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Isolation of Bacterial Endophytes from *Carica papaya, Tamarindus indica* and *Moringa oleifera* Leaves

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ABSTRACT

The study was carried out for isolation and characterization of the potentially isolated strains of the bacteria endophytes from Carica papaya, Tamarindus indica and Moringa oleifera leaves. The leaves of Carica papaya, Tamarindus indica and Moringa oleifera are a source for endophytes that may be a source of new natural products useful in food and agroindustry, little is known about isolation and characterization of endophytes with bacterial origin in these plants. The leaves of the above mentioned plants were collected, surface sterilized and then inoculated in King's B Media for isolation of bacterial endophytes. Six pure isolates of bacteria were isolated and subjected to PCR and sequencing of the PCR products to molecularly characterize them as bacterial endophytes. The percentage similarity and classification of endophytic bacterial strains were done with help of NCBI BLASTDNA against the recorded sequences of the database. The bacterial endophytes of Moringa oleifera demonstrated a 99 % coverage respectively for Staphylococcus pasteuri coverage and the bacterial endophytes of Carica papaya and Tamarindus indica revealed 95 % respectively for coverage of Lysinibacillus sphaericus and Paenibacillus azotifigens.

Keyword: Bacterial endophyte, synergistic, *Carica papaya, Tamarindus indica* and *Moringa oleifera*.

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INTRODUCTION

Endophytic microbes are a source of many important bioactive molecules. The Endophytes are classified as microbes which grow in the plant tissues without causing any expression of disease. Are briefly pathogenic. Endophytes have a niche so they are known to produce a wide range of bioactive compounds. The need for new sources of antimicrobial metabolites has been great due to the ongoing development of pathogen resistance to current health care treatments and medicines. Therefore, investigation for endophytic microbes, which could be used for producing new and unique quorum sensing inhibitors, is very essential. Papaya (Carica papaya) is a fruit crop which is very popular in the tropical and subtropical fruit trees. There is a growing interest in the study of the papaya as it is much faster to mature than many of its tropical and subtropical counterparts. Considerable research has been done on the papaya fruit's nutritional value and the medicinal properties of the papaya plant are known. But, the Papaya plant has not been studied in the field of plant endophyte research. As papaya contain a lot of sugar for example sucrose, rich in fructose and glucose, the endophytes associated with the papaya might be a good study of what papaya has to offer. Tamarindus indica is an African tree which is common throughout the Indian subcontinent. A versatile plant. The pulp of fruits was used as a spice in Asian cooking particularly in the southern part of India.

The research work done in the recent past focusses on the discovery of novel natural sources and the associated natural compounds with the potential of pharmaceutical application. The success results which have obtained from this area would be a great success in the battle against animal bacterial pathogens. Secondary metabolites produced by plants can be beneficial to treat diseases. Moringa oleifera is the most common species in the Moringaceae family. They grow well in tropical climate. In India and Nigeria this plant is grown as a crop, and is also referred to as Drumstick. It is used for foods and medicine, and a popular traditional medicine plant. The biological activities reported for Moringa oleifera are: antimicrobial, regulation of thyroid hormone, antidiabetic, antihyperglycemic and hypotensive. This wide range of properties of Moringa oleifera, warranted an investigation of the endophytes.

MATERIAL AND METHODS

Plant Sample Collection

Fresh, mature, and healthy leaves of *Carica papaya*, *Tamarindus indica*, and *Moringa oleifera* were collected for this study. After procurement, all samples were immediately transported to the laboratory and processed within 4-6 hours to prevent contamination and ensure the viability of endophytic bacteria.

Preparation of Culture Media

King's B (KB) media was prepared according to the manufacturer's instructions for the initial isolation of endophytic bacteria. The media components were mixed thoroughly using a hot plate to ensure complete dissolution. The prepared media was then sterilized by autoclaving at 121°C for 15-20 minutes at 15 lbs pressure. To verify sterility, the media was poured into 90 mm sterile Petri plates and incubated at 37°C for 24 hours. The absence of microbial growth confirmed the sterility of the prepared media.

