MOLECULAR CHARACTERIZATION OF *Erwinia* SPECIES CAUSING RHIZOME ROT IN BANANA

By

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"Molecular characterization of *Erwinia* species causing rhizome rot in banana"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"Molecular characterization of *Erwinia* species causing rhizome rot in banana"** is a record of research work done independently by **Ms. Geethu Gokul G** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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ABBREVIATIONS

%	Percentage
≤	Less than or equals to
±	Plus or minus
≥	Greater than or equals to
+ve	Positive
-ve	Negative
Nm	Nano metre
µm	Micro metre
mm	Milli metre
cm	Centi metre
ng	Nano gram
µg	Micro gram
mg	Milli gram
g	Gram
µL	Micro litre
mL	Milli litre
min	Minutes
s	Seconds
h	Hour, Hours
mm^2	Milli metre square
cm^2	Square centi metre
°C	Degree celcius
pH	Hydrogen ion Concentration
$CFUmL^{-1}$	Colony forming unit per millilitre
ppm	Parts per million

rpm	Revolutions per minute
lbs	Pound
M	Molar
bp	Base pair
Kbp	Kilo base pair
ssp.	Species
subsp.	Subspecies
E.carotovora	*Erwinia carotovora*
E.chrysanthemi	*Erwinia chrysanthemi*
DNA	Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
rRNA	Ribosomal Ribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
SDS	Sodium dodecyl sulphate
STE	Sodium chloride Tris EDTA
NaOH	Sodium hydroxide
HCl	Hydrogen chloride
CsCl	Cesium chloride
NaCl	Sodium chloride
KOH	Potassium hydroxide
CO_2	Carbon dioxide
H_2O_2	Hydrogen peroxide
NA	Nutrient agar
CVP	Crystal violet pectate
YGC	Yeast extract glucose calcium carbonate
YDC	Yeast extract dextrose calcium carbonate

LB	Luria Bertani
OD	Optical Density
UV	Ultra Violet
PCR	Polymerase Chain Reaction
TAE	Tris Acetate EDTA
ISR	Inter spacer region
V	Volume
S	Svedberg unit
T	Temperature
G	Gradient
BLAST	Basic Local Alignment Search Tool
NCBI	National Centre for Biotechnology Information
MEGA	Molecular Evolutionary Genetic Analysis

Introduction

1. INTRODUCTION

Banana or plantain (*Musa* sp.) known as 'Apple of Paradise' is one among the ancient fruits known to humankind. After wheat, rice and corn, banana is considered to be the fourth most consumed food item in the world. The origin of banana is from the tropical region of South East Asia. India is the largest producer of banana and this contributes a part of Indian economy (FAO, 2016).

The major banana growing states in India are Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, Gujarat, Madhya Pradesh, Bihar, Orissa, West Bengal, Assam, Manipur and Tripura. Among the banana producing states in India, Tamil Nadu ranks first in production (8.02 million tonnes) and area (1.25 lakh hectare). Maharashtra ranks second in production (4.10 million tonnes) and area (0.82 lakh hectare) followed by Gujarat with a production of 4.04 million tones and an area of 0.65 lakh hectare (NHB, 2016).

India has diverse agro climatic zones where different varieties of banana; especially some local varieties are cultivated. From the past, banana is known for its nutrition and antique nature, thereby it plays a major role in Indian culture and heritage and it is treated as the symbol of 'prosperity and fertility'. In India, banana is known as 'Kalpatharu' which means plant of virtues. Other than the nutritious values banana possess medicinal value also as it contain vitamins and minerals which are used for curing various diseases like kidney stones, asthma, bronchitis and also for curing heart and stomach related problems.

Even though the crop has high significance, it's yield is decreasing drastically due to several biotic and abiotic problems. Among these problems, the invasion of various diseases like Fusarium wilt, Eumusae leaf spot (Sigatoka leaf spot), Rhizome rot and viral diseases plays a major role in yield loss. In the past, rhizome rot also known as soft rot or tip over or pseudostem rot were not prevalent in the banana field but now the condition got reversed and this disease is causing heavy loss in commercial banana cultivation. A perusal of literature revealed the occurrence of rhizome rot disease of banana in India since 1973 (Edward *et al.*, 1973).

The rhizome rot disease becomes more prevalent during the past few years in the southern states of India including Tamil Nadu, Kerala and Karnataka. (Khan and Nagaraj, 1998; Nagaraj *et al.*, 2002; Rani *et al.*, 2002). The major cultivating varieties viz., Grand Naine, Nendran and Rastali are susceptible to banana rhizome rot disease.

The disease occurs during the entire period of crop growth and is mainly observed during late summer and rainy season. The disease spreads through infected plants parts, soil and water. It has been reported that the banana rhizome rot is mainly caused by *Erwinia* spp. belonging to *Enterobacteriaceae* family. Two species of *Erwinia* namely *Erwinia carotovora* and *Erwinia chrysathemi* are reported to cause the disease.

The rhizome rot disease shows symptoms in both below ground and above ground parts of the crop. Below ground symptoms include, rotting of the rhizome followed by brown discoloration from the periphery towards the lateral core of the rhizome. The disease affected tissue became soft, mushy and turns black to brown. The disease infection progresses from rhizome to pseudostem through the collar region. There by the plants became weak, dwarf with pale yellow lusterless withered leaves. During the later stages of disease infection, the pseudostem completely weakens and breaks down from the top of the rhizome and due to this symptom the rhizome rot disease is otherwise known as 'tip-over' disease. The severity levels of infection on the plants are mainly depend on the extent of infection in the rhizome. Sometimes the newly emerging leaves failed to open and showed brown necrotic symptoms.

Different *Erwinia* species synonymously known as *Pectobacterium* species are reported to cause rhizome rot in banana (Kwon *et al.*, 2000) and hence understanding the correct etiology of the disease is becoming necessary. Molecular characterization is the major tool for studying variability and characteristation of the pathogen. Sequencing of the 16S rRNA gene loci of various isolates can be used to find the phylogenetic relationship with other reported species.

Therefore characterization of the pathogen causing rhizome rot in banana with special reference to molecular characterization can help to understand the etiology of the

disease and variability of pathogen. Hence, the present study was carried out with the following objectives:

- Purposive survey in various locations of Kerala and Tamil Nadu
- Collection and isolation of pathogen from rhizome rot infected samples
- Cultural and morphological characterization of pathogen
- Biochemical and physiological characterization of pathogen
- Molecular characterization of pathogen including genomic DNA isolation, 16S rRNA gene amplification and sequencing, homology and phylogenetic analysis.

Review of literature

2. REVIEW OF LITERATURE

Banana and plantains are the staple food of various tropical countries, but now a days they are affected by fungal, bacterial, viral diseases and pests which caused severe yield loss (Jones, 2000). As banana plants were severely invaded by various diseases, crop improvement program like International Network for Improvement of Banana and Plantain (INIBAP) cannot be established properly (Buddenhagen, 1993; Ploetz, 2004). The major disease affecting banana plantations are Sigatoka leaf spot, Fusarium wilt, banana bunchy top disease etc. The significance of rhizome rot disease in banana plants for yield loss was less in the past but now it is increasing severely (Thwaites *et al.* 2000).

Lakshmanan and Mohan (1986) reported that the yield loss due to banana rhizome rot disease is nearly 60 to 80 per cent. Sathiamoorthy (1994) reported that in recent days the only bacterial disease which became an alarming threat to banana cultivation in India is the rhizome rot disease. Leifert *et al.* (1994) and Thangavelu (2009) reported that the incidence of rhizome rot disease on tissue culture banana plants became a major limiting factor for the establishment of micro propagated plantlets. The bacteria that contaminated the *in vitro* banana propagation was reported as *Erwinia carotovora* and caused heavy loss for the commercial tissue culture labs. At the time of secondary hardening two to five per cent death of the plants was due to the incidence rhizome rot disease (Thomas *et al.*, 2011).

Rhizome rot also known as soft rot caused by *Erwinia* spp. synonymously known as *Pectobacterium* spp. is one of the major emerging problem in the establishment of banana nurseries and young plantations in many countries including India (Manoranjitham *et al.*, 2010; Singh *et al.*, 2010; Thammaiah *et al.*, 2010; Patel *et al.*, 2011). The casual organism was reported as *Erwinia carotovora* and *Erwinia chrysanthemi* and they can invade the plants through wounds, mainly spread through water and infected plant materials (Ravishankar, 2010). According to Thangavelu (2009) the rhizome rot disease was observed three to four months after planting during rainy season and late summer conditions. The bacteria which cause rhizome rot disease are the overreaching residents in the soil where banana plant grows.

Arun *et al.* (2012) reported that the name of the disease can describe the typical symptoms of disease. The 'soft rot' pathogen infected plants showed rotting of rhizome followed with yellowing and resultant drying of leaves and finally the pseudostem breaking down at collar region there by the name 'tip over disease'.

Stover (1959) reported that the rhizome rot disease of banana was primarily observed in the Agun Valley of Honduras in 1948. Later the disease incidence was reported from Panama and Central America in variety 'Gros Michel' (Loos, 1962; Hildreth, 1963).The rhizome rot disease caused by the bacteria *Erwinia carotovora* was reported from Israel for the first time by Volcani and Zutra (1967). Fernandez-Borrero (1967) reported the first incidence of rhizome rot disease from Columbia. The second report of the disease incidence from Central America was given by Wardlaw (1972). Stover (1972) reported the primary incidence of disease from Latin America. Afterwards the disease was reported from several places such as Venezuela (Ordosgoitty *et al.,* 1974), Jamaica (Shillingford 1974), Cuba (Rivera-Docando 1978), Papua New Guinea (Tomilson *et al.,* 1987), Tarjon (Choi *et al.,* 1988), Brazil (Periera and Nunes 1988), Iran (Hassanzadeh 1990), Locanto (Guzman and Sandoval 1996). Gomez Caicedo *et al.* (2001) reported that the soft rot disease in 'Cachaco' plantain from Iconozo in Columbia.

In India the rhizome rot disease occurrence was first observed in Basrai variety of banana from Allahabad and it was reported by Edward *et al.* (1973). Chattopadhyay and Mukherjee (1986) reported the disease incidence on the Cavendish variety of banana from West Bengal. The banana rhizome disease had caused a heavy loss in yield (40 to 60 percent) in the Cavendish banana cultivation in Tamil Nadu (Lakshmanan and Mohan, 1986).

KAU (1990) reported the rhizome rot disease first time from in Kerala. The rhizome rot disease incidence was reported from Gujarat by Singh (1990) and Patel *et al.,* (2011). In Karnataka, first rhizome rot incidence was reported from Kolar and Banglore districts (Khan and Nagaraj, 1998) and later from Tamil Nadu by Manoranjitham *et al.* (2010), from Bihar by Singh *et al.* (2010) and from Karnataka by Thammaiah *et al.* (2010).

2.2 SYMPTOMATOLOGY:

Stover (1959) reported that the infection occur mainly at the growing stage of the plant. The symptoms observed were the failure of sprouting, rotting of newly planted rhizomes, dark brown colour and water soaked tissues on the rhizome surface. The inner tissues decayed leaded to the production of cavities surrounded with dark brown colour, decayed spongy tissues and finally the plant toppled at the shooting stage. He also reported that if disease severity level is high, then the newly emerging suckers will also rot.

Edward *et al.* (1973) reported that disease called tip over or bacterial head rot noticed in Basrai variety showed symptoms of discoloration and soft rot of rhizome and suckers, scanty roots with dark brown lesions and necrotic tips. Chattopadhyay and Mukherjee (1986) and Periera and Nunes (1988) reported yellowing of leaves, rotting of pseudostem and progress of infection upward destroying the leaf bases and downward resulting gallies rotting on surface and rotting of tissues emits foul smell.

According to Chattopadhyay (1987), the soft rot disease mainly affected during the emergence of banana plant. The soft rotting was mainly characterized by massive soft and odorous rot of the rhizome. The rotting progressed up the pseudostem from the rhizome and caused internal decay as it destroyed the growing point. The external symptom sometime resemble like Fusarium wilt as the leaf became yellow colour and wilted. He reported that the rhizome rot disease became a major threat for the 'Cavendish' variety of banana cultivation.

Buddenhagen (1994) reported that yellowish to brownish water soaked areas with black border develop in cortex of rhizome which later become soft with foul smell. The rhizome rot infected suckers showed yellowing of younger plants otherwise poor sprout emergence. Guzman and Sandoval (1996) reported that in FHIA-01 and FHIA-02 hybrids, infection moves from the rhizome to upper part of pseudostem.

Thwaites *et al.* (2000) reported that in mature banana plants, bacterial head rot caused "tip-over" or "snap-off "symptom in which the pseudostem weakened and broken down from the top of the corm. Internally the cortex contains water soaked brown or

yellow regions and in severely affected rhizomes the tissue became soft and cavities formed. He also reported that according to the severity of infection the external appearance of banana plant changes. Bacterial soft rot of rhizome and pseudostem rot were mainly characterized with an extensive corn-shaped rot in both rhizome and pseudostem. Rotting destroyed the growing point and surrounding tissues, which resulted in the collapse of lower leaves followed by the appearance of yellow colour and wilting of younger leaves.

Gomez Caicedo *et al.* (2001) reported that symptoms were first observed in 'Cachaco' plantain showing chlorosis of lower leaves followed by wilting of the petiole, watery rot of pseudostem with unpleasant odour. Based on the survey conducted in various districts of Karnataka, Thammaiah *et al.* (2005) reported that rotting of the rhizomes of newly planted plantlets, failure of sprout emergence, stunted growth and yellowing of leaves were the initial symptom of rhizome rot. Toppling over of mature fruited plants in which the rhizome remains attached with the pseudostem also occurs and because of this symptom, the disease is also called tip over or snap off disease. They also observed yellow or dark brown discoloured patches in the vascular regions in the rhizome and pseudostem cross-sections of infected plant.

According to Snehalatharani and Khan (2010) bacterial heart rot, rhizome rot or tip-over disease mainly affects two to six months old banana plants. Characteristic symptoms are produced on rhizome, pseudostem and leaves and the infection was more in rhizome. Massive soft rot accompanied by disagreeable foul smelling rot of the rhizome, internal decay of the pseudostem as the infection spread upward. The disease affected plants were stunted, water soaked appearance on the leaf base followed by the yellowing of leaf eventually withered and showed scorched appearance.

Nagaraj *et al.* (2012) reported the above ground and below ground symptoms of disease. According to them, the disease infection was more in two varieties of banana: G-9 and Robusta. The chief symptom of disease was rotting of the rhizome followed by brown discoloration from the periphery extended towards the lateral core of the rhizome. The disease affected tissue became soft, mushy and turned black to brown. They also reported that the disease infection progressed from rhizome to pseudostem through the

collar region. There by the plants became weak, dwarf with pale yellow lusterless withered leaves. The severity level of infection on the plants mainly depended on the extent of infection in the rhizome. Sometimes the newly emerging leaves failed to open and showed brown necrotic symptoms.

Nagrale *et al.* (2013) reported that the young leaves became yellow initially and later withered slowly, then it started blackening and rotting of central and peripheral leaf, as the rotting extended upward the pseudostem resulted in the decaying of whole plant, foul odour were also produced from the rotted collar region.

2.3 CASUAL ORGANISM

Stover (1959) isolated *Erwinia carotovora* from the pesudostem and rhizome of infected banana plants. According to Wardlaw (1972) the causal organism of banana "tip-over disease was *Erwinia carotovora.* Edward *et al.* (1973) reported that the bacterium in the ooze obtained from rotting parts of the banana plant was identified as *Erwinia carotovora.* Lakshmanan and Mohan (1986) and Choi *et al.* (1988) reported that *Erwinia carotovora* subsp. *carotovorum* is the chief organism causing tip over disease of banana. According to Gomez Caicedo *et al.* (2001) the bacterium causing vascular rot in plantain was identified as *Erwinia carotovora* as per the cultural and chemical characterization results. Thomas *et al.* (2008) reported that *Pectobacterium carotovorum* or *E.carotovora* caused soft rotting disease symptoms during or post acclamatization in micropropagated banana.

Shillingford (1974); Dickey (1979) conducted study on rhizome rot infected plants from Jamaica and Central America; the bacteria from both isolates were identified as *Erwinia chrysanthemi.* Dickey and Victoria (1980) reported that according to the results obtained from various morphological and biochemical test, the soft rot causing phytopathogenic bacteria in banana were *Erwinia chrysanthemi.* According to Chattopadhyay and Mukherjee (1986) the organisms which cause pseudostem rot in banana when isolated in pure culture on Potato Dextrose Agar were *Erwinia chrysanthemi* pv. *paradisica.* The collar and rhizome rot causing bacteria were

characterized by various morphological, biochemical, physiological and pathogenicity tests and identified as *Erwinia chrysanthemi pv paradisica* (Nagrale *et al.*, 2013).

The biochemical and pathogenicity test of the 10 strains of banana soft rot causing *Erwinia carotovora* revealed that they were the intermediates between *E.carotovora* and *E.chrysanthemi* (Hasssanzadeh, 1990). Snehalatharani and Khan (2010); Nagaraj *et al.* (2012) conducted biochemical and physiological characterization of nine isolates of *Erwinia* spp. causing rhizome rot in banana and reported that the disease was mainly caused by *Erwinia carotovora*, as the seven isolates showed similar characters. One isolate was similar to *Erwinia chrysanthemi* and another was intermediate between *E.carotovora* and *E.chrysanthemi.*

2.4 ISOLATION OF PATHOGEN

Disinfected rhizome bits were squeezed and the ooze was streaked on nutrient agar (NA) and greyish white bacterial colonies were obtained (Stover, 1959). Creamy yellow mucoid, shiny, small *E.carotovora* ssp. *carotovora* colonies were obtained on NA when streaked with bacterial suspension obtained from infected rhizome samples of banana (Nagaraj *et al.*, 2002; Rani *et al.*, 2002). Snehalatharani and Khan (2010) reported that colonies of *E.carotovora* from rotten rhizomes of banana on NA was creamish yellow, mucoid, glistening, convex, round to irregular shape. Nagaraj *et al.* (2012) reported that well separated shiny, creamy white, mucoid and regularly shaped colonies of *Erwinia* spp. were obtained by streaking the bacterial ooze from rotted banana rhizome on the NA medium.

Cupples and Kelman (1974) reported that soft rot causing *Erwinia* spp. produced characterstic depressions in sodium polypectate containing crystal violet pectate medium (CVP) when the bacterial ooze was streaked on it, as the *Erwinia* spp. posses pectolytic activity. Soft rotted rhizome and pseudostem bits of banana were taken and outer tissues were removed and sterilized, dried and the bits were squeezed along with 2 to 3mL sterile water to obtain bacterial ooze and the bacterial suspension were streaked on CVP medium (Tomlinson *et al.*, 1987). Liao and Wells (1986) reported that a loop full of rotted bacterial suspension from rotted vegetable samples were streaked on CVP medium and

typical fissure forming *E.carotovora* colonies were obtained. Dickey and Kelmen (1981) reported the isolation of *Erwinia* spp. from infected potato samples by crushing the rotted sections with sterile distilled water after surface sterlization and the resultant suspension after 5 to 30 min was streaked on the CVP medium.

Kado and Heskett (1970) reported a selective medium for *Erwinia* spp. called yeast extract glucose calcium carbonate agar (YGC or YDC). The *Erwinia* spp. produced dull white, muciod, flat sometimes convexed colonies.

Gomez-Caicedo *et al.* (2001) isolated bacterium from infected banana samples after crushing surface sterilized rhizome bits and streaking the suspension on Luria bertani medium.

Nagrale *et al.* (2013) reported that the rhizome and collar rot affected samples of banana were collected, surface sterilized, dried and crushed using 5mL of sterile water and the suspension were streaked on nutrient agar and sucrose peptone agar.

2.5 PATHOGENICITY

Stover (1959) had given a standardized protocol for proving the Koch's postulates. A piece of tissue was removed from the healthy rhizome by cork boring and inoculated with bacterial culture and planted in sterile soil. The rhizomes were cut opened after 25 days and noticed that the rotting had spread about 3.5 cm from the point of inoculation. He also reported that culture re-isolated from the infected sample yielded culture similar to original isolate.

Rahman *et al.* (1994) reported that the banana rhizome bits inoculated by pin pricking with 48 h old bacterial culture inoculated from diseased sample produced rotting symptom after seven days of inoculation with water soaked dark brown discolouration around the inoculated area with the emission of undesirable odour. According to Usha (2003) the banana rhizome bits inoculated with 48 h old bacterial culture along with nutrient agar produced rotting after eight days of inoculation.

Usha (2003) reported that the banana rhizome inoculated with 48 h old bacterial suspension by drenching method produced rotting after 56 days of inoculation. When the

rhizome was cut opened, dark brown discolouration with water soaked appearance was observed along with the emission of foul smell. Pre incubated 60 days old banana plant of G-9 variety was inoculated near base of stem with bacterial suspension (7x 10^5 $CFUmL^{-1}$) and plants were kept in glass house to observe the symptoms (Snehalatharani & Khan, 2010). For establishing the pathogenicity 48 h old bacterial culture (7x 10^7 $CFUmL^{-1}$) was inoculated in 30 days old seedling of G-9 variety on the collar region of pseudostem (Nagaraj *et al.*, 2012).

Nagrale *et al.* (2013) conducted pathogenicity test by hypodermic syringe method in plants grown on earthen pots. Bacterial suspension of $3x10^8$ $CFUmL^{-1}$ was injected into healthy rhizome using hypodermic syringe. In another method from the surface sterilized rhizomes a plug of tissue was removed and inserted with 3 to 4 mm^2 nutrient agar having 48 h bacterial culture and the wound was coated and planted in sterile soil.

According to Thomas *et al.* (2008) the micropropagated banana showed symptoms like yellowing and basal rotting when inoculated with bacterial suspension of *Erwinia carotovora* through soil drenching method. *In vitro* cultures of banana 'G-9' variety grown on Banana Propagation Medium when inoculated with bacterial ooze from soft rot infected rhizome showed symptoms like yellowing of leaves followed by the death of plants in 1-4 weeks.

2.6 CHARACTERIZATION OF PATHOGEN

Several researchers reported that banana rhizome rot is caused by the bacteria belonging to *Enterobacteriaceae* family, the *Erwinia spp.* In order to identify bacterial species causing this disease, the characterization in cultural, morphological, biochemical, physiological and molecular aspects were carried out.

2.6.1 CULTURAL CHARACTERIZATION

Presence of greyish white colonies on nutrient agar (NA) was reported by Stover (1959), when streaked with bacterial suspension of *Erwinia* spp. obtained from rhizome rotted samples. According to Lellilot (1956) and Bradbury (1977b) bacterial colonies of *E. chrysanthemi* were creamy white to greyish white, round with margin becoming

undulate to feathery and smooth colonies on NA. The bacterial colonies on PDA having pH 6.5 when incubated for 3 to 6 day produced flat to slightly rasied colonies having fried egg appearance. Dickey (1979) reported that after 24 h incubation of *E.chrysanthemi* streaked NA Petri plates produced bacterial colonies and the characters were slightly to moderately irregular and undulate, convex, pale coloured colonies with 1.5 to 2.5 mm diameter. According to AngelsRamos *et al.* (1982); Rani *et al.* (2002); Nagaraj *et al.* (2002) and Usha (2003) the bacterial colonies of *E.carotovora* ssp. *carotovora* on NA were cream to pale yellow in colour, mucoid, convex, slightly raised and fluidal with entire margin.

According to Snehalatharani and Khan (2010) the colonies of *Erwinia* spp. were light yellow to cream in colour, convex, mucoid, glistening and round to irregular in shape on NA medium. Bacterial suspension of *E.carotovora* causing tip over disease in banana produced well separated shiny, creamy white, muciod regularly shaped colonies on NA medium (Nagaraj *et al.*, 2012). According to Nagrale *et al.* (2013) bacterial colonies of *E.chrysanthemi* causing rhizome rot were white, creamy, butyrous and dome shaped in NA.

Cupples and Kelman (1974) reported that softrot causing *Erwinia* spp. produced cup like depressions in the CVP medium and the colonies of *Erwinia* were iridescent, translucent and criss crossed with internal markings. Bacterial suspension of rhizome rot causing *E.carotovora* when streaked on CVP medium produced blue coloured colonies with characteristic fissures or hallow due to pectolytic activity (Liao and Wells, 1986). Ma *et al.* (2007) cultured the *Pectobacterium* and *Dickeya* species belonging to *Enterobacteriace* family on CVP to check the pectolytic ability and the isolates which created pits were then streaked on Luria Bertani agar. Bhupendra and Yogendra (2015) reported that the soft rot causing bacterial ooze of *E.chrysanthemi* from infected samples when streaked on CVP medium produced characteristic deep pit when incubated for five days at 28°C.

According to Starr *et al.* (1966) soft rot causing bacteria of *Erwinia chrysanthemi* group produced traces of a blue, extra cellular, water-insoluble pigment called indigoidine when cultured on YDC agar medium. Bradbury (1977a) reported that *E. chrysanthemi*

incubated at 27°C on YDC after 5 to 10 days produced characteristic dark insoluble pigment and *E.carotovora* ssp. *carotovora* incubated at 25 to 30°C on NA for 24 hours produced smooth, slightly raised, grayish white, round and glistening colonies. Dickey and Victoria (1980) reported that the characters of bacterial colonies of *E.chrysanthemi* observed on YDC were irregular, convex, umbonate, butyrous with 2.5 to 3.5 mm in diameter. According to Khan and Nagaraj (1998) the *E.carotovora* isolated from infected banana rhizome samples produced convex, cream coloured and irregular fluidal colonies on YGC agar medium. Kado (2006) reported that *Erwinia* spp. produced yellow semi mucoid colonies with a clear zone around each colony due to the acid secretion which liberate carbohydrate as CO_2 which can be observed as small bubbles in the colonies when cultured on YGC medium.

Erwinia carotovora ssp. *carotovora* when streaked and incubated at 27°C for 24 h produced pink to purple colonies with 1.5 mm diameter as the bacteria reduced tetrazolium in the medium, where as *Erwinia carotovora* ssp. *atroseptica* colonies were colourless and 0.5 mm diameter. Bacterial colonies of *Erwinia chrysanthemi* on Logans media were completely red to purple with 2 mm diameter (Logan, 1966 and Fahy and Hayward, 1983). Usha (2003) reported that when 48 h old bacterial culture streaked on the logans medium yielded purple coloured colonies and reported as *Erwinia carotovora.* The OD values of *E.carotovora* inoculated nutrient broth after 72 h was reported in the range of 0.720 to 0.800.

Schaad and Bernner (1977) conducted cultural character studies in six different media such as nutrient agar (NA), Miller and Scroth agar (MS), yeast extract calcium carbonate agar (YDC), Bact Brillliant Green Agar (BGA), Bioquest XLD agar (XLD) and Bacto m Endo agarLES (mEndo). After incubation of bacterial suspension of *E.chrysanthemi* on nutrient agar at 30°C for 24 h produced effuse to low convex, mucoid with entire margin, transparent, colourless with 4 mm diameter colonies. On MS media after 48 to 72 h incubation bacterial colonies appeared were smooth, slightly raised, undulate and clear, 2 to 3 mm diameter with an orange centre. Bacterial colonies on YGC agar after 72 h incubation were white to light tan colour, slightly convex, finely granular, undulate with 5 mm diameter.

Chattopdhyay and Mukherjee (1986) reported that bacterial colonies of rhizome rot *E.chrysanthemi* on NA incubated at 27°C possessed the characters such as convex, slightly to moderately irregular, undulate and pale cream in colour in PDA. Tomlinson *et al.* (1987) conducted study in cultural characterization on two different media such as potato dextrose agar (PDA) and sucrose peptone agar (SPA). He found that *E.chrysanthemi* produced blue colour colonies whereas *E.carotovora* didn't produce any colored colonies.

According to Janse and Ruissen (1988) two types of *E.chrysanthemi* colonies appeared when incubated at 27°C for 2 days on yeast peptone glucose agar (YPGA) were gray to cream yellow, glistening, irregular colonies with raised center, irregular margin having 4 mm diameter as well as mucoid, lobate, flat, round colonies with entire margin. The *Erwinia* spp. causing rhizome rot in banana when isolated in Luria Bertani medium produced creamish, buttery colonies and also possessed foetid odour (Gomez-Caicedo *et al.*, 2001). According to Rafiei *et al.* (2015) bacterial suspension of *Erwinia* spp. streaked on nutrient agar sucrose after incubation at 25°C for 24 hours produced single colony with white to creamy colour colonies with irregular margins and on eosin methylene blue (EMB) bacterial colonies were emerald green in colour.

2.6.2 MORPHOLOGICAL CHARACTERIZATION

Pathogen causing rhizome rot in banana was identified as motile, gram negative and rod shaped (Stover, 1959). Blenden and Goldberg (1964) had given a detailed procedure of flagellar staining. A smear of bacterial culture was prepared using sterile water, then it was then covered with reagent A (100 mL of distilled water containing 5 g of tannic acid, 1.5 g of ferric chloride, 2.0 mL of 1570 formalin, and 1.0 mL of 1 % sodium hydroxide) for 2 to 4 min. After rinsing with sterile water, it was stained with reagent B (100 mL of 2% silver nitrate solution) for 30s. Again rinsed with sterile water, air dried and observed under oil immersion microscope.

Bottone and Schneierson, (1972) and Dickey (1978, 1980) reported that microscopically, the *Erwinia* isolates were short, stout, non-sporulating, actively motile using peritrichous flagella, Gram-negative bacilli with rounded ends that were not

capsulated. The *E.carotovora* present in the bacterial ooze of rhizome rot of banana were gram negative, short, rod shaped, peritrichously flagellated, non spore forming and non capsular (Edward *et al.*, 1973; Liao and Wells, 1980). According to Chattopadhyay and Mukherjee (1986) rhizome rot causing bacteria *Erwinia chrysanthemi* pv. *paradisiaca* were motile, gram negative and had the size of 0.7x 1.65mm. Jaap and Ruissen (1988) reported that the morphological characters of *Erwinia chrysanthemi* were gram negative, motile straight rods, measuring 0.5-0.7x1-3 µm.

The bacteria causing tip over disease in banana were small rods occurred singly, peritichously flagellated, non capsulated, gram negative and non spore forming (Nagaraj *et al.*, 2012; Nagrale *et al.*, 2013). According to Snehalatharani and Khan (2010) *Erwinia* spp. causing tip over disease in banana were gram negative, rod shaped, non capsulated, peritrichously flagellated and non spore forming in nature.

2.6.3 BIOCHEMICAL CHARACTERIZATION

Some of the important biochemical tests such as solubility in KOH, ability to cause rot in potato and carrot, sensitivity to various anitibiotics, growth at 3 and 4 per cent sodium chloride and catalase test were carried out by various workers for identifying the pathogen causing rhizome rot in banana.

Suslow *et al.* (1981) developed a new technique to detect the gram reaction of bacteria other than gram staining. This technique involved the solubility in 3 percent KOH solution. The procedure involved is mixing of a loopful of bacterial culture from the agar medium with 50 µL of 3 per cent potassium hydroxide solution with rapid circular agitation. After 5 to 8 s the loop was alternately raised and lowered. For the gram negative strains of bacteria the suspension become viscous and mucoid thread is formed where as gram positive bacteria dispersed into KOH and do not produced mucoid thread. Ngwira and Samson (1990); Gomez-Caicedo *et al.* (2001) and Akbar *et al.* (2015) confirmed the gram reaction of bacteria causing vascular rot in banana as gram negative, by observing the viscous, mucilaginous thread formation in the suspension of 3 per cent KOH and bacterial culture.

The soft rotting of potato and carrot by *Erwinia* spp. was reported by several workers. Lelliott *et al.* (1966) developed a protocol for potato soft rot test by *Pseudomonas* spp. Potato slices were placed in petri dish which halfway flooded with sterile distilled water. Surface sterilized potato slices were inoculated with bacterial culture and developed rotting after 3 days at room temperature.

Schillingford (1974) conducted soft rotting test in potato, tomato, carrot and onion. The rotting symptom produced in tomato and potato was rapid, after 48 to 72 h but in carrot and onion the symptom development was slow. According to Dickey and Victoria (1980) the potato slices inoculated with *Erwina* spp. isolated from banana produced rotting after an incubation of 72 h.

