Analysis of Endophytic Bacteria in Selected Medicinal Plants: *Piper longum* L. and *Piper chaba* Hunter

DISSERTATION

Submitted to the University of Calicut in partial fulfilment of the requirement for the award of degree of Master of Science in Botany

Submitted by

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ABSTRACT

Endophytic bacteria play an essential role in the growth and development of medicinal plants by producing bioactive compounds that contribute to their medicinal properties. This project aims to analyze the endophytic bacteria present in two selected medicinal plants, *Piper longum* and *Piper chaba*. Using molecular methods like 16S rRNA gene sequencing, we will isolate endophytic bacteria from the leaves of these plants and identify their diversity. Additionally, we reviewed the bioactive potential of these endophytic bacteria by screening for antimicrobial, antioxidant, and anticancer activities. The findings of this study will shed light on the endophytic bacterial population associated with these therapeutic plants and their possible applications in the agricultural and pharmaceutical sectors. Certain endophytic bacteria identified in the study are known to have beneficial effects on plant development and health. For example, some bacteria produce substances like indole acetic acid that promote plant growth, while others have antibacterial properties that may help protect plants from disease. Both *Piper longum* (long pepper) and *Piper chaba* (chaba pepper) belong to the Piperaceae family and have been widely used in Ayurvedic therapy due to their numerous health benefits, including anti-inflammatory, antioxidant, and antibacterial properties. Research on *Piper longum* revealed that endophytic bacteria from the Bacillus genus can produce antibiotic substances that inhibit harmful bacteria. Similarly, research on *Piper chaba* identified endophytic bacteria that produce antioxidant compounds with potential pharmaceutical applications. This study found a diverse population of bacteria in the tissues of *Piper longum* and *Piper chaba*, identified using molecular methods such as DNA sequencing. Some of these endophytic bacteria have been found to have positive effects on plant health and growth, producing compounds like indole acetic acid and possessing antibacterial properties that help protect the plants from diseases.

Keywords: *Piper longum*, *Piper chaba*, Endophytic Bacteria, 16S rRNA

INTRODUCTION

An endophyte is an endosymbiont, typically a bacterium or fungus, Endophytes are non-pathogenic organisms that live within plant tissues for a portion of their life cycle (Rosenblueth & Martínez-Romero. (2006). Endophytic bacteria, which interact closely with their host, are an essential part of the plant microbiome, These interactions enhance plant tolerance to environmental changes as well as promote plant growth, thus they have become attractive targets for increasing crop production (Pinski, A., Betekhtin, A., Hupert-Kocurek, K., Mur, L. A. J., & Hasterok, R. (2019).Some endophytes may promote host development and nutrient acquisition while also improving the plant's ability to handle abiotic conditions such as drought and reducing biotic stresses by increasing plant resistance to insects, diseases, and herbivores.Although endophytic bacteria and fungi are well studied, endophytic archaea are increasingly being investigated for their function in plant growth promotion as part of a plant's core microbiome(chow *et al.*(2022). Endophytic bacteria colonize the interior tissues of all plants investigated. Endophytic bacteria, unlike phytopathogens, do not harm the host. Instead, they can benefit the plant by providing defense against pests. infections and enhanced growth and development by the generation of plant growth-promoting chemicals and/or nitrogen fixation from the atmosphere (Glick, 2012; Mercado Blanco & Lugtenberg, 2014).

Environmental elements, including temperature, illumination, moisture, soil conditions, and soil fauna, greatly impact medicinal plant quality and production (Wu *et al*. (2021). Medicinal plants can be significantly impacted by their interactions with particular bacterial endophytes, according to recent research. (Ek-Ramos *et al*., 2019).Endophytic bacteria and pathogenic microbes share a habitat in plants, making inoculation with these bacteria an effective way to suppress infections (Senthilkumar *et al*., 2011). Medicinal plant-isolated bacterial endophytes can create bioactive compounds and stimulate secondary metabolite synthesis in host plants. The host-endophyte connection is dynamic, with endophytic bacteria changing their gene expression and producing diverse metabolites in response to modest changes in host plant growth, and vice versa (Ek-Ramos *et al*., 2019).

Medicinal plants and their associated endophytic bacteria were collected. Our study and analysis revealed mutually beneficial connections between bacterial endophytes.The collected plant samples included members from piperaceae family, *piper longum & piper chaba. P. longum*, a member of the Piperaceae family, is a popular medicinal plant in India. It is also

called Long Pepper in English and Pippali in Hindi. This blooming vine is cultivated for its dried fruit, which is utilized for both culinary and medicinal purposes. Long peppers have a taste *Piper longum* is a hotter relative of *Piper nigrum* that produces secondary metabolites like piperine and piperlongumine (Liu *et al*., 2009). The smooth, hairless, scrambling shrub known as *Piper chaba Hunter* is found in India. (Kirtikar K. R & Basu B.D., (1987). This plant is referred to as choi or Java long pepper.The plant offers medicinal properties for several diseases such as asthma, bronchitis, piles, colic pain, dyspepsia, and gastralgia.(Haque *et al*.,2018). *piper longum* is used as an adulterant instead of *piper chaba* (Kumar P.S (2014).

Adulteration is the substitution of a drug with inferior therapeutic and chemical properties, such as low grade, spoiled, or spurious drugs, or a completely different drug that is similar to the original (Kokate *et al*., 2007). Adulterants are similar to the original medicine in terms of morphology, chemical composition, and therapeutic properties, but are of lower quality and less expensive. This is the most prevalent sort of adulteration (Kokate *et al*., 2007; Dubey *et al*., 2004). The adulterated drugs is found to that there are adverse event reports, that are present due to the usage of adulterant herbs (De S *et al*., 1992). In such cases it is essential to sensitize the masses of the plant based medicine systems helps to identify the plant properly (Thakur R,*et al* ., 2018).

The study is relevant as endophytic bacteria plays a crucial role in growth and development of medicinal plants, by understanding the diversity and function of Endophyte bacteria in these plants can provide valuable insight into their potential medicinal properties and help in development of new drugs. It is also relevant in context of the increasing use of medicinal plants as alternative medicine, it can ensure the quality and authenticity of these plants by identifying potential adulterants.

The significance of the study lies in it's potential to contribute to the conservation and sustainable use of medicinal plants by identifying and characterizing endophyte bacterias that may be beneficial for plant growth and health. study can have implications for the pharmaceutical industry by providing valuable information on potential bioactive compounds produced by endophyte bacteria in medicinal plants.

OBJECTIVES

- To identify and characterize the endophyte bacteria present in the piper sp. that have been used as adulterant in medicinal plants.
- To assess the diversity and composition of Endophyte bacteria in these plants and compare them with those in authentic medicinal plants.
- To investigate the potential bioactive compounds produced by endophyte bacteria in piper species and their potential medicinal properties.
- To assess the impact of Endophyte bacteria on growth and health of piper species and their potential role in plant defense mechanism.

