2.1 Introduction

Identification of plant pathogens

Plant diseases create challenging problems in commercial agriculture and pose real economic threats to both conventional and organic farming systems. Plant pathogens are difficult to manage for several reasons. First of all, plant pathogens are hard to identify because they are so small. The positive identification of a pathogen often requires specialized equipment, training, and in some cases accurate diagnosis in the field is difficult (Nostro et al., 2000). Plant pathogens are constantly changing and mutating, resulting in new strains and new challenges to growers (Steven et al., 2000).

Bacterial diseases in Centella asiatica

Centella asiatica has a historical reputation for boosting mental activity and for helping a variety of systemic illness, such as high blood pressure, rheumatism etc. Due to high demand, this plant is being cultivated in many places of Tamilnadu both for use within India and for exporting purposes (Zheng and Qin, 2007). Centella suffers from many diseases like powdery mildew, downy mildew, bacterial leaf spot etc. Different pathogenic organisms including fungi and bacteria have been reported from the infected leaves of Centella plants (Manoharachary et al., 2003; Dubey and Pandey, 2008; Rakotoniriana et al., 2007).

Centella leaf spot disease

A bacterial leaf spot disease caused by Xanthomonas campestris pv centellae (Xcpvc) has been the major problem in cultivation of C. asiatica. The quality of the plant is very much affected by this and it affects marketing and income. Bacterial leaf spot of Centella
incited by the bacterium *Xcpvc* has resulted to 40-80% crop loss every year. The bacterium gets entrance into the leaves through the water pores or wounds and progress to the vascular system. Veins are blackened with leaf tissue browning in irregular shapes (Singh, 1998; Romain and Raemakers, 2001). It has a wide range of hosts especially of *Centella* plants. This disease is a direct effect of toxins produced by the bacterium. The bacteria persist for a few months in crop refuge and enter through stomatal cavities (Nardozzi and Kopiski, 2003).

A critical problem in the study of bacterial pathogens is the correct identification of the infectious agent. The study of diseases in plants is an important aspect of assessing plant health. Hence this chapter deals with the identification and isolation of *Xanthomonas campestris pv centellae* from the infected plant, *C. asiatica*. 


2.2 Review of Literature

Araujo and Robbs (2000) reported that the new bacterial disease on grapevine (*Vitis vinifera*) in Brazil causing severe damage to local crops. Further, they identified the causal agent by morphological, physiological and biochemical test as *Xanthomonas campestris pv. viticola*.

Dubey and Pandey (2008) described a new leaf spot disease of *Centella asiatica* L. was encountered during a survey for diseases of medicinal plants in nurseries and gardens of Jabalpur. The incidence of disease was during monsoon season and damaged 60-70% of the plants. The pathogen responsible for the disease was identified as a new species of *Pseudocercospora* namely *P. centelli* sp. nov.

Hayward (1983) studied the bacterium (*Xanthomonas campestris pv. viticola*) isolated from the samples (leaves, petioles, canes and bunches) on nutrient agar by streak plate method. Maximum populations (46 colonies/plate) recovered from canes and petioles after three days of incubation. Very low population (2 colonies/plate) was recovered from infected leaf.

Karavina et al. (2008) conducted the experiments to detect and characterize *Xanthomonas phaseoli* (E. F. SM) in seed lots collected from commercial seed dealers and farmers who retain common bean (*Phaseolus vulgaris* L) seeds for future planting. The experiments confirmed the presence of seed borne *X. phaseoli* in both retained and certified seedlots in Zimbabwe, with the former having significantly higher bacterial population levels.
**Malavolta et al. (1999)** described symptoms of bacterial leaf spot of grape as dark and angular leaf spots that causing necrotic areas and leaf blight. Cankers were often observed on petioles. Further, based on characterized by biochemical, cultural, physiological and pathogenicity tests identified as *Xanthomonas campestris pv. viticola*.

**Manoharachary et al. (2003)** studied the taxonomy and biodiversity of micro fungi in the leaves of *Centella asiatica* which showed spots with chestnut brown margins and a pale central region. They identified the pathogen as *Cercospora* sp. The microscopic characteristics of the pathogen did not match any of the known species including a new host. They described new species *Cercospora centellae* sp.nov.

