5.1. Introduction

The control of plant pathogens relies on the ability to identify infecting species. Therefore, alternative molecular methods have been developed. Studies of the 16S ribosomal RNA (rRNA) gene (Hauben *et al.,* 1997; Moore *et al.,* 1997a, 1997b) and the 16S–23S intergenic region (Gonçalves and Rosato 2002) can only be used to identify strains at the genus level. Ribosomal DNA (rDNA) is suited for phylogenetic studies because the degree of conservation that varies between the different rDNA components. The conserved sequences of the16S rRNA have led to the development of conserved primers for the detection of eubacteria. Despite the fact that rRNA genes are universal to all living organisms, the correspondence between genome composition and rRNA phylogeny remains poorly characterized.

Single-strand conformation polymorphism (SSCP) is a rapid and sensitive approach for characterization of DNA sequences. This method, first described by Orita *et al*. (1989) has been used for the detection of various alterations in DNA sequences including substitutions, deletions, insertions, and rearrangements. SSCP analysis resolves DNA sequences based on a characteristic size and shape of the folded structure, which is determined by the inter-molecular interaction of single stranded DNA (ssDNA). The SSCP technique was initially developed for examining point mutations in human DNA (Orita *et al.,* 1989), and has been extended to the study of the variability of plant pathogens, including viruses (Kong *et al.,* 2000), nematodes (Clapp *et al.*, 2000) and fungi (Kumeda and Asao, 1996). PCR products are now routinely used for SSCP analysis. After PCR amplification of the target sequence, the amplified product is denatured to two single-stranded DNAs and subjected to non-denaturing polyacrylamide gel electrophoresis. The mobility of the ssDNA under non-denaturing conditions depends on the secondary structure of the amplified product ssDNA that is determined by the nucleotide sequence. The ssDNA bands at different positions on the gel indicate different sequences. PCR-SSCP is capable of detecting more than 90% of all single base substitutions in 200-bp fragments (Hayashi, 1991; Widjojoatmodjo *et al.,* 1994).

The random amplified polymorphic DNA (RAPD) technique is based on the amplification of genomic DNA with a single primer of arbitrary nucleotide sequence (William *et al.*, 1990; Welsh and McClelland, 1990). RAPD is a rapid technique to

screen for nucleotide sequence polymorphisms, and has been developed to screen for genetic variants. RAPD technology has gained widespread acceptance and application because it is a relatively simple tool for genetic analysis in biological systems. RAPD is the preferred assay when the nucleotide sequence is not known. DNA amplification approaches using arbitrary primers have been employed in a large number of genetic studies including the estimation of genetic relationships (Wilde *et al.,* 1992). Furthermore, Kim *et al.* (1993) reported that PCR has been widely and successfully employed for the DNA sequence diversification analysis of important plant pathogenic bacteria and fungi.

5.2. Materials and Methods

5.2.1. Isolation of plant pathogenic bacteria

The different isolates of *Xanthomonas campestris* pv. *campestris* along with other phytopathogenic bacteria *viz., Xanthomonas axonopodis* pv. *vesicatoria*, *Ralstonia solanacearum*, and *Xanthomonas oryzae* pv. *oryzae* from Department of Biotechnology, the University of Mysore were used in the present studies. *Bacillus subtilis* and *Escherichia coli* were used for the comparative study. All bacteria were subjected to biochemical characterization to confirm their identity. The bacterial isolates were subjected to conventional diagnostic tests using standard protocols for cultural, biochemical, physiological, pathogenic characterization, including a test for the hypersensitive response (Roohie and Umesha, 2012).

5.2.2. Genomic DNA extraction

Genomic DNA was extracted from the following bacteria: *Xanthomonas campestris* pv. *campestris* (ten isolates), *Xanthomonas axonopodis* pv. *vesicatoria*, *X. oryzae* pv. *oryzae*, *R. solanacearum*, *E. coli*, and *B. subtilis*. The total genomic DNA extraction was performed following the protocol described by Gabriel and De Feyter (1992). A loopful of each isolate was suspended in 500 µl of phosphate buffered saline (PBS) in a microfuge tube, mixed by vortexing, and centrifuged at 12000 rpm for 15 min. The supernatant and the viscous material were discarded, and the pellet was washed with 1 ml buffer (50 mmol ethylenediaminetetraacetate (EDTA), 0.15 mol l^{-1} NaCl, pH 8.0) and repeatedly centrifuged. Proteinase K to a final concentration of 150 µg ml^{-1} and 30 µl of sodium dodecyl sulfate (SDS) were added and the

