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Phenotypic and Molecular Characterization of Endophytic Bacteria Isolated from Banana

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MM, SN, NK and AS designed the study, supervised and facilitated the research and wrote the first draft of the manuscript. Author GR performed the experiments and analyzed the results obtained in the study. All authors read and approved the final manuscript.

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ABSTRACT

Several species of endophytic bacteria has been reported from various plants. In the present study, samples of roots, rhizome, pseudostem, petiole and leaves of healthy Banana plant were collected from variety Yangambi km5 (*AAA*). In total, 38 endophytic bacteria were isolated. Amongst 16 isolates were selected based on compatibility by cross streak method. The 16 strains tested by paper disc method, Lf4, Lf5, Lf10, Pt4 and Ps7 showed inhibitory effects against *Pectobaterium carotovorum* subsp*. carotovorum* under *in vitro* conditions. The Lf4 and Lf5 showed similar results for the biochemical characteristics studied. The isolate Lf10 showed a slight difference with regard

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to oxidase test, Methyl red, Urease test and H_2S production. The isolates Pt4 and Ps7 showed similar result except nitrate reduction, KOH test and Pigment production. *Bacillus subtilis* (Bs) strain (culture collected from Department of Plant pathology, TNAU) was used as reference culture. The total DNA extracted from selected five isolates was identified by partial sequencing of the 16S rRNA gene and phylogenetic tree was constructed using MEGA 6.0. The results confirmed that isolates Lf4 and Lf5 were *Bacillus subtilis*, isolate Lf10 was an *Ochrobactrum daejeonense*, isolate Pt4 was an *Achromobacter xylosoxidans* and Ps7 was a *Pseudomonas aeruginosa*. The study revealed that all the five strains have biocontrol potential against soft rot pathogen.

Keywords: Endophytic bacteria; Banana cv. Yangambi km5 (AAA); biochemical characterization; Pectobaterium carotovorum subsp. carotovorum; 16S rRNA; phylogenetic analysis.

1. INTRODUCTION

Plants are normally associated with diverse microorganisms, some of which may stimulate plant defence responses and development [1]. Bacteria that associate with plants include rhizobacteria, epiphytic and endophytic bacteria. Endophytic bacteria colonize the internal tissues of the plants with no apparent negative effects on their host. Beneficial endophytic bacteria have attracted interest in the last few years due to the reports of potential positive influences on crop production [2]. Furthermore, numerous reports have shown that endophytic bacteria can act as biocontrol agents against plant pathogens or enhance plant growth. Endophytic bacteria are known to colonize the intercellular region in diverse plants with roots suggested to be the major point of entry and niche [3]. They are also known to enter the aerial parts of plants through natural openings like stomata and wounds and been isolated from various organs including roots, stems, leaves, buds, flowers, and tender fruits of diverse plants [4]. Very interestingly, some of the previous reports categorized bacterial endophytes associated with banana cv. Grand Naine into three groups [5]. The first group which includes organisms like *Klebsiella*, *Ochrobactrum* and *Bacillus* spp. were categorized as easily culturable with obvious colony growth. The second group which includes organisms likes *Methylobacterium*, *Alcaligenes*, *Brevundimonas*, *Pseudomonas*, and *Oceanobacillus*. One of the major *banana cultivars*, Grand Naine (G9), is highly susceptible to soft rot disease but the Yangambi km5 (*AAA*) variety is resistant. Based on these, the current research was designed to investigate the endophytic bacteria associated with tissue cultured banana cv. Yangambi km5 (*AAA*). The aims of the present work were as follows: (i) the isolation and molecular identification of endophytic bacteria associated with Banana (ii) evaluation of biological control potential against the bacterial pathogen *Pectobaterium carotovorum* subsp*. carotovorum* using *in vitro* techniques.

