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In vitro efficacy of endophytic bacteria against *Pectobacterium carotovorum* subsp. *carotovorum* causing soft rot disease in banana

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Abstract
The study was carried out to evaluate the endophytic bacteria isolated from banana leaf, pseudostem and roots against banana soft rot pathogen. Among the twenty endophytic bacteria tested, strain EBV2 showed maximum inhibitory activity and recorded with 660.18 mm^2 of mean inhibition zone. This effective endophytic strain was further characterized based on cultural, biochemical and molecular techniques. The culture characters showed circular, smooth/powdery form, creamy colour with wavy serrated margins. The biochemical characterization showed positive reaction for gram's staining, catalase test, citrate utilization test and growth in 5% NaCl and negative reaction for oxidase, KOH, indole and pigment production test and characterized the effective strain EBV2 as *Bacillus* sp. The PCR based molecular characterization using 16S rRNA universal primer and partial sequencing confirmed the effective strain EBV2 as *Bacillus subtilis*.

Keywords: Endophytic bacteria, banana soft rot, *Pectobacterium carotovorum* subsp. *carotovorum*, *Bacillus subtilis*, Agar-well diffusion method

Introduction
Banana is an important fruit crop cultivated worldwide. In recent years, the soft rot incited by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) is a dreadful disease causing severe yield loss wherever banana crop is growing. The management of this disease using chemicals is hazards to environment, creating health hazards and develops quick resistance. Hence, many researchers have shifted their research to identify new biocontrol agents for the management of plant diseases which are safe and promising alternative to pesticides (Meshanki Bamon *et al.*, 2018) [11]. Endophytic bacteria act as an effective colonizer of intercellular plant parts *viz.*, roots and other plant parts and affect the pathogen to enter inside the plant system (Ardanov *et al*., 2012) [2]. Numerous endophytic bacteria were identified in single host with the presence of antimicrobial genes (Rosenblueth and Martinez-Romero, 2006) [15]. The most commonly identified endophytic bacteria are *Bacillus, Pseudomonas* and Enterobacteria (Hallman *et al*., 1997) [7]. Endophytic bacteria prevent plant pathogens by various mechanisms as antibiotic production, growth promotion, induced systemic resistance, parasitism and signal interfering of quorum sensing activities (Mansoori *et al*., 2013; Eljounaidi *et al*., 2016) [10, 4]. Based on the above, research work was undertaken for the isolation and characterization of endophytic bacteria against *Pectobacterium carotovorum* subsp. *carotovorum.*

Materials and Methods
Collection and isolation of endophytic bacteria from plant sample
The isolation of banana endophytic bacteria was done as per the method described by Anjum and Chandra (2015) [1] with slight modifications. Sixty to hundred day old healthy banana plants were collected from different districts of Tamil Nadu. Collected plant samples were constantly washed with running tap water to remove soil, organic matter and epiphytotic microorganisms. Plant parts were separated into individual organs *i.e.*, leaves, pseudostem and roots by cutting with sterile scalpel.

Tissues were dissected into 1 cm^2, transferred to sterile Petri plates containing 70% ethanol and allowed for 5 minutes and sequentially immersed in 1% NaOCl for 1 minute. Finally, tissues were washed three times with sterile distilled water. The samples were macerated with sterile pestle and mortar using 0.1 M phosphate buffer (pH 7.0) under aseptic conditions. Serial dilutions of ground samples were made up to 10^{-5} using 9 ml sterile water in test tubes under aseptic conditions and 1 ml from each dilution was transferred to sterile Petri plates. The molten nutrient agar medium was poured into the Petri plates and allowed to solidify. The plates were incubated at room temperature for endophytic bacterial colony growth and three replications were maintained for each dilution. Water washings from 3rd and 7th washings served as control. Absence of bacterial colony growth in 7th water wash control confirmed the presence and isolation of bacterial entophytes from banana samples.