REVIEW OF LITERATURE

Endophytic bacteria, which reside within plant tissues without causing harm, play a critical role in plant growth, health, and the production of bioactive compounds. The study of these bacteria has progressed significantly over the years, revealing their potential applications in agriculture, medicine, and biotechnology.

War (2019) and Shabong (2023) emphasized the ecological role and interactions of plant-associated bacteria, including endophytes, for biotechnological applications. Their studies highlighted the ability of endophytic bacteria to produce growth-promoting characteristics and inhibitory compounds against phytopathogens. These bacteria can produce a variety of bioactive substances, such as hydrogen cyanide, siderophores, phosphate solubilization compounds, ammonia, and indole-3-acetic acid, which are beneficial for plant growth and health. Additionally, endophytic bacteria can inhibit the growth of plant pathogens through the production of enzymes and antimicrobial compounds, such as chitinase, cellulase, protease, and HCN. Understanding these interactions and the molecular mechanisms involved can lead to the development of biotechnological applications in agriculture and medicine. The research underscores the potential of utilizing endophytic bacteria for sustainable agricultural practices and the production of bioactive compounds for therapeutic purposes. This body of work highlights the importance of integrating ecological and biotechnological approaches to harness the benefits of endophytic bacteria in various fields.

Gupta *et al*. (2013) and Jagadesan and Subhash (2013) provided valuable insights into the natural occurrence and identification of endophytic bacteria in various plants. These studies emphasized the potential of endophytic bacteria in promoting plant growth and health through the production of beneficial compounds and enzymes. The identification of endophytic bacteria using molecular techniques such as 16S rRNA sequencing has revealed a diverse array of microbial communities residing within plant tissues. These bacteria produce a range of bioactive compounds, including antibiotics, hormones, and enzymes, which can enhance plant growth and protect against pathogens. The research highlights the potential of harnessing these natural microbial resources for developing biofertilizers and biopesticides, reducing the dependence on synthetic agrochemicals. Understanding the ecological roles and interactions of these bacteria with their host plants can lead to more sustainable and environmentally friendly

agricultural practices. This body of work underscores the importance of integrating endophytic bacteria into crop management strategies to improve yield and quality.

Kobayashi and Columbo (2000) and **Hamayun *et al.* (2010)** conducted comprehensive reviews on the effects of bacterial endophytes on plants and their applications in agriculture. They emphasized the significance of secondary metabolites secreted by endophytic bacteria, which play crucial roles in promoting plant growth and health. These metabolites include hormones, antibiotics, and other bioactive compounds that enhance plant resistance to pathogens and environmental stresses. The secretion of these compounds by endophytes can improve nutrient uptake, enhance root growth, and stimulate overall plant development. Additionally, the reviews highlighted the potential of utilizing endophytic bacteria as biocontrol agents in agriculture, reducing the reliance on chemical pesticides and fertilizers. Understanding the specific mechanisms and pathways through which these secondary metabolites exert their effects can lead to innovative agricultural practices. This body of work underscores the importance of integrating endophytic bacteria into sustainable farming systems to boost crop productivity and health.

Ware *et al.* (2023) characterized the bioactive potential of secondary metabolites isolated from Piper sarmentosum Roxb., identifying key components with antifungal, anticancer, anti-inflammatory, and antioxidant properties. The study revealed that the Piper genus contains a variety of bioactive compounds, including amide alkaloids, lignans, neolignans, and phenylpropanoids. These compounds have demonstrated significant biological activities, making them valuable candidates for developing new therapeutic agents. The research highlights the importance of secondary metabolites in the medicinal properties of plants and their potential applications in pharmaceuticals. Understanding the biosynthetic pathways and regulatory mechanisms involved in the production of these compounds can lead to the discovery of novel drugs. This study underscores the therapeutic potential of endophytic bacteria-associated compounds, contributing to the development of natural and sustainable pharmaceuticals. The findings also emphasize the need for further research into the bioactive potential of secondary metabolites from medicinal plants.

Lee *et al.* (2019) characterized various Bacillus strains as human probiotics, detailing their safety, microbiome interactions, and potential as probiotic carriers. The study identified strains such as *B. subtilis*, *B. polyfermenticus*, *B. clausii*, certain *B. cereus*, *B. coagulans*, *B. pumilus*, and *B. licheniformis*, which are commonly used in commercial probiotic formulations. These

strains have demonstrated various health benefits, including the promotion of gut health, enhancement of immune responses, and inhibition of pathogenic bacteria. **Dobrzyński *et al.* (2023)** further explored the biocontrol potential of *Bacillus pumilus* against fungal phytopathogens, highlighting its capability to suppress harmful fungi and promote plant health. The research suggests that *Bacillus pumilus* can produce lipopeptides and enzymes that are essential in the suppression of fungal pathogens and the promotion of induced systemic resistance (ISR) in plants. This body of work underscores the commercial viability of Bacillus strains as probiotics for both human health and agricultural applications, offering eco-friendly alternatives to chemical treatments.

Nandagopal *et al.* (2015) documented the diversity of traditional medicinal plants used by rural communities in Tamilnadu, India, highlighting the extensive use of these plants to treat a variety of illnesses. Their study recorded the use of 144 plant species across 102 genera and 45 families, emphasizing the rich biodiversity and ethnobotanical knowledge of these communities. The research underscores the importance of understanding the interactions between these medicinal plants and their associated bacterial endophytes. These interactions can lead to the discovery of novel bioactive compounds with therapeutic potential. The study also highlights the need for conserving this traditional knowledge and the biodiversity of medicinal plants, which are vital for developing new pharmaceuticals. Understanding the cultural and ecological context of these plants can inform sustainable harvesting and conservation practices. This research contributes to the growing body of knowledge on ethnobotany and the potential of medicinal plants as sources of novel bioactive compounds for pharmaceutical applications.

Compant *et al.* (2010) detailed the role of plant growth-promoting bacteria (PGPB) in the rhizo- and endosphere of plants, focusing on their colonization mechanisms and utilization prospects. These bacteria enhance plant growth through various mechanisms, including nitrogen fixation, phosphate solubilization, and the production of growth hormones. The study highlighted advanced techniques such as microarrays, chips, metagenomics, metaproteomics, metatranscriptomics, and meta-proteogenomic analysis to uncover the complex molecular interactions between bacterial endophytes and their hosts. **Dudeja *et al.* (2021)** corroborated these findings, emphasizing the importance of multidisciplinary approaches in understanding the intricate relationships between endophytes and plants. These advanced techniques allow for a comprehensive analysis of the genetic, proteomic, and metabolic profiles of endophytic

bacteria, providing insights into their functional roles. The ability to manipulate these interactions can lead to the development of biofertilizers and biopesticides, contributing to sustainable agricultural practices. This body of research underscores the transformative potential of PGPB in enhancing crop productivity and resilience.

