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Bacillus pumilus - A Potent IAA Producing Plant Growth-Promoting Rhizobacteria with *In Vitro* PGP Traits and Antagonism Against *Fusarium equiseti*

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Abstract

Background Plant growth promotion through microbial mediation has garnered increasing focus in sustainable agriculture because of its capacity to enhance agricultural yield as well as resilience. *Bacillus pumilus,* a PGPR, is known for synthesizing key phytohormones such as IAA, which play a crucial role in modulating plant development. Tryptophan, a precursor for IAA biosynthesis, is another essential compound linked to the plant-bacteria interaction. HPLC serves as a powerful tool for the precise quantification of these metabolites, offering insights into the microbial mechanisms promoting plant growth. Additionally, antagonism against plant pathogens, such as *Fusarium equiseti*, further highlights the potential of *Bacillus pumilus* in crop protection.

Results In this study, a bacterial isolate identified as *Bacillus pumilus* was evaluated for its PGP traits. The isolate demonstrated the production of significant amounts of IAA, as confirmed through HPLC analysis, revealing its metabolic capacity for enhancing plant growth. Alongside IAA production, the isolate exhibited other *in vitro* PGP activities, such as phosphate solubilization, and siderophore production, which are critical for improving nutrient availability to plants. Furthermore, *Bacillus pumilus* showed strong antagonistic activity against *Fusarium equiseti*, a notorious soil-borne pathogen, suggesting its role in biocontrol.

Conclusion The study underscores *Bacillus pumilus* as a potent IAA-producing PGPR with multiple *in vitro* plant growth-promoting traits and effective antagonism against *Fusarium equiseti*. The use of HPLC for accurate quantification of IAA and tryptophan provides valuable insights into the microbial mechanisms driving plant growth promotion. These findings emphasize the

potential of *Bacillus pumilus* as a bioinoculant in sustainable agriculture, offering a dual benefit of enhancing crop productivity and providing natural protection against plant pathogens. This research highlights the role of microbial-mediated strategies in advancing environmentally sustainable farming practices.

Keywords: Plant growth-promoting bacteria, indole-3-acetic acid, tryptophan, HPLC, microbial interactions.

Abbreviations

PGPR: Plant Growth-Promoting Rhizobacteria, IAA: Indole-3-Acetic Acid, HPLC: High-performance liquid chromatography, PGP: Plant Growth-Promoting.

Introduction

In the pursuit of sustainable agriculture and enhanced ecosystem health, the use of PGPR has gained increasing importance. PGPR boost nutrient uptake, increase plant growth, and provide resistance against various biotic and abiotic stresses, making them essential allies for modern agriculture. Among these beneficial microbes, *Bacillus pumilus* stands out as a potent producer of phytohormones and other PGP traits. This bacterium has the ability to synthesize key compounds, such as IAA and tryptophan, which play significant roles in promoting plant growth and development.

IAA, an essential auxin, is involved in critical plant physiological functions, such as differentiation, elongation, and cell division. It plays a central function in the growth of roots, shoot elongation, and overall plant architecture. B.p. produces IAA through the microbial metabolism of tryptophan, an essential amino acid that serves as a precursor for auxin biosynthesis. The presence of bacteria that can produce and release of IAA into the rhizosphere can have a significant impact on plant growth, nutrient uptake, and resilience to environmental stresses, positioning B.p. as a valuable PGPR (Jiang et al 2023).

In addition to IAA production, B.p. exhibits other beneficial traits, such as phosphate solubilization and siderophore production, which contribute to plant nutrition by increasing the bioavailability

of essential nutrients like phosphorus and iron. Moreover, B.p. displays antagonistic activity against various plant pathogens, including *Fusarium equiseti,* a significant phytopathogen responsible for crop diseases. The bacterium's biocontrol potential makes it an attractive candidate for integrated pest management strategies in sustainable agriculture (Ratnaningsih et al 2023).

To accurately assess the plant growth-promoting potential of B.p., it is crucial to quantify IAA and tryptophan production. HPLC is a sensitive and reliable analytical technique employed to measure these compounds. HPLC provides precise quantification, enabling researchers to evaluate the capacity for metabolism of the separated microorganisms and their effect on the growth of plants.

