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Exploring Stress-Tolerant Endophytes From Leucas Aspera Root For Climate-Resilient Agriculture

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Abstract
In agriculture, endophytic bacteria are becoming more environmentally friendly than chemical inputs because of their capacity to increase plant development and resilience to stress. Thirty bacterial isolates were isolated from Leucas aspera roots for the present study. After a preliminary screening for plant growth-promoting (PGP) characteristics, four isolates with robust activity were chosen. Following biochemical characterisation, these isolates were assessed for their ability to withstand biotic (antagonism against phytopathogens) and abiotic (temperature, salinity and drought) stress. Followingly, the impact of the chosen isolates on tomato seed germination and seedling development was evaluated. Two isolates out of the four showed notable growth-promoting activity: LAR04 was more successful in root formation and LAR25 consistently increased shoot biomass indicating isolate-specific advantages. LAR25 was identified as Bacillus pumilus by molecular identification using 16S rRNA gene sequencing. Along with resilience to abiotic stresses and antifungal activity against phytopathogens, both isolates exhibited a variety of PGP characteristics such as phosphate solubilization, indole-3-acetic acid synthesis, urease activity and oxidase activity. On comparison to uninoculated controls, inoculating tomato seedlings with these isolates greatly increased biomass accumulation, root and shoot length and general vigor. The work highlights the potential of endophytes derived from Leucas aspera as viable bioinoculants for sustainable tomato production and offers a basis for creating environmentally friendly microbial formulations to improve crop resilience and productivity.
Keywords: *Endophytic bacteria, PGP traits, Abiotic stress, Biotic stress, Bioinoculant.*

1. INTRODUCTION

Endophytes are referred to microorganisms that reside within plant tissues without causing harm, forming symbiotic relationship. Unlike epiphytic bacteria which inhabit plant surface and are easily removed by chemical disinfectants like sodium hypochlorite and ethanol while endophytes persist within internal plant tissue and are unaffected by this treatment [1]. These microorganisms are increasingly recognized for their crucial role in enhancing plant resilience and productivity. They help plant in various ways by promoting plant health, increasing mineral nutrient availability, fixation of atmospheric nitrogen, production of phytohormones such as auxin and gibberellins and imparting stress tolerance against biotic and abiotic stresses [2]. The population density of endophytic bacteria can vary from 10^2 to 10^9 colony-forming units per gram of tissue, depending on several factors including the plant species, tissue type, developmental stage, genotype, environmental conditions and microbial interactions phytopathogens [3]. The interaction between endophytic bacteria and their host plant is not completely understood. Given their adaptability and functional versatility, endophytic bacteria have gained attention as promising microbial resources. They are now considered a focal point in fields such as botany, microbiology, plant breeding and plant protection [4]. Biological control (biocontrol) refers to the use of beneficial microbes or their metabolites to suppress plant pathogens in an eco-friendly way. Endophytic bacteria, especially spore-forming strains like Bacillus offer advantages in colonization, stability and field performance, though few have reached commercial use due to survivability challenges. Successful application depends on host immunity, pathogen stage, plant physiology and environmental conditions [5]. Recent studies have demonstrated that endophytic bacteria and fungi such as Bacillus velezensis, Pseudomonas sp. and Penicillium brevicompactum can significantly reduce plant diseases like wilt, root rot and blight by activating host defense and producing antifungal compounds. These findings highlight the promising role of endophytes as eco-friendly biocontrol agents in sustainable crop protection [6].

The medicinal plant Leucas aspera is commonly known as “thumba” or “thumbai” belonging to the family Lamiaceae. It harbours various pharmacological activities like antifungal, antioxidant, antimicrobial, antinociceptive and cytotoxic activity. It is known to produce numerous phytochemicals such as triterpenoids, oleanolic acid, ursolic acid, b-sitosterol, nicotine, sterols, glucoside, diterpenes and phenolic compounds [7]. Medicinal plants are valuable sources of bioactive compounds with therapeutic potential,

but their natural production often occurs at extremely low concentrations. This limited yield poses significant challenges in terms of extraction, purification and large-scale application [8]. Interestingly, recent studies have shown that endophytic microorganisms living within these plants can produce the same or structurally similar bioactive metabolites as their host [9]. These endophytes, by mimicking or sharing biosynthetic pathways with their plant partners, offer an alternative and sustainable source for these important compounds. This opens up exciting possibilities for biotechnological applications where endophytes could be harnessed to produce high-value plant-derived compounds more efficiently and sustainably by passing the limitations of traditional plant harvesting.

Today's agricultural productivity faces challenges that were never before seen because of growing disease pressure, soil degradation and climate variability. Conventional methods that mostly rely on chemical pesticides and fertilizers are no longer viable and frequently result in diminished soil fertility and environmental damage over time. The majority of current research focuses on particular characteristics or well-known agricultural systems, despite the fact that microbial-based solutions have demonstrated considerable promise. This creates a large vacuum in the identification of multifunctional microorganisms that can flourish under challenging stress circumstances. Endophytic bacteria have shown promise as agents for sustainable agriculture in this context. However, there is still a need to investigate lesser-known plant species for novel endophytes with plant growth-promoting properties, abiotic stress tolerance and biocontrol potential. Due to their natural bioactive qualities, medicinal plants like Leucas aspera may remain as undiscovered sources of these helpful microorganisms. In order to find promising candidates that could support climate-resilient and environmentally friendly farming practices, this project will isolate and characterize stress-tolerant endophytes from Leucas aspera. These multipurpose endophytes have the potential to be sustainable substitutes for synthetic inputs, enhancing crop productivity while preserving the environment. In this study, we aimed to explore the endophytic bacteria residing in the roots of Leucas aspera and evaluate their potential to enhance plant growth. Our objectives included isolating and characterizing these bacterial strains, assessing their plant growth-promoting traits such as indole-3-acetic acid production, phosphate solubilization and enzymatic activities and testing their ability to withstand abiotic stresses like salinity, drought and high temperature. Additionally, we investigated their antifungal potential against important phytopathogens and examined the effects of the most promising isolates on the growth and biomass of tomato seedlings under controlled conditions.

2. METHODOLOGY

2.1 Sample collection and isolation of bacterial endophytes

Infection free healthy plant roots of Leucas aspera were collected from three different sites in Tirunelveli district to ensure maximum diversity of endophytic bacterial isolates. The first site was an agricultural field near Ramayanpatti, representing cultivated soils exposed to regular fertilizer and irrigation practices. The second site was a semi-arid uncultivated land in Radhapuram, characterized by dry, nutrient-poor soils and harsh environmental conditions. The third site was a moist riverbank ecosystem along the Tamirabarani River which provided a contrasting environment rich in organic matter and microbial diversity and transported to the Microbial Biotechnology Laboratory. The plant roots were washed repeatedly under tap water to remove adhering soil particles, debris and epiphytic microorganisms. The samples were surface sterilized by sodium hypochlorite and inoculated on nutrient agar plates for isolation of bacterial endophytes. Roots were carefully surface-sterilized using 70% (v/v) ethanol for 30 sec and with 3% (v/ v) sodium hypochlorite for 3 min, finally the samples were washed thrice with sterile double distilled water for 2 min. The final wash was spread on nutrient agar plate as a control plate, to ensure proper surface sterilization. All the procedures were performed under aseptic conditions [10].

Surface sterilized plant roots were dried on sterile filter paper and cut into of about 2-3 mm and placed on nutrient agar plates in triplicate and incubated at 37°C for 72 h with daily observation. Other roots per individual plant were crushed in 10 mL sterile saline solution using a sterile homogenizer and 1 mL of the suspension was serially diluted until 10^{-3} from which a 0.1 mL aliquot was spread onto each of the three Petri plates containing LB medium and incubated in the dark at 35 ± 2°C [11]. After incubation few representative colonies appearing on the Petri plates were picked-up using sterile loop. Morphologically distinct colonies were selected and purified by sub-culturing. Pure cultures of these isolates were processed for further morphological, biochemical and molecular characterization. Individual

bacterial colonies of each isolate were cultured on nutrient broth and stored in 20% sterile glycerol at -80°C for further studies.