suspension was incubated at 50° C for 1 h. An equal volume of a mixture chloroform-phenol-isoamyl alcohol (24:25:1) buffered with 10 mmol l^{-1} Tris-HCl (pH 8.0) was added. The suspension was mixed by vortexing and centrifuged at 12,000 rpm for 5 min to separate the layers. The upper layer was transferred to a fresh microfuge tube and 0.1 volume of 3 mol l^{-1} sodium acetate and 1x volume of isopropanol was added and mixed by vortexing. The precipitate was treated with 1 ml of 70% ethanol, and then centrifuged for 2 min at 12000 rpm. The ethanol was subsequently removed, and the pellet was resuspended in nuclease-free water. The DNA was quantified using a Nanodrop spectrophotometer (Beckman coultier, CA, USA), and the purity of the DNA was evaluated by electrophoresis in 0.8% agarose gel.

5.2.3. PCR assay

The PCR assay was carried out using 16S rRNA-gene based primer set (Lee *et al.,* 2009) which was custom synthesized (Sigma Aldrich, USA) with the sequences : 16S-Forward (5′-CCAGACTCCTACGGGAGGCAGC-3′) and 16S-Reverse (5′-GCTGACGACAGCCAT GCAGCACC-3′). The PCR amplification was performed with a thermal cycler (LabNet, USA) in a 25-µl reaction mixture containing 1 µl of genomic DNA, 0.35–0.5 µmol l^{-1} of each primer, 0.25 mmol l^{-1} of each deoxynucleoside triphosphate, and 1 µl of reaction buffer (1.5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl, pH 8.8), and 2.0 U of Taq DNA polymerase (BangaloreGenei, India). The following cycling conditions were used: 1 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. The reaction mixtures were stored at 15° C until they were used for analysis. The amplified DNA was detected by electrophoresis in 1% agarose gels (Himedia, India) in 1x TAE buffer.

5.2.4. PCR-SSCP analysis

After the evaluation of the PCR products by agarose gel electrophoresis, 5 µl of an individual PCR product was mixed with 2.5 µl of denaturing buffer (95% formamide, 20 mmol l-1 EDTA, 0.05% bromophenol blue). The mixtures were heated at 96° C for 10 min and then immediately chilled on ice. The denatured PCR products (30 µl of each sample) were loaded onto 6% acrylamide–bisacrylamide non-denaturing gels and electrophoresed in pre-chilled 1x TBE buffer at 150 V for 3 h at

room temperature. A DNA ladder was loaded to facilitate comparison of the SSCP patterns. After electrophoresis, silver staining of the polyacrylamide gels was carried out according to the method of Bassam *et al.* (1991). Briefly, the gels were fixed in 10% acetic acid for 20 min at room temperature and then washed with deionized water three times each for 2 min Silver impregnation was performed for 30 min at room temperature with 0.1% silver nitrate and 0.056% formaldehyde. The gels were then washed for 20 s with deionized water, and color development was performed for 2–10 min with a mixture of 30 g l^{-1} sodium carbonate, 0.056% formaldehyde, and 2 mg l^{-1} sodium thiosulfate. The color reaction was then stopped with 10% acetic acid.

5.2.5. Restriction fragment length polymorphism of SSCP analysis

The restriction enzymes used for restriction fragment length polymorphism (RFLP) analysis in this study included *Alw*I, *EcoR*II, and *Fok*I (New England Biolabs, USA). They were chosen in principle on the basis of the 16S rDNA nucleotide sequences available from *Xanthomonas* sp. databases (http://www.xanthomonas.org/). The amplified rDNA fragment was digested with a restriction enzyme in the buffer recommended by the manufacturer with 6–10 U of enzyme per sample at 37° C for 2 to 3 h with a total volume of 50µl of PCR product. The reaction was stopped by mixing with the loading buffer described above. The digested PCR products were subjected to electrophoresis in a 2% agarose gel. Each product was also electrophoresed on a 6% polyacrylamide minislab gel as described above.

