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RESEARCH ARTICLE

Multifunctional Endophytic *Pseudomonas aeruginosa* LAL25 leaves Exhibiting Plant Growth Promotion, Salt Tolerance and Heavy Metal Bioremediation Potential

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Abstract: Endophytic Bacteria are essential for promoting plant development, increasing stress tolerance and supporting environmental cleanup. Twenty-eight endophytic bacteria isolates from the leaves of the medicinal plant *Leucas aspera* were used in this investigation and their salt tolerance, heavy metal resistance and important plant growth promoting (PGP) characteristics were assessed. IAA production, ammonia generation, nitrogen fixation, phosphate solubilization and hydrogen cyanide synthesis were among the advantageous traits displayed by a number of isolates. Isolates LAL25 showed the best multifunctional activity among them. LAL25 demonstrated robust phosphate solubilization, positive ammonia and HCN generation and high amount of IAA (84.24±0.29 µg mL^{-1}). It also displayed remarkable halotolerance, maintaining growth up to 7% NaCl in both plate and liquid assays. Heavy metal tolerance assays revealed that LAL25 sustained significant growth in the presence of Pb^{2+}, Cu^{2+} and Co^{2+} upto 3-4 mM, outperforming all other isolates. Based on 16S rRNA gene sequencing, LAL25 was identified as *Pseudomonas aeruginosa*. The multifunctional properties of *P. aeruginosa* LAL25 highlight its potential as a promising bioinoculant for sustainable agriculture and as an effective candidate for the bioremediation of heavy metal contaminated environments.

Keywords: *Leucas aspera*, Plant Growth Promotion, Salt Tolerance, Bioremediation, Heavy metal Tolerance, Multifunctional endophytes.

INTRODUCTION

Global agricultural productivity is seriously threatened by external factors such as drought, salt, heavy metal poisoning and severe temperatures. Food security and environmental sustainability are under risk due to these abiotic stresses which also limit plant development, lower yield and disturb soil and ecosystem health. Abiotic stressors such as osmotic stress, drought, salt, cold, heat, heavy metals etc. are frequently encountered by plants [1]. Drought results in ionic imbalance and physiological sensitivity, while salinity produces cell toxicity and osmotic stress [2]. Since traditional chemical and physical repair methods tend to be expensive and can result in secondary pollution, finding novel approaches is necessary to address these concerns in a sustainable manner [3]. Microbial-based strategies, particularly those incorporating beneficial microbes linked with plants have attracted interest as economical and environmentally friendly substitutes. It is well accepted that endophytic bacteria that live in plant tissues are essential to the host's ability to adjust to biotic stressors and unfavorable conditions. Gaining further insight into the biology of bacterial endophytic populations and their close links to the plant genetic network is helping to advance our understanding of how microbes affect plant stress response, tolerance and adaptability [4]. These bacteria enhance plant growth by encouraging nutrient uptake, producing phytohormones like indole-3-acetic acid (IAA), solubilizing phosphates, producing siderophores that chelate iron and modulating ethylene levels. Additionally, by controlling osmotic equilibrium, antioxidant responses and metal detoxification processes, endophytes help plants withstand abiotic stressors like salt and heavy metals [5], [6]. Endophytes are attractive agents for sustained bioremediation because of their capacity to tolerate and biotransform heavy metals through biosorption, bioaccumulation and enzymatic reduction. For example, *Pseudomonas* species are well known for their diverse metabolic repertoire, which allows them to both promote plant development in saline and metal-contaminated environments and withstand extreme conditions. They also mediate the detoxification of metals like zinc, cadmium and chromium [7]. Novel and multipurpose endophytes can be found in medicinal plants that thrive in challenging conditions. A common therapeutic herb in tropical and subtropical areas, *Leucas aspera* has remarkable stress tolerance, flourishing in poor soils and challenging climates. *L. aspera* has long been recognized for its antibacterial and antioxidant qualities and its inherent resilience indicates that it contains distinct endophytic populations that have evolved to a variety of stressors [8].

Nevertheless, limited research has been performed on its endophytic bacterial diversity, especially those that have the ability to promote plant development, withstand salt and bioremediate heavy metals. Investigating multifunctional endophytes from *L. aspera* can uncover powerful bioinoculants that may

accelerate crop development in contaminated and saline soils, offering long-term substitutes for artificial fertilisers and remediation agents. This combination of characteristics can simultaneously reduce environmental contamination and improve plant fitness. Isolating strains like *Pseudomonas aeruginosa* from stress-adapted plants like *L. aspera* is a smart move because these strains have demonstrated promising characteristics in heavy metal detoxification combined with plant growth promotion under abiotic stress [5]. The current study aims to isolate and characterize the multifunctional endophytic *Pseudomonas aeruginosa* strain LAL25 from *Leucas aspera* leaves, with particular attention to its potential for heavy metal bioremediation, plant growth promotion and salt tolerance. In stress-affected soils, this study aims to assess the strain's viability as a sustainable bioinoculant for improving crop resilience and environmental remediation.