Testing the antibacterial activity of endophytic bacteria against *Pcc*

The isolated endophytic bacteria were evaluated for its antibacterial activity against *Pectobacterium carotovorum* subsp. *carotovorum* using agar – well diffusion method (Valgas *et al*., 2007) [20]. For this, 24 hrs old soft rot pathogen and bacterial antagonists were allowed to grow on nutrient broth in shaker to maintain equal volume of 2 X 10^8 CFU/ml. The NA medium along with *Pcc* was allowed free in laminar chamber for 20 to 30 minutes. A well with a size of 9 mm diameter was made with the help of sterile cork borer on the corner of the plate in three places just 1cm away from the periphery of the plates. The 48 h old nutrient broth multiplied endophytic bacterial antagonists were poured in the well at the rate of 100 µl / well and incubated for 24 h at 28 ± 2 ºC. Each treatment was replicated three times. The efficacy of antagonist was assessed by measuring the area of inhibition zone (mm^2) after 48 h of incubation at 28 ± 2 ºC (Suganyadevi *et al.*, 2016) [18] and sterile water used as negative.

Cultural, morphological and biochemical characterization of effective endophytic bacterium

Identification of the effective endophytic bacterial isolate was done based on cultural, morphological characterization and biochemical analysis. For cultural and morphological characterization, the colony growth characters were observed on Nutrient Agar medium based on Bergey's manual of systemic bacteriology (Wahyudi *et al.*, 2011) [22]. Biochemical characterization *viz.*, gram's reaction, catalase test, citrate utilize test growth in 5% NaCl, oxidase/KOH test and indole/pigment production test were carried out as per the methods described by Schaad *et al*. (2001) [17]

Molecular characterization of effective endophytic bacterium

The genomic DNA from the effective bacterial isolate was isolated using the standard protocol of CTAB method proposed by Knapp and Chandlee (1996) [9] with slight modifications. The amplification of genomic DNA was performed by 50 µl reaction (25 µl master mix, 5µl forward primer, 5µl reverse primer, 10 µl H_2O and 5µl genomic DNA) Eppendorf Master Cycler nexus gradient S (Eppendorf, A G, Hamburg, Germany). The PCR settings used were as follows: a hold of 2 min at 95°C, 40 cycles of 1 min at 95°C, 1 min at 55°C and 1min at 72°C and a final extension of 5min at 72°C. The PCR products were resolved on 1.5% agarose at 100 V stained with ethidium bromide (0.5µg/ml), photographed and analyzed using gel documentation system (Bio-Rad, USA). The partial gene sequencing was performed by outsourcing at Chromos Biotech Pvt. Ltd. Bangalore, India.

Statistical analysis

The data were statistically analyzed by SPSS (Statistical Package for Social Sciences) (Nie *et al.*, 1975) [12] and AgRes (Gomez and Gomez, 1984) [5]. Laboratory experiments were carried out under Completely Randomized Block Design (CRD).

Results and Discussion

Isolation and *in vitro* screening of endophytic bacteria against *Pcc*

Endophytes are ubiquitous colonizer of internal plant tissue which does not cause any morphological changes in disease sign (Schulz and Boyle, 2006) [19]. Twenty endophytic bacteria from banana leaves, pseudostem and root region were isolated (Table 1).

Table 1: Endophytic bacteria isolated from banana

S. No	Plant region	Isolate code	Number of isolates
1.	Leaf	EBM1, EBV1, EBT1, EBTI1, EBS1, EBTR1	6
2.	Pseudostem	EBM2, EBV2, EBT2, EBTI2, EBS2, EBTR2 and EBMT	7
3.	Root	EBM3, EBV3, EBT3, EBTI3, EBS3, EBTR3 and EBC	7