2.2 Characterization of Bacterial Endophytes based on Plant Growth Promoting (PGP) Traits

2.2.1 Screening for Indole-3-acetic acid (IAA) production

Gordon and Weber's (1951) method was used to determine IAA production qualitatively with slight modifications. Yeast Peptone-Mannitol (YPM) broth with tryptophan (100 mg /ml) were inoculated with bacterial isolate and incubated at 30°C with continuous shaking at 100 rpm under dark conditions after 72 h culture broth was centrifuged for 10 min at 10,000 rpm. About 2 ml of Salkowski reagent (A mixture of 0.5 M ferric chloride ($FeCl_3$) and 70% perchloric acid ($HClO_4$)) was added to 1 ml of culture supernatant and incubated at dark room for 30 min, appearance of pink color confirmed the production of IAA by bacterial endophytes. The test was performed for qualitative analysis (development of color) indicated the IAA production abilities. After incubation optical density was measured at 530 nm. A calibration curve was made with 0, 0.5, 1, 2, 5, 10, 15, 20, 25, 30 µg of IAA/mL [12].

2.2.2 Ammonia production and nitrogen fixation

Isolated endophytic bacterial isolates were inoculated into Peptone Water Broth medium and incubated at 28 ± 2°C, 150 rpm for 48 h. The bacterial cells were removed by centrifugation at 8,000 rpm for 5 min. To the supernatant, Nessler's reagent was added in a 2:1 ratio (v/v). Yellow to brown color development indicated ammonia production. Ammonium sulfate (0.1-5.0 µmol mL^{-1}) was used as a standard to quantify the amount of ammonia production and the absorbance of which was measured at 450 nm by a multimode reader and expressed as µmol mL^{-1}. The experiment was done in triplicates [13].

Nitrogen-fixing abilities of the isolates were determined qualitatively by culturing in Nitrogen-free (NF) medium containing (g L^{-1}) mannitol 20 g, K_2HPO_4 0.2 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.2 g, K_2SO_4 0.1 g, $CaCO_3$ 5 g and agar 20 g and Jensen's agar [14]. The growth of the isolates was monitored after incubation at 28 ± 2°C for 48 h.

2.2.3 Hydrogen Cyanide (HCN) Production

Production of HCN was detected according to the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine L^{-1} and 2.0 M NaCl. Bacteria were streaked on agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36±2°C for four days. Changes in the color of the filter paper from yellow to light brown color or reddish-brown indicated the HCN production activity [15].

2.2.4 Phosphate Solubilization

The bacterial isolates were screened for phosphate solubilising property, based upon visual observation using the procedure described by Xiaomei Yan et al., 2018. Phosphate solubilization activity was determined by using Pikovskayas agar (glucose 10 g, $Ca_3(PO_4)_2$ 5g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, KCl 0.2 g, $FeSO_4.7H_2O$ 0.002 g, yeast extract 0.5 g, $MnSO_42H_2O$ 0.002 g, agar 20, $d.H_2O$ 1L). The media inoculated with the isolates were incubated for 48 h. After incubation, phosphate-solubilizing isolate would form a clear halo zone around the bacterial colony [16].

2.3 Abiotic and Biotic Stress Tolerance Activity

2.3.1 NaCl Tolerance Test

Endophytic isolates were screened for salt-tolerance properties using by culturing them in nutrient broth and nutrient agar (NA) media supplemented with various levels of NaCl, i.e., 0.5, 1.5, 3, 5, 7 and 9% (w/v). Bacterial culture without salt was used as control. The plates were inoculated with fresh culture following the streak plate method and incubated for 48 h at 30°C and the growth on the NaCl-supplemented plates was compared with the control plate. The Optical density for the selected isolates at 600 nm were compared with the control. Triplicates were taken for each isolate [17].

2.3.2 High Temperature

All the isolates were tested for growth at high temperature on YMA plates. Freshly grown isolates were spotted four times as replications for a single isolate on YMA plates and incubated at 60°C and 70°C for 72 h. Bacterial growth was measured based on visible changes in growth and colony size on YMA plates [18].

2.3.3 Drought Tolerance

Drought-stress tolerance was screened using Trypticase Soy Broth (TSB), supplemented with different concentrations of Poly Ethylene Glycol (PEG) to provide the water potential of -1.0 MPa and -1.5 MPa.

These were inoculated with the overnight grown broth cultures of selected organisms with adjusted optical density (OD) of 0.1 at 600 nm. Growth of the isolates at various stress levels was estimated by measuring the OD at 600 nm after the incubation at 28°C for 24 h [19].

2.3.4 Antifungal Activity

Bacterial endophytes were tested in vitro for their antagonistic activity towards the plant pathogenic fungi, Bipolaris spicifera and Alternaria alternata obtained from the Department of Microbial Technology, Maduri Kamaraj University, Madurai. Potato dextrose agar was prepared (as per standard procedure) by Caleb Erhonyota et al., 2023 and poured into the Petri dish which was swabbed with Bipolaris spicifera and Alternaria alternata growing culture for 24 h. A cork borer was used to create the wells (10 mm in diameter). The endophytic strains were loaded in the wells at different concentrations (5 µg, 10 µg, 20 µg, 50 µg) according to the labelled concentrations. The plates were further incubated at room temperature for 24 h and the inhibition diameter was measured. All the assays were carried out in triplicates [20].

2.4 Biochemical Characterization

Based on morphology, the standard identification protocols were performed to characterize the bacterial isolates. Morphology, arrangement and appearance of the bacterial cell wall was determined by Gram's staining technique [21]. Biochemical activities of bacterial endophytes viz, oxidase test, urease activity, citrate utilization test, methyl red, Voges Proskauer test and gas production were performed following the procedures by Cappuccino and Sherman (2002)**.**

2.5 Effect of the elected isolates on seed germination of Tomato Seeds

Surface-sterilized tomato (Solanum lycopersicum) seeds were used to conduct seed germination assay. The seeds were disinfected with 70% ethanol for 1-2 min, followed by treatment with 2% sodium hypochlorite for 2-3 min and subsequently rinsed several times with sterile distilled water to remove any residual sterilant. They were then inoculated with bacterial suspensions of selected isolates (10^8 CFU/mL) for 2-4 hours, while control seeds were soaked in sterile distilled water. The treated seeds were transferred to sterile Petri plates lined with moistened Whatman filter paper and incubated at 25 ± 2°C under a 12 h light/dark cycle [22]. Although this assay was performed, the results are not enclosed in this manuscript. Following this, a plant growth assay was carried out. Germinated seeds were sown in sterilized soil pots and maintained in a greenhouse under controlled conditions (25-28 °C, 12 h light/12 h dark). Plants were watered at regular intervals and after 30 days of growth, parameters such as Root Length (RL), Shoot Length (SL), Fresh Root Weight (FRW), Dry Root Weight (DRW), Fresh Shoot Weight (FSW) and Dry Shoot Weight (DSW) were recorded. Each treatment was performed in triplicates with ten plants per replicate.

2.6 Molecular Characterization

The identification of the isolates LAR04 and LAR06 were carried out by complete 16S rRNA gene sequence analysis and phylogenetic studies (MacroGen Inc., South Korea) through Sanger's DNA sequence method using the universal primer i.e., 27F and 1492R for gene amplification. The 16S rRNA gene sequences were compared to sequences in the public database using the Basic Local Alignment Search Tool (BLAST) [23] on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov). The gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. Bootstrap analysis was done using MEGA 6 (**www.megasoftware.net**) [24]. The phylogenetic tree was constructed by maximum parsimony method.