5.2.6. RAPD analysis with M13 primers

Genomic DNA from the different isolates of *X. campestris* pv. *campestris* was used as a template for PCR fingerprinting using a M13 minisatellite core sequence (Huey and Hall, 1989) with the following sequence 5′-GAGGGTGGCGGTTCT-3′. The amplification reactions were performed according to the optimized protocol previously described (Giraffa *et al.,* 2000). One cycle of 94° C for 2 min was followed by 40 cycles of 94° C for 60 s (denaturing), 42° C for 20 s (annealing), and 72° C for 2 min (extension). A final extension was carried out at 72° C for 10 min. The PCR profiles were visualized by electrophoresis in 1.5% agarose gels and staining with ethidium bromide. A 1-kb plus DNA Ladder (BangaloreGenei, India) was used as a DNA molecular weight marker.

5.2.7. Data analysis

Each of the amplification products was identified by its size and number of DNA fragments. The presence or absence of individual bands in the amplified product was scored. A difference matrix was determined using a computer program NTSYS-PC (Applied Biostatistics Inc. CA, USA).

5.2.8. DNA sequencing and sequence analysis

The 16S rRNA gene PCR products were purified and sequenced on an ABI 3730 sequencer (Applied Biosystems, USA). Sequencing was carried out on selected PCR products. Sequences were analysed with the Classifier tool provided by the Ribosomal Database Project (Cole *et al.,* 2009). The 16S sequence obtained in this study has been deposited in GenBank.

5.3. Results

5.3.1. Isolation and biochemical classification of bacterial strains

The different isolates of *X. campestris* pv. *campestris* were confirmed based on typical morphological characters. On Tween B medium *X. axonopodis*. pv. *vesicatoria* colonies exhibited typical morphological characteristics such as yellow colonies with zones indicating starch hydrolysis, and *R. solanacearum* colonies appeared as typical mucoid creamy-white with pink centers on triphenyltetrazolium chloride (TZC) medium. These pathogens were further purified by streaking on the respective semi-selective media. *X. oryzae* pv. *oryzae* colonies showed yellow mucoid shining appearance around the seed and plant material. The isolated bacterial isolates showed gram-negative characteristics as determined by both gram staining and KOH solubility testing. All the isolates were subjected to gram staining, tests for starch hydrolysis, catalase activity, oxidase activity, asparagine medium growth, xanthomonadin pigment production, hypersensitivity to *Nicotiana tobaccum* and pathogenicity to *Brassica oleracea* var *capitata* (cv. *Golden acre* – a susceptible cabbage cultivar) (Table 5.1).

Table 5.1. Biochemical characterization of different bacterial isolates

Biochemical tests	Results					
	Xcc	*Xav*	*Xoo*	*Rs*	*E.coli*	*B.subtilis*
Gram reaction	-	-	-	-	-	+
KOH solubility	+	+	+	+	+	-
Starch hydrolysis	+	+	+	+	-	-
Lipase activity	+	+	+	+	-	-
Xanthomonadin pigment	+	-	+	-	-	-
Gelatin hydrolysis	+	+	+	+	-	-
Hypersensitivity to *Nicotiana tobacum*	+	+	+	+	+	+
Pathogenicity test	+	-	-	-	-	-

All the tests were conducted in replicates and repeated twice. '+' indicates positive reaction, '-'indicates negative reaction, '*Xcc'* indicates *X. campestris* pv. *campestris*, '*Xav'* indicates *X. axonopodis* pv. *vesicatoria*, '*Rs'* indicates *R. solanacearum*, '*Xoo'* indicates *X. oryzae* pv. *oryzae*, *E. coli* indicates *Escherichia coli.*

5.3.2. PCR analysis using the 16S rRNA primer

Using the 16S rRNA primer an approximately 730 bp PCR product was amplified from the genomic DNA of all the 10 isolates of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, *X. oryzae* pv. *oryzae*, *E. coli*, and *B. subtilis* tested (Figure 5.1).

