrol agents against fungal wilt disease in various plant species. Further studies will also be needed to analyse these strains under greenhouse and agricultural field conditions to reveal their ability as a good absorbant of heavy metals in heavy metal-contaminated soil and biocontrol agents against various fungal pathogens.

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CRediT authorship contribution statement

Kumar Shreshtha: Data curation, Methodology, Writing – original draft. **Satyam Raj:** Data curation, Methodology. **Arun Kumar Pal:** Data curation, Methodology. **Pooja Tripathi:** Conceptualization, Formal analysis, Supervision. **Krishna Kumar Choudhary:** Formal analysis. **Debasis Mitra:** Validation. **Anju Rani:** Validation. **Sergio de los Santos-Villalobos:** Writing – review & editing. **Vijay Tripathi:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

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