Araujo *et al*. (2005) conducted a pivotal study on the interactions between endophytic bacterial populations and the pathogen Xylella fastidiosa in citrus plants. Their research highlighted the potential of endophytes as sources of pharmacologically active compounds that could mitigate plant diseases. **Rosenblueth** and **Martinez-Romero (2006)** expanded on this by exploring the safe haven that endophytic bacteria find within the apo-plastic intercellular spaces of plants. This microenvironment allows for intimate and beneficial interactions between endophytes and their host plants, fostering the exchange of nutrients and signaling molecules. These interactions are essential for the production of bioactive compounds that can enhance plant health and resilience. Understanding the molecular dialogues that occur within these spaces can lead to the development of new strategies for harnessing endophytic bacteria in agriculture. The production of unique bioactive metabolites by endophytes underscores their potential in pharmaceutical and agricultural applications, paving the way for innovative solutions to plant health management.

Reinhold-Hurek and Hurek (1998) explored the role of diazotrophic endophytes in grasses, shedding light on their significant impact on plant health and development. These endophytes, a subclass of rhizospheric bacteria known as plant growth-promoting rhizobacteria (PGPR), have the remarkable ability to fix atmospheric nitrogen, a critical nutrient for plant growth. By colonizing the internal tissues of their plant hosts, these endophytes establish intimate interactions that go beyond the rhizosphere, penetrating deeper into plant tissues. This invasion is not harmful but rather beneficial, as these bacteria enhance nutrient acquisition, improve resistance to stress, and promote overall plant vigor. The intimate association between endophytic bacteria and their plant hosts involves complex molecular signaling pathways, ensuring a symbiotic relationship that is mutually advantageous. The study highlights that these beneficial microbes can enhance the efficiency of nutrient uptake and increase biomass production, making them valuable allies in sustainable agriculture. Furthermore, understanding the mechanisms by which these bacteria promote plant growth can lead to innovative agricultural practices, reducing the need for chemical fertilizers and enhancing crop

productivity. This research underscores the potential of harnessing diazotrophic endophytes to develop eco-friendly solutions for improving crop yield and resilience.

Czygan *et al*. (1993) discussed the historical use of medicinal plants by our ancestors to treat various contagious and deadly ailments. These early uses were deeply rooted in traditional knowledge passed down through generations, often documented in ancient texts and oral traditions. Modern research has validated many of these traditional uses, demonstrating the high therapeutic efficacy and minimal side effects of using herbs. Studies have shown that many medicinal plants contain active compounds that effectively combat pathogens, reduce inflammation, and promote overall health. The importance of medicinal plants in primary health care is particularly pronounced in many developing countries, where access to modern medical facilities and pharmaceuticals is often limited. In these regions, medicinal plants offer a more accessible and affordable option for health care, being readily available, locally cultivable, and requiring minimal processing.

Clarridge (2004) underscored the revolutionary impact of 16S rRNA gene sequence analysis in the field of clinical microbiology and infectious diseases. This gene, approximately 1,550 base pairs long, contains both conserved and variable regions, making it an ideal candidate for phylogenetic studies and bacterial identification. The conserved regions allow for the comparison across different species, while the variable regions provide the specificity needed for precise identification. **Rhoden *et al*. (2015)** further demonstrated the utility of 16S rRNA analysis in identifying a diverse array of endophytic bacterial genera within medicinal plants. Their study revealed the presence of genera such as Pantoea, Bacillus, Microbacterium, Pseudomonas, and Staphylococcus, showcasing the broad spectrum of bacteria that can reside within plant tissues. These advancements have not only enhanced our understanding of microbial diversity but also facilitated the exploration of microbial functions and interactions within their hosts. The ability to accurately identify and classify bacteria using 16S rRNA gene sequencing has profound implications for both environmental microbiology and biotechnology.

MATERIALS AND METHODOLOGY

1. Sample collection:

Plant sampling was carried out from a selected site of Kottakkal Arya Vaidya shala herbal garden. Selected plants chosen for the studies are ***piper longum*** and ***piper chaba*** is stored in plastic bags and carried to laboratory for further analysis.

2. Isolation of Endophytes:

2.1 Surface sterilization:

Collect 4-5 unfrozen leaf samples were surface sterilized to isolate and identify bacteria based on culture (Correa-Galeote *et al*. (2018). In Culture-dependent identification of microbial-community samples were completely washed with distilled water, blot dried, and submerged in 1.5% sodium hypochlorite for 10 minutes,wash in nucleus free water (NFW),treat in 70% ethanol (v/v) for one minute.Secondly, immersed in 1% sodium hypochlorite for about 10 minutes,wash in NFW twice (Nalini.S M *et al*.(2014).Sterilising agents commonly used include sodium hypochlorite: 1-5% for 2-10 minutes (Gardner JM.*et al*.(1982),ethanol: 70-95% for 30 seconds to 4 minutes (Dong Z.(1994), hydrogen peroxide (McInroy JA & Kloepper JW.(1994), and mercuric chloride 0.05-0.2% for 2-5 minutes (Maroof A. (2012),Following a thorough literature review, the surface sterilization agent for the current investigation was 1-1.5% sodium hypochlorite, 70% ethanol, and Nucleus free water at various treatment durations and combinations,followed by four consecutive washes with sterilized distilled water for surface sterilization and Use 100 µl of the fourth washing nucleus free water to plate on NA (nutrient agar) plates. To check sterilization efficacy, these plates were incubated for 10-15 days at 28-30°C (M. Ali *et al*. (2021).

2.2 Preparation of leaf Extraction:

The surface sterilized plant material, leaves are grinded into fine extract using mortar & pestle, add few drops of buffer PBS into it to avoid the leaf from dehydration. collect the leaf extract in microcentrifuge tubes.

3. Isolation of pure culture of Endophyte bacteria:

Endophytic bacteria are isolated, purified, and subcultured after the surface sterilization of plant material is done in this method.70 µl of the leaf extract were placed on a nutritional agar medium plate enriched with agents,the drop placed in plate is spread using L-rod in spread plate methodology.The control that washed the leaf sample in Nucleus free water is also plated.The plates were wrapped in clean wrap cling film and incubated at 22∘C with 12 h light and dark cycles for up to 6 to 8 weeks (Nalini.S M *et al*.(2014).Bacteria growing in striation were moved to new plates with Luria broth medium (LB) and agar to begin the purification process. The bacteria in the stock remained in LB at 4°C until DNA extraction. (S.A. Rhoden *et al*.(2015).

4. DNA Extraction:

Bacterial DNA isolation was carried out using column based DNA extraction.