2. MATERIALS AND METHODS

2.1 Collection of Plant Sample and Isolation of Endophytic Bacteria

Three months old banana cv. Yangambi km5 (*AAA*) was collected from an orchard, Tamil Nadu Agricultural University (TNAU) during February 2018. Plant samples were placed in plastic bags and either processed immediately or kept in a refrigerator for a short period of time. Samples were washed with tap water and the plant parts were cut separately, such as root, rhizome, pseudostem, petiole and leaf. Later 1g of plant tissues were taken and washed with tween 20 for 30 min, then washed with sterile distilled water two times to remove excess tween 20. All the plant tissues were surface sterilized with 5% NaOCl for 20 min. Then washed two times with sterile distilled water to remove excess NaOCl. After that wash with 70 per cent ethanol for 30 sec and all the plant tissues were washed 8 times with sterile distilled water, from the 8th wash 1 ml was taken and plated in Nutrient Agar (NA) medium. This was maintained as a sterile check. From each sample two replications were maintained, the plant tissues were homogenized using pestle and mortar by sterile peptone salt (1 g of peptone +1 g of NaCl in 1 litre of water) [6] 10 ml was used for maceration per sample, allow it to withstand for 20 min, and 1 ml of supernatant was taken from each sample for serial dilution. Three decimal (10^1 to 10^3) serial dilutions were carried out for each sample separately. From 10^1 and 10^3 dilution were (1 ml) taken and spread on the NA, from each dilution two replications were maintained. The NA pates were incubated at 37°C for a week and observed for the bacterial colony growth. Thereafter, the NA plates were

monitored at room temperature up to one month [7].

2.2 Antibacterial Activity of Endophytic Bacterial Isolates under *in vitro*

All 38 endophytic bacterial strains isolated from banana cv. Yangambi km5 (*AAA*) were checked for its antibacterial activity against *Pectobaterium carotovorum* subsp. *carotovorum* using perpendicular or cross streak method [8]. For this, the pathogen was initially grown for 5 days at the centre of nutrient agar plate as single line streak. After incubation, the endophytic bacterial isolates were inoculated perpendicular to the pathogen and incubated at 37°C for overnight and observed for any inhibition. The bacterial isolates which showed broad antimicrobial activity were further subjected to dual-culture assay. Three hundred microlitres suspension (approx. 2×10^8 CFUml^{-1}) of the bacterial pathogen *Pectobaterium carotovorum* subsp. *carotovorum* was poured into petri dishes of NA and maintained at room temperature for 5 min. A paper disc (about 10 mm) was immersed in a suspension of each endophytic bacteria (density adjusted (OD_{600} nm) to approximately 1×10^8 CFU ml^{-1}) and placed on the pathogen inoculated dishes. Cultures were incubated at 27°C for 48–72 hr and the width of any inhibition zones were measured [9]. Among the bacterial isolates which showed highest inhibition zones were further subjected to biochemical characterization.

2.3 Characterization of Effective Endophytic Bacterial Isolates

Identification of the endophytic bacterial isolates was based on morphological and biochemical analysis. Catalase test, oxidase activity, Gram reaction, Citrate utilization, Nitrate reduction and Methyl Red and Voges Proskauer test [10], Growth in 5% NaCl, Urease test, Indole production, Pigment production H_2S production and KOH test [11] were carried out as previously described by Schaad et al. [12].

