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Effect of Plant Growth Promoting Endophytic Bacteria on Rice Seedling Growth and Development

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Abstract

Bacterial endophytes were isolated from the seeds of traditional rice varieties from Tamil Nadu, specifically Arupatham Kuruvai (AK), Karuppu Kavuni (KK), and Kalanamak (KN1 and KN2), with an emphasis on their potential for vertical transmission across generations. The isolated strains were identified through 16S rRNA sequencing as *Stenotrophomonas rhizophila* (AK), *Pseudomonas* sp. (KK), *Stenotrophomonas* sp. (KN1), and *Pseudomonas reactans* (KN2). Various plant growth-promoting traits, including Indole Acetic Acid (IAA) production, hydrogen cyanide synthesis, siderophore production, phosphate solubilization, ammonia production, nitrogen fixation ability, and strain compatibility were assessed to identify the most effective strain for field trials. Additionally, the antagonistic activity of these strains was tested against bacterial pathogens (*Xanthomonas oryzae* pv. *oryzae*, *Bacillus subtilis, Escherichia coli*) and fungal pathogens (*Macrophomina phaseolina*, *Fusarium oxysporum* and *Colletotrichum* sp), with inhibition percentages recorded. Bioassay studies conducted using the blotter method on popular rice variety BPT 5204 and RNR 15048 revealed distinct growth patterns in inoculated seeds compared to controls. The findings demonstrate that the effectiveness of bacterial endophytes in promoting rice seedling growth varies significantly, highlighting their potential as natural bio-inoculants for sustainable rice cultivation.

Key words : Bacterial endophytes, *Pseudomonas* sp., *Stenotrophomonas rhizophila*, growth promotion.

Rice is the world's third most cultivated cereal crop, with its primary production centres in countries such as India, China, Indonesia, the Philippines, Thailand, Bangladesh, Vietnam, and Pakistan[31]. Meeting the increasing demand for rice production to sustain a growing global

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population is a critical challenge, particularly for developing nations like India. Research efforts continue to focus on enhancing rice yield through various agronomic practices, including the application of chemical fertilizers. However, balancing high productivity with environmental sustainability remains a significant challenge for researchers and plant breeders, necessitating innovative and eco-friendly solutions for sustainable rice cultivation. Thus, the development and adoption of eco-friendly approaches to maximize rice production are essential for long-term agricultural viability.

Bacterial endophytes are microorganisms residing in the inner parts of the various plant parts like root, stem, flowers, fruits and leaf, offer resistance to the host against several biotic and abiotic stress conditions[37,42]. Seed endophytes own the potential to get transferred vertically from one generation to another[9,13,25,39]. Endophytic plant growth promoting traits enable plants to utilize insoluble nutrients available in the soil by favoring better absorption to promote growth. Bacterial seed endophytes from crop plants such as rice, wheat, cotton, corn and tomato facilitate plant development and seedling establishment[11,15,39,41]. *Bacillus* sp. and *Pseudomonas* sp. are the most common genera observed as endophytes in several crop plants[38]. Benefits of bacterial endophytes are numerous as they enhance the growth of the plant by suppressing harmful phytopathogens and offer better resistance against the invasion of fungi, nematodes, insects and viruses[23].

Traditional rice varieties exhibit a range of beneficial traits, such as resistance to pests and pathogens, resilience to climatic changes, enhanced yield, and antimicrobial and medicinal properties. These properties are primarily attributed to the phytochemicals present in the plants, which provide natural protection against environmental stressors[24]. Additionally, the endophytes residing within the plant tissues are believed to play a crucial role in these protective mechanisms, possibly transferring beneficial traits to their host plants[1]. Given these advantages, there has been a growing interest in isolating bacterial endophytes from traditional rice varieties and characterizing them for their potential use as bioinoculants. These bioinoculants could be applied to popular rice varieties to enhance their growth and yield, offering a sustainable alternative to chemical fertilizers and pesticides[33]. Application of such potential bacterial endophytic strains will help to conquer and improve performance level of the crop plants while minimizing the continuous utilization of chemical pesticides that deplete soil microbiome which leads to environmental degradation and soil pollution[30].