(XploregenTM Bacterial Extraction kit, Cat. no. XPBAD22-50).

4.1 Bacterial culture plate:

Cultured Endophytic bacteria isolates were used to take the DNA extraction. Take a pinch of culture with a sterile loop.

4.2 Bacterial Broth:

DNA extraction is followed by the Xploregen kit method; Take 1 ml of sample in a 1.5 ml tube and centrifuge at 10,000 rpm for 5 minutes. Discard supernatants. Add 1 ml of **XBA 1** to the pellet and pipette mix, then add the whole solution to the beaded vial, Horizontal vortex the vial at maximum speed for 10 minutes, Add about 300 µl of **XBA 2** to the vial and Horizontal vortex the vial at maximum speed for 7 minutes. Centrifuge the tube at 10,000 rpm for 2 minutes at room temperature (RT).Transfer 950 µl of supernatant to a sterile 2 ml vial and Add 200 µl of **XBA 3** solution and vortex for 5 seconds. Centrifuge at 10,000 rpm for 2 minutes, Transfer 800 µl Supernatant to a clean sterile 2ml vial,Continue Adding 700 µl **XBA 4** solution to the supernatant and vortex for 5 Seconds and Transfer 700 µl of Lysate to the spin column (Do not discard the residual lysate) and Centrifuge at 10,000 rpm about 2 minutes and Discard the flow through.Repeat the above (12 and 13) steps to collect all the Lysate.(Ensure the entire

lysate is processed via the spin column).Add 600 µl of **XBA 5** to the spin column and Centrifuge at 10,000 rpm for 2 minutes,Discard the flow through and Add 600 µl of **XBA 6** to the spin column Centrifuge at 10,000 rpm for 2 minutes and discard the flow through.Centrifuge the empty spin column for 5 minutes at 10,000 rpm and Place the spin column into a sterile 1.5 ml vial and incubate for 2 minutes.Add 30 µl **XBA 7** to the center area of spin column and centrifuge for 5 minutes at 10,000 rpm,Place the spin column to a new sterile 1.5 ml vial.Discard the spin column and store both elution tubes for further processing.

4.3 DNA quantity analysis:

DNA quantification is a crucial step in many processes where knowing the amount of DNA present is required, such as when doing PCR techniques (Linacero, R., Rueda, J., Vázquez, A.M. (1998). The concentration of DNA is checked using a qubit fluorometer, adding drops of 1X dsDNA HS buffer into isolated DNA for the concentration analysis.

5.DNA amplification using polymerase chain reaction (PCR)

Polymerase Chain Reaction was carried out using TaKaRa Ex Taq Hot Start Version (RR006A) and was performed in PCR VERITI THERMO

SCIENTIFIC.Antibody-mediated repression is released during the initial DNA denaturation step of PCR. These enzymes can be used with standard PCRconditions.

5.1 DNA Amplification:

Total 50µl of general reaction mixture is required, PCR was done in a solution containing 5 µL buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCI), 4 µL 2.5 mM dNTPs, 0.2-1 µM 10-50 Pmol of each primer (Primer 1&2): (As most PCR products amplified with TaKaRa Ex Taq HS have one A at the 3'-termini, the obtained PCR products can be directly cloned into a T-vector.), 0.25 µL 5 U/µL of TaKaRa Ex Taq HS. Add Taq DNA polymerase, 4µL 2.5mM dNTP mixture, up to 50µL sterile purified water, and 2 µL of previously extracted sample DNA (10-20 ng/µl), under PCR conditions mentioned in Table.1.

PCR Conditions:

STEPS	TEMPERATURE	TIME	CYCLE
Initial Denaturation	98°C	30min	1cycle
Denaturation	98°C	10min	32cycle
Annealing	50°C	30min	32cycle
Extension	72°C	45min	32cycle
Final Extension	72°C	7min	1cycle
	10°C		

Table.1

5.2 Agarose Gel Electrophoresis:

After amplification, PCR products and 100bp DNA ladder (solution composed of DNA molecules of varying lengths) were loaded in 2% agarose gel for electrophoresis at 150 V. Band was observed at 1500 bp.

6. Purification and sequencing of 16S rRNA

6.1 Purification of PCR Products:

The amplified 16S genes were purified using ExoSap-IT Reagent. Enzymatic cleanup is a very efficient method for PCR purification. Adding ExoSAP-IT reagent directly to the PCR product eliminates transfer steps to tubes and conserves PCR amplicons, helping reduce the chance of cross contamination; no further processing needed.

6.2 Sanger Sequencing :

The sequencing was done by using Bigdye x Terminator V3.1 kit ,sample DNA is used as a template in a polymerase chain reaction (PCR).A mix of normal bases (dNTPs) and chain terminating bases (ddNTPs) is used in the PCR reaction,This will generate DNA fragments of different lengths.The DNA fragments are then separated by size using Capillary

Electrophoresis was done by Applied Biosystems 3730 Genetic Analyser.A laser is used to excite these fluorescently labeled bases at the end of each fragment.In the sequence, shorter fragments appear first, then progressively longer fragments. A chromatograph indicating which base is present at each place along the DNA fragment is created by recording the fluorescence of the base that ended each length of the segment.

7. Phylogenetic Analysis:

After the sequencing, the FASTA sequence of the samples were analyzed in the Nucleotide Blast programme of

NCBI (www.ncbi.nlm.nih.gov).

List of chemicals:

Sodium hypochlorite, Nuclear free water ,70% Ethanol, PBS buffer, XBA 1, XBA 2, XBA 3, XBA 4, XBA 5, XBA 6, XBA 7 (buffer), 1X dsDNA HS buffer, TaKaRa Ex Taq Hot Start Version (RR006A) kit, ExoSap-IT Reagent, Bigdye x Terminator V3.1 kit,

Equipments:

Autoclave, Laminar air flow, sterile plate, beaker with several measurements, Inoculation loop, L-rod, Micropipette, mortar & pestle, Burner, sterile cotton, Incubator, vortex apparatus, microcentrifuge tubes, centrifuge machine, qubit fluorometer, PCR VERITI THERMO SCIENTIFIC, Agarose Gel Electrophoresis, Applied Biosystems 3730 Genetic Analyser.

RESULT

Isolation of Endophytes from Pure culture:

Bacterial endophytes were isolated using fresh plant material (leaf) from the medicinal plants *Piper longum* and *Piper chaba*. Sterilisation of the surface was an essential step in eliminating epiphytic bacteria from the explant samples. Our analysis found that this step was satisfactory because the control plate (Fig.1) did not exhibit any development. As illustrated in (Fig. 2), an adequate number of colonies were found in the explants extraction (leaf) on the medium. These isolates were regarded as bacterial endophytes of the plant because there was no growth on the control plate. The obtained bacterial species were isolated on the basis of the unique features of colonies.A prominent bacterial colony from leaf tissue bacterial endophytic species were isolated.Endophytic bacteria are isolated, purified, and subcultured after the surface sterilization of plant material is done.The findings showed that the two piper species' isolated bacterial species were distinct from one another.The culturing is continued until we reach a pure culture of Endophyte bacteria is illustrated in (Fig.3) of each plant species.