**Mariano and Gama (2005)** observed that the bacterial colonies were yellow, mucoid, slimy, glistening, convex and round in shape, isolated from infected leaves in case of *Xanthomonas campestris pv. viticola* in grape.

**Mitrev and Kovacevic (2006)** have isolated the bacteria from the spots of pepper plants, surveys in open-field during 1996-2001 in Macedonia. Symptoms similar to natural symptoms were reproduced following inoculation on pepper leaves. The strains were negative for amylolytic activity and ability to utilize cis-aconitate and belong to type A of *Xanthomonas axonopodis pv. vesicatoria*, and were identified to race P0 and race P2. All strains were sensitive to copper sulfate and streptomycin sulfate and were homogeneous according to their Polymerase Chain Reaction (PCR).

**Rakotoniriana et al. (2007)** isolated fungal endophytes from leaves of *Centella asiatica* (Apiaceae) collected at Angoro (Middle Eastern region of Madagascar, 200 km from Antananarivo). Forty- five different taxa were recovered. The overall foliar colonization rate was 78%. The most common endophytes were the non-sporulating species 1 (IF 19.2%)
followed by *Colletotrichum* sp.1 (IF 13.2%), *Guignardia* sp. (IF 8.5%), *Glomerella* sp. (IF 7.7%), an unidentified ascomycete (IF 7.2%), the non-sporulating species 2 (IF 3.7%) and *Phialophora* sp. (IF 3.5%).

Schaad and White (1974) found that the bacterial colonies on nutrient agar are white, round, smooth, glistening with entire margin. On SX agar produced a clear starch digestion zone (3–4 mm). Bacterium is gram negative, rod shaped with rounded ends, motile by single polar flagellum and 0.4 - 1.2 × 2 – 3 μm in size.

Syeda et al. (2010) studied the production, characterization and identification of virulence factors produced by virulent isolates of *Pseudomonas syringae* pv. *sesami* (Psse-08/NARC 1) and *Xanthomonas campestris* pv. *sesami* (Xcs-08/NARC). Identification was made on the basis of earlier published reference data. Previously, toxins were detected by the phytotoxic as well as growth inhibition assays.

Viana (2006) found that, bacterial colonies were deep yellow, slimy, irregular to round in shape, when isolated on yeast dextrose calcium carbonate agar medium (YDCA) from infected leaves and cane of bacterial blight of grapes.
2.3 Materials and Methods

Classification of *Centella asiatica* (L.) Urban

Class        : Dicotyledenae  
Sub class:     Polypetalae  
Series:          Calyciflorae  
Order:           Umbellales  
Family:         Umbelliferae (Apiaceae)  
Genus:          Centella  
Species:        asiatica

Description of *Centella asiatica*

Habit-Perennial herb, Root-The plant consists of vertical root stalk, Stem- is prostrate slender, creeping with long stolens and nearly glabrous or hairy on young parts. This filiform stems have long internodes and rooting at nodes, Leaves- are cordate or hastate or orbicular or reniform or sub entire or palmately lobed consisting of long petiole and small stipules. Crowded leaves can be seen at nodes, consisting of very long stalks and sheathing leaf bases Leaf blades are dentate, crenate with thick radiate veins and dark green in colour Leaves are glabrous on both surfaces (Plate 1). Flowers-Flowers are small, sessile and dark pink in colour. They arise as simple umbels of 3-6 flowers at the ends of slender peduncles arising from the axils of leaves and much shorter than petioles supported below by an involucre of 2-boat shaped membranous persistent bracts, Fruits-Clusted at joints; Carpels oblong, sub-cylindric curved and less in length much laterally compressed, readily separating into 2
indehiscent halves (mericarps) united by a very narrow plane of junction. Vittae are seen in the furrows of each mericarp. The fruits when bruised have an aromatic odour, pungent, nauseous and bitter taste, Seeds-Solitary in each mericarp, pendulous embryo, laterally compressed.