METHODOLOGY

Sample collection and isolation of bacterial endophytes

Healthy, infection-free plant of *Leucas aspera* were collected from three distinct sites in Tirunelveli District, Tamil Nadu to maximize the diversity of endophytic bacterial isolates. The first sampling site was an agricultural field at Ramayanpatti, representing cultivated soil regularly exposed to fertilizers and irrigation. The second site at Radhapuram, comprised semi-arid and uncultivated land with dry, nutrient-poor soil conditions. The third site was located along the Thamirabarani River bank characterized by moist soil rich in organic matter and microbial diversity. Freshly collected plant samples were transported to the Microbial Biotechnology Laboratory under sterile conditions. The collected leaf samples were rinsed properly in running tap water to remove soil and dust particles followed by surface sterilization to eliminate epiphytic microorganisms. The sterilization procedure involved immersion in 70% ethanol for 30 seconds, followed by 3% sodium hypochlorite for 3 minutes and finally three rinses with sterile double-distilled water (2 minutes each). To confirm the efficiency of sterilization, the water from the final rinse was spread on nutrient agar plates as a control. After drying on sterile filter paper, the surface-sterilized leaf segments (2-3 mm) were aseptically placed on nutrient agar plates in triplicate and incubated at 37°C for 72 h with daily observation. After incubation, distinct bacterial colonies that appeared on the plates were picked using a sterile loop. Morphologically different colonies were purified by repeated sub-culturing on nutrient agar. Pure cultures of the isolates were maintained on nutrient agar slants at 4°C for short-term storage and preserved in 20% sterile glycerol at - 80°C for long-term use [9], [10]

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

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

The screening for IAA production was determined as described by Patten and Glick (2002). The isolates were grown in 100 ml flasks containing 50 ml Luria broth (LB) supplemented with L-tryptophan (100 mg/ml) for 48 h on a rotary shaker. Later the cultures were centrifuged at 10,000 g for 15 min and the supernatants collected. Two ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) with one ml of the supernatant was allowed to react with at 28 ± 2°C for 30 min. Pink colour developed indicating the presence of IAA was determined by measuring the absorbance in a spectrophotometer at 530 nm at the end of the incubation. A standard curve was plotted with IAA and Salkowsky reagent dissolved in LB medium to quantify the IAA ml^{-1} present in the culture filtrate [11].

Ammonia production and nitrogen fixation

Ammonia production was analyzed using the qualitative method of Singh *et al.,* 2018. Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 mL peptone water and incubated for 72 h at 35°C. Nessler's reagent (1 mL) was added to each tube as a colorimetric reagent for ammonia production. The colour change to faint yellow indicated the minimum ammonia production, while the colour change from deep yellow to a brownish colour indicated the maximum ammonia production [12].

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 (Kifle and Laing, 2016). The growth of the isolates was monitored after incubation at 28 ± 2°C for 48 h [13].

Hydrogen Cyanide (HCN) Production

Production of HCN was estimated qualitatively according to the methodology described by Lorck (1948). The isolates were grown in LB agar supplemented with glycine (4.4 g L^{-1}). One sheet of the sterilized whatman filter paper was immersed in 1% picric acid in 10% sodium carbonate for 1 min and struck underneath the Petri dish lids. The plates were sealed with parafilm and incubated at 28 ± 2°C for 2 days. Development of reddish-brown colour on the Whatman filter paper indicated production of HCN [14].

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 [15].

Salt Tolerance Test

Plate Assay (Qualitative)

Endophytic bacteria were inoculated onto Nutrient agar medium supplemented with different concentrations of NaCl (0.5%, 1.5%, 3%, 5%, 7% and 9%). All the plates were incubated at 28°C for 5 days and bacterial growth was observed at every 24h [16].

Liquid Kinetics (Quantitative)

The salt tolerance of endophytic bacteria was checked using NB supplemented with 0.5%, 1.5%, 3%, 5%, 7% NaCl (w/v) and a control without additional NaCl. Log phase culture of Endophytic bacteria (10^8 CFU/ml) was inoculated (1 ml) into the medium and incubated for 48 h in shaking incubator (150 rpm) at 28 ± 2°C. The growth was measured every four hours up to stationary phase by taking optical density at 600 nm using UV-Vis spectrophotometer and growth curve was prepared [17]. The sample were taken in triplicates.