DNA Extraction:

Bacterial DNA isolation was carried out using column based DNA extraction. Cultured Endophytic bacteria isolates were used to take the DNA extraction.Extracted DNA using XBA 1,2,3,4,5,6 & 7 reagents and stored the elution tubes for the further analysis. DNA quantification is a crucial step in many processes where knowing the amount of DNA present is required, the analysis shows the concentration of DNA extracted in *Piper chaba* is **3.6** & as for the *Piper longum* is **3.7**.

DNA amplification using polymerase chain reaction (PCR):

previously extracted sample DNA (10-20 ng/µl), under PCR conditions mentioned in Table.1, PCR is done by the universal primer 16s rRNA.After amplification PCR, PCR products and 100bp ladder were loaded in 2% agarose gel for electrophoresis at 150 V. Band was observed at 1500 bp as illustrated in (Fig.4).

Sequencing & Molecular identification of Endophytic bacteria:

The strain was identified by phylogenetic association using the PCR-amplified 16s rRNA gene .By measuring the fluorescence of the base that ends each length of the segment, a chromatograph is constructed that shows which base is present at each location along the DNA fragment is illustrated in (Fig.5).There are two Sanger processes involved in sequencing a DNA region: forward and reverse for each piper species.The trimmed FASTA of the forward and reverse sequence of *piper chaba* ; Forward FASTA contains 901

bp(ATACATGCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCG
GACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA
AACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGA
CGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGGGG
TAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTAACTGCTCGCA
CCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG
AAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGT
AACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTG
CTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAA
CTCAAGGGAATTGACGGGGGCCCGCACAAGCGGTGG)

as for the Reverse FASTA contains 867 bp

(GGCTGGCTCCATAAAGGTTACCTCACCGACTTCGGGTGTTGCAAACTCTCGTGGT
GTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATC
CGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAA
CTGAGAACAGATTTATGGGATTGGCTAAACCTTGCGGTCTTGCAGCCCTTTGTTC
TGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGA

ATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGG
AAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGC
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGC
TGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGT
GGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTC
AGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCA
TTTCACCGCTACACGTGGAAATTCCACTCTCCTCTTCTGCACTCAAGTTTCCCAGT
TTCCAATGACCCTCCCCGGGTTGAGCCGGGGGCTTTCACATCA)

And the trimmed FASTA of *piper longum* contains 885 bp

(TGCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
AGCTAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTC
GGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCG
TAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGCACCTTGA
CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTT
TCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACT
GGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATG
CGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG
ACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCA
GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAA
AGGAATTGACGGGGGCCCGC)

of forward FASTA and 881 bp

(GGCTGGCTCCATAAAGGTTACCTCACCGACTTCGGGTGTTGCAAACTCTCGTGGT
GTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATC

CGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAA
CTGAGAACAGATTTGTGGGATTGGCTAAACCTTGCGGTCTCGCAGCCCTTTGTTC
TGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGT
CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGA
ATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCGAAGGG
AAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGC
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGC
TGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGT
GGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTC
AGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCA
TTTCACCGCTACACGTGGAAATTCCACTCTCCTCTTCTGCACTCAAGTTTCCCAGT
TTCCAATGACCCTCCCCGGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAAC
CG) of Reverse FASTA.

The sequencing result is Illustrated in Table 2.The amplified 16s rRNA gene was sequenced using the chromatogram illustrated in Fig .4 as mentioned above and matched using BLAST search with strain sequences that were similar and found in the NCBI database.Several similarly resembling sequences were produced by the multiple sequence alignment process that was received from the NCBI database.The findings show the similarity of isolated endophyte bacteria in sample 1 ,*piper chaba* is 99.89% Identical to *Bacillus Pumilus* and Sample 2 *Piper longum* is 99.89% Identical to *Bacillus Aerius*.Using the basic local alignment search tool (BLAST) search, the nucleotide sequences with the closest identities were obtained from the NCBI database for analysis. The sequences that shared the most similarities with each other were chosen for phylogenetic analysis after the 16S rRNA gene sequence underwent filter search. The dendrogram was generated in accordance with the similarities within the species as illustrated in Fig.6 & 7.

Sample name	Primer name	Raw length (bp)	Trimmed length (bp)	Trim start	Trim end	Average QV score
Piper chaba	Forward	1191	901	17	918	51
Piper chaba	Reverse	1155	867	18	885	50
Piper longum	Forward	1187	885	22	907	49
Piper longum	Reverse	1178	881	17	898	50

Table.2

Fig.1 control plate

Fig. 2 colony of multiple endophytic bacteria in culture plate **(a)** *piper chaba* & **(b)** *piper longum*

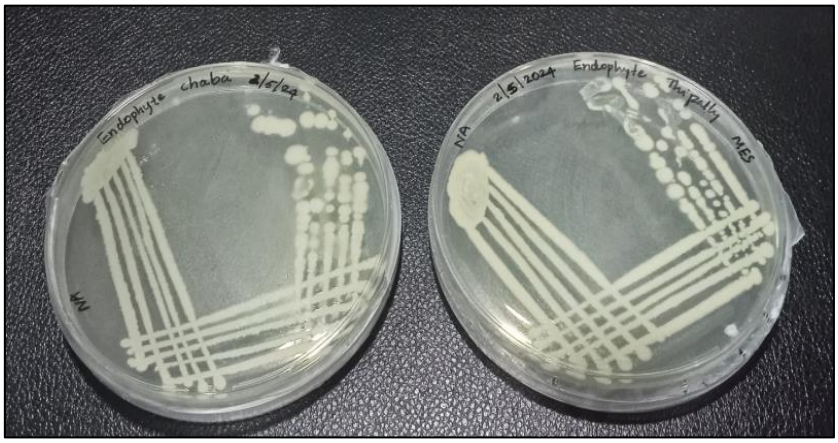

Fig.3 pure cultured endophyte bacteria in medium **(a)** *piper chaba* & **(b)** *piper longum*