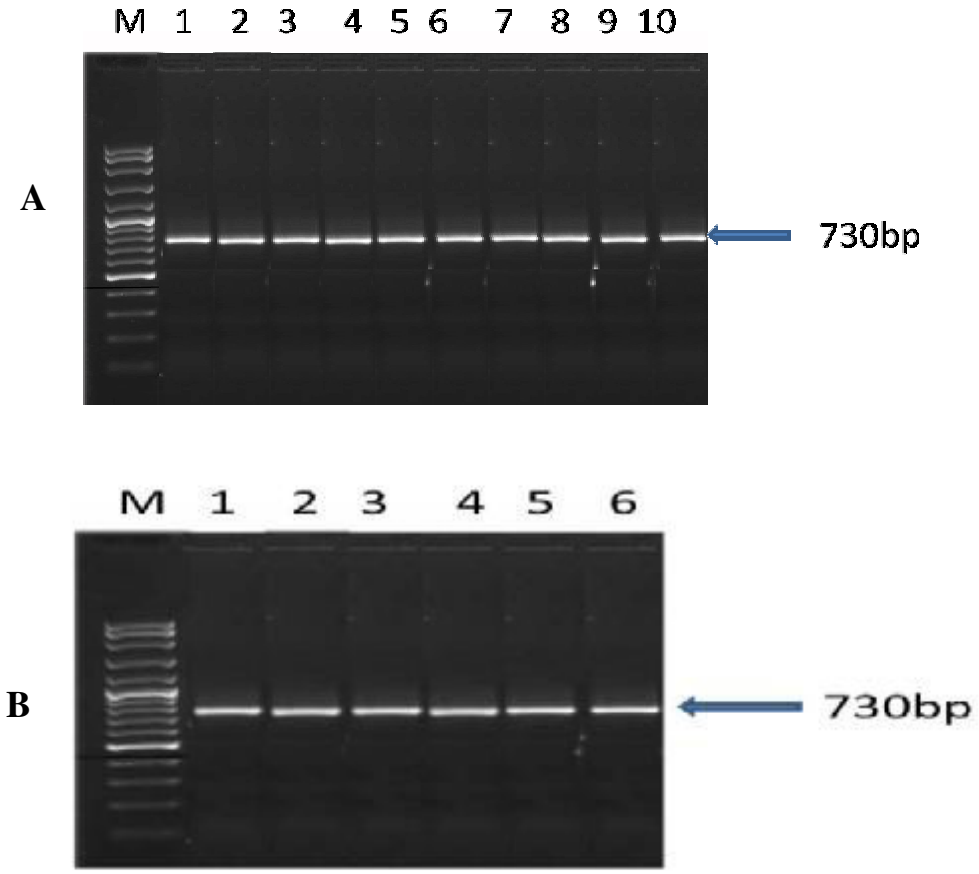

Figure 5.1. A: Agarose gel (1%) electrophoresis of PCR products amplified with the 16S rRNA primer set. Lanes: 1 – 10, *X. campestris* pv. *campestris* isolates. **B:** Lanes: 1 - *X. campestris* pv. *campestris*, Lane 2 - *Xanthomonas axonopodis* pv. *vesicatoria,* Lane 3 - *Xanthomonas oryzae* pv. *oryzae*, Lane 4 - *Ralstonia solanacearum,* Lane 5- *Bacillus subtilis* strain , Lane 6 - *Escherichia coli* strain. Lane M: 100 bp DNA ladder.

5.3.3. SSCP analysis

The denatured PCR products amplified by 16S rRNA primer yielded a single conformer. A band shift was observed only in *Bacillus subtilis*. The identical size of PCR product (730 bp) was obtained in all the isolates including the *E. coli* and *B. subtilis* bacteria (Figure 5.2). Electrophoresis of the denatured PCR amplification product of 730 bp showed only single conformer which was difficult to distinguish among various phytopathogens. This could resulted from the large amplicon size. Hence the PCR products were subjected to the RFLP analysis followed by SSCP.

5.3.4. RFLP-SSCP

Based on the 16S rRNA sequence from the ribosomal database, the sequences were analysed for the presence of the restriction sites. Only those enzymes which could once cleave were chosen based on the analysis in NEB cutter V2.0 (http://tools.neb.com/NEBcutter2/). The three-restiction enzymes *viz., Eco*RII, *Alw*I, and *Fok*I were used in the RFLP analyses. The restriction fingerprint obtained by *Eco*RII, *Alw*I and *Fok*I digestion was analysed. The *X. campestris* pv. *campestris* gave three bands, *X. axonopodis* pv. *vesicatoria* gave two bands, the *X. oryzae* pv. *oryzae* gave five bands, *R. solanacearum* gave three bands, *E .coli* gave three bands and the *B.subtilis* gave a single band. All of them showed a unique banding pattern upon restriction digestion. (Figure 5.3). Each band was considered as equivalent independent characters and all the bands were scored as present or absent for each isolate. Banding patterns were converted into binary tables. The data was analyzed using genetic data analysis software, Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.2. A dendrogram was generated using Unweighted Pair-Group Method with Arithmetic Averages (UPGMA).