Heavy Metal

Heavy Metal Tolerance Test (MIC determination)

The minimum inhibitory concentration (MIC) of heavy metals for the endophytic bacterial isolates from *Leucas aspera* leaves was determined using the broth dilution method. Luria-Bertani (LB) broth was supplemented individually with different concentrations (1.0 to 5.5 mM) of copper (Cu (II) as $CuCl_2$), cobalt (Co (II) as $CoCl_2 \cdot 6H_2O$) and lead (Pb (II) as $PbCl_2$). Each tube containing 10 mL of metal-supplemented broth was inoculated with 1% (v/v) of an overnight bacterial culture and incubated at 25°C for 24-72 h under shaking conditions (120 rpm). Bacterial growth was determined by measuring the optical density (600 nm) (UV-1800, Shimadzu, Japan) at prescribed time intervals (0, 8, 12, 24 and 48 h). All the experiments were performed in triplicates. Growth of the isolate without metals was considered as the control for this experiment. The MIC value was defined as the lowest metal concentration that completely inhibited visible growth or showed no increase in absorbance compared to the control (without metal). All experiments were carried out in triplicate to ensure accuracy [18].

DNA Extraction, 16S rRNA Amplification and Sequencing

The selected LAL25 strains were identified by determination of 16S rRNA gene sequences. Colony PCR was performed from live cells cultured on solid LB medium and the 16S rDNA were amplified by PCR using the following primers 27 f (50 -GAGTTTGATC ACTGGCTCAG-30) and 1492r (50 - TACGGCTACCTTGTTACGACTT-30) [19]. Amplification was performed for 30 PCR cycles with denaturation at 94 C for 1 min, annealing at 55°C for 1 min and extension at 72° C for 1.5 min. PCR products were purified using a standard PCR clean-up kit and subsequently sequenced in-house using Sanger sequencing with the ABI BigDye Terminator v3.1 system on an ABI 3730xl DNA Analyzer. The 16S rDNA sequence was compared against the GenBank database using the NCBI Blast program.

Statistical Analysis

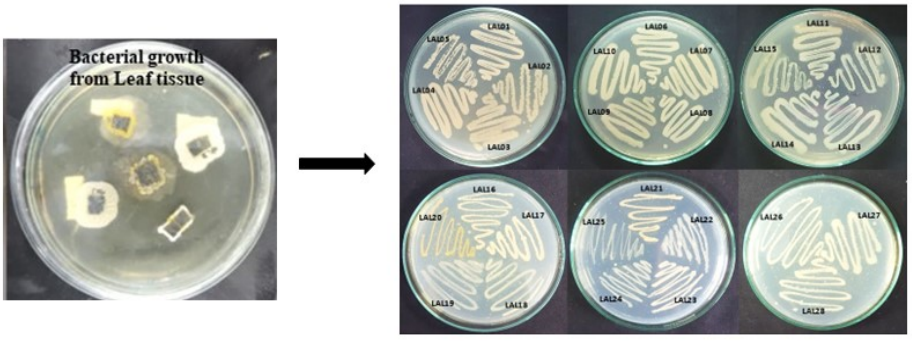
All experiments were conducted in triplicates and the results were expressed as mean ± Standard Error of the Mean (SEM). Statistical analysis was performed using GraphPad Prism (version 10.6.1).

RESULTS

Isolation of Bacterial Endophytes

Fresh leaves of the medicinal plant *Leucas aspera* were chosen in order to isolate endophytic bacterial isolates. The leaves yielded a total of 28 endophytic bacterial isolates. As seen in Fig. 1 bacterial endophytes were found on the plant leaves margins. A total of twenty-eight morphologically distinct bacterial colonies were isolated. Six isolates (LAL01-LAL06) were collected from Abishekapatti, near Manonmaniam Sundaranar University. From Tamirabarani, fourteen isolates (LAL07-LAL20) were obtained. Finally, eight isolates (LAL21-LAL28) were isolated from Ramayanpatti. The absence of bacterial colonies in the control plates confirmed that the isolates were indeed endophytes rather than surface contaminants. To accurately differentiate real endophytes from epiphytic and ambient microorganisms, a number of studies emphasise the crucial significance of stringent surface sterilisation procedures and the use of suitable plating controls. To guarantee the accuracy of endophyte isolation in medicinal plants, for example, research by Hallmann *et al.,* (1997) and subsequent investigations have established standardised techniques [20].