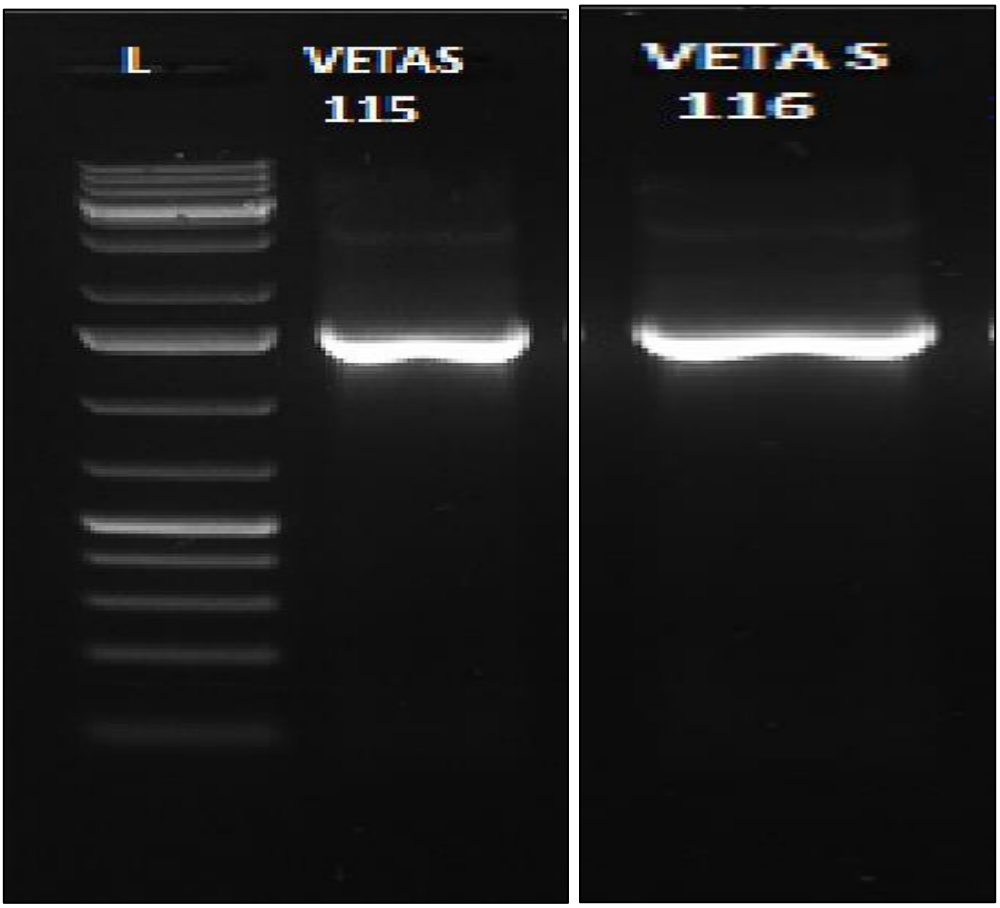

Fig.4 Agarose gel electrophoresis image of isolated gDNA (genomic DNA) of different bacterial isolates on 2% (w/v) agarose gel band appears to be 1500 bp.

VETAS : Endophytic bacterial isolates.**LAD:** Marker (100 base pair DNA ladder).

VETAS 115 - *Piper chaba*, **VETAS 116 -** *Piper longum*.

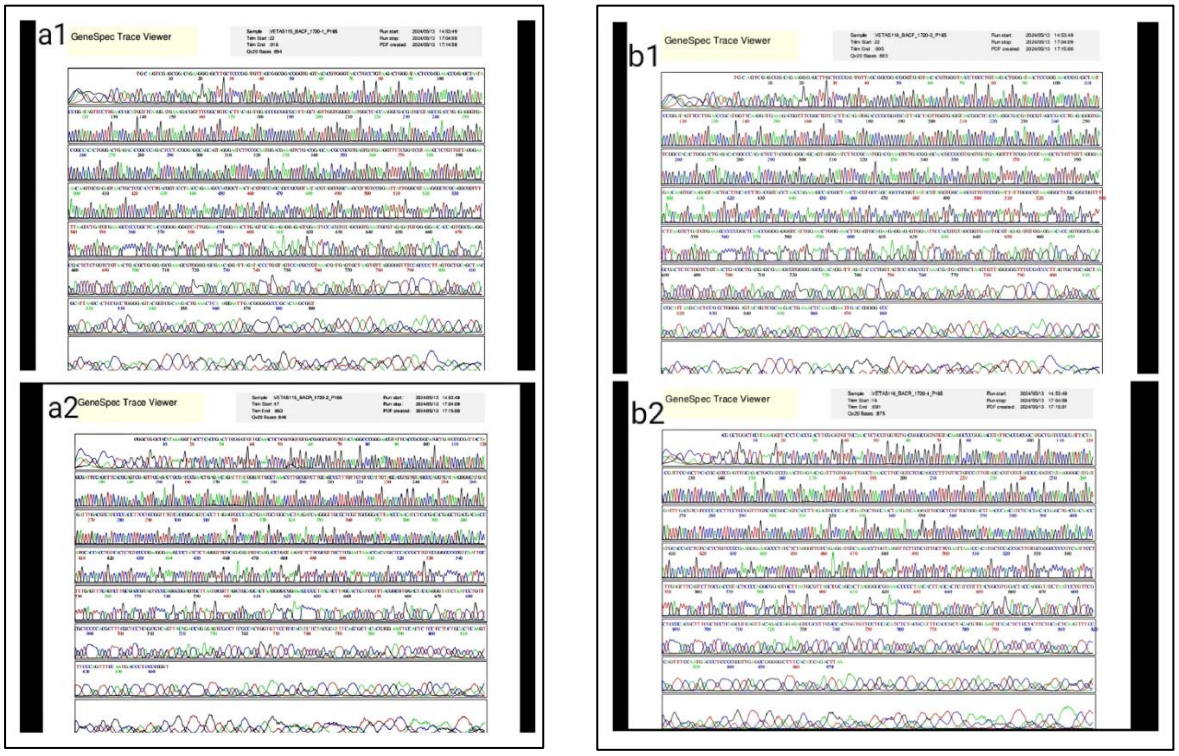

Fig 5 Chromatogram of DNA fragments isolated from *Piper chaba* and *piper longum* respectively.

(a1.) Forward of *piper chaba*, **(a2.)** Reverse of *piper chaba*. & **(b1.)** Forward of *piper longum*, **(b2.)** Reverse of *piper longum* .

