3.1. Introduction

In India, the black rot disease was first reported on cabbage from Bombay (Patwardhan, 1928). It was later reported from Katrain (Kullu district) area of Himachal Pradesh (Patel *et al.,* 1949; Rao and Srivastava, 1964). Since then the occurrence of the disease has also been reported from other parts of the country. Black rot of cabbage is a very destructive disease and causes losses by premature defoliation and also affects the quality of heads in cabbage with a crop loss upto 50%. The severity of the disease was recorded in cabbage susceptible cultivars like *Golden acre* and *Pride of India*. The plants may be affected at any time during the growth period *i.e.,* from the stage of young seedling till maturity. In cabbage, the earliest symptoms occur as necrotic lesions on cotyledons or the lower leaves of the seedling.

Xanthomonas campestris pv. *campestris* (Pammel) Dowson is the causal agent of black rot of crucifers, which is possibly the most important disease of crucifers worldwide (Williams, 1980). *X. campestris* pv. *campestris* is a small, rod shaped, aerobic, gram-negative, nonspore-forming bacterium (Onsando, 1992). The bacterial cells infect cabbage through hydathodes at the leaf margins, causing V-shaped lesions, or through stomata, causing round lesions. Another study shows that *X. campestris* pv. *campestris* normally gains entry into plants via hydathodes. Once inside the plant, *X. campestris* pv. *campestris* colonizes the vascular system where it produces an extracellular polysaccharide (EPS) called xanthan, which can obstruct the xylem vessels, causing tissue necrosis and severe leaf wilting (Williams, 1980; Onsando, 1992). In field-grown cabbages containing the major *f*-gene for black rot resistance derived from the cultivar *Early fuji*, the symptoms are either a localized marginal necrotic lesion of variable size, with a distinct dark rim at a minute dark area at the infected hydathode. The primary sources of the bacterium are infected plant residues (Dane and Shaw, 1994; Arias *et al.,* 2000) and seeds, in which *X. campestris* pv. *campestris* cells can survive up to three years (Clayton, 1925). The pathogen colonizes the vascular system and is able to move systemically in the plant. The secondary spread of the bacterium, proceeding from infected plants, is supported mainly by blowing rain, sprinkler irrigation (Kocks *et al.,* 1999), transmission by a wide range of host plants including cruciferous weeds (Schaad and Alvarez, 1993)

and by an epiphytic survival also on non-cruciferous crops acting as a shelter for the pathogen in the field (Arias *et al.,* 2000).

Our approach was to establish protocols based on PCR for detection of *X. campestris* pv. *campestris* in cabbage seeds since PCR has the advantage of specificity, sensitivity and ability to detect the pathogen in a short time. Further, this study attempts to correlate the PCR data with disease incidence. Culturing of bacteria is a time-consuming process, particularly for fastidious or slow-growing bacteria. An alternative is to study the genetic variation of bacterial ribosomal genes amplified by PCR. The 16S rRNA gene is highly conserved and is found in all bacteria. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hyper variable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification. Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species, or even genera. However, it is also contains variable regions that can be used to discriminate between bacteria of different genera and species. It can be used to screen for unknown sequence variations to genotype known sequence variations or to detect sequence variations to detect both known and unknown variations. Hypersensitive reaction and pathogenicity *(hrp*) proteins are the main components of the Type III Secretion System (TTSS) in plant pathogenic bacteria (Lindgren, 1986) and are required for delivery of the secreted substrate proteins, which consist of virulence effectors and the so-called avirulence (avr) proteins. The *hrp* gene cluster of *Xanthomonas* spp. contains genes for the assembly and function of a type (TTSS). The *hrpF* genes reside in a region between *hpaB* and the right end of the *hrp* cluster.

Multiplex PCR allows the simultaneous and sensitive detection of different DNA or RNA targets in a single reaction. On the other hand, PCR detection protocols can be designed to verify the presence of more than one pathogen in plant material by looking for common specific sequences in two or more of them, or to detect related viruses or bacteria on multiple hosts.

3.2. Materials and Methods

3.2.1. Isolation and characterization of *Xanthomonas campestris* pv. *campestris* using biochemical and molecular methods.

The plant materials were collected from different fields of Mysore district and the seed samples were procured from local seed agencies were subjected to screeing the laboratory following different seed health testing methods *viz.*, direct plating method, liquid assay method and growing on test.The plant materials were collected from different fields of Mysore district. While collecting the plant specimen information regarding the sowing date, fertilizer used, pesticides used was also recorded. These samples were brought to laboratory for isolation of pathogen. Thin sections of leaf or stem lesions with a drop of sterile distilled water was observed under microscope for the presence of typical bacterial ooze. The suspension was aseptically recovered from the slide and plated onto Nutrient agar (NA) medium (peptone 5.0 g/l; beefs extract 3.0 g/l; agar 20.0 g/l). The isolated pathogens were subjected to biochemical and molecular characterization. Small pieces of the infected leaves were cut aseptically and surface sterilized in 1% sodium hypochlorite and washed thrice with sterile distilled water. The bacterial suspension was serially diluted in 9 ml sterile distilled water. Then one ml of the diluted bacterial suspension was poured onto nutrient agar plates. The inoculated plates were kept at 28° C for 72 h. Observations were recorded for the typical colony characteristics on nutrient agar medium.

The different cabbage cultivars were collected from the commercial seed traders in Mysore. The seeds were subjected to various seed health testing methods in accordance with International Seed Testing Association (ISTA, 2009) guidelines. The conventional testing methods followed were Direct plating method (DPM), Grind and soak method (G and S), growing-on test (GOT) to screen the different cabbage cultivars for the presence of *Xanthomonas campestris* pv. *campestris.*

Direct Plating method: Seed assays were performed on all cabbage cultivars by plating the seeds on nutrient agar (NA) and Star's minimal (SX) agar (a semi-selective medium), as described by Schaad (1988). The seeds were sterilized by dipping in 1% sodium hypochlorite solution for 30 s and rinsing the seeds in sterile

distilled water (SDW) for three minutes to remove traces of sodium hypochlorite. The seeds were then placed on sterile blotter paper to dry. Twnty five sterilized seeds were plated in each petriplate. Each seed sample was replicated four times. After three days, the plated seeds were visually assessed for the presence of *X. campestris* pv. *campestris* colonies under a stereomicroscope (Schaad, 1988).

The Grind and Soak method: From each cabbage cultivar, 15 randomly selected seeds were surface sterilized in 1% sodium hypochlorite for 30 s, rinsed in sterile distilled water (SDW) for 2-3 minutes and then dried on sterile filter paper. The dried seeds were ground, and then soaked in 5ml of phosphate buffer saline (PBS), pH 7.4, for three hours. The resultant supernatant collected after filtering was used to prepare serial dilutions and were plated onto NA and SX and incubated at 28 ±1° C for two days (Schaad, 1988).