In the current study, three such traditional rice varieties from Tamil Nadu, namely Arupatham Kuruvai (AK), Karuppu Kavuni (KK) and Kalanamak (KN) were selected owing to their remarkable adaptability to climate change, nutritional profile and medicinal properties. The main objective of the study was to isolate endophytic bacterial strains from these traditional rice varieties and utilize the potential strains to improve the yield and overall growth of the local variety rice plants BPT 5204 and RNR 15048.

Sample Collection :

Seeds of traditional rice cultivars are collected from Swami Dayananda Educational

trust farm, Manjakudi and Valanadu Substainable Agriculture Producer Company Limited, Sirkazhi, Mailaduthurai district. Similarly local rice variety seeds BPT 5204 and RNR 15048 were obtained from TANSEDA, Arakkonam. These seeds were stored at 4ºC until future use.

Isolation of Bacterial Endophytes :

Seeds of traditional rice cultivars namely Arupatham Kuruvai (AK), Karuppu Kavuni (KK), Kalanamak (KN) were washed in running tap water to remove the surface contaminants. Seeds were dipped in 70% ethanol for 30 seconds and immediately rinsed twice with sterile distilled water. The seeds were then immersed in 2% sodium hypochlorite for 5 minutes and washed twice with sterile distilled water. All the seeds were blot dried in sterile filter papers. Once the process of surface sterilization is done, seeds were laid over nutrient agar plates and observed for isolation of endophytic strains[6]. Bacterial cells are streaked on plates to appear as individual colonies. Isolated colonies were named and stored in nutrient agar slants for further studies.

Identification of Endophytic Bacterial Isolates :

DNA Extraction :

DNA extraction from the isolated endophytic bacterial strains was performed following the methodology of Sharma and Singh[34]. The extracted endophytic bacterial DNA from traditional rice seeds was then processed for PCR amplification.

16s r RNA sequencing: PCR Amplification Mix:

The amplified DNA was then sequenced to confirm the identity of the isolates after purification. The total volume of the PCR amplification mix was 25 µL. The following universal primers were used for PCR amplification: 27F: 5' AGAGTTTGATCC-TGGCTCAG 3' and 1492R: 5' GGTTACCTTGTTACGACTT 3'. The reaction mixture consisted of forward primer (1 µL), reverse primer (1 µL), Isolated template DNA (1 µL), Master mix (12.5 µL) and Sterile water (9.5 µL). After PCR amplification, the sequenced isolates were identified and deposited in BANKIT-GENBANK by comparing them with the available NCBI database. The identified strains and their corresponding GenBank accession numbers were *Stenotrophomonas rhizophila*-AK PP938938, *Pseudomonas* sp KK - PP938939, *Stenotrophomonas* sp KN1-PP938940 and *Pseudomonas reactans* KN2-PP938941 (Table-1).

Plant Growth Promoting Traits of Endophytic Bacterial Isolates :

The isolated endophytic strains were maintained in nutrient broth at 28 ± 2ºC and 18 h cultures were used for studying the plant growth promoting characters. The strains were characterized based on their ability to produce indole acetic acid (IAA)[4], ammonia[5], Siderophore[32], hydrogen cyanide[20] and solubilization of Phosphate[10].

Nitrogen Fixation Activity :

Twenty-four-hour cultures grown in nutrient broth were streaked onto Ashby's nitrogen free mannitol Agar medium. The growth of the cultures on this medium indicated their ability to fix nitrogen, classifying them as

nitrogen-fixing (nitrofixer) isolates[2].