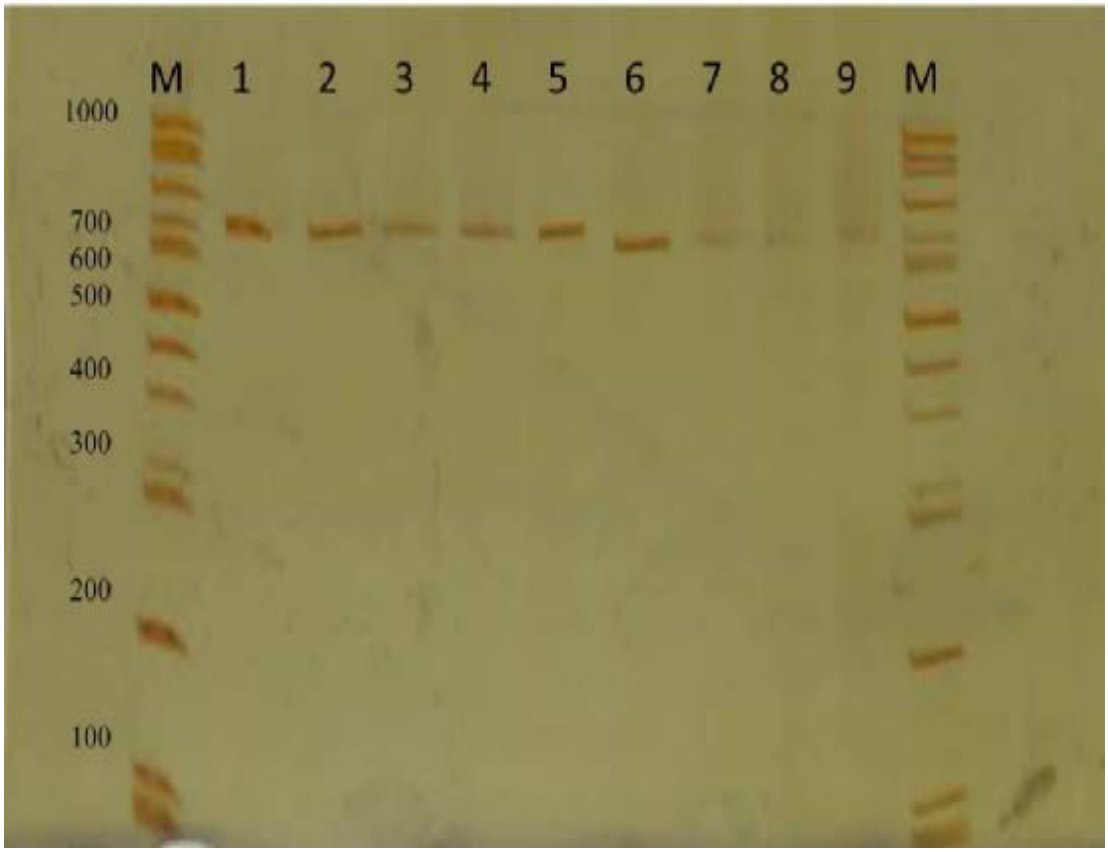

Figure 5.2. Non denaturing polyacrylamide gel (6%) of denatured PCR products amplified with the 16S rRNA primer set. Lanes: 1 - *X. campestris* pv. *campestris*, Lane 2 - *Xanthomonas axonopodis* pv. *vesicatoria*, Lane 3 - *Xanthomonas oryzae* pv. *oryzae*, Lane 4 - *Ralstonia solanacearum,* Lane 5 - *Escherichia coli,* Lane 6 - *Bacillus subtilis*, Lane 7, 8 and 9 - *X. campestris* pv. *campestris* isolates, Lane M: 100 bp DNA ladder.