Figure 1: Isolation and Purification of Endophytic bacterial colonies from *Leucas aspera* Leaves



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

Indole Acetic Acid (IAA) Production

The most common, well-characterized and physiologically active auxin in plants is indole-3-acetic acid (IAA) which has been thoroughly investigated among plant growth regulators [21]. In plants, IAA can induce long-term responses like cell division and differentiation as well as quick ones like enhanced cell elongation. In this study, two bacterial isolates cultivated in Yeast Peptone-Mannitol (YPM) broth with tryptophan (100µg mL^{-1}) were able to produce Indole-3-acetic acid (IAA). The bacterial endophytes synthesis of IAA was confirmed by the emergence of a pink colour. IAA production was indicated in LAL09 (34.79±0.17 µg mL^{-1}), LAL11 (21.38±0.26 µg mL^{-1}) and LAL25 (84.24±0.29 µg mL^{-1}) strains as shown in the Fig. 2. According to Goswami *et al.,* (2016), these values are within the range frequently reported for endophytic and rhizobacteria with potent plant growth-promoting activities. LAL25 relatively larger IAA production indicates that it has a greater capacity to modify plant root characteristics, improve nutrient uptake and contribute to plant growth by producing phytohormones [22].

Ammonia Production

Peptone water broth was used to assess the isolated endophytic bacterial strains capacity to generate ammonia. These microorganisms can emit ammonia which plants can use as an instant nitrogen supply to enhance root growth and overall plant productivity [23]. When Nessler's reagent was added, the culture supernatant turned dark indicating the formation of ammonia, whereas the uninoculated controls remained to appear yellow. The two examined isolates viz., LAL09 and LAL25 showed a strong positive reaction suggesting that they could provide ammonia as a source of nitrogen for plant growth. Similar findings have been documented in other bacteria associated with plants including *Bacillus*, *Pseudomonas* and *Enterobacter*, where increased nitrogen nutrition and higher plant growth have been directly connected to ammonia production [24]. The strong ammonia production capacity of LAL25 increases its significance as a multipurpose PGP endophyte which is profoundly used in sustainable agriculture.

Nitrogen Fixation

The potential of the endophytic bacterial isolates from *Leucas aspera* leaves to fix atmospheric nitrogen was demonstrated by their growth on Jensen's nitrogen-free medium. Strong nitrogen-fixing capacity was confirmed by the rapid development of LAL01, LAL02, LAL08, LAL11, LAL13, LAL15, LAL18, LAL23, LAL25, LAL26, LAL27 and LAL28 among the tested isolates. While the remaining isolates showed no discernible growth and were considered as non-nitrogen fixing, isolates LAL04, LAL07, LAL12 and LAL16 showed moderate or partial growth indicating limited nitrogen fixation. Endophytes frequently exhibit variation which is frequently impacted by microbial genetics, environmental adaptation and plant host selection processes [25]. Endophytic diazotrophs greatly increase plant nitrogen nutrition, boost root development and encourage biomass accumulation under both normal and stressful conditions, according to a number of studies [26]. In order to reduce chemical nitrogen inputs in agriculture, nitrogen-fixing isolates are therefore interesting candidates for the creation of environmentally friendly biofertilizers.

HCN Production

Hydrogen cyanide (HCN) production by bacterial isolates functions as a crucial part in inhibiting plant pathogens by interacting with their cellular metabolism and electron transport processes inevitably leading to cell death. Endophytic bacteria have a competitive advantage due to their capacity to produce HCN which enhances their potential as potent biocontrol agents. The isolates LAL09, LAL11, LAL13 and LAL25 showed good HCN production in the current study suggesting the existence of a potential function in the reduction of plant diseases. Similar results have been documented in other agricultural and medicinal plants where HCN-producing endophytes greatly enhanced plant health and decreased disease incidence [27].

Phosphate Solubilization

Phosphate-solubilizing bacteria are essential for transforming insoluble phosphate forms including rock phosphate into soluble forms that plants can absorb. In the current study, Pikovskaya's agar medium was used to screen endophytic bacterial isolates from *Leucas aspera* leaves for phosphate solubilisation. Three of the twenty-eight isolates that were tested displayed different degrees of phosphate solubilisation as visualized by distinct halo zones surrounding the colonies. Significant phosphate-solubilizing activity was shown by isolates LAL09, LAL14 and LAL28 indicating their potential as effective endophytes that promote plant growth. According to earlier research on medicinal plant-associated endophytes, the variation in halo diameter reflects variations in the isolate's metabolic capacities and organic acid synthesis [28]. Similar research has demonstrated that endophytic PSB enhances nutrition while also boosting biomass, chlorophyll content and overall crop health [29]. With the goal reduce reliance on chemical phosphatic fertilisers, these isolates are therefore interesting candidates for environmentally friendly biofertilizer formulations.