2.7Statistical Analysis

All experiments were conducted in triplicates and the results were expressed as mean ± Standard Error of the Mean (SEM). Statistical analysis were performed using GraphPad Prism (version 10.6.1). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine significant differences among treatments for tomato seedling growth parameters. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 Isolation of Endophytic Bacteria

Fresh root tissue of the medicinal plant, Leucas aspera was used for the isolation of bacterial endophytes. Surface sterilization was a critical step for removing the epiphytic microbes from sample explants. This step proved satisfactory in our study due to the absence of any growth on the control plate. Adequate

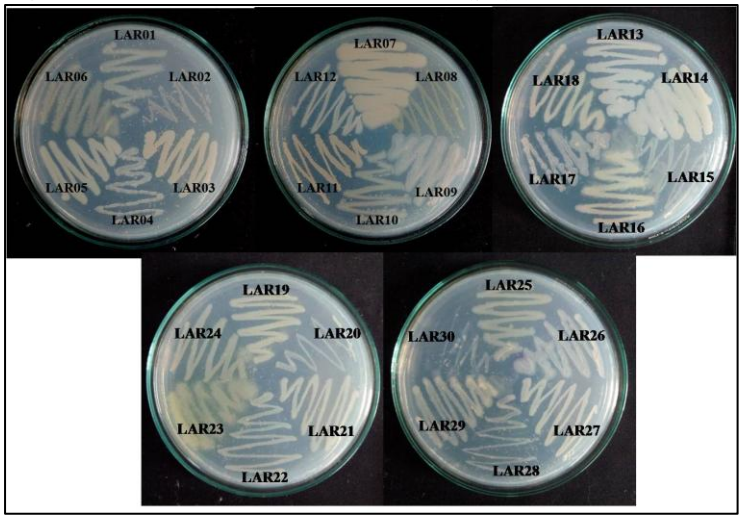
number of colonies were observed on the edges of plant root on LB agar media and these isolates were considered as bacterial endophytes of Leucas aspera plant as no growth existed on the control plate. Colonies with different morphology were selected and characterized further. Altogether, thirty bacterial colonies were isolated and designated as LAR01 to LAR30 respectively. From Abishekapatti, located near Manonmaniam Sundaranar University, eight isolates (LAR01-LAR08) were obtained. From Tamirabarani, twelve isolates (LAR09-LAR20) were recovered. Lastly, from Ramayanpatti, ten isolates (LAR21-LAR30) were obtained (Table 1, Figure 1). The bacterial isolates were confirmed to be endophytes as no bacterial colonies were observed in the control plates.

Table 1. Distribution of endophytic bacterial isolates from different collection sites of Leucas aspera

Site	Location	Number of Isolates	Isolate Codes	Remarks
1.	Abishekapatti	8	LAR01-LAR08	Likely lower microbial load
2.	Tamirabarani River	12	LAR09-LAR20	High microbial diversity
3.	Ramayanpatti	10	LAR21-LAR30	Moderate microbial diversity

Table 1 shows the isolate recovery varied by site with the highest diversity at Tamirabarani River, moderate diversity at Ramayanpatti and the lowest at Abishekapatti.

Figure 1. Pure culture of Isolated Endophytic Bacteria



3.2 Characterization of Bacterial Endophytes based on Plant Growth Promoting (PGP) Traits

3.2.1 IAA Production

Auxin are among the most widely studied plant growth regulators with indole-3-acetic acid (IAA) being the best known and most active form. IAA plays a central role in plant development by triggering quick responses such as cell elongation, as well as longer-term effects like cell decision and differentiation. Six bacterial isolates in our study were able to produce Indole-3-acetic acid (IAA) while growing in Yeast Peptone-Mannitol (YPM) broth with 100 mg/ml of tryptophan. The emergence of pink colour confirmed the production of IAA by the bacterial endophytes. Among all 30 isolates maximum IAA production was recorded in isolates LAR06 (87.90±0.43µg mL^{-1}), LAR21 (77.11±0.15µg mL^{-1}), LAR22 (67.55±1.21µg mL^{-1}), LAR23 (37.53±0.48µg mL^{-1}), LAR24 (25.13±0.45µg mL^{-1}) and LAR25 (84.6±0.82 µg mL^{-1}) are shown in the (Figure 2).

3.2.2 Ammonia Production

Ammonia is a secondary metabolite that microorganisms make by hydrolysing urea into ammonia and CO_2 which is essential for plant growth and development. The addition of Nessler's reagent caused the inoculation broth to turn brown instead of yellow indicating that the isolates LAR04, LAR06, LAR21 and LAR25 generated ammonia. The outcome is shown in Figure 2.

3.2.3 Nitrogen Fixation

Jensen's agar mediums are nitrogen-free media used to identify bacteria that fix nitrogen. The ability of these bacteria to fix atmospheric nitrogen is demonstrated by the development of endophytic bacteria on medium. Twenty-three isolates were observed to be positive for nitrogen fixation. Growth was examined on streaking the isolates on Jensen media of which 23 isolates exhibited full growth in LAR01, LAR02, LAR06, LAR11, LAR12, LAR13, LAR16, LAR17, LAR18, LAR23, LAR26, LAR27 and LAR30, while LAR03, LAR04, LAR07, LAR09, LAR14, LAR15, LAR20, LAR22, LAR25 and LAR29 rendered partial growth, while the remaining isolates exhibited absence of growth.

3.2.4 HCN Production

Qualitative screening of Hydrogen Cyanide (HCN) production revealed a distinct color shift of the filter paper from orange to dark brown indicating positive results and the absence of color change was considered negative. Ability for hydrogen cyanide synthesis was observed in the isolates LAR01, LAR02, LAR03, LAR04, LAR06, LAR12, LAR13, LAR21, LAR25 and LAR28.

3.2.5 Phosphate Solubilization

On Pikovskaya's agar medium, the endophytic bacterial isolates' capacity to solubilize phosphate was evaluated. Positive phosphate solubilization activity was shown by the development of distinct halo zones surrounding the bacterial colonies. LAR01, LAR04, LAR06, LAR21 and LAR25 were the examined isolates that showed phosphate-solubilizing capacity, the other isolates were deemed negative for this characteristic which was exhibited through absence of appearance of discernible halo zones.

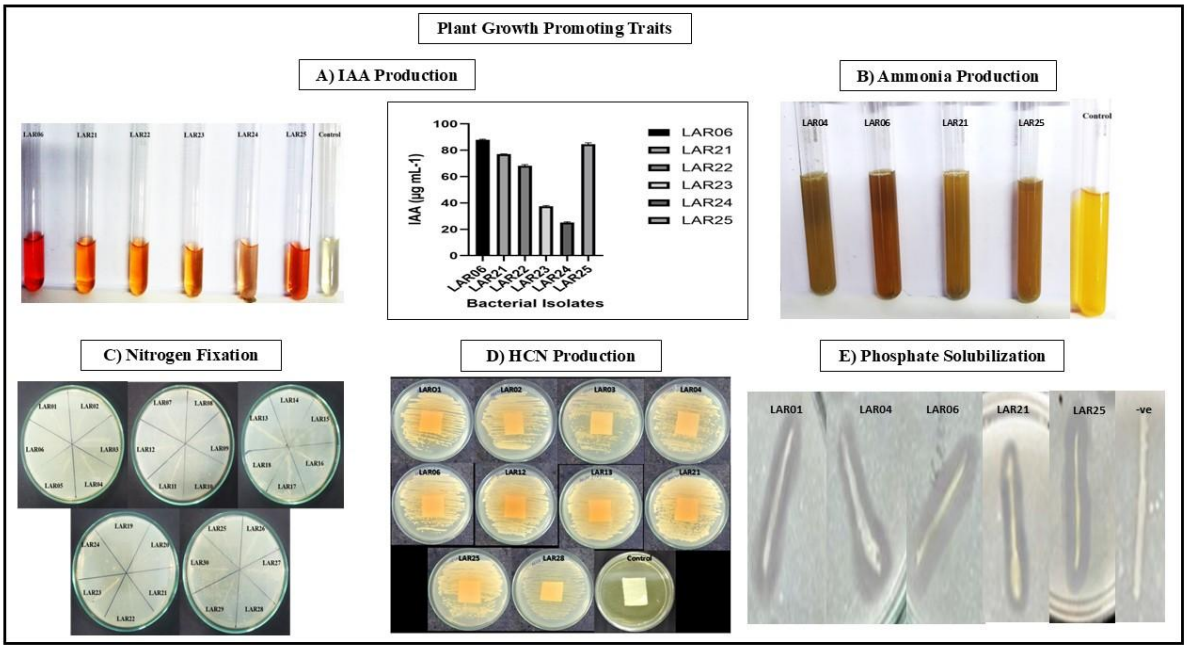
Table 2: Plant Growth Promoting (PGP) traits of endophytic bacterial isolates from Leucas aspera

