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IDENTIFICATION AND GROWTH PROMOTION EFFECT OF RHIZOSPHERE PROBIOTICS IN KIWIFRUIT

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Abstract

This study assessed rhizosphere probiotics' isolation, identification, and growth-promoting effects in kiwifruit. Kiwifruit seedlings were collected from diverse fields and transported to the lab under sterile conditions. Rhizosphere soil was air-dried, weighed (1g), and serially diluted for bacterial isolation at 27°C for 48 hours. The isolates displayed circular, greenish-yellow colonies and were all Gram-negative. Biochemical tests showed catalase-positive reactions, with variable results for other tests. Dextrose fermentation was observed in all isolates. Among the seven (LPN1–LPN7), LPN5 exhibited strong antifungal activity against *Fusarium solani*, inhibiting 40% of its radial growth after eight days. All isolates produced indole-3-acetic acid (IAA), with LPN6 generating the highest levels. LPN5 demonstrated potential as a bio-inoculant due to its IAA production, phosphate solubilization, HCN, ammonia production, and biocontrol properties, making it promising for bio-fertilization and biological control in kiwifruit and other crops.

Introduction

The term "rhizosphere" is used to describe the soil environment around root system of a plant, which is vital to the plant's growth and sustenance (Majumder *et al.* 2014, Ali *et al.* 2022). Certain strains of *Pseudomonas* have been observed to improve the plant health of many crops, including Kiwi (Almaghrabi *et al.* 2013, Goswami *et al.* 2013, Aarab *et al.* 2015, Kalita *et al.* 2015). They are the most metabolically and functionally varied group of bacteria, and they are the dominating bacterial group in the rhizosphere (Li *et al.* 2021). Rhizosphere interactions between plants and soil microbes may promote, inhibit, vary, or even harm plant development (Mohanram and Kumar 2019).

The rhizosphere of a plant is an essential part of the soil ecosystem since it is where roots and microbes interact. Depending on the microorganisms present, the plant's defence systems, the soil's nutritional state, and the environment, microbial colonisation of the root zone due to parasitic interactions within the host plant (Ahmad *et al.* 2019). Pseudomonads outnumber all other types of bacteria combined and play an important role in ecosystems. *Pseudomonas* strains are also known as plant growth-promoting rhizobacteria due to their ability to secrete auxins, gibberellins, and cytokinins, as well as to solubilize phosphate, potassium, and zinc, and to produce siderophores, HCN, and lytic enzymes. Several isolates of the non-pathogenic rhizobacteria such as *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas aureofaciens* were able to inhibit the growth of soil-borne pathogens thanks to the production of secondary metabolites like siderophore, HCN, protease, and antibiotics like phenazine-1-carboxylic acid (PCA), pyocyan (Almaghrabi *et al.* 2013, Patel *et al.* 2015). Both greenhouse and field-produced kiwies are susceptible to significant illnesses including root rots and wilt caused by soil-borne fungal infections like *Rhizoctonia solani* and *F. solani*. in the warm vegetable-producing zones of the globe. Chemical fungicides are the major tool for eradicating these pests (Savian *et al.* 2020).

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Extreme usage of these chemicals, however, poses risks to both human and environmental health. As a result, it is important to stress the use of non-chemical strategies for preventing and treating plant diseases. The use of rhizobacteria, which aid plant development and have biocontrol capability, might be a viable option. Several researchers have shown that Pseudomonads may be used to prevent illness in Kiwi. As plant growth promoting rhizobacteria (PGPR) has been shown to increase plant Nitrogen and Phosphorus absorption and play a substantial role in the bio-fertilization of crops, it may have considerable ramifications for plant nutrition (Almaghrabi *et al.* 2013, León *et al.* 2015, Patel *et al.* 2015). The rhizosphere of the kiwi plant is teeming with various N_2-fixers, with the most prevalent diazotrophs being members of the Enterobacteraceae and Azotobacteraceae families. There is evidence from studies that inoculating kiwi plants with PGPR strains results in significant improvements in plant height, plant dry weight, root length and weight, yield, leaf area, and the uptake of nitrogen, phosphorus, potassium, iron, zinc, manganese, and copper. The impact of PGPR inoculations on plant development and crop yields is affected by a wide range of abiotic and biotic parameters, such as soil texture, pH, temperature, moisture content, type, amendment, plant nutritional condition, species, age, microbial competition, and predation.