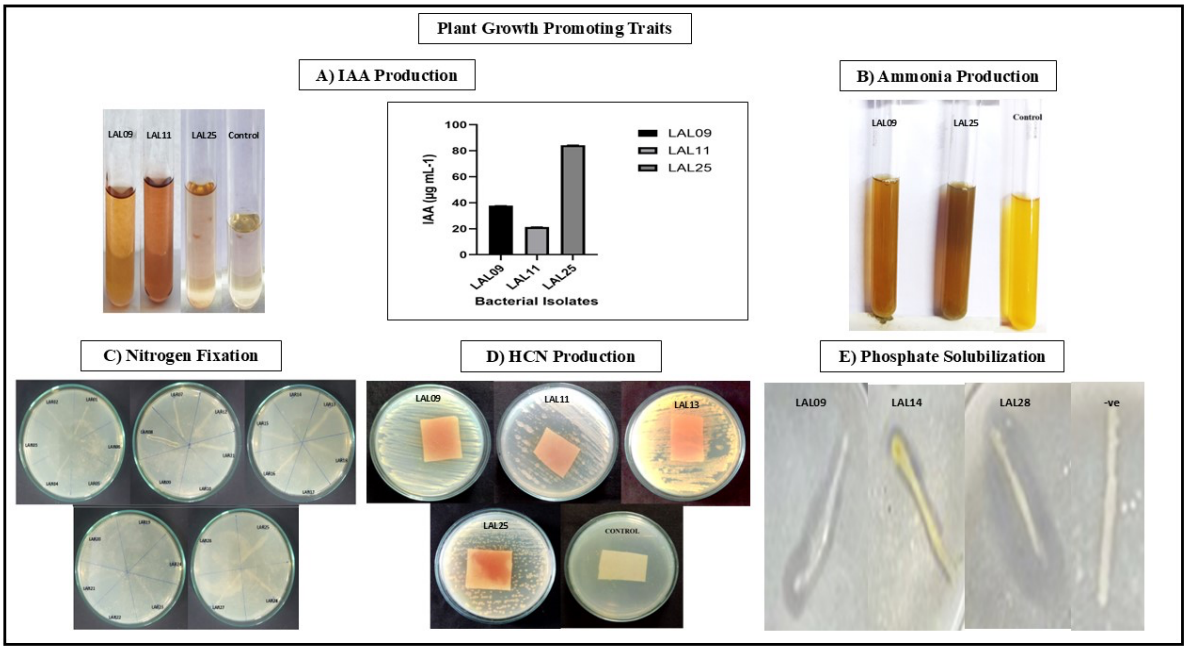
Table 1: Plant Growth Promoting (PGP) traits of endophytic bacterial isolates from *Leucas aspera* leaves

Bacterial Isolates	Plant Growth Promoting Traits				
	IAA Production	Ammonia	Nitrogen Fixation	HCN Production	Phosphate Solubilization
LAL01	-	-	+	-	-
LAL02	-	-	+	-	-
LAL03	-	-	-	-	-

LAL04	-	-	+	-	-
LAL05	-	-	-	-	-
LAL06	-	-	-	-	-
LAL07	-	-	+	-	-
LAL08	-	-	+	-	-
LAL09	+	+	-	+	+
LAL10	-	-	-	-	-
LAL11	+	-	+	+	-
LAL12	-	-	+	-	-
LAL13	-	-	+	+	-
LAL14	-	-	-	-	+
LAL15	-	-	+	-	-
LAL16	-	-	+	-	-
LAL17	-	-	-	-	-
LAL18	-	-	+	-	-
LAL19	-	-	-	-	-
LAL20	-	-	-	-	-
LAL21	-	-	-	-	-
LAL22	-	-	-	-	-
LAL23	-	-	+	-	-
LAL24	-	-	-	-	-
LAL25	+	+	+	+	-
LAL26	-	-	+	-	-
LAL27	-	-	+	-	-
LAL28	-	-	+	-	+

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* leaves. 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* leaves. (A) IAA production, (B) Ammonia production, (C) Nitrogen fixation, (D) HCN production and (E) Phosphate solubilization

Based on the number and diversity of plant growth-promoting traits, isolates LAL09, LAL11, LAL13, LAL25 and LAL28 were selected for subsequent screening of salt tolerance and heavy metal resistance. These isolates exhibited strong or multiple PGP traits such as IAA production, ammonia generation, nitrogen fixation hydrogen cyanide synthesis and phosphate solubilization indicating their potential as multifunctional bioinoculants.

