

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.811.218>

**Molecular Detection of the Causative Agent of Soft Rot
(*Pectobacterium carotovorum* subsp *carotovorum*) in Banana (*Musa* sp.)**

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A B S T R A C T

Soft rot disease of Banana caused by *Pectobacterium carotovorum* subsp. *Carotovorum* is found to be a serious disease and cause considerable yield loss. Common cultivars viz., Grand Naine, Rasthali and Nendran are extremely prone to this disease. Understanding the disease diagnosis and pathogen virulence is important in management programs. The pathogen was isolated from infected rhizomes from five locations of Theni district of Tamil Nadu. Pathogenicity of all isolates was confirmed by *in vitro* inoculation in rhizome bits and *in vivo* inoculation in pseudostems of three months old tissue cultured plants of cv. Grand Naine. The virulent isolates Icb₄ and Ikg₂ were characterized according to the morphological and microscopic characteristics, along with the physiological and biochemical assays; which indicates that the bacteria are gram negative, rod shaped, peritrichously flagellated. The colonies form characteristic deep cavities or pits in Crystal violet pectate medium (CVP). Results of biochemical tests showed that isolates Icb₄ and Ikg₂ have a positive reaction for catalase, H₂S production and nitrate reductase and a negative reaction for oxidase test, Indole test and gelatin liquefaction. The results determining the optimal temperature for the growth of isolates Icb₄ and Ikg₂ have revealed that the temperature at 30 °C - 37 °C were suitable to obtain profuse growth of *Pectobacterium*. Total DNA extracted from the isolates Icb₄ and Ikg₂ were subjected to PCR analysis of 16S-23S intergenic transcribed spacer region. PCR amplified 1.5 kb amplicons were sequenced. Sequence Homology and phylogeny analyses had shown that the isolates Icb₄ and Ikg₂ were belonged to *Pectobacterium carotovorum* subsp. *carotovorum*.

Keywords

Banana (*Musa* sp),
Pectobacterium
carotovorum subsp.
carotovorum, Soft
rot

Article Info

Accepted:
12 October 2019
Available Online:
10 November 2019

Introduction

Banana (*Musa* sp) is one of the ancient cultivated tropical fruit crop in India next to mango in both area and production (d'Hont *et al.*, 2012). Recently, rhizome rot or soft rot has come forth as a major disease, particularly in southern states of India (Usha, 2003; Snehalatharani and Khan, 2010; Nagaraj *et al.*, 2012). Soft rot disease is cited as a major protective factor in tissue culture banana during the hardening phase and infield plants during the Post rainy season. The disease is incited by *Pectobacterium carotovorum*, a soil-borne pathogenic bacterium (Thangavelu, 2009). Currently, soft rot of banana is ahead importance in southern states of India (Snehalatharani and Khan, 2010) though earlier the disease was considered as of negligible importance. Accepted tissue culture banana cultivars Grand Naine and Nendran are susceptible to this disease. This disease is more pronounced in young suckers leading to soft rot accompanied by a bad tempered foul odour and rotting of the crown region followed by epinasty of leaves, which dry out abruptly. In the initial stage of infection, dark brown water soaked appearance in the cortex area. In advance stage, the internal lesions may rot to such extent that hollow space surrounded by dark soft tissues are formed and finally toppling over the plant (Nagaraj *et al.*, 2012). Yeast Extract Glucose Calcium Carbonate (YGC) medium is a specific medium for *Pectobacterium* species; which produces acid. A clear zone surrounding each colony confirms from acid secretion, which releases the carbonate as carbon dioxide (Kado, 2006). Rapid and accurate diagnosis is essential for successful treatment and control of diseases. Diagnostic techniques of *Erwinia* spp. include the use of both traditional and modern molecular techniques. Different methods are followed in order to detect, identify and differentiate soft rot causing bacteria to species and subspecies level, of

these methods: microscopy, isolation, biochemical characterization, serological techniques, pathogenicity and bioassay tests.

All of these methods are time consuming, insensitive, inaccurate and not suitable for routine work to test a large number of samples (Czajkowsk *et al.*, 2009). In the last 30 years, Polymerase Chain Reaction (PCR) has been used for specific, rapid detection and identification of pathogen isolates. PCR techniques greatly enhance detection sensitivity, simplicity and rapidity compared with other methods of identification and are based on specific amplification of a target DNA sequence that is unique to a bacterial genome (Kang *et al.*, 2003). The objective of the present study was to confirm the identity of *Pectobacterium carotovorum* subsp *carotovorum* as the causal organisms of soft rot of Banana through biochemical and molecular techniques.