The isolated twenty endophytic bacteria from banana plants were screened *in vitro* for their efficacy against *Pectobacterium carotovorum* subsp. *carotovorum.* The highest inhibition zone of 660.18 mm^2 was recorded in the isolate EBV2 and it was followed by isolate EBT2 with the inhibition zone of 415.26 mm^2 collected from the stem region. The remaining isolates recorded less inhibition zone of 78.50 to 379.94 mm^2 (Table 2; Plate 1; Figure1). Many researches have investigated the efficacy of endophytic bacterial antagonists against soft rot pathogen. Cui *et al*. (2019) [3] isolated the bacterial antagonist *Bacillus amyloliquefaciens* (KC-1) from cabbage and identified its antibacterial action towards soft rot pathogen *Pcc*. Rajamanickam *et al*. (2018) [14] reported that endophytic strains *Bacillus subtilis* PP and CL3 inhibited the growth of banana soft rot pathogen to an maximum extent over an area of 924 mm^2 and 908 mm^2 by agar well diffusion method. Similarly, Ragavi *et al*., (2019) [13] also evaluated the antibacterial activity of endophytic bacteria against banana soft rot *Pcc* and identified the isolates as *Bacillus subtilis, Ochrobactrum daejeonense, Achromobacter xylosoxidans* and *Pseudomonas aeruginosa.*

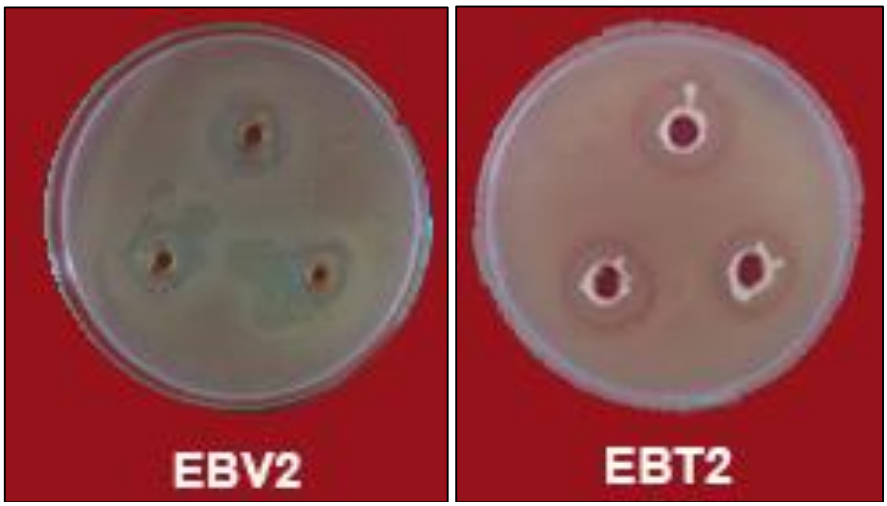

Plate 1: *In-vitro* efficacy of endophytic bacteria against *Pcc*

Table 2: *In-vitro* efficacy of endophytic bacteria against *Pectobacterium carotovorum* subsp. *Carotovorum*

S. No	Isolate code of Endophyte	Mean inhibition zone (cm)	Mean area of inhibition (mm^2)
1.	EBM1	1.37	147.33^{de} (12.13)
2.	EBM2	1.0	78.5^{f} (8.86)
3.	EBM3	1.6	200.96^{d} (14.17)
4.	EBV1	1.2	113.04^{de} (10.63)
5.	EBV2	2.9	660.18^{a} (25.68)
6.	EBV3	1.5	176.62^{d} (13.28)
7.	EBT1	1.06	88.20^{e} (9.39)
8.	EBT2	2.3	415.26^{b} (20.37)
9.	EBT3	1.06	88.20^{e} (9.39)
10.	EBTI1	1.9	283.38^{c} (16.83)
11.	EBTI2	1.86	271.57^{c} (16.47)
12.	EBTI3	1.46	167.33^{d} (12.93)
13.	EBS1	1.9	283.38^{b} (16.83)
14.	EBS2	1.3	132.66^{de} (11.51)
15.	EBS3	2.2	379.94^{a} (19.49)
16.	EBTR1	1.23	118.76^{de} (10.89)
17.	EBTR2	1.46	167.33^{c} (12.93)
18.	EBTR3	1.8	254.34^{b} (15.94)
19.	EBMT	1.73	234.94^{b} (15.32)
20.	EBC	1.3	132.66^{cd} (11.51)
21.	Control	0.0	0.00^{f} (0.52)