Surface Sterilization of Plant Material

Leaf disinfection and isolation of endophytic bacteria were performed following the method described by Mahajan et al. (2014) with modifications. All procedures were conducted in a laminar air flow chamber using sterilized scalpels and forceps. The sterilization protocol involved multiple steps:

The leaves were initially rinsed with double distilled (dd) water for 2-3 minutes. Surface disinfection was achieved by treating the leaves with 0.1% sodium hypochlorite for 5 minutes, followed by another dd water wash for 2-3 minutes. The leaves were then sprayed with Bavistin at a concentration of 500 ml/ha and 0.01% disinfectant. Subsequently, the leaves were submerged in dd water for 5 minutes and treated with 0.05% streptomycin solution, followed by another dd water rinse. Finally, the leaves were treated with 70% ethanol and left in dd water for an additional 5 minutes. After completing the sterilization procedure, the leaves were air-dried in the laminar air flow chamber.

Sterility Check

To confirm complete surface sterilization, 1 ml of the sterilized distilled water from the final rinse was poured onto nutrient agar medium and incubated at 37°C for 24 hours. The absence of bacterial growth on these plates confirmed successful surface disinfection of the leaves.

Isolation of Endophytic Bacteria

The surface-sterilized leaves from each plant species were placed onto KB media plates and incubated at 37°C for 24 hours. Following incubation, bacterial colonies were characterized based on morphological observation and Gram's staining. Single colonies were then inoculated into Brain Heart Infusion (BHI) broth and incubated for 24 hours.

Purification of Endophytic Bacteria

Endophytic bacterial purification was performed by sub-culturing a loopful of BHI broth culture onto 5% sheep blood agar plates. The plates were incubated at 37°C for 24 hours. A single colony was then transferred to fresh BHI broth and incubated at 37°C for 24 hours. Gram's staining was performed to verify culture purity, and the purified cultures were preserved for further studies.

Identification of Endophytic Bacteria
Gram's Staining

Bacterial identification was initiated through Gram's staining. Heat-fixed smears were prepared on slides and placed in a staining tray. Crystal violet was applied for 1 minute, followed by rinsing with distilled water. Gram's iodine was then added and left for 1 minute. After rinsing, the slides were decolorized with 95% ethyl alcohol for 5-10 seconds and immediately washed with distilled water. Safranin counterstain was applied for 1 minute, followed by final rinsing and air drying. The slides were examined under a microscope at 100X magnification.

DNA Extraction

Genomic DNA was extracted using the Instagene Matrix (Bio-Rad) kit. An isolated bacterial colony was grown overnight, picked, and suspended in 1 ml of autoclaved water in a microcentrifuge tube. The suspension was centrifuged at 10,000-12,000 rpm for 1 minute. The supernatant was discarded, and 200 µl of Instagene Matrix was added to the pellet. The mixture was incubated at 56°C for 30 minutes, vortexed, and placed in a boiling water bath at 100°C for 8 minutes. After vortexing for 10 seconds, the sample was centrifuged at 10,000-12,000 rpm for 2-3 minutes. The extracted DNA was stored at -20°C for future use.

Polymerase Chain Reaction

PCR was performed using universal bacterial primers 8F and 1541R as described in Table 1.

Table 1: Details of primers used for endophytic bacteria

Primer	Sequence	Product Size	Reference
8F	AGAGTTTGGATCTGGCTCAG	1533 bp	Zhou et al., 1997
1541R	AAGGAGGTGATCCAGCCGCA		

Table 2: Composition of PCR reaction mix for endophytic bacteria

Component	Volume
Taq Master mix	12.5 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
DNA template	2.0 µl
Nuclease-free water	8.5 µl
Total	25 µl

Table 3: PCR cycle conditions for endophytic bacteria

Temperature	Time	No.ofcycles
95°C	2 min	1
95°C	30 sec	25
50°C	30 sec	
72°C	2 min	
72°C	10 min	1
4°C	∞	Hold

Table 4: Summary of bacterial endophytes isolated

Plant Species	Isolate Code	Number of Isolates
Carica papaya	CP1, CP2	2
Moringa oleifera	MO1, MO2	2
Tamarindus indica	TI1, TI2	2
Total		6

The PCR reaction mixture (25 µl) was prepared as follows:

The reaction mixture was gently mixed and centrifuged to collect all components at the bottom of the tube. PCR amplification was performed in a thermal cycler under the following conditions:

Agarose Gel Electrophoresis

PCR products were analyzed by agarose gel electrophoresis. A 1.0% agarose gel was prepared in 1x Tris Borate EDTA (TBE) buffer (35 ml) and melted in a microwave oven. After cooling to 55°C, ethidium bromide (0.5 µg/ml) was added. The gel was poured into an electrophoresis tray and placed in the gel electrophoresis assembly with 1x TBE buffer. Samples were prepared by mixing 1 µl of gel loading buffer with 5 µl of PCR products. The samples were loaded along with a 1 kb DNA ladder. Electrophoresis was performed at 70 volts for 10 minutes, followed by 50 volts for 2 hours. The agarose gels were visualized under a UV trans-illuminator and photographed.

