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Variability of *Pectobacterium carotovorum* causing rhizome rot in banana

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ABSTRACT

The rhizome rot or tip over is a major and emerging disease of banana, causing substantial economic losses. Common cultivars such as Grand Naine, Rasthali and Nendran are highly susceptible to this disease caused by different *Pectobacterium* species. Understanding the disease etiology and pathogen variability are essential in management programs. Bacterium *Pectobacterium carotovorum* was isolated from infected rhizomes from 18 locations of Kerala and Tamil Nadu states of India. Pathogenicity of all isolates were confirmed by *in vitro* inoculation in rhizome bits and *in vivo* inoculation in rhizomes of three months old plants and pseudostems of two months old tissue cultured plants of cv. Nendran. Isolates were characterized by cultural, morphological, biochemical, physiological and molecular methods. Cultural characterization was carried out on Nutrient agar (NA), Yeast Extract Glucose Calcium Carbonate (YGC), Logan's medium and Nutrient broth. Morphological characterization was carried out by Gram staining, capsule staining and flagellar staining. Biochemical characterization was done through potato and carrot soft rot test, intrinsic antibiotic resistance, growth of bacteria in three and four per cent NaCl and growth of bacteria in CVP medium. Physiological characterization was carried out by growing the bacterium at different temperatures and pH. Based on these results, 18 isolates were allocated to six groups.

16S rDNA regions of representative isolates of each group have been PCR amplified and the 1.5 kb amplicons were sequenced. Homology and phylogeny analyses had shown that all the groups belong to *Pectobacterium carotovorum*, and two subspecies *carotovorum* and *brasiliense* were identified.

1. Introduction

Banana or plantain (*Musa* sp.) known as 'Apple of Paradise' is one among the ancient fruits known to humankind. Recently, rhizome rot has emerged as a major disease, especially in southern states of India (Usha, 2003; Snehalatharani and Khan, 2010; Nagaraj et al., 2012). Popular banana cultivars Grand Naine, Nendran and Rastali are susceptible to this disease. Symptoms of the disease include massive soft rot accompanied by disagreeable foul smelling rot of the rhizome and internal decay of the pseudostem as the infection spread upward. Infected plants show stunted growth, water soaked appearance on the leaf base, yellowing of leaf and finally toppling over of the plant (Nagaraj et al., 2012).

Different *Erwinia* species (syn. *Pectobacterium*) cause rhizome rot in banana (Kwon et al., 2000). Usha (2003) reported that in Kerala state of India, *Pectobacterium carotovorum* is the pathogen causing rhizome rot in banana. This study reveals the extent of variability and the subspecies of this pathogen present in humid tropics of India, through cultural, morphological, biochemical, physiological and molecular methods.

2. Materials and methods

2.1. Development of bacterial pure cultures

Infected rhizome samples at the initial stage of rotting (10% rotting) were collected from 18 banana fields from Kerala and Tamil Nadu states of India. Type of soil varied between sandy loam to laterite and the disease was observed mainly under conditions of high soil moisture. Samples were collected in sterile polythene bags and brought to the laboratory for pathogen isolation (Fig. 1).

Rhizomes have been washed, cleaned thoroughly and small rhizome

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Fig. 1. Symptoms of rhizome rot of banana **A -** Initial rotting of rhizome, **B** and **C -** Completely rotten rhizome, **D -** Pseudostem rotting, **E -** Toppled plant, **F -** Sample collection.

bits were surface sterilized using 1% sodium hypochlorite for 45 s and washed thrice in sterile distilled water. Sterile distilled water (1–2 drops) has been added to the bits, squeezed to obtain the bacterial ooze and a loop full of bacterial ooze was streaked on the nutrient agar (Usha, 2003). After streaking, Petri plates were kept for incubation at 28 ± 1 °C and observed for the development of bacterial colonies after 48 h (Fig. 2). Isolated single colonies were picked and streaked on nutrient agar medium for developing pure cultures. Single colonies picked from the pure cultures were streaked on the Crystal Violet Pectate (CVP) medium, the specific medium for *Pectobacterium* spp.

2.2. Pathogenicity tests

2.2.1. In-vitro tests

Surface sterilized rhizome bits of banana variety 'Nendran' were inoculated with 48 h. old culture (Fig. 3) (Rahman et al., 1994).

2.2.2. In vivo tests

Two methods were used under *in-vivo* condition; first by inoculating rhizomes of three months old healthy banana plants of cv. Nendran (Snehalatharani and Khan, 2010) and second by inoculating in the pseudostem of two months old tissue cultured banana plants of cv. Nendran, with 48 h. old bacterial culture (Thomas et al., 2008).

Controls were maintained on nutrient agar with no bacterium in the rhizomes. Inoculated samples along with controls were kept in the moist chamber and observed for symptom development (Figs. 4 and 5). Pathogen was re-isolated from the infected rhizome samples and cultured in specific medium.

2.3. Bacterial characterization

Characterization of the pathogen was carried out by cultural, morphological, biochemical, physiological and molecular methods.

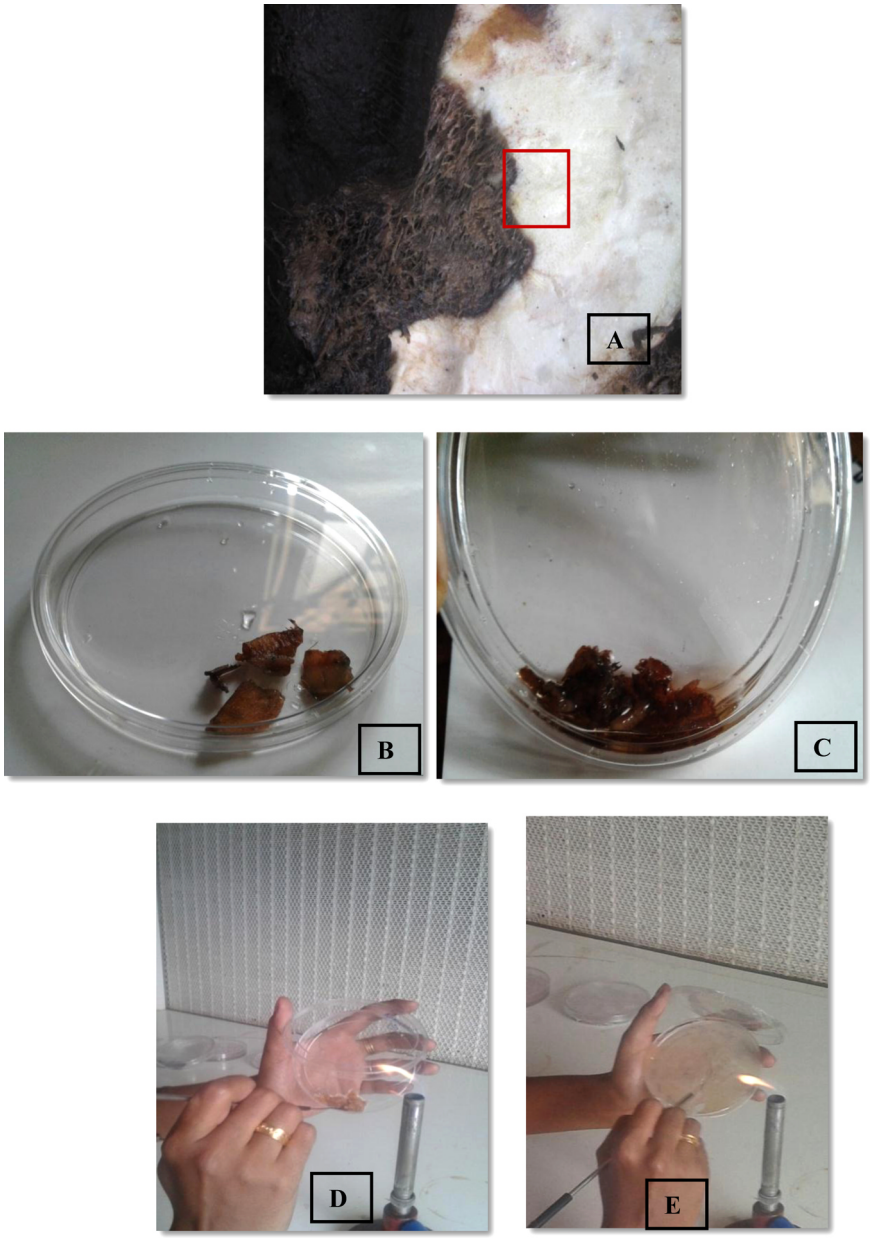

Fig. 2. Isolation of pathogen **A -** Rhizome bits were taken from the marked region, **B -** Surface sterilized rhizome bits, **C -** Rhizome bits macerated with 1 ml sterile distilled water, **D** and **E -** Loop-full of bacterial ooze was taken and streaking in Nutrient agar medium.

2.3.1. Cultural characterization

2.3.1.1. Characterization on NA medium. Nutrient agar medium recommended for culturing wide range of bacteria was prepared (Stover, 1959; Snehalatharani and Khan, 2010), streaked with 48 h. old bacterial culture and incubated at 28 ± 1 °C for 24–48 h.

2.3.1.2. Characterization on YGC medium. YGC medium is a specific medium for *Pectobacterium* species; which produces excessive acid. A clear zone surrounding each colony results from acid secretion, which liberates the carbonate as carbon dioxide (Schaad and Brenner, 1977; Kado, 2006). The medium was inoculated with 48 h. old bacterial culture, incubated at 28 °C for 24 h. and observed for acid and carbon dioxide production.

2.3.1.3. Characterization on Logan's medium. Logan's medium, a differential medium for *Pectobacterium* spp., has the same composition as nutrient agar medium, additionally containing a dye 2, 3, 5 triphenyl tetrazolium chloride (Logan, 1966; Fahy and Hayward, 1983). The media was streaked with 48 h. old bacterial culture, incubated at 28 ± 1 °C for 48 h. and the colonies were observed.

2.3.1.4. Characterization in nutrient broth. Five ml of sterile nutrient broth was taken in a sterile oakridge tube and inoculated with a loop-full of 48 h. old bacterial culture of each isolate. Control was kept in the similar manner but with no bacterial inoculum. The broth was incubated at 28 ± 1 °C with daily shaking, for uniform growth and the optical density was measured after 24, 48 and 72 h. at 620 nm (Usha, 2003).