2.4 Bacterial Identification Using 16S rRNA Gene Sequence Analysis

Bacterial genomic DNA was isolated from the effective bacterial cultures by lysis method [13]. The partial 16S rRNA gene amplification was performed through PCR using 50 µl reaction mix containing 2 µl of each primer, 25 µl of master mix (Genei, Bangalore, India) 19µl of H_2O and 2 µl of DNA template (5 µg/µl). Thermal cycler (Eppendorf Master cycler) universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R-Y (5'-GGYTACCTTGTTACGACTT-3'; Y = C/T) as described [14]. The thermocycling conditions included initial one denaturation step of 94°C for 5 min followed by 35 amplification cycles of 94°C for 30 s, 60°C for 40 s, 72°C for 40 s followed by a final extension at 72°C for 5 min [15]. The PCR products were electrophoresed and separated on 1.2% agarose gel and photographic using image analyser. The 1.5 Kbp PCR products were gel eluted using the QIAGEN gel extraction kit and sequenced at *Eurofins Genomics,* Whitefield Bangalore, India. The sequences were subjected to BLAST analysis and aligned using ClustalW [16]. Alignments were manually adjusted when necessary. Neighbour-joining phylogenetic analysis was performed using (MEGA 6.0) and a Phylogenetic tree constructed based on bootstrap analysis with 1000 replicates in the same program.

2.5 Statistical Analysis

Mean differences in inhibition zones were evaluated with ANOVA using CRD at 5% significance. All the data were statistically analyzed with IRRI star software and consequently interpreted.

3. RESULTS AND DISCUSSION

3.1 Isolation of Endophytic Bacterial Isolates

Endophytic bacteria are ubiquitous colonizer of the internal plant tissues where they do not usually cause any significant morphological change and disease sign [17]. In total, 38 endophytic bacteria were isolated from leaf, pseudostem, petiole, rhizome and root of banana cv. Yangambi km5 (*AAA*) (Table 1). The absence of bacterial colonies in a sterile check culture plate confirmed that the isolates obtained were endophytes. The bacterial endophytes were isolated from Banana cv. Grand Naine consist of almost 50 species as reported by by Thomas and Soly [18]. In this accordance of the earlier studies on banana which revealed the ubiquitous association of bacterial endophytes in the shoot-tips of cv. Grand Naine and the intracellular colonization by the endophytic bacteria [14]. Similarly, 47 endophytic bacterial strains were isolated from the shoot tips of Banana cv. Grand Naine [7].

Table 1. Endophytic bacteria isolated from Banana cv. Yangambi Km 5 (AAA)

S.No.	Plant part	Isolates	Total number of isolates
1	Leaf	YEB Lf_1; YEB Lf_2; YEB Lf_3; YEB Lf_4; YEB Lf_5; YEB Lf_6; YEB Lf_7; YEB Lf_8; YEB Lf_9; YEB Lf_{10}	10
2	Petiole	YEB Pt_1; YEB Pt_2; YEB Pt_3; YEB Pt_4; YEB Pt_5; YEB Pt_6; YEB Pt_7; YEB Pt_8	8
3	Pseudostem	YEB Ps_1; YEB Ps_2; YEB Ps_3; YEB Ps_4; YEB Ps_5; YEB Ps_6; YEB Ps_7; YEB Ps_8; YEB Ps_9	9
4	Rhizome	YEB Rh_1; YEB Rh_2; YEB Rh_3; YEB Rh_4; YEB Rh_5; YEBRh_6	6
5	Root	YEB Rt_1; YEB Rt_2; YEB Rt_3; YEB Rt_4; YEB Rt_5	5
	Total		38

3.2 *In vitro* Screening of the Endophytic Bacterial Isolates

Preliminary screening of antibacterial activity against *P. carotovorum* subsp*. carotovorum* was done by perpendicular or cross streak method. The 16 strains were identified based on compatibility between bacterial isolates and selected for secondary *in vitro* screening by paper disc method; inhibition zone was measured as a radius from the outer edge of the paper disc (Table 2).

The results revealed that, isolate Lf4 recorded maximum inhibition zone of 14.0 mm and isolate Ps7 as 13.3 mm followed by Lf10 of 12.1mm and the minimum inhibition zone recorded in isolates Lf3 (6.7mm), Ps5 (5.1mm) followed by Rh6 (5.8mm) against *P. carotovorum* subsp*. Carotovorum* (Fig. 1). The results of the experiments are in line with findings of earlier worker [19], Screening of endophytic bacteria against *Pseudomonas syringae* pv. *syringae* in dual-culture assay by paper disc method. *B. subtilis* is having bactericidal activity against soft rot pathogen *P. carotovorum* subsp. *Carotovorum* [20].