Salt Tolerance Test

Plate Assay (Qualitative)

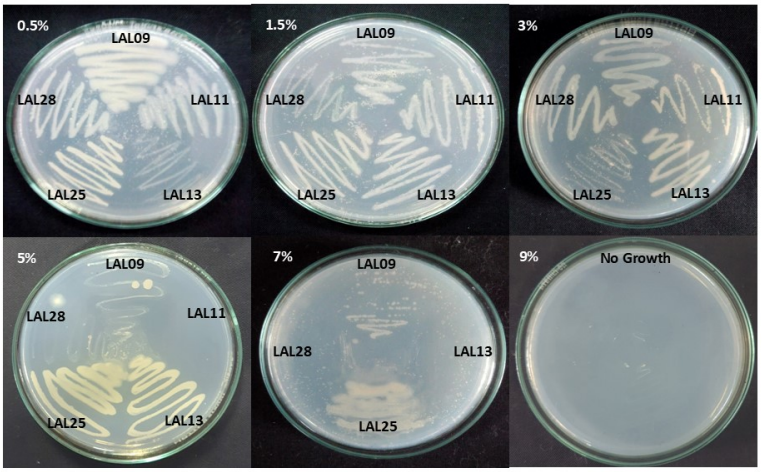
The salt tolerance of five selected endophytic bacterial isolates from *Leucas aspera* leaves were evaluated on nutrient agar supplemented with varying NaCl concentrations (0.5-9% w/v). All isolates exhibited luxuriant growth at 0.5% NaCl indicating normal tolerance under low-salt conditions. At 1.5% and 3% NaCl, isolates LAL09, LAL13, LAL25 and LAL28 showed good growth, whereas LAL11 displayed moderate growth. With increasing salinity (5% NaCl), the growth of most of the isolates declined and only LAL09, LAL13 and LAL25 showed visible colonies. At 7% NaCl, LAL25 maintained moderate growth, demonstrating strong halotolerance, while all other isolates exhibited very weak or no growth. None of the isolates survived at 9% NaCl. Among the tested strains, LAL25 showed the highest salt tolerance, sustaining growth up to 7% NaCl, followed by LAL13 and LAL09 which tolerated up to 5%. In contrast, LAL11 and LAL28 were inhibited beyond 3% NaCl, suggesting moderate to low salt tolerance. The strong performance of LAL25 indicates the existence of physiological and molecular adaptations that have been documented in halotolerant PGPR linked to medicinal plants such as osmoprotectant accumulation, ion homeostasis mechanisms or stress-responsive enzymes [30]. By generating phytohormones, encouraging nutrient absorption and modifying stress-responsive signalling pathways, these endophytes can improve plant salt tolerance [16]. LAL25 is therefore a viable option for additional research on the reduction of salt stress in plants (Table 2 & Figure 3).

Table 2: Salt tolerance of selected endophytic bacterial isolates at different NaCl concentrations

Isolate	NaCl Concentration					
	0.5%	1.5%	3%	5%	7%	9%
LAL09	+++	++	++	+	+	-
LAL11	+++	++	+	-	-	-
LAL13	+++	+++	+++	+++	-	-
LAL25	+++	+++	+++	++	++	-
LAL28	+++	++	++	-	-	-

Note: +++ = luxuriant growth; ++ = moderate growth; + = weak growth; – = no growth

Figure 3: Differential salt tolerance of endophytic bacteria from *Leucas aspera* leaves as observed on Nutrient agar plates with graded NaCl levels



Liquid Kinetics (Quantitative)

The salt tolerance of five selected endophytic bacterial isolates from *Leucas aspera* leaves were quantitatively assessed in nutrient broth containing different concentrations of NaCl (0-9% w/v). All isolates exhibited normal growth in control (0%) and 0.5% NaCl with optical density (OD_{600}) values ranging from 1.02 to 1.20 after 48 h of incubation indicating no inhibitory effect at low salinity levels. A gradual decline in growth was observed with increasing NaCl concentration. At 1.5% and 3% NaCl, isolates LAL09, LAL13 and LAL25 maintained moderate growth (OD_{600} between 0.78 and 1.10), while LAL11 and LAL28 showed reduced turbidity compared to the control. Further increase to 5% NaCl resulted in marked inhibition in most isolates with only LAL25 retaining significant growth (OD_{600} = 0.81 ± 0.02) followed by LAL09 (0.52 ± 0.02). At 7% NaCl, detectable growth was observed only for LAL25 (OD_{600} = 0.60 ± 0.03), however, all other isolates displayed total suppression which is in line with previous