Screening of different cabbage cultivars for black rot incidence under green house conditions

A glasshouse pot experiment was conducted to determine the presence of *X. campestris* pv. *campestris* in seedlots through the "Growing-On" detection technique by observing symptoms on growing plants. Seeds were planted in pots that contained heat-sterilized coir pith. Three seeds were planted in each pot in five replicates of each cultivar at a depth of 10 mm. The seed were assessed for emergence success. The seedlings were thinned down to one plant per pot. Necessary control measures were used to control aphids and other leaf-suckers. An occasional spray of Neem (*Azadirachta indica*) was given at an interval of every 15 days. The pots were covered with a clear polythene sheet at 14 days after crop emergence. A hand-held laboratory sprayer was used to atomize distilled water to create a fine mist for maintaining a high humidity environment inside the polythene sheet conducive for disease development. After a 10 to 14-day incubation period, the polythene plastic was removed, and the leaves and stems assessed for *X. campestris* pv. *campestris*-induced symptoms. The disease spread toward midrib was recorded on the basis of 0-5 scale: The following rating scales for the visual disease estimation were employed as per Singh *et al.* (1987): 0 - no visible symptoms (Immune I); 1) 1-5% infection (Resistant, R); 2) 6 -15% infection (Moderately resistant MR); 3) 16-30% infection (Moderately susceptible, MS); 4) 31 - 50% infection (Susceptible, S) and 5- >50%

infection (Highly susceptible, HS). The plants were kept in the glasshouse for further observations on symptom development. The colonies that developed from both the leaf material and those isolated from cabbage seeds were subjected to a number of morphological, biochemical tests, for the confirmation of the pathogen.

3.2.2. Biochemical characterization of bacterial isolates

Identification and characterization of the black rot pathogen was carried out by subjecting the bacterial isolates to various biochemical tests, such as Gram staining, potassium hydroxide (KOH) solubility test, Kovac's oxidase test (Hilderbrand and Schroth, 1972) starch hydrolysis, Lipase activity and Arginin dehydrogenase test (Lelliot and Stead, 1987), gelatin hydrolysis, oxidative/fermentative metabolism of glucose and catalase tests. The strains were also subjected to the hypersensitive reaction in tobacco (*Nicotiana tobaccum*) plants (Carlton *et al*., 1998) and pathogenicity test (Kauffman and Rao, 1972). Each test was conducted with four replicates and repeated twice.

Gram's staining: The Gram reaction of each isolate was determined following the staining procedure as follows. First, thinly spread bacterial smear was prepared on a clean slide. The slide was dried in air and fixed by heating. The dried smear was flooded with crystal violet solution for one minute and washed in tap water for few seconds. It was again flooded with iodine solution for one minute and washed and blot-dried. It was then decolorized with 95% ethyl alcohol by applying drop by drop until no more colour flows from the smear, the slide was washed and blot dried. Finally slides were counter stained for about 10 seconds with safranin, washed and examined under compound microscope with different magnifications.

Potassium hydroxide (KOH) solubility test: Gram staining results were confirmed by potassium hydroxide test (KOH) 3% (Suslow and schroth, 1982). The main principle behind this test is that the lipopolysaccharides found in the cellwall gets dissolved in 3% KOH solution and forms a mucoid thread. A loop full of The bacteria were aseptically removed from culture plates with sterilized tooth pick, placed on glass slide in a drop of 3% KOH solution and stirred for ten second using a quick circular motion of hand, then the tooth pick was raised a few centimetres above the slide and observed for the formation of viscous strand represent the bacterium as

gram-negative.

Kovac's oxidase test: A 24 h-old-bacterial colony on nutrient agar, supplemented with 1% glucose was used in this assay. A loopful of the inoculum was rubbed on filter paper impregnated with 4-5 drops of freshly prepared aqueous solution of Tetramethyl-p-phenylene diamine dihydrochloride and observed (Kovacs, 1956; Hilderbrand and Schroth, 1972).

Starch hydrolysis test: Starch is an insoluble polymer of glucose, some bacteria possess the ability to produce amylase that breaks starch into maltose and the amylase is an extra cellular enzyme which is released from microorganism. Starch agar plates (soluble starch 2.0 g/l; peptone 5.0 g/l; beefs extract 3.0 g/l; agar 20.0 g/l dissolved the nutrient agar powder in water by heating. Dissolve the starch in 10ml distilled water and add to molten agar) were inoculated by streaking the bacterial isolates and incubated for 4 days at 27±2° C. Plates were flooded with Lugol's iodine solution and observed for appearance of clear zone of hydrolysis around the bacterial growth indicates starch has been hydrolyzed (Lelliot and Stead, 1987).

Lipase activity: Tween 80 is a lipid and this can be hydrolyzed only by lipase producing bacteria.Tween 80 medium was prepared, (Peptone10.0 g/l; NaCl 5.0 g/l; $CaCl_2$, H_2O 0.1 g/l; Agar 15.0 g/l; Water 1000 ml) sterilized and poured into sterilized petri plates. Tween 80 agar plates were inoculated with the bacterial isolates and incubated up to 7 days (Lelliot and Stead, 1987).

Catalase test: Nutrient agar (Peptone5.0 g/l; Beef extract 3.0 g/l; Agar 20.0 g/l; distilled water 1000 ml) was prepared and sterilized it. Media was poured into sterilized petri plates. Bacterial culture was streaked on to plates and were incubated at 28-30° C for 2 days. After incubation plates were flooded with 3% hydrogen peroxide. Few drops of 3% hydrogen peroxide was added on the surface of 48 h-old-culture of each isolate and bubble formation was recorded as positive for catalase activity (Dickey and Kelman, 1988).

Gelatin Hydrolysis: Test tubes containing gelatin medium (Yeast extract 3.0 g/l, Peptone 5.0 g/l, Gelatin 120.0 g/l), was homogenised and dispensed into the test tube to a depth of 5 cm. and autoclaved at 121°C for 15 mins, were stab inoculated with the bacterial isolates and incubated for 7-14 days. Every 2-3 days the

liquefaction of the medium was recorded. On the final day, tubes were cooled at 5° C for 30 minutes before reading the results (Lelliott and Stead, 1987).

Hypersensitive Reaction: Most of the bacterial plant pathogens can induce hypersensitive reaction when injected into the tissue of a non-susceptible host plant. Non-pathogenic bacteria and some plant pathogens do not incite this hypersensitive response. Aqueous bacterial suspension was prepared from 24-48 h old bacterial culture using distilled water. The optical density was approximately adjusted to 0.3-0.4 at A_{610} nm which gives 10^8-10^9cfu/ml with UV-visible spectrophotometer (Hitachi. U-2000, Japan). The suspension was infiltered into the leaf lamina of tobacco plant using hypodermic needle. Rapid collapse and water soaking of inoculated tissue within 24 h to 48 h followed by a dry, light brown localized necrosis within 3 days indicates the positive reaction (Lelliot and Stead, 1987).

Pathogenicity Test: Cabbage seeds of known susceptible cultivars were sown in earthen pots (10 cm dia) and seedlings were raised in green house conditions following normal agronomical practices. The bacterial isolates were grown on YDC broth and 24 h old culture were centrifuged at 3500 rpm for 5 min and the pellets were redisolved in sterile saline. Cell suspension was adjusted to 1x 10^8 cfu/ml by adjusting OD at A_{610} nm to 0.45 in an UV-visible spectrophotometer (UV-visible spectrophotometer, Hitachi. U-2000, Japan) and used for cabbage seedlings. Fully expanded leaves of 4-5 weeks old plant were spray inoculated. Sterile water used as control. All inoculated plants were covered with polythene bags and maintained in green house condition. After 7-14 days, plant was observed for expression of typical symptoms of black rot (Lelliot and Stead, 1987).

3.2.3. Molecular characterization of *X. campestris* pv. *campestris*

Genomic DNA extraction: The DNA was extracted cabbage leaves, infected leaf material and seed samples using cetyl trimethyl ammonium bromide (CTAB) method. The CTAB extraction buffer (1% CTAB, 700 mM NaCl, 10 mM Tris-HCl pH 8 and 50 mM EDTA pH 8.0), for 30-40 s. After an initial homogenization, 350 µl of DNA extraction buffer (pre-warmed up to 65° C) were added and vortexed for 60 s. The samples were incubated at 65° C for 20 min for cell lysis.0.7 volume of chloroform/isoamyl alcohol (24:1) was added to the samples, mixed by hand for 5

min, and then centrifuged at 14,000 rpm for 5 min at 4° C. The supernatant was transferred to a fresh microfuge tube and extracted another time with 0.7 volume of cold isopropanol alcohol. The pellet was dried, and resuspended in sterile sterile water. The aliquots were placed in -20° C. The DNA was quantified using UV-Visible spectrophotometer (Hitachi, U-2000, Tokyo, Japan).