The purpose of this work is to look into the plant growth-promoting traits of B.p., particularly its production of IAA and tryptophan, phosphate solubilization, siderophore production, and antagonistic activity against *Fusarium equiseti*. By characterizing the interaction between B.p. and plants in terms of their molecules, This study adds to the growing efforts to leverage microbial solutions for enhancing crop productivity and promoting sustainable farming methods. Utilizing the capabilities of B.p. offers promising prospects for reducing the reliance on artificial pesticides and fertilizers, ultimately supporting environmental sustainability and agricultural resilience (Lata et al 2023).

Materials and Methods

Bacterial Isolation and Culture

Sample Collection Samples of soil were taken from areas in Uttar Dinajpur that cultivate mustard, specifically targeting the rhizospheric soil associated with mustard plants, to isolate PGPR. For collection, sterile polythene bags were utilized, and the samples were transported to the laboratory. The samples were labeled and stored at 4°C for subsequent analysis (Clare et al. 2022).

Isolation Procedure The serial dilution technique was used to isolate bacteria from the collected soil samples. The soil suspensions were prepared in distilled sterile water and diluted in steps. Diluted samples were plated on nutrient agar media supplemented with tryptophan to promote the growth of IAA-producing bacteria.

Incubation The inoculated plates were incubated at 28–30°C for the next 24–48 hours to allow for the growth of bacterial colonies.

Pure Culture Selection Distinct bacterial colonies were chosen based on their morphology. The selected colonies were streaked to produce pure cultures on newly prepared nutrient agar plates (Rychshanova et al. 2022). Pure cultures were maintained on agar slants at 4°C for future analysis.

Screening for Indole-3-Acetic Acid (IAA) Production

Inoculum Preparation Pure bacterial cultures, including *Bacillus pumilus*, were inoculated into tryptophan-supplemented broth and incubated at 28°C for 48-72 hours. Tryptophan, as a precursor, was added to enhance IAA production by the bacterial isolates.

Sample Preparation The dried residue was resuspended in methanol, and the solution was filtered using a 0.45 μm membrane filter. The specimens were kept at -20°C until analyzed by HPLC (Rychshanova et al. 2022).

HPLC Analysis for IAA Quantification

Instrument Setup Using an HPLC system outfitted with a C18 reverse-phase column and a UV-Vis detector calibrated at 254 nm, the ideal wavelength for IAA detection, the IAA concentration in bacterial extracts was measured.

Standard Preparation Pure IAA standard solutions were made at predetermined concentrations, and a calibration curve was generated by plotting peak areas against standard concentrations.

Chromatographic Conditions Methanol and water (80:20, v/v) at a flow rate of 1.0 mL/min made up the mobile phase. A 20 μL injection volume of the sample was used.

Sample Injection and Analysis Standard solutions and IAA Samples were added to the HPLC device. IAA concentrations were ascertained by comparing the sample peak regions with the standard calibration curve (Zhang et al. 2024).

Tryptophan Quantification

Tryptophan Extraction We used acidified ethanol to extract tryptophan from bacterial cultures. The obtained supernatant was used for solvent extraction after the cultures were centrifuged.

Sample Preparation The extracted tryptophan was passed through a membrane filter measuring 0.45 μm and diluted appropriately for HPLC analysis.

HPLC Analysis Tryptophan concentrations were quantified using HPLC under the same conditions as the IAA quantification, except for detector adjustments for optimal tryptophan detection. A calibration curve using pure tryptophan standards was generated to calculate sample concentrations (Girgin et al. 2024).

Screening for *In Vitro* Plant Growth-Promoting (PGP) Traits

Phosphate Solubilization *Bacillus pumilus* was tested for its ability to solubilize phosphate by growing the bacteria on Pikovskaya's agar medium. Clear halo zones around the colonies indicated phosphate solubilization.

Siderophore Production The bacterial isolates were grown on Chrome Azurol S (CAS) agar plates in order to measure the generation of siderophores. The colonies' orange halo was thought to be a sign that siderophore production was proceeding well.

Antagonism Against Fusarium equiseti

Dual Culture Assay The antagonistic activity of *Bacillus pumilus* against *Fusarium equiseti*, a common phytopathogen, was tested using a dual culture assay. The bacterial isolate and *Fusarium equiseti* were co-inoculated onto potato dextrose agar (PDA) plates. After incubating at 28°C for 5–7 days, the fungal growth was shown to be inhibited.