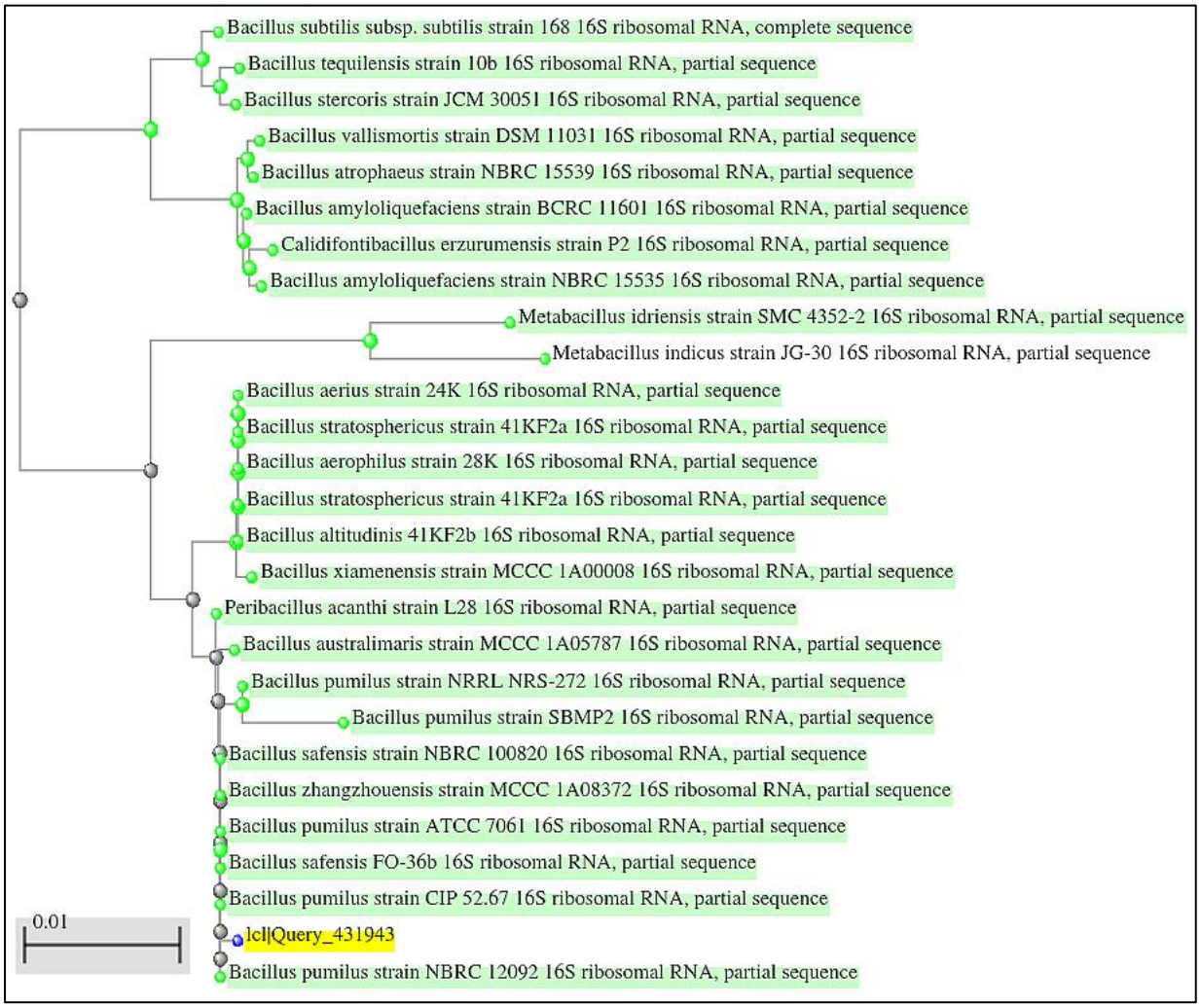

Fig.6 Dendrogram representing *piper chaba*. Phylogenetic tree retrieved from BLAST analysis showing the evolutionary relationship of *Bacillus pumilus* with its closest BLAST hits based on multiple sequence alignment.

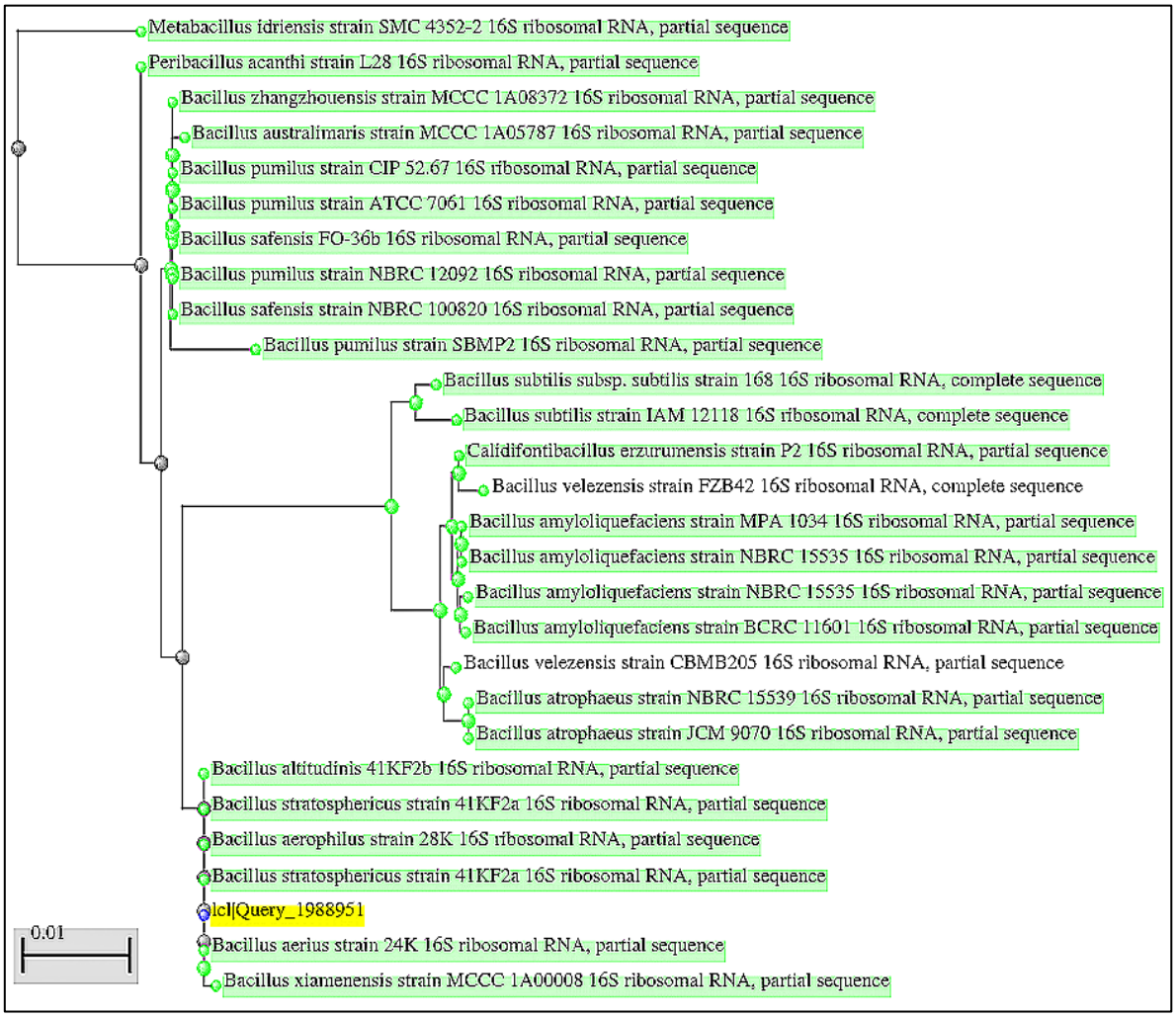

Fig.7 Dendrogram representing *piper longum* Phylogenetic tree retrieved from BLAST analysis showing the evolutionary relationship of *Bacillus aerius* with its closest BLAST hits based on multiple sequence alignment.