The isolates of *X. campestris* pv. *campestris* which were positive in various tests were taken up for the molecular assays. The total genomic DNA extraction was done following the protocol in Gabriel and De Feyter *et al.* (1992). A loopful of each isolate was suspended in 500 µl phosphate buffered saline (PBS) in microfuge tube and mixed by vortexing and centrifuged in 12000 rpm for 15 min. The supernatant and the viscous material were discarded and the pellet was washed with 1ml buffer (50 mM Ethylene diamine tetra acetate (EDTA), 0.15 M NaCl, pH 8.0) and centrifuged repeatedly. Proteinase K to final concentration of 150 µg/ml and 30 µl of Sodium Dodecyl Sulphate (SDS) were added to the suspension and incubated at 50º C for 1 h. Equal volume of Chloroform-Phenol-Isoamyl alcohol (24:25:1) buffered with 10mM Tris-HCl pH 8.0 was added for extraction. The suspension was mixed by vortexing and centrifuged at 12000 rpm for 5 min to separate the layers. The upper layer was transferred to a fresh microfuge tube and 0.1 vol, 3M Sodium acetate and 1 vol of Iso-propanol was added and mixed by vortexing. The precipitate was spooled out and rinsed with 1 ml of 70% Ethanol. It was then centrifuged for 2 min at 12000 rpm and the ethanol was removed and the pellet was dissolved with sterilized distilled water. The purity of the extracted DNA was evaluated by running on 1% agarose gel.

Polymerase chain reaction for the confirmation of *X. campestris* pv. *campestris*

The PCR was done by using primer for 16S rRNA, estA according to Lee *et al.* (2009) and *hrpF* according to Berg *et al*. (2005).

16S rRNA amplification: For determination of the identities of the colonies, a PCR assay using 16S-F3 (5'-CCAGACTCCTACGGGAGGCAGC-3') and 16S-R1 (5'-GCTGACGACAGCCATGCAGCACC-3') as primers. The PCR assay was carried out using the primer set for 16S rRNA which was custom synthesized by Sigma (Sigma Aldrich, MO, USA). The PCR amplification was performed with a thermal cycler (LabNet, NJ, USA) in a 30 µl reaction mixture containing 1µl of cell lysate,

0.35 to 0.5 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate, 1 µl reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris-HCl; pH 8.8), and 2.0 U of *Taq* DNA polymerase (BangaloreGenei, India) by using the following program: one cycle of denaturation for 1 min at 94° C and 35 cycles consisting of denaturation at 94° C for 30 s, annealing at 59° C for 30 s, and extension at 72° C for 1 min. Reaction mixtures were stored at 15° C until they were used for analysis. Amplified DNA was detected by electrophoresis in 2% agarose (Agarose I, Amresco Inc., Solon, OH, USA) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

PCR analysis with *estA* primer: The PCR assay was carried out using the primer set for *estA*. The primer sequence was as follows, Forward: 5'-TTGAGCAGGCATTCCCATGGCTTCAAC-3' and Reverse: 3'-GCCTTGGGCGCAATGCGTGGCGCACATGAC-5' were custom synthesized by Sigma (Sigma Aldrich, MO, USA). The PCR amplification was performed with a thermal cycler (LabNet, USA) in a 30 µl reaction mixture containing 1 µl of cell lysate, 0.35 to 0.5 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate, 1µl reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris-HCl; pH 8.8), and 2.0 U of *Taq* DNA polymerase (BangaloreGenei, India) by using the following program: one cycle of denaturation for 1 min at 94° C and 35 cycles consisting of denaturation at 94° C for 30 s, annealing at 58° C for 30 s, and extension at 72° C for 1 min. Reaction mixtures were stored at 15° C until they were used for analysis. Amplified DNA was detected by electrophoresis in 2% agarose (Agarose I; Amresco Inc., Solon, OH, USA) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

PCR analysis with *hrpF* primer: PCR was conducted according to Berg *et al.* (2005) using BE1(5′-CCGTAGCACTTAGTGCAATG-3′) and BE2 (5′-GCATTTCCATCGGTCACGATTG-3′) primers. The primers were custom synthesized from Sigma (Sigma Aldrich, USA). A master mix was prepared using the 10x buffer, dNTPs, *Taq* polymerase, this master mix was pipetted into each PCR tubes. To each PCR tubes 2 µl of primer was added (1 µl of forward primer and 1 µl of reverse primer) and 2.5 µl of purified 100 ng. DNA was added and the total concentration of each tube was made upto 25 µl using water PCR reagent or sterile

distilled water. The thermal cycle consisted of initial denaturation at 94° C for 5 min, annealing at 58° C for 1 min, and extension at 72° C for 10 min, followed by 30 cycles of denaturation at 94° C for 15 s, annealing 58° C for 15 s, elongation step 72° C for 15 s, and a final extension at 72° C for 10 min. Finally annealing temperature was standardized to 58° C for 15 s. The PCR tubes were given a small spin, and were placed in a PCR thermocycler (Labnet, NJ, USA).

PCR analysis with ITS primer: The ITS region of the *Brassica* spp was amplified using primers BE3 (5′-CCCGGCACGAAAAGTGTCAAG-3′) and BE4 (5′-CCTTAGCTCGGATTTTGGCC-3′). The primers were custom synthesized from Sigma (Sigma Aldrich, MO, USA).The BE3 and BE4 primers were used to amplify the ITS region of the cabbage cultivars.

Multiplex PCR: A multiplex PCR was performed using the *hrpF* (BE1 and BE2) and ITS (BE3 and BE4) primers for the simultaneous detection of pathogen in the infected leaf material collected and from the seeds of all ten cultivars. A master mix was prepared using the 10x buffer, dNTPs, *Taq* polymerase, this master mix was pipetted into each PCR tubes. To each PCR tubes 2 µl of primer was added (1 µl of forward primer (BE1, BE3) and 1 µl of reverse primer of (BE2, BE4), and 2.5 µl of purified 100 ng DNA was added and the total concentration of each tube was made upto 25 µl using water PCR reagent or sterile distilled water. Cycling conditions were: 3 min at 95° C, 40 cycles of 40 s at 95° C, 40 s at 63° C to 58° C over the first six cycles, 40 s at 72° C, followed by 5 min at 72° C. Aliquots (5 µL) of PCR products were run at 100 V for 1 h on 2% agarose gels with 1x TBE buffer containing 0.5 mg mL-1 ethidium bromide. A standard 100-bp DNA ladder (Fermentas, USA) was included on each gel. The bands were visualized under UV light.

Gel elution and DNA sequencing: The PCR product was subjected to DNA sequencing after eluting from the gel using QIAquick gel extraction kit (Qiagen, Germany). The purified samples were sent to a commercial agency (Chromous Biotech, Bangalore, India). The products were sequenced based on the Sangers' dideoxy termination method using ABI-Prism (Applied Biosystems, USA) genetic analyzer. The sequence was then analyzed using Sequence Manipulation suite (www. Bioinformatics.org).

3.2.4. Fatty acid methyl ester analysis (FAME)

The bacteial isolate confirmed by the biochemical and molecular methods was further subjected to gas chromatographic analysis of fatty acid methyl esters (GC-FAME). The bacterial cultures was sent to Royal life Sciences Pvt. Ltd, Hyderabad, India (affiliated to MIDI Sherlock, USA). The identification of the bacterial sp. was confirmed based on the unique fatty acid profiles as generated by the Sherlock Microbial Identification System.