Antimicrobial Activity :

Antimicrobial activity of the isolated bacterial endophytes was evaluated against bacterial and fungal pathogens using nutrient agar and potato dextrose agar medium. Antibacterial activity was proceeded with *Bacillus subtilis., Escherichia coli*, *Xanthomonas oryzae* pv. *oryzae* using agar spot assay[14]. Freshly inoculated bacterial cultures were used for the assay, and the zone of inhibition was measured after 24 h to assess antibacterial efficacy. Antifungal activity was carried out with *Macrophomina phaseolina*, *Colletotrichum* sp. and *Fusarium oxysporum* using dual culture method and the results were recorded after 7 days and the percentage inhibition was calculated[22].

Bioassay Germination Studies :

In the current study, two local rice varieties, BPT 5204 and RNR 15048, were used to analyze the effects of bacterial endophytes for growth promotion. Growth parameters were evaluated by inoculating each variety with endophytic bacterial strains (AK, KK, KN1, and KN2) using blotter method via seed dipping for two hours[7]. Following inoculation, the seeds were placed in petriplates, Germination percentage and growth parameters like root length and shoot length were recorded at five-day intervals until 10 days after sowing (DAS). The performance of endophytes varied between varieties, and within each variety, different strains exhibited distinct growth patterns and the results were statistically analyzed with one way ANOVA using Microsoft Excel 2010.

Compatibility Test between the Endophytic Bacterial Strains :

The compatibility test was conducted to evaluate how well the isolated endophytic bacterial strains interact with one another so that they can be used as a consortium to promote synergistic effect on growth promotion in tested rice cultivars. This test helps in assessing the cooperative, mutualistic, or antagonistic behaviour among the isolates, as described by Batra *et al.,*[3]

Plant Growth Promoting Traits of Endophytic bacterial strains :

Bacterial endophytes employ multiple mechanisms to promote crop plant growth. Quantitative analysis of IAA production by endophytic bacterial isolates, with and without tryptophan supplementation, revealed that all four isolates are capable of producing IAA and the presence of tryptophan as a precursor significantly influenced the production levels compared to those without supplementation. IAA was found to be maximum in KN1 with 4.83 µg/mL followed by KN2 (3.51 µg/mL) and AK (1.73 µg/mL) and the least among the isolated strains was recorded with KK (1.69 µg/mL). Findings were in accordance with the study conducted by Khianngam *et al.,*[18] where endophytic bacterial strains namely *Enterobacter hormaechei* and *Bacillus aryabhattai* promoted the growth of rice plants gradually and could be a better replacement for synthetic IAA. In a study by Herlina *et al.,*[12] sixteen bacterial endophytes from groundnut were capable of producing IAA as they enhanced the growth of lateral roots profusely in mungbean and also support in better nutrient absorption.

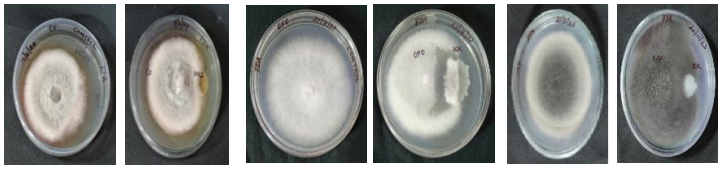
Ammonia production, phosphate solubilization, hydrogen cyanide (HCN) production and siderophore production were tested for all the four endophytic bacterial strains. Ammonia production and hydrogen cyanide was found in all the four endophytic bacterial isolates. Ammonia production aids in nitrogen absorption from the soil and HCN production contributed to plant protection by deterring pests and pathogenic attacks. Phosphate solubilization was detected in two strains - *Pseudomonas* sp. (KK) and *Pseudomonas reactans* (KN2). Phosphate solubilisation was qualitatively analysed for their efficiency and Phosphate solubilisation index (PSI) with diameter of clear zone and colony diameter. Phosphate solubilisation efficiency was about 283.3 % and 128.6 % and PSI was about 3.83 and 2.29 for KK and KN2 isolates respectively. Phosphate solubilization plays a crucial role in making inorganic phosphate available to plants, an essential macronutrient for growth promotion. However, siderophore production was not observed in all analysed strains. The combined plant growth-promoting traits of these endophytic bacteria contribute to crop growth by offering protection against various biotic and abiotic stress conditions. According to the results of Fouda *et al.,*[8] fifteen endophytic bacterial strains (PI-1 to PI-15) were isolated from healthy leaves of *Pulicaria incisa*. Among them, four strains demonstrated phosphate solubilization and ammonia production capabilities *Agrobacterium fabrum* PI-1, *Acinetobacter radioresistens* PI-3, *Bacillus cereus* PI-8, and *Bacillus subtilis* PI-10 those were in accordance with our present study. Twenty bacterial endophytes were isolated from the root, stem and leaves of *Chromolaena odorata* and found that not all the strains were capable of producing IAA and Phosphate solubilisation but interestingly found that few strains were producing strong moderate to weak production of the plant growth promoting traits[21] and several insoluble forms of nutrients are available in inorganic forms due to the poor solubility in soil[27]. Thirteen out of thirty two endophytic bacterial isolates from the roots of *Withania somnifera* were capable of hydrogen cyanide production suggests a promising avenue for natural plant protection strategies[19].