DISCUSSION

Study relies on the Endophyte bacterial analysis in selected study area Kottakkal Arya Vaidya Sala, the selected plants are important in Medicinal Plants, such as *Piper chaba* and *Piper longum* belongs to the piperaceae family. Endophytes are known to occupy intercellular gaps based on their microecology. (Bacon and Hinton, 2002; Chanway, 1998; McCully, 2001)They have the ability to produce significant bioactive metabolites that are used in modern medicine.The study's plant subjects have historically been employed as traditional medicines.Chaba exhibits several biological properties such as anti-inflammatory, analgesic, anti-diarrheal, anti-diabetic, anti-microbial, anti-parasitic, anti-malarial, anti-leishmanial, adipogenic, cytotoxic/anticancer, gastro-protective, anti-ulcer, diuretic, depressed, anti-hypertensive, and antipyretic characteristics. (Godecke, T., Kaloga, M. & Kolodziej, H. A (2023). *Piper longum* is used to treat a variety of conditions, including respiratory infections, spleen illnesses, tumors, chronic malaria, diarrhea, cholera, asthma, constipation, gonorrhea, paralysis of the tongue, and bronchitis. (Kumar, S., Kamboj, J., Suman, & Sharma, S. (2011). These plants were not found to be studied on the Endophyte bacterias consisting in it.The *Piper chaba* is used as an adulterant instead of *Piper longum* (Kumar P.S (2014). The piper species were kept for additional research on the isolation of endophytic bacteria, Numerous Endophyte colonies are seen in both plants.16S rRNA sequencing was used to isolate and better identify a variety of species. prominent endophyte bacteria have been isolated and identified.The 16S rDNA gene sequence of each bacterium is species-specific and hence can be utilized for accurate bacterial identification (Jin *et al*. (2014).The isolated endophytic bacteria in *Piper chaba* is *Bacillus Pumilus* and as for *Piper longum* is *Bacillus aerius*.These sequences, together with those that were aligned from databases, were used to build a phylogenetic tree.The similarity of endophyte bacteria isolated from researched medicinal plants is displayed by the dendrogram. The dendrogram of *Bacillus pumilus* & *Bacillus aerius* represent the relationships of similarity among a group of entities.61 Indian medicinal plants from 33 distinct families that are used to treat a range of infectious diseases, were examined for their antimicrobial properties. Screening was carried out at 1000 and 500 μg/ml concentrations by agar dilution method against *Bacillus cereus* var mycoides, *Bacillus pumilus*, *Bacillus subtilis*, *Bordetella bronchiseptica*, *Micrococcus luteus*, *Staphylococcus aureus*, etc (Kumar P.V., Chauhan S.N., Padh H., & Rajani M.,(2006).Plant

Growth-Promoting Bacteria (PGPB) are a viable substitute for traditional fertilization. One of the more intriguing PGPB strains, among the spore-forming bacteria of the phylum Firmicutes, is *Bacillus pumilus.B. pumilus* is one of the most intriguing Bacillus species that can be utilized for the biocontrol of fungal phytopathogens because of its variety of biocontrol properties,*B. pumilus* can promote plant growth regardless of whether it alters the native microbiota or not.(Dobrzyński, J., Jakubowska, Z., & Dybek, B. (2022).The antagonistic characteristics of *B. xiamenensis* and *B. pumilus* endophytic strains in preventing downy mildew infection in grapevine seedlings(Zhang *et al*.2017).The endophytic Bacillus species are becoming significant strains as a plant growth stimulant and as a biological control agent for the prolonged maintenance of plant health. In enhancing wheat development and biologically controlling plant diseases (Zhao *et al*. 2015).*B. aerius* CMCPS1, isolated from hot springs, revealed the maximum hydrolytic activity.The multifunctional cellulase complex of *B. aerius* CMCPS1 is a potential biocatalyst for application in lignocellulosic biomass-based biorefineries.(Ganesan, M., Mathivani Vinayakamoorthy, R., Thankappan, S. *et al*. (2020).Thermophilic bacteria, especially from the genus Bacillus, make up a huge potential source of novel enzymes that could be applicable for biotechnological applications. In this work, we described the cellulose and hemicellulose-related enzymatic activities of the hot spring *Bacillus aerius* CCMM B940 from the Moroccan Coordinated Collections of Microorganisms (CCMM), and revealed its potential for hemicellulosic biomass application. The strain was also capable to grow on agriculture waste such as orange and apple peels as the sole carbon source (Maski, S., Ngom, S.I., Rached, B. *et al*. (2021).

CONCLUSION

The research on *Piper longum* and *Piper chaba* has revealed a diverse population of endophytic bacteria that play significant roles in the growth, development, and health of these medicinal plants. The study highlighted the presence of beneficial endophytes, such as those from the Bacillus genus, known for producing bioactive compounds like indole acetic acid, which promotes plant growth, and antibiotic substances that protect against harmful bacteria. These findings underscore the potential applications of endophytic bacteria in agriculture and pharmaceuticals, offering new avenues for developing natural biofertilizers, biopesticides, and therapeutic agents.

FINDINGS

- A diverse population of endophytic bacteria was identified in the tissues of *Piper longum* and *Piper chaba* using 16S rRNA gene sequencing.
- Certain bacteria, such as those from the Bacillus genus, produce bioactive compounds with antimicrobial, antioxidant, and anticancer properties.
- Indole acetic acid produced by some endophytic bacteria promotes plant growth.
- Endophytic bacteria with antibacterial properties were found, which may help protect the plants from diseases.

FUTURE STUDIES

- Further research is needed to explore the specific mechanisms by which these endophytic bacteria enhance plant health and productivity.
- Investigate the potential for scaling up the application of beneficial endophytes in agricultural practices.
- Study the ecological impacts and sustainability of using endophytic bacteria in agriculture.
- Explore the development of new pharmaceutical agents based on the bioactive compounds produced by these endophytes.
- Address the issue of interspecies adulteration by thoroughly characterizing the endophytic bacterial communities of closely related medicinal plants.

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