SED: 0.03
CD (P=05): 0.07

Values are mean of three replications; Values in parentheses are square root transformed values In a column, means followed by a common letter are not significantly different at 5% levels by LSD

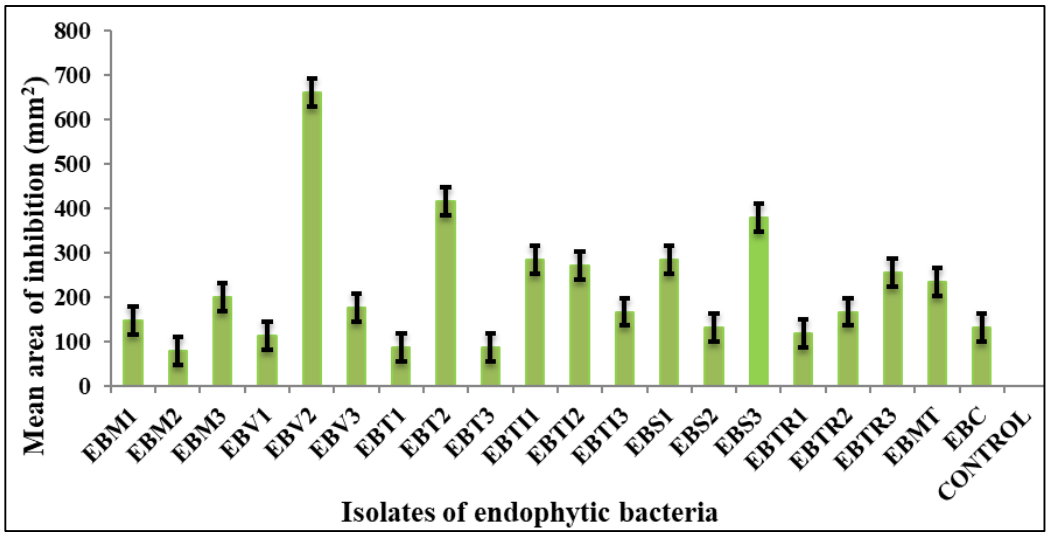

Fig 1: *In-vitro* efficacy of endophytic bacteria against *Pcc*

Cultural, morphological and biochemical characterization of effective endophytic bacterial antagonist

The effective endophytic bacterial antagonist EBV2 was characterized based on cultural character showing circular, smooth and powdery form, creamish colour and serrated wavy margins are observed (Plate 2; Table 3). The results are similar with Sampath kumar *et al*., (2018) [15] reported that endophytic *Bacillus* subtilis show creamy colour, smooth/slimy in appearance and serrated margins. Gupta *et al*. (2015) [6] studied the biochemical characterization of endophytic *Bacillus* sp. and identified positive reaction to gram staining reaction, catalase test, amyloytic activity and negative result to urease production. Similar results are in coincidence with our study isolate EBV2 showing positive reaction for gram's reaction, catalase test, citrate utilization test and growth in 5% NaCl and negative reaction for oxidase, KOH, indole and pigment production test (Table 3). Ragavi *et al*. (2019) [13] also revealed similar cultural and biochemical characterization for the identification of endophytic bacteria and identified the isolate as *Bacillus subtilis*.