B. subtilis IIHR BS-2 showed an inhibitory effect on *P. carotovorum* in the 100% concentration [21]. Clear inhibition zones were observed under *in vitro*, indicating that *B. amyloliquefaciens* KC-1 influenced a strong effect against Pcc E1 on agar plates [22]. The *Ochrobactrum lupine* was having antibacterial activity against *E. carotovora* subsp*. carotovora* and of pepper against *Xanthomonas axonopodis* pv. *vesicatoria* but the present study showed that *Ochrobactrum daejeonense* also having antibacterial activity against *P. carotovorum* subsp*. Carotovorum* [23]. Amongst 16 strains, five strains were selected with maximum inhibition zone for further biochemical characterization. Identification of endophytic bacterial strains was based on catalase production, oxidase activity, and Gram reaction, Citrate utilization, Nitrate reduction and Methyl Red and Voges Proskauer test etc., (Table 3) showed that isolates were clustered into different phenotypic groups. The results of biochemical test showed that the isolates Lf4 and Lf5 revealed that rod shaped gram positive bacteria has a positive reaction for Gram staining, Catalase test, VP (Voges Proskauer) test, Growth in 5% NaCl, Nitrate reduction and Citrate utilization and a negative reaction for KOH test, Oxidase activity, Urease test, MR-VP test, H_2S production, Indole production. However, the isolate Lf10 revealed that rod shaped gram negative bacteria have a positive reaction for Oxidase activity, MR (Methyl Red), Urease test, H_2S production and negative reaction for Citrate utilization, Nitrate reduction, Indole production and pigment production.The isolate Pt4 exposed that rod shaped gram negative bacteria has a positive reaction for Catalase test, Oxidase activity, Growth in 5% NaCl and showed negative reaction for Nitrate reduction, VP (Voges Proskauer), Pigment production. The isolate Ps7 revealed that gram negative rod shaped bacteria has a positive reaction for Pigment production, Nitrate reduction, KOH test and negative reaction for MR- VP test, Urease test and Indole production (Figs. 2, 3 and 4).

The isolate Lf10 only positive for urease test, this showed their ability to break down urea, to ammonium ($NH4^+$) which can be readily absorbed by the plants to promote growth. This is an important aspect in growth and development of bananas in the case where fertilizers are applied, as the bacteria have shown potential to convert urea to simpler forms. The results of the experiments are in line with findings of earlier worker [24] who investigated

on occurrence and distribution of endophytic bacteria in a legume community. All the isolates were Catalase positive. This result indicated that they can produce Catalase enzyme. Similar result was observed by Son et al. [25] who had conducted experiment on isolation, identification and screening of endophytic bacteria. Catalase is the enzyme which helps bacteria to avoid cellular toxicity. *Bacillus subtilis* (Bs) strain (culture collected from Department of Plant pathology, TNAU) used as reference culture.

Table 2. Inhibition of *Pectobacterium carotovorum* subsp *carotovorum* by promising endophytic bacteria

S. No	Isolates	Inhibition of *Pectobacterium carotovorum* subsp *carotovorum* (radius in mm)	
		16-18h-old culture [a]	Cross Streak method [b]
1	Lf10	12.1^{m}	+
2	Pt2	11.0^{hj}	+
3	Pt4	11.0^{k}	+
4	Lf3	6.7^{c}	+
5	L9	9.1^{e}	+
6	Ps7	13.3^{n}	+
7	Lf5	11.3^{kl}	+
8	Pt1	10.6^{h}	+
9	Rh5	11.2^{hi}	+
10	Ps5	5.1^{a}	+
11	Lf4	14.0^{o}	+
12	Ps8	8.4^{d}	+
13	Rh6	5.8^{b}	+
14	Rt2	9.3^{eg}	+
15	Rt4	9.1^{ef}	+
16	Bs	14.2^{oo}	+
SE±(m)		1.21	
CD (p=0.05)		2.46	

