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Research Article

Harnessing Soil Rhizobacteria from Gwalior region for Multiple Plant Growth Promoting traits and Heavy Metal Resistance

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ABSTRACT

Rhizospheric soil bacteria play a significant role in influencing plant development by employing multiple growth enhancing strategies and are crucial in alleviating heavy metal stress as well as other abiotic challenges faced by plants. Plant growth enhancing rhizobacteria (PGER) are extensively applied as bioinoculants to boost crop advancement, health, and yield. This study aimed to isolate and characterize plant growth enhancing rhizobacteria (PGER) from agricultural soils of the Gwalior region for their multifunctional traits and heavy metal resistance. Total five bacterial isolates were screened for their abilities to synthesize of indole-3-acetic acid (IAA), solubilization of phosphate and zinc, the biosynthesis of siderophores, production of hydrogen cyanide (HCN) and ammonia. Among the tested isolates, two bacterial strains, MR2D and A8D, exhibited the most promising plant growth enhancing traits after characterization. Additionally, these two selected strains were further evaluated for their tolerance to multiple heavy metals, including Zn, Cu, Cr, Ni, Co and Pb at varying concentrations (i.e., 0.1%, 0.5% and 1.0%) respectively. Molecular characterization through 16S rRNA gene sequencing identified A8D as Leclercia adecarboxylata and MR2D as Pantoea agglomerans. Both isolates demonstrated the dual functionality by promoting plant growth and tolerating heavy metals. Therefore, the study suggests the applicability of these strains in sustainable agriculture and bioremediation. These findings highlight the potential of PGER as eco-friendly bioinoculants to improve crop productivity as well as soil health under a stressed environment.

Keywords: *HCN, Siderophore, Auxin Production, Phosphate solubilization, PGER, Heavy metals.*

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INTRODUCTION

Soil is a vital natural resource that all organisms, including humans, rely on, either through direct or indirect means. Different microorganisms flourish on abundantly present nutrients in soil and perform a noteworthy role in biogeochemical cycles and pedogenesis through multiple interactions (Ahemad & Kibret, 2013). Among contaminants, abundant heavy metals in the environment (like Cd, Cu, Pb, Cr, which are known to be harmful in nature) pollute soils through numerous natural and manmade activities (Liu et al., 2013). Plant growth enhancing rhizobacteria are found in the rhizospheric region on the surface of plants, within their tissues, and also promote crop advancement or biocontrol. These rhizobacteria can increase plant development directly by improving nutrient cycling, including biological fixation, phytohormone production and phosphate solubilization, among others (Backer et al., 2023). Application of Plant growth enhancing rhizobacteria nutritional supplements to soil or plants is continuously expanding in agriculture, providing substitute i.e., artificial fertilizers, antibiotics, herbicides, pesticides (Calvo et al., 2014; Chaudhary et al., 2023; Goswami et al., 2022). Microbial agents, on the other hand, are a promising alternative in environmentally friendly agriculture (Santoyo et al., 2016; & Vinale et al., 2008). Several examples of crop growth-enhancing rhizobacteria have been identified and improved over the past decade, primarily due to the growing importance of the rhizosphere as a key ecological unit in biosphere operations and the in-depth study of PGER methods of action (Kumar et al., 2024). A probable rhizobacteria is considered efficient if it can demonstrate strong competitive abilities over the current rhizosphere communities by having a favourable effect on the plant after inoculation. By Antoun and Prevost (2005), rhizobacteria typically make up 2-5% of rhizosphere bacteria. PGERs are a trend for the future as well as prospective tools for environmentally friendly agriculture. A soil is considered naturally nutritious when its microbes release inorganic minerals from the biological reserves at a rate that supports the rapid development of plants. Bacterial genera like *Bacillus*, *Pseudomonas* and *Azotobacter* remain often predominant in the rhizosphere because they grow rapidly in soils with diverse organic content and can withstand common agricultural fertilizers used in seed treatments (Vijaypal, 1998). This study focused on isolating, characterizing and evaluating beneficial PGERs that support plant growth and development and heavy metal tolerance.