According to Togashi (1988) and Nabhan *et al.* (2006) sterile potato slices inoculated with soft rot causing *Erwinia* spp. incubated at 30°C showed complete rotting of potato slices within 2 to 3 days. Alcron *et al.* (1991) conducted soft rotting test in carrot and potato slices prepared from surface sterilized healthy tubers. These slices were placed in Petri plates containing moisten filter paper, a small notch was made on the center of each slice and two drops of bacterial suspension of *Erwinia* spp. was added to the notch after that the plates were sealed with parafilm and incubated at 30°C.

Snehalatharani and Khan (2010) reported that *Erwinia* species causing tip over disease in banana, produced soft rot in the inoculated potato slices. Nagaraj *et al.* (2012) reported that soft rotting in potato slices were produced by the isolates of *E. carotovora* and *E.chrysanthemi.* A different nature in rotting was observed by an isolate which showed characters in between *E.carotovora* and *E.chrysanthemi.*

Nagrale *et al.* (2013) conducted potato soft rot testing in 1 cm thick slices of potato, the surface sterilized slices were placed in petri dish containing sterilized filter paper impregnated with 2 mL water. The potato slices were inoculated by pin pricking and bacterial culture of *E.chrysanthemi* were placed and incubated under moisture condition. Rotting of potato slices were observed after 24 to 48 h. Sandipan (2014) carried out the potato soft rot testing using *Erwinia carotovora* using healthy potato tubers. The potato slices were prepared and inoculated with bacterial culture and

incubated at 28°C for 24 to 48 h and reported that inoculated potato slice samples developed softened, slimy and watery mass accompanied with foul odour. Rafiei *et al.* (2015) conducted potato soft rot test of *Pectobacterium* spp., by puncturing of 10 µL (10^8 $CFUmL^{-1}$) of culture to the surface sterilized potato tubers. The inoculated tubers were incubated in moist chamber, showed rotting symptoms after 72 h. Doolotkeldieva *et al.* (2016) carried out the carrot and potato soft rot test using healty slices of potato and carrot and created a slight cut on the centre of slice and inoculated with bacterial culture of *Erwinia carotovora* ssp. *carotovora* and observed complete rotting symptom in potato after 4 days and in carrot after 14 days.

Bottone and Schneierson (1972) reported that *Erwinia* spp. were generally resistant to ampicillin, cephalothin, erythromycin and tetracycline and were variably susceptible to chloramphenicol and colistin sulfate. The antibiotic sensitivity of *Erwinia* spp. was reported by several workers. According to Bradbury (1977a, b) the soft rot causing bacteria *E.carotovora* is resistant to the antibiotic erythromycin where as *E.chrysanthemi* were sensitive to erythromycin. Dickey and Victoria (1980) reported that the susceptibility of bacteria isolated form *Musa paradisica* varies based on the species to erythromycin, as *E.carotovora* were resistant where as *E.chrysanthemi* were susceptible to erythromycin.

Liao and Wells (1986) reported that *E.carotovora* containing media when impregnated with antibiotic erythromycin at different concentration didn't produce inhibition zone around the disc containing erythromycin as it revealed that *E.carotovora* were resistant to erythromycin. According to Gomez-Caicedo *et al.* (2001) the bacteria causing vascular rot in plantain were susceptible to antibiotics like streptomycin, tetracycline as they observed transparent hallow, free from bacterial growth around the disc with antibiotic. Trokenheim *et al.*(2006) conducted a study on the antibiotic resistance of bacteria of the family *Enterobacteriaceae* and reported that the *Erwinia carotovora* species are susceptible to the antibiotic streptomycin.

Snehalatharani and Khan (2010) reported that *Erwinia* spp. causing tipover disease in banana showed varying nature to erythromycin sensitivity. One group of isolates were resistant to erythromycin as it belonged to *E.carotovora*, second group were

susceptible to erythromycin as it belonged to *E.chrysanthemi* and the third group showed intermediate characters between other two groups. Tavasoli *et al.* (2011) reported that 90 percent of soft rot causing *Pectobacterium* spp. are resistant to antibiotic erythromycin. According to Nischwitz (2012) *Erwinia* spp. are susceptible to streptomycin as they observed characteristic inhibition zone when bacterial culture containing media is incorporated with streptomycin at different concentrations.

Nagaraj *et al.* (2013) conducted antibiotic sensitivity test by seeding the bacterial culture of *E.carotovora* into molten nutrient agar (50°C) and poured in petriplates, sterile filter paper disc of 8mm were dipped in different concentration of antibiotics and placed on solidified media containing bacteria. He reported that bacteria were susceptible to streptomycin sulphate and streptocycline as characteristic inhibition zones were observed. Nagrale *et al.* (2013) reported that bacteria causing collar and rhizome rot in banana were *E.chrysanthemi* and they were susceptible to erythromycin. According to Akbar *et al.* (2015) the soft rot causing *Erwinia* spp. were resistant to antibiotic erythromycin as he conducted the antibiotic sensitivity test using erythromycin disc and no inhibition zone were formed. Rafiei *et al.* (2015) reported that *Pectobacterium* species were resistant to the antibiotic erythromycin by performing antibiotic sensitivity assay procedure given by Schaad *et al.* (2001)

The salt tolerance of *Erwinia* spp. was reported by several workers. According to Dickey (1978); Dickey and Victoria (1980) both *E.carotovora* and *E.chrysanthemi* grew in the medium contain 5 percent sodium chloride. Jaap and Ruissen (1988) checked the salt tolerance of *Erwinia* spp. after growing it in 5 percent NaCl containing nutrient broth, and observed positive results after 48 h. Gomez-Caicedo *et al.* (2001) conducted bacterial growth test and reported that pathogen causing vascular rot in banana grown normally on nutrient broth with 3 and 4 percent sodium chloride.

Akbar *et al.* (2015) reported that soft rot causing bacteria grow normally in 5 per cent NaCl containing agar media when incubated at 27°C for 24 h which revealed that the *Erwinia* spp. were salt tolerant. Rafiei *et al.* (2015) conducted an experiment by growing bacteria in 4 and 5 per cent NaCl at 28°C and reported that growth of bacteria in the media agreed with *Pectobacterium* spp.

The pectin degrading ability of *Erwinia* spp. in crystal violet pectate medium was reported by several workers. Dickey (1978); Dickey and Victoria (1980) reported that both *E.carotovora* and *E.chrysanthemi* has the ability to reduce the pectate present the media on which the bacteria grows.

Mc. Feeters *et al.* (1992) reported bacteria belonging to the family *Enterobacteriace* had pectolytic activity as the bacteria grown in solid medium containing pectin or pectate substrate, which were degraded by pectolytic or pectinolytic enzymes. Pectate degradation was observed by the depressions formed in the gel where the pectin had degraded and also observed the presence of precipitate solution with opaque gel containing non degraded pectin or pectin substrate.

Ngwira and Samson (1990) observed the pectate degrading nature of *E.chrysanthemi* when grown in modified Sutton's medium. Bdlyia *et al.* (2004) developed a modified crystal violet pectate medium to detect the *Erwinia* spp. As the *Erwinia* spp. had the ability to produce pectolytic enzymes, it reduced the pectin or polypectate present in the medium, there by produced cavity in the medium.

Snehalatharani and Khan (2010) reported that the *Erwinia* spp. causing tipover disease in banana has the ability to degrade the sodium polypectate present in the crystal violet pectate medium (CVP) as the characteristic pits were produced by the bacteria.

According to Nagaraj *et al.* (2013) the three groups of bacteria *E.carotovora, E.chrysanthemi* and the intermediate group between these two had the ability to degrade pectate present in the medium. Rafiei *et al.* (2015) reported that soft rot causing *Pectobacterium* spp. had pectolytic ability to degrade pectin and pectate substrates present in the medium.

The catalase activity of *Erwinia* spp. was reported by several workers. Lelliott *et al.* (1966) checked the catalase reaction, picked a loopful of bacterial culture of *Erwinia* spp. from a 24-48 h nutrient agar slant was smeared on a slide and covered with 20V Hydrogen peroxide and observed the bubble formation and reported the catalase positive activity

According to Dickey (1978) and Cappuccino and Sherman (1992) *Erwinia* spp. produced gas bubbles when the bacterial smear was covered with 30V Hydrogen peroxide as the bacteria produces catalase enzyme. Nagrale *et al.* (2013) reported that *E.chrysanthemi* causing collar and rhizome rot in banana were catalase positive.

According to Snehalatharani and Khan (2010) and Nagaraj *et al.* (2012) the three group of *Erwinia* spp. reported to cause tip over disease in banana showed positive catalase reaction. Raiefi *et al.* (2015) reported that *Pectobacterium* spp. isolated from north-west of Iran showed positive catalase activity.

2.6.4 PHYSIOLOGICAL CHARACTERIZATION

The effect of temperature on growth of *Erwinia* spp. was reported by several workers. Goto and Matsumoto (1987) reported that the *Erwinia* spp. can grow and survive at 36°C when cultured on peptone water. Gallois *et al.* (1992) reported that soft rot causing *Erwinia carotovora* and *Erwinia chrysanthemi* can surivie at 37°C.

Hadas *et al.* (2001) reported that the *E.carotovora* species grown on yeast salt medium at 36°C to 37°C. Yap *et al.* (2004); Akbar *et al.* (2015) observed that the *Erwinia carotovora* species can grow at 37°C on Luria Bertani broth.

Usha (2003) reported that the growth of *E. carotovora* was maximum in the nutrient broth incubated at 28°C and the bacteria survived up to 40°C. Shrestha *et al.* (2005) studied the effect of temperature on the growth of *Erwinia* spp. The maximum temperature for bacterial growth was recorded as 36°C and the optimum growth was recorded at 27-28°C.

The effect of pH on growth of *Erwinia* spp. was reported by several workers. Usha (2003) reported that the growth of *E. carotovora* was maximum in the nutrient broth with neutral pH and minimum in the nutrient broth with acidic and alkaline pH. Shrestha *et al.* (2005) reported that none of the pathogens can grow at pH $\leq$ 5.0 and 10.0 $\geq$. The most favorable pH for the growth of *Erwinia* spp. was recorded as 7.5.

Doolotkeldieva *et al.* (2016) conducted the characterization of *E. carotovora* spp. in terms of morphological, physiological and biochemical characters and reported that morphologically the bacteria was rod shaped with round ends and gram negative. The cultural characters were convex, mucoid and creamy white colonies, and also the cavity production on CVP medium. He also reported that the bacterial growth was observed at 28°C, 30°C and 36°C on the nutrient broth.

2.6.5 MOLECULAR CHARACTERIZATION

The DNA isolation from *Erwinia* spp. was reported by several workers. Marmur (1961) developed a procedure for bacterial DNA isolation using Cesium Chloride for the lysis of bacterial cells, sodium dodecyl sulphate as a detergent for easy disruption of gram negative bacteria cells, EDTA as chelating agent to inhibit activity of nucleases and 1M perchlorate for dissociating DNA from protein. Dickey and Victoria (1980) had isolated the bacterial DNA of the *Erwinia* spp. of *Musa paradisica.*L as per the method given by Marmur (1961).

Staskawickz *et al.* (1984); Goto and Mantumoto (1987) isolated the bacterial DNA of *Pseudomonas* spp. and *E.carotovora* using the procedure given by Marmur (1961) with slight modification in the extraction of DNA. They used sodium acetate before precipitating DNA with isopropanol and DNA was purified using cesium chloride.

Bereswill *et al.* (1992) extracted the bacterial DNA by lysing the cell with lysozyme and sodium dodecyl sulphate and the purification of the nucleic acid was done by repeated washing with phenol and then with chloroform isoamyl alcohol.

Neumann *et al.* (1992) developed a rapid method of DNA isolation from gram negative bacteria by using sodium chloride Tris EDTA (STE) buffer. The main advantage of this method as compared to other methods was efficient recovery of DNA even in smaller quantities, with less RNA contamination.

Kwon *et al.* (1997) carried out DNA isolation by extraction of lysates using chloroform for two times in order to remove the excess phenol present in it. Nishiguchi *et al.* (2002) had given a modified protocol of Ausubel *et al.* (1998) for the bacterial

genomic DNA extraction using lyzozyme and SDS for lysis of bacterial cell and extraction using STE buffer.

Cheng and Jiang (2006) and Rafiei *et al.* (2015) used the same improved standard phenol/chloroform method using STE buffer based on the procedure given by Neumann *et al.* (1992) but they eliminated the lysis step using SDS, lyzozyme or proteinase K and used phenol directly for lysis.

The importance of 16S rDNA gene and its sequence in phylogenetic studies were reported by several workers. Ribosomes are of two types, 70S and 80S. Prokaryotic cell possesses 70S ribosome where as eukaryotic cell posses 80S ribosome. The bacterial ribosome is of 70S which is subdivided into 30S (smaller subunit) and 50S (larger subunit). The 30S subunit of ribosome is a complex of 16S ribosomal RNA and 22 proteins. The part of DNA most commonly used for taxonomy is the gene coding for 16S rRNA called 16S rDNA gene or 16S rRNA gene. Nearly 60,000 16S rRNA sequences are available in Ribosomal Database (Maidak *et al.*, 2001)

According to Janda and Abbott (2001) the 16S rRNA gene sequences are the common housekeeping marker genes for the taxonomic and phylogenetic studies as this gene possess various features such as present in most bacteria sometimes as multigene family or operons, the function of this gene was not changed from time to time so it can be used to find evolutionary relationships and 16S rRNA gene is nearly 1500 to 2000 bp so it is large enough for bioinformatic studies.

The identification of new genus can be easily done by the comparison of 16S rRNA sequences and DNA-DNA hybridization. There by it helped in the taxonomic classification by calculating similarity at species level (Christensen *et al.*, 2001).

Patel (2001) reported the potential use of 16S rRNA gene sequence for the identification of genus and species of organisms which can't be identified by various biochemical tests and he also reported that these genes are tolerant to mutation.

According to Clarridge (2004) 16S rRNA gene sequencing can improve clinical microbiology as it helped in identifying poorly described, rarely isolated and

biochemically aberrant strains. The gene sequencing is robust, accurate, reproducible there by novel organisms can be identified.

Brands *et al.* (2010) carried out PCR amplification of 16S rRNA gene using broad range of universal primers followed by gene sequencing to study microbial diversity.

According to Thapa *et al.* (2011) 16S rRNA sequence analysis is a useful tool to study phylogenetic relationship among microorganisms as the sequences are highly conserved and can be used as a taxonomic marker.

The polymerase chain reaction for 16S rRNA genes was carried out and reported by several workers. Weisberg *et al.* (1991) reported that fD1 and rP2 universal primers amplified the 16S rRNA gene of *Enterobacteria* species using polymerase chain reaction along with the total bacterial genomic DNA.

Beriswill *et al.* (1992) carried out the polymerase chain reaction for specific species detection of *Erwinia* spp. He used 2 µL of isolated total genomic DNA as template in the PCR reaction mixture of 25 µL. Kwon *et al.* (2000) reported that the universal 16S rRNA primer fD1 and rP2 amplified the 16S rRNA gene of *Erwinia* spp. Turner *et al.* (1999) used 16S rRNA universal primer set 8F and 1492R to amplify small subunit of rRNA genes of bacteria and reported that the amplicon length was 1.5 Kb.

Ma *et al.* (2007) extracted the total genomic DNA from *Pectobacterium* and *Dickeya* belonging to *Enterobacteriaceae* family and conducted PCR and amplified the portion of seven genes and found out the host range and molecular phylogenies. The 16S rRNA universal primers like 8F and 1522R were used to amplify the 16S rRNA genes of bacterial species (Frank *et al.*, 2008)

Lima *et al.* (2011, 2012) carried out the amplification of 16S rRNA gene using 8F and 1522R universal primer set as reported by Edward *et al.* (1989) to study microbial dynamics and diversity. Ramachandran *et al.* (2015) isolated the total bacterial genome from *Erwinia carotovora* from overnight culture in the nutrient agar broth using DNeasy® Plant Mini Kit (Qiagen, United Kingdom) and used fD1 and rP2 universal primers to amplify the 16S rRNA gene using PCR.

The colony PCR of *Erwinia* spp. were carried out and reported by several workers. Yap *et al.* (2004) conducted colony PCR of *Erwinia carotovora* using ITS primers by picking colony grown overnight on LB agar and suspended in 500 µL sterile water and denatured it for 5 min and the debris were removed by centrifuging and the supernatant was taken to carry out the PCR along with the reaction mixture containing dNTP, forward and reverse primer, taq DNA polymerase.

Rahmani *et al.* (2006) used colony PCR to amplify 16S rRNA gene by taking the supernatant of the bacterial solution of *E.coli* and *P.aeruginosa* after lysis and used as the DNA template along with PCR reaction mixture containing 16S rRNA universal primers U1 and U2.

According to Devereux and Wilkinson (2004) the amplification of 16S rRNA gene can be done by using the extracted genomic DNA from the bacteria or otherwise by using small amount of bacterial cell culture through cell lysis by denaturation method. He used universal primers fD1 and rP2 to amplify the 16S rRNA genes.

Lee *et al.* (2006) carried out the colony PCR of *E.chrysanthemi* by picking small amount of 24 h colony grown in agar medium and suspended in 50 µL sterile water and denatured at 100°C for min and centifuged to remove the cell debris. The supernatant were used as the DNA template in the 30 µL PCR reaction mixture.

Direct colony PCR is a fast and widely applied PCR amplification technique for bacterial strains using bacterial cell culture either in the solid agar medium or in the broth by adopting cell lysis by denaturation technique and using the supernatant as the DNA template for PCR cycle (Walch *et al.*, 2016)

2.7 PHYLOGENETIC STUDIES

According to Ma *et al.* (2007) phylogenic constructions based on the sequence analysis of seven genes of soft rot causing bacterial species indicated monopoly of *Pectobacterium* and *Dickeya* spp. lineages belonging to *Enterobacteriaceae* family.

Kwon *et al.* (1997) carried out the study on phylogenetic relationship of *Erwinia* spp. by comparative analysis of 16S rRNA gene sequences. He reported that by the

sequence and phylogenetic tree analysis considerable heterogeneity was observed in the species belonging to *Erwinia*.

The phylogenetic analysis of *Pectobacterium* species belonging to *Enterobacteriaceae* family was carried out by amplifying and sequence analysis of 16S-23S rRNA Inter Spacer Regions (Kown *et al.*, 2000). He reported that sequence analysis of ribosomal DNA can clarify the phylogenetic relationship between various taxa. He had also given the detailed procedure for sequence analysis and phylogenetic tree construction. Using Clustal W software (Thompson *et al.*, 1994) the ISR sequences were aligned and as per the methodology given by Jukes and Cantor (1969) an evolutionary distance matrix were created. Considering the guidelines of neighbor joining method given by Saitou and Nei (1987) using neighbor joining program in MEGA software (Kumar *et al.*, 1993) an evolutionary tree was constructed. Bootstrap technique was used to analyze the stability of relationships between the species.

Tamura *et al.* (2011) developed software called MEGA5 for the construction of phylogenetic tree. It is the newest version of Molecular Evolutionary Genetic Analysis software which have special features like collection of maximum likelihood (ML) analyses for interfering evolutionary trees, can select best suited substitution models for nucleotide and aminoacid and can infer ancestral and evolutionary changes associated with the species.

Thapa *et al.* (2011) constructed phylogenetic tree of *Erwinia* spp. by comparing the amplified 16S rRNA sequences along with the 16S rRNA sequence already present in the gene bank. They reported that the 16S rRNA gene sequence of *E.carotovora* and *E.chrysanthemi* were 92% similar.

Materials and methods

3. MATERIALS AND METHODS

3.1 COLLECTION OF SAMPLE AND ISOLATION OF PATHOGEN:

3.1.1 Collection of diseased samples

The infected rhizome samples at the initial stage of rotting were collected from banana fields of Kerala and Tamil Nadu. The samples were collected in sterile polythene bags and brought to the laboratory for further analysis. The symptoms of rhizome rot observed during collection were given in Plate.1

3.1.2. Isolation of pathogen from infected plant samples

In order to detect the presence of pathogen, ooze test of infected samples were carried out. For isolation of pathogen, the collected samples were washed with water to remove the adhered soil particles. Small bits of rhizomes were taken from the sample after removing the surface layer. The rhizome bits were then surface sterilized using one per cent sodium hypochlorite solution for 45 s followed by washing the samples using sterile distilled water thrice. After the surface sterilization the rhizome bits were transferred into sterile Petri plates.

One or two drops of sterile distilled water were added to the bits and then the bits were squeezed along with the water to obtain the bacterial ooze. A loopfull of bacterial ooze was taken and streaked on the nutrient agar containing Petri plates. After streaking, the Petri plates were sealed using parafilm and kept for incubation at a temperature of 28±1°C for 48 h (Plate. 2).

Isolated single bacterial colonies were picked for further sub-culturing, by streaking the bacterial colonies on the nutrient agar medium for obtaining bacterial pure culture. The single colony of bacterium was picked from the pure culture and streaked on the crystal violet pectate (CVP) medium (Annexure I), specific medium for *Erwinia* spp. (*Pectobacterium* spp.).

Plate 1. Symptoms of rhizome rot disease. A-Initial rotting of rhizome. B and C-Completely rotted rhizome. D-Pseudostem rotting. E- Toppled plant. F-Sample collection.

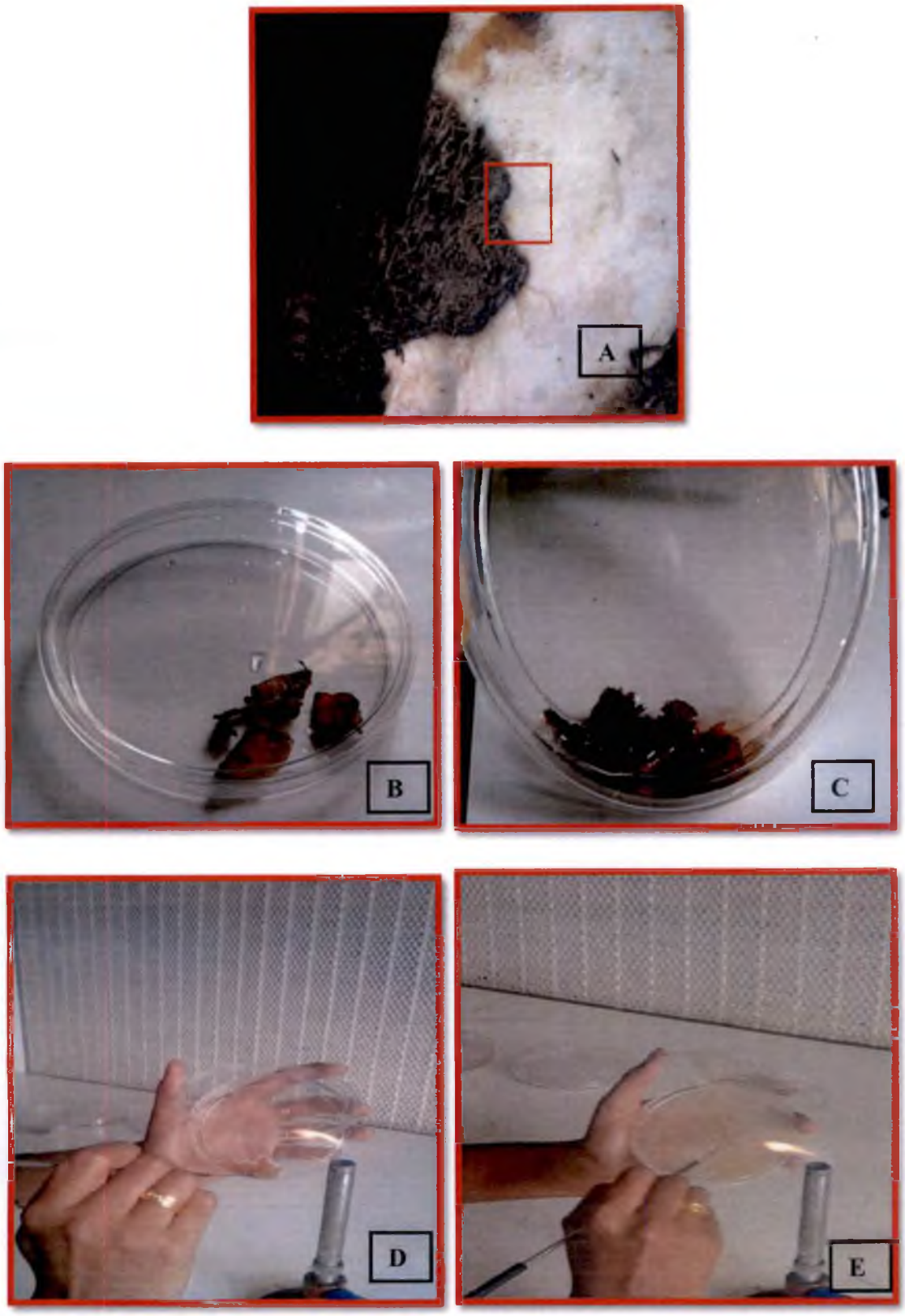


Plate 2. Isolation of pathogen. A-Rhizome bits taken for isolation. B-Surface sterilized rhizome bits. C-Rhizome bits macerated with 1ml sterile distilled water D and E-Loop full of bacterial ooze and streaking in nutrient agar medium.

3.2 PATHOGENICITY STUDIES

3.2.1 Pathogenicity test under *in-vitro* conditions

The healthy rhizome of var. 'Nendran' was cut into three to four cm thick bits and then these bits were surface sterilized with one per cent sodium hypochlorite and washed thrice with sterile distilled water. Using sterile needle pin pricks were made in a circle of 1cm diameter at the center of rhizome bit. A single 48 h old bacterial colony was taken along with the nutrient agar and placed to the pin pricked region. Wet cotton dipped in sterile distilled water was kept over the inoculated region in order to maintain the moisture. Control was also maintained in the similar manner without bacterial culture (Plate. 3).They were incubated in the moisture chamber and observed for development of symptom. The pathogen was re-isolated from the infected rhizome bit and cultured in specific medium.

3.2.2 Pathogenicity test under *in-vivo* conditions

The pathogenicity test was carried out in 'Nendarn' variety of banana. Three months old healthy banana plants with rhizome were taken and washed thoroughly with water for removing the adhering soil particles. The rhizomes were surface sterilized by dipping in one per cent sodium hypochlorite solution for about 30 s followed by dipping in sterile distilled water thrice. Using a sterile stainless steel blade, a piece of one cm^2 was removed at a depth of 1.5 cm from the collar region. A single 48 h old bacterial colony was taken and placed inside the wound and the tissue was re-placed. Moist cotton dipped in sterile distilled water was kept over it in order to maintain the moisture. Control was also maintained in the similar manner by placing nutrient agar without the bacterium (Plate. 4). Inoculated samples along with control were kept in the moist chamber and were observed for development of symptoms. The pathogen was re-isolated from the infected rhizome sample and cultured in specific medium.

The pathogenicity test was also carried out in the pseudostem of two months old tissue culture banana plant var. 'Nendran'. Using sterile needle, pin pricks were made in a circle of 1cm diameter on the pseudostem. A single 48 h old bacterial colony was taken along with the nutrient agar was placed on the wounded area. Control plants were also

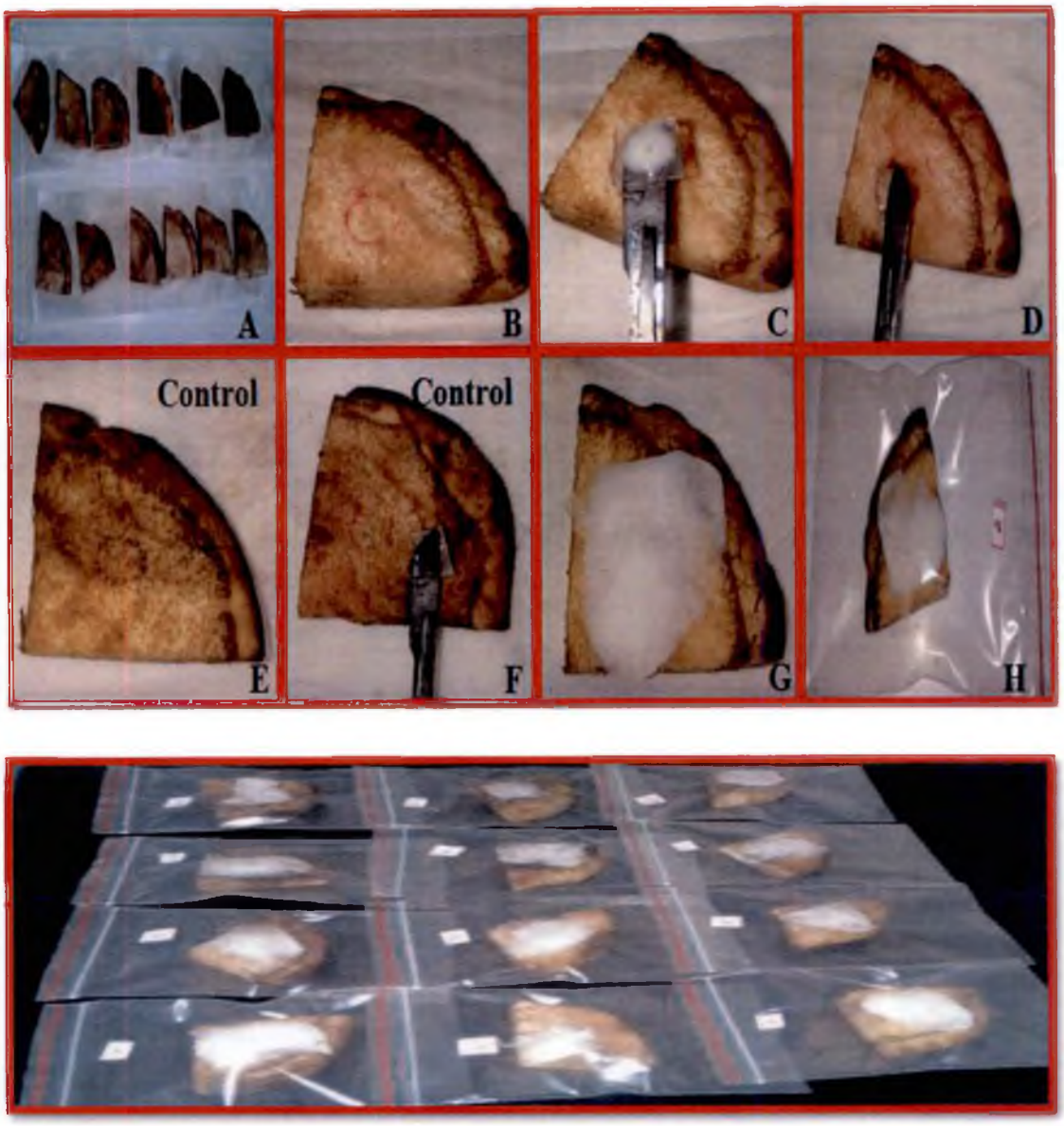


Plate 3. Pathogenicity test-*in-vitro*. A- Surface sterilized rhizome bits. B- Pin pricks made in a circle of 1cm diameter. C, D-Pin pricked portion inoculated with 48 h old bacterial culture. E, F- Control pin pricked portion covered with Nutrient agar only. G, H, I- Inoculated region covered with moist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

Plate 4. Pathogenicity test- *in-vivo* (Rhizome). A and B - Three month old banana plant. C- One cm^2 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, I- Inoculated region covered with moist cotton and incubated by covering with sterile news paper to maintain moisture.

