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Biofertilizer Potential of *Pseudomonas parafulva* Isolated from the Chickpea Rhizosphere

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Abstract

Rhizobacteria play a crucial role in enhancing plant growth and improving crop yield. This study focuses on the plant growth-promoting and biofertilizer potential of rhizobacteria isolated from the chickpea (*Cicer arietinum*) rhizosphere. The evaluation involved both primary and secondary screening processes to assess the key biochemical traits. The primary screening focused on ammonia and auxin production, where ammonia production enhances nitrogen availability for plants and auxins, particularly indole-3-acetic acid (IAA), contribute to root formation and overall plant growth. Secondary screening examined the production of siderophores and hydrogen cyanide (HCN). Siderophores facilitate iron uptake by plants, while HCN acts as a biocontrol agent by inhibiting soilborne pathogens. The study's findings showed that, among the five bacterial isolates one isolate (B67) had high auxin and siderophore-producing abilities it also exhibited ammonia and HCN producing abilities. Inoculation of this rhizobacterial isolate on chickpea seeds exhibited positive effects on seed germination and seedling growth. Isolate B67 has been identified as *Pseudomonas parafulva* using 16S rRNA gene sequencing. These findings suggest that *Pseudomonas parafulva* isolated from the chickpea rhizosphere possesses multiple plant growth-promoting traits positively influences growth of plants. Therefore, it might be used as an effective bioinoculant and biocontrol agent, offering a sustainable alternative to chemical fertilizers and pathogen management strategies.

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Abbreviation

IAA : Indole-3-acetic acid
HCN : Hydrogen cyanide
PGPR : Plant growth promoting rhizobacteria.

Introduction

The rhizosphere, the narrow zone of soil influenced by plant roots, hosts a diverse community of micro-organisms that interact with plant roots to influence plant growth and health. These interactions play a critical role in sustainable agricultural practices, with a particular emphasis on plant growth promoting rhizobacteria (PGPR). PGPR are bacteria which directly and indirectly enhance plant growth by promoting nutrient availability, improving plant health and acting as biocontrol agents against plant pathogens.[1,2] Their beneficial effects occur through various mechanisms, including nitrogen fixation, phytohormone synthesis and improving plant resistance to abiotic conditions including drought and salinity.[3,4]

The chickpea (*Cicer arietinum*) rhizosphere is a highly dynamic environment, enriched with a variety of microorganisms, like N_2 fixing bacteria and rhizobacteria (PGPR), which collectively influence the plants growth, nutrient uptake and resistance to environmental stresses. The interaction between chickpea roots and rhizospheric microorganisms is central to its growth, particularly in nitrogen-deficient soils where PGPRs can enhance nutrient availability and plant vigour.[3,5]

The positive impact of PGPR in the chickpea rhizosphere is largely attributed to their ability to enhance nutrient cycling and improve plant stress tolerance. Several PGPRs, including *Pseudomonas* and *Bacillus* species, produce phytohormones like IAA, which induce root elongation and improve plant growth under drought and salinity stress.[6,7] These microorganisms also help solubilize phosphorus, an essential nutrient for plant metabolism and productivity, which often remains unavailable due to soil conditions.[8] In addition, PGPRs can suppress soil-borne pathogens by producing antimicrobial compounds competing with pathogens for nutrients and activating plant defence mechanisms.[1,9,10] One of the most common mechanisms through which PGPRs exert biocontrol activity is the synthesis of antibiotics can stop a variety of plant diseases from growing.[11] *Pseudomonas* and *Bacillus* species produce a broad spectrum of antibiotics, such as phenazines and bacillomycin, which help prevent the establishment of harmful pathogens in the rhizosphere.[12,13]

In addition to symbiotic nitrogen fixers like *Rhizobium leguminosarum* and *Bradyrhizobium*, free living nitrogen fixers like *Azotobacter* and *Azospirillum* help in improving soil fertility by changing atmospheric N_2 in plant-usable form. These bacteria improve soil nitrogen levels, release ammonia and secrete phytohormones that stimulate root growth and enhance nutrient absorption. Unlike symbiotic bacteria, free-living nitrogen fixers function independently, making them valuable for chickpea cultivation in nitrogen-deficient soils. However, recent studies suggest the presence of PGPRs in the chickpea rhizosphere can further enhance the nodulation process and increase the overall nitrogen fixation efficiency, leading to improved crop yields.[5]