DNA Sequencing

The PCR products of all six bacterial endophytes were sent to Eurofins Genomics India Pvt. Ltd, Bangalore for Sanger sequencing using the forward and reverse primers.

Phylogenetic Analysis

The sequences obtained for the six bacterial endophytes were subjected to phylogenetic analysis. BLAST (Basic Local Alignment Search Tool) was used for sequence alignment, and Clustal-W (CLUSTAL 2.1) was employed for multiple sequence alignment reports. Phylogenetic trees were constructed using Molecular Evolutionary

Table 1: Gram's Reaction of Bacterial Endophytes

S. No.	Isolate Name	Plant Source	Gram's Reaction
1	P1	*Carica papaya*	Gram positive rods
2	P2	*Carica papaya*	Gram positive rods
3	M1	*Moringa oleifera*	Gram positive rods
4	M2	*Moringa oleifera*	Gram negative rods
5	T1	*Tamarindus indica*	Gram positive cocci in clusters
6	T2	*Tamarindus indica*	Gram positive rods

Table 2: Genotypic Characterization of Bacterial Endophytes

S. No.	Sample	16S rRNA Amplification	Product Size
1	P1	Positive	~1533 bp
2	P2	Positive	~1533 bp
3	M1	Positive	~1533 bp
4	M2	Positive	~1533 bp
5	T1	Positive	~1533 bp
6	T2	Positive	~1533 bp

Genetic Analysis (MEGA 11) version 11. The Neighbor-Joining method was used for tree construction with the Bootstrap resampling method for statistical support.

RESULTS

Isolation of Endophytic Bacteria

Endophytic bacteria were successfully isolated from surface-disinfected leaves of *Carica papaya*, *Moringa oleifera*, and *Tamarindus indica* using King›s B media. After incubation at 37°C for 24 hours, distinct bacterial colonies emerged on the culture plates. Based on morphological differences, colonies were purified by repeated streaking (five times) onto 5% sheep›s blood agar. Purified colonies were examined microscopically, and based on colony morphology and Gram›s staining characteristics, two colonies were selected from each plant species.

Gram's Staining Results

The results demonstrated that the majority of isolates (5 out of 6) were Gram-positive rods, which is consistent with earlier studies reporting that approximately 90% of endophytic bacteria are Gram-positive rods (Reference 21). However, some studies have also reported the presence of