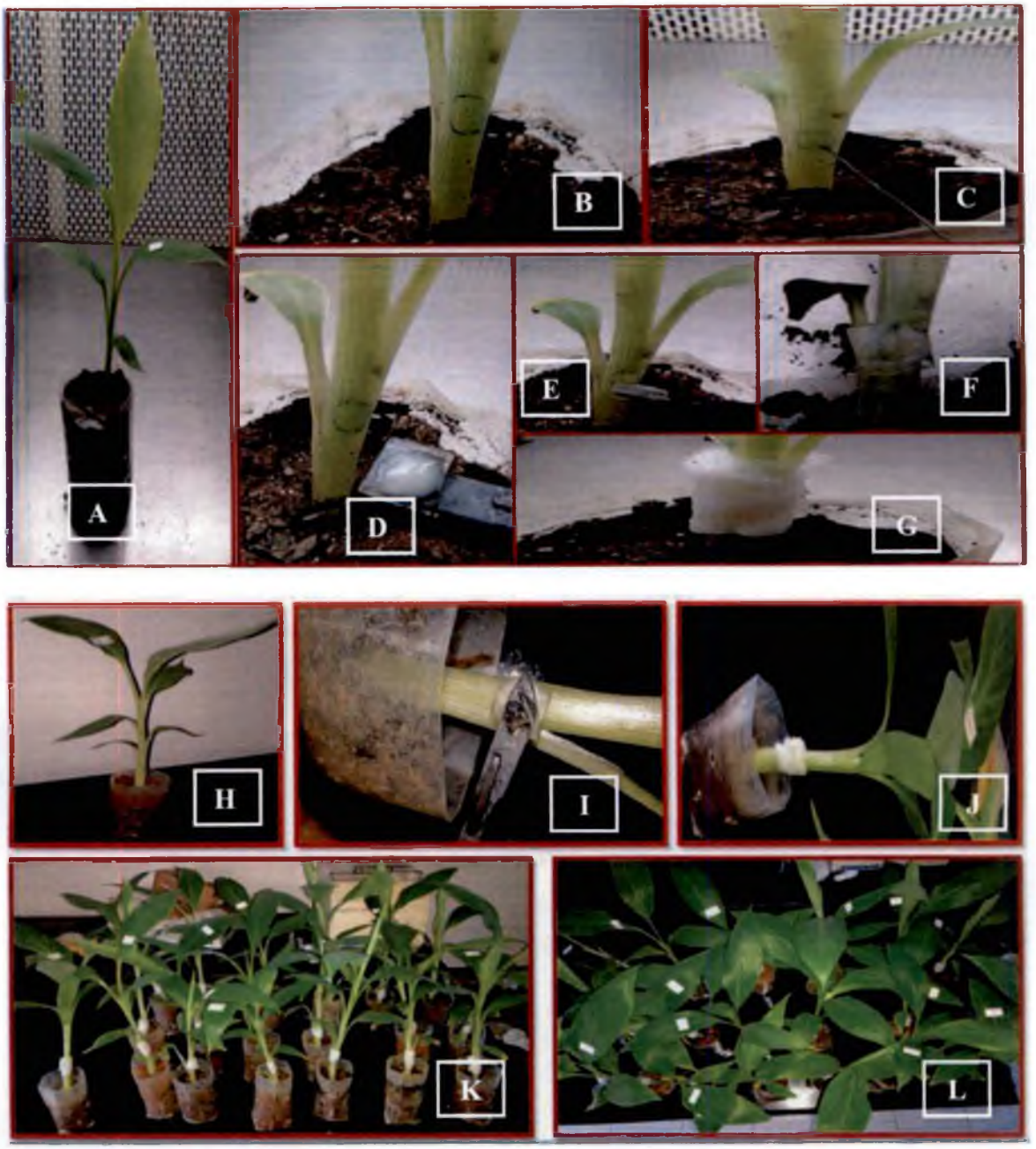


Plate 5. Pathogenicity test *in-vivo* (Pseudostem). A- Two months old TC banana plant. B, C- Pin pricks made in a circle of 1cm diameter on pseudostem. D, E, F- Pin pricked portion inoculated with 48 h old bacterial culture. G- Inoculated portion covered with moist cotton. H, I, J- Control, pin pricked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

maintained in similar manner without bacterial culture. The inoculated portion was covered with moist cotton dipped in sterile distilled water and incubated under moist condition (Plate. 5). The pathogen was re-isolated from the infected pseudostem and cultured in specific medium.

3.3. CHARACTERIZATION AND IDENTIFICATION OF PATHOGEN

3.3.1 Cultural Characterization

The 48 h old bacterial culture was used for cultural characterization studies. The colony morphology and cultural characters were studied by growing the bacterial culture in different media like nutrient agar, yeast extract glucose calcium carbonate (YGC), Logan's medium and nutrient broth.

3.3.1.1 Growth of bacterium in nutrient agar

Nutrient agar is the medium which is highly recommended for culturing wide range of bacteria. The medium was prepared by weighing the required quantity of the various components (Annexure I) and these components except agar were mixed in one litre water. When whole components got dissolved, required quantity of agar was added and boiled till the agar melts completely. The medium was sterilized by autoclaving at 15lbs pressure at 121°C for 25 minutes. After autoclaving the medium was poured on the Petri plates and allowed to solidify, then the medium was streaked with 48h old bacterial culture and the incubated at 28±1°C for 24 to 48h.

The details of characters used for observation are:

Size: Pinpoint, small, moderate and large

Pigmentation: Color of colony

Form: Shape of colony

Circular: unbroken peripheral edge

Irregular: Intended peripheral edge

Rhizoid: Rock like spreading growth

Margin: The appearance of the outer edge of the colony

Entire: Sharply defined, even

Lobate: Marked indentation

Undulate: Wary Indentation

Serrate: Tooth like appearance

Filamentous: Thread like spreading edge

Elevation: The degree to which the colony growth is raised on the agar surface

Flat: Elevation not discernible

Raised: Slightly elevated

Convex: Dome shaped elevation

Unbonate: Raised with elevated, convex central region

Colony nature:

Mucoid

Non mucoid

3.3.1.2 Growth of bacteria in the Yeast Extract Glucose Calcium Carbonate (YGC) agar medium

Yeast Extract Glucose Calcium Carbonate Agar (Annexure I) is a specific medium for the isolation and cultivation of *Erwinia* (*Pectobacterium*) species; which produce excessive acid. A clear zone surrounding each colony results from acid secretion, which liberates the carbonate as CO_2.

The components required to prepare YGC medium except agar were taken, measured and mixed in 1000 mL of distilled water. After the components were completely dissolved in water, agar was put into the medium and boiled till it got melted. Due to presence of calcium carbonate, the prepared medium forms opalescent solution with white precipitate. After it got completely melted it was sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes. After sterilization while the media is hot, it was poured into the Petri plates and allowed to solidify and inoculated with 48 h old bacterial culture. The Petri plates were incubated at 28° C for 24 h to observe the acid and CO_2 production.

3.1.1.3 Growth of bacteria in Logan's medium

Logan's medium (Annexure I) is a differential medium for *Erwinia* (*Pectobacterium*) spp. The composition of media is similar to nutrient agar medium, in addition it contains a dye called 2, 3, 5 tri-phenyl tetrazolium chloride. The components of Logan's medium except agar and dye were taken, measured and mixed in 1000 mL of distilled water. After the components were completely dissolved in water, agar was put into the medium and boiled till it got melted. The medium was sterilized by autoclaving at 15 lbs pressure at 121°C for 25 minutes. After autoclaving the media was poured on the Petri plates and allowed to solidify. 100 µL of 0.5% 2, 3, 5 tri- phenyl tetrazolium chloride dye was spread over the solidified medium in the Petri plate. After that the media was streaked with 48 h old bacterial culture and then incubated at 28±1°C for 48 h. The characters of bacterial colony were observed.

3.3.1.4 Growth of bacteria in the nutrient broth

The composition of nutrient broth is similar to nutrient agar medium except agar (Annexure I). The medium was prepared and autoclaved. 5 mL of sterile nutrient broth was taken in a sterile oak ridge tube and inoculated with a loop full of 48 h old bacterial culture. The control was kept in the similar manner without bacterial inoculation. The inoculated broth along with the control was shaken daily at a temperature of 28±1°C. The optical density was measured after 24, 48 and 72 h at a wave length at 620 nm.

3.3.2 Morphological characterization

The 48 h old bacterial cultures grown in nutrient agar was used to carry out morphological characterization using Gram stain, capsule stain and flagellar stain.

3.3.2.1 Gram staining

Gram staining is a useful tool for identifying and classifying bacteria into two major groups, Gram negative and Gram positive. In the gram staining process, the heat fixed bacterial smear is subjected to four different reagents in the order likewise crystal violet (primary Stain), iodine solution (mordant), alcohol (decolorizing agent) and Safranin (counter stain).

The bacteria which retain primary stain appear dark blue/violet and called Gram positive bacteria whereas those loose crystal violet and get the stain of safranin appear red and referred as Gram negative bacteria.

Materials required:

48 h old bacterial culture, gram staining reagents, wash bottle containing distilled water, droppers, inoculation loop, glass slide, blotting paper, Bunsen burner

Procedure

* 48 h old bacterial culture was used for this experiment

* At first, made a thin smear of bacteria on a sterile glass slide and allowed it to air dry

* When it was air dried, heat fixed using the flame from Bunsen burner

* The smear was then stained with crystal violet for 30 s

* Washed the slide with distilled water for few seconds using wash bottle

* Then flooded the smear with Grams Iodine solution for 60 s

* Washed the slide with 95% alcohol which acted as the decolorizing agent, added drop by drop until no more color flows from the smear

* Washed the slide with distilled water and then drained

* Stained the slide with safranin solution for 30 s

* Then washed the slide with distilled water to remove the excess stain

* Then the slide was air dried; visualized and the size was measured under oil immersion microscope

3.3.2.2 Capsule staining

Capsule staining is a negative staining method in which the background is stained by using an acid stain that carries negative charge on its surface. In negative staining the

capsule appears as a clear zone between cell wall and dark background under phase contrast microscope.

Materials required:

48 h old bacterial culture, Nigrosin/Indian ink, absorbent paper, clear glass slide, isolation loop, cover slip, Bunsen burner

Procedure:

* Made a suspension of bacterial culture in one mL distilled water and incubated the suspension at room temperature for 10-15 min

* A drop of Nigrosin was put close to one end of the clean glass slide

* Added two loops full of broth culture into the drop of stain and mixed it with loop

* Prepared a smear of the mixture using the edge of a second slide at 30° angle and pushed away the other end of the slide.

* Air dried the slide and visualized using phase contrast microscope

3.3.2.3 Falgellar staining

This staining was done to study the presence of flagella which is fine thread like filamentous appendage projecting out from the cell wall which helps in movement.

Materials required

48 h old bacterial culture, flagella mordant (Iodine solution), 0.25% Ziehl's carbol fuchsin solution, clear glass slide, 1mL distilled water blank, dichromate solution, alcohol

Procedure

* Prepared grease free slide by taking the slides and dipped in dichromate solution washed with water and then rinsed with 95% alcohol. Wiped the slide and dried by passing the slide through flame from Bunsen burner and allowed it cool.

* Made a suspension of bacteria in one mL distilled water and incubated the suspension at room temperature for 10-15 min

* Placed two drop full of suspension towards the end of the slide

* Tilted the slide and allowed the broth to form a thin film on glass slide an air dried at room temperature not heat fixed

* Covered the slide with flagella mordent that is iodine solution for 10 min

* Washed the slide gently with distilled water with the help of wash bottle

* Flooded the glass slide with 0.25% Ziehl's carbon fusion for 5 min.

* Then again washed the slide with distilled water with the help of wash bottle

* Air dried the slide at room temperature and visualized the slide using oil immersion microscope

Summary of observations recorded in morphological characterization

* Gram reaction: +ve or –ve

* Size: length and width

* Shape: rod, coccus, spiral

* Arrangement of cells: single, chain

* Presence or absence of capsule

* Presence or absence of flagella: Monotrichous, Peritrichous, Amphitrichous

3.3.3 Biochemical characterization

3.3.3.1 Solubility in three per cent KOH

Sterile three per cent KOH was prepared and from that two drops was taken and placed on the center of a sterile glass slide. A loop full of 48 h old bacterial culture was taken from the nutrient agar medium and placed on it and mixed properly. After 6 to 7 s

the loop was slightly raised and lowered little above the glass slide in order to observe the formation of viscous filament (Plate. 6A).

3.3.3.2 Potato soft rot test

Good and pure potato tubers without any infection was taken, washed with distilled water, peeled and cut into slices. A small piece of 0.5 cm^2 was removed from the centre of the slice using sterile stainless steel blade. Then the slices were surface sterilized by dipping in 30% alcohol and flamed. The sterilized slices were placed in the Petri plate containing sterile distilled water up to half of the slice and then inoculated with 48 h old bacterial culture. A control was maintained similarly without bacterial culture (Plate. 6B). The inoculated plates along with the control were incubated at room temperature for 3-4 days to observe the rotting (Lelliott *et al.*, 1966). The pathogen was re-isolated from the rotted potato slices and cultured in the specific media.

3.3.3.3 Carrot soft rot test

Fresh carrot without any infection was taken and washed with distilled water. After peeling, it was cut into slices of equal thickness. A small piece of 0.5 cm^2 was removed from the centre of the slice using sterile stainless steel blade. Then the slices were surface sterilized by dipping in 30% alcohol and flamed. The sterilized slices were placed in the Petri plate containing sterile distilled water up to half of the slice and then inoculated with 48 hours old bacterial culture. A control was maintained similarly without bacterial culture (Plate: 6C). The inoculated plates along with the control were incubated at room temperature for 8-9 days to observe the rotting (Lelliott *et al.*, 1966). The pathogen was re-isolated from the rotted carrot slices and cultured in the specific media.

3.3.3.4 Intrinsic antibiotic resistance

Nutrient agar containing one per cent dextrose was used to carry out this experiment. Filter paper disc of 1cm diameter were prepared and dipped in 100% alcohol and then air dried to make it sterile. One mL of bacterial suspension was prepared and kept at room temperature for 10 to 15 min. The medium was poured into sterile Petri plates and after solidifying, 0.1 mL of bacterial suspension was added and bacterial lawn

was prepared. Air dried sterile filter paper discs were dipped in 200 ppm streptomycin and erythromycin antibiotic solutions. These filter paper discs containing antibiotic solution were placed above the bacterial lawn in the Petri plate (Plate. 6D to G). After placing the discs, the Petri plates were incubated at room temperature (28±1°C) for 48 h. Observations were recorded on bacterial growth and formation of inhibition zone.

3.3.3.5 Growth at three and four percent NaCl containing medium

Three and four percent NaCl containing peptone water is used as the medium for this experiment. Ten mL of prepared medium, were taken in an oak ridge tube and autoclaved. To the autoclaved medium a single colony of 48 h old bacterial culture was inoculated. A control for three and four per cent NaCl containing peptone water without bacterial inoculation was also maintained. These tubes were incubated at 28±1°C with shaking at 100 rpm for 48 h. The growth of bacteria was checked by measuring the OD value at 620 nm and was compared with control.

3.3.3.6 Pectate degradation

Crystal vioet pectate medium is recommended for cultivation of pectolytic micro-organisms, which can degrade sodium polypectate in the medium. This is the specific medium for *Erwinia* species (Annexure I). As *Erwinia* (*Pectobacterium*) spp. posses pectolytic activity it degrade the sodium polypectate present in the CVP medium and produce characteristic pits where the pectin is degraded.

Procedure

Suspended 24.96 grams of dehydrated medium (Readymade media from Himedia) in 1000 mL distilled water. Placed it on magnetic stirrer with no heat while stirring and ensured that each particle is wetted. When all particles are uniformly wetted in the suspension, then the heater was turned on and brings to almost boiling state with continuing mixing. While the medium is hot, checked the pH and adjusted if necessary with 1M NaOH or 1M HCl (Added drop by drop and not overshooted).Then heated the solution to dissolve the particles in the medium completely. Caped the flask with aluminium foil rather than cotton plug and sterilized the media by autoclaving at 15lbs

pressure at 121°C for 25 min. Avoided foam production and poured directly into the Petri plates as soon as possible while it is hot.

The pectolytic activity of *Erwinia* spp. bacteria was checked by inoculating the 48h old bacterial culture in the Petri plate containing crystal violet pectate medium. The Petri plates were incubated at 37°C for 5 days to observe the pectate degradation.

3.3.3.7 Catalase test

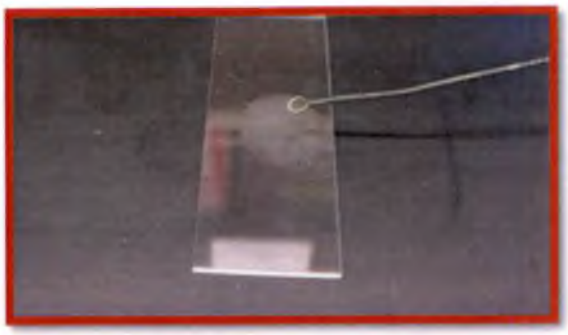
Fourty eight h old bacterial culture was taken and smeared on a sterile glass slide containing one drop of distilled water and the smear was covered with 30V H_2O_2 solution, in order to observe the catalase activity of the bacteria.

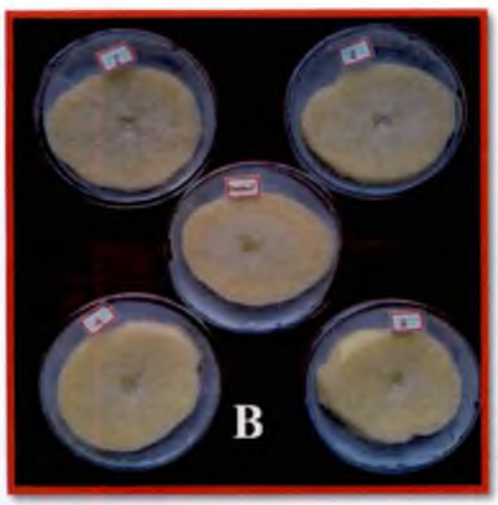
3.3.4 Physiological characterization

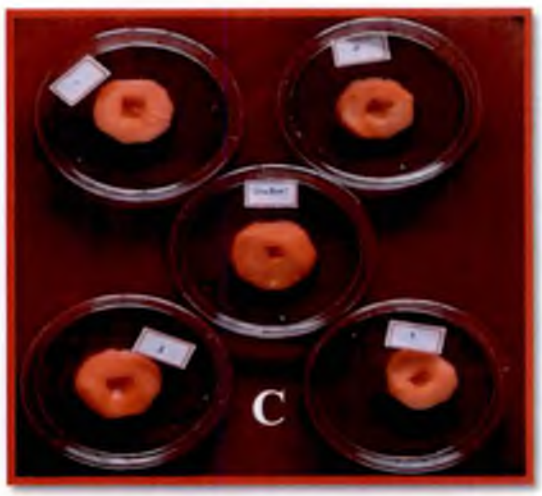
The growth of bacterium under different temperature and pH were studied for physiological characterization.

3.3.4.1 Growth of bacterium at different temperatures

The 48 h old bacterial colony was picked from nutrient agar medium and inoculated into sterile nutrient broth in an oak ridge tube and then incubated at different temperatures such as 27°C, 29°C, 31°C, 33°C, 35°C, 37°C and 39°C with shaking at 120 rpm for 48 h. The control was maintained similarly by keeping nutrient broth without bacterial inoculation. The observations were measured in terms of optical density, at a wavelength of 620 nm after 24 and 48 h.







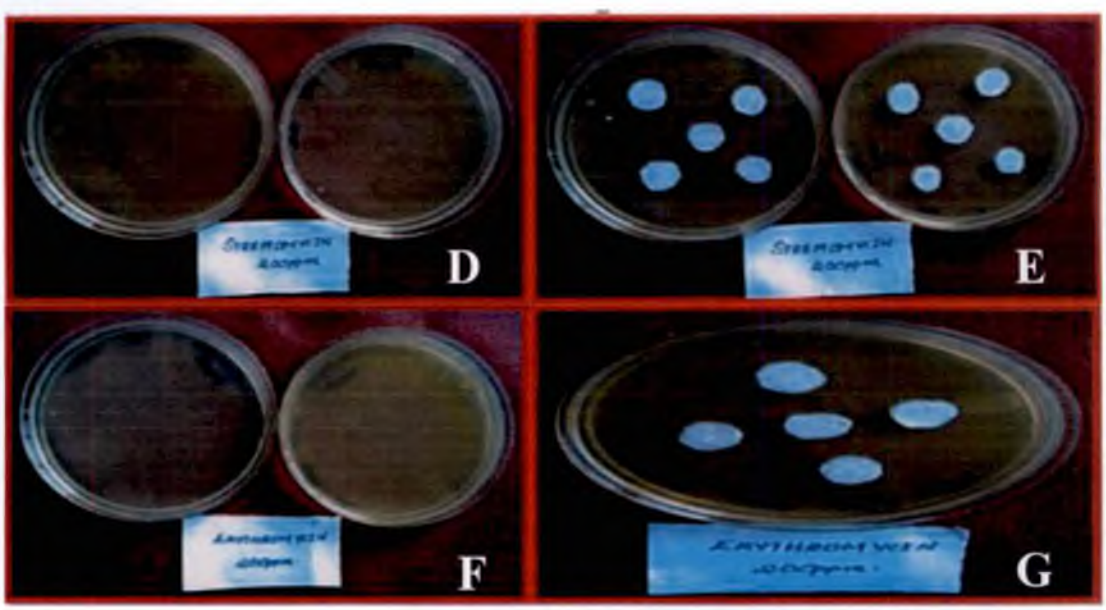


Plate: 6. Biochemical tests. A- Solubility in 3% KOH. B- Potato soft rot test. C- Carrot soft rot test. D- Bacterial lawn prepared to check streptomycin resistance. E- Filter paper discs dipped in 200 ppm streptomycin placed over the bacterial lawn. F- Bacterial lawn prepared to check erythromycin resistance. G- Filter paper disc dipped in 200 ppm erythromycin placed over the bacterial lawn.

3.3.4.2 Growth of bacteria at different pH

The study was conducted to check whether the bacteria can grow under acidic, neutral and alkaline pH conditions. The pH of nutrient broth was adjusted by adding drop by drop of 1M NaOH or 1M HCl. The sterile media at different 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 was also maintained without bacterial inoculation. The inoculated culture as well as the control was maintained at a temperature of 28°C with shaking at 120 rpm for 48 h. The observations were measured in terms of optical density, at a wavelength of 620nm after 24 and 48 h.

3.3.5 Molecular characterization

Molecular characterization is one of the major tool for studying variability and characteristics of pathogen through the various procedures including total genomic DNA isolation and PCR amplification of 16S rRNA gene. Sequencing of the identified loci of various isolates can be used to find the phylogenetic relationship with the other reported species.

3.3.5.1 DNA Isolation

Reagents:

* NaCl buffer:

    i. 150 mM NaCl – 0.876 g in 100 mL

    ii. 100 mM EDTA – 3.72 g in 100 mL

    iii. Tris HCL 10 mM – 0.1576 g in 100 mL

The reagents prepared separately and mixed together to make NaCl buffer and autoclaved before using.

* 10% SDS – 10 g in 100 mL

* Phenol : Chloroform (1:2) – 7 mL + 14 mL

Procedure

i. Fourty eight h old bacterial cultures grown on nutrient agar medium was used. A single colony was picked and inoculated into a conical flask containing 20 mL of autoclaved sterile Luria Bertani (LB) broth. (Annexure I)

ii. This conical flask was kept for overnight incubation at 37°C for 150 rpm in a shaker.

iii. Took 2 mL of culture in an Eppendorf tube and centrifuged at 4°C for 5 min at 10000 rpm

iv. Decanted the supernatant to obtain the pellet which is settled down at the bottom of the tube

v. To this pellet added 100 µL of NaCl buffer and dissolved the pellet. To this solution 250 µL of SDS was added and mixed well. SDS is considered as lysis buffer which breaks the cell well.

vi. This mixture was incubated at 60°C for 15 min in water bath. After the incubation, added equal volume of Phenol : Chloroform solution. Phenol : chloroform solution helps to remove the debris and protein contents.

vii. Then centrifuged it at 12,000 rpm for about 10 min. After the centrifugation three layers were observed. Top aqueous layer contain DNA, an intermediate layer with cell debris and a bottom layer which contain phenol.

viii. The aqueous layer which contains DNA was transferred into a new Eppendorf tube. To this solution equal quantity of ice cold isopropanol was added. This solution was mixed properly by slightly inverting the tube in order to precipitate the DNA.

ix. The reaction mixture was incubated at -20°C for 2 h.

x. After the incubation, the solution was centrifuged at 10,000 rpm for 10 min at 4°C. Pellet of DNA was settled at the bottom. The supernatant was poured without disturbing the pellet.

xi. 20μL of 70% ethanol was added to the pellet. Ethanol helps in removing the salts which are present in the pellet.

xii. Centrifuged the solution at 10,000 rpm for 5 min and discarded the ethanol to obtain the pellet.

xiii. Air dried the pellet and dissolved the DNA pellet in distilled water.

3.3.5.2 Qualitative analysis of DNA using Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method for the detection, separation, identification and qualification of nucleic acid. Agarose is a linear polymer extracted from seaweed, it forms gel by hydrogen bonding. The pore size mainly depends upon the concentration of agarose used. Higher the concentration of agarose lower the will be the pore size. This is a simple, rapid way to perform and capable of resolving DNA fragments of lower concentration that cannot be separated adequately by other procedures.

Procedure

i. Wiped the gel casting tray and comb using 70% ethanol

ii. Prepared the gel casting unit and placed on a horizontal surface and the comb is placed in the gel tray in such a way that it is about 1 inch away from one end of the tray. The position of the comb is vertically, such that the teeth were about 1-2mm above the lower surface of the tray.

iii. 100 mL of 0.8% agarose solution was prepared by taking 0.8g of agarose and dissolved in 100 mL 1X TAE buffer (Annexure III) by boiling using microwave oven.

iv. Allowed the solution to cool at 42-45°C and added two drops of ethidium bromide to the solution and mixed well.

v. The warm gel solution was poured into the already prepared gel casting unit and allowed the gel to solidify for about 30-45 min at room temperature.

vi. Cleaned the electrophoresis unit by wiping with 70% ethanol and the electrophoresis tank is filled with 1X TAE buffer.

vii. When the gel got solidified the comb was removed and the tray was separated from the casting unit and placed in the electrophoresis unit containing buffer in such a way that the wells are towards the cathode.

viii. Before loading the samples into the well, 5µL of sample was mixed with 1µL of loading dye and this mixture was loaded into the consecutive wells leaving first well.

ix. 5µL of λDNA *Eco*R1/*Hind*III double digest ladder was also loaded in the first well.

x. Electrophoresis unit was connected to a power pack and the voltage was adjusted to 80V and pressed the 'Start' or 'Run' button.

xi. Electrophoresis was carried until when the dye has migrated up to two third of the gel, pressed the 'Stop' button and taken the gel from the electrophoresis unit for visualization of bands.

3.3.5.3 Visualization of DNA bands

DNA bands were visualized using UV transilluminator as well as Gel documentation system.

UV transilluminator

* Before placing the gel on the visualization screen it was cleaned by wiping with 70% ethanol.

* Carefully removed the gel from the casting tray and gel was placed on the visualization screen of UV transilluminator by raising the cover.

* After placing the gel on the screen, the lid was lowered and switch on the UV transilluminator for the visualization of DNA bands.

Gel documentation system

Gel documentation was done with Bio-Rad gel documentation XR system using 'Quantity one' software. 'Quantity one' is a software package for imaging and analyzing electrophoresis gels. 'Quantity one' can acquire the images of gel by capturing the images

using the controls in the imaging device window displayed on the computer screen. The scanned image can be used for further analysis.

Procedure

* The loading frame of gel doc XR was cleaned by wiping with 70% ethanol.

* Carefully separated the gel from the tray and placed on the frame

* The gel doc XR camera will be in live/focus mode. Focused the camera by looking the sample in the display window and positioned the sample within the frame.

* Selected the trans-UV illumination mode and adjusted the time of exposure

* Acquired the images by clicking on the exposure after the automatic exposure time had completed. The clear image was displayed on the screen. Then click on the 'freeze' button to stop the UV exposure. It can also be done by manual exposure mode.

* The image of the gel was optimized by using various display controls for adjusting the appearance of the image.

* After analyzing the image selected the 'output' option and then saved the image for further use.

3.3.5.4 Quantification of DNA using spectrophotometer (Nano Drop)

This is a simple and an accurate method for estimating the concentration of the nucleic acid present in a sample. This technique is mainly based on the Beer-lamberts' law in which; when a light beam passes through a medium, some amount of light is absorbed and some amount gets reflected and rest is transmitted through the medium. If the concentration is more, then the absorbance will be more. Observations were made on absorbance at 260 nm (A_{260}), 280 nm (A_{280}), ratio of A_{260}/A_{280} and concentrations of DNA. The nucleic acid shows absorbance peak at 280 nm whereas the protein shows absorbance peak at 260 nm. The ratio of absorbance at 280 nm to 260 nm A_{260}/A_{280} represents the purity of DNA. When the value is 1.8 to 2.0 it represents pure DNA and free from contaminants. When A_{260}/A_{280} value is lower than 1.8, it represents protein

contamination and if the value is above 2.0 it represents RNA contaminations.

Procedure

i. The Nano Drop spectrophotometer was connected to a system containing software ND-1000

ii. The arm and measurement pedestal was cleaned by wiping with sterile tissue paper.

iii. Opened the software ND-1000 and started the option nucleic acid

iv. Before measuring the sample it is necessary to run blank using distilled water

v. Loaded one mL of distilled water to the sampling arm and lowered the pedestal and then clicked the blank option

vi. Reading of the blank was zero, after taking the measurement the pedestal and arm was cleaned with tissue paper.

vii. One mL of sample was loaded to the sampling arm and lowered the pedestal and clicked the 'measure' option

viii. Recorded the readings such as concentration, absorbance at 280 nm and 260 nm and the ratio of A_{260}/A_{280}

ix. When the measurement was complete, opened the sampling arm and wiped the sample from both upper and lower pedestals using tissue paper. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

3.3.5.5 PCR amplification of isolated genomic DNA

The PCR is an *in-vitro* method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strand and flank the region of interest in the target DNA. PCR amplifies a specific segment of DNA that lies between two known primer sequences. Typically PCR results in millions – fold amplification of target DNA.

Thermo cycling – Composition and thermal profile

The PCR was carried out to amplify the 16S rRNA gene using the universal 16S rRNA primer sets fD1 and rP2.

(a) Composition of 25 µL reaction mixture

i. Primer

Forward primer – fD1: 0.1 µL of 10 mM

Reverse primer – rP2: 0.1 µL of 10 mM

ii. dNTP – 1.25 µL of 10mM (contain mixture of dATP, dGTP, dCTP, dTTP)

iii. Template DNA – 2 µL

iv. Taq polymerase – 0.2 µL

v. 10 X Taq buffer – 2.5 µL (with 15mM $MgCl_2$)

vi. Distilled water – 18.85 µL

(b) Standardization of thermo cycling program

Thermo cycling program was carried out as reported by (Kown *et al.*, 1997) and the annealing temperature was standardized by gradient PCR. PCR reaction mixture was prepared using DNA of same samples in 12 tubes and gradient temperature was given. The temperature (T) given was 57°C and the gradient (G) was 9. The first grid had a temperature of 48°C and the last grid had a temperature of 66°C.

(c)The thermo cycle program used to amplify the 16S rRNA gene was

i. Initial denaturation – 94°C for 4 min
ii. Denaturation - 94°C for 1 min
iii. Primer annealing – 64.2°C for 1 min } (35 cycles)
iv. Primer extension - 72°C for 3 min
v. Final extension - 72°C for 10 min
vi. Final storage of sample - 4°C for 5 min

3.3.5.6 Colony PCR using 48 hours old bacterial culture

Other than PCR using genomic DNA, colony PCR was also carried out to amplify the 16S rRNA gene using a pair of universal 16S rRNA primer set 8F and 1522R (Frank *et al.*,2008).

Procedure:

* 48 hours old bacterial colonies cultured in nutrient agar medium was used for carrying out colony PCR

* A single colony was picked with the help of sterile inoculation loop and swirl into 50 µL of sterile distilled water in PCR tubes

* Denatured the sample directly by keeping in PCR machine at 98°C for 2 min and spinned the sample to separate cell debris

* Transferred the supernatant into a new micro centrifuge tube

* Used 2 µL of the supernatant as template in 25 µL of PCR reaction mixture

* Prepared master mix by adding the reagents in the sequential order as shown below in master mix composition

* Mixed the components by spinning

* 23 µL aliquots of the master mix were added to each PCR tube containing 2 µL of DNA template which is the supernatant after denaturation of the bacterial colony suspension

* Immediately PCR tubes were kept in PCR machine and set the PCR program and later run the PCR

Thermo cycling – composition and thermal profile

The PCR was carried out to amplify the 16S rRNA gene using the universal 16S rRNA primer sets 8F and 1522R.