MATERIALS AND METHODS

Study area, collection and isolation of samples

Three soil samples have been collected from different vegetable crops cultivated on agricultural land in the nearby Gwalior region. Soil was gently scraped to a depth of 10-15 cm from the rhizospheric region of selected plant species. Each soil sample was kept under a sterile polybag and stored at 4^0 °C temperature for further analysis. Soil samples were processed using the serial dilution approach, as outlined by Johnson and Curl (1972), to isolate bacterial strains exhibiting plant growth-enhancing traits. For that, 1gm of soil was taken in 0.855 normal saline water, up to (10^{-2} to 10^{-9}) fold dilution was made, and serially diluted. The dilutions were spread on Nutrient Agar medium. After Incubation at 37^0 °C for 24 hr hence, fine isolated single colonies were picked up from pure culture. All the selected isolates were maintained at 4^0C as glycerol stock. Preliminary identification of bacterial colonies was carried out using microscopic examination and Gram staining, following the method described by Ying (2021).

In Vitro Assessment of PGPR Activities.

Evaluation of auxin (IAA) production

The capacity of bacterial strains to synthesize indole-3-acetic acid (IAA) was examined; for this, the isolates were inoculated in nutrient broth accompanied by tryptophan (50ug/mL) at 28 °C for 48h at 200rpm. After the incubation period, cultures were centrifuged and supernatant collected. IAA was

determined spectrophotometrically by mixing 2ml of supernatant with 4ml of Salkowski mixture (1ml of 0.5M $Fecl_3$ in 50ml of 35% HClO4) and incubating for 30 min. Moreover, observed for the development of pink colour (Gorden & Weber, 1951).

Screening of phosphate solubilization by the plate assay method

Phosphate production by the isolates were measured on Pikovaskya agar plates. Bacterial cultures were inoculated on an agar plate and incubated for 7 days at a temperature of 280 °C. Formation of a clear halozone surrounding the bacterial colonies was taken as a positive phosphate solubilizers (Pikovaskya, 1948).

Zinc solubilization potential of rhizobacterial strains

Zinc solubilization capability for selected isolates was evaluated using spot inoculation method on Tris-minimal medium (composition per liter: Tris-HCl 6.06 g, NaCl 4.68 g, KCl 1.49 g, NH_4Cl 1.07 g, Na_2SO_4 0.43 g, $MgCl_2 \cdot 2H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 30 mg), as defined by Fasim et al. (2002). For 14 days, the inoculated plates had been kept at 30°C, and growth of clear halo zones around the bacterial colony was monitored (Sharma et al., 2012).

HCN, Siderophore and Ammonia Production of rhizobacterial isolates

Selected isolates of bacteria were streaked over King's B media treated with 0.4% (w/v) glycine in order to assess HCN generation. The development of a reddish-brown coloration on the filter paper, indicative of HCN release, was monitored over four days, ensuring the method given by Miller and Higgins, (1970).

The Chrome Azurol S (CAS) assay approach, created by Schwyn and Neilands (1987), was used to assess the production of siderophores. After being spot-inoculated on CAS Agar plates, the bacterial colonies cultured for four days at 28 degrees Celsius. An indication of bacterial isolates that produced siderophores was the formation of an orange yellow halozone surrounding the growth (Schwyn & Neilands, 1987).

Evaluation of ammonia synthesis was tested with the combination of Nesseler's reagent with the bacterial cultures in peptone water broth, which changes slightly yellow to brown in colour, indicating ammonia production (Cappuccino & Sherman, 1992).

Heavy metal tolerance of the selected rhizobacterial isolates

Luria Bertani agar plates were employed in the plate diffusion method to examine the bacterial isolates' tolerance to heavy metals. Cultures that were 24 hours old were swabbed onto LB plates. Inoculated plates then incubated at 37degree celcius for 24 to 48 hours after 100 µl of solutions of the proper heavy metal salts i.e., zinc ($ZnSO_4.7H_20$), copper ($CuSO_4.5H_2O$), chromium ($K_2Cr_2O_7$), nickel ($NiCl_2$), lead Pb $(NO_3)_2$ and cobalt ($Cocl_2$) concentrations of 1%, 0.5%, and 0.1% were added to each well independently. Following the duration of Incubation, the resistance was assessed (Hassen et al., 1998).

Characterization of selected PGPRs

The morphological traits of bacterial isolates, including motility, cell shape, and Gram's character, were examined. Additionally, biochemical characterization of the selected traits was carried out (i.e., for urease, catalase, oxidase, methyl red, Voges-Proskeur, citrate consumption and H_2S generation) (Cappuccino & Sherman, 1992).

Molecular Identification of the two most potent PGPR Isolates by 16S rRNA Gene Sequencing

The two selected rhizobacterial strains underwent molecular characterization. Molecular identification was performed by 16S rRNA gene sequencing using universal primers, and phylogenetic trees were constructed using MEGA11 software (Tamura et al., 2021). After being verified, the isolate sequences were then submitted to GenBank (NCBI) and assigned accession numbers.