Non-nodulating PGPR also contributes to plant health by producing siderophores which binds with iron and increases its availability to plants while restricting its accessibility to iron for harmful soil-borne pathogens. Additionally, species like *Serratia* and *Enterobacter*, play a role in organic matter decomposition, releasing essential nutrients that promote chickpea growth.[4,14,15]

The biocontrol potential of PGPR is particularly significant for chickpea (*Cicer arietinum*), which is susceptible to soil borne pathogens like *Fusarium* and *Rhizoctonia*. Recent *studies* have shown that PGPRs such as *Bacillus subtilis* and *Pseudomonas fluorescens* effectively reduce pathogen incidence while promoting plant growth.[16] Additionally, the application of these PGPR not only reduces disease symptoms but also improves seedling vigor, leading to improved plant establishment and productivity. Similarly, it was also found that *Bacillus subtilis* and *Pseudomonas fluorescens* enhance water retention in chickpea, reduce water loss through improved root structure and increase chlorophyll content, thereby supporting the plants growth under water-limited conditions.[6,17]

The ability of PGPR to enhance plant growth under adverse environmental factors, such as drought, salinity and nutrient deficiencies, is a growing area of interest in sustainable agriculture. With the increasing need to reduce chemical fertilizers and pesticides, rhizospheric microorganisms offer an eco-friendly alternative for enhancing plant productivity and maintaining soil health for the long term.[18-20] Understanding the interactions between plants and beneficial microbes is essential for harnessing their full potential in agricultural applications.

The isolation of indigenous PGPR is particularly valuable in agricultural practices due to their natural adaptation to specific soil and environmental conditions. Unlike commercially avaialable non-native strains, indigenous PGPR are naturally adapted to the specific environmental and soil conditions of a given region, making them more effective and resilient.[21] The objective of this research article is to explore the plant growth promoting and biofertilizer potential of rhizobacteria isolated from chickpea rhizosphere of agricultural field of Mandsaur district, Madhya Pradesh. The study aims to assess their impact on plant growth, soil health and biocontrol activities against soil-borne pathogens, thereby contributing to sustainable agricultural practices.

Materials and Methods

Sample Collection

Samples of soil were collected from *Cicer arietinum* (chick pea) rhizosphere in Mandsaur district Madhya Pradesh, India. Samples were collected from different locations in the field by gathering the soil adhering to the roots and packing it in sterile polythene bags and safely delivering it safely to the laboratory and storing it at room temperature till the further analysis.[22]

Isolation and Purification of Rhizospheric Bacterial Cultures

Rhizobacteria were obtained from soil samples using serial dilution and spread plate methods. 100 ml of sterile distilled water and one gram of soil were mixed together and the mixture was vigorously shaken for 15 minutes using a vortex mixer. One ml of soil suspension was mixed with 9 ml sterile water and successive dilutions were made up to 10^{-6}. The diluted soil suspension (0.2 ml of 10^{-6} dilution) was spread on Nutrient agar medium and kept at 37°C temperature for 48 hours. The colonies having different morphological characteristics were selected and purified by the streak plate method. The purified single colonies were stored in Nutrient agar slants at 4°C temperature for further analysis.[23]

Identification of Rhizospheric Bacterial Cultures

All the rhizobacterial cultures were identified with the help of colony characteristics like shape, color, margin, elevation as well as biochemical tests like MR test, VP test, Simmon citrate test, Motility test, Triple sugar iron test, Phenylalanine deaminase test, Hydrogen sulphide production test, Sugar fermentation test and Nitrate broth test. The bacterial cultures were gram stained and examined under microscope to determine their gram characteristics, bacterial shape and arrangement.[24,25]

Plant Growth Promoting and Biocontrol Activities of Rhizospheric Bacteria

The growth promoting and biocontrol activities of rhizobacterial cultures were evaluated using primary and secondary screening methods. Primary screening was based on auxin and ammonia production assay and secondary screening was based on the biocontrol activities like siderophore and HCN production assay.