(a) Composition of 25 µL reaction mixture:

Master Mix:

i. Primers

Forward primer – 8F: 0.1 µL of 10 mM

Reverse primer – 1522R: 0.1 µL of 10 mM

ii. dNTP mix –1 µL of 10mM

iii. 10 X Taq buffer – 2.5 µL (with 15 mM $MgCl_2$)

iv. Taq DNA polymerase – 0.4 µL

v. Sterile water – 18.9 µL

Total volume = 23 µL

vi. Template DNA = 2 µL

Total volume of reaction mixture = 25 µL

(b) Thermo cycle program used for colony PCR is as follows

i. Initial denaturation - 95°C for 3 min

ii. Denaturation - 94°C for 90 s
iii. Primer annealing - 55°C for 40 s } (35cycles)
iv. Primer extension - 72°C for 90 s

v. Final extension - 72°C for 20 min

vi. Final storage of sample - 4°C for 10 min

Details of primers used in PCR amplification were given in Annexure IV

3.3.5.7 Qualitative analysis of PCR products using Agarose gel electrophoresis

Procedure

i. Wiped the gel casting tray and comb using 70% ethanol

ii. Prepared the gel casting unit and placed on a horizontal surface and the comb was placed in the gel tray in such a way that it is about one inch away from one end of the tray and the comb was positioned vertically in such a way that, the teeth are about 1-2 mm above the surface of the tray.

iii. 100 mL of 2% agarose solution was prepared by taking 2 g of agarose and dissolved in 100 mL 1X TAE buffer by boiling using microwave oven.

iv. Allowed the solution to cool at 42-45°C and added two drops of Ethidium bromide to the solution and mixed well.

v. The warm gel solution was poured into the already prepared gel casting unit and allowed the gel to solidify for about 30-45 min at room temperature.

vi. Cleaned the electrophoresis unit by wiping with 70% ethanol and the electrophoresis tank was filled with 1X TAE buffer.

vii. When the gel got solidified, the comb was removed and the tray was separated from the casting unit and placed in the electrophoresis unit containing buffer in such a way that the wells are towards the cathode.

viii. Before loading the samples into the well, 10 µL of the PCR product was mixed with 1 µL of loading dye and this mixture was loaded into the consecutive wells leaving first well.

ix. 5 µL of 1 Kbp plus ladder was also loaded in the first well.

x. Electrophoresis unit was connected to a power pack and the voltage was adjusted to 80V and pressed the 'Start' or 'Run' button.

xi. Electrophoresis was carried until when the dye has migrated up to two third of the gel, pressed the 'Stop' button and taken the gel from the electrophoresis unit for visualization of bands.

3.3.5.8 Visualization of PCR products

DNA bands were visualized using UV transilluminator as well as Gel documentation system.

UV transilluminator

* Before placing the gel on the visualization screen, it was cleaned by wiping with 70% ethanol.

* Carefully removed the gel from the casting tray and gel was placed on the visualization screen of UV transilluminator by raising the cover.

* After placing the gel on the screen, the lid was lowered and switch on the UV transilluminator for the visualization of bands

Gel documentation system

Gel documentation was done with Bio-Rad gel documentation XR system using 'Quantity one' software. 'Quantity one' is a software package for imaging, analyzing and data basing electrophoresis gels. 'Quantity one' can acquire the images of gel by capturing the images using the controls in the imaging device window displayed on the computer screen. The scanned image can be used for further analysis.

Procedure:

* The loading frame of gel doc XR was be cleaned by wiping with 70% ethanol.

* Carefully separated the gel from the tray and placed on the frame

* The gel doc XR camera will be in live/focus mode. Focused the camera by looking the sample in the display window and positioned the sample within the frame.

* Selected the trans-UV illumination mode and adjusted the time of exposure

* Acquired the images by clicking on the exposure after the automatic exposure time had completed. The clear image gets displayed on the screen. Then click on the 'freeze' button to stop the UV exposure. It can also be done by manual exposure mode.

* The image of the gel was optimized by using various display controls for adjusting the appearance of the image.

* After analyzing the image selected the 'output' option and then saved the image for further use.

3.3.5.9 Grouping of bacterial isolates

Based on the results of cultural, morphological, biochemical and physiological character, the 18 bacterial isolates were clarified manually and by the help of dendrogram.

3.3.5.10 Sequencing of PCR products

Using two sets of universal 16S rRNA primers, the DNA samples of 18 bacterial isolates were amplified and amplicons were sequenced by an outsourcing agency (Sci. Genome Lab Pvt. Ltd, Kochi). One representative sample of each group was used for sequencing.

3.4. DATA ANALYSIS:-

3.4.1 Merging the forward and reverse sequence

The sequences obtained in the 'FASTA' format after sequencing were analyzed, the reverse complement of the respective reverse sequences were generated by using the software 'Reverse complement' and the forward sequence and the reverse complement were merged using 'merger' tool in EMBOSS GUI and the 'FASTA' sequences of respective samples were generated for further analysis.

3.4.2 Homology analysis of PCR product sequences

In order to find the homology of the sequences, BLASTn (especially for nucleotide sequences) of Basic Local Alignment Search Tool (BLAST) software of NCBI was used. The organism (*Erwinia carotovora/Pectobacterium carotovorum*) specific BLAST was carried out by using already prepared reverse FASTA sequences of the samples for homology analysis. The FASTA sequence of each sample acted as the query sequence. The query sequence was compared with the already existing sequence in the database and the most sequence resembling with the query sequence were identified.

3.4.3 Phylogenetic analysis

Phylogenetic analysis of 16S rRNA sequence of the samples was done. 'ClustalW' tool was used to identify barcode gaps and MEGA6 software was used for the phylogenetic analysis. Phylogenetic tree was constructed using 'Phylogeny' tool of MEGA6 software.

3.4.5 Sequence submission to GenBank (NCBI database)

Submission tool 'BankIt' was used for the deposition of nucleotide sequence.

Procedure:

- Accessed the NCBI website containing MyNCBI page and logged in to the account by providing username and password at http://www.ncbi.nlm.nih.gov/guide/howto/submit-sequence-data/website.
- The 'fasta' sequence of 16S rDNA was submitted through BankIt.
- The additional information like date of public release, basic information about author and working title, name of organism and other necessary information were also provided to generate the accession numbers from NCBI.

Results

4. RESULTS

4.1 COLLECTION OF SAMPLE AND ISOLATION OF PATHOGEN

4.1.1 Collection of diseased samples

The rhizome rot infected samples were collected from banana fields of 14 different locations in the districts, Thrissur, Palakkad, Ernakulam, Kollam and Trivandrum of Kerala and also from four different locations in the districts Kanyakumari, Madurai and Tuticorin of Tamilnadu. The details of sample collection were presented in Table. 1.

Table: 1. Description of sample collection

Sample No.	Date of collection	District	Location	Isolate no.
1	02-04-2016	Trivandrum	Kazhakuttam	1_{TrKz}
2	05-04-2016	Trivandrum	Neyyatinkara	2_{TrNy}
3	05-04-2016	Trivandrum	Nedumangad	3_{TrNd}
4	10-04-2016	Kollam	Adoor	4_{KoAo}
5	10-04-2016	Kollam	Paravoor	5_{KoPr}
6	03-05-2016	Palakkad	Karimba	6_{PaKr}
7	03-05-2016	Palakkad	Kalladikod	7_{PaKl}
8	19-05-2016	Palakkad	Mannarkad	8_{PaMn}
9	25-05-2016	Thrissur	Mannuthy	9_{ThMn}
10	30-05-2016	Thrissur	Koratti	10_{ThKr}
11	21-07-2016	Eranakulam	Kalamassery	11_{ErKl}
12	06-08-2016	Thrissur	Mala	12_{ThMl}
13	21-09-2016	Thrissur	Kannara	13_{ThKn}
14	21-09-2016	Thrissur	Achankunnu	14_{ThAh}
15	24-09-2016	Kanyakumari	Nagercoil	15_{KaNg}
16	24-09-2016	Tuticorin	Tuticorin	16_{TuTt}
17	27-09-2016	Madurai	Madurai	17_{MaMd}
18	27-09-2016	Kanyakumari	Thakkala	18_{KaTa}

4.1.2 Isolation of the pathogen

The isolation of pathogen from the infected rhizome samples on the nutrient agar medium yielded small bacterial colonies which were cream to yellowish colour, slightly raised and mucoid. Single isolated colonies were picked and sub cultured to obtain bacterial pure culture.

Further inoculation of 48 h old bacterial culture on crystal violet pectate (CVP) medium, produced pits characteristic of *Erwinia/Pectobacterium* spp. The isolate no. for each sample was given by using abbreviations of location and districts (Table. 1; Plate. 7).

4.2 PATHOGENICITY STUDIES

4.2.1 Pathogenicity test under *in-vitro* condition

The rhizome bits of the 'Nendran' variety which were inoculated with bacterial colonies and incubated under laboratory conditions produced rotting symptoms after 8 days of inoculation. The characteristic symptoms observed were discolouration and rotting. The inoculated area became sunken, water soaked with dark brown in colour and emitted unpleasant foul smell. No rotting symptoms were observed in the control.

The pathogen was re-isolated from the samples and the bacterial colonies were exactly similar to the original isolates. Culturing of the bacteria on the *Erwinia* spp. specific CVP medium produced characteristic pits (Plate. 8).

4.2.1 Pathogenicity test under *in-vivo* condition

The pathogenicity test carried out in three months old 'Nendran' variety banana by inoculating with bacterial culture at collar region produced rotting of rhizome after 37 days of inoculation. The bacterial infections progressed from the collar region to the rhizome and caused softening and rotting of the tissue. When the rhizomes were cut opened, the tissues were dark brown, water soaked and emitted foul smell. No symptoms of rotting were observed in the control. Variation was observed between isolates in the extent of rhizome rotting (Plate. 9). The pathogen was re-isolated and confirmed by

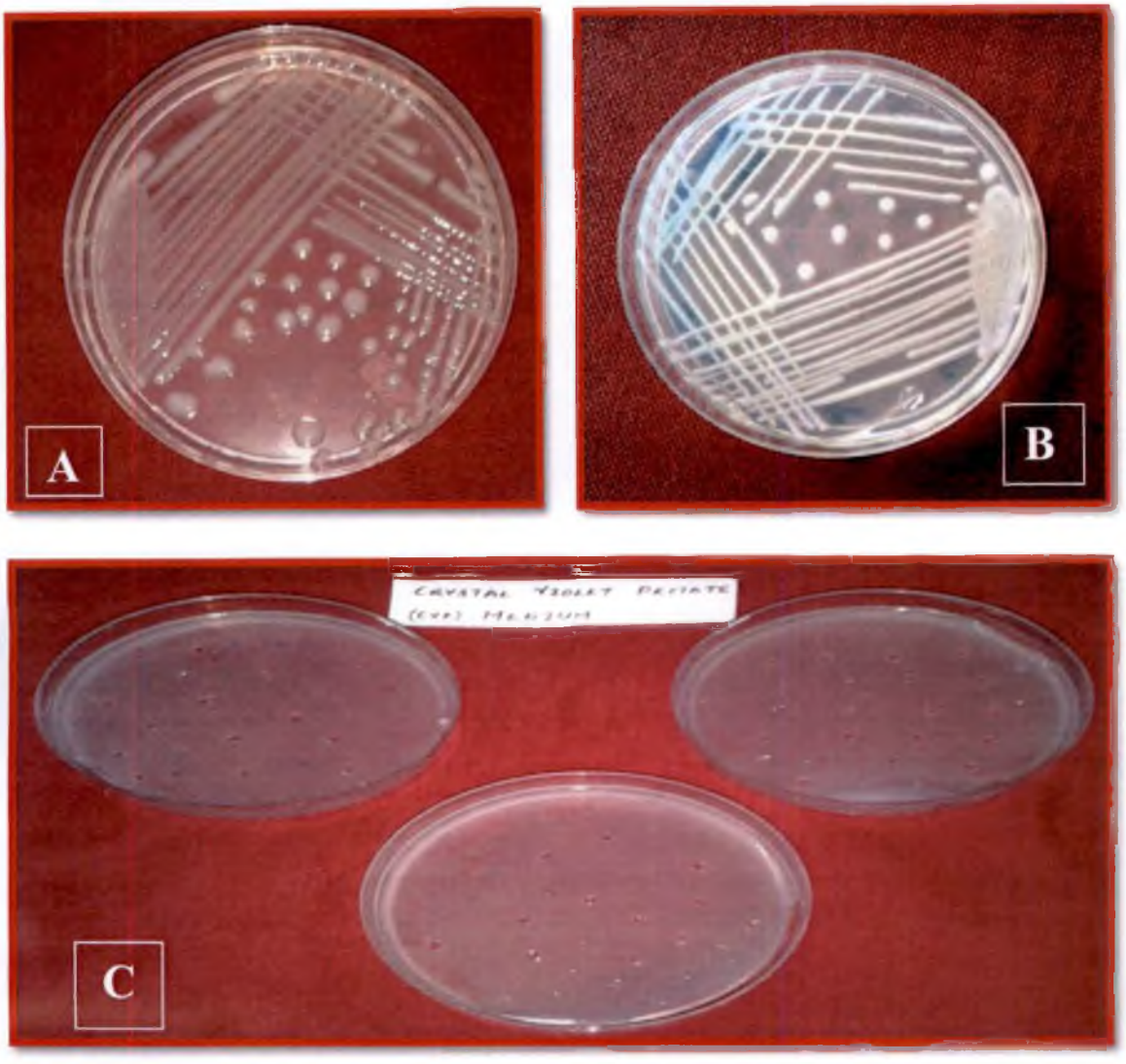
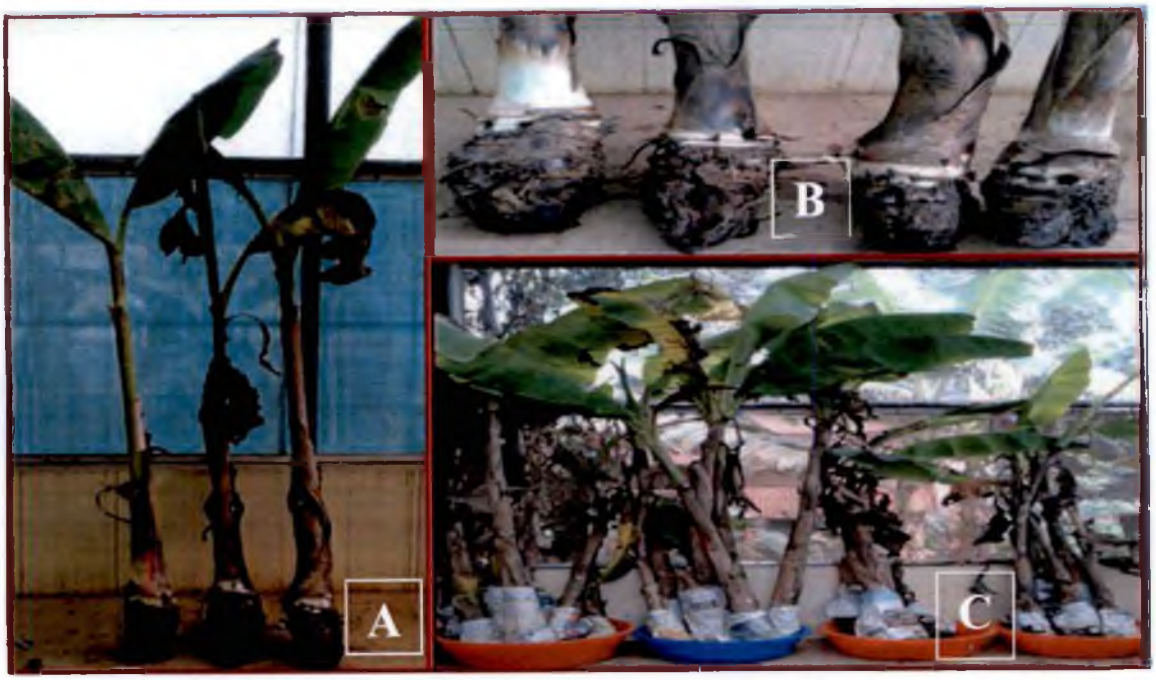


Plate: 7. Pathogen isolated in nutrient agar and crystal violet pectate medium. A, B- Bacterial colonies on nutrient agar medium. C- Fissures formed in crystal violet pectate medium.

Plate: 8. Proving of pathogenicity *in-vitro*. A, B- Rotted rhizome bits along with control. C-Re-isolated bacterial colonies in nutrient agar medium. D, E- Fissures formed in crystal violet pectate medium by re-isolated culture.



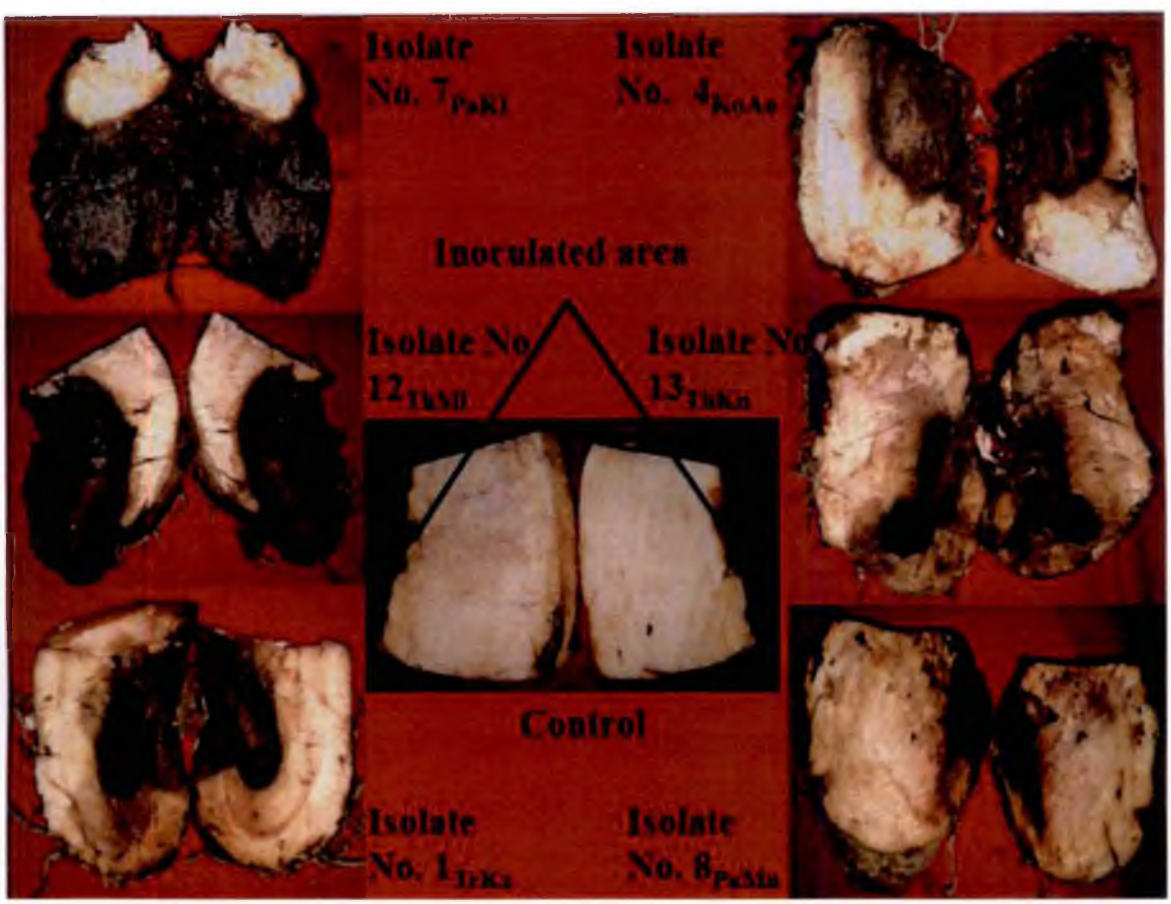


Plate: 9. Proving of pathogenicity *in-vivo* (Rhizome). A, B, C- Symptoms observed after two weeks of inoculation. D- Rotted rhizomes along with control after 37 days of inoculation

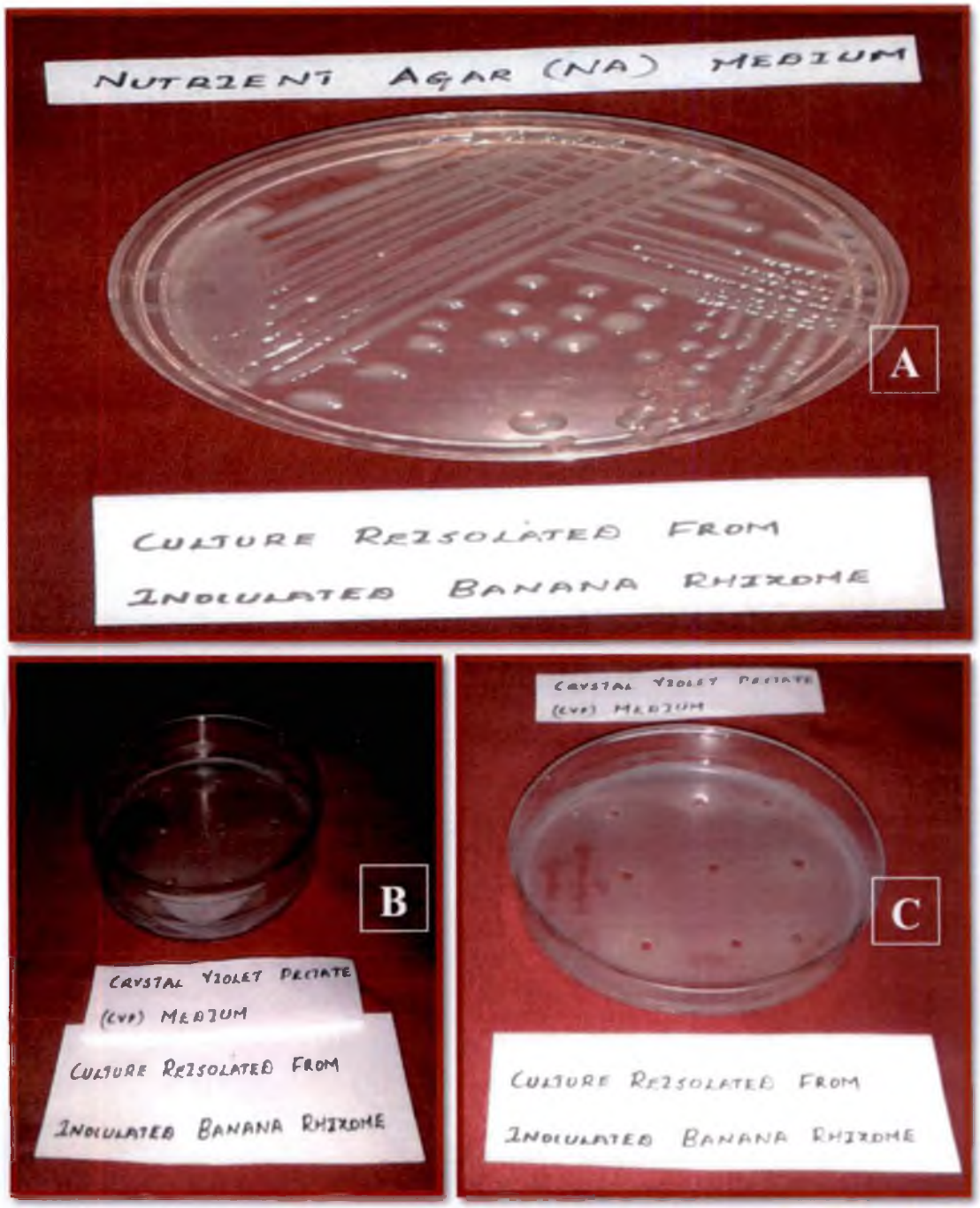


Plate: 10. Re-isolation of bacteria from infected rhizome. A- Re-isolated bacterial colonies on nutrient agar medium from infected rhizome. B and C- Fissures formed in crystal violet pectate medium by re-isolated culture.

culturing in the CVP medium produced characteristic pits similar to the original isolates (Plate. 10).

Pathogenicity test in the pseudostem of two months old tissue culture plants produced characteristic symptoms after six days of inoculation. The initial symptoms observed were yellowing of the lower leaves and later progressed to upper leaves. The pseudostem was weakened and was completely rotted and the plants fell down by 15 days with emission of foul smell. There was no symptom in the control (Plate. 11). The pathogen was re-isolated and confirmed by culturing in the CVP medium. Culturing of the bacteria on the *Erwinia* spp. specific CVP medium produced characteristic pits similar to the original isolates (Plate. 12).

Variation was observed in the time taken for the rotting of pseudostem by different isolates. The minimum time taken for rotting was 7 days by isolate no. 3_{TrNd}, 7_{PaKl} and 17_{MaMd} followed by 10 days by isolate no.5_{KoPr}, 12_{ThMl} and 14_{ThAh}. The maximum time for rotting was 15 days by isolate no.2_{TrNy} and 8_{PaMn}, followed by 13 days by isolate no. 10_{ThKr}, 13_{ThKn} and 18_{KaTa} (Table. 2).

4.3 CHARACTERISATION AND IDENTIFICATION OF PATHOGEN

4.3.1 Cultural characterization

4.3.1.1 Growth of bacteria in nutrient agar

After 48 h of incubation at 28±1°C, bacterial growth was observed in the inoculated Petri plates. The colour and size of the bacterial colonies showed variation between isolates. The observations were recorded and given in the Table. 3 and in the Plate. 13. The characteristics of the colony observed were recorded and given below:

- Size: small, colonies with 2.3-2.5 mm diameter
- Pigmentation: Cream, creamy yellow, slightly yellowish
- Form: circular
- Margin: Entire
- Elevation: Slightly raised, convex
- Colony nature: mucoid

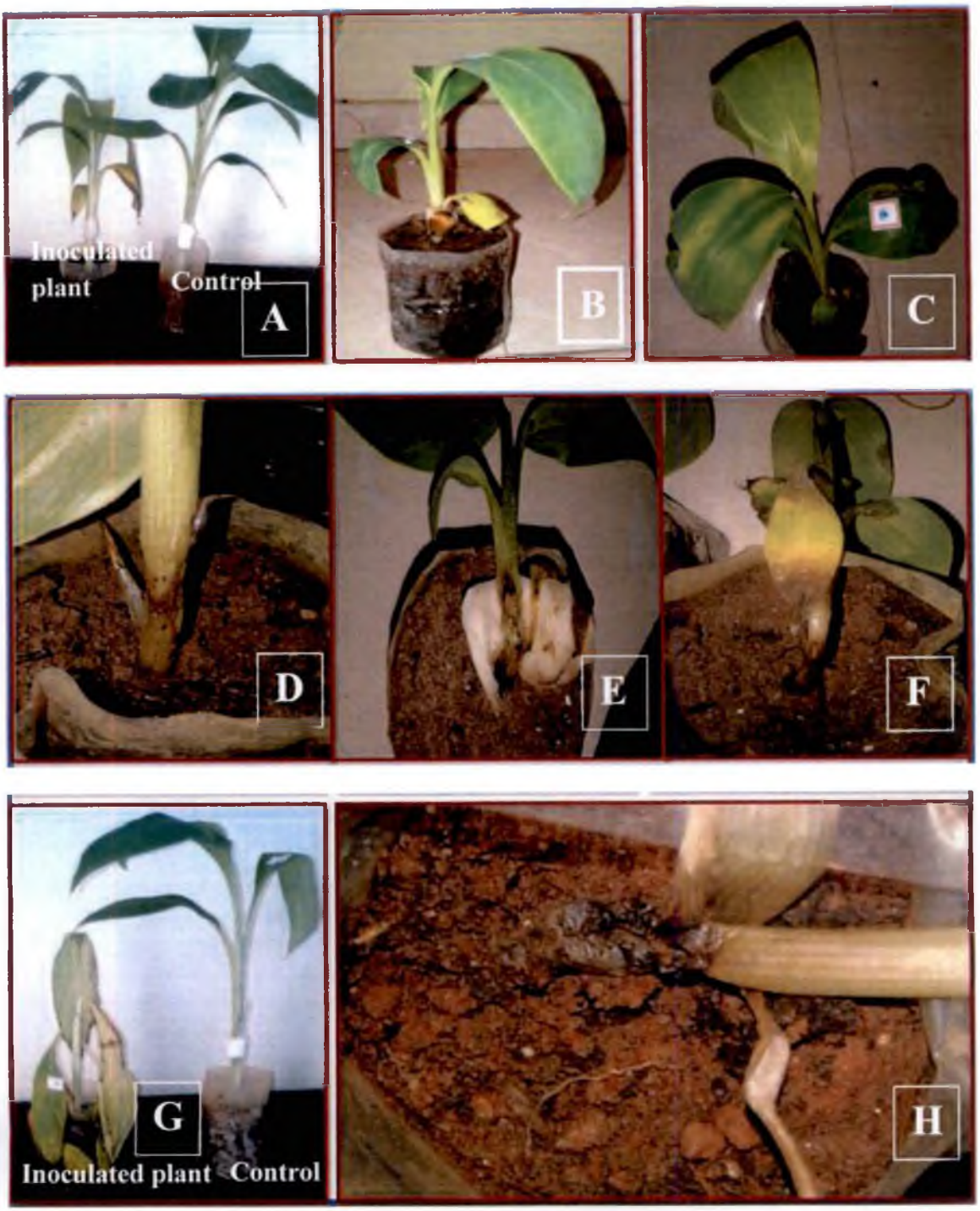


Plate: 11. Proving of pathogenicity *in-vivo* (Pseudostem). A- Initial symptoms observed after one week of inoculation along with. B and C- Yellowing of the leaves progressed to upper leaves. D, E and F- Stages of rotting in the pseudostem. G-Completely wilted plant along. H- Completely rotted pseudostem

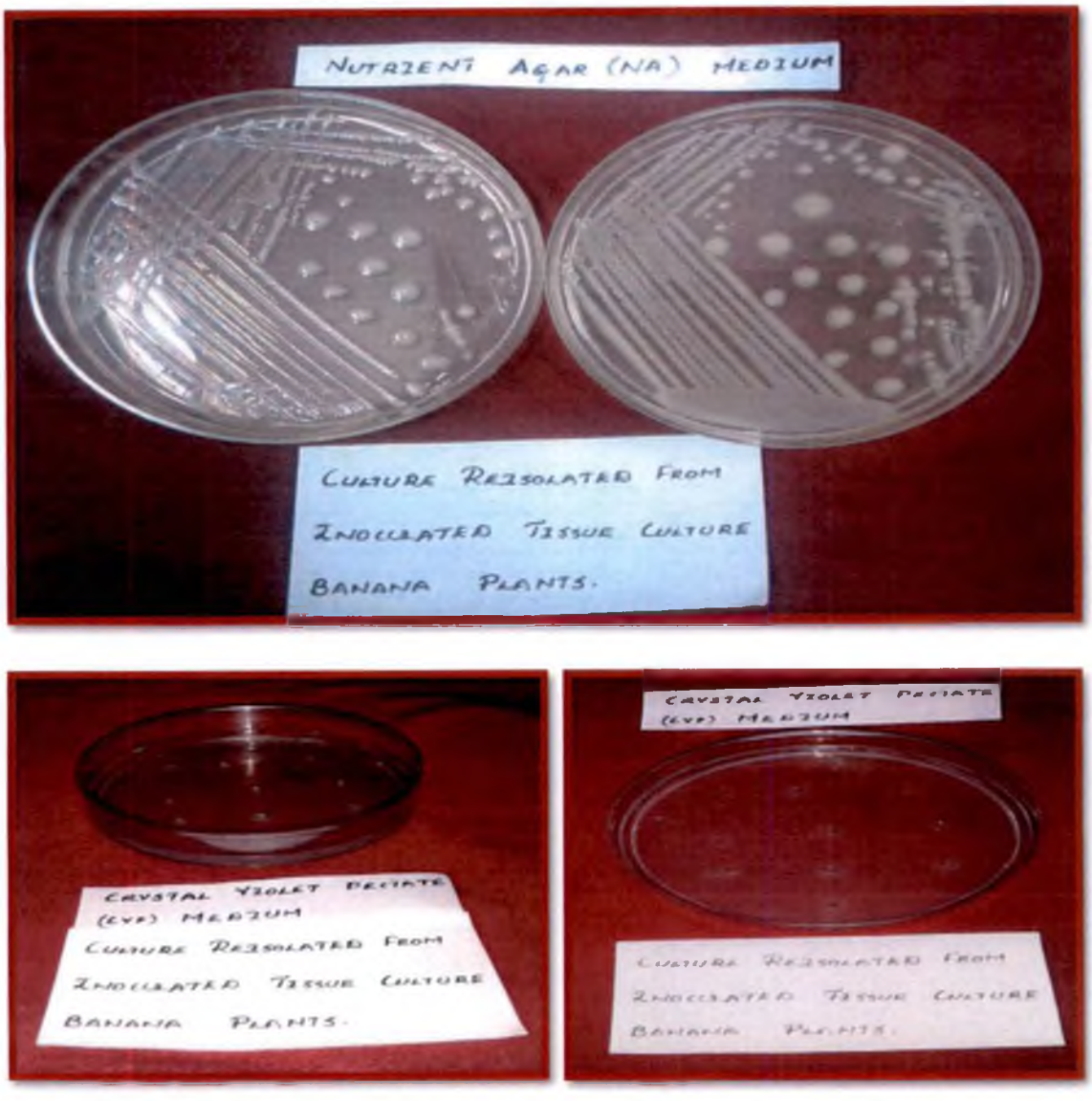


Plate: 12. Re-isolation of bacteria from infected pseudostem. A- Re-isolated bacterial colonies in nutrient agar medium from infected TC plants. B, C- Fissures formed in crystal violet pectate medium by re-isolated culture.