Nitrogen Fixation Activity :

Endophytic bacterial isolates KK and KN2 demonstrated growth on mannitol ashby agar, indicating their nitrogen-fixing ability. Similarly nitrogen fixing *Pseudomonas stutzeri* A15 endophytic rhizobacteria influenced the growth of rice seedlings when compared to untreated control[26]. Rice plants are highly efficient nitrogen feeders therefore; the application of bioinoculants capable of nitrogen fixation can serve as a sustainable alternative to chemical nitrogen fertilizers. This approach not only enhances crop growth and yield but also mitigates the environmental impact associated with synthetic fertilizers[29].

Antimicrobial Activity of the Bacterial Endophytes :

In the present study, none of the endophytic bacterial strains demonstrated inhibitory activity against the tested bacterial pathogens, including *Bacillus subtilis*, *Escherichia coli*, and *Xanthomonas oryzae* pv. *oryzae*. The antagonistic potential of four endophytic bacterial isolates was evaluated against three fungal phytopathogens: *Colletotrichum* sp., *Fusarium oxysporum*, and *Macrophomina*



A B C

Fig. 1. Inhibition of mycelial growth by endophytic bacterial isolates against fungal phytopathogens after seven days of co-culture.
A) KN2 against *Colletotrichum* sp B) KK against *Fusarium oxysporum*
C) KK against *Macrophomina phaseolina*

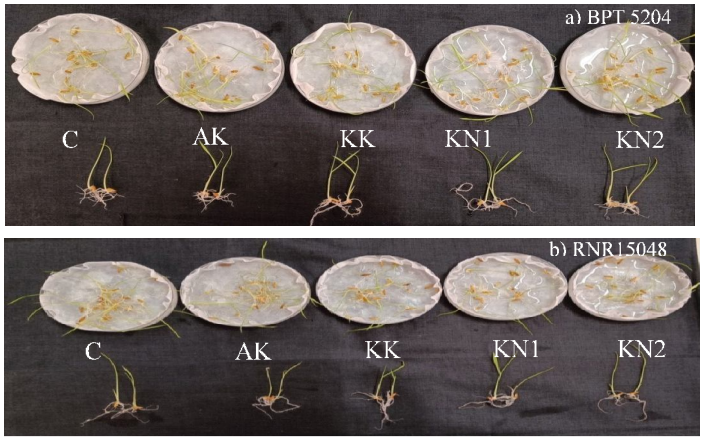


Fig. 2. Effect of bacterial endophytes on local rice varieties a) BPT 5204 and b) RNR 15048 using blotter method