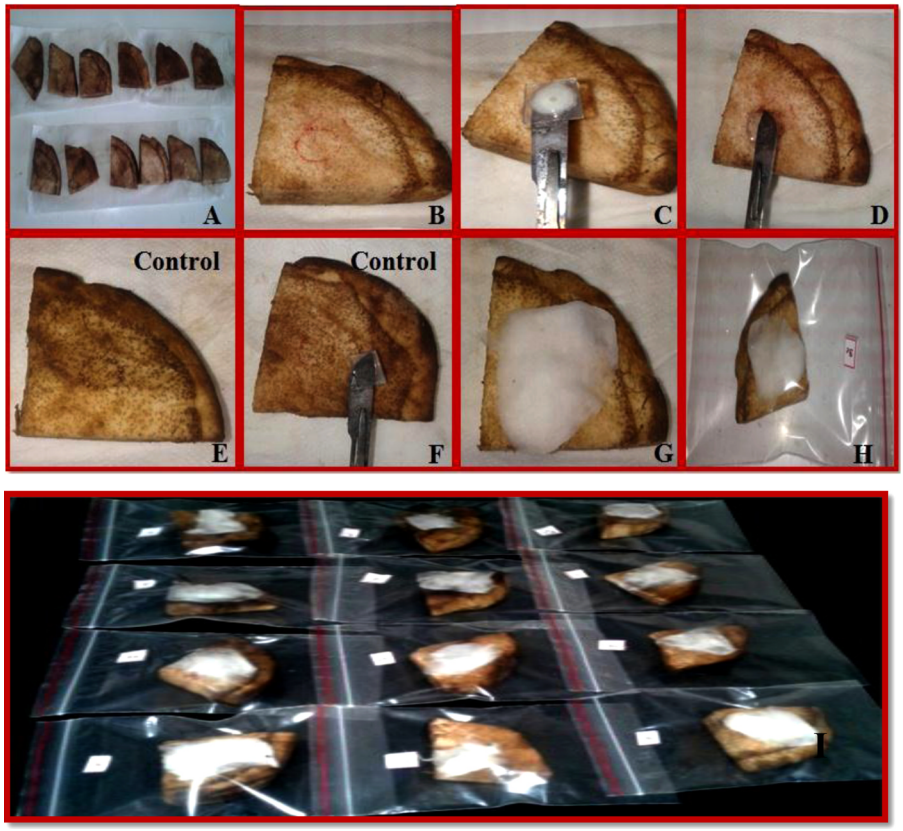

Fig. 3. Pathogenicity test *in-vitro* **A -** Surface sterilized rhizome bits; **B -** Pin pricks - circle of 1 cm diameter; **C** and **D -** Pin pricked portion inoculated with 48 h. old bacterial culture. **E** and **F -** Control (pin pricked portion covered with Nutrient agar only); **G, H** and **I -** Inoculated region covered with moist cotton and incubated by keeping in sterile plastic bag to maintain moisture.

2.3.2. Morphological characterization

2.3.2.1. Gram staining. Bacterial culture (48 h. old) was gram stained (Pelczar, 1957). Glass slides were air dried; visualization and the size measurement were done under oil immersion microscope.

2.3.2.2. Capsule staining. For all the isolates, the background was stained using an acid stain that carries negative charge on its surface (Breakwell et al., 2009). Glass slides were air dried and visualized under phase contrast microscope.

2.3.2.3. Flagellar staining. Flagellar staining was carried out (Blenden and Goldberg, 1964) to understand the presence of filamentous flagella. Air dried slides were visualized using oil immersion microscope.

2.3.3. Biochemical characterization

2.3.3.1. Potato and carrot soft rot test. Potato and carrot slices were inoculated with bacterium and the plates were incubated at room temperature (Lelliott et al., 1966). Pathogen was re-isolated from the rotten potato and carrot slices and cultured in specific medium. (Figs. 6A and 6B)

2.3.3.2. Intrinsic antibiotic resistance. Nutrient agar containing 1% dextrose was used (Nagaraj et al., 2013). After placing the filter paper discs containing antibiotic, the Petri plates were incubated at 28 ± 1 °C for 48 h. Observations were recorded on bacterial growth and formation of inhibition zone (Figs. 6C and 6D).

2.3.3.3. Growth on three and four per cent NaCl containing media. Salt tolerance of *Pectobacterium* spp. was evaluated by culturing bacterium in 3% and 4% NaCl containing PEPTONE broth. Tubes have been incubated at 28 ± 1 °C with shaking at 100 rpm for 48 h (Gomez-Caicedo et al., 2001) and the bacterial growth was measured as OD value at 620 nm.

2.3.3.4. Pectate degradation. Cultures were grown on CVP medium recommended for pectolytic microorganisms, which can degrade sodium polypectate in the medium and produce characteristic pits where the pectin is degraded (Cuppels and Kelman, 1974; Mc Feeters et al., 1992). Pectolytic activity of bacteria was checked by inoculating the 48 h. old bacterial cultures in CVP medium and incubating the Petri plates at 37 °C for 5 days.

2.3.4. Physiological characterization

2.3.4.1. Growth of bacterium 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.

2.3.4.2. Growth of bacteria at different pH. Sterile media with pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 were inoculated with loop-full of 48 h. old bacterial cultures. The control had no bacterial inoculums. Cultures were maintained at 28 °C with shaking at 120 rpm for 48 h and optical density at 620 nm was measured after 24 and 48 h (Shrestha et al., 2005).

Grouping of bacterial isolates was done using the results of cultural,

Fig. 4. Pathogenicity test - *in vivo* (Rhizome) **A** and **B -** Three months old banana plants **C -** 1 sq.cm tissue removed from the collar region; **D -** Wounded portion inoculated with 48 h. old bacterial culture; **E -** Control (wounded portion covered with Nutrient agar only) **F, G, H** and **I -** Inoculated region covered with moist cotton and incubated by covering with sterile news paper to maintain moisture.

morphological, biochemical and physiological characters and also with the help of dendrogram derived from the characters showing variability.

2.3.5. Molecular characterization

DNA was isolated from the cultures (Neumann, 1992) and the 16S rRNA gene was amplified through PCR using the universal 16S rRNA primer set 8 F and 1522 R (Lima et al., 2011, 2012). The reaction mixture had 2 µl template DNA (25 ng/µl), 0.1 µl each of forward and reverse primers (10 mM), 1 µl of dNTP mix (10 mM), 0.4 µl Taq DNA polymerase (2.5 unit), 2.5 µl, Taq buffer and the final volume was made up to 25 µl. Thermal cycling included initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 90 s, primer annealing at 55 °C for 40 s and primer extension at 72 °C for 90 s and final extension was at 72 °C for 20 min. Products were electrophoresed on 2% agarose gel.

The 1.5 Kbp marker of representative samples from each group were sequenced (AgriGenome, Kochi, India). Forward and reverse complement sequences of each sample were merged (merger' tool in EMBOSS GUI). Homology of the sequences were identified (*Pectobacterium carotovorum* specific BLASTn). Phylogenetic analysis was done (MEGA 6.0) and barcode gaps were identified using alignment in Clustal Omega.

3. Results and discussion

Details on the isolates collected are presented in Table 1. Pathogen isolated from the infected rhizome samples have yielded small bacterial colonies on NA medium, which were cream to yellowish colour, slightly raised and mucoid with a diameter of 2.3–2.5 mm (Figs. 7A and 7B). On CVP medium, which is specific for *Pectobacterium*, characteristic fissures or pits were produced (Fig. 7C).

A, B - Bacterial colonies on NA medium, **C -** Fissures formed in CVP medium

Koch's postulates were proved for all 18 bacterial isolates by three

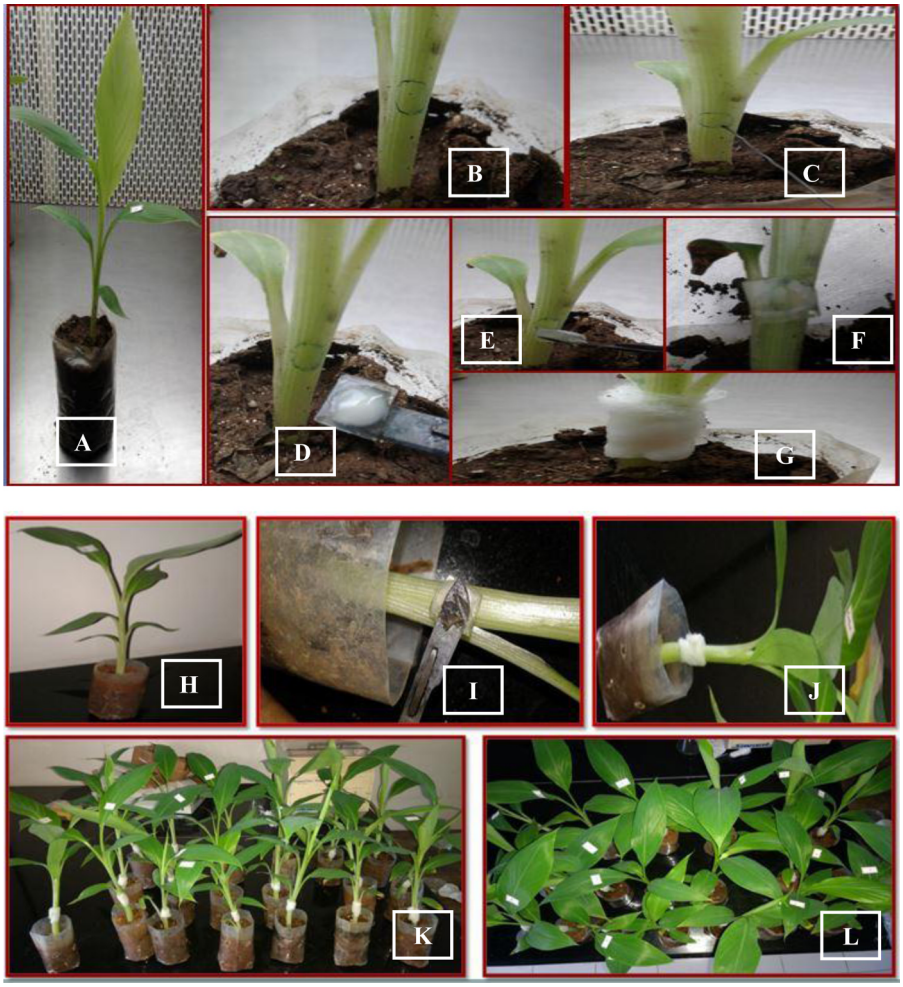

Fig. 5. Pathogenicity test *in vivo* (Pseudostem) **A -** Two months old tissue cultured banana plants **B** and **C -** Pin pricks made in a circle of 1 cm diameter on pseudostem. **D, E** and **F -** Pinpricked portion inoculated with 48 h. old bacterial culture.; **G -** Inoculated portion covered with moist cotton; **H, I** and **J -** Control (pin pricked portion covered with Nutrient agar only; **K** and **L -** Inoculated plants along with control incubated in the moisture chamber.

methods. Rotting of inoculated rhizome bits of banana variety Nendran was observed in 8 days under *in-vitro* condition; the pathogen was re-isolated in NA medium and CVP medium and produced characteristic pits similar to the original isolates (Fig. 8). Stover (1959) and Usha (2003) also reported that the inoculated rhizome bits under artificial condition produces rotting, discolouration and foul smell after 7 and 8 days of inoculation, respectively. Rotting of healthy rhizome was observed after 37 days of inoculation under *in vivo* condition; pathogen was reisolated in NA and CVP media which produced characteristic pits similar to the original isolates (Figs. 9 and 10). Nagaraj et al. (2012) and Nagrale et al. (2013) reported similar observations. Under *in vivo* condition, the bacterium has produced characteristic symptoms on tissue culture plants, after 6 days of inoculation. Symptoms observed were yellowing of lower leaves and the yellowing progressed to upper leaves. Weakening of the pseudostem has started and finally the plant fell down as the pseudostem got completely rotten and emitted foul smell, after 16 days of inoculation. Thomas et al. (2008) carried out the pathogenicity test in micropropagated banana and reported that they are susceptible and produced rotting symptom within two weeks after inoculation, which was similar to that obtained in the present study.