Bacterial Isolates	Plant Growth Promoting Traits				
	IAA Production	Ammonia	Nitrogen Fixation	HCN Production	Phosphate Solubilization
LAR01	-	-	+	+	+
LAR02	-	-	+	+	-
LAR03	-	-	-	+	-
LAR04	-	+	-	+	+
LAR05	-	-	-	-	-
LAR06	+	+	+	+	+
LAR07	-	-	-	-	-
LAR08	-	-	-	-	-
LAR09	-	-	-	-	-
LAR10	-	-	-	-	-
LAR11	-	-	+	-	-
LAR12	-	-	+	+	-
LAR13	-	-	+	+	-
LAR14	-	-	-	-	-
LAR15	-	-	-	-	-
LAR16	-	-	+	+	-
LAR17	-	-	+	+	-
LAR18	-	-	+	-	-
LAR19	-	-	-	-	-
LAR20	-	-	-	-	-
LAR21	+	+	-	+	+
LAR22	+	-	-	-	-
LAR23	+	-	+	-	-
LAR24	+	-	-	-	-
LAR25	+	+	-	+	+
LAR26	-	-	+	-	-
LAR27	-	-	+	-	-
LAR28	-	-	-	+	-

LAR29	-	-	+	-	-
LAR30	-	-	+	-	-

The table summarizes the plant growth promoting (PGP) traits including indole-3-acetic acid (IAA) production, ammonia production, nitrogen fixation, hydrogen cyanide (HCN) production and phosphate solubilization exhibited by the endophytic bacterial isolates from Leucas aspera root. The symbol (+) indicates the presence of a trait, while (-) indicates absence.

Figure 2: Plant Growth Promoting Traits

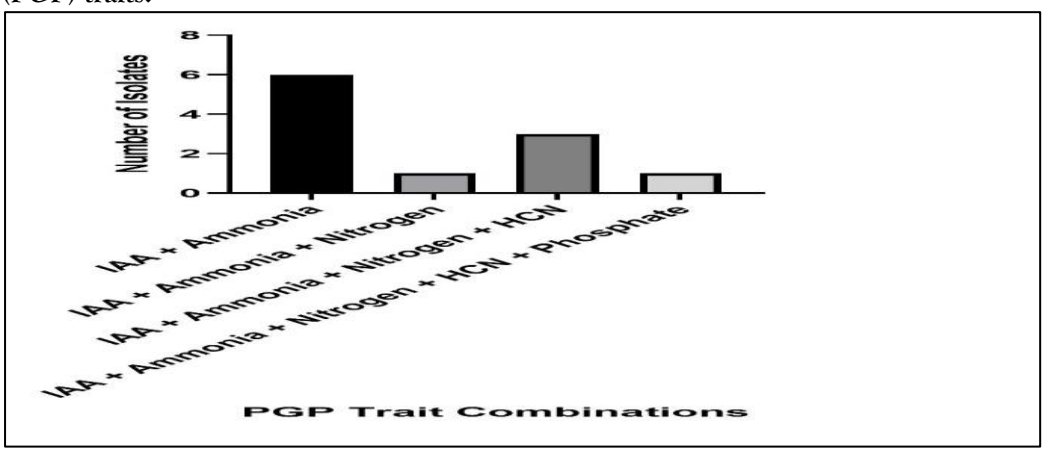


Plant growth-promoting traits of endophytic bacterial isolates from Leucas aspera root. (A) IAA production, (B) Ammonia production, (C) HCN production, (D) Siderophore production, (E) Phosphate solubilization, and (F) Nitrogen fixation.

Table 3: Distribution of Bacterial Isolates Based on Combined Plant Growth-Promoting (PGP) Traits

PGP Traits	Isolates	No. of Isolates
IAA + Ammonia	LAR02, LAR12, LAR13, LAR16, LAR17, LAR23	6
IAA + Ammonia + Nitrogen	LAR01	1
IAA + Ammonia + Nitrogen + HCN	LAR04, LAR21, LAR25	3
IAA + Ammonia + Nitrogen + HCN + Phosphate	LAR 06	1

Figure 3: Distribution of Endophytic bacterial isolates on combinations of plant growth-promoting (PGP) traits.



3. Abiotic stress tolerance activity

3.3.1 NaCl tolerance activity

Upon evaluating the bacterial isolates for growth in varying NaCl concentrations, all the four isolates grew luxuriantly in 0.5% (w/v) concentration of NaCl. At a concentration of 1.5% (w/v) NaCl, isolates LAR06 and LAR25 exhibited minimal development, whereas isolates LAR06 and LAR25 had noticeably inadequate development at a concentration of 7% (w/v). All isolates exhibited growth in the medium up to 9% (w/v) concentration of NaCl, but absence of growth was observed at 10% (w/v) of NaCl (Table 4).

Table 4: Salt Tolerance Ability of Endophytic Bacterial Isolates

Isolate	NaCl					
	0.5%	1.5%	3%	5%	7%	9%
LAR04	+++	+++	+++	+++	+++	++
LAR06	+++	++	++	++	++	+
LAR21	+++	+++	+++	++	++	+
LAR25	+++	+++	+++	++	++	++

+++ = strong activity; ++ = moderate activity; + = weak activity; – = no activity

3.3.2 High Temperature

After 24 hours of growth on nutrient agar medium, the bacterial isolates were examined for growth at 60°C and 70°C. The bacterial isolates' capacity to tolerate high temperatures was demonstrated by their growth at 60°C. At 70°C, however, no development was seen indicating their upper thermal limit. These findings demonstrate how they might be used in heat-stressed environments.

3.3.3 Drought Tolerance

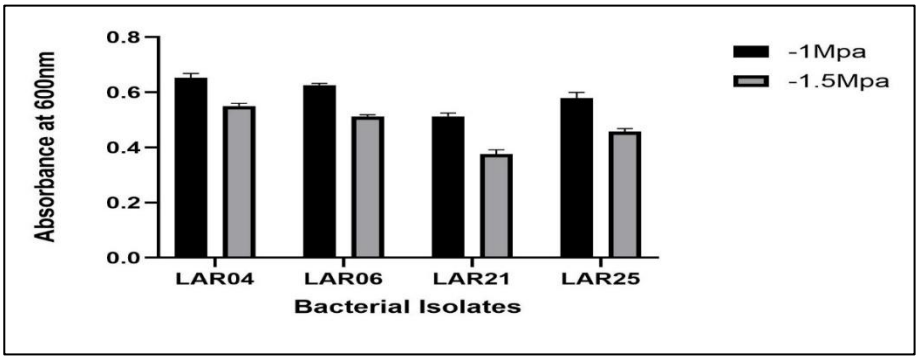
Polyethylene glycol (PEG)-induced osmotic stress was used to test the isolated bacterial strain ability to withstand drought. 25% PEG (-1 MPa osmotic pressure) and 30% PEG (-1.5 MPa osmotic pressure) were the two concentrations evaluated. Optical density (OD) at 600 nm was used to measure the growth response. Isolates LAR04 (0.65±0.01), LAR06 (0.62±0.004), LAR21 (0.51±0.009) and LAR25 (0.58±0.01) showed relatively greater absorbance values at -1 MPa (25% PEG), suggesting superior resistance to drought stress. The same isolates, LAR04 (0.55±0.008), LAR06 (0.51±0.004), LAR21 (0.37±0.01) and LAR25 (0.45±0.009) also exhibited significant growth at -1.5 MPa (30% PEG) indicating their capacity to tolerate increased osmotic stress (Table 5 & Figure 4).