Materials and Methods

Carefully removed from diverse fields, these kiwifruit seedlings were transported to the lab in sterile polythene bags to ensure their continued health and viability. All samples were kept in the fridge at 4°C (Degree Celsius which is 39.2 Fahrenheit) until further analysis. Kiwifruit's rhizospheric soil was extracted and air-dried. Soil was weighed at 1 gram, then diluted serially and plated to grow at 27°C (80.6 Fahrenheit) for 48 hrs. Before being used, pure cultures were kept on slants in a Nutrient agar medium at 5°C (41 Fahrenheit).

Genomic DNA was extracted from the isolated bacterial strains using the DNeasy UltraClean Microbial Kit (QIAGEN, USA) following the manufacturer's protocol. The quality and concentration of extracted DNA were assessed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis. The purity of the DNA was measured based on the A260/A280 ratio, ensuring values between 1.8 and 2.0.

For metagenomic sequencing, the 16S rRNA gene (V3-V4 region) was amplified using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCT TGTTACGACTT-3'). PCR amplification was carried out in a 50 µl reaction mixture containing 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, 1.25 U Taq DNA polymerase (Thermo Fisher Scientific, USA), and 50 ng of template DNA. The PCR conditions were as follows: (i) Initial denaturation: 95°C for 3 min; 2. Denaturation: 95°C for 30 sec. (ii) Annealing: 55°C for 30 sec; (iv) Extension: 72°C for 1 min (v) Final extension: 72°C for 5 min

The PCR product was verified using 1.5% agarose gel electrophoresis and purified using the QIAquick PCR Purification Kit (QIAGEN, USA). Sequencing of the 16S rRNA amplicons was performed using the Illumina MiSeq platform (paired-end 2x250 bp sequencing). The obtained sequences were analyzed using QIIME2 (Quantitative Insights into Microbial Ecology) for taxonomic classification and diversity analysis.

The test culture was spread out thinly over the clean slide and then heat-fixed. Crystal Violet was dripped over the smear for roughly 60 sec. The slide was cleaned using flowing water. We used a lot of Gram's iodine and left the stain for a full minute and a half. Ethyl alcohol (95%) was used in a dropwise fashion to remove the discolouration. Some safranine was poured in for a whole minute and twenty seconds. Wash the slide with water. Take the culture on the sterile slide

and add 3% H_2O_2 for observation of bubble production if the bubble is present then the test is positive.

Each bacterial culture was inoculated into its own autoclaved test tube containing 5 ml of tryptone broth. The uninoculated control tube was kept for further use. Each tube, including the control, received 1 ml of Kovac's reagent after incubation for 48 hrs. After waiting 12-16 min, the tubes were misted. The reagent was able to climb to the top when the tubes were allowed to stand upright.

Five millilitres of Methyl Red-Voges-Proskauer (MRVP) broth were placed in each vial, and then they were all autoclaved. Two uninoculated test tubes were saved for later use as a reference. For 48 hrs, the test tubes were kept at 27°C. Each test tube, including the control, received six drops of MR indicator, and the resulting colour change was recorded. For the subsequent test tubes, including the control, we added 10 drops of VP (Voges-Proskauer) reagent I and 4-5 drops of VP-II reagent to the incubator. There was a noticeable difference in tube colour between the test samples and the control group.

A plan to make Simmon's citrate agar slants and streak them with culture was created. Test tubes were held at 27°C for 48 hrs. It was shown that colours may be affected by the angle of view. After 48 hrs of growth at 29°C, plates of starch agar were inoculated with thriving cultures. Iodine solution was dropped onto plates with viable bacteria growth and left there for 30 sec. The unused iodine solution was drained. Observed the results in the presence of zone. Tubes were loaded with a broth mixture that included 0.5% of four different sugars. Durham tube was contained in each of the tubes. The test tubes were kept in an incubator at 27°C for a full day. A change in colour or the formation of bubbles indicated the possible presence of acid or gas, so the tubes were examined further.