3.3. Results

3.3.1. Isolation and characterization of *Xanthomonas campestris* pv. *campestris* using biochemical and molecular methods.

Among the thirty six isolates obtained from infected leaf material, cabbage seeds, only ten isolates were found to be positive for various biochemical tests. The colonies of positive isolates appeared as appeared as opaque, glistening with yellow, mucoid, circular and convex on the yeast dextrose calcium carbonate agar (YDC) medium. The results of the ten cultivars subjected to screening for the presence of seed-borne *X. campestris* pv. *campestris* using direct plating method, grind and soak method and growing on test method are depicted in the table (Tables 3.1and 3.2). The typical symptoms of black rot disease in cabbage seedlings and the colony morphology of *X. campestris* pv. *campestris* were observed and photographed (Figures 3.1; 3.2 and 3.3).

All the eleven cultivars screened which were brought from different seed agencies were subjected to screening for the black rot incidence under green house conditions. Out of eleven cultivars only one cabbage cv. *Pusa mukta* recorded 4% black rot incidence hence it was categorized as resistant (R) cultivar. Five cabbage cultivars *viz*., *Indam krishna*, *Indan saina*, *Gaurav*, *Unnati* and *NS 43* recorded 9 to 14% black rot incidence and categorized as moderately resistant (MR). Only two cabbage cultivars *viz*., *Quisto* and *Golden acre* recorded 43% and 45% black rot incidence and categorized as susceptible (S) cultivars. The other two cultivars *F1 bhima* and *NBH boss* recorded 63% and 66% black rot incidence, hence they were categorized as Highly susceptible (HS) cultivars (Table 3.3).

Based on the morphological criteria, the suspected colonies were subcultured onto YDC medium and subjected to characterization for further studies.

3.3.2. Biochemical characterization of bacterial isolates

Gram's staining: On gram's staining, the isolated bacterial cells were found to retain a pink to red colour stain of safrnin and were considered as gram negative (Figure 3.4 and Table 3.2). Known gram positive bacteria showed violet colour, which was compared with our isolates.

Potassium hydroxide (KOH) solubility test: In the KOH solubility test, mucous thread formation was observed with in ten second of mixing when lifted up with the tooth pick. Thus the bacterium was considered as gram negative (Figure 3.5 and Table 3.2) known gram positive bacteria strain did not shown the mucous thread formation when compared with our test isolates.

Kovac's oxidase test: There was no change in colour of the test isolates to violet after few seconds on the whatman no. 1 filter paper, impregnated with 1% aqueous solution of N, N, N,' N – Tetramethyl-paraphenylene diamine dichlroride which indicateed the bacterium was negative for kovacs oxidase test. (Figure 3.6 and Table 3.2)

Starch hydrolysis test: Starch molecules are too large to enter into the bacterial cells, so some bacteria will secrete exoenzymes that will degrade starch into subunits that can be then easily utilized by the organism. Starch agar is a simple nutritive medium with starch added. Since no colour change occurs in the medium when organisms hydrolyze starch, iodine solution is added to the plate after incubation. Iodine turns blue, purple, or black (the colour depends on the concentration of the iodine used) in the presence of starch. A clear zone of hydrolysis was formed around the bacterial colonies, when the plates were flooded with Lugol's iodine. Hence the bacterium indicated positive for starch hydrolysis (Figure 3.7 and Table 3.2).

Gelatin Hydrolysis: After 2 to 3 days of incubation, test isolates showed the liquefaction of the gelatin media when compared to the control, hence the bacterium indicated positive for gelatin hydrolysis (Figure 3.8 and Table 3.2).

Lipase activity: The inoculated Tween 80 agar plates showed the presence of white precipitate around the colonies of the bacteria, hence the bacterium indicated positive for lipase activity (Figure 3.9 and Table 3.2).

Catalase test: Catalase production and activity was observed and the indicating a positive test for *X. campestris* pv. *campestris*.

Hypersensitive Reaction: Necrosis was observed in tobacco leaves within 24 h of infiltrated with bacterial suspension, whereas sterile distilled water infiltrated leaf regions did not show any change in the leaf colour, which served as control. Thus it shows positive result for hypersensitive reaction (Figure 3.10 and Table 3.2).

Pathogenicity Test: Cabbage seedlings spray inoculated with *X. campestris* pv. *campestris* suspension showed typical symptoms 'V'- shaped lesions. Control plants inoculated with distilled water did not show any symptoms (Figures 3.11, and Table 3.2).

3.3 Molecular characterization

PCR Assay: The DNA was isolated from all the positive ten isolates, good quality DNA was observed. The isolated DNA was quantified using Spectrophotometer (Beckman Coultier, USA). The DNA was later used for amplication using three different primers. The isolates were later subjected to molecular characterization by PCR using 16S rRNA, *estA* gene and *hrpF* genes.

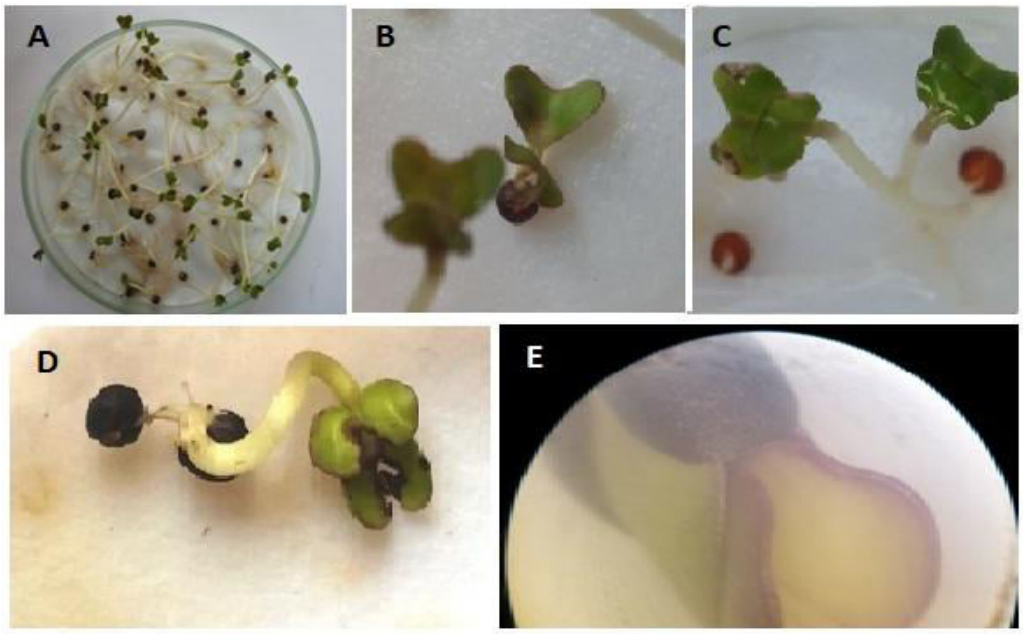

Figure 3.1. Typical symptoms of Black rot infection of cabbage seedlings.A-Germinated cabbage seedling showing vigorous growth; B and C- Cabbage seedlings showing V-shaped lesions towards the internal veins; D-showing chlorosis and blackness of nerves; E- Microscopic observation of infected cabbage seedlings showing spread of black rot infection.