Variation was observed in days taken for the rotting of tissue cultured plants by different isolates. Minimum time taken for rotting was 7 days by the isolate no. 3_{TrNd}, 7_{PaKl} and17_{MaMd}, followed by 10 days by 5_{KoPr}, 12_{ThMl} and 14_{ThAh}. Maximum time of 15 days was taken by 2_{TrNy} and 8_{PaMn}, preceded by 13 days by 10_{ThKr}, 13_{ThKn} and 18_{KaTa} (Table 2, Fig. 11 and 12).

In NA medium, bacterial colonies were cream to yellowish in colour, circular with 2.3 − 2.5 mm in diameter with entire margin, mucoid and slightly raised in nature (Fig. 13A). In YGC medium, cream to yellow coloured colonies were produced with a clear zone after 24 h of incubation. Small bubbles were observed on the top of each bacterial colony due to the emission of carbon dioxide from the YGC medium which is the characteristic feature of *Pectobacterium carotovorum* (Fig. 13B). In Logan's medium, small to medium sized colonies (1.6–2.0 mm) of purple colour were produced after 24 h. of incubation. The colonies were slightly raised and mucoid in nature. The pathogen was identified as *Pectobacterium carotovorum* by observing the purple colour of colonies (Fig. 13C).

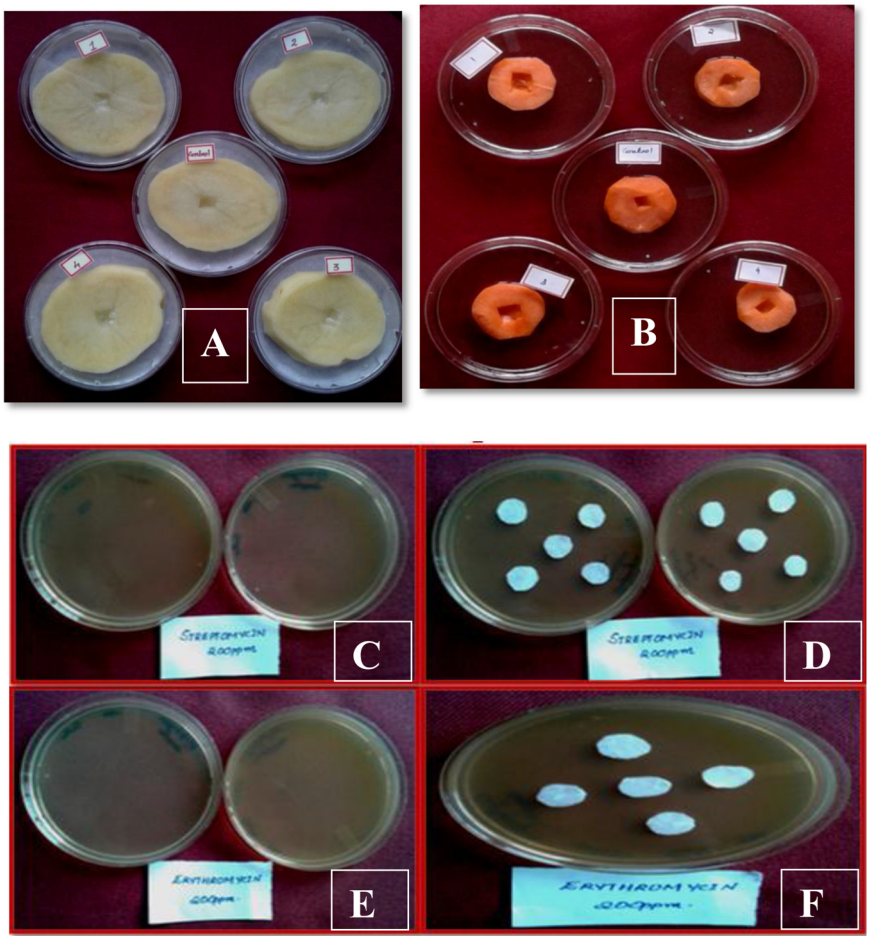

Fig. 6. Biochemical tests. **A -** Potato soft rot test, **B -** Carrot soft rot test, **C** - Bacterial lawn prepared to check streptomycin resistance, **D -** Filter paper discs dipped in 200 ppm streptomycin placed over the bacterial lawn, **E** - Bacterial lawn prepared to check erythromycin resistance, **F -** Filter paper disc dipped in 200 ppm erythromycin placed over the bacterial lawn.

Table 1
Details on the samples used in this study.

Sample no.	District, state	Location	Isolate no.
1	Trivandrum, Kerala	Kazhakuttam	1_{TrKz}
2	Trivandrum, Kerala	Neyyatinkara	2_{TrNy}
3	Trivandrum, Kerala	Nedumangad	3_{TrNd}
4	Kollam, Kerala	Adoor	4_{KoAo}
5	Kollam, Kerala	Paravoor	5_{KoPr}
6	Palakkad, Kerala	Karimba	6_{PaKr}
7	Palakkad, Kerala	Kalladikod	7_{PaKl}
8	Palakkad, Kerala	Mannarkad	8_{PaMn}
9	Thrissur, Kerala	Mannuthy	9_{ThMn}
10	Thrissur, Kerala	Koratti	10_{ThKr}
11	Eranakulam, Kerala	Kalamassery	11_{ErKl}
12	Thrissur, Kerala	Mala	12_{ThMl}
13	Thrissur, Kerala	Kannara	13_{ThKn}
14	Thrissur, Kerala	Achankunnu	14_{ThAh}
15	Kanyakumari, Tamil Nadu	Nagercoil	15_{KaNg}
16	Tuticorin, Tamil Nadu	Tuticorin	16_{TuTt}
17	Madhurai, Tamil Nadu	Madhurai	17_{MaMd}
18	Kanyakumari, Tamil Nadu	Thakkala	18_{KaTa}

Bacterial growth in terms of OD value in the nutrient broth was recorded after 24, 48 and 72 h. of incubation. Difference was observed in the growth rate of isolates. After 72 h., maximum OD value was recorded in 17_{MaMd} (0.840), followed by 3_{TrNd} (0.838) and the minimum was in 2_{TrNy} (0.555), preceded by 8_{PaMn} (0.558) (Fig. 14).

Variability in OD values shows the presence of variation in growth rate of isolates. Previously, Usha (2003) has reported the variability in *Pectobacterium carotovorum* through this method.

After Gram staining, red coloured short rods were observed under oil immersion microscope, indicating the Gram negative nature of this bacterium. Variation in size of the bacterial rods was also observed among the isolates. Length of the bacterium was in the range of 0.95–3.10 μm and width in the range of 0.57–1.08 μm (Fig. 15). Maximum length (3.10 μm) was in 7_{PaKl} followed by 3_{ErKl}, 3_{TrNd} and 17_{MaMd} whereas; minimum length (0.95 μm) was in 2_{TrNy} followed by 8_{PaMn}. Maximum width (1.10 μm) was in 17_{MaMd} followed by 3_{TrNd} and minimum width was in 2_{TrNy} followed by 8_{PaMn} (Table 3).

Capsule staining (negative staining) showed that the bacteria is non-capsular in nature (Figs. 16A, 16B and 16C). The peritrichous nature of the flagella was observed through flagellar staining (Figs. 16D, 16E and 16F).

Potato and carrot slices, inoculated with bacterial cultures, have produced rotting symptoms with the emission of characteristic foul smell after 3–4 and 5–7 days, respectively. In similar studies, potato soft rot symptoms are reported to be initiated in three days (Lelliott et al., 1966; Togashi, 1988; Nabhan et al., 2006). Rotting in carrot was slow and complete rotting was observed after 14 days only (Schillingford, 1974; Doolotkeldieva et al., 2016).

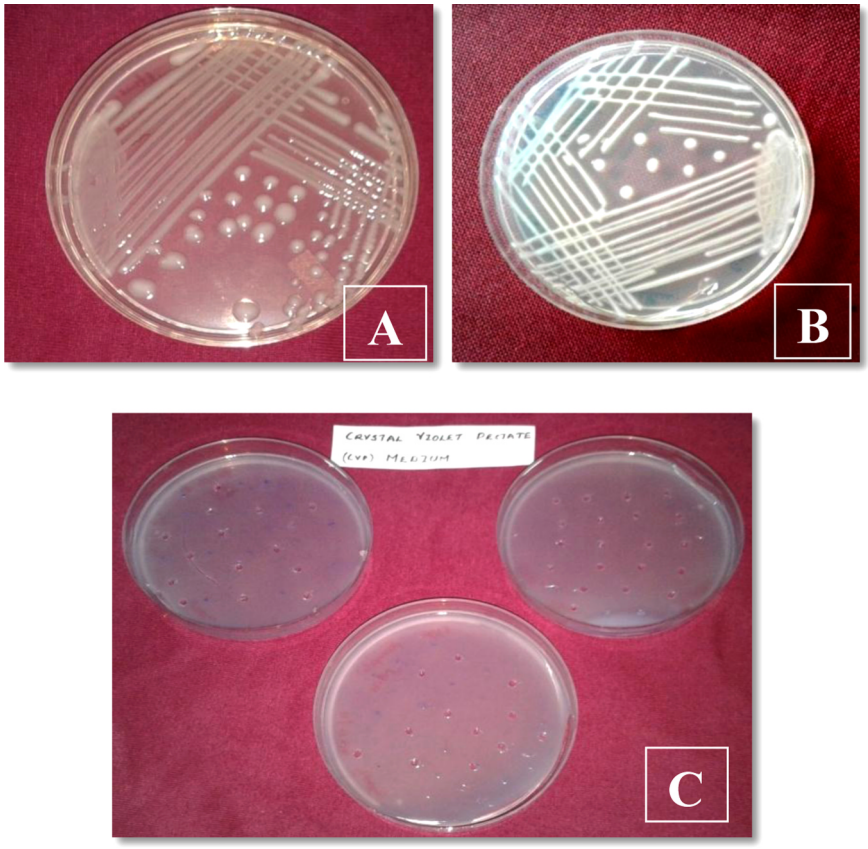

Fig. 7. Pathogen isolated in Nutrient agar (NA) and Crystal Violet Pectate (CVP) media.

Variation was observed in time for rotting of potato and carrot by different isolates. Minimum time taken for rotting was two and four days in potato and carrot, respectively by isolates 1_{TrKz}, 3_{TrNd}, 5_{KoPr} and 7_{PaKl} whereas, maximum time taken for rotting was six and seven days by 2_{TrNy} and 8_{PaMn} (Table 4, Fig. 17 and 18).

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 where as *P. chrysanthemi* is susceptible to erythromycin as well as 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* (Fig. 19).

Bacterial growth was observed in the inoculated peptone broth containing three and four per cent sodium chloride. Results showed that the isolates tolerate the salt at these levels. Variation was observed in the OD values of bacterial isolates grown in the medium containing three and four per cent NaCl. Maximum OD value was recorded with the isolate 11_{ErKl} and minimum with 8_{PaMn} (Table 5 and 6). Salt tolerant nature of *Pectobacterium* sp. is 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. is proven to have the pectate degrading ability (Bhupendra, Yogendra, 2015), similarly, the isolates in this study are found to degrade the sodium polypectate present in the CVP medium, leading to the formation of fissures in five days. In few isolates such as 2_{TrNy}, 10_{ThKr} and 13_{ThKn}, the pits were surrounded by opaque gel containing non-degraded pectin or pectin substrate as reported by Mc Feeters et al. (1992) (Fig. 20).