Table: 2. Time for development of symptoms in tissue culture 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

Plate: 13. Bacterial colonies in nutrient agar medium

Table: 3. Size and colour of bacterial colonies of different isolates

Isolate No.	Diameters (mm) of bacterial colony after 48 h	Colour
1_{TrKz}	2.4	Cream
2_{TrNy}	2.3	Light Yellow
3_{TrNd}	2.5	Creamy Yellow
4_{KoAo}	2.4	Light Yellow
5_{KoPr}	2.4	Creamy Yellow
6_{PaKr}	2.5	Cream
7_{PaKl}	2.3	Creamy Yellow
8_{PaMn}	2.3	Light Yellow
9_{ThMn}	2.4	Light Yellow
10_{ThKr}	2.3	Cream
11_{ErKl}	2.4	Creamy Yellow
12_{ThMl}	2.5	Creamy Yellow
13_{ThKn}	2.4	Cream
14_{ThAh}	2.5	Creamy Yellow
15_{KaNg}	2.5	Cream
16_{TuTt}	2.3	Light Yellow
17_{MaMd}	2.5	Creamy Yellow
18_{KaTa}	2.4	Cream

4.3.1.2 Growth of bacteria in Yeast extract Glucose Calcium Carbonate (YGC) medium

The bacteria inoculated YGC medium produced cream to yellow coloured semi mucoid colonies with a clear zone around each colony after 24h of incubation. Small bubbles were observed on the top of each bacterial colony due to the emission of CO_2 from the YGC medium which is the characteristic feature of *Erwinia carotovora/Pectobacterium carotovorum* (Plate. 14A).

4.3.1.3 Growth of bacteria in Logan's differential medium

The bacteria inoculated Logan's medium produced small to medium colonies of 1.6 to 2mm diameter with purple colour having entire margin after 24 h of incubation at 28±1°C, The colonies were slightly raised and mucoid in nature. The pathogen was identified as *Erwinia carotovora/Pectobacterium carotovorum* by observing the purple colour of colonies (Plate. 14B).

4.3.1.4 Growth of bacteria in nutrient broth

The bacterial growth was observed and was recorded in terms of OD value The maximum OD value observed was 0.840 whereas the minimum OD value recorded was 0.555 after 72 h of incubation.

Variation was observed in the OD values of isolates. The maximum OD value was observed by the isolate no.17_{MaMd} (0.840), followed by isolate no.3_{TrNd} (0.838) and the minimum OD value was recorded by the isolate no.2_{TrNy} (0.555), followed by isolate no. 8_{PaMn}(0.558) after 72 h of incubation (Table. 4).

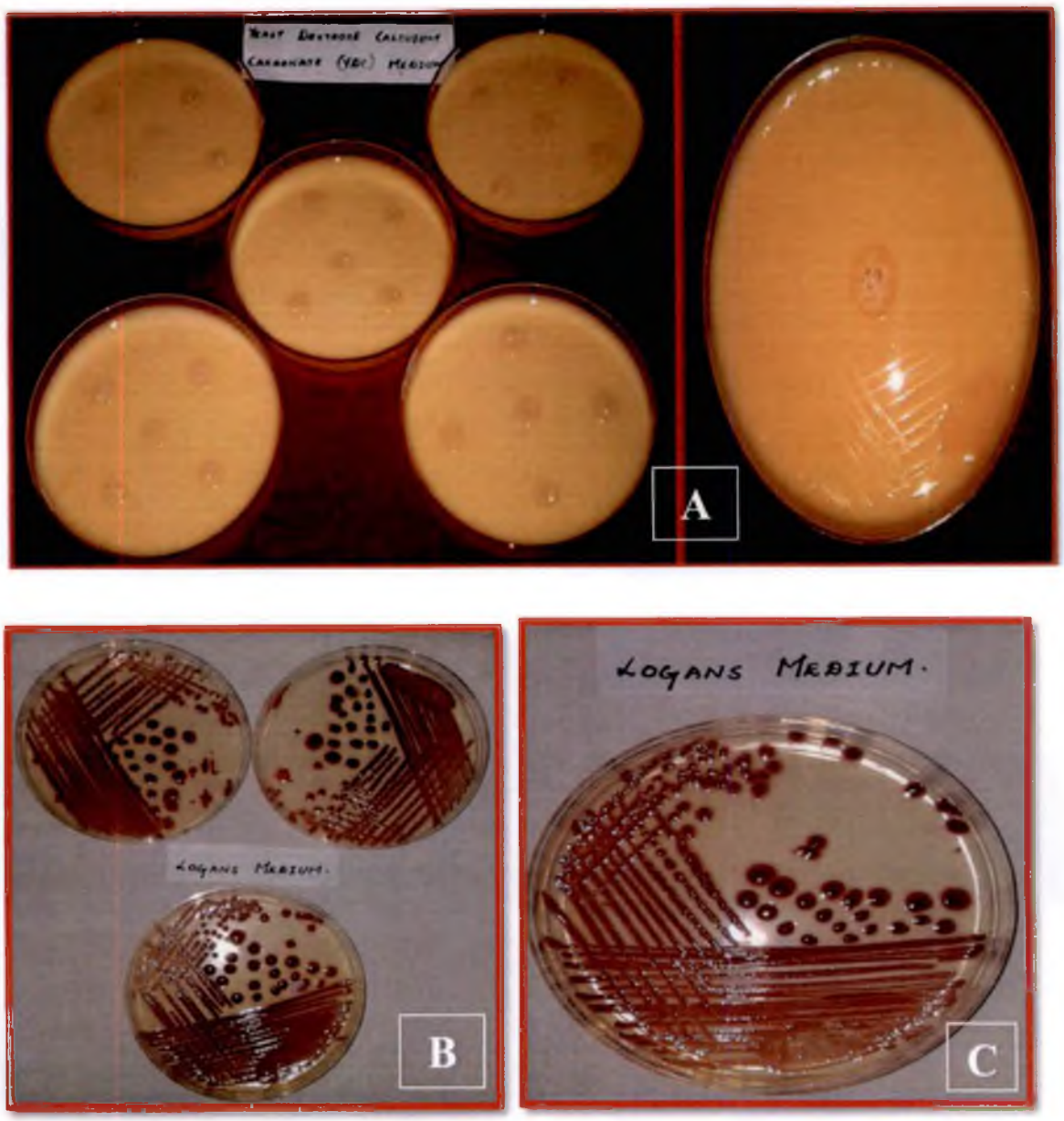


Plate: 14. Bacterial colonies in yeast extract glucose calcium carbonate and Logan's medium. A-Acid secretion and CO_2 release in YGC medium. B, C- Purple colour colonies in Logan's medium

Table: 4. Growth of bacterial isolates in nutrient broth

Isolate No.	OD values		
	After 24 h	After 48 h	After 72 h
1_{TrKz}	0.712	0.720	0.731
2_{TrNy}	0.542	0.548	0.555
3_{TrNd}	0.819	0.826	0.838
4_{KoAo}	0.641	0.648	0.656
5_{KoPr}	0.618	0.624	0.635
6_{PaKr}	0.704	0.717	0.725
7_{PaKl}	0.809	0.813	0.827
8_{PaMn}	0.540	0.550	0.558
9_{ThMn}	0.643	0.651	0.659
10_{ThKr}	0.572	0.582	0.590
11_{ErKl}	0.815	0.821	0.833
12_{ThMl}	0.622	0.629	0.638
13_{ThKn}	0.557	0.569	0.578
14_{ThAh}	0.626	0.633	0.742
15_{KaNg}	0.710	0.719	0.728
16_{TuTt}	0.638	0.646	0.653
17_{MaMd}	0.821	0.832	0.840
18_{KaTa}	0.568	0.577	0.587
Control	0.038	0.062	0.087

4.3.2 Morphological characterization

4.3.2.1 Gram staining:

After the completion of gram staining test, the bacteria lost the colour of crystal violet (primary stain) and attained the colour of safranin (counter stain) and appeared red coloured short rods when observed under oil immersion microscope indicating the Gram negative nature. The width of the bacterium was in the range of 0.57-1.08 µm and length in the range of 0.95-3.06 µm.

Significant variation in size was observed between isolates. The maximum length (3.10 µm) recorded was in by isolate no. 7_{PaKl} which was on par with isolates viz., 3_{ErKl}, 3_{TrNd}, 17_{MaMd}. The minimum length (0.95 µm) was recorded in isolate no. 2_{TrNy} which was on par with 8_{PaMn}. The maximum width (1.10 µm) was recorded in isolate no.17_{MaMd} which was on par with isolate no. 3_{TrNd}. The minimum width was recorded in isolate no. 2_{TrNy} which was on par with 8_{PaMn}. (Plate. 15, Table. 5)

4.3.2.2 Capsule staining:

The negative staining method used for capsule staining didn't produce a clear zone between the cell wall and the background when observed under phase contrast microscope. This indicated that the bacteria is non-capsular in nature (Plate: 16).

4.3.2.3 Flagellar staining:

After the flagellar staining, by using various reagents, the slide was observed under oil immersion microscope. Flagella were present around the bacterial rods (peritrichous flagella), indicating the motile nature of bacteria (Plate: 16).

Summary of parameters observed in morphological characterization

* Gram reaction: Gram negative

* Size: 0.57-1.08 µm in width, 0.95-3.06µm in length

* Shape: rod shape

* Arrangement of cells: single as well as in chains

* Presence or absence of capsule: non-capsular

* Flagella: Peritrichous

Table: 5. Size of different 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

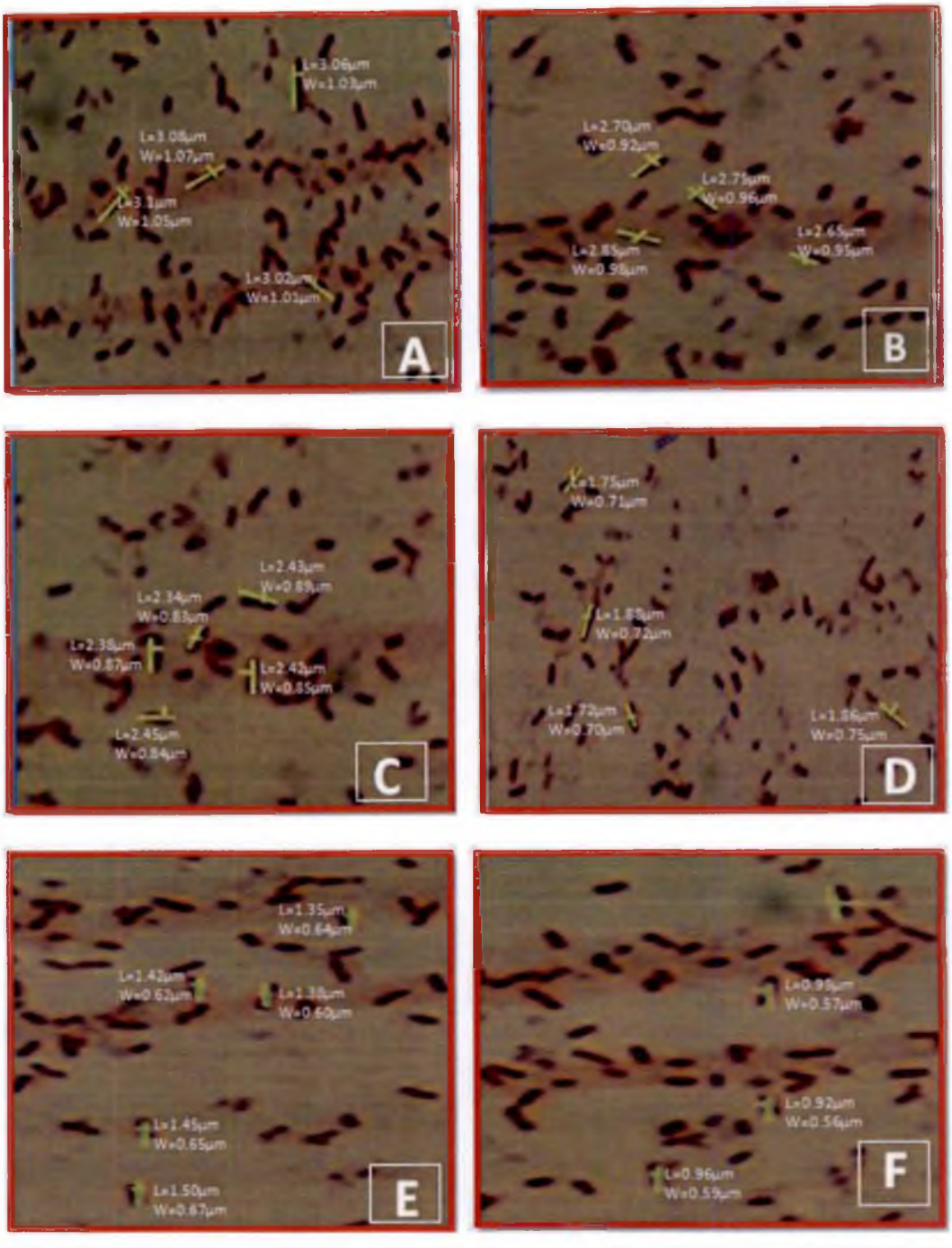


Plate: 15. Gram staining. A, B, C, D, E and F- Red coloured bacterial rods with varying sizes.

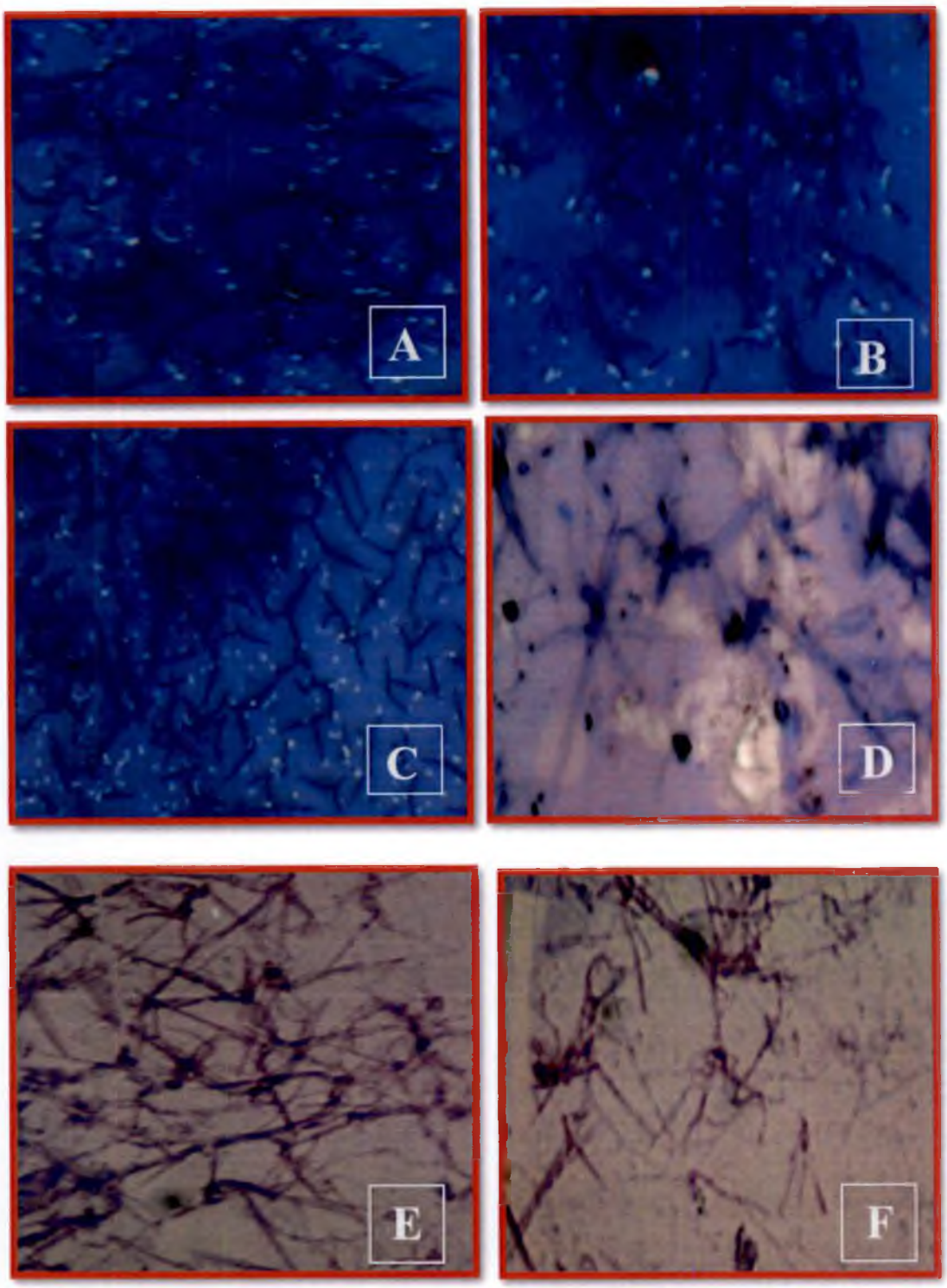


Plate: 16. Capsule and flagellar staining. A, B and C- Capsule staining, no clear zone present around the bacterial rods indicating non-capsular nature of bacteria. D, E and F- Flagellar staining, several flagella present around each bacterial rod indicating peritrichous nature.

4.3.3.1 Solubility in three percent KOH

The bacterial suspension mixed with the three per cent KOH on the glass slide resulted in viscous filament formation after 6 to 7 s and confirmed the gram negative nature of the bacteria. The Gram negative bacteria don't get dispersed with KOH solution leads to the formation of viscous filament whereas Gram positive disperses in KOH (Plate. 17A)

4.3.3.2. Potato soft rot test

The potato slices inoculated with bacterial culture incubated in the Petri plates containing sterile distilled water at room temperature produced the rotting symptoms with the emission of characteristic foul smell in three to four days. There was no rotting in control (Table. 6, Plate. 17B, C). The pathogen was re-isolated from the sample and confirmed by culturing it in the specific media viz. CVP medium, YGC medium and Logan's medium. The bacteria produced characteristic pits in CVP medium, CO_2 and acid in YGC medium and purple coloured colonies in Logan's medium which were similar to that of original isolate (Plate. 17D, E and F)

4.3.3.3 Carrot soft rot test

The carrot slices inoculated with bacterial culture incubated in the Petri plates containing sterile distilled water at room temperature produced the rotting symptoms with the emission of characteristic foul smell in five to seven days after inoculation. There was no rotting in the control (Table. 6 and Plate. 18A, B). The pathogen was re-isolated from the sample and confirmed by culturing it in the specific media viz. CVP medium, YGC medium and Logan's medium. They produced characteristic pits in CVP medium, CO_2 and acid in YGC medium and purple coloured colonies in Logan's medium which were similar to that of original isolate (Plate. 18C, D and E).

Variation was observed in time for rotting of potato and carrot by different isolates. The minimum time taken for rotting was two and four days in the case of potato and carrot respectively by isolate no. 1_{TrKz}, 3_{TrNd}, 5_{KoPr} and 7_{PaKl}. The maximum time taken

for rotting was six and seven days in the case of potato and carrot respectively by isolate no.2_{TrNy} and 8_{PaMn} (Table. 6).

4.3.3.4. Intrinsic antibiotic resistance

Antibiotic resistance was studied by keeping filter paper disc containing 200ppm antibiotic solution over bacterial lawn in nutrient agar medium. Inhibition zone was observed around the filter paper disc containing streptomycin whereas no zone was observed around the filter paper disc containing erythromycin (Plate. 19). The result confirmed that the bacterial isolates were *Erwinia carotovora / Pectobacterium carotovorum* as they were resistant or insensitive to erythromycin but susceptible or sensitive to streptomycin.

4.3.3.5 Growth at three and four per cent NaCl containing medium

Bacterial growth was observed in the inoculated peptone broth containing three and four percent NaCl. The observations were recorded in terms of OD value of the inoculated nutrient broth in comparison with un inoculated control after 24 and 48 h. The results showed that the pathogen could tolerate the salt concentrated medium. The OD value recorded in the medium containing three percent NaCl was in the range of 0.524-0.805 and with four percent NaCl was in the range of 0.445-0.802 after 48 h of incubation.

Variation was observed in the OD values of bacterial isolates grown three and four percent NaCl. In three per cent NaCl containing medium, the maximum OD value of 0.825 was recorded by the isolate no.11_{ErKl} and the minimum OD value of 0.530 was recorded by the isolate no.8_{PaMn} after 72 h of incubation (Table.7).

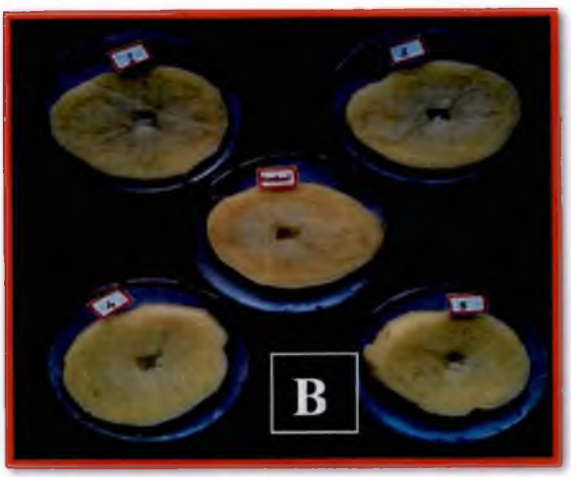
In the case of four percent NaCl containing medium the maximum OD value of 0.819 was recorded by the isolate no.11_{ErKl} and the minimum OD value of 0.453 was recorded by the isolate no.8_{PaMn} after 72 h of incubation (Table. 8).

4.3.3.6 Pectate degradation

The CVP medium inoculated with bacterial culture and incubated at 37°C, produced characteristic fissures or pits after five days of incubation. The pit formation was mainly due the pectolytic ability of the *Erwinia* spp. The bacteria degraded the sodium polypectate present in the CVP medium and produced depressions in the medium. In some of the isolates like 2_{TrNy}, 8_{PaMn}, 10_{ThKr}, 13_{ThKn} and 18_{KaTa}, the pits were surrounded by opaque gel containing non degraded pectin or pectin substrate (Plate. 20A, B).

4.3.3.7 Catalase test

The bacterial smear covered with 30V of H_2O_2 solution on glass slide produced air bubbles due to the gas production. The result indicated the positive catalase activity of the bacterium (Plate. 20C).



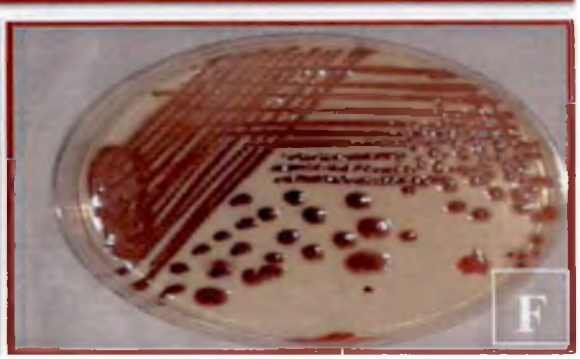


Plate: 17. Biochemical test- I. A- Viscous filament formation. B, C- Inoculated potato slices compared along with the rotted potato slices. D, E and F- Pathogen re-isolated and confirmed by culturing in YGC, CVP and Logan's media

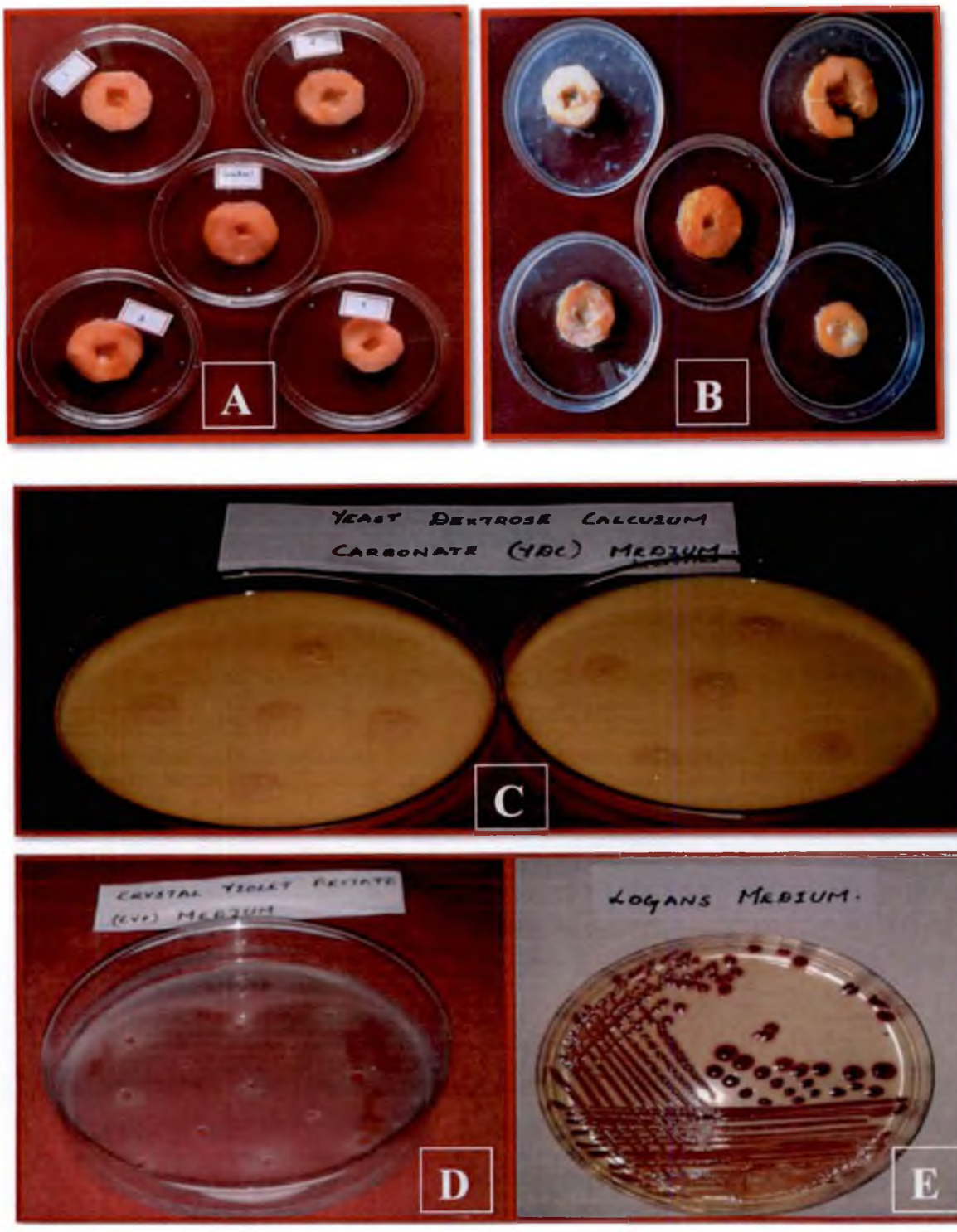


Plate: 18. Biochemical test- II. A, B- Inoculated carrot slices compared along with the rotted carrot slices. C, D, E- Pathogen re-isolated and confirmed by culturing in YGC, CVP and Logan's media

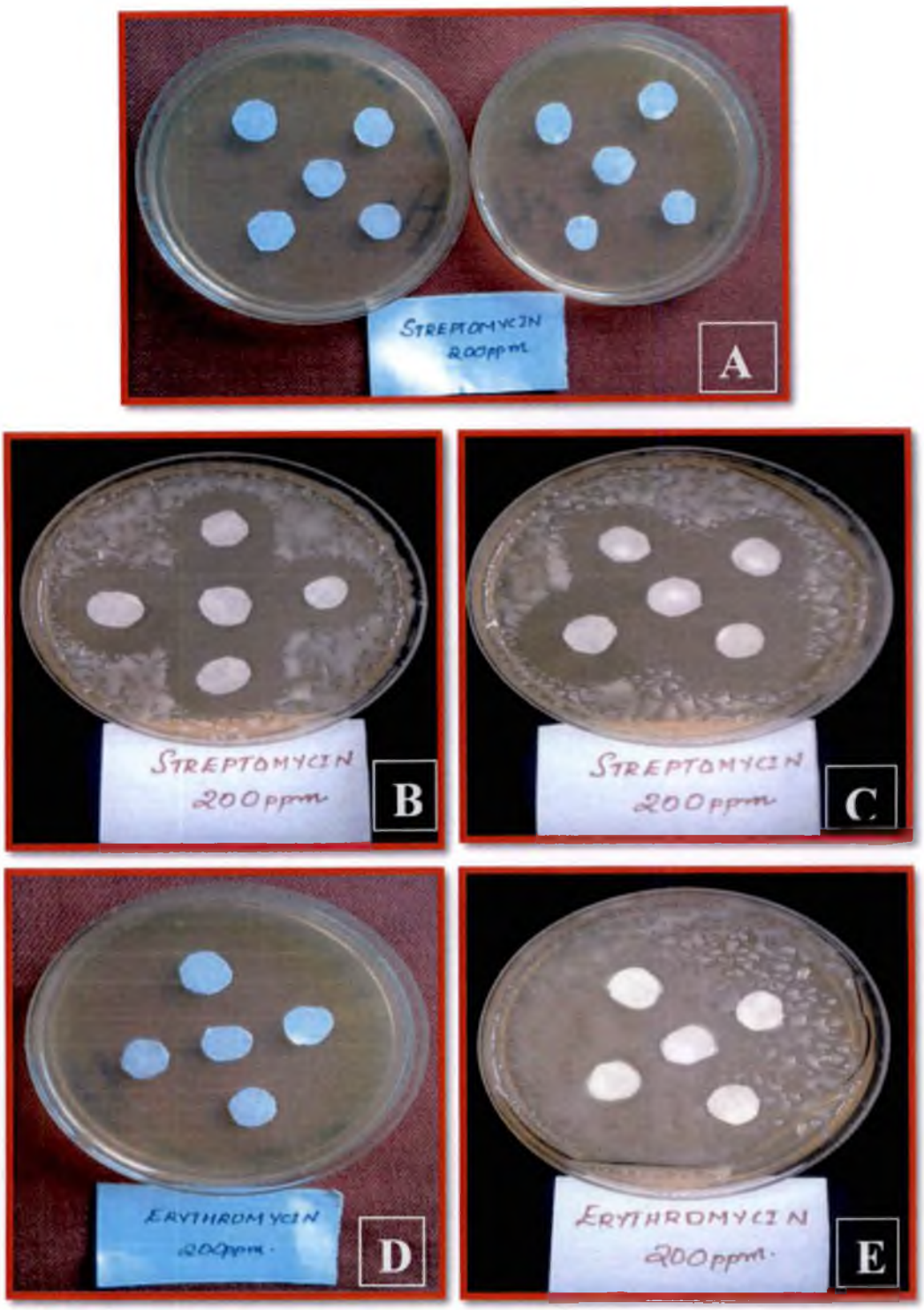


Plate: 19. Biochemical test- III. Intrinsic antibiotic resistance. A-Bacterial lawn with filter paper discs dipped in 200ppm streptomycin. B and C-Inhibition zone formed around filter paper discs containing streptomycin. D-Bacterial lawn with filter paper discs dipped in 200ppm erythromycin. E- No inhibition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

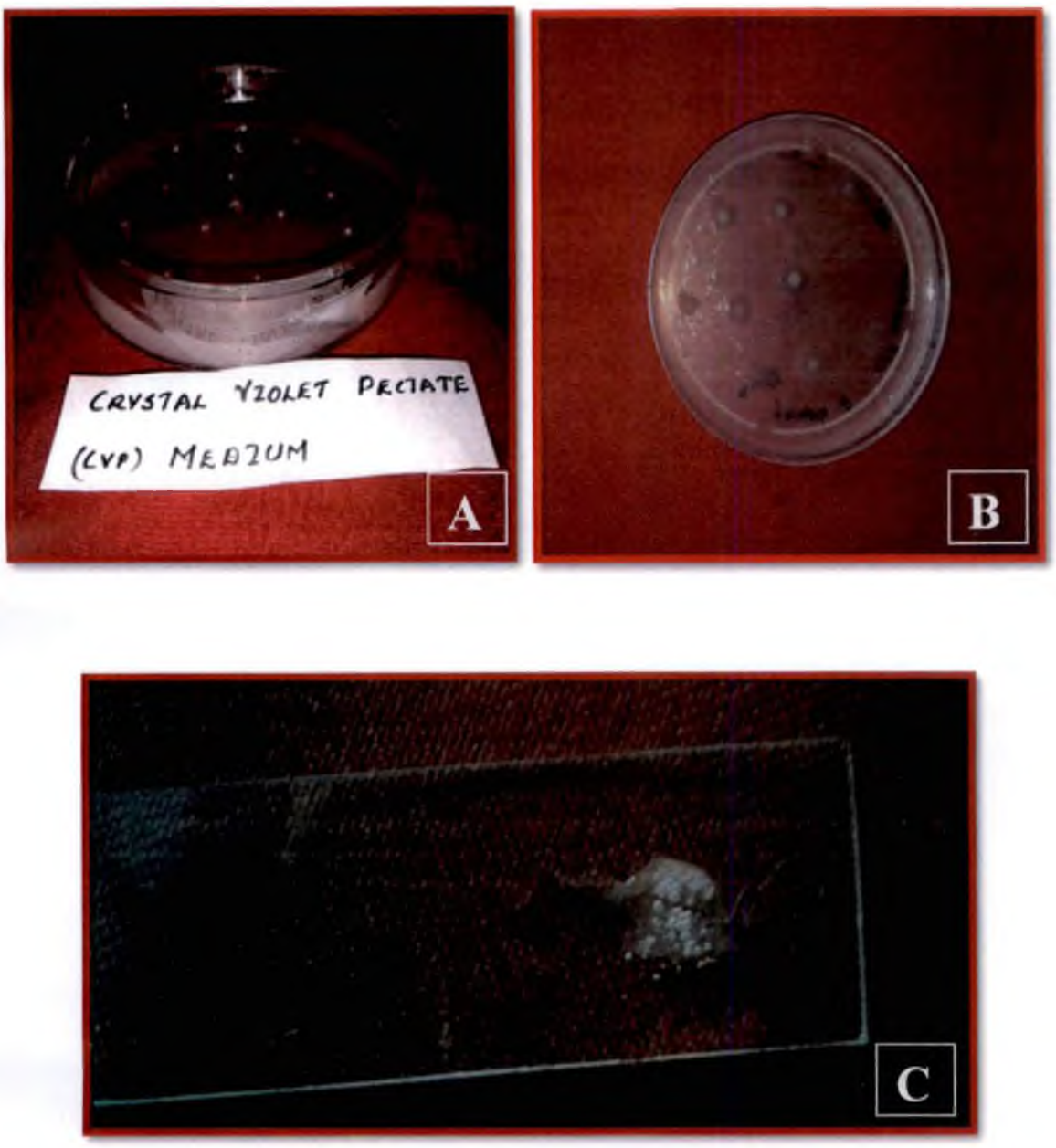


Plate: 20. Biochemical test- IV. A- Fissures formed in CVP medium. B- Opaque gel formed around fissure showing non-degraded pectin. C- Air bubble formation indicating positive catalase test.