Primary Screening

Auxin Production

Rhizobacterial cultures were grown in nutrient broth containing 0.1% tryptophan and incubated at 37°C for 48 hours. After incubation Salkowski reagent (2 ml 0.5M $FeCl_3$ in 49 ml H_2O and 49 ml 70% $HClO_4$) was added in each test tube. The mixtures were kept at room temperature for 30 minutes. The appearance of pink color showed the production of IAA. Uninoculated medium was used as control.[26]

Ammonia Production

Peptone broth (4%) was used for detection of ammonia production in rhizobacterial cultures. Rhizobacterial cultures were inoculated in 5 ml peptone broth and kept at 37oC temperature for 48 hours. After incubation 0.5 ml Nessler's reagent was added to each test tube. Ammonia production was detected by the color turning from brown to yellow. Uninoculated medium was used as control.[27]

Secondary Screening

Hydrogen Cyanide Production

The rhizobacterial cultures were spot inoculated on Nutrient agar plates containing 4% glycine. A sterilized Whatman filter paper no. 40 soaked in 2% of Na_2CO_3 in 0.5% picric acid solution was placed on the lid of Petri plates and sealed with paraffin and incubated at 37°C for 7 days. When the color of the filter paper changed from yellow to orange, it indicated that the rhizobacterial cultures were producing HCN. No change in color showed absence of HCN production.[28]

Siderophore Production

The Chrome azurol S (CAS) agar plate method was used to detect the siderophore formation. The 24 hours fresh rhizobacterial cultures were inoculated on CAS agar plates and incubated at 30°C temperature for 5-6 days. Siderophore production was shown by the cultures that had an orange zone surrounding the colony. This assay was based on the ability of siderophore to bind with ferric acid with high affinity.[29]

Effect of Selected Rhizobacteria on Seed Germination and Seedling Growth of Chickpea

The seeds of chickpea (Ujjain local kanta chana variety) were used for germination test and they were procured from local market of Ujjain, Madhya Pradesh. Quality of the seeds was checked by observing physical appearance and uniform healthy seeds were selected for experiment. The seeds were surface sterilized with 0.1% $HgCl_2$ for two minutes then they were washed three times with sterile distilled water. A 24 hours old bacterial culture diluted in 1:10 ratio with sterile distilled water was used for seed treatment. Surface sterilized chickpea seeds were soaked in bacterial suspension for 30 minutes and five seeds were placed on moistened filter paper in sterilized Petri plate with equal spacing and incubated at 28 ± 2°C for 5 days. After 48 hours percentage germination was recorded and after 5 days root length, shoot length and dry weights of root and shoot were recorded. Vigor index was calculated using the following formula.[30]

Vigor index = Seed germination (%) x [Mean Root Length + Mean Shoot Length]

Seeds treated with distilled water served as control and this experiment was performed in triplicates.[26]

Statistical Analysis

To evaluate the significance of the results, statistical analysis was performed on the seedling growth data. The experiment was conducted in triplicates and the findings were presented as mean ± standard deviation (SD). The mean value at $p \leq 0.01$ was considered significant.

Molecular Characterization of Rhizobacterial Culture B67 by 16S rRNA Gene Sequencing

The molecular characterization of rhizobacterial culture B67 was conducted through 16S rRNA gene sequencing to determine its taxonomic identity. The gene sequencing of rhizospheric bacteria was performed by Agharkar Research Institute (ARI), Pune, India. A total genomic DNA was extracted by GeneElute Genomic DNA isolation kit (Sigma, USA) and 16S rRNA gene was amplified by polymerase chain reaction (PCR) using 27F and 1492R primers. The PCR product was purified using a Magnetic bead-based method to ensure high-quality sequencing. Sequencing was carried out using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained sequence was analyzed and compared with reference sequences in the NCBI database to determine sequence identity and phylogenetic affiliation.

Results

Isolation and Characterization of Rhizobacterial Cultures

Five rhizobacterial cultures were successfully isolated from the rhizospehric soil samples. The morphological characterization revealed that isolates B33, B65 and B26 have white colour colonies, B67 has yellow colour colonies and B168 has orange colour colonies in old cultures and light-yellow colour in fresh colonies. All isolates show circular colonies and smooth margins on Nutrient agar plates while elevations of the colonies varied, B168 and B67 have convex elevation, B33 and B65 have drop like elevation while isolate B26 has raised elevation.

It was observed that four cultures (B33, B168, B65 and B26) were gram positive and one culture (B67) was gram negative. All rhizobacterial cultures were cocci in shape. Culture B67 tested positive for the Simmon's citrate test, but showed negative results in the MR test, VP test, Nitrate broth test, Motility test, TSI test, Sugar fermentation test and Phenylalanine test. Culture B33 showed negative reactions in all biochemical tests. Culture B168 gave positive result for the Nitrate broth test and was motile. B65 was MR positive and motile, while B26 was MR positive, VP positive and Nitrate broth test positive. It was also motile and gave a positive result in the Phenylalanine test. The results of the biochemical reactions are shown in Table 1.