Materials and Methods

Source of bacterial strains

Survey on the disease incidence was done to know the status of the disease in major banana growing areas of Theni district viz., Kamayagoundanpatti, Cumbum, Pudupatti, Erasakkanaickanur and Gokilapuram. Infected plant parts showing typical symptoms of soft rot disease of banana were collected from farmers' field at mentioned locations (Table 1). The survey is conducted by Rover's method. Per cent Disease Incidence (PDI) can be calculated by, Per cent disease incidence = No. of plants infected/ Total No. of plants examined x 100. Soil type varied between sandy loam to laterite and the disease was observed mainly under conditions of high soil moisture and humidity. The affected rhizomes in the initial stages of rotting were collected in sterile polythene bags and brought to the laboratory for pathogen isolation (Fig. 1).

Isolation and maintenance of culture

The affected part of the rhizome and pseudostem of banana was washed in the tap water, dried in blotter paper and then were cut into small pieces with a sterile scalpel and these were disinfected with 70 per cent alcohol for 45 sec and washed thrice in sterile distilled water so as to remove traces of alcohol. The samples were dried by sterilized blotting paper. The small bit of sample was crushed in 5 ml sterilized distilled water using sterilized mortar and pestle and kept for 15 minutes to come out bacterial ooze. CVP medium is the indicative media for *Erwinia* spp, so the pour plate method was done to get single colonies (Totagi, 2011). Isolated single colonies were picked and streaked on nutrient agar medium for developing pure cultures. The inoculated plates were incubated at 28° C for 48 hours. The pure cultures of the bacterial isolates were maintained in 70% glycerol stock in refrigerator at -20°C for further studies

Pathogenicity tests

***In-vitro* tests**

Healthy rhizome bits of banana variety 'Grand Naine' were surface sterilized in 70 per cent alcohol for 30 sec and washed with double sterile water to wash excess alcohol and the rhizome bits were inoculated with 48 h. old pathogenic bacteria culture under aseptic conditions (Rahman *et al.*, 1994).

***In vivo* tests**

The pseudostem of 60 days old tissue-cultured banana plants of cv. Grand Naine, were taken and make a small cut with help of a sterilized scalpel, after that the plants were inoculated with agar plugs contained 48 h. old bacterial culture grown in NA medium and covered with sterile moist cotton (Thomas *et al.*,

2008). The pseudostem of Banana plants were inoculated with only agar plugs without any bacterial cultures served as control. The inoculated plants were kept in a glasshouse and were watered regularly. Observations were recorded for the development of symptoms such as pale yellow and brown discoloured necrotic leaves and the development of rotting at the rhizome region. Pathogen was reisolated from the infected rhizome and pseudostem samples and cultured in a specific CVP medium.

Virulence test

The virulence determination tests of bacterial isolates were determined by potato and carrot soft rot test with slight modifications by using a healthy potato and carrot. The potato and carrot were washed thoroughly in tap water, before being submerged in a container filled with 70% ethanol. After about two minutes they were removed, allowed to dry in the air, and cut these potato and carrot tubers into slices with a sterilized scalpel. The slices of potato and carrot placed in a sterilized Petri dish by inoculating with agar plugs contained 24 h. old bacterial culture grown in NA medium and covered with sterile moist cotton and incubated at 30 °C. The observations were taken seven days after incubation. The rotting of potato and carrot tissue was an indication of the pectolytic activity (Perombelon, 1979; Pérombelon and Kelman, 1980). The pathogen was reisolated from the rotten potato and carrot slices and cultured in a specific medium.

Bacterial characterization

Characterization of bacterial pathogen was carried out by based on the virulence of pathogen on the rhizome, potato and carrot respectively, the highly virulent cultures of *Pectobacterium carotovorum* subsp *carotovorum* designated as isolates Icb₄ and

Ikg₂ were Characterized by morphological, biochemical, physiological and molecular level (Snehalatharani and Khan, 2010).