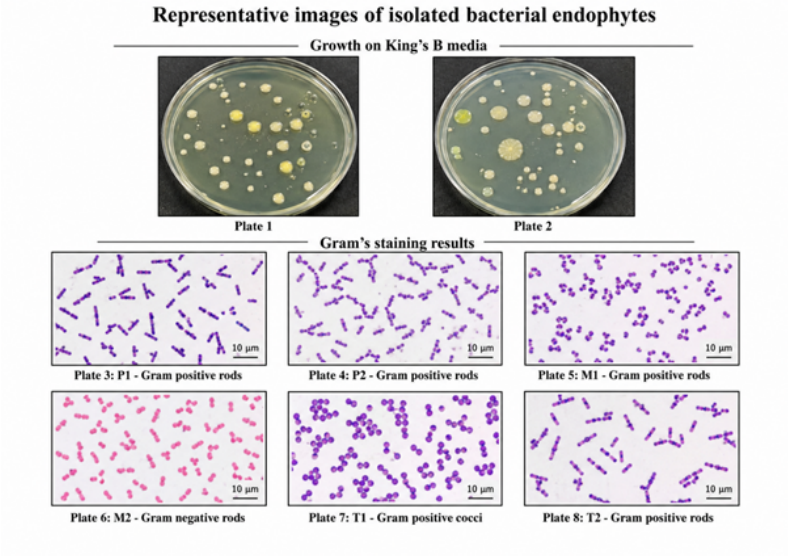


Fig. 1: Representation of isolated bacterial endophytes

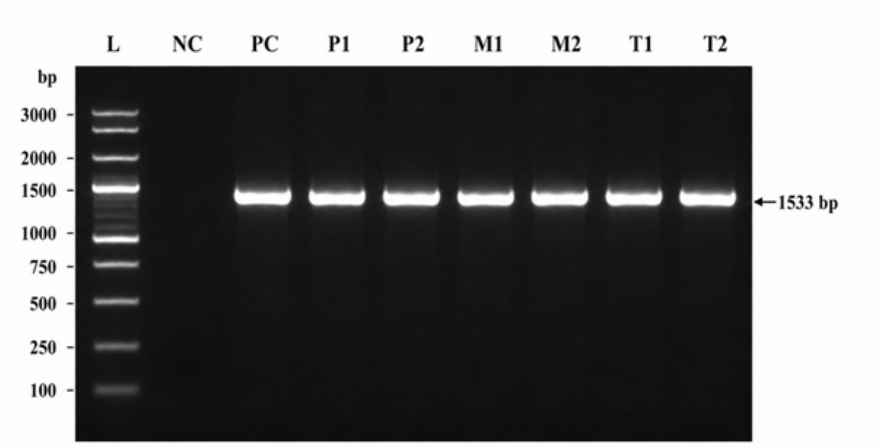


Fig. 2: Agarose Gel Electrophoresis of PCR Products

endophytic Gram-positive cocci , as observed in isolate T1 (*Staphylococcus pasteuri*).

Discussion on Isolation

Endophytic bacteria represent a highly diverse group of microorganisms. With approximately 300,000 plant species on Earth, each plant species, and often each individual plant, can harbor one or several endophytes. Many endophytes can inhabit multiple different plant hosts (Reference 23). Endophytes are potentially hyper-diverse, with estimates suggesting there could be nearly 1,000,000 endophytic bacterial taxa. This diversity likely varies geographically, from country to country, region to region, and among different climatic zones. The successful isolation of six distinct bacterial endophytes from three different plant species in this study supports the concept of endophytic diversity.

Molecular Characterization of Isolated Endophytic Bacteria

PCR Amplification of 16S rRNA Gene

All six bacterial endophyte isolates underwent genotypic characterization using the 16S rRNA gene. PCR amplification was performed using universal bacterial primers 8F and 1541R, which successfully generated amplicons of approximately 1533 bp for all isolates, in accordance with the method described by Reference 24 with minor modifications.

Phylogenetic Analysis and Species Identification

The 16S rRNA gene sequences of all six isolates were compared with deposited sequences in the NCBI GenBank database using the BLAST tool. Phylogenetic trees were constructed using the Neighbor-Joining method with Bootstrap resampling in MEGA 11.

Bacterial Endophytes of *Carica papaya*

Sequence Analysis: The nucleotide sequence of isolate P1 showed 98.66% similarity to *Lysinibacillus sphaericus* with a query cover of 95%.

Phylogenetic Placement

The phylogenetic tree (Plate 10) demonstrated that isolate P1 clustered closely with *Lysinibacillus sphaericus* reference strains, confirming its identification.

Discussion

Lysinibacillus sphaericus is a well-known endophytic bacterium with mosquitocidal properties and plant growth-promoting activities. Its isolation from *Carica papaya* leaves in this study is consistent with previous findings. Hlaing and Soe (2018) isolated *Bacillus* and *Staphylococcus* species from papaya leaves, while Kushwah (2019) identified *Bacillus subtilis*, *Bacillus wiedmannii*, and *Staphylococcus pasteuri* as bacterial endophytes of papaya plants. The presence of *Lysinibacillus* species in papaya adds to the known endophytic diversity of this plant.

Isolate P2

Sequence Analysis

The nucleotide sequence of isolate P2 showed 99.38% similarity to *Paenibacillus illinoisensis* with a query cover of 97%.

Phylogenetic Placement

The phylogenetic tree (Plate 11) placed isolate P2 within the *Penicillus* clade, closely related to *P. illinoisensis* reference strains.

Discussion

Paenibacillus illinoisensis is a Gram-positive, endospore-forming bacterium known for its plant growth-promoting properties, including phosphate solubilization and production of antimicrobial compounds. The high similarity (99.38%) strongly supports the identification of P2 as *P. illinoisensis*. This represents a notable finding, as *Paenibacillus* species are increasingly recognized as important plant-associated bacteria with biotechnological potential.