**Values are mean of three replications, a- Inhibition zone was measured as radius from the outer edge of the paper disc b- "+" Indicates the pathogen inhibition at the meeting point. Different letters in superscripts indicate significantly different values*

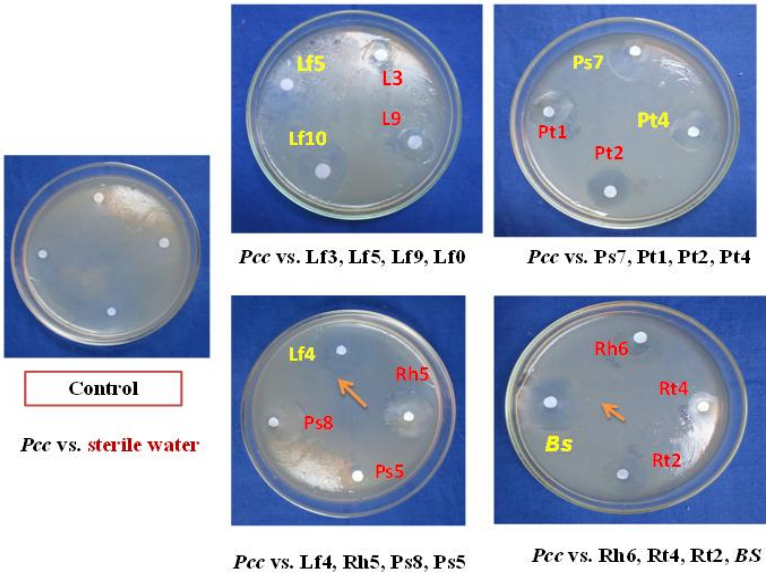

Fig. 1. Screening for the antibacterial activity of endophytic bacteria against soft rot pathogen (*Pectobacterium carotovorum* subsp *carotovorum*)

Table 3. Morphological and biochemical characterization of Endophytic bacteria

S.No	Charecteristics	Effective bacterial endophytes					
		Lf4	Lf5	Lf10	Pt4	Ps7	Bs
1	Morphology	Rod	Rod	Rod	Rod	Rod	Rod
2	Gram staining	+	+	-	-	-	+
3	Catalase test	+	+	+	+	+	+
4	Oxidase activity	-	-	+	+	+	-
5	Citrate utilization	+	+	-	+	+	+
6	Nitrate reduction	+	+	-	-	+	+
7	MR (Methyl Red)	-	-	+	-	-	-
8	VP (Voges Proskauer)	+	+	+	-	-	+
9	Growth in 5% NaCl	+	+	+	+	+	+
10	KOH test	-	-	-	-	+	-
11	Urease test	-	-	+	-	-	-
12	H_2S production	-	-	+	-	-	-
13	Indole production	-	-	-	-	-	-
14	Pigment production	-	-	-	-	+	-