Cultural characterization

Cultural Characterization was done on Nutrient Agar (NA) and Yeast Extract Glucose Calcium Carbonate (YGC) medium. Nutrient agar medium was prepared and streaked with a single colony of 48 h. old bacterial culture and incubated at 28±1°C for 48 h (Snehalatharani and Khan, 2010). Yeast Extract Glucose Calcium Carbonate YGC medium was prepared and spot inoculated with 100µl of 24 h. old bacterial culture and incubated at 28 ± 1 °C for 48 h and observed for acid and carbon dioxide production (Kado, 2006; Snehalatharani and Khan, 2010).

Morphological features of soft rot bacterium

General morphology

The gram stain usually allowed observation of cell size, shape and arrangement.

Gram staining (Skerman, 1967)

A smear was prepared from a young 24 h. old bacterial culture, air-dried and gently heat fix by lamp flaming on the underside of the slide. Slide was flooded with crystal violet for 1 min and washed for 3-4 seconds in a gentle flow of tap water. This was flooded with iodine solution for 1 min and washed again with tap water. The stain was decolourized by applying 95 per cent ethanol dropwise to smear until no more colour runs from the lower edge (usually 10-20 seconds). The slide was again washed and counterstained with safranin for 1 min. the slide was again washed in tap water, air-dried and observed under oil immersion microscopy.

Flagella staining

The Leifson (1951) technique is used for flagella staining.

Stain procedure

The cleaned new slides were flamed and an area 2/3 of the side was marked with a grease pencil. 24 h. old young bacterial cultures were used. Sterilized distilled water was added gently on 24 h. young bacterial slants and the motile bacteria were allowed to dispense in water.

A drop was placed on one edge of greased ring and tilt to allow a drop to glide downslide, air-dried without heat. Exactly 1 ml of Leifson stain was added to within the ring and stains for exactly 14 min and then washed with running tap water and air-dried. It was examined under oil immersion. Stain intensity may be varied by altering staining time.

Biochemical characterization

The physiological and biochemical characteristics of strain Icb₄ and Ikg₂ were studied for pectate degradation, gelatin liquefaction, urease production, H₂S production, nitrate reductase, sensitivity to erythromycin, Motility test, catalase, oxidase test, indole test methyl red test, Gelatin liquefaction, Caesin hydrolysis, growth at 3% NaCl and Growth at 5% NaCl (Table 4). The tests were conducted as per the methods described by Ploetz *et al.*, (2003), Seo *et al.*, (2002) and Fiori *et al.*, (2005).

Growth rate of bacteria at different temperatures

Bacterial colonies (48 h. old) were inoculated into sterile nutrient broth in an Oakridge tube and incubated for 48 h. at different

temperatures 27, 29, 31, 33, 35, 37 and 39 °C, with shaking at 120 rpm (Shrestha *et al.*, 2005). Control was maintained using nutrient broth with no bacterial inoculation. Observations on optical density at 620 nm were read after 24 and 48 h.

Molecular characterization

DNA was isolated from the bacterial cultures (Icb₄ and Ikg₂) using lysis method (Chen and Kuo, 1993) and the 16S rRNA gene was amplified through Polymerase chain reaction (PCR) using the universal 16S rRNA primer set 27 F and 1492 R (Meng *et al.*, 2017).

PCR was performed using 50 µl reaction mix containing 2 µl of each primer, 25 µl of master mix (Genei), 19µl of H₂O and 2 µl of DNA template (5µg/µl).

Thermal cycler (Eppendorf Master cycler) included initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1min and primer extension at 72 °C for 1min and final extension was at 72 °C for 10 min.

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. The Phylogenetic tree was constructed using MEGA 6.0 software. The bacterial isolates Icb₄ and Ikg₂ were used as reference out group.

Statistical Analysis

Mean differences of the disease incidence were evaluated with ANOVA using Duncan's Multiple Range-Test at 5% significance (Gomez and Gomez, 1984). All the data were statistically analyzed with SPSS software and consequently interpreted.

Results and Discussion

Survey and isolation of the pathogen from Banana

Roving survey was conducted during 2017 to assess the disease incidence of soft rot of banana in Theni districts (Table 1). The per cent disease incidence was noticed in all the locations surveyed, with a range from 32.4 to 62.6 per cent. The maximum disease incidence was observed in Cumbum taluk followed by Kamayagoundanpatti village. The survey is in line with the studies conducted by Snehalatharani (2001) and Nagaraj and Khan (2002). Similarly, Thammaiah *et al.*, (2005) and Vasundhara and Thammaiah, (2017) were conducted Roving survey major banana growing areas of Belgaum, Bagalkot and Bijapur districts of Northern Karnataka during 2012.