RESULTS

Five distinct rhizobacterial isolates designated as A8D, M2RD, MR3B, A6B and KC2A, were obtained from agricultural soil samples collected from the Gwalior region. Morphological examination revealed that most isolates were rod-shaped and Gram-positive, while isolate A8D displayed a coccoid form. Qualitative phosphate solubilization assays showed that isolates A8D and MR2D had the highest solubilization efficiency, forming clear halozones on Pikovskaya's agar medium. These isolates also demonstrated multiple plant growth-promoting attributes, including the production of indole-3-acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN) and siderophores. Zinc solubilization analysis further identified isolate MR2D as the most efficient zincsolubilizer, producing a clear solubilization zone measuring 32 mm as described in (Table 1).

Furthermore, the heavy metal tolerance of two selected isolates A8D and MR2D was evaluated against six metal ions (i.e., $ZnSO_4$, $CuSO_4$, $NiCl_2$, $CoCl_2$, $Pb(NO_3)_2$ and $K_2Cr_2O_7$ at three varying concentrations (0.1%, 0.5%, and 1.0%). Isolate A8D exhibited the highest resistance across all tested metals, indicating strong adaptability to metal-contaminated environments as shown in (table 2,3 and 4).

Table1. Bacterial isolates showing production of various plant growth promoting traits. ('+++' very good growth, '++' good growth, '+' fair growth, '-' no growth)

Isolates	Auxin	Phosphate	Ammonia	Zinc	Siderophore	HCN
A8D	++	++	+	+	+++	-
MR2D	++	++	+	++	++	-
MR3B	-	+	+	-	+	-
A6B	+	+	+	-	+	-
KC2A	-	-	+	-	++	-

Table2. Heavy metal tolerance (HMT) of isolate at 1% concentration of selected metals

Culture	$ZnSo_4$(1%)	$CuSo_4$(1%)	$NiCl_2$(1%)	$CoCl_2$(1%)	$Pb(No_3)_2$(1%)	$K_2Cr_2O_7$(1%)
MR2D	12.48±0.25	20.8±0.416	12.48±0.25	17.68±0.354	R	29.12±0.583
A8D	R	R	R	R	R	R

Table3. Heavy metal tolerance (HMT) of isolate at 0.5% concentration of selected metals

Culture	$ZnSo_4$(0.5%)	$CuSo_4$(0.5%)	$NiCl_2$(0.5%)	$CoCl_2$(0.5%)	$Pb(No_3)_2$(0.5%)	$K_2Cr_2O_7$(0.5%)
MR2D	10.4±0.208	8.32±0.167	10.4±0.208	11.44±0.229	R	20.8±0.416
A8D	R	R	R	R	R	R

Table4. heavy metal tolerance (HMT) of isolate at 0.1% concentration of selected metals

Culture	$ZnSo_4$(0.1%)	$CuSo_4$(0.1%)	$NiCl_2$(0.1%)	$CoCl_2$(0.1%)	$Pb(No_3)_2$(0.1%)	$K_2Cr_2O_7$(0.1%)
MR2D	R	R	R	R	R	R
A8D	11.44±0.229	R	R	13.52±0.271	R	R

Moreover, biochemical characterization of both selected isolates, A8D found as cocci and MR2D found as Bacilli type of rhizobacteria (Table 5). Molecular identification using 16S rRNA gene sequencing confirmed that A8D showed high similarity to *Leclercia adecarboxylata* and MR2D to *Pantoea agglomerans*. Phylogenetic trees constructed using the neighbor-joining method in MEGA11 software confirmed close evolutionary relationships with reference strains. The GenBank accession numbers PQ632568 and PQ632651 were assigned to A8D and MR2D, respectively, as shown in Figures 1 and 2.

Table5. Morphological and Biochemical Characterization of two selected bacterial isolates

Tests **(Morphological)**	**A8D**	**MR2D**
Color	Cream	Yellow
Shape	spherical	Rod
Gram staining	Streptococci	Bacilli
Motility	-ve	-ve
Endospore formation	+	+
Biochemical		
Catalase test	+	+
Urease test	+	-ve
Voges-Proskauer (VP) test	-	+
Methyl red (MR) test	-	+
Citrate utilization	-ve	+ve