'+' indicate positive test. '-' indicate negative test

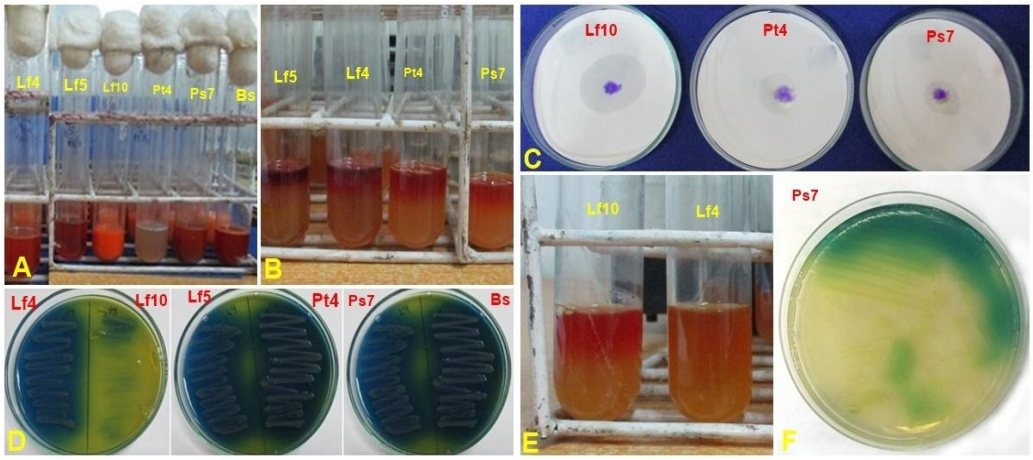

Fig. 2. Biochemical characterization of endophytic bacteria: A- Nitrate reduction ,B- VP (Voges Proskauer), C- Oxidase activity, D- Citrate utilization, E- MR (Methyl Red), F- Pigment production

Compare to Bs, isolates Lf4 and Lf5 were identified as *Bacillus* sp and the Ps7 was identified as *Pseudomonas* sp., based on blue-green pigment productions. Endophytic bacterial isolates in Kenyan Bananas (*Musa Spp*.) with the general biochemical properties of the isolates were determined using the following assays; Urease test, Nitrate reduction test, Citrate utilization test, Catalase test, Methyl Red-Voges-Proskauer test (MR-VP), Indole production test, Hydrogen sulphide test [26,27].

Genomic DNA extracted from five effective bacterial strains was subjected to PCR analysis. PCR amplification of 16S rRNA gene resultant in the formation of 1,500 bp product as identified by 1.2% agarose gel electrophoresis (Fig. 5). The BLAST analysis of nucleotide sequence had more than 99% similarity with existing isolates in NCBI. The nucleotide sequence of our isolates submitted in NCBI database (Table 4). Similarly, *Bacillus* up to species level identified through 16s rRNA gene sequencing [28].

Sequence analysis of nearly full length of 16S rRNA gene sequences indicated that isolated endophytic strains Lf4 and Lf5 were intimately related to *Bacillus subtilis*. Strain Lf10 homologous with *Ochrobactrum daejeonense*. Strains Ps7 were homologous with *Pseudomonas aeruginosa* and strain Pt4 with *Achromobacter xylosoxidans*.

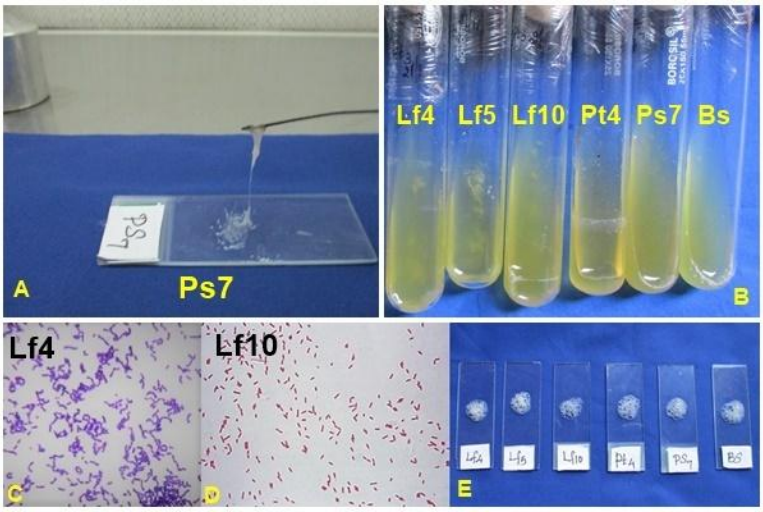

Fig. 3. Morphological and biochemical characterization of Endophytic bacteria: A- KOH test,B- Growth in 5% NaCl, C- Gram positive reaction, D- Gram negative, E- catalase test