The causal organism *Pectobacterium carotovorum* subsp. *carotovorum* was isolated from infected rhizome and pseudostem showing characteristic symptoms of soft rot. Pathogen isolated from the infected samples have given small bacterial colonies on the NA medium, which were cream to white colour, to some extent raised and mucoid with a diameter of 2mm. Previously Snehalatharani and Khan (2010) and Totagi (2012) have reported the colony characters of *Erwinia carotovora* subsp. *carotovora* (*Pectobacterium carotovorum*). CVP medium, which is specific for *Pectobacterium* sp, characteristic small pits were formed.

Pathogenicity and virulence test

The results of Pathogenicity revealed that all the bacterial isolates were produced characteristics symptom of soft rot upon artificial inoculation. Rotting of inoculated rhizome bits of banana cv. Grand Naine was observed in 7 days under *in-vitro* condition the

pathogen was reisolated in NA medium and CVP medium and produced characteristic pits similar to the original isolates. Usha (2003) also reported that the inoculated rhizome bits under artificial conditions produce rotting, discoloration and foul smell after 7 and 8 days of inoculation, respectively.

Under *in vivo* condition, the bacterium has produced symptoms on tissue culture plants, 6 days after inoculation. Symptoms observed were yellowing of lower leaves and the yellowing gently spread to upper leaves. Deteriorating of the pseudostem has started and finally the plant chop down as the pseudostem got entirely rotten and emitted foul smell, after 16 days of inoculation.

Nagaraj *et al.*, (2012) and Nagrale *et al.*, (2013) reported that complete rotting of healthy rhizome bits were observed after 37 DAI under *in vivo* condition. The similar plant symptoms were observed by Thomas *et al.*, (2008) who demonstrated that plants inoculated with pathogenic *Pectobacterium carotovorum* subsp. *carotovorum* produced rotting symptoms within 7-12 days after inoculation. Deviation was observed in days taken for the complete rotting of grand naine plants by different isolates. The minimum time taken for rotting was 4- 7 days by the isolate no. Icb₄ and Ikg₂ followed by 8-10 days by Ipp₆ and Ien₁₁. Maximum time of 10-12 days was taken by Igp₉ (Table 2).

The results of virulence determination revealed that potato and carrot slices were inoculated with bacterial strains has been produced rotting symptoms with the emission of characteristic foul smell after 2–7 days (Table 3), respectively. In similar studies, potato inoculated with bacterial pathogen produced the soft rot symptom after 3 days of inoculation (Togashi, 1988; Nabhan *et al.*, 2006). Variation was observed in time for the rotting of potato and carrot by different

isolates. Bacterial isolates Ikg₂ and Icb₄ recorded a minimum of 3 days for complete rotting of potato. Maximum time of 4-5 days by isolates Ipp₆ Igp₉, and Ien₁₁ respectively (Table 3). Minimum time taken for complete rotting was 4 days in carrot by isolates Ikg₂ and Icb₄. Whereas, maximum time taken for complete rotting of carrot was 5 and 7 days by Ipp₆ Igp₉, and Ien₁₁, (Table 3). Rotting in carrot was slow and complete rotting was observed after 14 days after inoculation (Schillingford, 1974; Doolotkeldieva *et al.*, 2016).

Cultural characterization

In YGC medium, Creamish yellow-coloured colonies were produced with a clear zone after 24 h of incubation. Small bubbles were observed on the apex of each bacterial colony due to the emission of carbon dioxide from the medium which is the characteristic feature of *Pectobacterium carotovorum*. Similarly, Usha (2003) has reported the cultural characters of *Pectobacterium carotovorum* in the YGC medium. Kim *et al.*, (2012) stated that *Pectobacterium* and *Erwinia* strains were incubated on nutrient agar (NA) and *Xanthomonas* strains were grown on YGC medium.

Morphological, biochemical and physiological characterization

The Gram staining is the first step in the preliminary identification of a bacterial organism. While Gram staining is a priceless analytic tool in research settings, not all bacteria can be classified by this method. It is used to discriminate and categorize bacterial species into two great groups (Gram positive and Gram negative). In the present study after Gram staining, red colored short rods were observed under an oil immersion microscope, indicative of the Gram-negative nature of this bacterium.