Quantification of Inhibition The percentage of fungal growth inhibition was calculated by measuring the radial growth of the pathogen in the presence and absence of the bacterial isolate. The formula used was:

$$\%Inhibition = \left(\frac{Control\ growth - Treated\ growth}{Control\ growth}\right) \times 100$$

This allowed for quantitative assessment of *Bacillus pumilus* antagonistic potential against *Fusarium equiseti*.

Statistical Analysis

Data Processing Agilent software was used to process data from the HPLC examination of the amounts of tryptophan and IAA. Standard deviations and mean values were computed as part of descriptive statistics.

Regression Equation

o The equation is followed by the calibration curves:

$y = ax + b$

In this case, y stands for the area or height of the instrument response.

x is a representation of the compound's quantity.

The coefficients a and b are found using the calibration data.

Inferential Statistics Student's t-test was used in the statistical analysis to determine the significance of the variations in antagonistic activity and IAA generation amongst the bacterial isolates. P-values less than 0.05 were considered statistically significant (Gharsallah et al. 2024).

Quality Control Measures

Instrument Calibration Standard IAA and tryptophan solutions were used to calibrate the HPLC system regularly to guarantee the precision and accuracy of the measurement.

Control Samples Positive and negative control samples were included in each analytical batch to ensure assay performance and identify any possible systematic errors.

Reproducibility To ensure the results could be repeated, all tests, including the antagonism assays and HPLC quantification, were run in triplicate (Mohammadi et al. 2024).

Results and Discussion

Isolation and Identification of *Bacillus pumilus* Isolates

To comprehend PGPR's involvement in sustainable agriculture, it is essential to identify and isolate them. The soil samples that were gathered from Uttar Dinajpur's mustard-growing regions were serially diluted and then plated on nutritional agar that contained 50 µg/mL of kanamycin. separate colonies emerged following a 48-hour incubation period at 30°C, showing diverse morphological

features, such as color, size, and texture . Colonies exhibited creamy white, yellow, and pink hues (Mustafa et al. 2024).

Initially, Gram staining and motility tests were used to identify the bacterial isolates, revealing that the majority of isolates were Gram-positive, rod-shaped, and motile. Biochemical assays, including catalase activity, IAA production , Siderophore Production and phosphate solubilization tests, were conducted to evaluate their plant growth-promoting traits (Table 1, Fig. 1 & Fig. 2). Among these, the isolate *Bacillus pumilus* demonstrated strong PGP potential.

For precise molecular identification via 16S rRNA gene sequencing was performed. The sequences were compared with reference sequences in NCBI databases, confirming that the isolate belonged to the species *Bacillus pumilus* (Fig. 3). This isolate was designated *Bacillus pumilus* MUSRH-05 and selected for further analysis of its PGP traits and antagonistic activity against *Fusarium equiseti*.

IAA and Tryptophan Production by *Bacillus pumilus*

One of the key plant growth-promoting traits of *Bacillus pumilus* MUSRH-05 is its ability to synthesize IAA, a vital phytohormone that enhances root development and overall plant growth. The isolate was cultured in tryptophan-supplemented broth to stimulate IAA and tryptophan production. After incubation, the IAA and tryptophan were extracted using ethyl acetate for subsequent quantification (Calatrava et al. 2024).

The extracts' HPLC examination verified that tryptophan and IAA were produced. The retention times for IAA and tryptophan were found to be 3.131 minutes and 2.644 minutes, respectively (Fig. 4). Quantitative analysis revealed that *Bacillus pumilus* MUSRH-05 produced IAA at a concentration of 303.333 ng/µL, while tryptophan production was quantified at 151.383 ng/µL, based on peak areas of 5704.8125 and 2027.7877, respectively.

The significant production of IAA by *Bacillus pumilus* highlights its potential to promote plant growth through the synthesis of auxin, which regulates various aspects of plant development. The production of tryptophan, a precursor to IAA, further underscores the metabolic capabilities of this strain in influencing plant-microbe interactions.

Antagonism Against *Fusarium equiseti*

The antagonistic activity of *Bacillus pumilus* MUSRH-05 against the phytopathogen *Fusarium equiseti* was evaluated *in vitro*. The purpose of the dual culture assays was to assess the inhibition of fungal growth by the bacterial isolate. The results showed that *Bacillus pumilus* MUSRH-05 exhibited strong antifungal activity, with a significant inhibition zone around the bacterial colony i.e., 80% (Fig. 5). This antagonism is likely due to the production of secondary metabolites by *Bacillus pumilus*, which interfere with the growth of *Fusarium equiseti*.