Bacterial Endophytes of *Moringa oleifera*

Sequence Analysis: The nucleotide sequence of isolate M1 showed 98.28% similarity to *Paenibacillus azotifigens* with a query cover of 95%.

Phylogenetic Placement: The phylogenetic tree (Plate 12) demonstrated that isolate M1 grouped with *Paenibacillus azotifigens* reference strains.

Discussion

Paenibacillus azotifigens is a nitrogen-fixing bacterium that contributes to plant nutrition and growth promotion. Its isolation from *Moringa*

oleifera leaves is significant, as *Moringa* is known for its nutritional and medicinal properties. Previous studies have reported the isolation of *Pectobacterium cypripedii* and *Pantoea agglomerans* from the *Moringa* rhizosphere (Berg et al., 2009). White (2017) identified *Bacillus pumilus*, *Citrobacter koseri*, and *Klebsiella pneumoniae* from *Moringa* seeds, while Ilmi et al. (2018) reported *Bacillus cereus* from *Moringa* stems. The identification of *P. azotifigens* from *Moringa* leaves in this study expands the known endophytic community of this important medicinal plant. Sequence Analysis: The nucleotide sequence of isolate M2 showed 82.93% similarity to *Cupriavidus taiwanensis* with a query cover of 80%.

Phylogenetic Placement

The phylogenetic tree (Plate 13) placed isolate M2 within the *Cupriavidus* clade.

Discussion

Cupriavidus taiwanensis is a Gram-negative, rod-shaped bacterium belonging to the family Burkholderiaceae. The relatively lower similarity (82.93%) and query cover (80%) suggest that M2 may represent a novel strain or species within the *Cupriavidus* genus. *Cupriavidus* species are known for their metabolic versatility, including heavy metal resistance and degradation of environmental pollutants. The isolation of this bacterium from *Moringa* leaves is noteworthy and warrants further investigation for its potential biotechnological applications.

Bacterial Endophytes of *Tamarindus indica*

Sequence Analysis

The nucleotide sequence of isolate T1 showed 98.23% similarity to *Staphylococcus pasteuri* with a query cover of 99%.

Phylogenetic Placement

The phylogenetic tree (Plate 14) placed isolate T1 within the *Staphylococcus* clade, closely related to *S. pasteuri* reference strains.

Discussion

Staphylococcus pasteuri is a Gram-positive coccus belonging to the genus *Staphylococcus*, commonly found on human skin but also reported as a plant endophyte. Kushwah (2019) previously identified *S. pasteuri* as an endophyte of papaya plants. The isolation of *S. pasteuri* from *Tamarindus indica* in this study indicates that this bacterium can colonize multiple plant hosts. The presence of Gram-positive cocci as endophytes has been reported though they are less common than Gram-positive rods.

Isolate T2

Sequence Analysis

The nucleotide sequence of isolate T2 showed 97.85% similarity to *Bacillus paramycoides* with a query cover of 99%.

Phylogenetic Placement

The phylogenetic tree (Plate 22) placed isolate T2 within the *Bacillus* clade, closely related to *B. paramycoides* reference strains.

Discussion

Bacillus paramycoides is a recently described species within the *Bacillus cereus* group, known for its biocontrol potential. Chigurupati et al. (2020) previously isolated *Bacillus velezensis* from *Tamarindus indica* leaves. The identification of *B. paramycoides* from the same plant species in this study suggests that *Tamarindus indica* harbors a diverse community of *Bacillus* species with potential plant growth-promoting and biocontrol properties.

CONCLUSION

This study successfully isolated and characterized six bacterial endophytes from *Carica papaya*, *Moringa oleifera*, and *Tamarindus indica* leaves. The identified endophytes—*Lysinibacillus sphaericus*, *Paenibacillus illinoisensis*, *Paenibacillus azotifigens*, *Cupriavidus taiwanensis*, *Staphylococcus pasteuri*, and *Bacillus paramycoides*—represent a diverse group of bacteria with significant potential for agricultural, biotechnological, and pharmaceutical applications. The research highlights the importance of exploring endophytic microbial communities associated with medicinal plants, as these microorganisms may serve as valuable sources of novel bioactive compounds and plant growth-promoting agents. The presence of potentially novel *Cupriavidus* strain (M2) warrants further investigation through whole-genome sequencing and comprehensive characterization.

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