*Xanthomonas campestris* (Pammel 1895) Dowson 1939

**Taxonomical position**

- **Kingdom:** Bacteria
- **Phylum:** Proteo bacteria
- **Class:** Gamma proteo bacteria
- **Order:** Xanthamonadales
- **Family:** Xanthomonadaceae
- **Genus:** Xanthomonas
- **Species:** campestris
- **Host:** Centella plant

**Description and *Xanthomonas campestris***

*X. campestris* is an aerobic, rod-shaped, Gram-negative bacteria characterized by its two cell walls and yellow pigment (Plate 1). It has a filamentous structure called hypersensitive response and pathogenicity (Hrp) pili. Host associated, over 20 different pathovars of *X. campestris* have been identified by their distinctive pathogenisity on a wide range of plants including crops and wild plants. This bacterium is mesophilic with optimal temperature at 25-30°C (77-85°F) and inactive at temperatures below 10°C (50°F) (Charles, 2007). The hypersensitive response and pathogenisity (Hrp) pili that help transfer effector proteins to decrease the host’s defense and glide through water (David and Charles, 2007).
They can live in a soil for over a year and spread through any movement of water including rain, irrigation and surface water (Weber et al., 2005).

**Isolation of Xcpvc**

**Sample collection**

The infected Centella leaves showing the typical symptoms of bacterial spot were collected during the year 2010 from farmer’s fields of major *Centella* growing areas of Tirunelveli and Tuticorin districts in Tamilnadu (Map 1 & Table 2.1).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Districts</th>
<th>Area of collection</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Tirunelveli</td>
<td>Cheranmahadevi</td>
<td>2010</td>
</tr>
<tr>
<td>2.</td>
<td>Tuticorin</td>
<td>Kurumboor</td>
<td>2010</td>
</tr>
</tbody>
</table>

The collected samples were transported to Plant Molecular Biology Research Unit (PMBRU) in fresh condition in plastic bags for further analysis. All the leaves showed typical external symptoms like yellowish brown spots, decolouration, minute water soaked lesions, cankers lesions and irregular yellow and brown patches.

**Isolation of Xcpvc from infected Centella leaves**

Small pieces of infected leaves were cut aseptically from the edge of typical spots along with a little portion of healthy tissue. The infected leaf bits were surface sterilized in 70% alcohol or 1% sodium hypochlorite and washed in three series of sterile water to remove traces of alcohol. The infected leaf bits were then suspended in a small test tube containing 3 ml sterilized distilled water for 10 min. When water became slightly turbid due to oozing of bacterial cells from the cut ends of the diseased tissue, the bacterial suspension was serially
diluted in 9 ml sterile distilled water. Then one ml of the diluted bacterial cell suspension was poured into sterilized petriplates containing nutrient agar. The plates were rotated gently in clockwise and anti-clockwise direction, so as to distribute the bacterial cell suspension uniformly in the plates and to obtain well separated bacterial colonies. The inoculated plates were incubated at 28ºC for 72 hours. Observations were made for development of well separated light yellow, convex, small bacterial colonies on the nutrient agar medium.

**Isolation of the bacteria on semi- selective media**

One ml of the diluted bacterial cell suspension was poured into sterilized separated petriplates containing two selective media mTBM and mMD5A. The plates were rotated gently in clockwise and anti-clockwise direction, so as to distribute the bacterial cell suspension uniformly in the plates and to obtain well separated bacterial colonies. The inoculated plates were incubated at 28ºC for 4-7 days. Observations were made for development of well separated light yellow, convex, mucoid, small bacterial colonies on the two semi- selective media.

**Purification of bacterial culture**

The suspected bacterial colonies were picked up with the help of sterilized inoculated loop and streaked onto the surface of yeast extract dextrose calcium carbonate agar (YDCA, Schaad and Stall, 1988). The inoculated plates were incubated at 28ºC for 48 to 72 hours and the observations were made for the development of well separated light yellow coloured bacterial colonies. The purified bacterial colonies were streaked on nutrient agar slants and stored at 5ºC in refrigerator and also in sterile distilled water taken in small culture tubes, by suspending 2-3 loops full of the bacterial culture for future use.
Identification of *Xcpve*

The morphological characteristics such as cell shape, gram reaction, capsule and spore staining characters of the isolate was studied as described by society of American Bacteriologists, Bradbury (1970) and Schaad and Stall (1988).

**Physiological and biochemical characters**

The physiological and biochemical characters of the isolated bacterium was studied for hydrolysis of starch, gelatin liquefaction, indole production, hydrogen sulphide production, urease production and acid from different sugars *viz.*, glucose, mannose, arabinose, galactose, fructose, lactose, maltose, sucrose and alcohol. The tests were conducted as per the methods described by Bradbury (1970) and Schaad and Stall (1988).