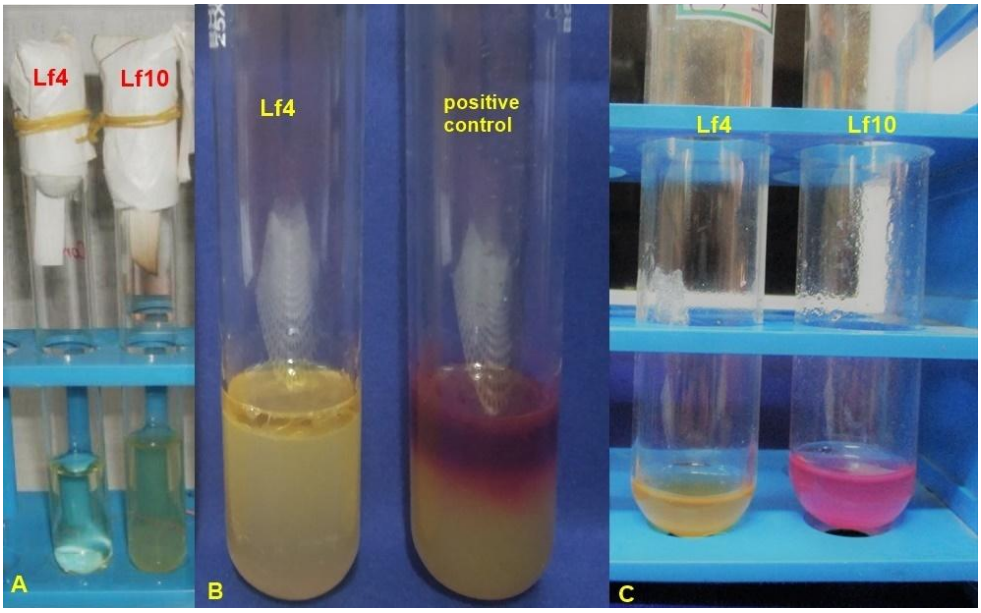

Fig. 4. Biochemical characterization of Endophytic bacteria: A- H_2S production, B- Indole production, C-Urease test

Table 4. Identification of endophytic bacteria from Banana cv. Yangambi km5 (*AAA*) by partial sequencing of 16s rRNA gene

S. No	Isolate no	% similarity	GenBank accession no
1.	Lf4	99-100	MK828245
2.	Lf5	99-100	MN089639
3.	Lf10	99-100	MN088831
4.	Pt4	99-100	MN088092
5.	Ps7	99-100	MN227375