Table 5: Growth response of drought-tolerant endophytic bacterial isolates under PEG-induced osmotic stress

Bacterial Isolates	-1Mpa	-1.5Mpa
LAR04	0.65±0.01	0.55±0.008
LAR06	0.62±0.004	0.51±0.004
LAR21	0.51±0.009	0.37±0.01
LAR25	0.58±0.01	0.45±0.009

Values are expressed as mean ± SEM from triplicate determinations. Values are non-significant.

Figure: 4 Drought Tolerances of Endophytic Bacteria



3.4 Biotic Stress Tolerance Activity

3.4.1 Antifungal Activity against Phytopathogen

At varying doses (5-50 µl), the antagonistic activity of the chosen endophytic bacterial isolates was evaluated against Alternaria alternata and Bipolaris spicifera.

All four strains demonstrated significant antifungal activity against A. alternata with different zones of inhibition. The highest inhibition was shown by strain LAR04, with zones increasing progressively from 17.5±0.40mm at 5 µl to 25.63±0.44mm at 50 µl. While LAR06 and LAR21 showed moderate inhibition, with maximal zones of 19.86±0.57mm and 20.33±0.47mm, respectively, LAR25 also showed a significant effect, reaching 24.5±0.40mm at 50 µl (Figure 5,6 & Table 6). The isolates exhibited differential activity in the case of B. spicifera. The two most successful were LAR06 and LAR25, which produced inhibition zones of 28.76±0.26mm and 24.73±0.30mm at 50 µl, respectively. With a peak of 23.53±0.26mm, LAR04 showed constant inhibition throughout concentrations. LAR21, on the other hand, displayed somewhat less activity, reaching a maximum of 20.56±0.30mm at 50 µl (Figure 7 Table 7). For the majority of isolates, the inhibitory effect was dose-dependent overall, with significant inhibition zones seen at higher doses. LAR06 and LAR25 demonstrated strong antifungal efficacy against B. spicifera, while LAR04 was notably very effective against A. alternata.

Figure 5: Antifungal Activity of Endophytic Bacteria Against Phytopathogens

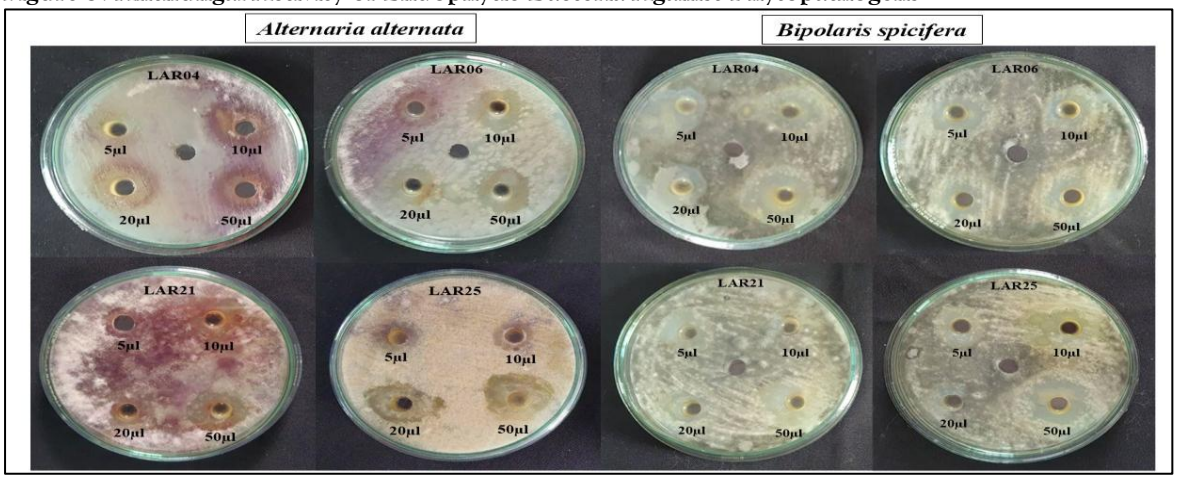


Table 6: Antifungal Activity of Endophytic Bacteria Against Alternaria alternata

S. No.	Bacterial Strains	Control	Alternaria alternata			
			Zone of Inhibition (mm)			
			5µl	10µl	20µl	50µl
1	LAR04	-	17.5±0.40	20.5±0.40	24.16±0.62	25.63±0.44
2	LAR06	-	14.93±0.32	18.36±0.38	16.96±0.77	19.86±0.57
3	LAR21	-	14.4±0.32	17.7±1.12	14.43±0.41	20.33±0.47
4	LAR25	-	14.83±0.23	14.6±0.43	18.73±0.52	24.5±0.40

Values are presented as mean ± SEM (n=3). Values are non-significant.

Figure 6. Antifungal activity of endophytic bacterial isolates from Leucas aspera against Alternaria alternata

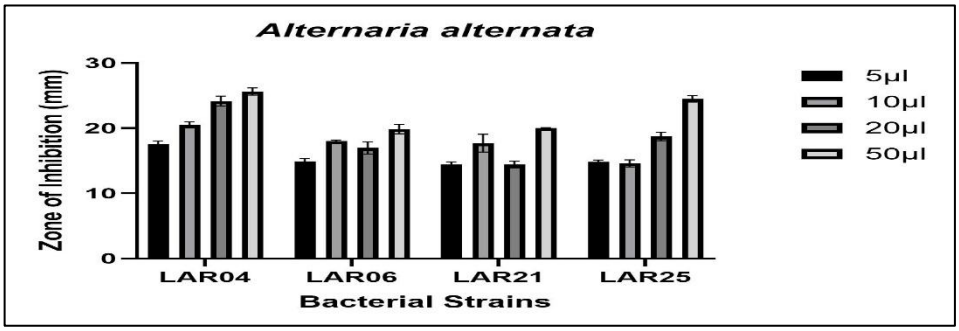
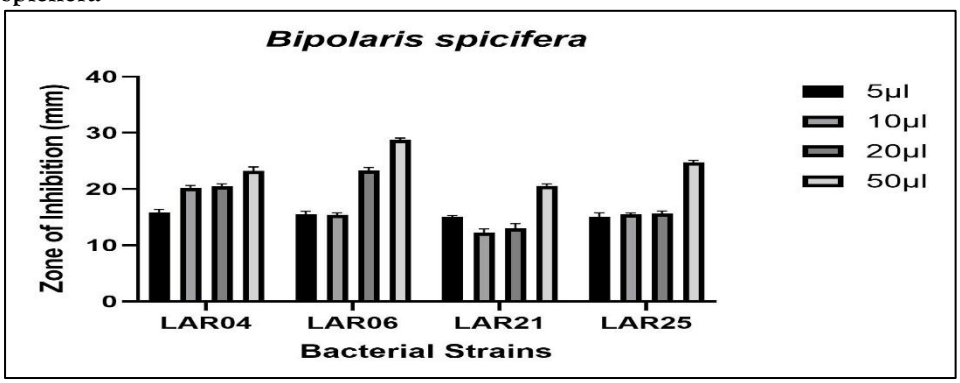


Table 7: Antifungal Activity of Endophytic Bacteria Against Bipolaris spicifera

S. No.	Bacterial Strains	Control	Bipolaris spicifera			
			Zone of Inhibition (mm)			
			5µl	10µl	20µl	50µl
1	LAR04	-	15.83±0.44	20.23±0.32	20.5±0.37	23.53±0.26
2	LAR06	-	15.53±0.41	15.43±0.28	23.26±0.246	28.76±0.26
3	LAR21	-	15.03±0.20	12.26±0.53	13±0.69	20.56±0.30
4	LAR25	-	15.06±0.57	15.5±0.21	15.63±0.36	24.73±0.30

Values are presented as mean ± SEM (n=3). Values are non-significant.