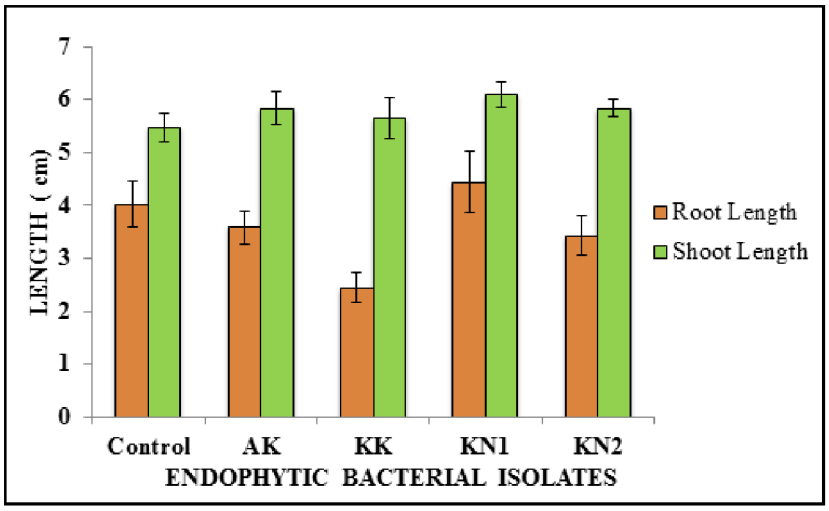
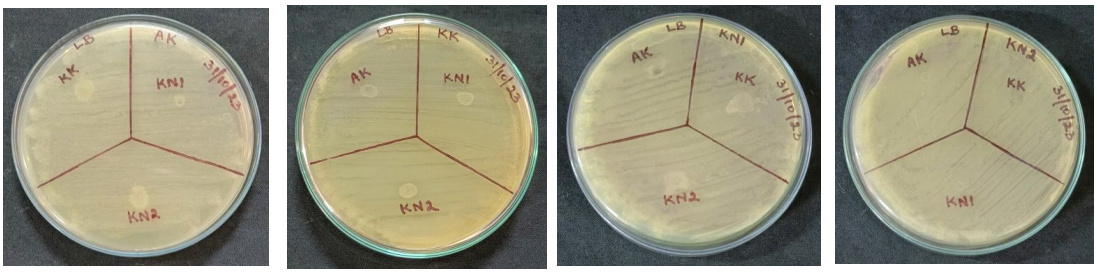


Fig 3: Effect of endophytic bacterial strains on rice variety BPT 5204



a) AK b) KK c) KN1 d) KN2

Fig. 4. Compatibility test between the isolated endophytic bacterial isolates

Table-1. 16s r RNA gene sequencing of endophytic bacterial strains

Plant Part	Source	Identified as	Strain name	Accession no.
Traditional Rice seeds	Endophyte	*Stenotrophomonas rhizophila*	AK	PP938938
Traditional Rice seeds	Endophyte	*Pseudomonas* sp	KK	PP938939
Traditional Rice seeds	Endophyte	*Stenotrophomonas* sp	KN1	PP938940
Traditional Rice seeds	Endophyte	*Pseudomonas reactans*	KN2	PP938941

Table-2. Antimicrobial activity of four endophytic bacteria against fungal phytopathogens.

Fungal pathogens	Endophytic bacterial strains inhibition percentage at 7 days			
	AK	KK	KN1	KN2
Colletotrichum sp	8.22±1.37	17.81±1.37	4.80±0.69	19.87±2.06
Fusarium oxysporum	0	50.59±2.35	0	33.53±0.59
Macrophomina phaseolina	0	27.57±0.65	0	20.51±1.28

phaseolina (Table-2, Fig. 1). Among these isolates, KN2 showed the highest inhibition against *Colletotrichum* sp. (19.87%), followed by KK (17.81%), while KN1 exhibited the lowest inhibition (4.80%). Notably, KK (identified as *Pseudomonas* sp.) and KN2 (identified as *Pseudomonas reactans*) demonstrated significant antagonistic activity against *Fusarium oxysporum* and *Macrophomina phaseolina*. The KK strain showed the highest inhibition, with 50.59% against *Fusarium oxysporum* and 27.57% against *Macrophomina phaseolina*. These findings align with the results of Singh *et al.*,[35] which suggest that endophytic bacterial isolates can inhibit fungal pathogens through the production of hydrogen cyanide (HCN). Furthermore, endophytic strains such as *Bacillus* sp. and *Stenotrophomonas* sp. have been reported to inhibit *Colletotrichum acutatum* and *C. gloeosporioides*, which cause leaf disease in rubber trees[36].