After 48 h. of incubation, maximum growth of bacteria was recorded at 27 °C (OD value 0.844) followed by growth at 29 °C (OD value 0.836). OD values of bacterial isolates grown in the medium at 27 °C has varied with maximum of 0.844 in isolate 17_{MaMd} and minimum of 0.557 in 2_{TrNy} (Fig. 21). Previous study by Shrestha et al. (2005), on the effect of temperature on the growth of *Pectobacterium* spp., has reported the optimum growth at 27–28 °C.

After 48 h. of incubation, among the bacterial cultures in media with different pH, maximum growth was recorded at pH 7.0 (OD value 0.629–0.655) followed by pH 7.5 (OD value 0.448–0.470). Maximum OD value of 0.655 was recorded in 11_{ErKl} and 17_{MaMd} and OD value of 0.629 was in 8_{PaMn} (Fig. 22). Growth of bacteria was very low under acidic (pH 5.0) as well as alkaline (pH 9.0) conditions (OD values nearer to that of control). According to Shrestha et al. (2005) the most

favorable pH for the growth of *Pectobacterium* spp. is 7.0 and no strain can grow at pH below 5.0 or above 10.0. Similar results were also reported by Usha (2003).

PCR using the 16S rRNA universal primers has yielded markers at 1.5 kb in all the isolates (Figs. 23A and 23B). The product size was similar to that reported by reported by Kwon et al. (2000)

Based on the pathogenicity test, cultural, morphological, biochemical and physiological characters, the 18 isolates collected from different locations were grouped into six groups manually and by constructing dendrogram (Fig. 24). The parameters used for grouping were *in vivo* pathogenicity test on TC plants, OD values in Nutrient broth, bacterial size, OD values in 3% and 4% NaCl, OD value at 27 °C, 37 °C and pH 7 (Tables 7a–7e).

The dendrogram constructed using the parameters viz, *in vivo* pathogenicity test on TC plants, OD values in Nutrient broth, bacterial size, OD values in 3% and 4% NaCl, OD value at 27 °C, 37 °C and pH 7 for grouping the 18 bacterial isolates (Fig. 24). At 16% similarity coefficient the 18 bacterial isolates branched out into several clusters and based on the clustering the 18 bacterial isolates were grouped into six groups.

Homology analysis on sequences of one isolate each of the groups (Group 1–11$_{ErKl}$; Group 2–5$_{KoPr}$; Group 3–15$_{KaNg}$; Group 4–9$_{ThMn}$; Group 5–13$_{ThKn}$; Group 6–8$_{PaMn}$). had shown two subspecies, *Pectobacteium carotovorum* ssp. *carotovorum* and *Pectobacteium carotovorum* ssp. *brasiliense* among the isolates (Table 8). Groups 1, 2, 3 and 4 belonged to *Pectobacteium carotovorum* ssp. *carotovorum* whereas, group 5 and 6 to *Pectobacteium carotovorum* ssp. *brasiliense.* Weisburg et al. (1991) and Kwon et al. (2000) also used this approach to establish

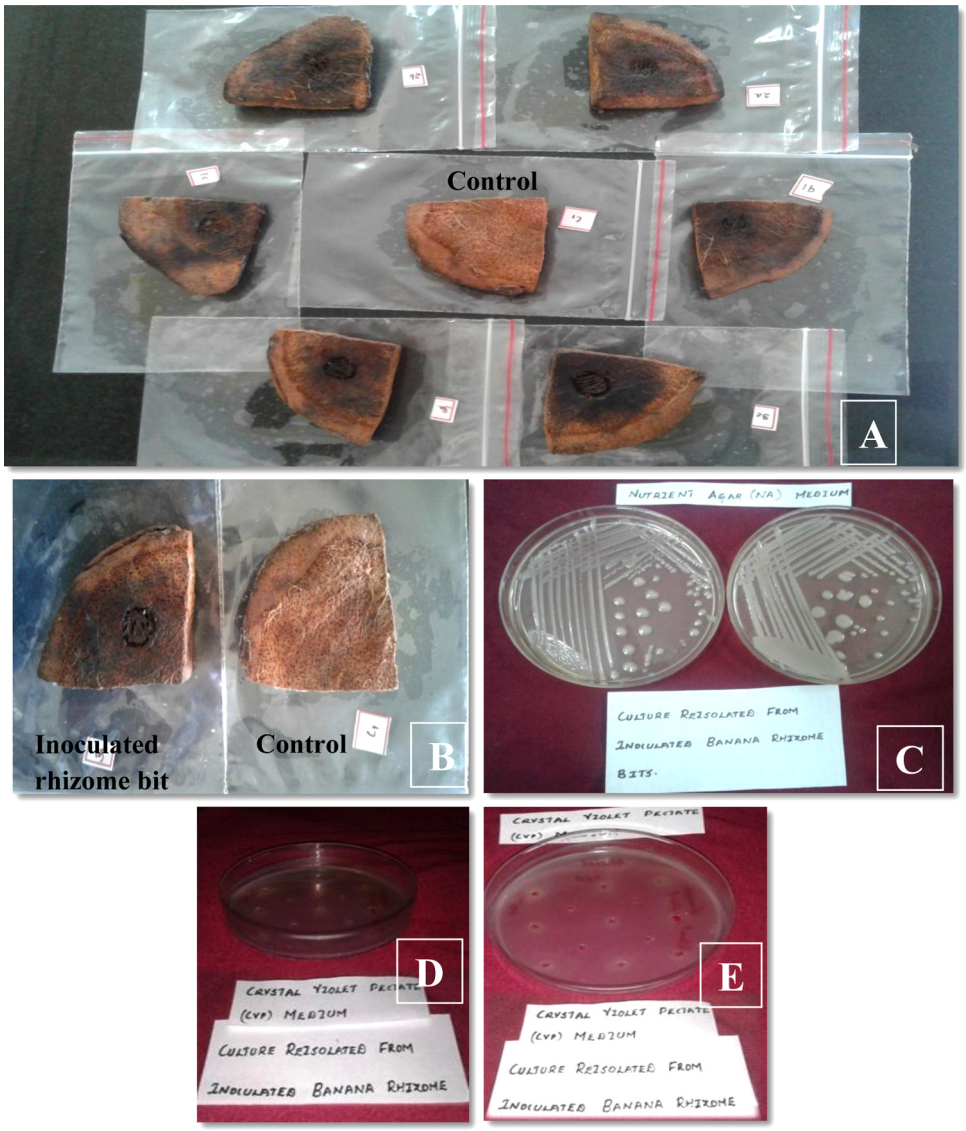

Fig. 8. Proving of pathogenicity *in vitro* **A** and **B -** Rotted rhizome bits along with control, **C -** Re-isolated bacterial colonies in NA medium, **D** and **E -** Fissures formed in CVP medium by re-isolated culture.

Fig. 9. Proving of pathogenicity *in vivo* (Rhizome) **A, B** and **C -** Symptoms observed after two weeks of inoculation, **D -** Rotted rhizomes along with control.

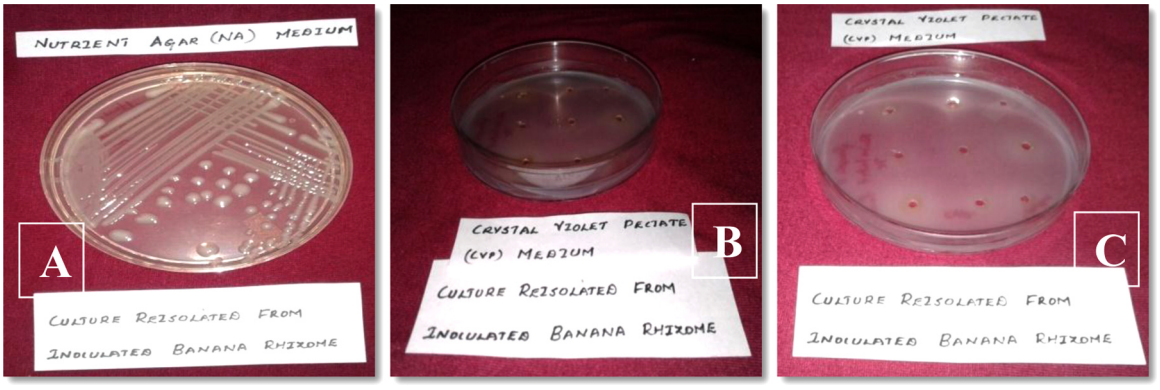

Fig. 10. Reisolation of bacterial culture from infected rhizome **A -** Re-isolated bacterial colonies obtained on NA medium from infected rhizome, **B** and **C -** Fissures formed in CVP medium by reisolated culture.

Table 2
Time of development of symptoms in tissue cultured banana plants.

Isolate no.	Lower leaf starts yellowing and rotting (days)	Complete rotting of the plant (days)	Falling down of plant (days)
1_{TrKz}	8	9	10
2_{TrNy}	12	13	14
3_{TrNd}	4	5	6
4_{KoAo}	9	10	11
5_{KoPr}	6	8	9
6_{PaKr}	8	9	10
7_{PaKl}	4	5	6
8_{PaMn}	12	13	14
9_{ThMn}	9	10	11
10_{ThKr}	10	11	12
11_{ErKl}	4	5	6
12_{ThMl}	6	8	9
13_{ThKn}	10	11	12
14_{ThAh}	6	8	9
15_{KaNg}	8	9	10
16_{TuTt}	9	10	11
17_{MaMd}	4	5	6
18_{KaTa}	10	11	12