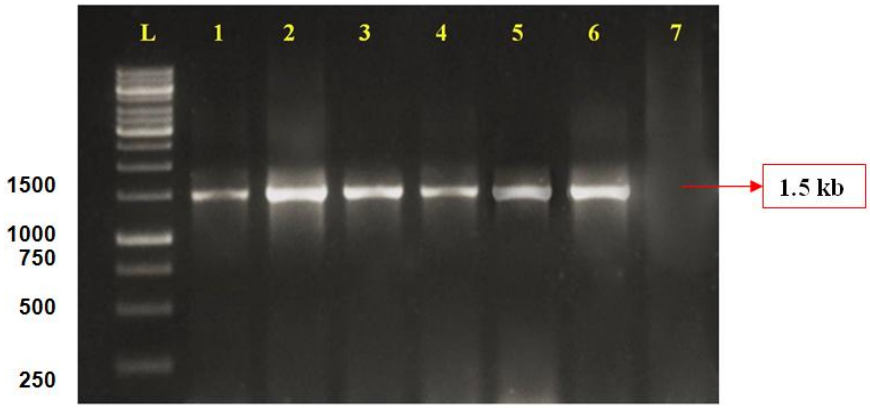

Fig. 5. Agarose gel electrophoresis for 16srRNA gene amplification

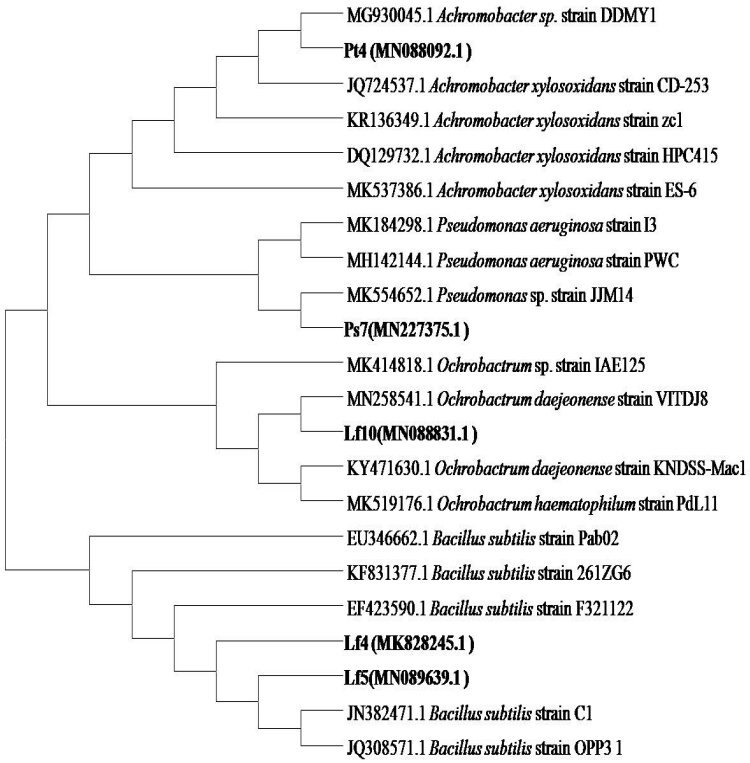

Fig. 6. Phylogenetic tree of partial 16S rRNA gene sequences of endophytic bacterial isolated from Banana

An evolutionary distance between bacterial strains were assembled with the partial 16S rRNA gene sequences of the endophytic bacteria by MEGA 6.0 and including representative bacterial type strains of related taxa generated by neighbour-joining method (Fig 6). Previously some genus has been reported as banana intercellular colonizers, including *Bacillus, Burkholderia cepacia, Citrobacter sp., Klebsiella spp., Klebsiella variicola, Ochrobactrum* [15]. Diverse endophytic bacterial species have been isolated from banana in the previous studies which include *Bacillus subtilis* and *Pseudomonas aeruginosa* [7]. Similarly, *Achromobacter xylosoxidans* BE 53 strain identified from Pseudostem of banana by 16srRNA gene sequences [29].

4. CONCLUSION

Bacterial endophytes are colonized in healthy plant tissues and exist inside a plant cells with no clear symptoms of disease. The present study indicates there is possibility of modulation of the in planta resistance to soft rot in banana by the bacterial endophytes as evident from the inhibition of growth of the pathogen by some of the endophytes especially isolated from banana cv. 'Yangambi Km 5'. Characterization of endophytic bacterial isolates was analyzed through morphological and biochemical methods. Through molecular characterization, a bacterium was confirmed at the species level, strains Lf4 and Lf5 were identified as *Bacillus subtilis*. Strain Lf10 was identified as *Ochrobactrum daejeonense*. Strain Ps7 was identified as *Pseudomonas aeruginosa* and strain Pt4 identified as *Achromobacter xylosoxidans*. The phylogenetic tree constructed with the 16S rRNA sequences had discriminated the bacterial isolates clearly. The strains characterized in the study can be expected to have promising application as plant biofertilizer or bioenhancer for plant growth improvement.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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