Figure 7: Antifungal activity of endophytic bacterial isolates from Leucas aspera against Bipolaris spicifera



3.5 Biochemical Characterization

The morphological characteristics of the isolates varied widely. The isolates had elevated colonies and a spherical appearance. They had a smooth, shiny surface with a smooth border and a rough, creamy surface with undulating to erosive borders. White and yellow colonies were seen. Microscopic examinations were conducted to examine the isolates morphology and Gram response, among other properties. Only one isolate had a cocci form, the other three were rod-shaped. Two isolates were found to be Gram negative by Gram staining.

Through biochemical analysis, the endophytes were identified. Gram staining was used to validate the morphological traits of the isolates. The indole and urease test was used to identify the single enzyme test. The Voges Proskauer and Methyl red tests were used to identify the assay for the metabolic pathway. The citrate utilization test verified the use of a single substrate. The Methyl red positive and Voges Proskauer negative test findings confirmed that most of the four isolates (LAR04, LAR06, LAR21 and LAR25) have the capacity to convert glucose into acidic end products such lactate, acetate and formate. Only one sample, LAR06, tested negative in the MR test, the other three isolates tested positive. Only one sample, LAR25, produced a positive result in the VP test; the other three isolates produced negative results. Citrate utilization activity was demonstrated by the three isolates (LAR06, LAR21 and LAR25). The urease test revealed that the isolates LAR4, LAR06 and LAR25 were positive. LAR21 was the only one to test positive for indole. Oxidase activity was detected in three isolates (LAR06, LAR21 and LAR25). The outcomes of the isolates are shown in Table 8.

Table 8: Morphological and Biochemical Characterization for the Selected Endophytes

Isolates	Gram Staining	Color of colony	Culture appearance after staining	Indole Test	Methyl Red	VP Test	Citrate Utilization	Urease Test	Oxidase Test
LAR04	+	White	Rod	-	+	-	-	+	-
LAR06	-	White	Rod	-	-	-	+	+	+
LAR21	+	White	Cocci	+	+	-	+	-	+
LAR25	-	White	Rod	-	+	+	+	+	+

3.6 Effect of the selected isolates on seed germination of Tomato Seeds

When tomato seedlings were treated with the endophytic bacterial isolates LAR04, LAR06, LAR21 and LAR25, their plant growth characteristics significantly improved in comparison to the uninoculated control. In comparison to the control, which had roots that were only 6.0 ± 0.41 cm long,

the LAR04-treated plants possessed the longest roots (10.5 ± 0.49 cm), followed closely by LAR06 (10.4 ± 0.67 cm) and LAR21 (10.2 ± 1.3 cm). A significant increase in shoot length was also seen, the control plants only reached 11.63 ± 0.86 cm, while the LAR25-treated plants recorded the highest value (22.8 ± 1.9 cm), followed by LAR04 (19.7 ± 1.8 cm), LAR06 (18.8 ± 1.04 cm) and LAR21 (17.5 ± 1.5 cm). With LAR25 exhibiting the highest fresh root weight (223 ± 5 mg) and dry root weight 49 ± 4.6 mg), followed by LAR06 (186.3 ± 4.59 mg and 42.7 ± 8.3 mg, respectively), fresh and dry root biomass significantly increased across all treatments. Similarly, there was a considerable increase in shoot biomass. LAR25 produced the highest fresh shoot weight (304 ± 10.2 mg), followed by LAR04 (300.7 ± 5.5 mg) and LAR21 (292.3 ± 19 mg), whereas the control produced 206 ± 7.2 mg. All treatments also showed an increase in dry shoot weight, the control only recorded 39 ± 3.0 mg, while LAR04 produced the highest value (65.7 ± 3.5 mg), followed by LAR25 (63.3± 8.4 mg) and LAR21 (60.3 ± 5.1 mg). Collectively, these results demonstrate that all the tested isolates promoted tomato seedling growth with LAR04 particularly enhancing root development, while LAR25 consistently increased shoot growth and overall biomass, highlighting isolate-specific effects on tomato growth (Table 9 & Figure 8).

Figure 8: Effect of Endophytic Bacteria inoculation on growth of Tomato plant

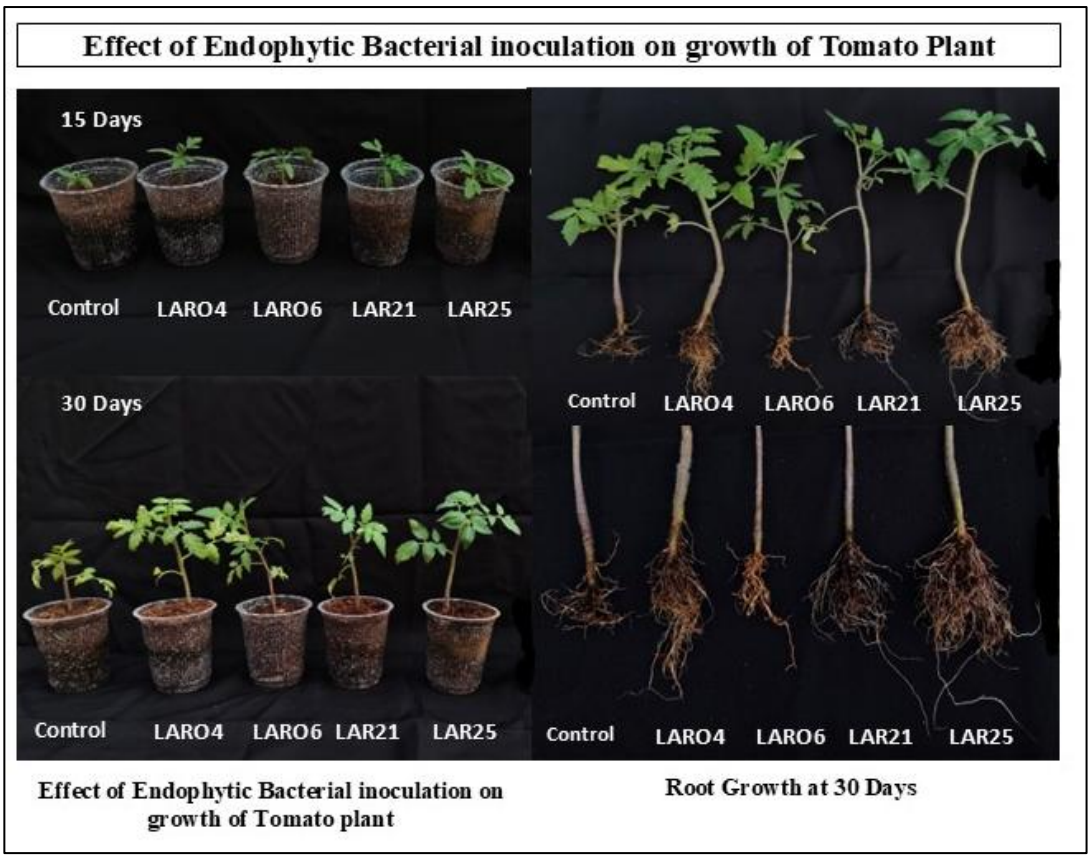


Table 9: Effect of Endophytic Bacterial inoculation on growth of Tomato seeds

Isolates	RL (cm)	SL (cm)	FRW (mg)	DRW (mg)	FSW (mg)	DSW (mg)
Control	6.0 ± 0.41^{d}	11.63 ± 0.86^{d}	125.7 ± 3.5^{d}	26.3 ± 0.58^{c}	206 ± 7.2^{c}	39 ± 3.0^{c}
LAR04	10.5 ± 0.49^{a}	19.7 ± 1.8^{b}	170 ± 9.5^{c}	36.7 ± 8.1^{b}	300.7 ± 5.5^{a}	65.7 ± 3.5^{a}
LAR06	10.4 ± 0.67^{a}	18.8 ± 1.04^{b}	186.3 ± 4.59^{b}	42.7 ± 8.3^{a}	243.7 ± 4.59^{b}	50.3 ± 1.5^{b}
LAR21	10.2 ± 1.3^{ab}	17.5 ± 1.5^{b}	149.3 ± 5.5^{c}	34.3 ± 6.1^{b}	292.3 ± 4.9^{a}	60.3 ± 5.1^{a}