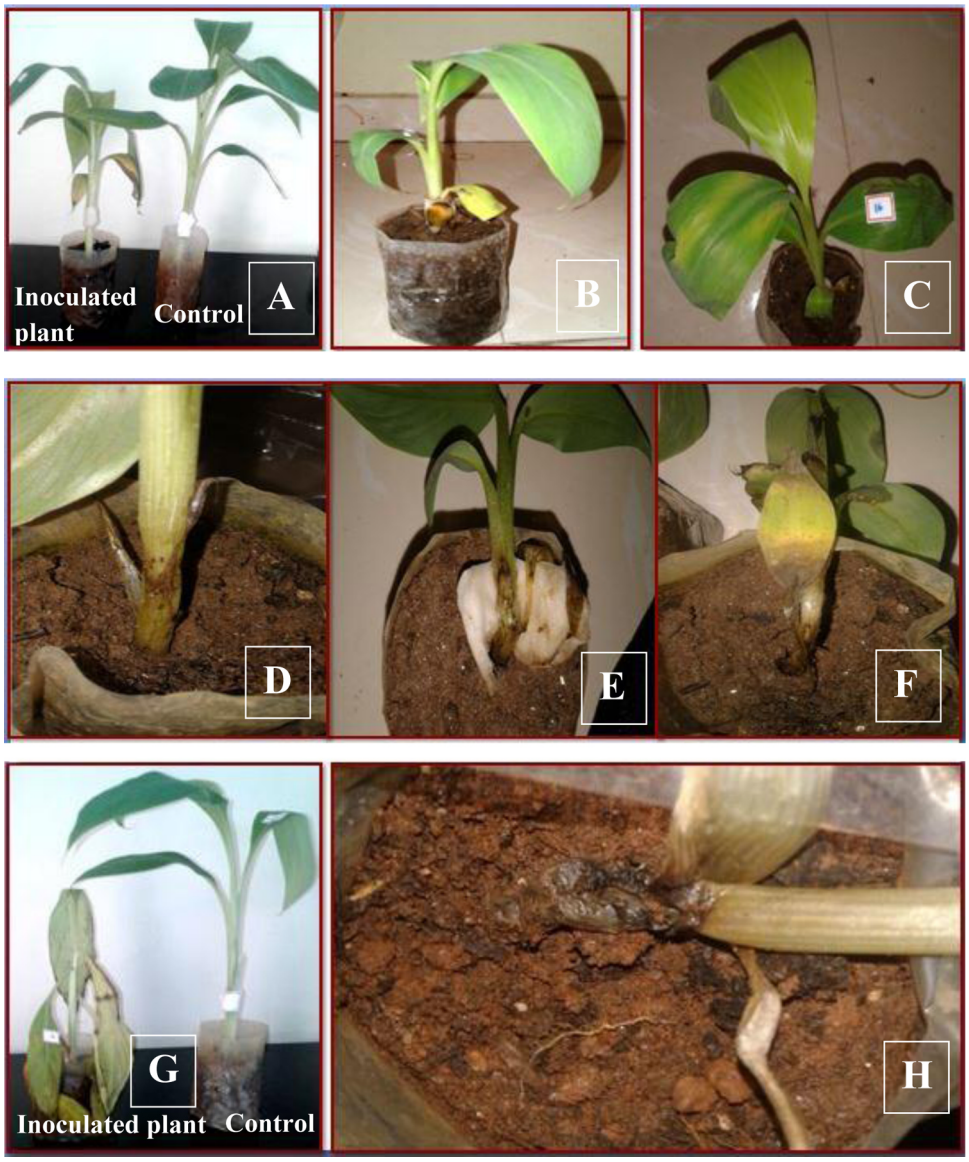

Fig. 11. Proving of pathogenicity *in vivo* (Pseudostem) **A -** Initial symptoms observed after one weeks of inoculation along with control, **B** and **C -** Yellowing of the leaves progressed to upper leaves, **D, E** and **F -** Stages of pseudostem rotting in the pseudostem, **G -** Completely wilted plant along with control, **H -** Completely rotted pseudostem.

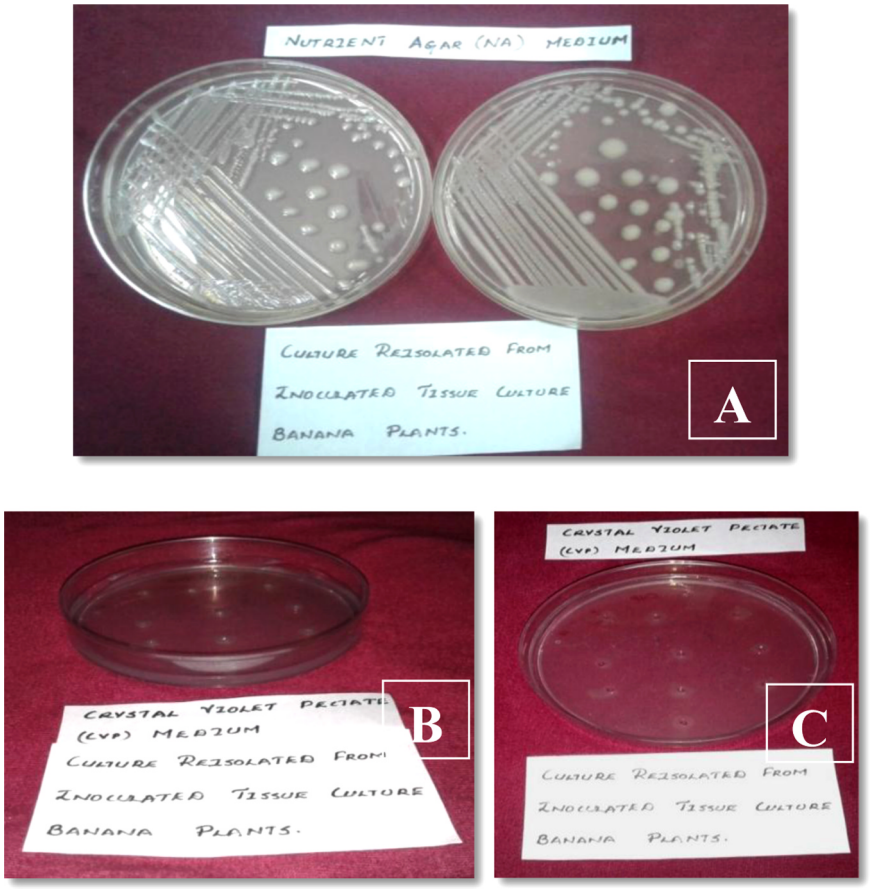

Fig. 12. Reisolation of bacterial culture from infected pseudostem. **A -** Reisolated bacterial colonies in NA medium from infected TC plants, **B** and **C-** Fissures formed in CVP medium by re-isolated culture.

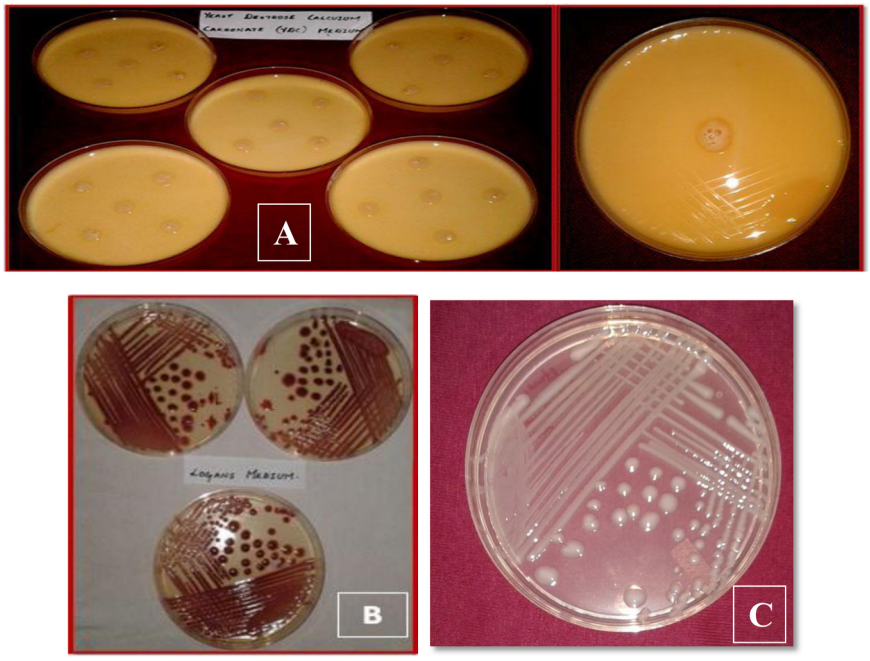

Fig. 13. Cultural characterization **A -** Acid secretion and carbon dioxide release in YGC medium, **B -** Purple colour colonies in Logan's medium, **C -** Mucoid colonies in NA medium.

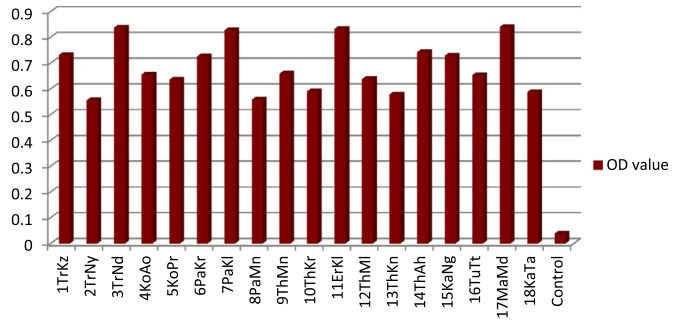

Fig. 14. Growth rate of bacterial isolates in nutrient broth at 72 h. of incubation.

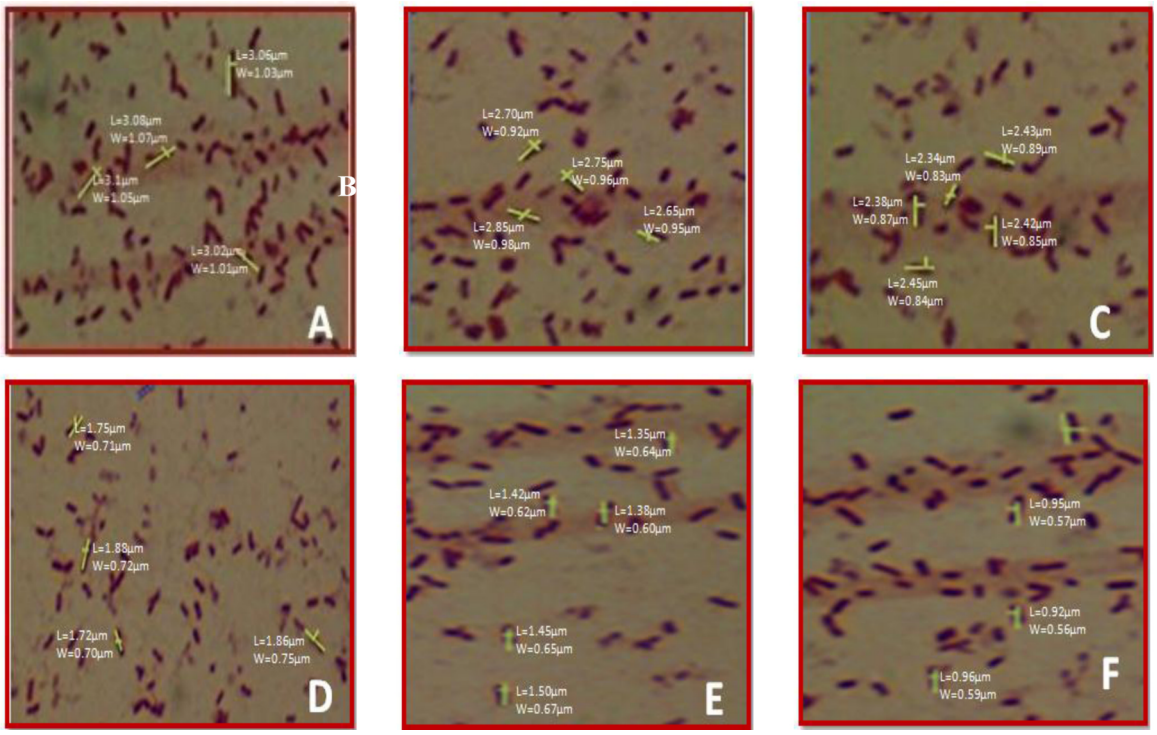

Fig. 15. Bacterial rods with measurements A, B, C, D, E and F - Representative isolates of each group.

Table 3
Variability in sizes of bacterial isolates.