Table 1: Biochemical characteristics of rhizobacterial cultures

S. No.	Name of culture	MR Test	VP Test	Nitrate broth test	Motility test	Simmon citrate test	TS test	Sugar fermentation			Phenyla -lanine test
								Lactose fermen tation	Mannitol fermen tation	Sorbitol fermen tation	
1.	B33	-	-	-	-	-	-	-	-	-	-
2.	B168	-	-	+	+	-	-	-	-	-	-
3.	B67	-	-	-	-	+	-	-	-	-	-
4.	B65	+	-	-	+	-	-	-	-	-	-
5.	B26	+	+	+	+	-	-	-	-	-	+

Plant Growth Promoting and Biocontrol Activities of Rhizospheric Bacteria

The plant growth-promoting and biocontrol activities of all the five cultures were determined and the results of the primary and secondary screenings are shown in Table 3.

Table 3: Plant growth promoting and biocontrol activities of different rhizobacterial cultures

S. No.	Name of culture	Plant growth promoting and biocontrol activities			
		Primary screening		Secondary screening	
		Auxin production	Ammonia production	HCN production	Siderophore production
1.	B33	+	-	-	-
2.	B168	+	-	-	+
3.	B67	+++	++++	++++	+
4.	B65	++	-	-	-
5.	B26	++	-	-	+

The results of the primary screening showed that, all the cultures exhibited the positive results of auxin production in which highest production was seen in B67, cultures B65 and B26 showed moderate auxin production, whereas cultures B33 and B168 showed the lowest production. Similarly, ammonia production was also tested and the results showed that only B67 exhibited the positive result during primary screening. The results of the secondary screening showed that the siderophore production was detected in three cultures (B67, B168 and B26) and two cultures B65 and B33 were not able to produce siderophore. The HCN production was only observed in B67 and all the four cultures showed the negative results. So, these results indicate that the isolate B67 is the most effective rhizobacteria for the plant growth promoting as well as biocontrol applications.

Effect of Selected Rhizospheric Bacteria on Seed Germination and Seedling Growth

The effect of selected plant growth-promoting rhizobacteria (PGPR) namely B67 on seed germination and seedling growth of chickpea seeds was evaluated using Petri plate assays. Several growth parameters were analyzed, including germination percentage, root and shoot length, dry weight and vigour index. The results showed that the germination percentage of control seeds was 80% whereas seeds treated with B67 exhibited 90% germination rate, indicating a positive influence on seed germination. The statistical analysis showed a highly significant difference ($p \leq 0.01$) between the treated and control groups, as presented in Table 4 and Figure 1.

In terms of root growth, the control seeds had a mean root length of 1.70 ± 0.69cm while eeds treated with B67 showed improved root growth, with mean root lengths of 3.84 ± 1.17 cm. The treatment with B67 demonstrated the significant improvement in root growth.

Similarly, shoot growth analysis revealed that control seeds had a mean shoot length of 1.93 ± 0.50 cm, whereas seeds treated with B67 had mean shoot lengths of 3.82 ± 0.66 cm. These findings confirm that B67 significantly enhances root and shoot growth, further establishing its role in promoting seedling development.

Table 4: Effect of rhizobacterial culture on germination and seedling of chickpea

S. No.	Name of culture	Percentage germination (%)	Effect on root growth		Effect on shoot growth		Vigor Index
			Root length (cm)	Dry weight of root (mg/ 5 seeds)	Shoot length (cm)	Dry weight of shoot (mg/ 5 seeds)	
01.	Control	80	1.70 ± 0.69	37 ± 1.52	1.93 ± 0.50	24 ± 0.7	290.4 ± 68.22
02.	B67	90	3.84 ± 1.17	43 ± 1.00	3.82 ± 0.66	77 ± 1.7	689.4 ± 120.92