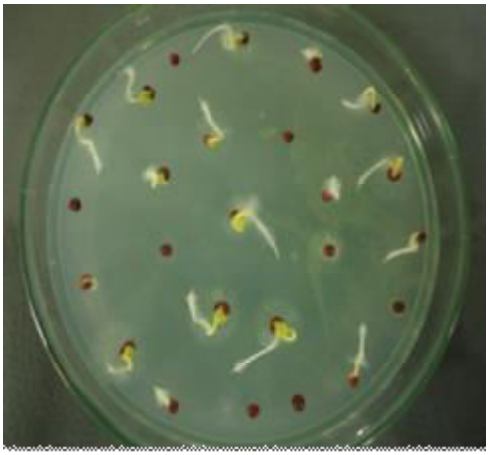

Figure 3.2. Direct plating method; Cabbage seeds showing seed-borne nature of *X. campestris* pv. *campestris* as yellow colonies.

Figure 3.3. Yellow, mucoid colonies of *X. campestris* pv. *campestris* on NSCA medium.

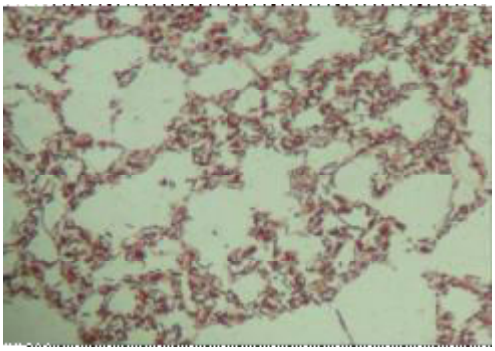

Figure 3.4. Gram's Reaction: pinkish red coloured bacterial cell showing gram negative nature of *X. campestris* pv. *campestris* cells.

Figure 3.5. Formation of mucoid thread, suggesting gram negative nature of *X. campestris* pv. *campestris.*

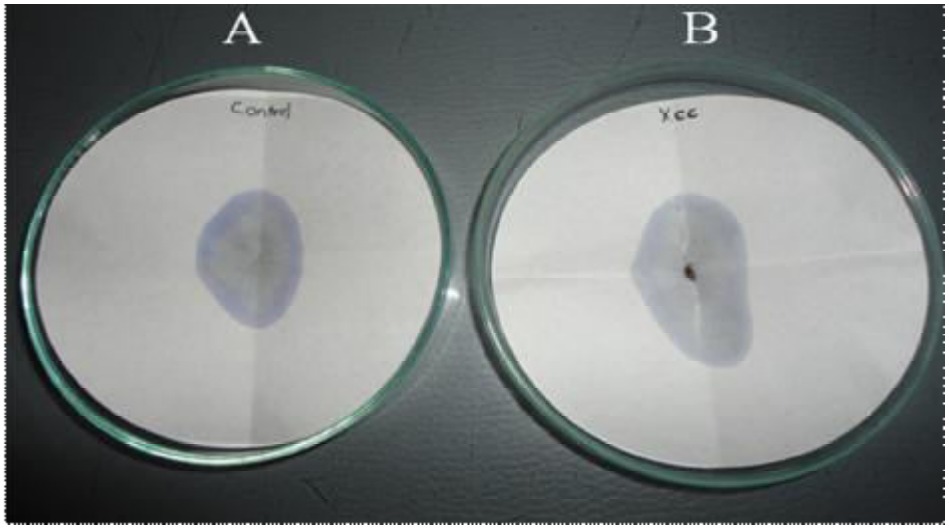

Figure 3.6. Kovac's oxidase test; A: negative; B:Change in colour to purple indicating positive nature of *X. campestris* pv. *campestris.*

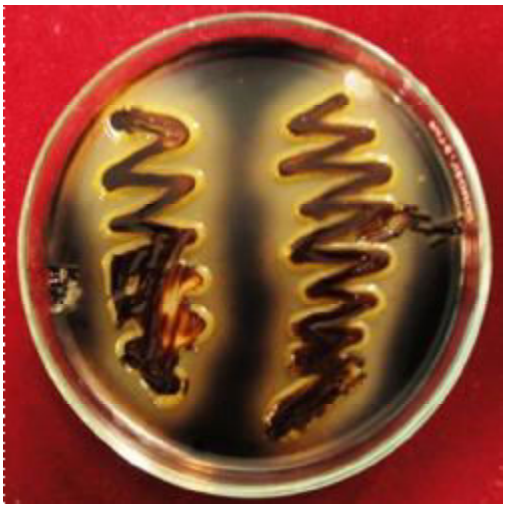

Figure 3.7. Starch hydrolysis test: A: clear zone around *X. campestris* pv. *campestris* indicating positive result.

Figure 3.8. Gelatin hydrolysis test A. Negative control showing no Liquefaction indicating Negative result. B: *X. campestris* pv. *campestris* showing Liquifaction indicating positive result.

Figure 3.9. Lipase activity A: positive control showing milky white precipitate around the colony. B. No milky white precipitate around *X. campestris* pv. *campestris* thus negative for the test.

Figure 3.10. Hypersensitive response test: Tobacco plant inoculated with *X. campestris pv. campestris* showing necrotic lesion.

Figure 3.11. Pathogenecity test: showing typical symptoms of V-shaped lesions in cabbage plant.

Table 3.1. Screening of different cabbage cultivars for *X. campestris* pv. *campestris*

Sl No	Cultivars	Results		
		DPM	G and S	GOT
1	Indam Krishna	-	-	-
2	F1 Bhima	+	+	+
3	Unnati	-	-	-
4	Golden Acre	+	+	+
5	Kiran	+	+	+
6	Gaurav	-	-	-
7	Indam Saina	-	-	-
8	Quisto	+	+	+
9	NBH boss	+	+	+
10	Ganesh Gol	+	+	+
11	Pusa mukta	-	-	-

'+' indicates presence of pathogen detected; '-' indicates pathogen has not been detected
DPM: Direct plating method; G & S: Grind and soak method; GOT: growing on test

Table 3.2. Biochemical characterization of *Xanthomonas campestris* pv. *campestris.*

Sl. No.	Biochemical test	Results
1	Gram's reaction	-
2	KOH test	+
3	Starch hydrolysis	+
4	Kovacs oxidase test	-
5	Lipase activity	+
6	Gelatin hydrolysis	+
7	Arginine test	+
8	Catalase	+
9	Hypersensitivity test	+
10	Pathogenicity test	+

All tests were conducted in four replicates and were repeated twice '+' indicates positive reaction, '-' indicates negative reaction.

Table 3.3. Screening of cabbage cultivars for the presence of black rot disease under green house conditions

Cultivars	Incidence of black rot (%)	Categorization
Pusa mukta	4±0.2	R
Indam krishna	14±0.2	MR
Gaurav	9±0.3	MR
Indam saina	13±0.4	MR
Unnati	9±0.3	MR
NS 43	9±0.4	MR
Golden acre	44±0.8	S
Quisto	43±0.6	S
F1 bhima	63±0.7	HS
NBH boss	66±0.8	HS

Values are the means ±SE of four replicates and repeated thrice.

PCR analysis 16S rRNA primer: The PCR analysis revealed that all the ten isolates of isolates of *X. campestris* pv. *campestris* isolates (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively) amplified a 730 bp region with the 16S rRNA primer set.The isolates amplified 730bp region (Figure 3.12).

PCR analysis *estA* primer: The *estA* primer which amplifies lipase gene in *Xanthomonas* sp. All the ten isolates showed a 777 bp amplification which is specific for the lipase gene (Figure 3.13).

PCR analysis *hrpF* primer: The *hrpF* primer amplifies a region of the *hrpF* gene. All the ten isolates showed a 619 bp amplification which is specific for *X. campestris* pv. *campestris* as no other *xanthomonas* spp could amplify the *hrp F* gene (Figure 3.14).