The antagonistic property of *Bacillus pumilus* is a valuable trait for biological control, providing a sustainable substitute for chemical fungicides in the control of soil-borne illnesses. The dual action of promoting plant growth through IAA production and controlling pathogens demonstrates the multifaceted benefits of *Bacillus pumilus* as a PGPR.

Comparison of HPLC and Spectrophotometric Methods for IAA Quantification

To validate the accuracy of HPLC quantification, the outcomes were contrasted with the spectrophotometric method's findings. While the spectrophotometric approach is commonly used for IAA measurement, it is less specific due to interference from other indolic compounds. In contrast, HPLC offers higher accuracy and precision by separating IAA from additional substances found in the sample. The HPLC method showed a higher correlation coefficient ($R^2 = 0.9987$) for IAA quantification compared to the spectrophotometric method ($R^2 = 0.840$), emphasizing the superiority of HPLC for accurate IAA analysis.

Reproducibility and Linearity of HPLC Quantification

The reproducibility and linearity of the HPLC method were evaluated by analyzing multiple replicates and constructing calibration curves. The relative standard deviation (RSD) values for both IAA and tryptophan quantification were consistently below 2%, demonstrating the high precision of the method. Calibration curves for IAA and tryptophan showed excellent linearity, with R^2 values of 0.9987 and 0.9991, respectively (Strieder et al. 2024), confirming the method's reliability for plant growth-promoting studies.

Therefore, Bacillus pumilus MUSRH-05 exhibits significant plant growth-promoting traits, including high levels of IAA production and effective antagonism against *Fusarium equiseti*. The accuracy and precision of HPLC analysis, combined with the robust antagonistic properties of this

bacterial isolate, suggest its potential as a bioinoculant for enhancing crop productivity and disease resistance. These findings demonstrate the utility of *Bacillus pumilus* in sustainable agriculture.

Conclusion

To sum up, this research indicates the noteworthy function of *Bacillus pumilus* as a potent PGPR with multiple beneficial traits, including the production of IAA, tryptophan, siderophores, and phosphate solubilization capabilities. The quantification of IAA and tryptophan using HPLC highlighted the metabolic capabilities of *Bacillus pumilus*, specifically the MUSRH-05 strain, in promoting plant growth through phytohormone production.

In addition to its phytohormone production, *Bacillus pumilus* exhibited efficient phosphate solubilization and siderophore production, both critical traits for enhancing nutrient availability in the rhizosphere. Phosphate solubilization facilitates the conversion of insoluble phosphate forms into bioavailable forms, contributing to improved plant nutrition. Siderophore production, on the other hand, enhances iron acquisition by plants and suppresses the growth of pathogenic microorganisms by sequestering iron, a vital nutrient. The antagonistic activity of *Bacillus pumilus* against *Fusarium equiseti* further underscores its potential as a biocontrol agent, offering protection against plant pathogens while promoting overall plant health.

The combined effects of IAA production, phosphate solubilization, siderophore production, and pathogen antagonism highlight the multifunctional nature of *Bacillus pumilus* as a biofertilizer and biocontrol agent. These attributes position *Bacillus pumilus* as a promising candidate in order to create sustainable agricultural solutions aimed at reducing the reliance on synthetic fertilizers and pesticides.

Future studies should concentrate on examining the cooperative impacts of microbial consortia, delving further into the mechanisms underlying interactions between plants and microbes, and testing the efficacy of *Bacillus pumilus* in diverse environmental conditions through field trials. The integration of *Bacillus pumilus* into biofertilizer formulations could contribute significantly to enhancing crop yields, improving soil health, and promoting sustainable agricultural practices.

This study highlights *Bacillus pumilus* as a key player in promoting plant growth, nutrient availability, and pathogen resistance. The findings underscore its potential in advancing sustainable agriculture, providing a natural and eco-friendly alternative to chemical inputs, and promoting environmental stewardship in the pursuit of agricultural resilience and food security.

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Conflict of Interest

Authors declare that there are no conflict of interest.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest

The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

No informed consent is required.

Data Availability Statement

No datasets were generated or analyzed during the current study.

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Consent of publication

I, Shambhu Swarnakar performed the experiment and surveyed the field for soil collection. The photo belongs to me. I have no objection if image is used for publication purposes in the article.

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