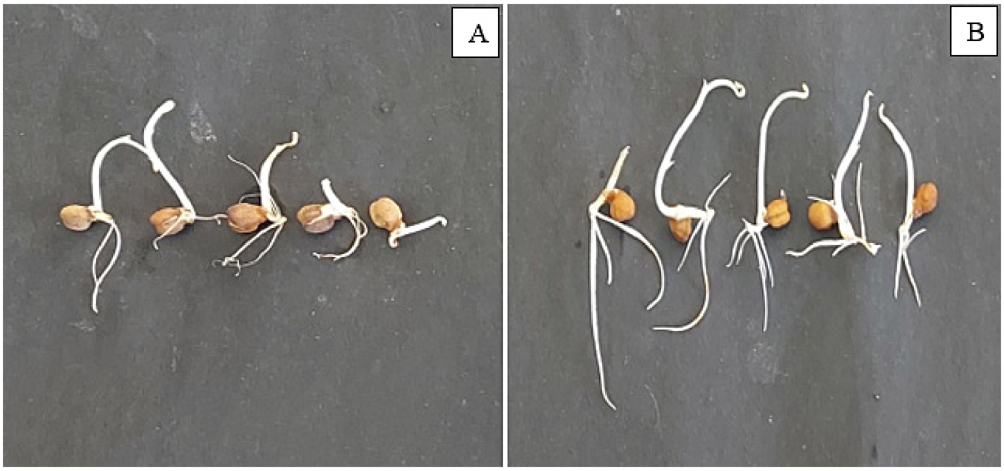

Fig. 1: Effect of plant growth promoting rhizobacteria B67 treatment on germination and seedling of chickpea A. Control B. B67 treatment

The mean dry weight of the control root was 37 ± 1.52 mg per 5 seeds, while seeds treated with B67 had significantly higher root dry weights of 43 ± 1.00 mg per 5 seeds. The higher root dry weight in seeds treated with B67, suggest superior root development. Similarly, the control seeds had a mean shoot dry weight of 24 ± 0.7 mg per 5 seeds, whereas seeds treated with B67 exhibited a significantly higher shoot dry weight of 77 ± 1.7 mg per 5 seeds. These results demonstrate that B67 significantly enhances shoot dry weight, further supporting its role in improving overall plant health.

The vigour index was also positively influences by the bacterial treatment. The vigour index of control seeds was 290.4 ± 68.22, while seeds treated with B67 exhibited the highest vigour index at 689.4 ± 120.92. These results suggest that B67 is the highly effective treatment and significantly enhances seedling growth and vigor compared to the control.

Overall, the results confirm that PGPR, especially B67, positively influences chickpea seed germination and seedling growth, highlighting its potential to improve crop establishment and early developmental stages.

Molecular Characterization of Rhizobacteria B67 by 16S rRNA Gene Sequencing

The isolate B67 was identified as *Pseudomonas parafulva* through 16S rRNA sequencing shown in Figure 2.

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>B67
GAGCGGATGAYGGGAGCTTGCTCCTKGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTG
CCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAG
CAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTTGTTGGTGAGGTAA
TGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGAC
ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCC
AGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTT
GTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGT
GGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGC
TAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGA
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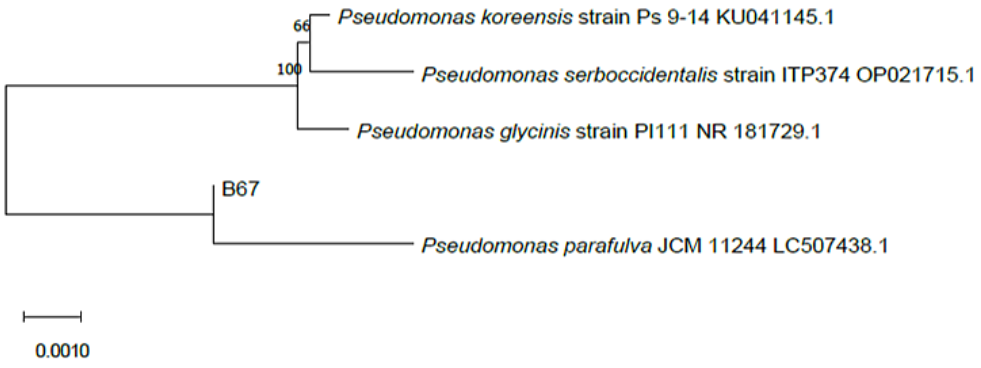

Fig. 2: Molecular characterization of rhizobacterial isolate B67 as *Pseudomonas parafulva*

Discussion

The results of this study highlight the significant potential of rhizobacteria in promoting plant growth and enhancing agricultural productivity. The rhizosphere of *Cicer arietinum* (chickpea) harbors a diverse microbial community of rhizobacteria, many of which exhibit plant growth-promoting characteristics.[31] In this study, both primary and secondary screening techniques were used to identify the most effective rhizobacterial strains with the potential to promote plant growth through different mechanisms.