Table.1 Survey on disease incidence of soft rot of banana in Theni district of Tamil Nadu

S. No	Isolate no	Place of collection	Districts	GPS Data of location in Latitude – longitude	Disease incidence (%)*
1.	Ikg ₂	Kamayagoundanpatti	Theni	“9.71-77.33”	54.1 (47.35)
2.	Icb ₄	Cumbum	Theni	“9.68- 77.24”	62.6 (52.3)
3.	Ipp ₆	Pudupatti	Theni	“9.76-77.30”	40 (39.23)
4.	Igp ₉	Gokilapuram	Theni	“9.78-77.34”	36.9 (37.41)
5.	Ien ₁₁	Erasakkanaickanur	Theni	“9.78-77.39”	32.4 (34.70)
CD (p=0.05)					2.67

Table.2 Period of development of symptoms in banana (cv. Grand Naine) plants

S. No	Isolate no	Yellowing of leaf and rotting (days)	Full rotting of the plant (days)	Falling down of plants (days)
1.	Ikg ₂	5	6	7
2.	Icb ₄	4	5	6
3.	Ipp ₆	8	9	10
4.	Igp ₉	10	11	12
5.	Ien ₁₁	6	7	8

Table.3 Virulence determinants of *Pectobacterium carotovorum* subsp. *carotovorum* by potato and carrot soft rot test

S. No	Isolates	Potato soft rot		Carrot soft rot	
		Initiation of rotting	Completion of rotting	Initiation of rotting	Completion of rotting
1.	Ikg ₂	2	3	2	4
2.	Icb ₄	2	3	2	4
3.	Ipp ₆	3	4	3	5
4.	Igp ₉	4	5	4	6
5.	Ien ₁₁	3	5	3	7

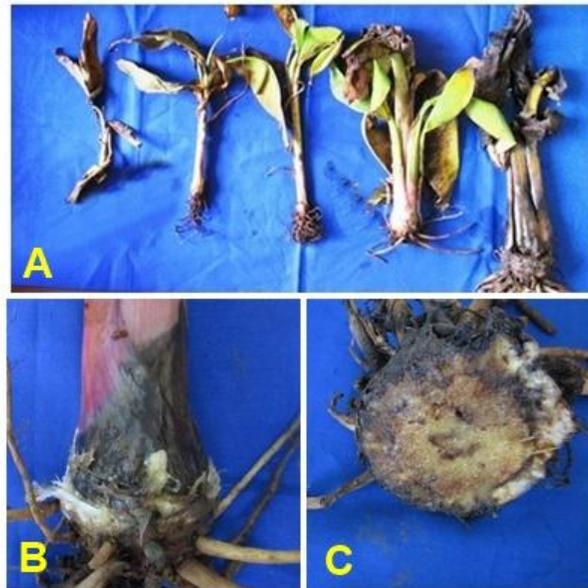
Table.4 Morphological, physiological and biochemical characterization of soft rot causing virulent bacterial strains

S.No	Characteristics features	<i>Pectobacterium carotovorum subsp. carotovorum</i>	
		Icb ₄	Ikg ₂
1.	Colony character	cream to yellowish color, slightly raised and mucoid	cream to yellowish color, slightly raised and mucoid
2.	Gram reaction	-	-
3.	Pectate degradation	+	+
4.	Pits formation on CVP medium	+	+
5.	Flagella	Peritrichous	Peritrichous
6.	Growth at 36-37°C	+	+
7.	Growth in presence of 5 % and 3% NaCl	+	+
8.	Motility test	+	+
9.	Urease production	-	-
10.	Nitrate reductase	+	+
11.	H ₂ S production	+	+
12.	Catalase test	+	+
13.	Oxidase test	-	-
14.	Indole test	-	-
15.	Gelatin liquefaction	-	-
16.	Methyl red test	+	+
17.	Casein hydrolysis	+	+
18.	Erythromycin sensitivity (200 ppm)	+	+

Table.5 Effect of different temperature on growth of bacterial strains under *in vitro*

S. No.	Temperature(°C)	OD value at 620 nm*	
		Strain I (Icb ₄)	Stain II (Ikg ₂)
1	27	0.587	0.542
2	29	0.668	0.652
3	31	0.709	0.737
4	33	0.819	0.885
5	35	0.931	0.988
6	37	1.465	1.204
7	39	0.435	0.445
8	40	0.157	0.164