Table: 6. Time for potato and carrot soft rot by bacterial isolates

Isolate No.	Potato soft rot		Carrot soft rot	
	Symptom initiation (days)	Completion of rotting (days)	Symptom initiation (days)	Completion of rotting (days)
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- No change after 3,4,5 days in potato and carrot slices				

Table: 7. Effect of three percent Sodium chloride on growth of bacterial isolates

Isolate No.	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: 8. Effect of four percent Sodium chloride on growth of bacterial isolates

Isolate No.	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

4.3.4 Physiological characterization

4.3.4.1 Growth of bacterium at different temperatures

Bacterial cultures was inoculated in nutrient broth and were incubated at varying temperatures such as 27°C, 29°C, 31°C, 33°C, 35°C, 37°C and 39°C. The OD values were recorded after 24 and 48 h. The maximum growth was recorded at 27°C with an OD value of 0.844 followed by 29°C with an OD value of 0.836 after 48h of incubation. The growth of bacteria was very low at 37°C and survived up to 39°C .

Variation was observed in the OD values of bacterial isolates grown in the medium at 27°C. The maximum OD value 0.844 was recorded by the isolate no.17_{MaMd} and the minimum OD value 0.557 was recorded by the isolate no.2_{TrNy}, after 48 h of incubation (Table. 9a, 9b. Figure. 1).

4.3.4.1 Growth of bacterium at different pH

Bacterial cultures were inoculated in nutrient broth at different pH like 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. The observations were recorded in terms of OD value of the nutrient broth inoculated with bacterial culture in comparison to control after 24 h and 48 h. The maximum growth was recorded at pH 7 with OD value in the range of 0.629-0.655 after 48 h of incubation. It was followed by pH 7.5 with an OD value in the range of 0.448-0.470 after 48 h of incubation.

The growth of bacteria was very low at pH of 5 and at pH of 9 as the OD values were almost near to that of control. Variation was observed in the OD values of bacterial isolates grown in the medium at pH. The maximum OD value 0.655 was recorded by the isolate no. 11_{ErKl} and 17_{MaMd} and the minimum OD value was 0.629 recorded by the isolate no.8_{PaMn} after 48 h of incubation (Table. 10a, 10b. Figure. 2).

Table: 9a. Effect of temperature on growth of bacterial isolates

Isolate no.	OD value at different temperature.						
	27°C	29°C	31°C	33°C	35°C	37°C	39°C
1TrKz 24h	0.731	0.727	0.720	0.715	0.622	0.363	0.119
48h	0.733	0.730	0.725	0.717	0.626	0.369	0.120
2TrNy 24h	0.555	0.553	0.549	0.545	0.432	0.261	0.089
48h	0.557	0.556	0.551	0.546	0.435	0.265	0.090
3TrNd 24h	0.838	0.832	0.829	0.815	0.752	0.429	0.127
48h	0.841	0.836	0.831	0.819	0.756	0.433	0.128
4KoAo 24h	0.656	0.651	0.648	0.642	0.548	0.342	0.109
48h	0.657	0.654	0.653	0.645	0.550	0.344	0.111
5KoPr 24h	0.785	0.630	0.624	0.618	0.528	0.387	0.108
48h	0.788	0.634	0.625	0.621	0.532	0.389	0.109
6PaKr 24h	0.725	0.720	0.716	0.704	0.615	0.367	0.116
48h	0.728	0.722	0.719	0.710	0.616	0.380	0.117
7PaKl 24h	0.827	0.823	0.819	0.808	0.750	0.428	0.123
48h	0.829	0.826	0.822	0.813	0.754	0.432	0.125
8PaMn 24h	0.558	0.554	0.551	0.546	0.437	0.264	0.090
48h	0.561	0.559	0.553	0.548	0.440	0.267	0.091
9ThMn 24h	0.659	0.655	0.652	0.647	0.553	0.321	0.113
48h	0.662	0.656	0.657	0.653	0.558	0.334	0.114

Table: 9b. Effect of temperature on growth of bacterial isolates

Isolate No.	OD value at different temperature.						
	27°C	29°C	31°C	33°C	35°C	37°C	39°C
10_{ThKr} 24h	0.608	0.586	0.581	0.577	0.457	0.285	0.096
48h	0.642	0.589	0.586	0.580	0.462	0.298	0.098
11_{ErKl} 24h	0.833	0.831	0.825	0.813	0.753	0.433	0.125
48h	0.837	0.835	0.828	0.817	0.757	0.435	0.127
12_{ThMl} 24h	0.792	0.638	0.633	0.626	0.536	0.383	0.114
48h	0.794	0.641	0.637	0.631	0.540	0.395	0.115
13_{ThKn} 24h	0.628	0.572	0.567	0.563	0.453	0.280	0.094
48h	0.631	0.576	0.572	0.568	0.455	0.292	0.097
14_{ThAh} 24h	0.788	0.635	0.629	0.622	0.532	0.380	0.110
48h	0.790	0.636	0.632	0.625	0.534	0.392	0.111
15_{KaNg} 24h	0.728	0.724	0.720	0.713	0.619	0.361	0.118
48h	0.733	0.725	0.723	0.715	0.621	0.369	0.120
16_{TuTt} 24h	0.653	0.649	0.644	0.640	0.542	0.337	0.105
48h	0.655	0.650	0.647	0.643	0.546	0.340	0.107
17_{MaMd} 24h	0.840	0.835	0.828	0.811	0.747	0.425	0.120
48h	0.844	0.836	0.831	0.814	0.751	0.428	0.122
18_{KaTa} 24h	0.608	0.565	0.561	0.559	0.450	0.297	0.091
48h	0.632	0.567	0.562	0.563	0.452	0.299	0.093
Control 24h	0.051	0.073	0.097	0.208	0.263	0.184	0.013
48h	0.076	0.106	0.114	0.217	0.271	0.192	0.020

Table: 10a. Effect of pH on growth of bacterial isolates

Isolate no.	OD value at different pH								
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
1_{TrKz} 24h	0.030	0.163	0.212	0.445	0.640	0.453	0.256	0.183	0.048
48h	0.033	0.167	0.216	0.448	0.645	0.456	0.258	0.185	0.052
2_{TrNy} 24h	0.016	0.148	0.200	0.426	0.629	0.449	0.249	0.172	0.039
48h	0.019	0.151	0.202	0.429	0.630	0.457	0.253	0.174	0.043
3_{TrNd} 24h	0.040	0.171	0.226	0.464	0.650	0.463	0.269	0.190	0.048
48h	0.042	0.174	0.227	0.469	0.654	0.467	0.272	0.193	0.053
4_{KoAo} 24h	0.026	0.159	0.206	0.438	0.636	0.456	0.255	0.181	0.045
48h	0.029	0.163	0.209	0.441	0.637	0.457	0.258	0.183	0.046
5_{KoPr} 24h	0.035	0.165	0.216	0.444	0.646	0.462	0.259	0.189	0.058
48h	0.038	0.167	0.217	0.448	0.649	0.465	0.261	0.192	0.061
6_{PaKr} 24h	0.033	0.165	0.210	0.440	0.642	0.452	0.257	0.184	0.050
48h	0.037	0.168	0.215	0.443	0.644	0.457	0.261	0.187	0.051
7_{PaKl} 24h	0.041	0.175	0.224	0.462	0.650	0.464	0.267	0.197	0.059
48h	0.043	0.177	0.226	0.467	0.654	0.469	0.270	0.199	0.062
8_{PaMn} 24h	0.015	0.146	0.201	0.427	0.623	0.445	0.245	0.171	0.037
48h	0.017	0.149	0.202	0.430	0.629	0.448	0.247	0.172	0.040
9_{ThMn} 24h	0.028	0.162	0.205	0.435	0.635	0.457	0.259	0.180	0.048
48h	0.030	0.164	0.206	0.438	0.639	0.460	0.262	0.184	0.051

Table: 10b. Effect of pH on growth of bacterial isolates

Isolate No.	OD value at different pH								
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
10_{ThKr} 24h	0.023	0.157	0.203	0.432	0.632	0.453	0.253	0.178	0.042
48h	0.029	0.159	0.206	0.437	0.635	0.456	0.254	0.180	0.044
11_{ErKl} 24h	0.042	0.170	0.221	0.464	0.653	0.467	0.262	0.191	0.061
48h	0.047	0.173	0.224	0.459	0.655	0.470	0.264	0.195	0.063
12_{ThMl} 24h	0.038	0.164	0.215	0.449	0.647	0.460	0.260	0.187	0.057
48h	0.042	0.169	0.220	0.453	0.649	0.464	0.262	0.189	0.060
13_{ThKn} 24h	0.040	0.153	0.201	0.433	0.630	0.451	0.252	0.179	0.041
48h	0.023	0.155	0.203	0.435	0.633	0.452	0.255	0.181	0.045
14_{ThAh} 24h	0.037	0.163	0.217	0.452	0.645	0.460	0.262	0.193	0.056
48h	0.039	0.168	0.219	0.457	0.648	0.465	0.265	0.194	0.059
15_{KaNg} 24h	0.031	0.160	0.214	0.441	0.640	0.450	0.256	0.182	0.052
48h	0.035	0.163	0.218	0.446	0.643	0.453	0.258	0.185	0.054
16_{TuTt} 24h	0.027	0.161	0.207	0.437	0.637	0.455	0.257	0.183	0.046
48h	0.029	0.164	0.210	0.439	0.638	0.458	0.259	0.185	0.049
17_{MaMd} 24h	0.042	0.172	0.222	0.454	0.651	0.456	0.265	0.190	0.053
48h	0.045	0.176	0.224	0.458	0.655	0.459	0.267	0.195	0.056
18_{KaTa} 24h	0.021	0.156	0.203	0.430	0.631	0.453	0.250	0.175	0.040
48h	0.023	0.158	0.205	0.443	0.634	0.456	0.253	0.177	0.043
Control 24h	0.008	0.023	0.045	0.113	0.164	0.104	0.072	0.031	0.009
48h	0.011	0.027	0.049	0.118	0.167	0.105	0.074	0.033	0.014

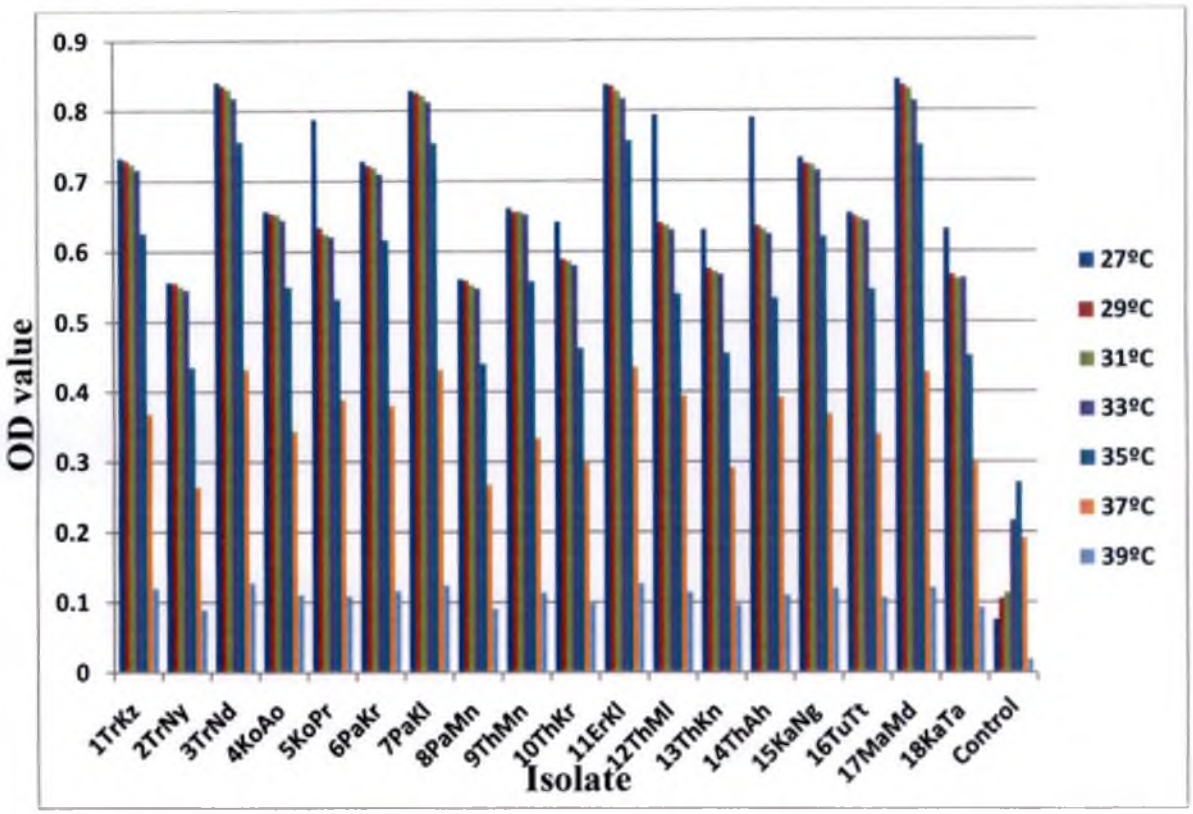


Fig. 1: Effect of temperature on growth of bacterial isolates

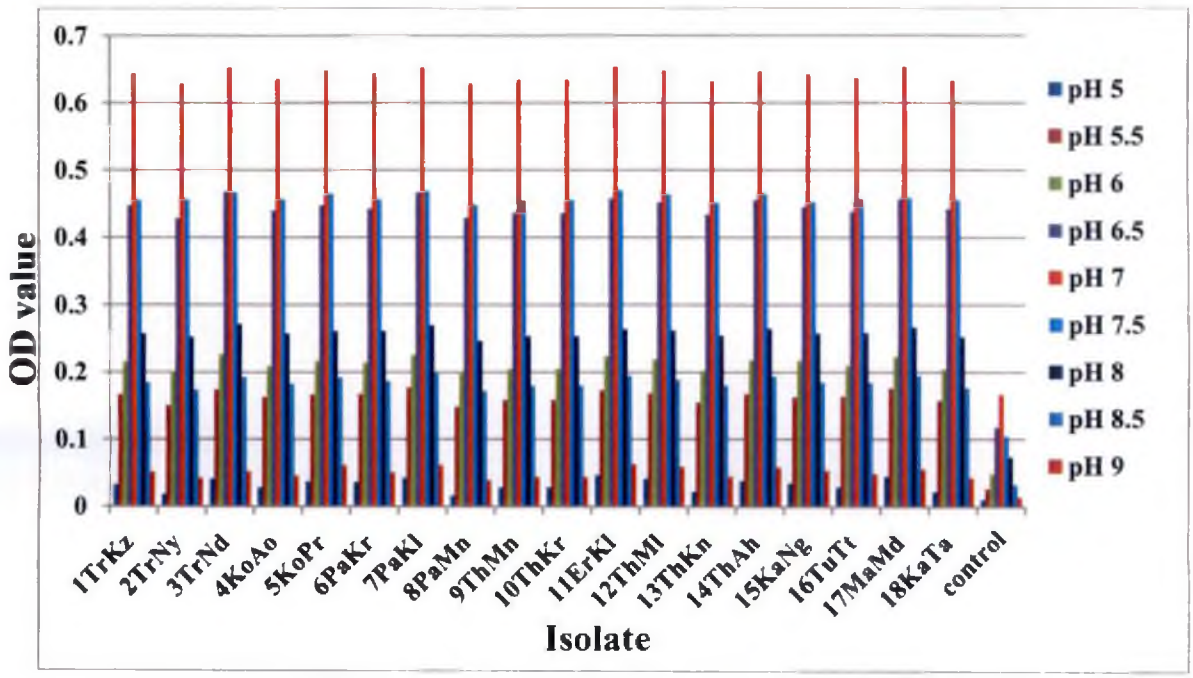


Fig. 2: Effect of pH on growth of bacterial isolates

4.3.6 Molecular characterization

4.3.6.1 DNA isolation

Total genomic DNA of the bacteria from different samples collected were isolated using Sodium Chloride Tris EDTA buffer (STE buffer/NaCl buffer). DNA pellets were obtained, after centrifugation of the mixture containing isopropanol and aqueous layer obtained after adding phenol chloroform. The pellets were ethanol washed, air dried and dissolved in distilled water and used for further analysis.

4.3.6.2 Qualitative analysis of DNA using Agarose gel electrophoresis and visualization of DNA bands:

When the loading dye had reached $3/4^{th}$ of the gel, the gel was taken out from the electrophoresis unit and visualized the bands using UV transilluminator and Gel documentation system. In the UV transilluminator the DNA bands produced fluorescence by intercalating with the ethidium bromide present in the gel. After that, the gel was taken and placed in the gel documentation system. The gel was exposed to the trans UV and the picture was taken by using the camera present in the gel documentation system. The DNA bands obtained were intact without any contamination and the gel picture was saved for further analysis. The gel picture of the DNA isolated from the 18 samples is given in Plate. 21.

4.3.6.3 Quantification of DNA using spectrophotometer (Nano Drop)

DNA samples of all isolates got $A_{260/280}$ in the range 1.79 to 1.86 and the inference derived from this was that the isolated DNA is having high purity. The observations recorded in terms of UV absorbance at 260 nm and 280nm, $A_{260/280}$ and DNA concentrations of all samples were presented in the Table. 11.

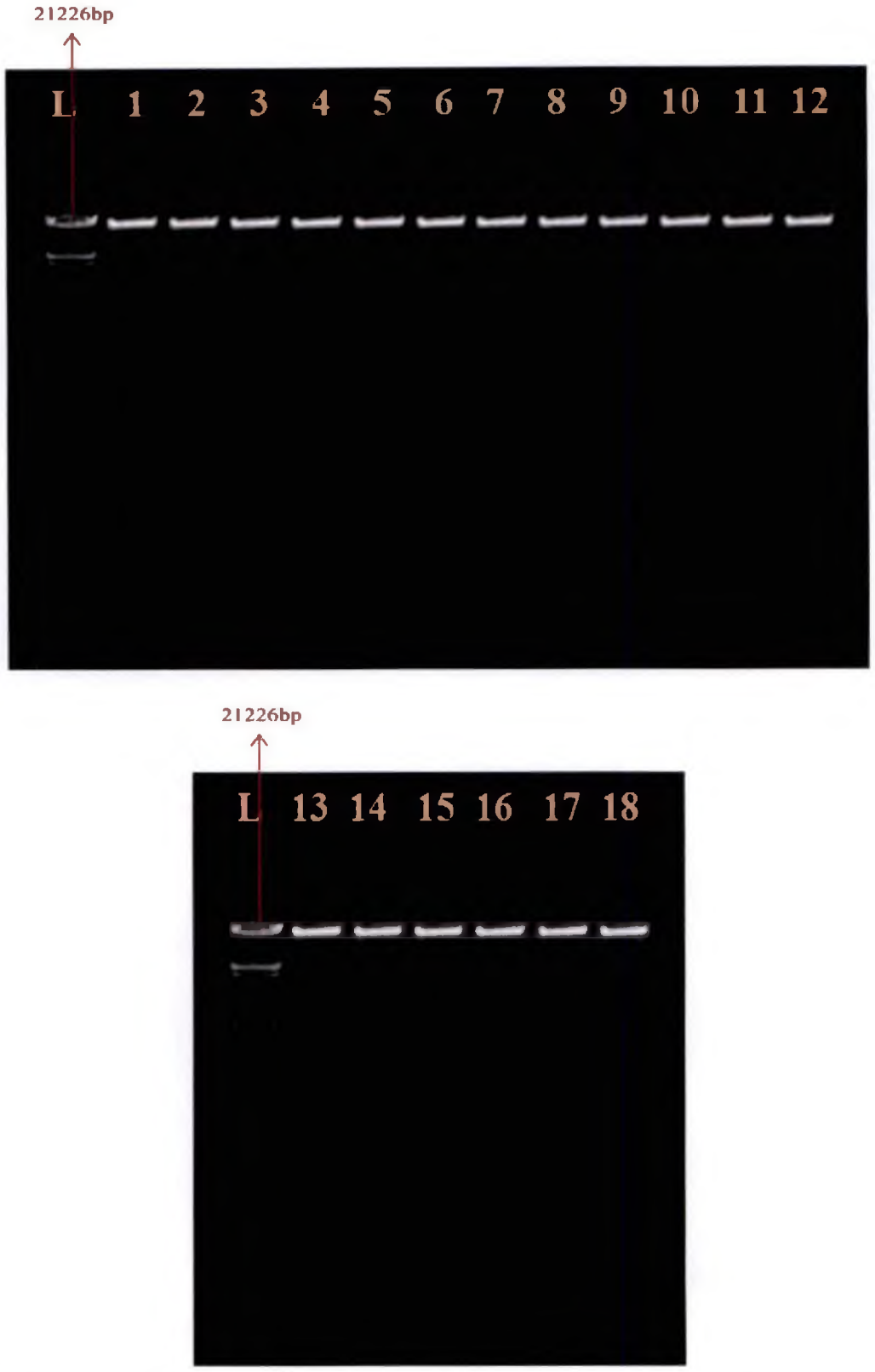


Plate: 21. DNA isolation. L- λDNA *Eco*R1/*Hind*III double digest ladder, 1 to 18- DNA of 18 bacterial isolates

Table: 11. DNA quantification of bacterial isolates

Isolate No.	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260/280}$	Concentration (ng/µL)
1_{TrKz}	2.054	0.910	1.84	690.5
2_{TrNy}	1.567	1.168	1.83	760.8
3_{TrNd}	2.220	1.220	1.80	867.1
4_{KoAo}	3.853	1.455	1.82	955.1
5_{KoPr}	2.056	1.145	1.79	905.1
6_{PaKr}	1.567	1.168	1.83	786.7
7_{PaKl}	2.345	1.289	1.81	1510.3
8_{PaMn}	2.054	0.910	1.84	1550.9
9_{ThMn}	2.586	0.715	1.85	1355.1
10_{ThKr}	4.954	2.586	1.82	892.0
11_{ErKl}	2.220	1.220	1.80	930.3
12_{ThMl}	0.889	0.478	1.86	1725.4
13_{ThKn}	1.567	1.168	1.83	1641.0
14_{ThAh}	2.072	1.129	1.80	1268.5
15_{KaNg}	2.072	1.129	1.80	1870.8
16_{TuTt}	4.956	2.588	1.82	951.2
17_{MaMd}	2.220	1.220	1.80	1237.2
18_{KaTa}	0.640	0.354	1.81	864.5

4.3.6.3 PCR amplification of isolated total genomic DNA

The genomic DNA containing 16S rRNA gene was amplified using a pair of 16S rRNA universal primer fD1 and rP2 by thermocycling of 35 cycles. There was no amplification obtained at the already reported annealing temperature. By doing gradient PCR the annealing temperature was standardized as 64.2°C (Plate. 22).

The PCR of 18 DNA samples were carried out using fD1 and rP2 primers at the standardized temperature (64.2°C). All showed amplicons of same size.

4.3.6.4 Colony PCR

Instead of using genomic DNA as the template in PCR, here the denatured bacterial colony suspension is used to carry out the PCR thermocycling. The 16S rRNA universal primer 8F and 1522R were used in colony PCR. The ampllicon size of the PCR products was 1.5 Kbp.

4.3.6.5 Qualitative analysis of DNA using Agarose gel electrophoresis and visualization of PCR products:

The PCR products were analyzed using two percent agarose gel. The 10 µL PCR product mixed with one µL of loading dye and loaded in the consecutive wells and in the second well a blank was also loaded. 5 µL of EcoR1 digested Hind III lambda marker was loaded in the first well. When the loading dye reached $3/4^{th}$ of the gel, the gel was taken out and visualized.

Under UV transilluminator PCR products produced fluroscence and clear DNA bands with no contamination with an amplicon size of 1.5 kb was obtained when the gel was visualized using Gel documentation system. The Gel pictures were saved for future use. The Gel pictures of PCR amplicons of two set of 16s rRNA universal primers are given in the Plate. 23, 24.

Plate: 22. Standardization of PCR. L - 100bp ladder, Temperature given at each well: 1 - 48°C, 2 - 49.2°C, 3 - 50.8°C, 4 - 51.6°C, 5 - 53.4°C, 6 - 55.2°C, 7 - 57°C, 8 - 58.8°C, 9 - 60.6 °C, 10 - 62.4°C, 11 - 64.2°C, 12 - 66 °C

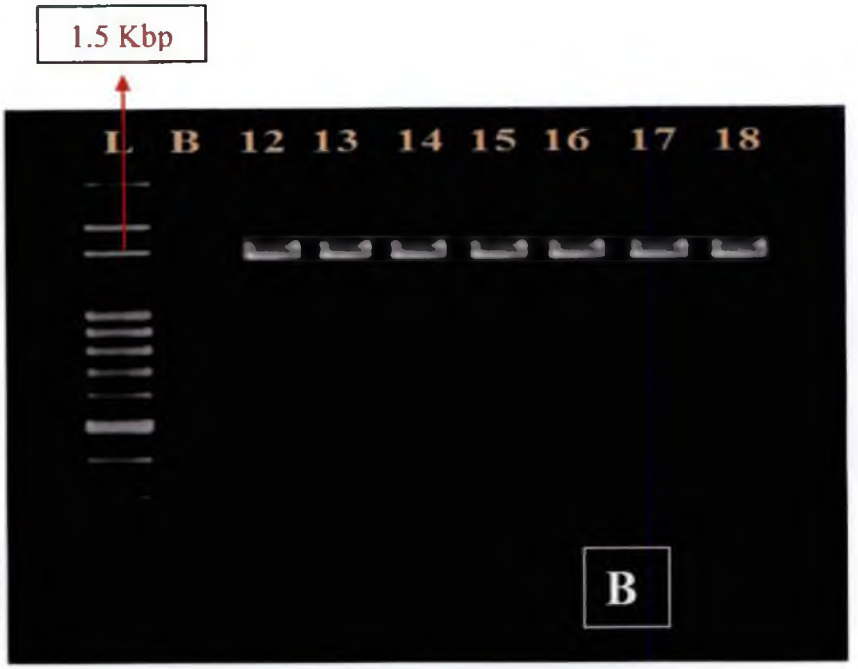


Plate: 23. A, B– PCR amplification of 16S rRNA gene using fD1and rP2 primer. L - 1 Kb plus ladder, 1 to 18 - Amplified DNA of 18 bacterial isolates.

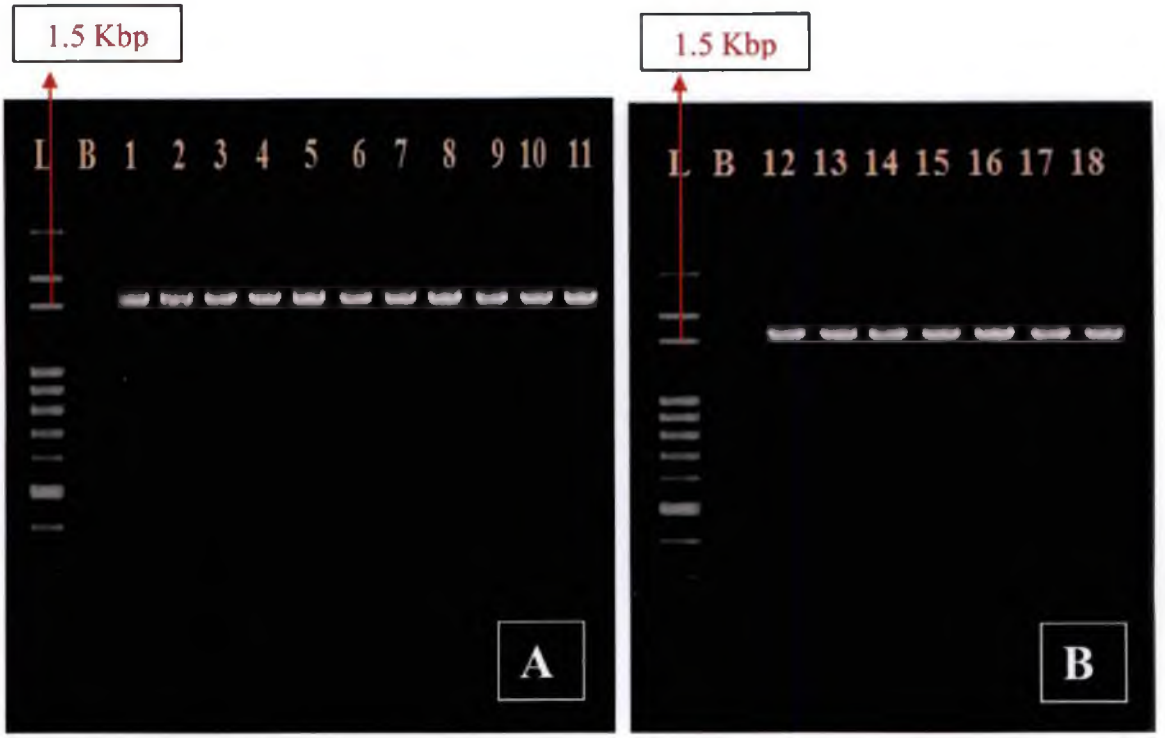


Plate: 24. A, B– PCR amplification of 16S rRNA gene using 8F and 1522R primer. L -1 Kb plus ladder, 1 to 18 - Colony PCR product of 18 bacterial isolates

4.3.6.6 Grouping of the isolates

Based on the results of cultural, morphological, biochemical and physiological characterization, the 18 isolates collected from different locations were grouped into six groups manually and by constructing dendrogram (Figure 3). The parameters used for grouping were OD values in nutrient broth, bacterial size, OD values in 3% and 4% NaCl, OD value at 27°C, 37°C and pH 7 (Table. 12, 13, 14 and 15).