**a. Utilization of glucose, sucrose, fructose for acid production:**

The acid and gas production by the isolate was tested by using basal medium supplemented with bromothymol blue. 10 ml of basal medium was dispensed in each test tube. Small Durham’s tubes were introduced into these tubes in inverted position. The complete set was sterilized in an autoclave for 15 minutes. To these tubes, filter sterilized carbohydrates *i.e.*, glucose, fructose, sucrose were added at 0.1% of 24 hours old bacterial culture and incubated at 28°C for 72 hours. Observations were made for gas production in Durham’s tube and change in colour of the medium in case of acid production.

**b. Utilization of asparagines as sole source of carbon and nitrogen**

Asparagine can be utilized both as carbon and nitrogen source by some of the phytopathogenic bacteria and not by others. The test was carried out in the following medium.
Solution 1: $K_2HPO_4$: 8 g, $KH_2PO_4$: 2 g, Distilled water: 100 ml.
Solution 2: $MgSO_4\cdot7H_2O$: 2 g, $FeSO_4$: 0.5 g, $NaCl$: 1 g, $MnSO_4$: 0.02 g, $H_2SO_4$: 1 drop, Distilled water: 100 ml.
Solution 3: $Na_2MoO_4$: 0.02 g; Distilled water: 100 ml.
Solution 4: $CuSO_4$ saturated solution in distilled water

10 ml of each solution was mixed in the order 3, 4, 2, 1 as described above and filtered and added 960 ml distilled water and 2 g per L asparagines. The medium was dispensed in 5 ml quantities in tubes and autoclave. Then the medium was inoculated with the test bacterium and incubated at 25-30 °C and examined for growth. Positive growth indicates utilization.

c. Catalase test

A loopful of 48 hours slant growth of the test bacterium was smeared on a slide and covered it with a few drops of 20 volume hydrogen peroxide. The reaction will found to be positive if gas bubbles are produced.

d. Gelatin liquefaction

The following medium (nutrient gelatin) was used.

The tested medium contained Peptone (10.0 g), beef extract (5.0 g), gelatin (20.0 g), water (1000 ml) and pH (7.0). All the ingredients were mixed together and heated over a water bath until the gelatin has dissolved. The medium was dispensed in tubes to a depth of about 4 cm and sterilized at 15 lb pressure for 20 min. The tubes were cooled and allowed to stand at 20°C for two days to check the sterility. These gelatin columns were inoculated by stabbing a straight inoculation needle charged with 48 hours growth of the test bacterium.
The tubes were incubated at 28ºC and observed for liquefaction of the gel column at intervals upto one month. The form in which the gel liquefied was recorded.

e. Methyl red reaction

The following medium (methyl red broth) was used for the test.

The employed medium contained Protease peptone (5.0 g), glucose C.P. (5.0 g), K$_2$HPO$_4$ (5.0 g), distilled water (1000 ml) and pH (7.0). The medium was dispensed in 5 ml quantities in tubes and sterilized by steaming for three min for three successive days. The tubes were inoculated with the test bacterium (48 hours culture) and incubated the tubes for 7 days at 28ºC and a few drops of 0.02% methyl red in 50% alcohol was added to the culture tubes. If a distinct red indicates methyl red positive (methyl red is orange-red at pH 4.5 and lower).

f. Reduction of nitrate to nitrite

Nitrate broth with following composition was used.

The tested medium contained Peptone (10 g), beef extract (5.0 g), KNO$_3$ (1 g) and distilled water (1000 ml). The ingredients were dissolved by heating in water bath. The tubes were dispensed to a depth of 5 cm and autoclaved. The broth was inoculated with the test bacterium and incubated at 28ºC. The nitrate reduction was tested upto 15 days at regular intervals. A few drops of sulphanilic acid (0.8% in 5N acetic acid) and dimethyl-alpha-naphthylamine (0.5% in 5 N acetic-acid) was added to the nitrate broth culture. Nitrite is present if the mixture becomes distinct pink or red. No colour would mean that nitrate is present as such or has been reduced to ammonia and free nitrogen. To confirm either of these two possibilities few zinc crystals were added to the above broth reagent mixture and shaked for a few minutes. Nitrates are present without reduction if the broth becomes pink or red.
No colour in either of the above two tests would mean that nitrate is reduced to ammonia or free nitrogen.

g. Urease test

The following basal medium was used.