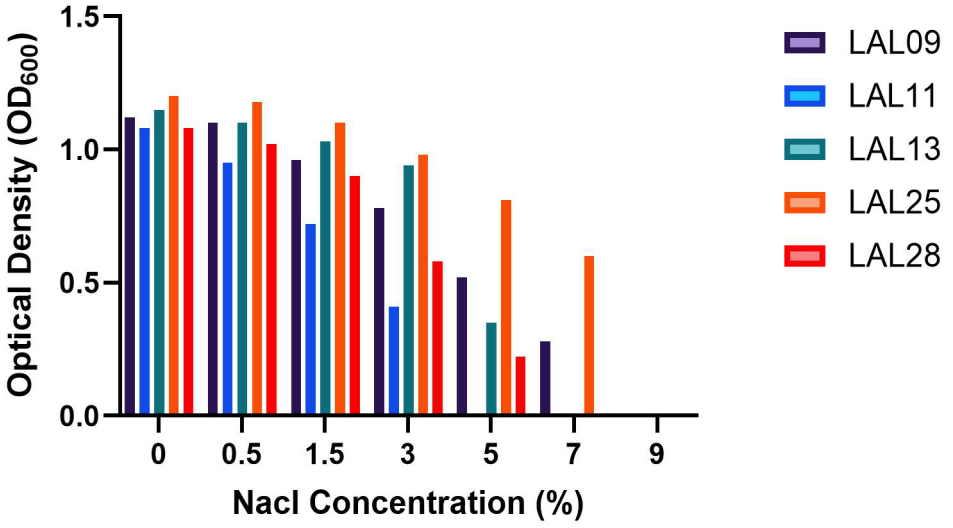
research demonstrating that the majority of plant-associated bacteria can withstand low-salt conditions without experiencing appreciable physiological stress [31]. At 9% NaCl, no isolate showed any growth (Table 3 & Figure 4). Growth gradually decreased as salinity escalated which is consistent with the known osmotic and ionic stress that NaCl places on bacterial cells [32]. Overall, the findings unequivocally show that LAL25 is the most halotolerant strain, able to maintain growth up to 7% NaCl, whereas the other isolates only showed tolerance up to 3-5%. This finding implies that LAL25 has efficient osmoregulatory systems and could help sustain plant growth in the face of salinity stress. Similar results have been documented for salt-tolerant endophytic bacteria that improve nutrient uptake in saline environments, produce phytohormones and increase plant development through mechanisms like ACC deaminase activity [33].

Table 3. Salt Tolerance of Endophytic Bacterial Isolates at Different NaCl Concentrations

Isolate	NaCl Concentration						
	0% (Control)	0.5%	1.5%	3%	5%	7%	9%
LAL09	1.12 ± 0.03	1.10 ± 0.05	0.96 ± 0.04	0.78 ± 0.03	0.52 ± 0.02	0.28 ± 0.02	ND
LAL11	1.08 ± 0.04	0.95 ± 0.03	0.72 ± 0.05	0.41 ± 0.02	ND	ND	ND
LAL13	1.15 ± 0.02	1.10 ± 0.03	1.03 ± 0.02	0.94 ± 0.03	0.35 ± 0.01	ND	ND
LAL25	1.20 ± 0.02	1.18 ± 0.02	1.10 ± 0.03	0.98 ± 0.02	0.81 ± 0.02	0.60 ± 0.03	ND
LAL28	1.08 ± 0.03	1.02 ± 0.03	0.90 ± 0.03	0.58 ± 0.02	0.22 ± 0.01	ND	ND

ND - No detectable growth; values represent mean ± SEM of triplicate readings

Figure 4: Salt tolerance of endophytic bacterial isolates at different NaCl concentrations



Heavy Metal

The minimum inhibitory concentration (MIC) assay demonstrated differential tolerance of the endophytic bacterial isolates toward heavy metals. At 1 mM concentration, all isolates exhibited normal growth across the three metals with optical density values ranging from 0.9 to 1.2 at 600 nm. This suggests that the isolates were not significantly harmed by low concentrations of these metals which is in line with previous findings that many environmental bacteria can withstand trace metal concentrations because they have natural detoxification processes (Nies, 2003). Growth progressively decreased as the concentration increased with most isolates showing inhibition beyond 2-3 mM. Among all, LAL25 consistently maintained strong growth up to 3 mM and moderate growth at 4 mM for all tested metals particularly lead (OD_{600} = 0.60 ± 0.01). In contrast, other isolates such as LAL11, LAL13 and LAL28 showed complete inhibition beyond 2 mM (Table 4). These findings show that LAL25 has the best potential for bioremediation applications in metal-contaminated areas since it has the strongest resilience to heavy metals. The presence of efficient metal-resistance mechanisms such as efflux pumps, metal-binding proteins or enzymatic detoxification pathways which have been extensively documented in metal-resistant *Pseudomonas* strains is suggested by LAL25 capacity to proliferate at metal concentrations that inhibited all other isolates [34].