Plate 2: Cultural and morphological characters of endophytic bacterium EBV2

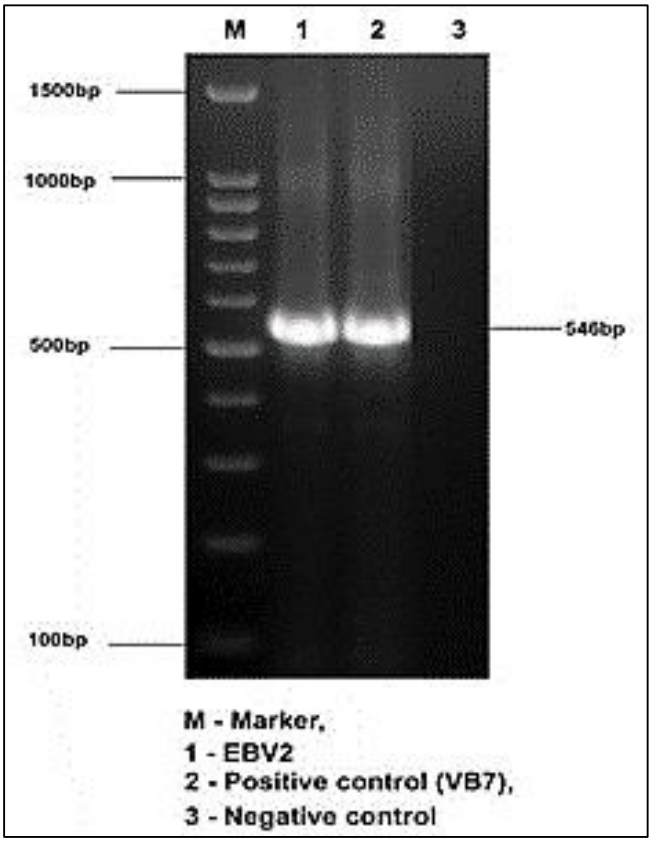

Plate 3: Agarose gel electrophoresis for 16srRNA gene amplification of EBV2

Table 3: Cultural, morphological and biochemical characterization of effective endophytic bacterial isolate EBV 2

S. No	Isolate code	Cultural and morphological characters		
		Colony colour	Colony form	Colony margin
1.	EBV2	Creamy	Circular, smooth and powdery	Deeply serrated with wavy edge
S. No	Biochemical characterization		Positive (+) / Negative (-)	
1.	Gram staining		+	
2.	Catalase test		+	
3.	Citrate utilization		+	
4.	Growth in 5% NaCl		+	
5.	Oxidase activity		-	
6.	KOH test		-	
7.	Indole production		-	
8.	Pigment production		-	

Molecular characterization of effective endophytic bacterial isolate EBV 2

Genomic DNA of effective bacterial antagonist EBV2 was subjected to PCR amplification using universal primer 16S rRNA BCF1 and BCR2. The amplication of product with amplicon size of 546 bp was identified (Plate 3). The amplicon product of 546 bp was sequenced in Chromos Biotech, Bangalore, India. Blast analysis was carried out in NCBI database and identified more than 99% similarity with other existing *Bacillus* isolates in NCBI database. Similar to the 16S rRNA primer used in our study, Vinodkumar *et al*. (2017) [21] used BCF1 and BCR2 primers for the identification of *Bacillus* species characterization from carnations, cotton, turmeric, and banana and revealed the bacterium as *Bacillus amyloliquefaciens*. Rajamanickam *et al*. (2018) [14] used 16S rDNA intervening sequence- specific BCF 1 (5′CGGGAGGCAGCAGTAGGGAAT3′) and BCR2 (5′CTCCCCAGGCG GAGTGCTTAAT3′) primers for the confirmation of effective endophytic banana isolates as *Bacillus subtilis* against *Pcc*. Ragavi *et al*. (2019) [13] used 16S rRNA universal primer for the characterization of banana endophytic bacteria and identified the effective strains as *Bacillus subtilis, Ochrobactrum daejeonense, Achromobacter xylosoxidans and Pseudomonas aeruginosa.*

Conclusion

The present study demonstrated the effectiveness of endophytic bacterial isolate EBV2 against soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) in banana. Based on the cultural, morphological and biochemical characterization, the effective strain EBV2 was identified as *Bacillus* sp. Through molecular characterization, the endophytic bacterium was further confirmed up to species level as *Bacillus subtilis*.

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