Fig.1 Symptoms of rhizome rot of banana



A – different stages of rotting of 1-3 months old plants, **B** - Initial rotting of collar region of pseudostem, **C** - Completely rotten rhizome

Fig.2 Effect of temperature on growth of bacterial isolates

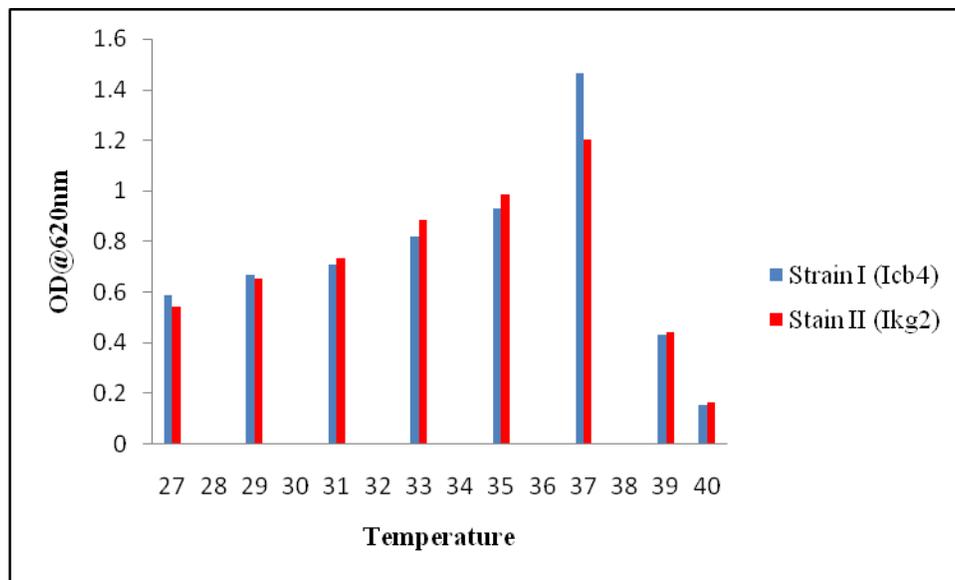
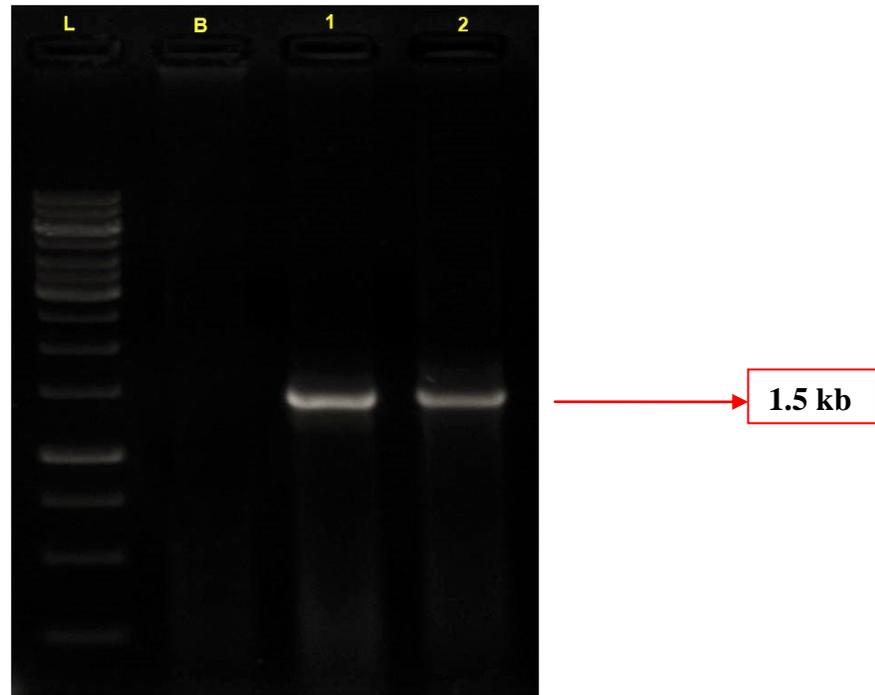
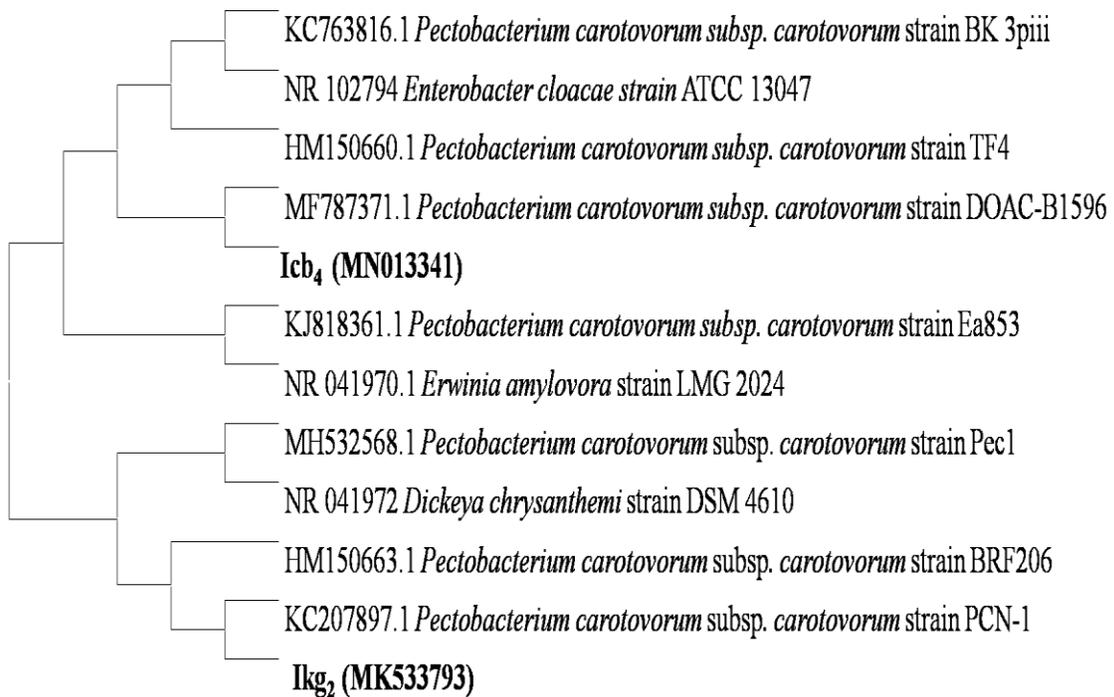