Table 4: Heavy metal tolerance of endophytic bacterial isolates based on MIC values at different metal concentrations

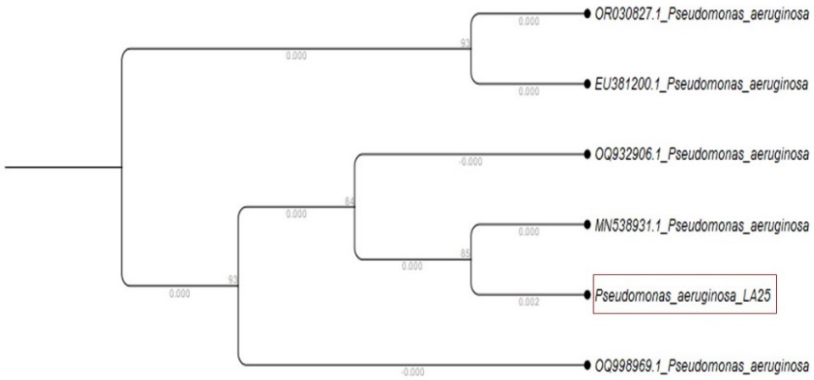
Isolate	etal (mM)	1 mM	2 mM	3 mM	4 mM
LAL09	**Pb**	1.12 ± 0.04	0.96 ± 0.02	0.28 ± 0.01	ND
	Cu	1.10 ± 0.05	0.80 ± 0.04	0.35 ± 0.02	ND
	Co	1.08 ± 0.08	0.94 ± 0.03	0.40 ± 0.08	ND
LAL11	**Pb**	0.96 ± 0.05	0.40 ± 0.08	ND	ND
	Cu	0.85 ± 0.05	ND	ND	ND
	Co	0.90 ± 0.06	0.35 ± 0.15	ND	ND
LAL13	**Pb**	1.05 ± 0.02	0.58 ± 0.05	ND	ND
	Cu	1.02 ± 0.03	0.54 ± 0.02	ND	ND
	Co	1.10 ± 0.04	0.70 ± 0.01	0.22 ± 0.01	ND
LAL25	**Pb**	1.20 ± 0.05	1.08 ± 0.03	0.90 ± 0.05	0.60 ± 0.01
	Cu	1.15 ± 0.02	0.96 ± 0.02	0.60 ± 0.03	0.38 ± 0.02
	Co	1.12 ± 0.01	1.00 ± 0.05	0.52 ± 0.04	0.25 ± 0.07
LAL28	**Pb**	0.98 ± 0.05	0.50 ± 0.06	ND	ND
	Cu	0.90 ± 0.01	ND	ND	ND
	Co	0.95 ± 0.02	0.42 ± 0.08	ND	ND

ND - No detectable growth; values represent mean ± SEM of triplicate readings

Molecular Characterization

Among the twenty-eight endophytic bacterial isolates collected from *Leucas aspera* leaves, LAL25 continuously demonstrated higher performance in all plant growth promoting (PGP) features, salt tolerance and heavy-metal resistance assays. LAL25 was selected for molecular identification using 16S rRNA gene sequencing due to its potent multifunctional activity. The nearly full-length 16S rRNA gene sequence of LAL25 was successfully amplified, purified and sequenced. The acquired sequences taxonomic identity was confirmed by BLAST analysis which showed 98% match to *Pseudomonas aeruginosa*. The sequence was submitted to the NCBI GenBank database and an accession number: OR135244 was obtained. The positioning of LAL25 within the *Pseudomonas aeruginosa* clade was further validated by phylogenetic analysis which showed its tight evolutionary relationship with previously identified strains (Figure 3). According to these findings, LAL25 is a powerful endophytic *Pseudomonas aeruginosa* isolate that promotes plant growth and exhibits exceptional resistance to abiotic stressors making it a viable option for upcoming biotechnological and agricultural uses. Several studies demonstrated that *P. aeruginosa* strains have a considerable potential as bioinoculants in stressful situations due to their ability to produce IAA, siderophores, HCN and show phosphate solubilization [35]. Since *Pseudomonas aeruginosa* species are known to tolerate and biotransform metals such as Pb, Cu and Co through mechanisms including biosorption and redox-mediated transformation, the remarkable heavy-metal resistance exhibited by our endophytic isolate LAL25 (*P. aeruginosa*) is particularly noteworthy [36].

Figure 5: Phylogenetic Tree



CONCLUSION

In this study, twenty-eight endophytic bacterial strains were isolated from *Leucas aspera* leaves and their ability to promote plant development, tolerate salt and withstand heavy metals was assessed. LAL25 continuously demonstrated the highest PGP activity of all isolates including phosphate solubilisation, HCN synthesis and the formation of IAA and ammonia. Additionally, it revealed superior resistance to Pb^{2+}, Cu^{2+} and Co^{2+}, as well as the best tolerance to salinity (up to 7% NaCl) suggesting its potential relevance in bioremediation and abiotic stress mitigation. *Pseudomonas aeruginosa* was identified as the isolate using molecular identification using 16S rRNA sequencing. Overall, *P. aeruginosa* LAL25 is a multifunctional endophyte exhibiting significant potential for use in environmental remediation and sustainable agriculture.

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