PCR analysis ITS primer: The PCR analysis using ITS primer specific for brassica sp. revealed that all the ten cultivars *viz., Indam Krishna, Unnati, Golden Acre, Kiran, Gaurav, Indam Saina, Quisto* and *NBH- boss* amplified 360 bp region of the internal transcribed spacer. A Non crucifer member was taken as negative control in which no amplification was observed (Figure 3.15).

Multiplex PCR: A multiplex PCR was performed in which an ITS primer specific to *brassica* spp was used along with the *hrpF* gene, when the infected material and the pathogen DNA were isolated and subjected to multiplex assay, a 360bp band specific for the *brassica* was observed along with a 619 bp band specific for *X. campestris* pv. *campestris.* The seed samples which proved to infected with *X. campestris* pv. *campestris* DPM, G and S , GOT methods also showed a typical 619 bp amplification which proved that the seed samples carry the infection (Figure 3.16).

The PCR assay revealed that the *hrpF* primer set is potent to detect the presence of pathogen as compared to other primers used in the study. Since the *hrpF* primers amplify the hypersensitivity and pathogenecity genes which are highly conserved and are pathogen specific. The ITS primers used in the study along with the *hrpF* gene primers are reliable for pathogen detection in seed samples, infected leaf material and also from the seed wash extracts. Under the present conditions, several cabbage cultivars described to be tolerant to black rot by seed companies proved to be susceptible. The cultivars '*Indam krishna'* and '*Unnati'* proved to be moderately

resistant and several others were either susceptible or highly susceptible to black rot pathogen. The study also established that cultivars like *Gaurav* and *Indam Saina* are moderately resistant. The present study has exploited the PCR method for the easy detection of the pathogen where in three primers were checked for the efficiency, of which 16S rRNA and the *estA* amplified in some *Xanthomonas* species other than *X. campestris* pv. *campestris* but the *hrpF* gene proved to be very specific. The laboratory experiments conducted showed that only two cultivars *viz.*, *Indam Krishna* and *Unnati* proved to be relatively tolerant to other cultivars. This study has given an insight into taking up two more cultivars *viz., Gaurav* and *Indam saina* could be taken up for production at large scale.

DNA sequencing

The *hrpF* partial coding sequence has been sequenced and submitted to nucleotide database of NCBI. *Xanthomonas campestris* pv. *campestris* strain Xcc1 HrpF protein (*hrpF*) gene, partial cds (600bp) with accession no. **KF939056.1.**

3.3.4. Fatty acid methyl ester analysis (FAME)

In FAME analysis about 40 mg of wet bacterial cells were saponified, methylated and the fatty acid methyl esters are extracted in a mixture of ether-hexane and analyzed by gas chromatography and the zones of the resulting peaks in the chromatograms were calculated and the bacterial isolate was identified as *X. campestris* pv. *campestris* based on the Sherlock Microbial Identification System . The gas chromatographic profile of the bacterial isolate is as given in figure (Figure 3.17).

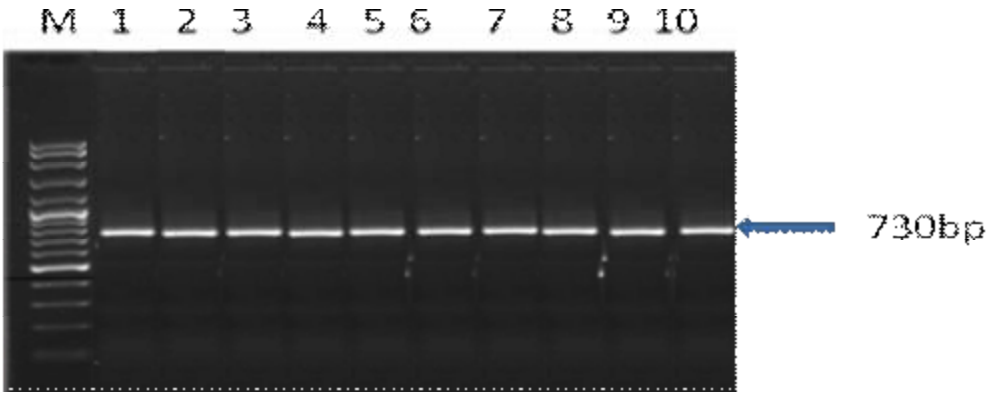

Figure 3.12. Agarose gel electrophoresis (2%) of PCR products amplified with the 16S rRNA primer set. Lanes: 1-10, *X. campestris* pv. *campestris* isolates (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively). Lane M: 100 bp DNA ladder.

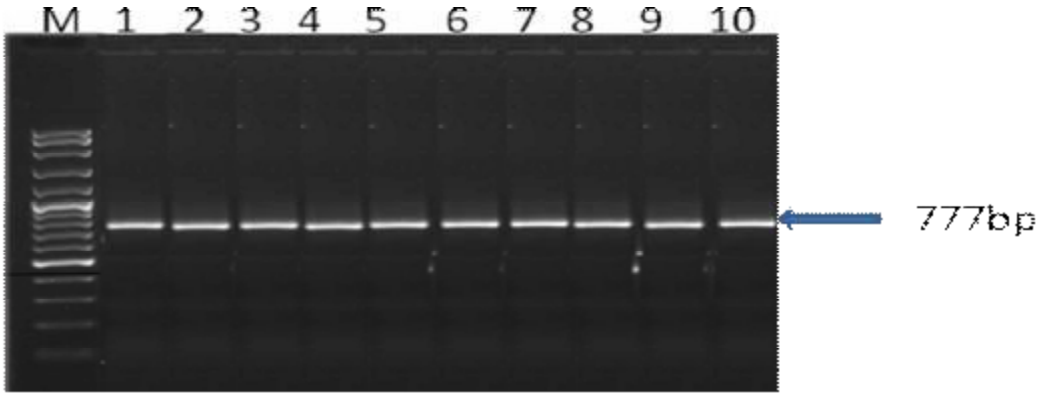

Figure 3.13. Agarose gel electrophoresis (2%) of PCR products amplified with *estA* primer set. Lanes: 1-10, *X. campestris* pv. *campestris* isolates (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively) Lane M: 100 bp DNA ladder.

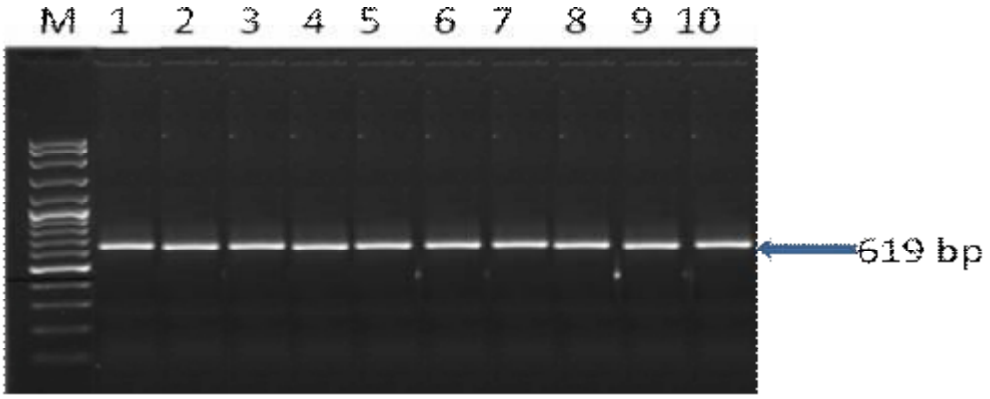

Figure 3.14. Agarose gel electrophoresis (2%) of PCR products amplified with *hrpF* primer set. Lanes: 1-10, *X. campestris* pv. *campestris* isolates (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively). Lane M: 100 bp DNA ladder.