Isolate no.	Length (µm)	Width (µm)
1_{TrKz}	2.42^{d}	0.89^{ef}
2_{TrNy}	0.95^{j}	0.57^{m}
3_{TrNd}	3.05^{a}	1.08^{ab}
4_{KoAo}	1.88^{f}	0.78^{h}
5_{KoPr}	2.60^{c}	0.92^{e}
6_{PaKr}	2.34^{de}	0.83^{g}
7_{PaKl}	3.10^{a}	1.05^{bc}
8_{PaMn}	0.98^{j}	0.59^{m}
9_{ThMn}	1.70^{g}	0.75^{hi}
10_{ThKr}	1.32^{i}	0.69^{jk}
11_{ErKl}	3.06^{a}	1.02^{c}
12_{ThMl}	2.75^{b}	0.98^{d}
13_{ThKn}	1.50^{h}	0.64^{l}
14_{ThAh}	2.84^{b}	0.96^{d}
15_{KaNg}	2.30^{e}	0.87^{f}
16_{TuTt}	1.82^{f}	0.72^{ij}
17_{MaMd}	3.03^{a}	1.10^{a}
18_{KaTa}	1.44^{h}	0.66^{kl}
CV %	3.30	3.24
CD (0.05)	0.102	0.039

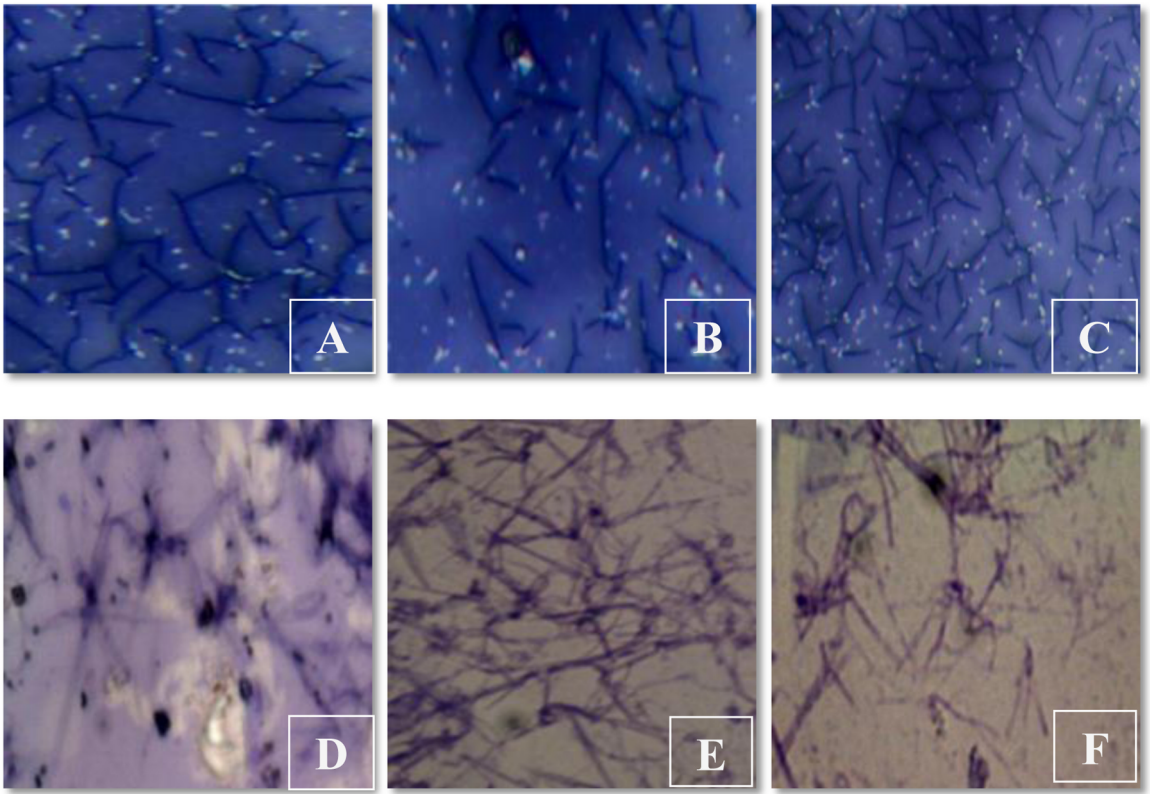

Fig. 16. Capsule and flagellar staining **A, B** and **C -** No clear zone present around the bacterial rods indicating non-capsular nature of bacteria, **D, E** and **F -** Several flagella present around each bacterial rod indicating peritrichous nature.

Table 4
Time taken (days) for initiation of potato and carrot soft rot by bacterial isolates.

Isolate	Potato soft rot		Carrot soft rot	
	Symptom initiation	Completion of rotting	Symptom initiation	Completion of rotting
1_{TrKz}	2	3	3	4
2_{TrNy}	5	6	6	7
3_{TrNd}	2	3	3	4
4_{KoAo}	3	4	4	5
5_{KoPr}	2	3	3	4
6_{PaKr}	2	3	3	4
7_{PaKl}	2	3	3	4
8_{PaMn}	5	6	6	7
9_{ThMn}	3	4	4	5
10_{ThKr}	3	4	4	5
11_{ErKl}	2	3	3	4
12_{ThMl}	2	3	3	4
13_{ThKn}	3	4	4	5
14_{ThAh}	2	3	3	4
15_{KaNg}	2	3	3	4
16_{TuTt}	3	4	4	5
17_{MaMd}	2	3	3	4
18_{KaTa}	3	4	4	5

Control- Ngo change after 5 days in potato and carrot slices.

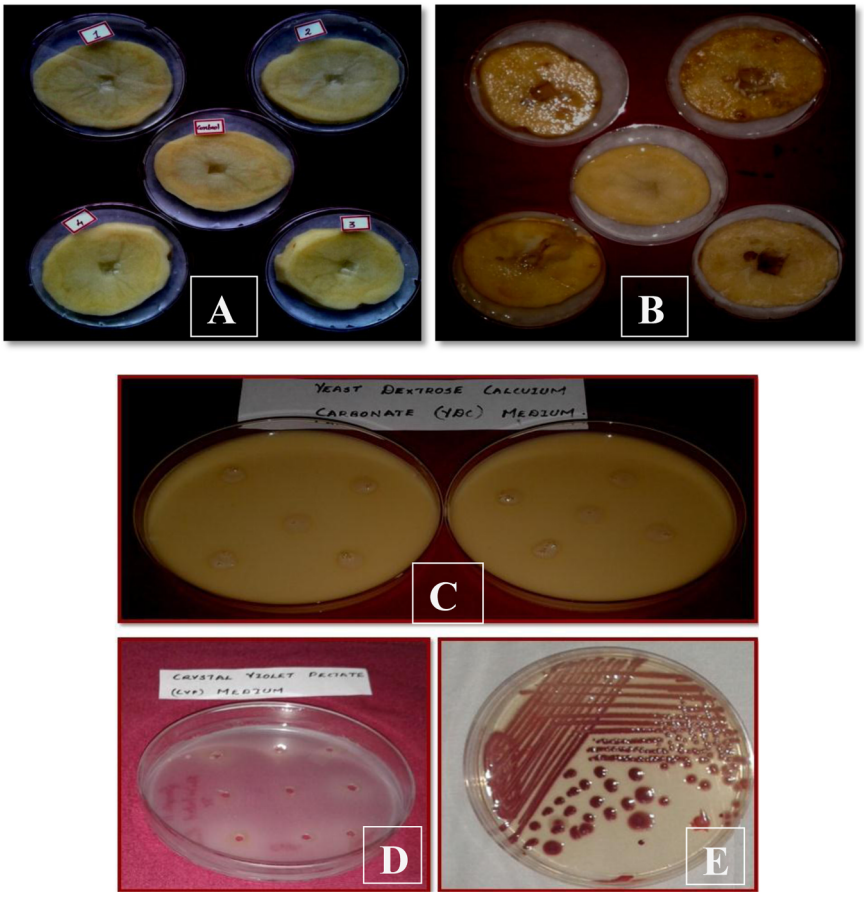

Fig. 17. Observations of Biochemical test- I. **A** and **B -** Inoculated potato slices compared along with the rotted potato slices, **C, D** and **E -** Pathogen reisolated and confirmed by culturing in YGC, CVP and Logan's media.

the identity of *Pectobacterium* species.

Barcode gaps (Fig. 25) were generated by aligning the six 16S rRNA sequences of two subspecies of *Pectobacteium carotovorum.* Two sequences of *Pectobacteium carotovorum* ssp. *brasiliense* showed considerable variability with four sequences of *Pectobacteium carotovorum* ssp. *carotovorum.* The sequences were deposited in NCBI GenBank and the accession numbers obtained are given in Table 8.

The phylogenetic tree constructed using the DNA sequences in MEGA 6.0 had shown that groups 5 and 6 branched out from the other subspecies (bootstrap value 100) (Fig. 26). In a similar study, Kwon et al. (2000) and Thapa et al. (2011) had constructed phylogenetic tree of *Pectobacterium* species and reported that *P. carotovorum* subsp. *carotovorum* is closer with *P. carotovorum* subsp. *oderiferum*.

4. Conclusion

Variability among 18 bacterial isolates was analyzed through pathogenicity test followed by cultural, morphological, biochemical and physiological characterization and based on these, the isolates were grouped into 6. Through molecular characterization, variation was confirmed at subspecies level, *Pectobacterium carotovorum* ssp. *carotovorum* and *Pectobacterium carotovorum* ssp. *brasiliense* (Table 9). Barcode gaps of *Pectobacterium carotovorum* ssp. *carotovorum* and *Pectobacterium carotovorum* ssp. *brasiliense* were also assessed by aligning six sequences. Phylogenetic tree constructed with the 16S rRNA sequences had discriminated the two subspecies clearly.

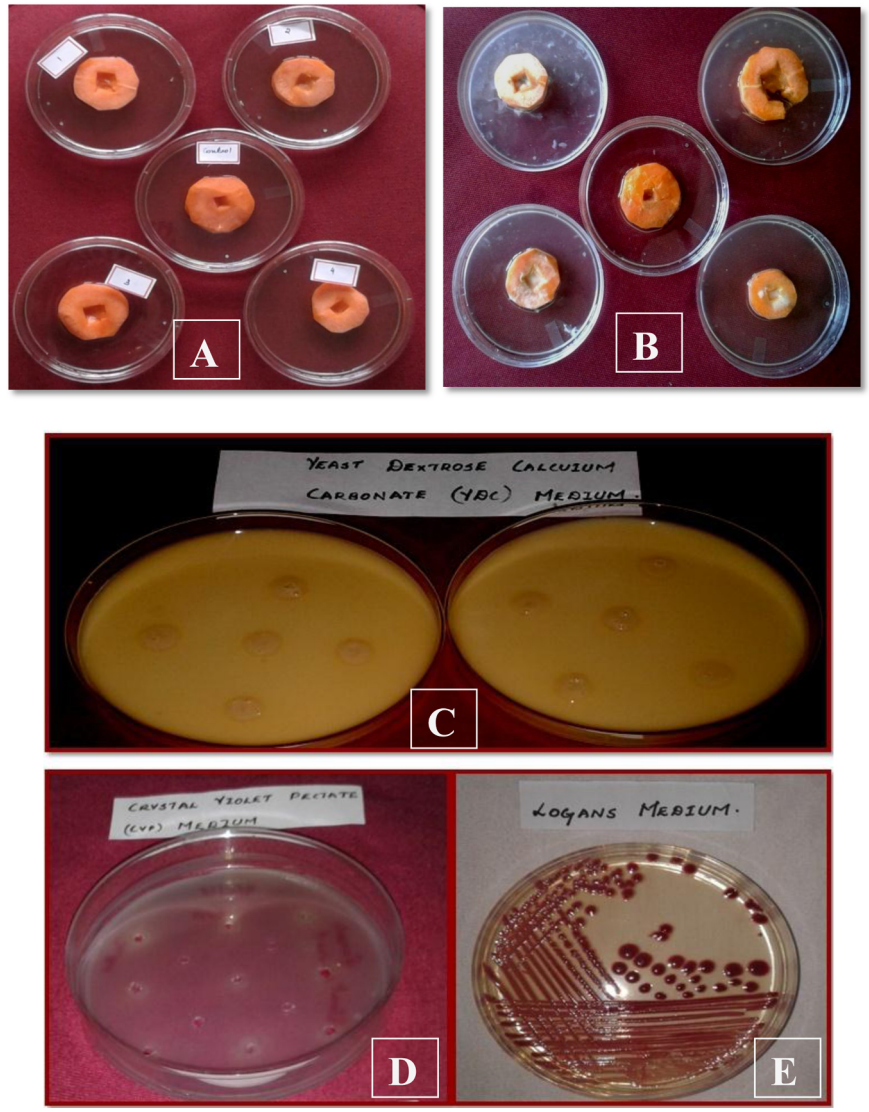

Fig. 18. Observations of Biochemical test- II. **A** and **B -** Inoculated carrot slices compared along with the rotted carrot slices, **C, D** and **E -** Pathogen re-isolated and confirmed by culturing in YGC, CVP and Logan's media.