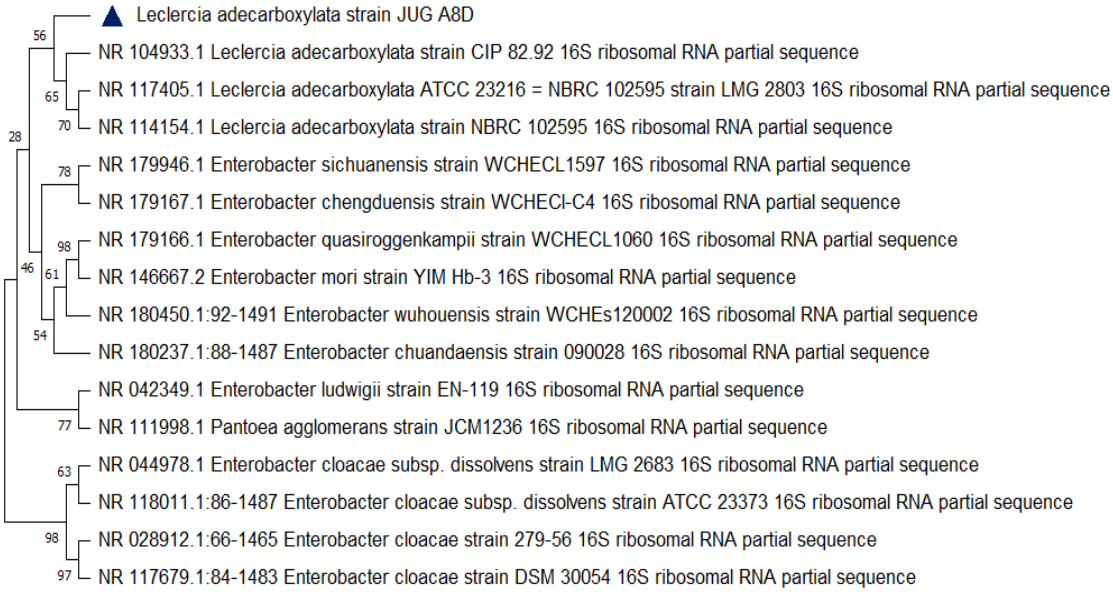

Figure1. Phylogenetic tree of *Leclercia adecarboxylata* strain JUG A8D buildup by neighbor joining technique using MEGA 11

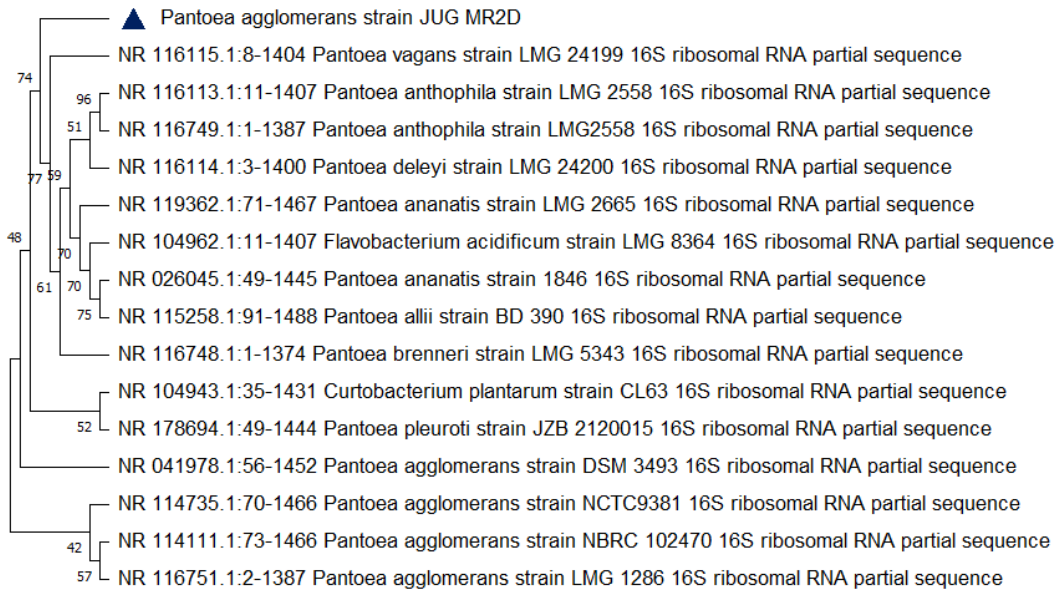

Figure2. Phylogenetic tree of *Pantoea agglomerans* strain JUG MR2D buildup by neighbor joining technique using MEGA 11

DISCUSSION

The isolated rhizobacteria exhibited promising plant growth-promoting characteristics. The presence of coccoid and rod-shaped Gram-positive bacteria among the isolates supports previous findings that bacteria with robust cell envelopes can better withstand adverse environmental conditions, including metal contamination (Gadd, 2010).