The process of making IAAs (indole-3-acetic acid) L-Tryptophan (0.1 g/l) in nutritional broth was used to inject 13 bacterial cultures. Cultures were centrifuged at 5°C for 15 min at 10000 rpm while developing exponentially. The supernatant was treated with Salkowski reagent, and the appearance of a pink colour confirmed the presence of IAA, following the method as described by Walpola *et al*. (2013). Phosphorus-solubilizing abilities of isolates were evaluated by plating them on Pikovskaya's agar. There were clearing zones after just three days at 27.2°C. By using the method outlined by Bapiri *et al.* (2012) the microorganisms were able to solubilize the zinc. Tris-minimal medium plates supplemented with zinc phosphate and bromophenol blue were used to cultivate the bacteria. After about a week of incubation at 27°C, the colonies on inoculated plates were surrounded by a clear zone caused by the bacterial solubilization of inorganic zinc.

Quantifying HCN output was done using a method by Bakker and Schippers. Isolates were streaked onto solid agar plates containing 4.4 g glycine/l and covered with filter paper soaked in 0.5% picric acid in 1% Na_2CO_3. Plates were wrapped in parafilm. The production of HCN was measured by colour change from white to brown. Ammonia production was tested by inoculating bacterial strains in peptone water and adding Nessler's reagent.

Each *Pseudomonas* isolate was tested for its ability to combat *Fusarium solani*. On a PDA plate incubated at 27°C, the development of test pathogens was stifled by *Pseudomonas* spp. LPN5. Increasing incubation time resulted in higher antifungal activity.

Seven distinct bacterial strains (LPN 1-7) were cultured in nutrient broth for 48 hrs on a shaker at 27.2°C. At 5°C, each culture was centrifuged for 16 min at 8000 rpm. Pellets were washed and resuspended in sterile distilled water to achieve a final concentration of $1x10^8$ cells/ml. Seeds were coated with bacterial suspensions and placed in pots with sterilized garden soil. Germination rate, root length, shoot length, root weight, and shoot weight were recorded up to 21 DAS (Days after Sowing).

Results and Discussion

From kiwifruits, rhizobacteria were extracted using the serial dilution technique using King's medium. All *Pseudomonas* isolates (LPN1-LPN7) were rod-shaped with round colonies. Colony colour varied among isolates, ranging from yellow-green, yellow, green, light green, dark green, to pink (Table 1). Growth rates were categorized as either fast (LPN1-3, LPN7) or slow (LPN4-6), as shown in Table 1. All *Pseudomonas* isolates (LPN1-LPN7) were identified as Gram-negative and exhibited catalase positivity. The seven Pseudomonas isolates (LPN1–LPN7) were subjected to standard biochemical tests for identification. All isolates tested negative for the Methyl Red, Indole, and Voges-Proskauer tests, while they were positive for Citrate utilization, Urease activity, and Starch hydrolysis. In terms of carbohydrate fermentation, all isolates metabolized Dextrose except LPN3 which did not isolates, while varying results were observed for Sucrose, Mannitol, and Lactose fermentation. Notably, LPN6 was the only isolate that could ferment all tested sugars, while LPN5 was unable to utilize Sucrose and Mannitol (Table 2). It was discovered that IAA was produced by all seven *Pseudomonas* spp. (Table 3). The highest levels of IAA generation were discovered in LPN6, which showed up as the pinkest colour.

Table 1. Morphological characterization of Pseudomonas species from the kiwifruit root zone.

LPN	Anatomy of cell	Colonial Societies and Their Subtypes	Development	Colour
1	Rod	Round	Fast	Yellow green
2	Rod	Round	Fast	Yellow
3	Rod	Round	Fast	Green
4	Rod	Round	Slow	Light green
5	Rod	Round	Slow	Dark green
6	Rod	Round	Slow	Pink
7	Rod	Round	Fast	Yellow green

LPN: Labelled *Pseudomonas* Number.