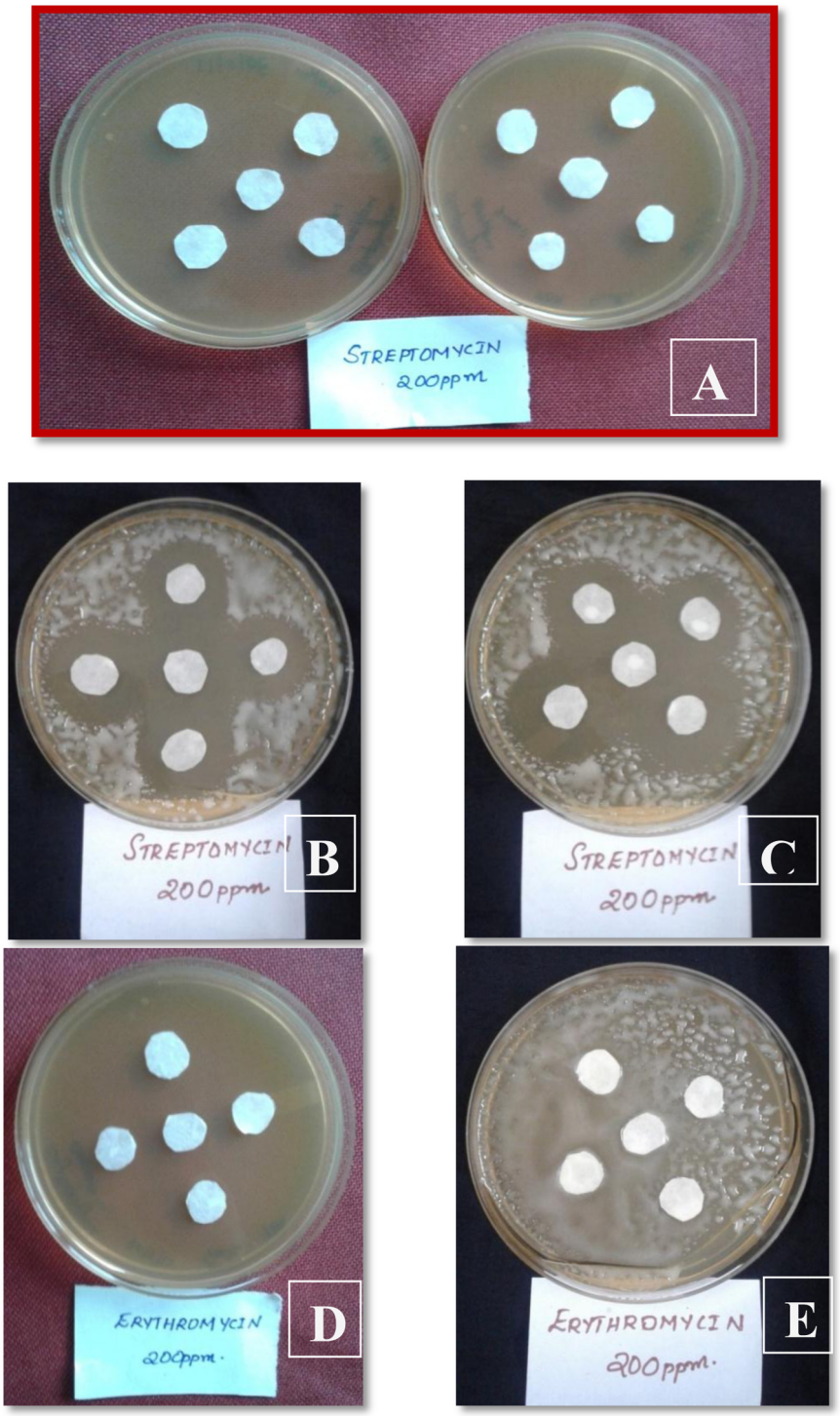

Fig. 19. Observations of Biochemical test- III Intrinsic antibiotic resistance. **A -**Bacterial lawn with filter paper discs dipped in 200 ppm streptomycin, **B** and **C -**Inhibition zone formed around filter paper discs containing streptomycin, **D -** Bacterial lawn with filter paper discs dipped in 200 ppm erythromycin, **E -** No inhibition zone around filter paper discs containing erythromycin.

Table 5
Effect of three percent sodium chloride on growth of bacterial isolates.

Isolate	OD values		
	After 24 h.	After 48 h.	After 72 h.
1_{TrKz}	0.688	0.654	0.690
2_{TrNy}	0.511	0.528	0.534
3_{TrNd}	0.785	0.804	0.823
4_{KoAo}	0.637	0.552	0.668
5_{KoPr}	0.711	0.739	0.754
6_{PaKr}	0.665	0.653	0.688
7_{PaKl}	0.786	0.802	0.822
8_{PaMn}	0.507	0.524	0.530
9_{ThMn}	0.636	0.550	0.666
10_{ThKr}	0.552	0.556	0.587
11_{ErKl}	0.789	0.805	0.825
12_{ThMl}	0.712	0.741	0.751
13_{ThKn}	0.555	0.558	0.609
14_{ThAh}	0.707	0.735	0.750
15_{KaNg}	0.663	0.648	0.695
16_{TuTt}	0.633	0.547	0.663
17_{MaMd}	0.779	0.800	0.818
18_{KaTa}	0.580	0.553	0.604
Control	0.062	0.078	0.164

Table 6
Effect of 4% sodium chloride on growth of bacterial isolates.

Isolate	OD values		
	After 24 h.	After 48 h.	After 72 h.
1_{TrKz}	0.603	0.652	0.668
2_{TrNy}	0.435	0.447	0.455
3_{TrNd}	0.707	0.799	0.811
4_{KoAo}	0.551	0.547	0.586
5_{KoPr}	0.663	0.735	0.743
6_{PaKr}	0.600	0.649	0.664
7_{PaKl}	0.702	0.800	0.815
8_{PaMn}	0.433	0.445	0.453
9_{ThMn}	0.578	0.542	0.583
10_{ThKr}	0.485	0.500	0.512
11_{ErKl}	0.709	0.802	0.819
12_{ThMl}	0.684	0.737	0.746
13_{ThKn}	0.487	0.503	0.515
14_{ThAh}	0.680	0.732	0.740
15_{KaNg}	0.602	0.650	0.667
16_{TuTt}	0.576	0.539	0.580
17_{MaMd}	0.705	0.797	0.814
18_{KaTa}	0.480	0.498	0.550
Control	0.059	0.076	0.157

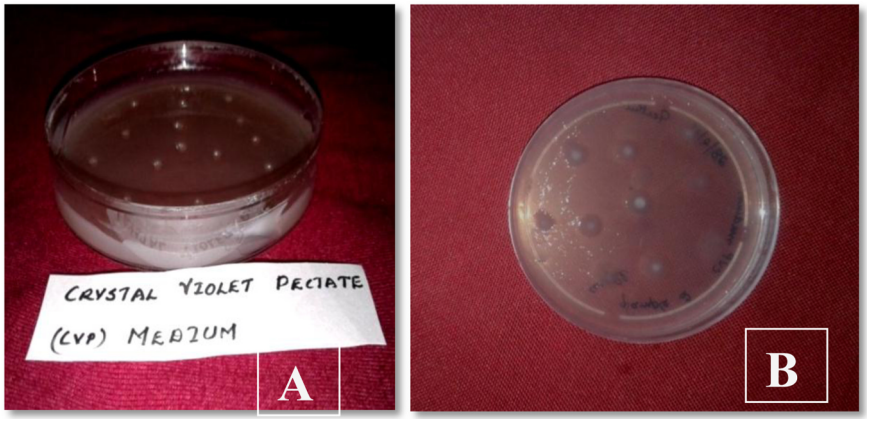

Fig. 20. Observations of Biochemical test- IV **A -** Fissures formed in CVP medium, **B -** Opaque gel formed around fissure showing non-degraded pectin, **C -** Air bubble formation indicating positive catalase test.

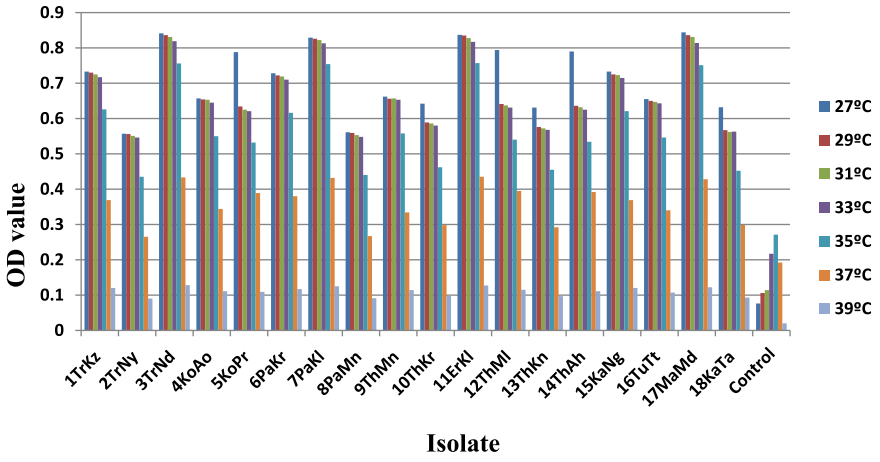

Fig. 21. Effect of temperature on growth of bacterial isolates.

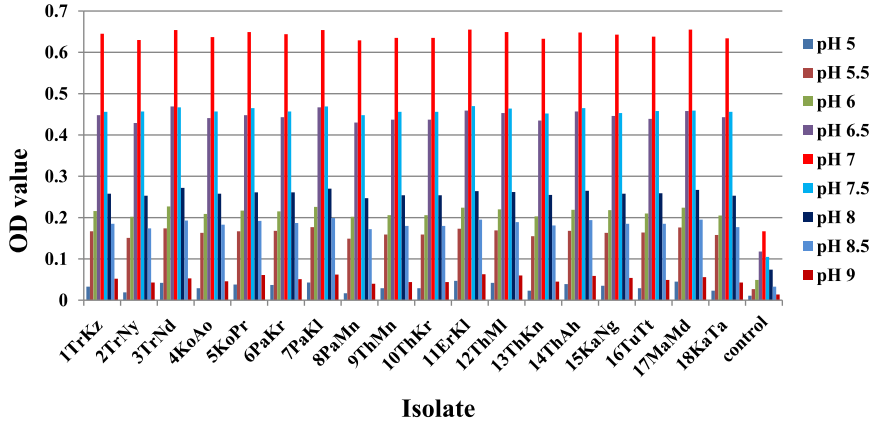

Fig. 22. Effect of pH on growth of bacterial isolates.