The employed medium contained NH$_4$H$_2$PO$_4$ (0.5 g), K$_2$HPO$_4$ (0.5 g), MgSO$_4$.7H$_2$O (0.2 g), NaCl (5.0 g), yeast extract (1.0 g), agar (20.0 g), phenol red (0.012 g) and pH (6.8).

The basal medium was dispensed in 90 ml quantities in flasks, autoclaved and cooled to 45°C. To each flask 10 ml of 20% filter sterilized urea solution was added, mixed well and dispensed in tubes in 5 ml quantities and allowed the medium to solidify in a slanting position. The slants were inoculated with the test bacterium, incubated and observations were recorded at regular intervals upto 15 days. If the medium changes from yellow to red, urease production is positive.

h. Indole production

Tryptophan broth medium was used in this test.

The tested medium contained Tryptophan or casein digest (10.0 g), NaCl (5.0 g), water (1000 ml) and pH (7.0). The medium was dispensed in tubes and autoclaved. To detect indole production the Gnezda oxalic acid test strips were prepared as follows. Whatman No. 1 filter strips (5 × 50 mm) were soaked in warm saturated solution of oxalic acid and cooled down. The strips gets covered with oxalic acid crystals and dried at room temperature and used without sterilizing. Tryptophan broth tubes were inoculated with the test organism and inserted an oxalic acid test strip as directed in H$_2$S test. The tubes were incubated at 28°C and
observed for colouration of oxalic acid crystals at regular intervals for 14 days. If indole is produced, the oxalic acid crystals on test strip become pink or red.

i. **Hydrogen sulphide production**

The following medium (peptone water) was used.

The employed medium contained Peptone (10 g), NaCl (5.0 g), water (1000 ml) and pH (7.0). The medium was dispensed in 5 ml quantities in tubes and autoclaved. To detect H$_2$S the lead acetate test strips were prepared as follows. Whatman No. 1 filter paper was cut into 5 × 50 mm strips which are then soaked in warm saturated solution of lead acetate. The strips were then dried, autoclaved and again dried at 60ºC. The medium in each tube was inoculated with a loopful of 48 hours slant growth of the test bacterium. After inoculation a test strip was inserted in between the plug and inner wall of the tube, so that it hangs just above the broth. The tubes are incubated at 28ºC and observations were recorded at regular intervals upto 14 days. The blackening of test strip indicates liberation of H$_2$S.

j. **Oxidase test**

For oxidase test, 48 hours slant growth of the test bacterium was streaked on a filter paper saturated with 1% tetramethyl-para-phenylene-diamino-dihydrochloride. The reaction will be positive if a red or purple colour appears within 10 seconds. The reaction will be delayed positive if the colour appears in 10 to 60 seconds.

k. **Starch hydrolysis**

The employed medium was referred to as starch broth contained peptone (10.0 g), beef extract (5.0 g), starch soluble (2.0 g), agar (20.0 g), water (1000 ml) and pH (7.0). The medium was sterilized by autoclaving and poured into sterilized petriplates. The test culture
was spot inoculated in four plates. The plates were inoculated at 25 °C and test for starch hydrolysis, one plate at a time, after 2, 4, 7 and 14 days as follows. The agar surface was flooded with Lugol’s iodine and allowed reacting a few minutes for development of colourless zone around the bacterial growth.

**Pathogenesis tests on Centella plants**

Pathogenesis test was carried out to find out whether the isolated bacteria was capable of producing typical symptoms of bacterial spot under artificial inoculation condition on *Centella* seedlings or not. *Centella* seedlings were raised in sterilized soil in nursery in small polythene bags. Thirty days to forty days old seedlings were transplanted to sterile soil contained in 11 inches plastic pots for the purpose of inoculation of the bacterial isolate. The strain of *Xanthomonas campestris pv centellae* from *Centella* was multiplied in nutrient broth taken in Earlenemayer’s flask by inoculating a loopful of bacterial culture to nutrient broth. The inoculated flask was incubated at 28 °C for 72 hours. Bacterial suspension was prepared by adjusting cell concentration to $5 \times 10^7$ CFU per ml in spectrophotometer at 480 nm (spectronic 20 D, Milton and Roy, USA). The 6 to 8 weeks old plants were pre incubated for 24 hours in humid tent made up of plastic sheets in which humidity was maintained between 60 to 80% before the inoculation. The leaves of plants were slightly injured by insect pin and sprayed with bacterial suspension. The inoculated plants were kept in the plastic tent for two days in which high humidity was maintained by spraying sterile water inside the tent at 25 to 30 °C. The plants were taken out from the plastic tent and kept in glasshouse. Observations were made for the development of symptoms of bacterial spot. Plants similarly, injured and sprayed with sterile water constituted control. The bacterium was reisolated and compared with the original culture of *Xcpvc* (reference strain MTCC 2286) by studying the type of staining morphology of the colonies and biochemical characters.
Inoculation methods