Table 2. Biochemical Characterization of *Pseudomonas* spp. from the Kiwi root zone.

LPN	MR test	Indole test	VP test	Citrate Use test	Urease test	Starch hydrolysis	Sucrose	Mannitol	Dextrose	Lactose
1	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
2	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve
3	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
4	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
5	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve
6	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
7	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve

LPN: Labelled *Pseudomonas* Number, MR: Methyl Red, VP: Voges-Proskauer.

On Pikovskaya's agar, all the isolates showed the ability to produce distinct haloes surrounding the spot inoculation. Such phosphate-clearing bacterial zones demonstrated phosphate solubilization capabilities (Table 3). No halo zones appeared surrounding colonies, indicating that no isolates could solubilize zinc (Table 3). Colour change in the filter paper indicated that all *Pseudomonas* spp. isolates, except LPN4, generated HCN. The dark brown hue of the filter paper indicates that the most HCN was generated by LPN5 (Table 3). Ammonia was also generated by all of the isolates, as shown by the presence of yellowish-brown precipitates in peptone broth

(Table 3). Each Pseudomonas isolate was tested for its ability to combat *F. solani*. On a PDA plate incubated at 27°C, the development of test pathogens was stifled by *Pseudomonas* spp. LPN5. Increasing the incubation time resulted in a higher level of antifungal activity. After 8 days in the incubator LPN5 was able to block 40% of radial growth of *F. solani*, as determined by recording the average percentage of inhibition (Table 3).

Table 3. Growth promotion and antagonism of *Pseudomonas* spp. from kiwifruit roots.

LPN	Production of IAA	Solution of phosphate	Solubilization of Zinc	Production of HCN	Ammonia Production[E]	Antagonistc against *F. solani*[F]
1	+ve effect	+ve effect	-ve effect	+ve effect	+ve effect	-ve effect
2	+ve effect	+ve effect	-ve effect	+ve effect	+ve effect	-ve effect
3	+ve effect	+ve effect	-ve effect	-ve effect	+ve effect	-ve effect
4	+ve effect	+ve effect	-ve effect	+ve effect	+ve effect	-ve effect
5	+ve effect	+ve effect	-ve effect	+ve effect	+ve effect	-ve effect
6	+ve effect	+ve effect	-ve effect	+ve effect	+ve effect	-ve effect
7	+ve effect	+ve effect	-ve effect	+ve effect	+ve effect	-ve effect

LPN: Labelled *Pseudomonas* Number, HCN: Hydrogen Cyanide.

Pseudomonas spp. isolates were found in kiwi seeds of a consistent size and shape (LPN1 to LPN7). Seeds bacterized with the aforementioned microbial inoculants showed induced vegetative characteristics 22 days after planting in containers (Table 4). LPN5 was shown to be the most effective in encouraging seed germination, plant growth, and root formation. The highest yields were observed in plants treated with *Pseudomonas* spp. In the control group, only 40% of seeds germinated. However, when compared to the control group, all seeds treated with Pseudomonas spp. showed statistically significant improvements in germination, growth, and development, with a significance level of 5%. Among the isolates, LPN5 showed the highest germination rate (Table 4).

Table 4. Growth and germination of kiwifruit seeds treated with *Pseudomonas* spp. (22 days).