Fig.3 PCR on isolates Ikg₂ and Icb₄– Amplification of 16S rRNA gene using 27 F and 1492 R primers



L - 1 Kb plus ladder, B – control, 1 and 2 - Amplified colony PCR product of bacterial isolates

Fig.4 Phylogenetic tree of partial 16S rRNA gene sequences of *P. carotovorum* subsp *carotovorum* isolated from Banana. Sequences of selected references strains are included



The length of the bacterium was in the range of 2.3–2.7 µm and width in the range of 0.6–0.9 µm. The flagella stain give a method for viewing bacterial flagella. Peritrichous nature of the flagella was observed through flagella staining.

The results of biochemical tests revealed that isolates Icb₄ and Ikg₂ have a positive reaction for Pectate degradation, Pits formation on CVP medium, Growth at 36-37°C, Motility test, Nitrate reductase, Catalase, H₂S production, Methyl red and Caesin hydrolysis and a negative reaction for Urease production, Oxidase test, Indole test and Gelatin liquefaction. Bacterial growth was observed in the inoculated peptone broth containing three and four per cent sodium chloride. Results showed that the isolates Ikg₂ and Icb₄ tolerate the salt at all the concentration. Salt tolerant nature of *Pectobacterium* was previously reported by Dickey (1979), Gomez-Caicedo *et al.*, (2001), Akbar *et al.*, (2015) and Rafiei *et al.*, (2015).

After 5 days of incubation, bacterial cultures inoculated on CVP medium produced characteristic fissures or depressions due to pectate degradation as reported by Cupples and Kelmen (1974), Ma *et al.*, (2007) and Snehalatharani and Khan (2010). *Pectobacterium* spp. proven to have the pectate degrading ability (Bhupendra and Yogendra, 2015), similarly, in the present study the isolates Ikg₂ and Icb₄ were found to degrade the sodium polypectate present in the CVP medium, leading to the formation of fissures in five days. Bacterial isolates were insensitive to erythromycin but sensitive to streptomycin. An inhibition zone was observed around the filter paper disc containing streptomycin solution whereas no inhibition zone was observed around the filter paper disc containing erythromycin. *P. carotovorum* is reported to be resistant to erythromycin and susceptible to streptomycin

(Dickey and Victoria, 1980; Snehalatharani and Khan, 2010; Nagrale *et al.*, 2013; Akbar *et al.*, 2015). Since the isolates in this study are resistant to erythromycin and susceptibility to streptomycin, it is confirmed that they belong to *Pectobacterium carotovorum*.