a. Vein inoculation

The middle and tertiary veins of the selected healthy Centella leaves were injected with $2 \times 10^8$ CFU/ml of bacterial cells with the help of hypodermic syringe. Later, plants were incubated in glasshouse and observed for the development of symptoms.

b. Pin pricking

Leaves of about 4 to 6 weeks old seedlings were selected and washed with tap water. Later leaves were punctured with sterilized pin at several place and the culture prepared as above ($2\times10^8$ CFU/ml) was applied to both the surface of leaves with the sterilized cotton wool and plants were incubated in glasshouse till symptoms developed.

Preparation of mMD5A agar medium

This medium was modification of the MD5A agar medium (Cubeta and Kuan, 1986) from which it differs in antibiotics composition and concentration. The amounts of phosphate salts have also been adjusted to achieve the correct pH without further adjustment.

Preparation of mTBM agar medium

This medium is a modification of Tween Medium B (McGuire et al., 1986) from which it differs in adding 10.0 g/l skim milk powder and leaving out 0.4 mg/l tobramycin and 0.25 g/l CaCl$_2$. 
2.4 Results and Discussion

The study of diseases in plants is an important aspect of assessing plant health. A critical problem in the study of bacterial pathogens of the plants is the correct identification of the infectious agent. But the isolation and identification of plant disease causative organisms was successfully done by the plant researchers. Pathovars of *Xanthomonas* are known to cause diseases on several vegetable and cash crops (Mandavia *et al.*, 1999). *Xanthomonas* is a very important kind of phytopathogenic bacteria, which causes the plant diseases all around the world.

**Isolation of Xcpvc from infected Centella leaves**

The ooze test was conducted by cutting a small bit of the leaf tissue from the infected leaves and suspending it in a drop of sterile water taken on a microscopic glass slide and observing under low power objective of the microscope. Within few minutes the bacterial ooze started jetting out from the cut ends of the infected tissue, thus revealing the association of bacteria with the disease.

Isolation was made from the bacterial ooze obtained from the infected leaf tissue in sterile distilled water followed by dilution plate technique on nutrient agar which produced typical *Xanthomonas* colonies within 72 hours. The colonies were yellow, slimy, glistening and round in shape (Plate 2).

**Isolation of the bacteria on semi-selective media**

Isolation was made from the bacterial ooze obtained from the infected leaf tissue in sterile distilled water followed by dilution plate technique on two semi-selective media
namely mTBM and mMD5A produced typical *Xanthomonas* colonies in 4-7 days. The colonies were pale yellow, mucoid, slimy, glistening and round in shape (Plate 2).

**Purification of bacterial culture**

Well separated out colonies isolated from the infected leaves were purified by streaking on the surface of YDCA medium. The culture was stored on nutrient agar slants at 5 °C and also suspended a few loopful of culture in sterile distilled water contained in ampules. These were kept as the stock cultures for further studies. The bacterial colonies on YDCA medium were deep yellow, slimy, highly viscous and irregular to round in shape (Plate 3).

**Identification of Xcpvc**

**Morphological, physiological and biochemical characteristics**

The results of the various morphological, physiological and biochemical tests are given in Table 2.2 and Plate 3. The isolated bacterium was found to be rod shaped, obligately aerobic, gram negative, oxidase negative and monotricously flagellated, but not utilized asparagines as a sole source of carbon and nitrogen. It was positive for catalase reaction utilized glucose, fructose, sucrose for acid production, liquefaction of gelatin and produced hydrogen sulphide and did not produce indole, in addition, the strain failed to reduce nitrate to nitrites. The isolated bacterium was compared with the original culture of *Xcpvc* (reference strain MTCC 2286) by studying the type of staining morphology of the colonies and biochemical characters (Plate 3).