	LPN						
	1	2	3	4	5	6	7
Root length (cm)	5.11	5.03	4.98	4.89	5.12	4.91	4.99
Shoot length (cm)	5.01	4.77	4.87	4.67	5.33	5.12	4.66
Seed germination (%)	65.88	65.74	62.58	75.85	77.95	71.98	69.85
Root weight (g)							
Fresh wt.	0.022	0.023	0.025	0.019	0.017	0.022	0.025
Dry wt.	0.011	0.014	0.010	0.011	0.015	0.014	0.017
Shoot weight (g)							
Fresh wt.	0.33	0.28	0.24	0.41	0.35	0.37	0.25
Dry wt.	0.24	0.22	0.21	0.33	0.27	0.28	0.19

This study represents the ongoing efforts to isolate and purify PGPR from the rhizosphere soil of kiwi plants. As root exudates provide a wealth of nutrients, the rhizosphere is an ideal habitat for soil microorganisms. The community of microorganisms within the rhizosphere of kiwi plants

is distinct and shaped by the specific physical and ecological conditions of the soil environment. Ouf *et al.* (2023) used King's medium to extract the same 10 strains of fluorescent pseudomonads from the soil around the rhizospheres of maize, rice, and bajra. From the soybean rhizosphere in Plumbon, Cirebon, Indonesia, 115 Pseudomonas spp. Isolates were collected by researchers led by Sharma *et al.* (2022) and Ouf *et al.* (2023). All of the samples tested positive for catalase, citrate utilisation, urease production, and starch hydrolysis but negative for indole synthesis and MRVP. The isolates were all Gram-negative rods. IAA was demonstrated to be produced by all investigated strains. The study of Kamble and Galerao (2015) reported the finding that various *Pseudomonas* species isolated from the rhizosphere of garden plants in Amravati, Maharashtra, produce IAA. It is believed that bacteria responsible for making IAA promote root growth and plant development (Ahmed and Hasnain 2014). Solubilization of phosphate by bacterial isolates is linked to the generation of organic acids such as gluconic, acetic, lactic, fumaric, and succinic acid. Soil pH drops as organic acids are broken down, with H^+ replacing Ca^{2+} and releasing HPO_4^{2-} into the soil solution. The author said that the predominant phosphate solubilizers were Pseudomonas and Bacillus (Singh 2022). Corn's growth and grain output were reported to have increased after being inoculated with phosphate-solubilizing bacteria, and the prices of fertilizer and emissions of greenhouse gases were also lowered (Nkebiwe *et al.* 2016). Seventy percent of the isolates found by Kaur and Sharma (2013) were able to increase chickpea growth by solubilizing phosphate at concentrations ranging from 5.08 to 13.45 mg/100 ml.

Except for LPN3, all of the isolates that turned yellow filter paper orange-brown were deemed to be HCN producers. According to research by Ramadan *et al.* (2016), many plant-associated fluorescent pseudomonads use HCN as a broad-spectrum antibacterial agent for the biological control of root infections. All of the samples isolated here were confirmed to be high ammonia producers. Ammonia was shown to be produced by 95% of *Bacillus* isolates and 94% of *Pseudomonas* isolates (Noumavo *et al.* 2018). Only LPN5 was shown to suppress the development of *Fusarium solani* in vitro testing of this study. It also found that *Pseudomonas* spp. had antifungal activity against *Fusarium solani.* Jabeen *et al.* (2020) observed that both *P. aeruginosa* and *P. lilacinus*, either used alone or in combination, significantly reduced *F. solani*. The root length, shoot length, fresh and dry weight of root and shoot, and total plant weight were increased significantly using kiwi seeds bacterized with *Pseudomonas* sp. LPN5 (Table 4). The findings are consistent with those of other authors who found that inoculating wheat seed with *P. fluorescens* NCIM 5096 increased germination by 10%. It also found that pre-treatment of rice seedlings with PGPR isolates improved germination (Jabeen *et al.* 2020).

This study highlights the potential of *Pseudomonas* spp. as plant growth-promoting rhizobacteria (PGPR) in kiwifruit cultivation. The isolates exhibited key traits such as indole-3-acetic acid (IAA) production, phosphate solubilization, and HCN generation, contributing to enhanced plant growth and biocontrol against *F. solani*. Among the tested isolates, LPN5 showed the highest antifungal activity and plant growth enhancement, making it a promising bio-inoculant. These findings support the use of *Pseudomonas* spp. as an eco-friendly alternative to chemical fertilizers and fungicides, promoting sustainable agriculture and soil health.

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