LAR25	8.8 ± 0.76^{c}	22.8 ± 1.9^{a}	223 ± 5^{a}	49 ± 4.6^{a}	304 ± 9.2^{a}	63.3 ± 8.4^{a}

Effect of endophytic bacterial isolates on tomato seedling growth parameters. Values are presented as Mean ± SEM from triplicate determinations. Different letters (a-d) within a column indicate significant differences between treatments according to one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). RL: Root Length; SL: Shoot Length; FRW: Fresh Root Weight; DRW: Dry Root Weight; FSW: Fresh Shoot Weight; DSW: Dry Shoot Weight.

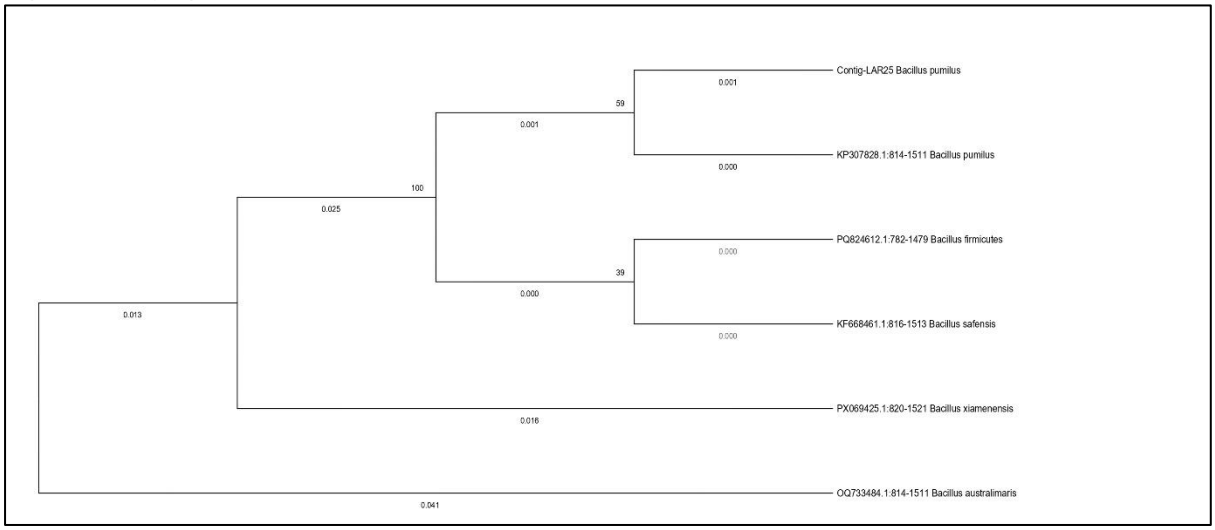
3.7Molecular Characterization

A total of thirty isolates were obtained from Leucas aspera roots. They were examined for their capacity to promote plant development in response to both abiotic and biotic stresses. The LAR25 strains that produced the most was selected for additional study. The strains were identified using the 16S rRNA gene sequence in addition to their physical and biochemical traits (Table 10). The 16S rRNA gene sequences have been deposited in NCBI GenBank. Strains LAR25 demonstrated the maximum similarity of 99% for Bacillus pumilus (Accession no. PX368833). Furthermore, a phylogenetic tree was constructed based on the 16S rRNA gene sequences to illustrate the evolutionary relationship of the isolates with closely related species (Figure 9).

Table 10: Molecular identifications of selected endophytic bacterial isolates

Bacterial Isolates	GenBank Accession Number	Similarity (%)	Closest Species
LAR25	PX368833	99	Bacillus pumilus

Figure 9: Phylogenetic tree



4. DISCUSSION

Thirty endophytic bacterial strains were successfully isolated from Leucas aspera roots, demonstrating the varied microbial population present in this therapeutic plant. The isolated strains are confirmed to be true endophytes by the lack of growth on control plates, which confirms the efficacy of the surface sterilizing technique. A diversified endophytic population that may represent a broad variety of functional capacities is indicated by the selection of colonies with unique morphologies for additional examination. This is consistent with techniques reported by Devi et al., (2016), who found that surface sterilization successfully eradicated epiphytic microorganisms, guaranteeing that the bacteria recovered are actual endophytes [25]. The presence of endophytic bacteria in Leucas aspera roots was initially reported by Dayamrita & Nivya Mariam Paul (2021) [7]. The primary emphasis of the study was on the characteristics of the isolated endophytic bacteria that promote plant growth and their ability to withstand biotic and abiotic stress. After being assessed for different PGP properties in vitro, 21 of the 30 isolates of endophytic bacteria in our investigation had at least one PGP trait, as indicated in Table 1. On the combined evaluation of all five PGP features (IAA, Ammonia, Nitrogen fixation, HCN production and Phosphate solubilization), three isolates (LAR04, LAR21 and LAR25) showed the capacity to produce four traits,

whereas LAR06 is capable of producing all five (Table 2 & Figure 3). IAA production was found in six of the 30 isolates, with isolate LAR06 producing the highest amount of IAA (87.93 µg mL^{-1}). The functional diversity within root-associated bacterial communities is highlighted by the specific endophytes propensity to produce IAA. For bacteria to biosynthesize IAA, L-tryptophan is a necessary component of the culture media [26]. In the current investigation, strain LAR06 considerably increased IAA production when culture medium was supplemented with L-tryptophan. The majority of the 23 endophytic bacterial isolates in the current investigation have the capacity to fix nitrogen and four of the 30 isolates tested positive for ammonia production. This suggests that nitrogen metabolism is a functional characteristic shared by the endophytes of Leucas aspera roots. According to Noar and Bruno-Bárcena (2018), the capacity of 23 isolates to fix nitrogen demonstrates their potential to augment plant nitrogen needs and lessen reliance on synthetic fertilizers, hence promoting sustainable crop production [27]. Ten isolates of Leucas aspera endophytes (LAR01, LAR02, LAR03, LAR04, LAR06, LAR12, LAR13, LAR21, LAR25 and LAR28) tested positive for hydrogen cyanide (HCN) generation in the current investigation, according to qualitative screening. As a biological control agent, HCN successfully combats plant pathogens. HCN causes the cell to die by blocking the electron transport chain, which cuts off the cell's energy source [28]. The fact that our investigation included HCN-positive isolates raises the possibility that these endophytes support Leucas aspera defensive systems by shielding the host from harmful fungi and other microorganisms.