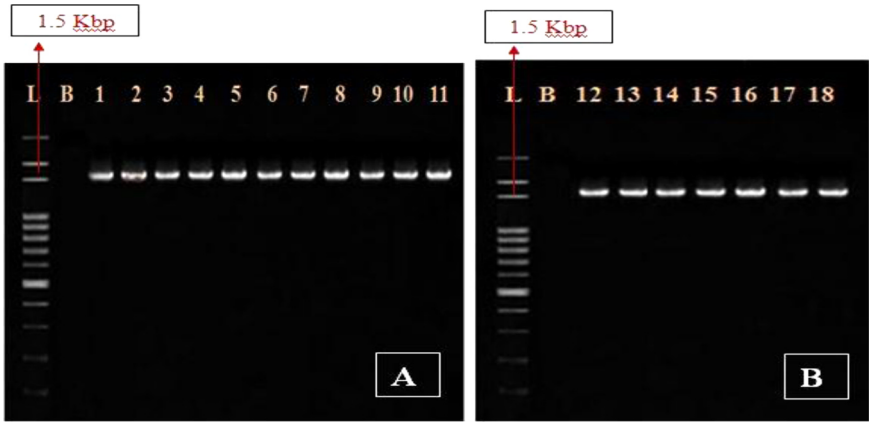

Fig. 23. PCR on isolates A and B – Amplification of 16S rRNA gene using 8 F and 1522 R primers. L - 1 Kb plus ladder, 1–18 - Amplified colony PCR product of 18 bacterial isolates.

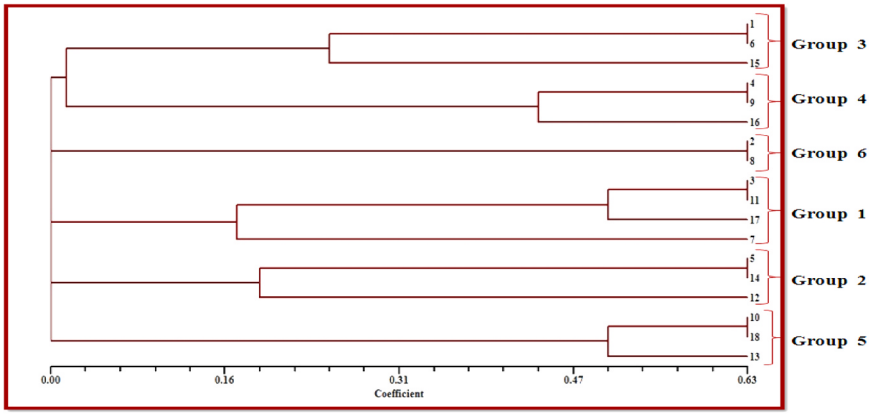

Fig. 24. Dendrogram of different isolates constructed using the parameters, growth of bacterial isolates in nutrient broth and size of bacterial rod.

Table 7a
Grouping based on the cultural parameters.

Group	*In vivo* pathogenicity test on TC plants		Isolate no.
	Yellowing of lower leaves (Days after inoculation)	Complete rotting and falling down of plant (Days after inoculation)	
Group 1	4	6	3_{TrNd}, 7_{PaKl}, 11_{ErKl}, 17_{MaMd}
Group 2	6	9	5_{KoPr}, 12_{ThMl}, 14_{ThAh}
Group 3	8	10	1_{TrKz}, 6_{PaKr}, 15_{KaNg}
Group 4	9	11	4_{KoAo}, 9_{ThMn}, 16_{TuTt}
Group 5	10	12	10_{ThKr}, 13_{ThKn}, 18_{KaTa}
Group 6	12	14	2_{TrNy}, 8_{PaMn}

Table 7b
Grouping based on the cultural parameters.

Group	Range of OD values in nutrient broth (72 h.)	Isolate no.
Group 1	0.790–0.840	3_{TrNd}, 7_{PaKl}, 11_{ErKl}, 17_{MaMd}
Group 2	0.740–0.790	5_{KoPr}, 12_{ThMl}, 14_{ThAh}
Group 3	0.690–0.740	1_{TrKz}, 6_{PaKr}, 15_{KaNg}
Group 4	0.640–0.690	4_{KoAo}, 9_{ThMn}, 16_{TuTt}
Group 5	0.590–0.640	10_{ThKr}, 13_{ThKn}, 18_{KaTa}
Group 6	0.540–0.590	2_{TrNy}, 8_{PaMn}

Table 7c
Grouping based on the morphological parameters.

Group	Range of bacterial size		Isolate no.
	Length (μm)	Width (μm)	
Group 1	2.90–3.30	1.0–1.1	3_{TrNd}, 7_{PaKl}, 11_{ErKl}, 17_{MaMd}
Group 2	2.50–2.90	0.9–1.0	5_{KoPr}, 12_{ThMl}, 14_{ThAh}
Group 3	2.10–2.50	0.8–0.9	1_{TrKz}, 6_{PaKr}, 15_{KaNg}
Group 4	1.70–2.10	0.7–0.8	4_{KoAo}, 9_{ThMn}, 16_{TuTt}
Group 5	1.30–1.70	0.6–0.7	10_{ThKr}, 13_{ThKn}, 18_{KaTa}
Group 6	0.90–1.30	0.5–0.6	2_{TrNy}, 8_{PaMn}

Table 7d
Grouping based on the biochemical parameters.

Group	Range of OD values (72 h)		Isolate no.
	3% NaCl	4% NaCl	
Group 1	0.780–0.830	0.780–0.850	3_{TrNd}, 7_{PaKl}, 11_{ErKl}, 17_{MaMd}
Group 2	0.730–0.780	0.710–0.780	5_{KoPr}, 12_{ThMl}, 14_{ThAh}
Group 3	0.680–0.730	0.640–0.710	1_{TrKz}, 6_{PaKr}, 15_{KaNg}
Group 4	0.630–0.680	0.570–0.640	4_{KoAo}, 9_{ThMn}, 16_{TuTt}
Group 5	0.580–0.630	0.500–0.570	10_{ThKr}, 13_{ThKn}, 18_{KaTa}
Group 6	0.530–0.580	0.430–0.500	2_{TrNy}, 8_{PaMn}

Table 7e
Grouping based on the physiological parameters.

Group	Range of OD values (48 h)			Isolate no.
	27 °C	37 °C	pH 7	
Group 1	0.800–0850	0.410–0.440	0.650–0.655	3_{TrNd}, 7_{PaKl}, 11_{ErKl}, 17_{MaMd}
Group 2	0.750–0.800	0.380–0.410	0.645–0.650	5_{KoPr}, 12_{ThMl}, 14_{ThAh}
Group 3	0.700–0.750	0.350–0.380	0.640–0.645	1_{TrKz}, 6_{PaKr}, 15_{KaNg}
Group 4	0.650–0.700	0.320–0.350	0.635–0.640	4_{KoAo}, 9_{ThMn}, 16_{TuTt}
Group 5	0.600–0.650	0.290–0.320	0.630–0.635	10_{ThKr}, 13_{ThKn}, 18_{KaTa}
Group 6	0.550–0.600	0.260–0.290	0.625–0.630	2_{TrNy}, 8_{PaMn}

Table 8
Homology analysis of 16S rRNA sequence using BLASTn.

GenBank accession number	Query coverage (%)	Identity (%)	Species	E-value
MF458978	98	93	*Pectobacterium carotovorum* subsp. *carotovorum*	0.0
MF458979	96	94	*Pectobacterium carotovorum* subsp. *carotovorum*	0.0
MF458980	95	94	*Pectobacterium carotovorum* subsp. *carotovorum*	0.0
MF458981	94	93	*Pectobacterium carotovorum* subsp. *carotovorum*	0.0
MF458982	98	88	*Pectobacterium carotovorum* subsp. *brasiliense*	0.0
MF458983	98	88	*Pectobacterium carotovorum* subsp. *brasiliense*	0.0

Fig. 25. Barcode gaps identified after aligning the four sequences *Pectobacterium carotovorum* ssp. *carotovorum* and two sequences of *Pectobacterium carotovorum* ssp.*brasiliense*.

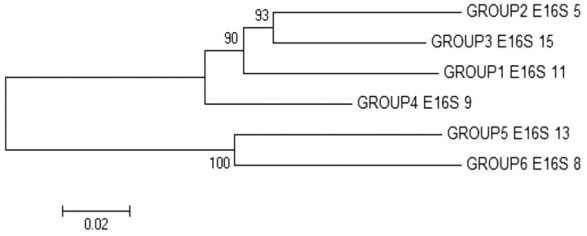

Fig. 26. Phylogenetic tree constructed from six 16S rRNA sequences.

Table 9
Pathogenicity, cultural, morphological, biochemical, physiological and molecular variations among the subspecies of *Pectobacterium carotovorum*.

Sl. no.	Character	*Pectobacterium carotovorum* subsp. *carotovorum*	*Pectobacterium carotovorum* subsp. *brasiliense*
1	Pathogenicity test		
(i)	*In vivo* method on tissue culture plants	Yellowing of lower leaves and complete rotting - falling down of plants occur in 4 and 7 days after inoculation respectively.	Yellowing of lower leaves and complete rotting - falling down of plants occur in 10 and 15 days after inoculation respectively.
2	Cultural characters		
(i)	On nutrient broth	The OD values observed was high (0.635–0.840)	The OD values observed was low (0.555–0.590).
3	Morphological characters		
(i)	Gram staining	The length (1.70 μm–3.10 μm) and width (0.95 μm–1.50 μm) of bacterial rod was high.	The length (0.72 μm–1.10 μm) and width (0.57 μm–0.69 μm) of bacterial rod was low.
4	Biochemical characters		
(i)	Growth on three and four per cent NaCl containing media	The OD values in the three (0.663–0.825) and four (0.580–0.819) per cent NaCl containing media were high.	The OD values n the three (0.530–0.609) and four (0.453–0.550) per cent NaCl containing media were low.
(ii)	Pectate degradation	Perfect fissures were formed in CVP medium due to complete degradation of pectin.	The fissures were covered with opaque gel like substance in CVP medium due to partial degradation of pectin
5	Physiological characters		
(i)	Growth of bacterium at different temperatures	After 48 h incubation at 27 °C the OD values (0.655–0.844) were high.	After 48 h incubation at 27 °C the OD values (0.655–0.844) were low.
(ii)	Growth of bacteria at different pH	After 48 h incubation at pH 7 the OD values (0.637–0.655) were high.	After 48 h incubation at pH 7 the OD values (0.629–0.635) were low.
6	Sequence variations at 16S rDNA	After aligning the 16S rDNA sequence of groups 1–6, the nucleotide sequences in of groups 1–4 were exactly similar to each other. Likewise in groups 5 and 6 were exactly similar to each other, but in particular regions they were different from those in groups 1–4 (barcode gaps were identified at subspecies level).	

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