Ammonia and auxin production were assessed in the primary screening, are key indicators of rhizobacteria that promote plant growth. Ammonia, a product of nitrogen fixation, is essential for enhancing nitrogen availability in the soil, thus improving soil fertility and plant growth.[32] In addition, auxins like indole-3-acetic acid (IAA), play a crucial role in root elongation and development, which can significantly influence nutrient uptake and overall plant health.33 The high levels of auxin production observed in *Pseudomonas parafulva* (B67) suggest that this strain has the potential to enhance root development and overall plant growth, a finding consistent with previous studies that highlight the auxin-producing ability of *Pseudomonas* species.[34,35]

Secondary screening focused on biocontrol activities, specifically siderophore and hydrogen cyanide (HCN) production. Siderophores are iron chelating compounds that increase iron availability in nutrient deficient soils promoting plant growth.[31] The siderophore-producing ability of isolate B67 further enhances its role in promoting plant growth by alleviating nutrient stress. Additionally, HCN production by *P. parafulva* contributes to its biocontrol activity by inhibiting the growth of soil-borne pathogens, a well-documented feature of *Pseudomonas* species.[36] The ability of B67 to produce both siderophores and HCN suggests its dual role as a plant growth promoter and a biocontrol agent.

The inoculation of chickpea seeds with *P. parafulva* (B67) resulted in improved seed germination, seedling growth and root development, which aligns with previous research showing that *Pseudomonas* strains can significantly enhance plant growth by various mechanisms.[33]

Molecular identification of isolate B67 using 16S rRNA gene sequencing confirmed its identity as *Pseudomonas parafulva*, a species known for its multifunctional plant growth-promoting and biocontrol capabilities, including nitrogen fixation, auxin production, siderophore synthesis and HCN production. These plant growth-promoting traits highlight its potential as an eco-friendly alternative to chemical fertilizers and synthetic pesticides, offering a sustainable solution for agricultural productivity.

Indigenous PGPR, such as *P. parafulva* play a vital role in sustainable agriculture by enhancing soil fertility, promoting plant growth, protecting crops from pathogens and improving stress tolerance. Their natural adaptation to local soil and environmental conditions makes them an effective alternative to chemical inputs, contributing to climate-resilient and environmentally sustainable farming. Future research and large-scale application of native PGPR bioformulations could significantly enhance agricultural productivity while maintaining soil health and ecological balance.[19,21,37,38]

In this study, *P. parafulva* has been isolated from agricultural fields in the Mandsaur district of Madhya Pradesh, specifically in the Malwa region. Given its adaptability to this region, it has potential for use in sustainable agricultural practices to enhance crop productivity. However, further studies are needed to evaluate its adaptability to other environmental conditions and its effects on different plant species.

Conclusion

In the present research, a total of five rhizobacterial cultures were isolated and characterized for their potential in plant growth promotion and biocontrol. Among them, isolate B67 was identified as *Pseudomonas parafulva* through 16S rRNA gene sequencing, exhibited the plant growth promoting activities. It produces high amounts of auxin, ammonia, HCN and siderophores, suggesting its potential as both plant growth enhancer and biocontrol agent.

When tested on chickpea seeds, treatment with *P. parafulva* (B67) significantly enhanced germination, root and shoot length and dry weight of roots and shoots, indicating its ability to promote seedling vigor. These results suggest that *P. parafulva* (B67) has great potential for use in agricultural applications to enhance plant growth and to provide biocontrol against plant pathogens especially in Malwa region of Madhya Pradesh. Thus, *Pseudomonas parafulva* (B67) could be developed as an effective microbial inoculant for improving crop productivity and sustainability in agricultural fields. Future studies should explore its adaptability across different environmental conditions and its efficacy on various crops to maximize its potential benefits in sustainable agriculture.

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Conflict of Interest
The authors do not have any conflict of interest.

Data Availability Statement
The manuscript incorporates all datasets produced or examined throughout this research study.

Ethical Statement
This research did not involve human participation, animal subjects or any material that requires ethical approval.

Author Contribution

- **Noureen Qureshi:** Data collection, experiment performing, analysis of data, compilation of the results and writing original draft.
- **Alka Vyas:** Supervision, visualization, review and editing.
- **Harsh Vyas:** Visualization, analysis and review

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