**a. Utilization of glucose, sucrose, fructose for acid production**

It was observed that after 72 hours of incubation, there was production of gas in Durhams tubes and colour of the medium changed to yellow.
b. Utilization of asparagines as sole source of carbon and nitrogen

After three days of incubation at 30ºC, no growth was observed. The bacterium showed negative reaction for this test.

c. Catalase test

After covering with a few drops of 20 volume hydrogen peroxide to a slide smeared by a loopful of test bacteria, there was production of gas bubbles.

d. Gelatin liquefaction

In this test, it was observed that liquefaction of the gel column in the test tube inoculated with test bacterium and no liquefaction of gel when kept as control.

e. Methyl red reaction

After adding a few drops of 0.02% methyl red in 50% alcohol to the culture tube. There was no distinct red colour. The bacterium showed negative reaction for this test.

f. Reduction of nitrate to nitrite

It was observed that, nitrate was not reduced by this bacterium and colour of the broth become pink.

g. Urease test

In this test, there was no change of medium colour in the inoculated slants from yellow to red. The bacterium showed negative reaction.
h. Indole production

There was no colour change of the oxalic acid crystals on the test strip.

i. Hydrogen sulphide production

It was observed that bacterium of test strips indicated liberation of H$_2$S. The bacterium showed positive reaction for this test.

j. Oxidase test

It was observed that no red or purple colour appeared within 10 seconds.

k. Starch hydrolysis

A colourless zone around the bacterial growth in contrast to the blue background of the medium was observed.

**Pathogenisity tests on Centella**

The strain of *Xcpvc* artificially inoculated to 30 days old *Centella* seedlings, by spraying the bacterial culture to pre-injured leaves. The plants started producing small water soaked lesions on the leaves. The lesions became necrotic leading to severe leaf spots of infected leaves. The first symptoms of the disease observed 7 days after inoculation of the plants. Re-isolations of the bacterium made from artificially inoculated plants yielded yellow coloured colonies on YDCA and the strain was confirmed as *Xanthomonas campestris pv centellae* (Plate 3).

Karavina *et al.* (2008) conducted the experiments to detect and characterize *Xanthomonas phaseoli* in seed lots collected from commercial seed dealers and farmers.
Olsen et al. (2002) have established the methods for detection of the bacteria *Xanthomonas translucens* causing bacterial blight on barley seed in Arizona. Popovic et al. (2010) conducted laboratory assays to detect and identify *Xanthomonas axonopodis* pv. *phaseoli* on bean seeds collected from naturally contaminated commercial crops. Said et al. (2003) identified and characterized *Xanthomonas campestris* pv. *campestris* (Xcc) strains causing Black rot on Cabbage from Tanzania. Mariano and Gama (2005) observed that the bacterial colonies were yellow, mucoid, slimy, glistening, convex and round in shape, isolated from infected leaves in case of *Xanthomonas campestris* pv. *viticola* in grape. Viana (2006) found that, bacterial colonies were deep yellow, slimy, irregular to round in shape, when isolated on yeast dextrose calcium carbonate agar medium (YDCA) from infected leaves and cane of bacterial blight of grapes. The similar result was observed in the present study.

Dubey and Pandey (2008) described a new leaf spot disease of *Centella asiatica* L. which was encountered during a survey for diseases of medicinal plants in nurseries and gardens of Jabalpur. The incidence of disease was during monsoon season and damaged 60-70% of the plants. The pathogen responsible for the disease was identified as a new species of *Pseudocercospora* namely *P. centelli* sp. nov. Rakotoniriana et al. (2007) isolated fungal endophytes from leaves of *Centella asiatica* (Apiaceae) collected at angoro (middle eastern region of Madagascar, 200 km from Antananarivo). Manoharachary et al. (2003) studied the taxonomy and biodiversity of micro fungi in the leaves of *Centella asiatica* showed spots with chestnut brown margins and a pale central region. They identified the pathogen as Cercospora sp. But in the present study *Xanthomonas campestris* pv. *centellae* which cause leaf spot disease was isolated from the leaves of *Centella asiatica*. Further, it was identified by morphological, physiological and biochemical tests.
2.5 References