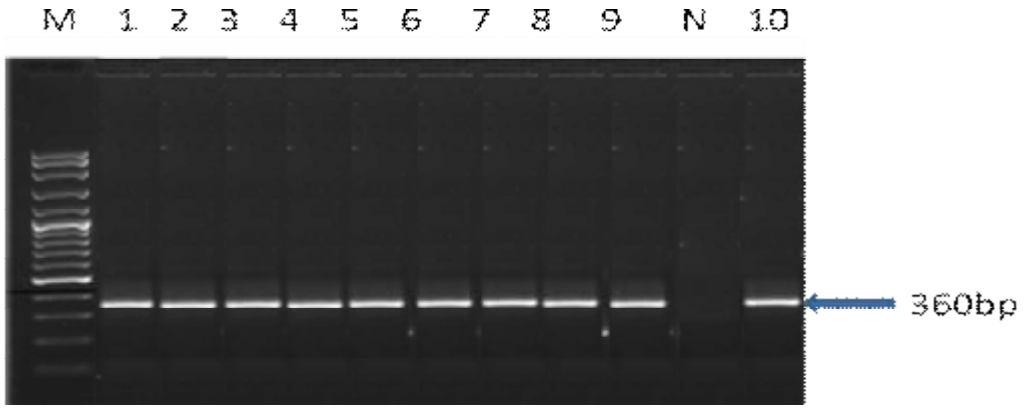

Figure 3.15. Agarose gel electrophoresis (2%) of PCR products amplified with the ITS primer set. Lanes: 1-10 the ten cvs *viz., Indam krishna*, *Unnati*, *Golden acre*, *Kiran*, *Gaurav*, *Indam saina*, *Quisto* and *NBH boss*. Lane N: Non brassica sp. Lane M: 100 bp DNA ladder.

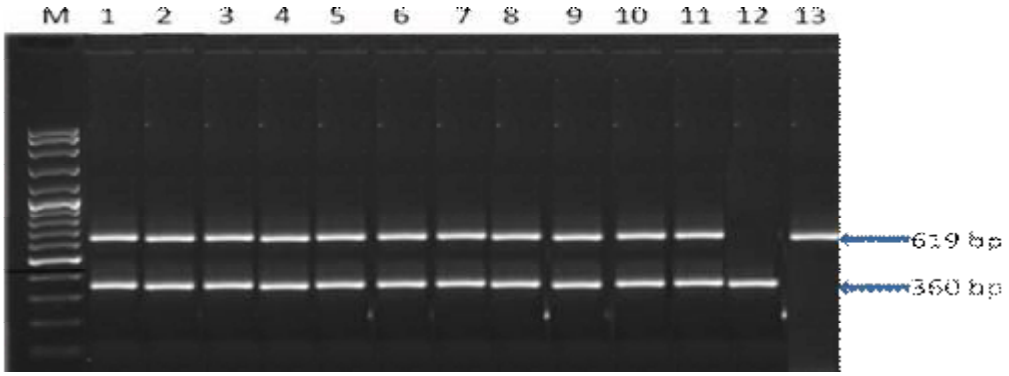

Figure 3.16. Multiplex PCR: DNA of infected cabbage leaves and DNA from *X. campestris* pv. *campestris* were amplified using gene specific primer for pathogen (hrpF) and ITS primers for Brassica. The 619 bp banding pattern indicates the presence of *X. campestris* pv. *campestris* and 360 bp banding pattern indicates the presence of host.

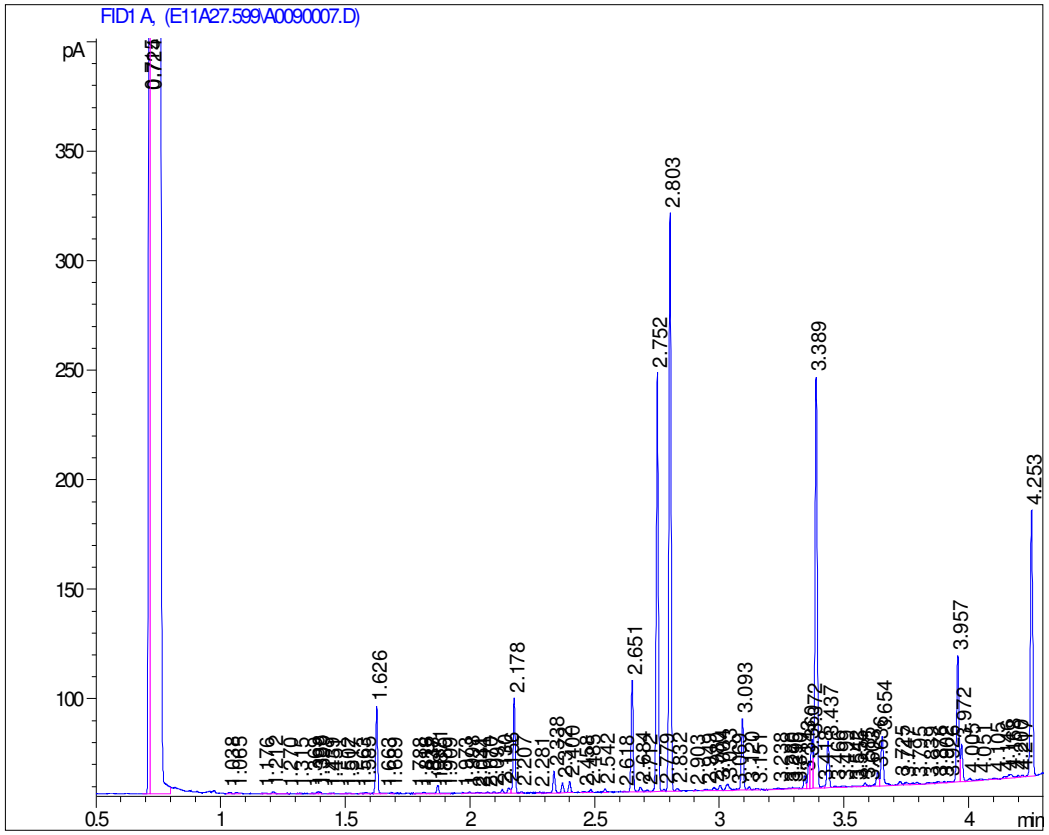

Figure 3.17. Gas chromatographic profile of fatty acid methyl esters of *Xanthomonas campestris* pv. *campestris.*

3.4. Discussion

Black rot of crucifers caused by *X. campestris* pv. *campestris* (Pammel) Dowson is considered to be one of the most important diseases of crucifers worldwide and is most serious in *Brassica oleracea*. It was a disease of minor importance has become a major disease among the vegetable crops in recent years. In India, the disease was first reported on cabbage from Bombay on cauliflower in 1949 (Patel *et al.,* 1949) subsequently it was reported from katrain area of Himachal Pradesh. Since then the occurrence of the disease has also been reported from other parts of the country. The black rot of cabbage is one of the major bacterial disease which leads to huge losses. The symptoms caused by *X. campestris* pv. *campestris* in cabbage depend upon several factors such as cultivar, plant age (Schaad and Alvarez, 1993), light and temperature, humidity, strain of the pathogen and even the method used for inoculation. However, in the true leaves, blackened vascular tissues and V-shaped chlorotic to necrotic lesions along the leaf margins are characteristic symptoms of

black rot (Cook *et al.,* 1952; Williams, 1980; Onsando, 1992; Alvarez, 2000). The data obtained in the present study reveals the black rot of cabbage caused by *X. campestris* pv. *campestris* is a disease of major importance among the vegetables. There has been a severe outbreak of the disease in all the cabbage growing areas of Karnataka has resulted in huge yield losses to the small holding farmers. Although the farmers have adopted all the available and possible management strategies, the disease remains unchecked due to faster inoculum build up and spread.