One of the most significant characteristics of endophytic bacteria that promotes plant growth is phosphate solubilization which increases the amount of phosphorus available in the soil. On Pikovskaya's agar medium enriched with tricalcium phosphate (TCP), 39.14% of isolates (119 total) showed halo zones, suggesting phosphate-solubilizing capacity [29]. The ability of the five isolates (LAR01, LAR04, LAR06, LAR21 and LAR25) to solubilize phosphate was also confirmed by the presence of distinct halo zones on Pikovskaya's medium. The similar results underscore the selective character of this trait by showing that only a small percentage of endophytic or rhizospheric bacteria have considerable phosphate-solubilizing capacity. The discovery of multifunctional strains that demonstrated all five PGP features including LAR04, LAR21, LAR25 and especially LAR06 indicates their potential as excellent bioinoculants. Isolates with several growth-promoting traits are frequently thought to be more effective at sustaining plant health in a variety of environmental settings. To determine their resilience and suitability for sustainable agriculture, these chosen strains were subsequently put through a thorough assessment of their biotic stress activity against phytopathogens and abiotic stress tolerance (such as salinity, drought and temperature). Plant growth is inhibited by osmotic and ionic stressors caused by salinity. Because high salt concentration negatively impacts agricultural productivity and soil health, soil salinity is a major global concern [4][30]. Since it allows them to live in saline environments and promote plant growth in stressful situations, salt tolerance is regarded as one of the most significant abiotic stress adaptations of endophytic bacteria. All four of the isolates that were chosen for this study were able to grow at low salt concentrations (0.5% NaCl), although isolates LAR06 and LAR25 showed poor growth at 7% NaCl and decreased growth at 1.5%. Interestingly, all the isolates were able to withstand saline levels as high as 9% NaCl, but at 10% the growth was hindered. Through a variety of processes, including osmotic adjustment, biomass accumulation, photosynthetic efficiency and enhanced ion homeostasis in saline environments, endophytic bacteria can stimulate plant growth and improve stress resilience. In bermudagrass under salt stress, for example, Enterobacter ludwigii B30 was demonstrated to enhance osmotic control, ion absorption and rhizosphere microbial structure, thereby mitigating the adverse effects of salinity on plant performance [31]. One important environmental factor affecting endophytic bacteria's ability to survive and function well is temperature. All of the isolates in this investigation showed growth at 60°C, demonstrating their resistance to high temperatures. At 70°C, however, no growth was visualized indicating that this was above their thermal tolerance threshold. According to Shaffique et al., (2022), microorganisms are essential for reducing heat stress in plants through the production of phytohormones, osmolytes and enhanced physiological resilience [32]. Our isolates demonstrated the possibility of utilisation of thermotolerant strains as biofertilizers or as a component of larger approaches to increase crop resilience in heat-stressed soils. Isolates LAR04, LAR06, LAR21 and LAR25 in this investigation showed considerable growth at both -1 MPa and -1.5 MPa, demonstrating their ability to tolerate drought-like conditions and their tolerance to PEG-induced osmotic stress. According to Dubey et al., (2021), Pseudomonas sp. AKAD A1-16 demonstrated strong ACC deaminase activity to mitigate ethylene-

induced growth inhibition. Bacillus cereus, Pseudomonas otitidis and Pseudomonas sp. were among the drought-tolerant soybean root endophytes that improved biomass, photosynthetic pigments and membrane stability under water-deficit stress [33]. Chen et al., (2017) also showed that by controlling osmolyte balance and inhibiting chlorophyll breakdown, Pantoea alhagi sp. enhanced drought tolerance in wheat [34]. These results support endophytic bacteria's potential as environmentally benign bioinoculants to increase crop resilience in drought-prone environments. The chosen isolates demonstrated potent antagonistic activity against phytopathogenic fungi in addition to their capacity to promote growth and withstand stress. Bipolaris spicifera was significantly inhibited by LAR06 and LAR25, whereas Alternaria alternata was observed to be controlled by LAR04. According to Do Quang Trung et al., (2021), Eleusine indica-derived Bacillus amyloliquefaciens EI-15 efficiently prevented Alternaria alternata in vitro and decreased pitaya twig lesion formation in vivo [35]. In a similar vein, Xie et al., (2025) observed the dramatic inhibition of A. alternata in pepper by B. amyloliquefaciens RaSh1 reducing disease incidence from 80% to 40% with improvement in defense enzyme activity, plant growth and physiological performance with lowered oxidative stress [36]. In a different study, H Jiang (2024) demonstrated that by triggering host biochemical defense mechanisms, phyllosphere endophytes such Stenotrophomonas maltophilia and Brevundimonas olei reduced the severity of Maydis leaf blight in maize [37]. All these research demonstrates the usage of endophytic bacteria as non-toxic and sustainable crop protection substitutes for chemical fungicides. In line with earlier publications on functionally varied endophytic bacteria, the morphological and biochemical differences among isolates LAR04, LAR06, LAR21 and LAR25 demonstrate their metabolic diversity and support their potential roles in biocontrol and plant growth promotion. When compared to the uninoculated control, the endophytic isolates LAR04, LAR06, LAR21 and LAR25 dramatically increased biomass and root and shoot growth in tomato seedlings indicating their efficacy as endophytes that promote plant growth. According to a number of studies, endophytes isolated from crops like tomatoes and chillies currently show a variety of plant growth-promoting (PGP) characteristics such as the formation of indole acetic acid (IAA), phosphate solubilization, ammonia generation and siderophore synthesis. Together, these characteristics improve root-shoot development, seedling vigor and overall plant growth [38], [39][38], [39]. Notably, LAR04 was very successful in fostering root development in our investigation, but LAR25 consistently increased shoot length and biomass. This suggests that various isolates may promote growth through different processes. Their PGP characteristics inclusive of IAA synthesis, phosphate solubilization and nitrogen fixation contributing to improved nutrient absorption and general plant vigor are probably responsible for these effects. Certain isolates, including Pseudomonas oleovorans, Bacillus species, Agrobacterium tumefaciens and Microbacterium species exhibited potent antagonistic activity against plant pathogens in addition to promoting growth indicating their dual function of promoting growth and suppressing disease. Additionally, gibberellins and IAA are synthesized by phytohormone-producing endophytes such as Sphingomonas sp. LK11 which further enhance plant development and biomass accumulation [40]. When taken as a whole, these results highlight the potential of endophytic bacteria as organic bioinoculants for sustainable agriculture, enhancing crop productivity and seedling establishment while lowering dependency on conventional pesticides and fertilizers. Bacillus pumilus (99% similarity) was the discovered isolate LAR25, respectively. With the help of spore formation and metabolite production, Bacillus species are well-established endophytes that have potent plant growth-promoting and biocontrol properties [41]. Numerous Bacillus strains have demonstrated phosphate solubilization, stress tolerance, IAA generation and phytopathogen suppression [42], which validates our findings regarding LAR25 PGP potential. The ability of Bacillus xiamenensis species to promote host growth in the face of abiotic stress is also becoming more widely acknowledged for instance, Bacillus sp. MN-54 enhanced plant performance in the presence of heavy metal stress [43]. Its potential function in nutrient cycling and stress reduction is highlighted by the discovery that Bacillus pumilus is LAR25. These results collectively suggest that Bacillus pumilus isolated from Leucas aspera holds the potential as bioinoculants for sustainable crop production.

5. CONCLUSION

In the present study, thirty endophytic bacterial isolates were isolated from the roots of Leucas aspera and their morphological, biochemical and plant growth-promoting (PGP) characteristics were extensively evaluated. LAR25, showed exceptional PGP characteristics and biotic and abiotic stress resistance, which

makes them viable options for additional uses. LAR25 was identified as Bacillus pumilus (99% similarity) using 16S rRNA sequencing. Under controlled conditions, both isolates demonstrated a variety of functional features viz., stress tolerance, enzyme activity and nutrient solubilization, all of which improved tomato seedling growth. This finding is significant as it reveals the way by which the endophytic bacteria from medicinal plants could be effectively utilised as bioinoculants for sustainable agriculture. Additional research in both greenhouse and field settings is necessary to confirm their effectiveness, clarify underlying mechanisms and evaluate their function in stress resilience and integrated disease management.

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7. Conflict of Interest Disclosure

The authors report no conflicts of interest in relation to this study.

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