Phosphate solubilization is a critical attribute of PGPR, as phosphorus is often present in insoluble forms in soil. The ability of isolates A8D and M2RD to solubilize phosphate likely involves mechanisms such as the release of organic acids, enzymatic hydrolysis, and chelation processes, which increase phosphorus bioavailability to plants (Rodríguez & Fraga, 1999; Sharma et al., 2013). These mechanisms enhance root growth and plant development, especially in nutrient-deficient soils (Singh & Reddy, 2020).

The production of IAA by A8D and MR2D suggests a potential role in modulating plant root architecture, including root elongation and lateral root formation. Enhanced root systems improve water and nutrient uptake, contributing to plant vigour (Susilowati et al., 2024). In addition, siderophore production by these isolates supports iron acquisition in the rhizosphere, especially under iron-limiting conditions, which is essential for chlorophyll synthesis and overall plant health (Ahmed & Holmström, 2014). The release of ammonia also contributes to the nitrogen pool in the rhizosphere, while HCN production may offer protection against root pathogens (Cappuccino & Sherman, 1992).

The zinc solubilization observed in MR2D demonstrates the capability of this isolate to mobilize insoluble zinc via organic acid production, thereby improving zinc uptake in plants (Saravanan et al., 2007; Kamran et al., 2017). Zinc is an essential micronutrient, and its bioavailability in soil is often a limiting factor in plant metabolism and growth.

Heavy metal tolerance in isolates A8D and MR2D further supports their potential application in bioremediation. The ability to grow in the presence of Zn, Cu, Ni, Co, Pb, and Cr at high concentrations suggests that these isolates may possess resistance mechanisms such as efflux systems, enzymatic detoxification, metal sequestration through exopolysaccharides, and siderophore-mediated

chelation (Rajkumar et al., 2012; & Gadd, 2010). These features make them suitable for use in phytoremediation and in supporting plant health in contaminated soils.

The identification of A8D as *Leclercia adecarboxylata* and MR2D as *Pantoea agglomerans* aligns with prior studies reporting their dual functionality in promoting plant growth and tolerating environmental pollutants (Ma et al., 2019; & Khan et al., 2020). Several genera of PGPR, including *Pseudomonas fluorescens*, *Bacillus subtilis*, *Azospirillum brasilense*, *Rhizobium spp.*, and *Enterobacter spp.*, have been widely studied for similar traits such as phosphate solubilization, nitrogen fixation, siderophore production, and phytohormone synthesis (Lugtenberg & Kamilova, 2009; Bashan et al., 2004; & Bhardwaj et al., 2014). The present findings add *Leclercia* and *Pantoea* to this list of multifunctional rhizobacteria with potential for use in sustainable agriculture.

The dual functionality of these isolates as both plant growth enhancers and metal-tolerant bacteria positions them as strong candidates for use as microbial inoculants. Their application can lead to improved nutrient uptake, stress resilience, and soil detoxification, critical components for modern, eco-friendly agricultural practices.

CONCLUSION

The study at hand investigated the plant growth enhancing potential of rhizobacterial isolates obtained from agricultural soils. Among the isolates, *Leclercia adecarboxylata* A8D (PQ632568) and *Pantoea agglomerans* MR2D (PQ632651) exhibited noteworthy growth enhancing capabilities. However, their functional efficiency may be affected by various environmental stressors, such as exposure to heavy metals and antibiotics. Other critical factors, including the origin of isolation, soil characteristics, fertilizer usage, and optimized cultivation conditions, also influence their overall performance.

Given their capacity to support plant growth, these bacterial strains hold promise for inclusion in microbial consortia aimed at bioremediation. With further enhancement and strain improvement, their role in sustainable agriculture could be significantly strengthened. The novel isolates characterized in this research offer valuable prospects for environmentally friendly agricultural applications, promoting soil fertility, increasing crop productivity, and contributing to long-term ecological sustainability.

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Conflict of interest

The authors declare no conflict of interest with any other work.

Author Contributions

The conception and design of the study were jointly developed by Mir Sajad Rabani and Mahendra K. Gupta, reflecting equal contribution. Anjali Pathak is responsible for writing the manuscript and performing data analysis. Mir Sajad Rabani and Mahendra K. Gupta contributed to proofreading. All authors reviewed and approved the final version of the manuscript.

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