After 48 h of incubation, the maximum growth of bacteria was recorded at 37 °C (OD@620nm value 1.465 and 1.204) followed by growth at 35 °C (OD@620nm value 0.931 and 0.988) in isolates Icb₄ and Ikg₂ (Table 5, Fig. 2). A previous study by De Boer and Kelman (2001), also reports that *E. carotovora* subsp. *carotovora* can grow at 37 °C while *E. carotovora* subsp. *atroseptica* fails to grow at such a high temperature. The temperature of 30°C and above is most favourable and destructive for the development of soft rot caused by *E. carotovora* sub sp. *Carotovora*. Similarly, Smadja *et al.*, (2004) demonstrated that all multiplication of eight strains of *E. carotovora* was high and able to grow up to 37°C. The temperature range 20-35°C enhanced the rotting ability of *E. carotovora* subsp *carotovora* was previously reported by Raju *et al.*, (2008), Bhat *et al.*, (2010) and Raan *et al.*, (2012).

DNA amplification and polymerase chain reaction (PCR)

The 16S rRNA universal primers were used to amplify DNA fragment enzymatically by polymerase chain reaction (PCR). The primer set which has a sequence 27F (5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R- (5'-GGTTACCTTGTTACGACTT-3'), during the electrophoresis process within (one and a half) hour, the results showed that selected isolates Icb₄ and Ikg₂ has yielded markers at 1.5 kb (Fig. 3). The BLAST analysis of nucleotide sequence had more than 99% similarity with existing isolates of *Pectobacterium carotovorum* subsp.

Carotovorum in NCBI. The nucleotide sequence of our isolates submitted in NCBI database, bearing Acc. No. MK533793 and MN013341. The phylogenetic tree constructed using the 16S rRNA gene sequences of bacterial pathogen and including representative bacterial type strains of related taxa generated by the neighbour-joining method in MEGA 6.0 (Fig. 4). Sequence analysis of 16S rRNA gene sequences indicated that the strains Ikg₂ and Icb₄ was homologous with *Pectobacterium carotovorum* subsp. *carotovorum* strain PCN-1 and strain DOAC-B1596 and were also closely related to *Pectobacterium carotovorum* subsp. *carotovorum* strain BRF206. In a similar study, Zhu *et al.*, (2010) reported that the length of the 32 16S rRNA sequences of almost all isolates was 1381 bp, it has at least a 98% nucleotide sequence similarity with those of other *Pectobacterium* strains present in GenBank. Thapa *et al.*, (2011) had constructed a phylogenetic tree of *Pectobacterium* species and reported that *P. carotovorum* subsp. *Carotovorum* is closer with *P. carotovorum* subsp. *oderiferum*. Caruso *et al.*, (2016) stated that the analysis of the 16S rRNA gene sequence revealed that the isolates from tomato belong to *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliensis*, respectively.

Characterization of bacterial isolates was analyzed through Pathogenicity test followed by cultural, morphological, biochemical and physiological methods. Through molecular characterization, a bacterium was confirmed at the subspecies level, *P. carotovorum* Ssp. *carotovorum*. The phylogenetic tree constructed with the 16S rRNA sequences had discriminated the pathogen clearly.

Acknowledgement

The authors thank the financial support provided by the Department of Science and

Technology (FIST), Government of India, New Delhi for accomplishing this work.

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How to cite this article:

Ragavi, G., M. Muthamilan, S. Nakkeeran, N. Kumaravadivel, U. Sivakumar and Suganthi, A. 2019. Molecular Detection of the Causative Agent of Soft Rot (*Pectobacterium carotovorum* subsp *carotovorum*) in Banana (*Musa sp.*). *Int.J.Curr.Microbiol.App.Sci.* 8(11): 1854-1868. doi: <https://doi.org/10.20546/ijcmas.2019.811.218>