Table: 12. 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: 13. 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: 14. 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: 15. 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}

The dendrogram constructed using the parameters viz, 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 (Figure: 3). 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.

4.3.6.7 PCR product sequencing:

The PCR products analyzed on the two percent agarose gel yielded single band of amplicons. The 16S rRNA sequences obtained after were listed in Annexure VI.

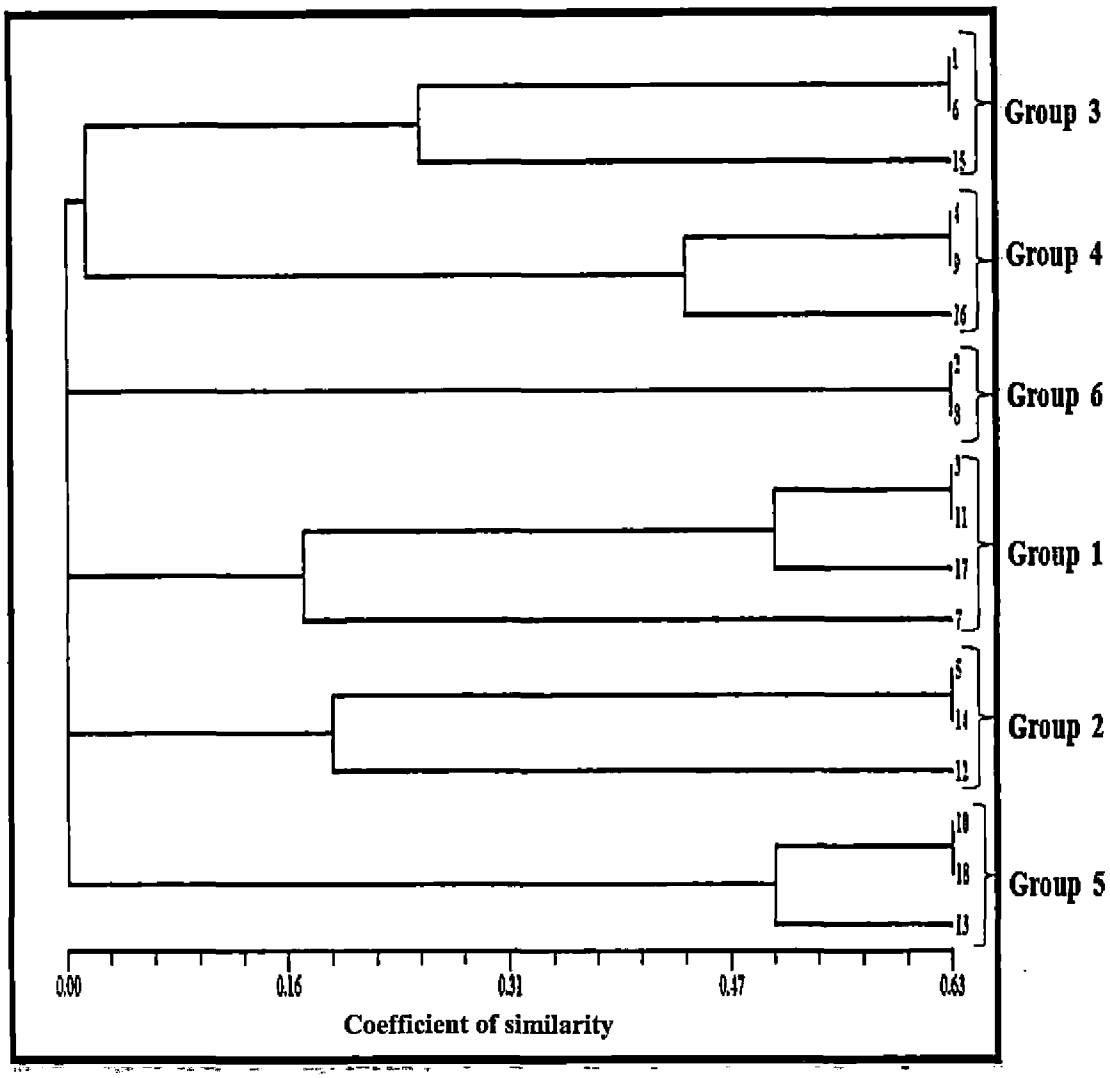


Fig. 3: Dendrogram constructed based on cultural, morphological, biochemical and physiological parameters. The 18 bacterial isolates were grouped into six groups

4.4 DATA ANALYSIS:

4.4.1 Homology analysis of PCR product sequences

After making the reverse complement of the reverse sequence, the reverse complement sequence was merged with the forward sequence. The merged sequences in the FASTA format were used to carry out the homology analysis using BLASTn software. The results obtained from the BLAST analysis were presented in the Table.16, Figure. 4.

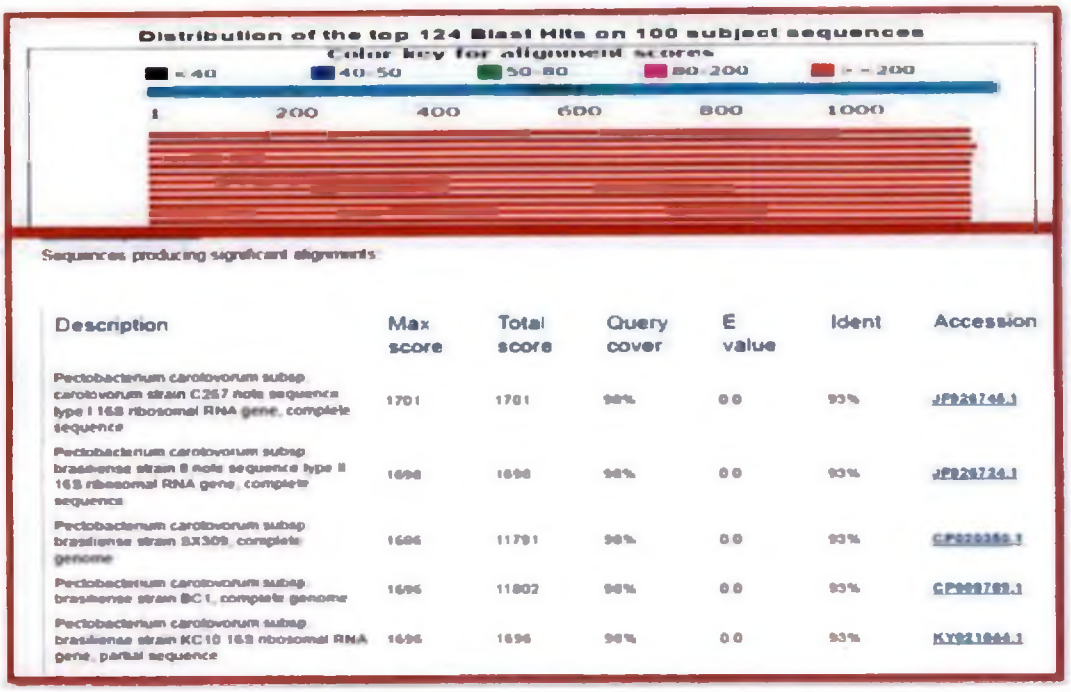
Table: 16. Homology analysis of sequence using BLASTn

Sequnce ID	Query coverage (%)	Identity (%)	Name of the species	E – value
G1_E16S_11	98	93	*Pectobacterium carotovorum* subsp.*carotovorum*	0.0
G2_E16S_5	96	94	*Pectobacterium carotovorum* subsp.*carotovorum*	0.0
G3_E16S_15	95	94	*Pectobacterium carotovorum* subsp.*carotovorum*	0.0
G4_E16S_9	94	93	*Pectobacterium carotovorum* subsp.*carotovorum*	0.0
G5_E16S_13	98	88	*Pectobacterium carotovorum* subsp.*brasiliense*	0.0
G6_E16S_8	98	88	*Pectobacterium carotovorum* subsp. *brasiliense*	0.0

4.4.2 Phylogenetic analysis

The barcode gaps were identified by aligning the six 16S rRNA sequences using 'Clustal W'software. The conserved regions in the sequences was indicated with '*' in the aligned sequence. The variability in the non-conserved sequences was checked manually and marked. The variability in the sequences of each subspecies of *Erwinia*

Sequence ID: G1_E16S_11



Sequence ID: G6_E16S_8

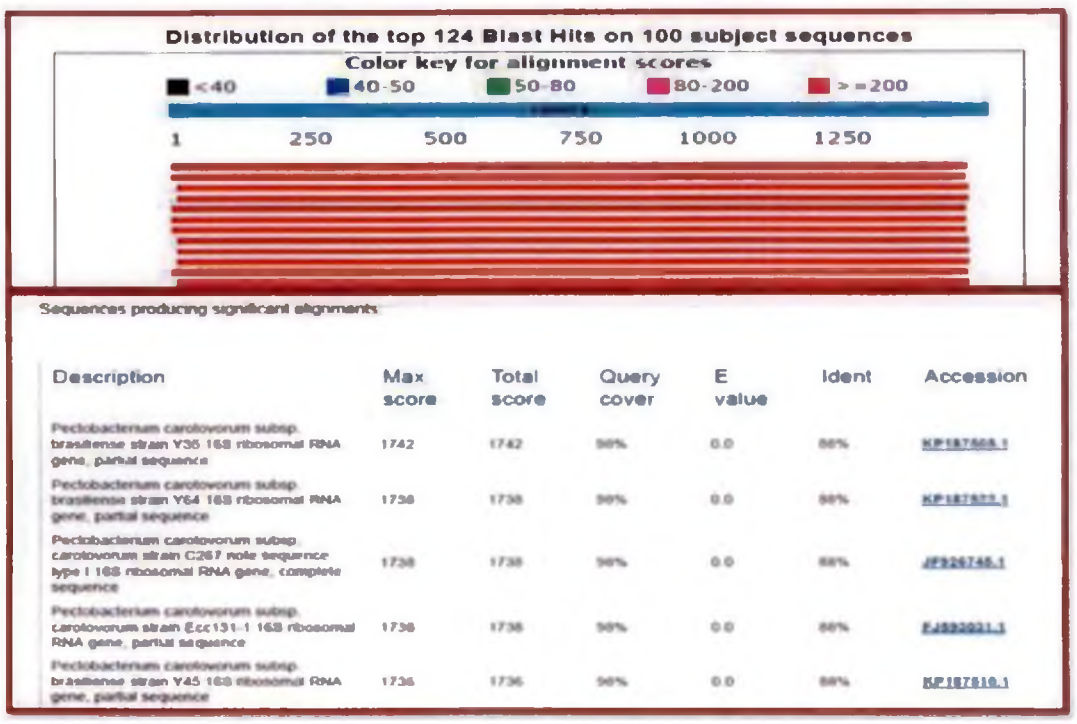


Fig. 4: Homology analysis. BLASTn results of the sequences of group 1 and group 6

carotovora/Pectobacterium carotovorum was noticed and there by barcode gaps was identified from the aligned sequences of *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *brasiliense*. The barcode gaps obtained were shown in the Figure. 5.

Phylogenetic tree was constructed using MEGA6 software by using the 16S rRNA sequences of six isolates. The group 5 and group 6 sequences of *Pectobacterium carotovorum* subsp. *brasiliense* branched out separately with a bootstrap value of 100 compared with other four sequences of *Pectobacterium carotovorum* subsp. *carotovorum* The group 2 and group 3 sequences branched out with a bootstrap value of 90 where as group 1 and group 4 with a bootstrap value of 93. The phylogenetic tree was shown in the Figure. 6.

4.4.3 Sequence submission to GenBank (NCBI database)

All six sequences from the *Erwinia carotovora/Pectobacterium carotovorum* were deposited in the GenBank (NCBI database). The accession numbers obtained from the NCBI for the sequences deposited were listed in the Table. 17.

Table: 17. Accession number generated for the 16S rRNA sequence deposited in GenBank (NCBI)

Sequence ID	Accession number
G1_E16S_11	MF458978
G2_E16S_5	MF458979
G3_E16S_15	MF458980
G4_E16S_9	MF458981
G5_E16S_13	MF458982
G6_E16S_8	MF458983

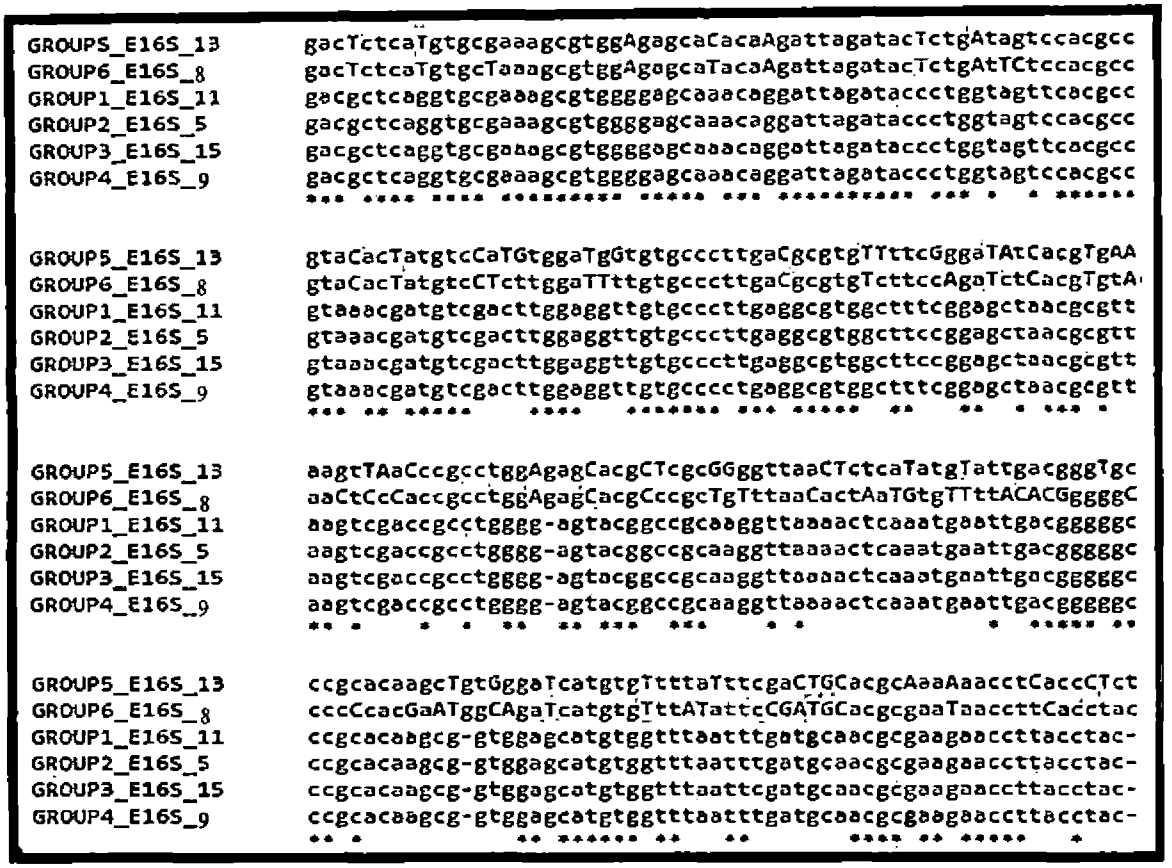


Fig. 5: Barcode gaps analysis – barcode gaps identified after aligning four sequences *Pectobacterium carotovorum. carotovorum* (Group 1, 2, 3 and 4) and two sequences of *Pectobacterium carotovorum.brasiliense* (Group 5 and 6)

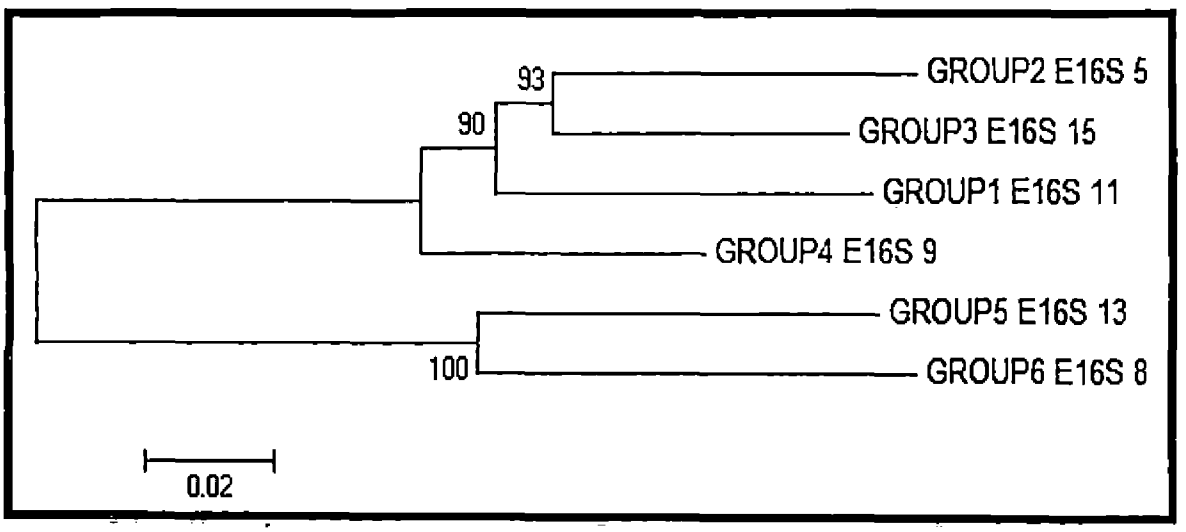


Fig. 6: Phylogenetic tree constructed for six 16S rRNA sequences

Discussion

5. DISCUSSION

Banana and plantains are the staple food of various tropical countries, but now a day they are affected by many diseases and pests which caused severe yield loss (Jones, 2000). The significance of rhizome rot disease in banana plants for yield loss was less in the past but now it is increasing severely (Thwaites *et al.* 2000). According to Snehalatharani and Khan (2010) bacterial heart rot, Rhizome rot, Soft rot or tip-over disease mainly affects two to six months old banana plants, produced characteristic symptoms on rhizome, pseudostem and leaves and mainly the infection was more in rhizome. Nagaraj *et al.* (2012) clearly reported the above ground and below ground symptoms of the disease. According to them the disease infection was more in two varieties of banana: G-9 and Robusta.

Lakshmanan and Mohan (1986) reported that the yield loss due to banana rhizome rot disease is nearly 60 to 80 per cent. Sathiamoorthy (1994) reported that in recent days the only bacterial disease which became an alarming threat to banana cultivation in India is the rhizome rot disease. At the time of secondary hardening two to five percent death of the plants were due to the incidence soft rot disease (Thomas *et al.*, 2011).

The rhizome rot disease becomes more prevalent during the past few years in the southern states of India including Tamil Nadu, Kerala and Karnataka. (Khan and Nagaraj, 1998; Nagaraj *et al.*, 2002; Rani *et al.*, 2002). Report says that the pathogen is invading tissue culture or micropropagated banana plants also. There are several varieties susceptible for the rhizome rot disease. The majorly cultivating 'Nendran' variety in Kerala is also susceptible to banana rhizome rot disease

Different *Erwinia* species synonymously known as *Pectobacterium* species are reported to be the pathogen of rhizome rot in banana. So understanding of the correct etiology of the disease is becoming necessary. Molecular characterization is the major tool for studying variability and characteristics of the pathogen. Sequencing of the 16S rRNA gene loci of various isolates can be used to find the phylogenetic relationship with other reported species.

Hence, the present study was carried out with the following objectives:

- Purposive survey in various locations of Kerala and Tamil Nadu
- Isolation of pathogen from rhizome rot disease infected samples
- Cultural and morphological characterization of pathogen
- Biochemical and physiological characterization of pathogen
- Molecular characterization of pathogen including genomic DNA isolation, 16S rRNA gene amplification, sequencing and phylogenetic analysis.

The samples were collected from the rhizome rot infected banana fields from 14 different locations of Thrissur, Palakkad, Ernakulam, Kollam and Trivandrum districts of Kerala and from four different locations of Tamilnadu in the districts Kanyakumari, Madurai and Tuticorin districts of Tamil Nadu. Total 18 samples were collected and used in the present study.

5.1 METHOD OF ISOLATION AND IDENTIFICATION OF PATHOGEN

The pathogen isolated from the infected rhizome samples by the standard tissue maceration method on nutrient agar medium yielded small bacterial colonies which were cream to yellowish colour, slightly raised and mucoid with a diameter in the range of 2.3 to 2.5 mm. Standard tissue maceration method for isolation of pathogen from disease infected rhizome bits were successfully done by several researchers. (Tomlinson *et* al., 1987; Gomez-Caicedo *et al.*, 2001; Nagaraj *et al.*, 2002; Rani *et* al., 2002; Usha 2003). Similar bacterial colonies were obtained on the nutrient agar medium when isolated from rhizome rot infected samples of banana by Stover (1959), Dickey and Victoria (1980), Usha (2003), Snehalatharani and Khan, (2010) and Nagaraj *et al.,* (2012).

Further streaking of 48 h old bacterial culture on the *Erwinia/Pectobacterium* spp. specific crystal violet pectate (CVP) medium, produced characteristic fissures or pits of *Erwinia* spp., as reported by several workers (Cupples and Kelman, 1974; Ma *et al.*, 2007; Bhupendra and Yogendra, 2015).

5.2 PATHOGENICITY STUDIES

Pathogenicity is defined as the capability of any pathogen to induce infection or disease in healthy plants. The pathogenicity test were carried out in three different ways

on the banana var. Nendran to prove the Koch's postulates viz. inoculation in rhizome bits, rhizome of three month old plants, pseudostem of two month old tissue culture plants, with 48 h old bacterial culture. Even though rotting was observed in all isolates, there was variation in the severity of rotting between isolates.

The rhizome bits of the 'Nendran'var. inoculated with bacterial culture which are incubated under *in-vitro* conditions produced rotting symptoms after eight days of inoculation. The characteristic symptom observed were discolouration and rotting around the inoculated portion. The rhizome bit became sunken, water soaked with dark brown colour around the inoculated area and it also emitted unpleasant foul smell. Stover (1959) while working with soft rot causing *Erwinia carotovora* ssp. *carotovora/ Pectobacterium carotovorum* ssp. *carotovorum* conducted *in-vitro* inoculation of rhizome bits and reported rotting by 7 days. Usha (2003) also reported that the inoculated rhizome bits under artificial condition produced rotting, discolouration and foul smell after 8 days of inoculation.

The pathogenicity test carried out under *in-vivo* condition using three month old 'Nendran' variety banana by inoculating with bacterial colony at collar region produced the rotting of rhizome in 37 days after inoculation. Usha (2003) also conducted pathogenicity test in pot culture by soil inoculation and reported that rotting was observed after 56 days of inoculation. She also reported dark brown discoloration with water soaked appearance along with the characteristic emission of foul smell of infected rhizomes. Nagaraj *et al.* (2012) and Nagrale *et al.* (2013) reported similar observations when they conducted pathogenicity test for rhizome rot.

Tissue culture plants of two months old when inoculated by pin pricking the pseudo stem with single bacterial colony produced characteristic symptoms after six days of inoculation. The symptoms observed were yellowing of the lower leaves and yellowing progressed to upper leaves, weakening of the pseudostem and finally the plant felled down as the pseudostem got completely rotted and emitted foul smell 16 days after inoculation. Thomas *et al.*, (2008) carried out the pathogenicity test in micropropagated banana to check whether they are resistant or susceptible to rhizome rot disease and reported that they are susceptible and produced rotting symptom two weeks after

inoculation which was similar to that obtained in the present study. Variation was observed in days for the rotting of tissue culture plants by different isolates. The minimum time taken for rotting was seven days by the isolate no. 3_{TrNd}, 7_{PaKl} and 17_{MaMd}; followed by 10 days by the isolates viz., 5_{KoPr}, 12_{ThMl} and 14_{ThAh} and the maximum time for rotting was 15 days taken by isolates viz., 2_{TrNy} and 8_{PaMn} which was preceded by 13 days by isolates viz., 10_{ThKr}, 13_{ThKn} and 18_{KaTa}.

The time required to produce the disease symptom was very less under *in-vitro* condition as compared to *in-vivo* condition. Stover (1959) also reported that under *in-vivo* condition plants took long time for the symptom development.

The pathogen was re-isolated from the samples and the bacterial species was confirmed by culturing it on *Erwinia/Pectobacterium* spp. specific crystal violet pectate (CVP) medium, which produced characteristic fissures or pits of *Erwinia* spp., as reported by several workers (Cupples and Kelman, 1974; Ma *et al.*, 2007; Bhupendra and Yogendra, 2015).

5.3 CHARACTERIZATION OF PATHOGEN

The characterization and identification of banana rhizome rot disease causing pathogen was carried out based on cultural, morphological, biochemical, physiological and molecular characters.

5.3.1 Cultural characterization

Cultural characterization was carried out in 5 different ways viz., culturing the 48 h old bacteria in nutrient agar medium, crystal violet pectate medium, yeast extract glucose calcium carbonate agar medium, Logan's medium and in nutrient broth.

The Petri plates containing bacterial culture isolated from the rhizome rot disease infected banana rhizome samples when incubated for 48 h on nutrient agar yielded cream to slightly yellowish, mucoid, slightly raised convex colonies with entire margin as reported earlier (Stover,1959; Dickey and Victoria, 1980; Usha, 2003; Snehalatharani and Khan, 2010; Nagaraj *et al.*, 2012).

The bacterial culture inoculated on CVP medium produced characteristic fissures or depressions due to pectate degradation as reported by Cupples and Kelmen (1974); Ma *et al.* (2007); Snehalatharani and Khan (2010). The pit formation was observed after five days of incubation. Bhupendra and Yogendra (2015) reported that *Erwinia* spp. has pectate degrading ability because the sodium polypectate present in the CVP medium were degraded by the bacteria and yielded the formation of fissures in five days. In some of the isolates like 2_{TrNy}, 10_{ThKr} and 13_{ThKn} in the present study, pits were surrounded by opaque gel containing non degraded pectin or pectin substrate as reported by Mc Feeters *et al.* (1992).The differences observed in the CVP medium shows the presence of variation in isolates.

The bacterial culture inoculated Petri plates containing YGC medium produced cream to yellow coloured semi mucoid colonies with a clear zone around each colony after 24 h of incubation at 28±1°C. Several other workers also got similar results (Schadd and Bernner, 1977; Dickey and Victoria, 1980; Khan and Nagaraj, 1998). The bacterium was confirmed as *Erwinia* spp. as small bubbles were observed on the top of each bacterial colony due to the emission of CO_2 from the YGC medium which is the characteristic feature of *Erwinia* spp. as reported by Kado (2006). The indigoidine pigment was not produced by any of the bacterial cultures on the YGC medium which is the characteristic feature of *Erwinia chrysanthemi* as reported by Starr *et al.* (1966) and Bradbury (1977b). Hence it is confirmed that all the bacterial isolates belong to *E. carotovora.*

The bacterial culture inoculated in Logan's medium and incubated at 28±1°C produced small to medium colonies of 1.6 to 2 mm diameter with purple colour having entire margin after 24 h. The pathogen was identified as *E. carotovora* by observing the purple coloured colonies as reported by Usha (2003). Logan's medium is differential medium for *Erwinia* spp. to distinguish between species of *Erwinia. E. carotovora* produce pink to purple colonies whereas *E. chrysanthemi* produce red to purple bacterial colonies (Fahy and Hayward, 1983).

The bacterial growth was observed in terms of OD value, in the nutrient broth after 72 h of incubation. Difference was observed in the growth rate of isolates, which

include both fast growing and slow growing isolates. The maximum OD value was recorded by the isolate no. 17_{MaMd} (0.840), followed by isolate no. 3_{TrNd} (0.838) and the minimum OD values was recorded by the isolate no. 2_{TrNy} (0.555), preceded by isolate no. 8_{PaMn} (0.558) after 72 h of incubation. The difference observed in the OD values shows the presence of variation in the growth rate of bacterial isolates (Table. 4). Similar observations were recorded by Usha (2003) for *Erwinia carotovora.*

5.3.2 Morphological characterization

Morphological characterization of the isolates was carried out by three different ways viz., Gram staining, capsule staining and flagellar staining.

The bacteria appeared as red coloured short rods after Gram staining when observed under oil immersion microscope indicating the Gram negative nature. There was significant variation in the size of the bacterial rods between isolates. The maximum length recorded was 3.10 µm in by isolate no. 7_{PaKl} followed by 3.05 µm in isolate no. 3_{TrNd} and the maximum width recorded was 1.10 µm in isolate no. 17_{MaMd} followed by 1.08 µm in isolate no. 3_{TrNd}. The peritrichous nature of flagella and non-capsular nature of bacterium observed in the present study was reported by several workers (Blenden and Goldberg, 1964; Gomez-Caicedo *et al.*, 2001; Edward and Stanely, 1971; Liao and wells, 1986; Nagaraj *et al.*, 2012). The difference in the size shows presence of variation in bacterial isolates (Table. 5).

5.3.3 Biochemical characterization

Biochemical characterization of the isolates were carried out by checking solubility in KOH, potato soft rot test, carrot soft rot test, intrinsic antibiotic resistance, growth in three and four per cent NaCl, pectate degrading ability and catalase test.

The bacterial suspension mixed with the three per cent KOH produced viscous filament and confirmed the gram negative nature of the bacterial isolates as reported by Suslow *et al.* (1982); Ngwira and Samson in (1990); Gomez-Caicedo *et al.* (2001); Akbar *et al.* (2015).

The potato and carrot slices inoculated with bacterial culture produced rotting symptoms with the emission of characteristic foul smell after three to four days and five to seven days respectively. Lelliott *et al.*(1966); Togashi (1988) and Nabhan *et al.* (2006) conducted potato soft rot test and reported that rotting symptoms were observed after three days. Schillingford (1974); Doolotkeldieva *et al.* (2016) reported that rotting in carrot were slow and complete rotting was observed after 14 days. Similar result was obtained in the present study also.

Variation was observed in time for rotting of potato and carrot by different isolates. The minimum time taken for rotting was two and four days by isolate no. 1_{TrKz}, 3_{TrNd}, 5_{KoPr} and 7_{PaKl} in the case of potato and carrot respectively. The maximum time taken for rotting was six and seven days by isolate no. 2_{TrNy} and 8_{PaMn} in the case of potato and carrot respectively. The difference observed in the time for rotting is an indication of variation in the virulence of isolates (Table. 6).

The pathogen was re-isolated from the inoculated potato and carrot slices and confirmed by culturing it in the specific media like CVP medium, YGC medium and Logan's medium and obtained the bacterial colonies similar to that of original isolate as reported by several researchers (Logan, 1966; Fahy and Hayward, 1983; Kado, 2006; Ma *et al.*, 2007; Bhupendra and Yogendra, 2015).

The bacterial isolates were resistant or insensitive to erythromycin but susceptible or 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. According to Dickey and Victoria (1980); Snehalatharani and Khan (2010); Nagrale *et al.* (2013); Akbar *et al.* (2015) *E.carotovora* is resistant to erythromycin and susceptible to streptomycin where as *E.chrysanthemi* is susceptible to erythromycin and streptomycin. Since in the present study, the bacterial isolates showed resistance to erythromycin and susceptibility to streptomycin, it is confirmed that they belong to *Erwinia carotovora/Pectobacterium carotovorum.*

Bacterial growth was observed in the inoculated peptone broth containing three and four percent NaCl. The results showed that the pathogen could tolerate the salt

concentrated medium. The salt tolerant nature of *Erwinia* spp. was reported earlier by different workers. (Dickey, 1979; Gomez-Caicedo *et al.*, 2001; Akbar *et al.*, 2015; Rafiei *et al.*, 2015).

Variation was observed in the OD values of bacterial isolates grown in the medium containing three and four percent NaCl. In the three and four per cent NaCl containing medium the maximum OD value was recorded by the isolate no.11_{ErKl} and the minimum OD value was recorded by the isolate no. 8_{PaMn} after 72 h of incubation. The differences observed in OD values of isolates at 3% and 4% NaCl containing medium shows the presence of variation between isolates (Table: 7,8).