In Karnataka the work related to black rot of cabbage is very less, therefore there is a need for detailed study of the disease in respect of the disease development, isolation and identification using PCR and management of the disease. The diagnostic procedures are based primarily on classical methods such as isolation on simple media and pathogenicity tests. PCR-based methods offer advantages over more traditional diagnostic tests, in that organisms do not need to be cultured prior to their detection and protocols are highly sensitive and rapid.

The detection of the pathogen depended on the conventional ways by plating techniques which are laborious but still are efficient. With the advent of DNA based techniques the process of pathogen detection has become less consuming. DNA based methods are powerful tools to identify and detect microbes with high sensitivity and specificity. Recent advances have led to new possibilities for pathogen detection that depend on the recognition of DNA sequences that are specific only to the pathogen genome or that of a particular strain of the pathogen that is to be detected. DNA amplification of PCR is a highly sensitive to detect the pathogen-specific DNA. DNA detection techniques have an advantage over cultural methods in short duration of time when compared to the longer durations required for purification and isolation of the organism under investigation. Specific primers have been developed for the early detection of the *X. campestris* pv. *campestris* based methods detect and identify the pathogen. PCR based seed assays the most reliable methods of determining whether or not seeds are infected with seedborne pathogens. Infected seeds of tolerant varieties may show no visible external symptoms.

In this study, duplicate isolates obtained from the same lesion produced similar PCR profiles, except in one case where two isolates belonged to different races. This suggests PCR can be used for rapid initial screening of isolates to select

non-identical ones for further analysis, including race-typing. The results in general indicate that most leaf lesions, from which isolations were made, were a result of infection by a single race of the pathogen. The experiments confirmed the presence of *X. campestris* pv. *campestris* as a seedborne pathogen certified seed.

The PCR assay described here is suitable for the rapid detection of *X. campestris* pv. *campestris* from *Brassica* seed, infected leaf and stem tissue, and for complementary testing of bacterial colonies. This sensitive and selective method circumvents many of the problems associated with existing detection techniques, enabling the reliable detection of *X. campestris* pv. *campestris* in the presence of microorganisms that overgrow or inhibit this pathogen on agar plates. The apparent requirement for the *hrpF* protein in disease development, and the species-specificity exhibited by the primers used in this assay, suggest that *hrpF* may be a useful target in the design of PCR assays specific for other xanthomonads.

To date, relatively few *hrpF* genes have been sequenced for members of this genus; as the *hrpF* sequences of more species become available, the suitability of this target for specific detection at the species (and perhaps pathovar) level will be revealed. PCR can be used to rapidly screen *Brassica* spp. seed batches for the presence of *X. campestris* pv. *campestris* pathovars. This assay provides a means for growers and the seed industry to be aware of the black rot status of their planting material, so that they may more effectively employ disease control measures or seed disinfection. The present results indicate the existence of *X. campestris* pv. *campestris* strains knowledge about the genetic diversity of *X. campestris* pv. *campestris* in the locality will be essential whenever disease management strategies are based on host plant resistance.

16S rRNA gene sequences allow bacterial identification that is more robust, reproducible, and accurate than that obtained by phenotypic testing. In the present study *X. campestris* pv. *campestris* was detected by molecular technique. PCR assay using primers specific to a region of the *hrpB* gene cluster to detect *X. campestris* pv. *campestris*. The use of these primers with PCR will allow for the early detection of the pathogen, which is critical due to its rapid and uncontrolled spread on symptomatic and asymptomatic crop material

The *hrpF* gene has proved a useful target for the PCR-based detection of *X. campestris* pathovars. Although this gene is too highly conserved to enable differentiation of the pathovars, primers targeting the 3′ end of *hrpF* successfully amplified a 619 bp product only from *X. campestris*. The product was not amplified from extracts of other bacterial genera, nor from *Xanthomonas* species isolated from noncruciferous plants (that are not members of the *campestris* species. The *hrpF* gene has proved a useful target for the PCR-based detection of *X. campestris* pathovars. Although this gene is too highly conserved to enable differentiation of the pathovars, primers targeting of *hrpF* successfully amplified a 619 bp product only from *X. campestris*. The product was not amplified from extracts of other bacterial genera, the black rot bacteria may be carried within the seed, often attached to the funiculus (infection), or simply associated with the surface (Cook *et al*., 1952). Both infected and infested seeds are capable of developing disease and acting as a reservoir of infection for surrounding plants. It is anticipated that seeds infected in either manner are detected by the assay. However, recovery of the pathogen from infested seeds is expected to be easier than from infected seeds. If necessary, washed seeds may be redried and subsequently sown without adverse affects on germination, although bacteria present in infected seed are likely to have spread during the wash step. When compared with the traditional plating method, the PCR assay was found to have a number of advantages. In particular, PCR offers enhanced sensitivity by virtue of the amplification reaction, enabling relatively low numbers of bacteria to be detected. Seed batches with low infection levels that were negative by the plating method were revealed to contain *X. campestris* pv. *campestris* when analysed by PCR. The *X. campestris* pv. *campestris*-specific primers offer greater selectivity than is possible with the plating method because other microflora present on seed are still capable of growth on the selective media (Randhawa and Schaad, 1984), requiring further evaluation of suspect colonies by pathogenicity testing. Additionally, the selective media is cumbersome to prepare and the antimicrobials limit its shelf-life, complicating the adoption of this technique by diagnostic laboratories conforming to stringent quality assurance requirements. PCR is a much quicker test to perform with a result achieved within two working days (less than one day when testing lesions on seedlings). The *hrpF* gene has proved a useful target for the PCR-based detection of *X. campestris* pathovars. Control of the disease is difficult and is usually attempted through the use of healthy planting material (seeds and transplants) and the

elimination of other potential inoculum sources (infected crop debris and cruciferous weeds). An alternative approach through the development and use of resistant cultivars has long been recognized, but in practice has had only limited success. Multiplex PCR is useful in plant pathology because different bacteria frequently infect a single host and consequently sensitive detection is needed for the propagation of pathogen-free plant material. Among molecular techniques, multiplex PCR is increasingly used because it improves the efficiency of diagnostic PCR (Johnson, 2000). In the near future multiplex PCR will probably be adapted for the simultaneous detection of viruses and bacteria of one particular crop and for the simultaneous detection of other major plant pathogens such as viruses, viroids, bacteria, and fungi in the same reaction, as already demonstrated for viruses and viroids or viruses and bacteria.

Black rot can be controlled using healthy plant material including seeds and transplants and by adopting cultural practices that limit bacterial spread. The most efficient form of disease control is using resistant cultivars; however, only a small number of useful sources of resistance are available. Symptoms of black rot were observed in all visited fields, in seedbeds, in newly transplanted crops and in mature crops. The disease is frequently overlooked by farmers and extension workers, who observe symptoms, but mistakenly think they are related to natural plant senescence. The increased and almost continuous production of *Brassica* crops in some areas, the ability of *X. campestris* pv. *campestris* to spread by seed movement and rain splash and to survive in plant debris left in the field may cause build up and maintenance of inoculum, thereby increasing the impact of the disease in the future, unless appropriate control measures are adopted. A significant proportion of cabbage seed is imported and it is important to ensure that such seed is tested and free of the pathogen to minimise the risk of introduction of additional pathotypes. Selection of cultivars with disease resistance is another means of control which should be considered in combination with crop rotation and sanitation schemes.