Bioassay Germination Assay :

Germination studies were conducted on two commonly grown rice varieties, BPT 5204 and RNR 15048, to assess the impact of the endophytic bacterial strains (Fig. 2). After five days, the germination percentage (%) was evaluated and found to be higher in all treated strains with 90% for KN1(*Stenotrophomonas* sp.) followed by AK (*S. rhizophila*) and KN2– with 95 %. *Pseudomonas* sp (KK) promoted seed germination in BPT 5204 rice variety up to 97.5 %. The effect of seed germination in RNR 15048 was less when compared to the other variety used in the study. The differential performance by the endophytes in root and shoot enhancement, compared to the control plants, indicates that the effectiveness of these strains varies across different rice varieties. Root and shoot lengths were measured at 5-day intervals up to 10 days after sowing (DAS). In BPT 5204, maximum root and shoot lengths of 3.37 cm and 2.04 cm, respectively, were recorded at 5 Days after sowing (DAS) when treated with *Stenotrophomonas* sp. KN1(Fig. 3). In contrast, the control plants showed significantly lowers growth, with root and shoot lengths of 1.71 cm and 1.63 cm, respectively. After 10th day, the root length in treated plants increased to 4.44 cm, while shoot length was increased to 6.09 cm, when treated with *Stenotrophomonas* sp. Similarly endophytic strains *Bacillus cereus* PI-8 and *B. subtilis* PI -10 when inoculated on seeds of *Zea mays* enhanced the growth of the plants in green house experiment[8]. It is also evident that bacterial endophytes from *Melia azedarach* - *Pseudomonas monteilii*, *Pseudomonas farsensis* and *Cupriavidus* sp. enhance its own growth especially over foliar region[28]. SLB4- *P. fluorescens*, SLB6- *Pseudomonas* sp. and SY1-*Pseudomonas* sp. promoted seedling development, rooting and shooting in rice seedlings and Bermuda grass, when isolated as endophytes from invasive *Phragmites australis*[40]. Similar results were observed with rice plants under salt stress with potent bacterial endophytes from selected plants inhabiting sand dunes at Pohang beach[17]. This study was initiated to check the efficiency of the plant growth promoting endophytic bacteria when applied to rice varieties and check effectiveness of growth promotion with respect to biomass enhancement when compared with control. Statistical analysis revealed that higher F-statistic value than F-critical value for root length indicating significant differences in

growth promoting ability of the varieties tested and the observed effects are real and not due to chance ($P < 0.05$) for the variety BPT 5204. The difference in shoot length among the two varieties was minimal when treated with the bacterial endophytic strains in 10 days of the growth period. However, the subsequent treatment with bacterial isolates might result in the increase of shoot length in the rice plants.

Compatability Test among Endophytic Strains :

Compatibility tests confirmed that all four strains were compatible with one another, as they grew over a lawn without forming a zone of inhibition around the spotted culture (Fig. 4). Compatibility helps the growth of the crop plants when applied as consortia over field trials in our future works which was found in accordance with the findings of James and Mathew[16].

The application of endophytic bacterial strains from traditional rice on widely used rice varieties of Tamil Nadu offers a promising solution for growth improvement as well as an effective alternative strategy to minimize chemical fertilizers thereby enhancing soil quality and fertility. Fostering such environmentally friendly microbes as potential bioinoculants will be a great boon to the agriculture industry.

Conflict of interest

The authors declare that there is no conflict of interest associated with the manuscript.

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