The bacterial culture inoculated on CVP medium produced characteristic fissures or depressions due to pectate degradation as reported by Cupples and Kelmen (1974); Ma *et al.* (2007); Snehalatharani and Khan (2010). The pit formation was observed after 5 days of incubation. Bhupendra and Yogendra (2015) reported the *Erwinia* spp. has pectate degrading ability, the sodium polypectate present in the CVP medium were degraded by the bacteria and yielded the formation of fissures in five days. In some of the isolates like 2_{TrNy}, 10_{ThKr} and 13_{ThKn} the pits are surrounded by opaque gel containing non degraded pectin or pectin substrate as reported by Mc Feeters *et al.* (1992).

The bacterial smear on the glass slide when covered with 30V of H_2O_2 produced air bubbles due to the gas production which indicated the positive catalase activity of the bacteria. Cappuccino and Sherman (1992); Snehalatharani and Khan (2010) and Raiefi *et al.* (2015) also got same result and reported the catalase positive nature of *Erwinia* spp.

5.3.4 Physiological characterization

The maximum growth of bacteria was recorded at 27°C with an OD value of 0.844 followed by 29°C with an OD value of 0.836 after 48 h of incubation. Variation was observed in the OD values of bacterial isolates grown in the medium at 27°C (Table: 9a,b and Figure: 1). The maximum OD value 0.844 was recorded by the isolate no. 17_{MaMd} and the minimum OD value 0.557 was recorded by the isolate no. 2_{TrNy}, after 48 h of incubation. Shrestha *et al.* (2005) based on the study on the effect of temperature on

the growth of *Erwinia* spp. reported that the optimum growth of bacteria was recorded at 27-28°C.

The growth of bacteria was very low at temperature of 37°C. At 37°C the maximum OD value (0.435) was recorded by isolate no. 11_{ErKl} and minimum OD value (0.261) by isolate no. 2_{TrNy}. This indicates difference in the temperature tolerance ability of the isolates. Goto and Mantumoto (1987); Gallois *et al.* (1992); Hadas *et al.* (2001); Yap *et al.* (2004); Akbar *et al.* (2015) also reported that the *Erwinia* spp. can survive at 36°C-37°C. The bacteria survived at temperature of 39°C and the OD values recorded were almost nearer to that of control.

The bacterial cultures were incubated in the medium with different pH and found that the maximum growth was recorded at pH 7.0 with an OD value in the range of 0.629-0.655 followed by pH 7.5 with an OD value in the range of 0.448-0.470 after 48 h of incubation. The growth of bacteria was very low at acidic condition (pH 5) and alkaline condition (pH 9) because the OD values recorded were almost nearer to that of control. This assures the role of liming in the management of rhizome rot. Similar results were also reported by Usha (2003). According to Shrestha *et al.* (2005) the most favorable pH for the growth of *Erwinia* spp. was recorded as 7.0 and none of the pathogens can grow at pH $\leq$ 5.0 and 10.0 $\geq$.

Variation was observed in the OD values of bacterial isolates grown in the medium with varying pH. The maximum OD value, 0.655 was recorded by the isolate no. 11_{ErKl} and 17_{MaMd} and the minimum OD value, 0.629 was recorded by the isolate no. 8_{PaMn} after 48 h of incubation (Table.10 a,b and Figure. 2). The differences observed in the OD value at varying pH containing medium showed the presence of variability in isolates.

Based on the results of cultural, morphological, biochemical and physiological characterization dendrogram was constructed and, the bacterial isolates were grouped into six groups. Group one includes four isolates viz. isolate no. 3_{TrNd}, 7_{PaKl}, 11_{ErKl} and 17_{MaMd}. Group two includes three isolates viz. isolate no. 5_{KoPr}, 12_{ThMl} and 14_{ThAh}. Group three includes three isolates viz. isolate no. 1_{TrKz}, 6_{PaKr} and 15_{KaNg}. Group four includes three

isolates viz. isolate no. 4_{KoAo}, 9_{ThMn} and 16_{TuTt}. Group five includes three isolates viz. isolate no. 10_{ThKr}, 13_{ThKn} and 18_{KaTa}. Group five includes two isolates, viz., isolate no. 2_{TrNy} and 8_{PaMn}. Similar grouping of the *Erwinia* isolates was carried out by Snehalatharani and Khan (2010) and Nagaraj *et al.* (2012).

5.3.5 Molecular characterization:

Molecular characterization is one of the major tool for studying variability and characteristics of pathogen. The procedures carried out for molecular characterization includes total genomic DNA isolation, PCR amplification of 16S rRNA gene, sequencing of the amplicons of isolates, homology and phylogenetic analysis.

Neumann *et al.* (1992) developed a rapid method of DNA isolation from gram negative bacteria by using Sodium Chloride Tris EDTA (STE) buffer. Nishiguchi *et al.* (2002) Cheng and Jiang (2006); Rafiei *et al.* (2015) used the improved standard phenol/chloroform method using STE buffer based on the procedure given by Neumann *et al.* (1992) but they eliminated the lysis step using SDS, lyzozyme or proteinase K and directly lysis was done using phenol. Same modified protocol for DNA isolation is followed in the present study also. Total genomic DNA of the bacteria from different samples collected was isolated using STE buffer/NaCl buffer. DNA pellets were obtained after centrifugation of the mixture of isopropanol and aqueous layer obtained after adding phenol chloroform. The pellets were ethanol washed, air dried and dissolved in distilled water and used for further analysis.

The purity and concentration of the isolated total genomic DNA of the bacteria were checked using the Nano Drop. UV absorbance is recorded at 260 nm (A_{260}) and 280 nm (A_{280}). In which nucleic acid shows absorbance peak at 280 nm whereas the protein shows absorbance peak at 260 nm. The ratio of absorbance at 260 nm to 280 nm ($A_{260/280}$) was calculated and thereby determined the purity of DNA. If the value of $A_{260/280}$ is in between 1.8 to 2.0 which means it is a pure DNA and free from contaminants whereas $A_{260/280}$ value is lower than 1.8 it represents protein contamination and if the value is above 2.0 it represents RNA contaminations.

All the DNA samples got $A_{260/280}$ in the range 1.8 and the inference derived from this was that the isolated DNA is having high purity without RNA and protein contamination. The DNA was visualized by agarose gel electrophoresis followed by observing the gel using Gel documentation system. The DNA bands obtained were intact without any contamination and the gel picture was saved for further analysis.

According to Janda and Abbott (2007) the 16S rRNA gene sequences are the common housekeeping marker genes for the taxonomic and phylogenetic studies. The function of this gene was not changed from time to time so it can be used to find evolutionary relationships and 16S rRNA gene is nearly 1500 to 2000bp so it large enough for bioinformatic studies.

The genomic DNA containing 16S rRNA gene was amplified using a pair of 16S rRNA universal primer fD1 and rP2 by thermocycling of 35 cycles as reported by Weisberg *et al.* (1991) and Kwon *et al.* (2000). There was no amplification at the already reported annealing temperature of 58°C. By performing gradient PCR the annealing temperature was standardized at 64.2°C.

The PCR of DNA samples of 18 isolates were carried out using fD1 and rP2 primers at the standardized temperature (64.2°C). The double stranded DNA is denatured and then annealed with a pair of 16S RNA primer such as fD1 and rP2 which are complimentary to the opposing strands of DNA. The enzyme DNA polymerase recognizes this small region of primer sequences of the duplex DNA as a substrate and in the presence of deoxy nucleotide tri phosphates it synthesizes the complementary strands.

Colony PCR was also carried out in which the denatured bacterial colony suspension was used to carry out the PCR thermocycling. The 16S rRNA universal primer 8F and 1522R were used in colony PCR as reported by Yap *et al.* (2004); Rahmani *et al.* (2006); Frank *et al.* (2008) and Lima *et al.* (2011, 2012)

The PCR products were analyzed using two percent agarose gel. Under UV transilluminator, PCR products produced fluorescence and clear DNA bands with no contamination with an amplicon size of 1.5 kb when the gel was visualized using Gel documentation system as reported by Kwon *et al.* (2000)

One representative samples from each group (Group 1- isolate no. 11_{ErKl}; Group 2- isolate no. 5_{KoPr}; Group 3- isolate no. 15_{KaNg}; Group 4- isolate no. 9_{ThMn}; Group 5- isolate no. 13_{ThKn}; Group 6- isolate no. 8_{PaMn}) was sequenced with specific set of primers. The specific set of universal 16S rRNA primer is already reported so cloning was not carried out in the present study. The sequencing was carried out by an outsourcing agency - Sci. Genome Lab Pvt. Ltd, Kochi.

5.4 DATA ANALYSIS:

5.4.1 Homology analysis of PCR product sequences

The merged sequences in the FASTA format were used to carry out the homology analysis using BLASTn software. The results obtained from the BLAST analysis showed that the bacterial isolates were similar to *Pectobacterium carotovorum* ssp. *carotovorum and Pectobacterium carotovorum* ssp. *brasiliense* with query coverage in the range of 94%-98% and identity in the range of 88%-94%. Samples belonging to group 1, 2, 3 and 4 showed similarity to *Pectobacterium carotovorum* ssp. *carotovorum*; Samples belonging to group 5 and 6 showed similarity to *Pectobacterium carotovorum* ssp. *brasiliense.* Weisberg *et al.* (1991) and Kwon *et al.* (2000) also carried out BLAST analysis to find the homology of *Erwinia/Pectobacterium* spp.

5.4.2 Phylogenetic analysis

Barcode gaps were generated by aligning the six 16S rRNA sequence of two sub species of *Pectobacterium carotovorum.* The two sequences of *Pectobacterium carotovorum* ssp. *brasiliense* showed considerable variability as compared with other four sequences of *Pectobacterium carotovorum* ssp. *carotovorum* and there by the barcode gaps were identified.

Kwon *et al.* (2000) had given the detailed procedure of phylogenetic tree construction. Same procedure was followed to construct the phylogenetic tree using MEGA6 software with six 16S rRNA sequences. Similarly various researchers had constructed the phylogenetic tree using MEGA software. Kwon *et al.* (2000) and Thapa *et al.* (2011) had constructed phylogenetic tree of *Erwinia* spp.

After analyzing the six 16S rRNA sequences, representatives of each group, variability at subspecies level was identified. The isolate no. 11_{ErKl} of group 1, isolate no. 5_{KoPr} of group 2, isolate no. 15_{KaNg} of group 3 and isolate no. 9_{ThMn} of group 4 belongs to *Pectobacterium carotovora* ssp. *carotovorum*. The isolate no. 13_{ThKn} of group 5 and isolate no. 8_{PaMn} of group 6 belongs to *Pectobacterium carotovora* ssp. *brasiliense*.

The findings of present study are useful for screening of banana germplasms for rhizome rot resistance. Generation of barcode of pathogen for quarantine related programmes is the future line of work.

Summary

SUMMARY

The study entitled "Molecular characterization of *Erwinia* species causing rhizome rot in banana was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara and Banana Research Station (BRS) during the period 2015-2017. The objectives of the present study were collection and isolation of rhizome rot pathogen, characterization of pathogen by cultural, morphological, biochemical, physiological and molecular (16S rRNA sequences) methods and variability of pathogen.

1. The rhizome rot infected samples were collected from banana fields of 14 different locations in the districts Thrissur, Palakkad, Ernakulam, Kollam and Trivandrum of Kerala and also from four different locations in the districts Kanyakumari, Madhurai and Tuticorin of Tamilnadu. Isolation of pathogen from the samples by the standard tissue maceration method yielded 18 isolates. The bacterial colonies were small, cream to yellowish in colour, slightly raised and mucoid with a diameter in the range of 2.3 to 2.5mm. To confirm the pathogen, 48 h old bacterial culture was streaked on the *Erwinia/Pectobacterium* specific crystal violet pectate (CVP) medium. It produced characteristic fissures or pits of *Erwinia* spp., and thereby confirmed the pathogen.

2. The pathogenicity tests were carried out in three different ways viz. inoculation in rhizome bits (*in-vitro*), rhizome of three months old plants (*in-vivo*), and pseudostem of two months old tissue culture plants (*in-vivo*) on banana var. Nendran with 48 h old bacterial culture. The rhizome bits inoculated with bacterial culture produced rotting symptoms after eight days of inoculation. Inoculation of three months old banana at collar region produced rotting of rhizome in 37 days after inoculation. Inoculations of two months old tissue culture plants produced characteristic symptoms in six days and complete rotting after 16 days of inoculation. The differences in time for symptom development and severity of symptoms were recorded between isolates.

3. Cultural characterization was carried out by culturing in four different media viz., nutrient agar (NA), yeast extract glucose calcium carbonate (YGC) agar medium, Logan's medium. Nutrient agar, yielded cream to slightly yellowish, mucoid, slightly raised convex colonies with entire margin. The bacterial growth in the nutrient broth was

recorded at different intervals and variation was observed between isolates. The maximum OD value recorded at 72h was of 0.840 and minimum OD value recorded was 0.555.

4. On the YGC medium cream to yellow coloured semi mucoid colonies with a clear zone around was developed after 24 h of incubation. Small bubbles were observed on the top of each bacterial colony due to the emission of CO_2 from the YGC medium which is the characteristic feature of *Erwinia/Pectobacterium* spp. The indigoidine pigment was not produced by any of the bacterial cultures on the YGC medium which is the characteristic feature of *Erwinia chrysanthemi.* Hence it is confirmed that all the bacterial isolates belong to *Erwinia carotovora.*

5. The bacterial culture inoculated in Logan's medium and incubated at 28±1°C produced small to medium colonies of 1.6 to 2mm diameter with purple colour having entire margin after 24 h. *E. carotovora* produces pink to purple colonies as the bacteria reduced tetrazolium in the Logan's medium whereas *E. chrysanthemi* produces completely red to purple colonies. Hence it is confirmed that all the bacterial isolates belong to *Erwinia carotovora/Pectobacterium carotovorum.*

6. Morphological characterization of the bacterium was carried out by staining viz., Gram staining, capsule staining, flagellar staining and microscopic examination. The bacteria appeared as red coloured short rods indicating the Gram negative nature. The size of the bacterial isolates varied significantly between 3.10 µm-0.95 µm x 1.10 µm-0.57 µm. The bacterium was found to be non-capsular and peritrichous in nature.

7. Biochemical characterization was carried out by various test viz, solubility in KOH, potato and carrot soft rot test, pectate degradation, growth in NaCl containing medium, pectate degradation and catalase test. The bacterial suspension mixed with the three per cent KOH produced viscous filament which also confirmed the gram negative nature. The potato and carrot slices inoculated with bacterial culture produced rotting symptoms with the emission of characteristic foul smell in three to four days and five to seven days after inoculation respectively. The bacterial isolates were resistant or insensitive to erythromycin but susceptible or sensitive to streptomycin which is a positive result for

Erwinia carotovora/Pectobacterium carotovorum. Bacterial growth was observed in the inoculated peptone broth containing three and four percent NaCl indicating salt tolerant nature of the bacterium.

8. The bacterial culture inoculated on CVP medium produced characteristic fissures or depressions in five days due to pectate degradation. In some of the isolates, the pits are surrounded by opaque gel containing non degraded pectin or pectin substrate. The bacterial smear on the glass slide when covered with 30 volume of H_2O_2 produced air bubbles due to the gas production which indicated the positive catalase activity of the bacteria.

9. Physiological characterization was done by growing the bacterium at different temperature and pH. The maximum growth was recorded at 27°C followed by 29°C after 48 h of incubation. The bacterial growth was very low at 37°C. The maximum growth was recorded at pH 7.0 followed by pH 7.5 after 48 h of incubation. The most favorable pH for the growth of *Erwinia/Pectobacterium* spp. was observed as 7.0. Differences were observed in OD value of isolates under varying temperature and pH.

10. The molecular characterization was carried out by total genomic DNA isolation followed by PCR amplification of 16S rRNA gene as well as by colony PCR. All the DNA samples got $A_{260/280}$ in the range 1.8 and the inference derived from this was that the isolated DNA is having high purity without RNA and protein contamination.

11. Based on the results of cultural, morphological, biochemical and physiological characterization dendrogram was constructed and the 18 bacterial isolates were grouped into six groups.

12. Total genomic DNA isolated was subjected to PCR analysis using universal 16S rRNA primers produced amplicons of 1.5 Kb size.

13. Sequencing of the amplicons of representative isolates of six groups were carried out and the sequences obtained are used for homology analysis, phylogenetic analysis and phylogenetic tree construction. All the six groups of bacterial isolates were belonged to *Erwinia carotovora/Pectobacterium carotovorum* but variation was observed at

subspecies level of the six groups, two subspecies viz., *Pectobacterium carotovorum* ssp. *carotovorum* and *Pectobacterium carotovorum* ssp. *brasiliense* were observed.

14. Barcode gap of *Pectobacterium carotovorum* ssp. *carotovorum* and *Pectobacterium carotovorum* ssp. *brasiliense* was also assessed by aligning the six sequences using 'Clustal W' software.

15. The phylogenetic tree was constructed with the six sequences using 'MEGA 6' software. The two subspecies of *Erwinia carotovora/Pectobacterium carotovorum* were discriminated in the phylogenetic tree.

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Annexures

ANNEXURE I

Composition of media used for culturing the pathogen

1) Composition of nutrient agar (NA) medium

Ingredients	gl^{-1}
Peptone	5.000
Beef extract	5.000
Glucose	5.000
NaCl	5.000
Agar	15.000
Final pH	6.8

2) Composition of crystal violet pectate (CVP) medium

Ingredients	gl^{-1}
Sodium polypectate	18.000
Sodium hydroxide	0.360
Sodium nitrate	2.000
Calcium chloride.H2O	0.600
Crystal violet	0.0015
Sodium lauryl sulphate	0.100
Agar	4.000
Final pH (at 25°C)	7.2±0.2

3) Compostion of yeast extract glucose calcium carbonate (YGC) agar medium

Ingredients	gl^{-1}
Yeast extract	10.000
Dextrose/glucose	20.000
Calcium carbonate	20.000
Agar	15.000

4) Composition of nutrient broth

Ingredients	gl^{-1}
Peptone	5.000
Beef extract	5.000
Glucose	5.000
NaCl	5.000
Final pH	6.8

5) Logan's medium

Ingredients	**gl^{-1}**
Nutrient Agar	28.000
Yeast extract	5.000
Glucose	5.000
0.5% 2,3,5 tri phenyl tetrazolium chloride	10mL
Final pH	6.8

6) Peptone water containing NaCl

Ingredients	**g / 100 mL**
Peptone	1.000
4% NaCl	4.000
3% NaCl	3.000

7) Luria Bertani (LB) broth

Ingredients	**g / 100 mL**
Casein enzymic hydrolysate	10.000
Yeastextract	5.000
Sodium chloride	10.000
Final pH	7.5

ANNEXURE II

Reagents used for DNA isolation

1) STE extraction buffer

i. 150 mM NaCl – 0.876 in 100 mL

ii. 100 mM EDTA – 3.72 in 100 mL

iii. Tris HCL 10 mM – 0.1576 in 100 mL

Separately prepared and mixed together to make NaCl buffer and autoclaved before using.

2) Sodium dodecyl sulphate (10%)

10 g of sodium dodecyl sulphate put in 100 mL distilled water and mixed well. The mixture was stored in refrigerator at 9°C.

3) Phenol : Chloroform (1 : 2 v/v)

To one part of phenol, one part of chloroform was added and mixed properly.

4) Ethanol (70%)

To seventy parts of absolute ethanol thirty parts of distilled water was added and mixed well. 70% alcohol stored at 9°C.

ANNEXURE III

Composition of buffers and dyes used for gel electrophoresis

1. TAE buffer (50X)

Tris base	: 242 g
Galcialaceitic acid	: 57.1 mL
0.5M EDTA (pH- 8)	: 100 mL

2. Loading dye (6X)

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol in water

3. Ethidium Bromide

The dye was prepared as a stock solution of Ethidium bromide (stock 10 mg/mL; working concentration 0.5 µg/mL (Genie) and was stored at room temperature in a dark bottle.

4. Agarose gel

Gels with two different compositions were made: 0.8 per cent for Genomic DNA and 2 per cent for PCR samples.

ANNEXURE IV

16S rRNAPrimers used in PCR

SI no.	Name	Sequence
1.	fD1	5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3'
2	rP2	5'CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT -3'
3	8f	5'- AGA GTT TGA TCC TGG CTC AG -3'
4	1522R	5'- AAG GAG GTG ATC CAG CCG CA -3'

ANNEXURE V

List of laboratory equipment used for the study

SI NO.	Equipment	Stage used	Company
1.	Laminar Air Flow Cabinet	Pathogen isolation and culture preparation	Rotek, B&C
2	Incubator shaker	Incubation and shaking	Lab companion SI-600
3	Accublock digital dry bath	DNA isolation	Labnet, Labnet international Inc.
4	Vortexer	DNA isolation	GeNei™
5	High speed refrigerated centrifuge	DNA isolation	Kubota 6500
6	Nanodrop®spectopotometer ND- 1000	Qualitative assessment of nucleic acids	GeNei
7	Laminar Air Flow Cabinet	Preparation of PCR reaction mixture	Rotek, B&C
8	Proflex PCR system	Polymerase chain reaction	Applied Biosystems
9	Electrophoresis unit	Agarose gel electrophoresis	GeNei
9	Gel Doc ™ XR+	Gel documentation	BIO-RAD
10	Ultra low temperature freezer	Storage of DNA samples	Haier BIO-MEDICAL

ANNEXURE VI

16S rRNA sequences of *Erwinia carotovora* (*Pectobacterium carotovorum*) submitted in NCBI

>GROUP1_E16S_11 (*Pectobacterium carotovorum* ssp. *carotovorum*)

AAATGTCCAATCCTACGGAGCACCAGTGGGATTTGCCATTGGGCAGCTGTATGCAGCATGCGGTGTATGGAGAGGCCTTCCGTT
GTAAGTACTTCCAGCCGGGAGGAAGTGCTGATGTATAAACCCAGCGAATGACGTACCCGCAGAAGAAGCCACTGGCTAACTCC
GTGCCAGCAGCCGCGGTAATACCGAAGGGTGCAAGCGTTATCGGAATTTCTGGGCGTAAGCGCACGCAGGCGGTCTGTCAAGTC
CGGATGTGAAATCCCCGGGCTTCAACCTGGGAACTGCATTCGAAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGGTAGAATTC
AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTTCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
CGTGGCTTTCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTTGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAG
ATGGATTGGTGCCTTTGGGAACTTTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTG
GGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGGTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGC
GAGAGCAAGCGGACCTCATAAAATGCGTTGTAGTCCGGATTGGAGTTTGCAACTTGACTCCATGAAGTTGGAATCGCTAGTAAT
TGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAG
TAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGGAAGTCGTAGCATTGATCTTGCCCC

>GROUP2_E16S_5 (*Pectobacterium carotovorum* ssp. *carotovorum*)

GCTGACTGAACCGGTCGATCTACGAGCAGCAGTGGGAATTGCCATGGCAAGCCTGATGCAGCCATGCGCGTTATGAAGAGCTCG
GTGTAGTACTTCAGCGGAGAAGTGTGAGTATAACTCAGCATTGACGTACCGCAGAAGAAGCACCGCTAACTCCGTGCAGCAGCC
GCGGTATACGAAGGGTGCAAGCGTAATCGAATTACTGGGCGTAAAGCGCACGCAGGCGTCTGTCAAGTCCGGATGTGAAATCC
CCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGGCTAGAGTCTGTAGAGGGGGGTAGAATTCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGC
TAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTTGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTT
TGGGAACTTTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAG
TCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC
CTCATAAAGTGCGTCGTAGTCCGGATTGGAGTTTGCAACTTGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATG
GTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACC
TTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGGAAGTCGAGTAATGAAGTACCCCCCC

>GROUP3_E16S_15 (*Pectobacterium carotovorum* ssp. *carotovorum*)

CTAGCGTCGAGAGCAGTCATGAAGCTGAACGTCAGATCCTACGAGCAGCATGGATATGGCCAATTAGGCCAGCTGATGCAGCAT
GCGCGGTTAGAGGAGCCTTCGGGTTGTAAGTACCTCAGCGGGAGGAAGGTGTGAGTATAACTCAGCAATTGACGTACCGCAGA
AGAAGCACCGCTACTCCGTGCAGCAGCCGCGGTATACGAGGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGC
AGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAG
GGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGA
CTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTTCACGCCGTAAACGATGTCGACTTGGAG
GTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCA
GAGAACTTTCCAGAGATGGATTGGTGCCTTTGGGAACTTTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAA
GAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTTTGCAACTTGACTCCATGAAGTC
GGAATCGCTAGTAATTGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
GGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATCATGACTGGGGGAAGTCGTAGCGATGA
GCAACGCCCCC

>GROUP4_E16S_9 (*Pectobacterium carotovorum* ssp. *carotovorum*)

AGTACGCATGACTGAACCGTTCCAAATCTAACGAAGCAGCATTGGAATTTGCACAATGGCAGCCTGATGCAGCAGGCCGTGTAT
GAAGAGCTTCGGTGTAAGTACTCAGCGGAGAAGGTGTGAAGTAATAACTCAGCAATTGACGTAACGCAGAAGAAAGCACGGCT
AACTCCGTGCAGCAGCCGCGGTATACGAAGGGTGCAAGCGGTAATCGAATTACTTGGCGTAAAGCGCACGCAGGCGGTCTGTCA
AGTCCGGATGTGAAATCCCCGGCTCAACCTGGGAACTGCATTCGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTT
CAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCCTGA
GGCGTGGCTTTCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTGATGCAACGCGAAGAACCTTACCTACTTTTGACATCCAGAGAACTTTCCA
GAGATGGATTGGTGCCTTTGGGAACTTTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCT
CGCGAGAGCAAACGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTTTGCAACTTGACTCCATGAAGTTGGAATCGCTAGT
AATTGTAGATCAGAATGGTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAG
AAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGGAAATTGGAAAAATTGATTTTTTTGCAC
CC

>GROUP5_E16S_13 (*Pectobacterium carotovorum* ssp. *brasiliense*)

ACATGCTGCGCAGCTACCATGCAGTCGAGCGGCAGCGGGGAGTACTTTGCTCCTTTGCCGGCGAGCGGCGGACGGGTGAGTAGT
GTCTGGAAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTATCTAATACCGCATAGCGTCGCGAGACCAAAGAGGGGG
ACCTTCGGGCCTCTTGCCATCATATGTGCCCATATGGTATTAGCTAGTAGGTGGGGTAGGGGCTCCCCTAGGCCACTATCCCTAG
CTGGTCTGAGAAGATGACCACCCACTCTGGATCTGACACACGGTCCACACTCCCACGGGAGGCAGCAGTGGGAAATATTGCACA
GTGGGCGCACGCCTGATGCACCCATGCCGCGTGTATGAAAAAGGCCTTCGGGTTGTAAAGTATTTTCAGCGGGGAAGAGGGTGT
TGAGGTTAATAACCTCCAATATTGATTTTACCCGCAAAAAAAGCCCCGGATAACTCCGTGCCAGCCGCCGCGGTAATACAGAGG
GTGCAAGCGTATCTCAAAATTACTGGGCGTAAAGCGCACGCACGCGGTCTGAAAAGTCAGATGTGAACTCCCCGGGCTCACCGT
GAAAACTGTTTTCAAAACTGGCAGGCTAGAGTCGTGTAGAGGGGGGTAAATTTACGTGTGTAGCGGTGAAGTGTGTAGAGATCT
GGAGGAATACCGGTGGAAAAGGCGTCCCCGTGGACACAGACTGACTCTCATGTGCGAAAGCGTGGAGAGCACACAAGATTAGA
TACTCTGATAGTCCACGCCGTACACTATGTCCATGTGGATGGTGTGCCCTTGACGCGTGTTTTCGGGATATCACGTGAAAAGTTA
ACCCGCCTGGAGAGCACGCTCGCGGGGTTAACTCTCATATGTATTGACGGGTGCCCGCACAAGCTGTGGGATCATGTGTTTTATT
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>GROUP6_E16S_8 (*Pectobacterium carotovorum* ssp. *brasiliense*)

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MOLECULAR CHARACTERIZATION OF *Erwinia* SPECIES CAUSING RHIZOME ROT IN BANANA

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The rhizome rot or tip over disease affecting the rhizome caused by *Erwinia* spp. is a major and emerging issue which leads to a substantial economic loss in banana. The widely cultivatedbanana varieties like Grand Naine, Rasthali and Nendran are very susceptible to the disease. Different *Erwinia* species are reported to cause the disease. Understanding the correct etiology of the disease and variability of the pathogen based on molecular characterization is very much important in crop improvement programmes.

Isolation of pathogen from infected rhizomes from different locations yielded 18 bacterial isolates. In nutrient agar medium, the bacterial colonies were small, cream to yellow in colour, slightly raised and mucoid. The bacterium when streaked on the crystal violet pectate medium produced fissures or pits characteristic of *Pectobacterium* spp.

The pathogenicity tests were carried out by inoculation in rhizome bits (*in-vitro*), rhizome of three months old plants (*in-vivo*) and pseudostem of two months old tissue culture plants (*in-vivo*) of banana var. Nendran. Inoculation of rhizome bits and inoculation at collar region of three months old banana plants produced rotting symptoms after eight and 37 days respectively. Pseudostem inoculation of two months old tissue culture plants var. Nendran also produced characteristic rotting symptoms after six days.

After proving pathogenicity, the 18 bacterial isolates were characterized by cultural, morphological, biochemical, physiological and molecular methods. Cultural characterization was carried out by using nutrient agar (NA), yeast extract glucose calcium carbonate (YGC), Logan's medium and nutrient broth. On nutrient agar, cream to slightly yellowish, mucoid, slightly raised convex colonies with entire margin varying in size were formed. The bacterial growth in the nutrient broth was recorded at different intervals and variation in OD values was observed between isolates. On YGC medium, cream to yellow coloured semi mucoid colonies were formed with a clear zone around each colony after 24 h of inoculation. Small bubbles were observed on the top of each bacterial colony due to the emission of CO_2 which is the characteristic feature of *Pectobacterium* spp. In Logan's medium small to medium colonies of 1.6 to 2 mm diameter with purple colour colonies were formed, which is the characteristic feature of *Erwinia carotovora*/*Pectobacterium carotovorum*.

Morphological characterization of the bacterium was carried out by staining viz., Gram staining, capsule staining, flagellar staining. The bacteria appeared as red coloured short rods indicating the Gram negative nature with 3.10 µm-0.95 µm x 1.10 µm-0.57 µm in size. The bacterium was non-capsular in nature with peritrichous flagella.

Biochemical characterization of the bacterial isolates were carried out by conducting different test viz., solubility in KOH, potato soft rot test, carrot soft rot test, intrinsic antibiotic resistance, growth in three and four per cent NaCl, growth in CVP medium and catalase test. The bacteria, in three per cent KOH produced viscous filament. The potato and carrot slices inoculated with bacterial culture produced rotting symptoms with the emission of characteristic foul smell in three to four days and five to seven days respectively and variation was observed between isolates. The bacterial isolates showed resistance to erythromycin and susceptibility to streptomycin. Bacterial growth was observed in the inoculated peptone broth containing three and four percent NaCl. The bacterial culture inoculated on CVP medium produced characteristic fissures or depressions due to pectate degradation. Catalase test was positive for all the isolates.

Physiological characterization of the bacterium was carried out by growing at different temperature and pH. The maximum growth was recorded at 27°C and at pH 7.0. Based on the results of cultural, morphological, biochemical and physiological characterization of the 18 bacterial isolates, dendrogram was constructed which classified the isolates into six groups. The molecular characterization of bacterial isolates was carried out by total genomic DNA isolation, PCR amplification of 16S rRNA gene and sequencing. Colony PCR of 18 bacterial isolates were also carried out. The sequences obtained for six isolates, representative of each group were used for homology analysis, phylogenetic analysis and phylogenetic tree construction. All the six groups of bacterial isolates were belonged to *Pectobacterium carotovorum* but variation was observed at subspecies level. Out of the six groups, there were two subspecies viz., *Pectobacterium carotovorum* ssp. *carotovorum* and *Pectobacterium carotovorum* ssp. *brasiliense*. The Barcode gap of *Pectobacterium carotovorum* ssp. *carotovorum* and *Pectobacterium carotovorum* ssp. *brasiliense* was assessed and the sequences were deposited in the NCBI GenBank.