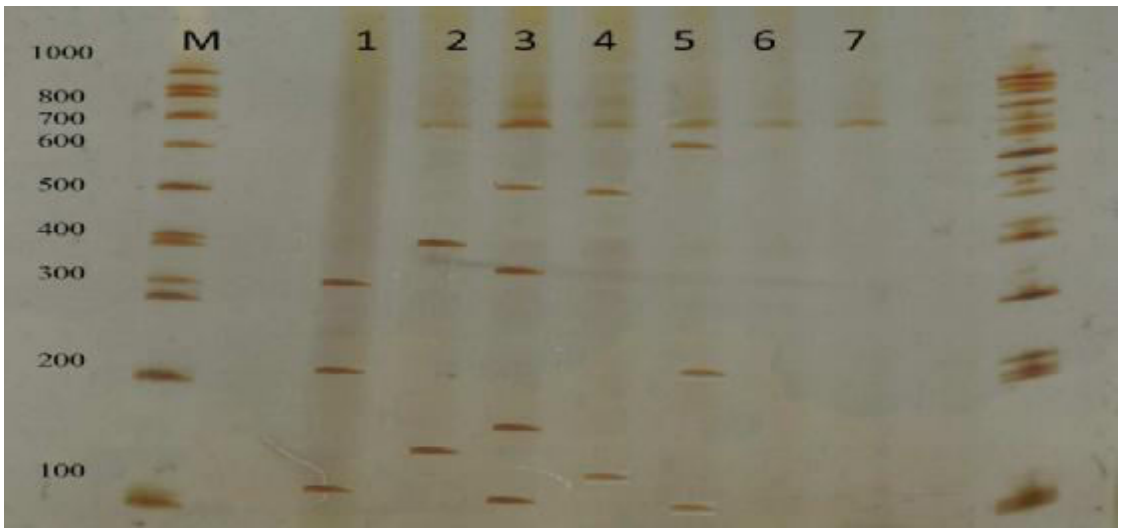

Figure 5.3. Non denaturing polyacrylamide gel (6%) electrophoresis of restriction fragments obtained by 16S rRNA PCR products digested with *EcoRII*. Lanes: 1 - *X. campestris* pv. *campestris*, Lane 2 - *Xanthomonas axonopodis* pv. *vesicatoria*, Lane 3 - *Xanthomonas oryzae* pv. *oryzae*, Lane 4 - *Ralstonia solanacearum,* Lane 5 - *Escherichia coli*, Lane 6 and 7– *Bacillus subtilis*. Lane M: 100 bp DNA ladder.

The dendrograms (Figures. 5.4 and 5.5) show clustering of all phytopathogens studied based on the RFLP patterns obtained with *Alw*I and *Fok*I restrictions, respectively. Four clusters were detected: cluster 1 containing the *Xanthomonas* species (*X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*), cluster 2 grouped the *X. axonopodis* pv. *vesicatoria* strains, cluster 3 comprising the *R. solanacearum* and cluster 4 *E. coli*. The cluster analysis resulted in grouping the *Xanthomonas* sp. into a prominent cluster compared to other phytopathogens. As a result of RAPD-PCR analysis of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *Ralstonia solanacearum*, *X. oryzae* pv. *oryzae*, and *Escherichia coli* the distinctive patterns were obtained that allowed to demonstrate a clear differentiation among the species considered in the study. Repetition of RAPD analysis assessed reproducibility of the RAPD-M13 patterns. The Figure 5.6 shows the dendrogram based on RAPD patterns of phytopathogens. Five clusters were detected. Cluster 1 contained the *X. campestris* pv. *campestris* and the isolate *X. campestris* pv. *campestris* (XCC2). Cluster 2 consists of the *X. oryzae* pv. *oryzae*. Cluster 3 consisted of grouped the *X. axonopodis* pv. *vesicatoria* strains only. The *R. solanacearum* was grouped as the

fourth cluster and the *E.coli* was grouped into the fifth cluster. The cluster analysis resulted in grouping the *Xanthomonas* sp into a prominent cluster compared to other phytopathogens. The *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* are major *Xanthomonas* sp. producing xanthomonadin pigment. The biochemical characteristics of the both were similar as proven by the phylogeny also.

The dendrogram obtained by RAPD-M13 (Figure. 5.6) was similar to the dendrograms obtained by *Eco*RII, *Alw*I and *Fok*I RFLP analyses. This study confirms the efficacy of RAPD markers. Similar finding has proved the successful application of RAPD markers for estimation of genetic variability.

5.3.5. DNA Sequencing and analyses

Sequences were edited to exclude the PCR primer binding sites and manually corrected with Chromas 2 (Chromas Version 2.22; w.technelysium.com.au/chromas. html). The sequence data of 16S rRNA gene fragment has been submitted to the GeneBank database with accession number KC855543. *In silico* analyses indicated that the submitted sequence had homology with the 16S rRNA gene sequence of *X. campestris* pv. *campestris* in the NCBI database. By using M13 core sequence a distinct band was obtained in *X. campestris* pv. *campestris* compared to other pathogens used in the study. Upon the sequencing and bioinformatic analysis it was found as hrp (hypersensitive reaction proteins) encoding gene, whereas the major band in *X. axonopodis* pv. *vesicatoria* and *X. oryzae* pv. *oryzae* was have been found to code for methyltransferase gene and peptidyl-aspartate metallo endopeptidase, respectively.

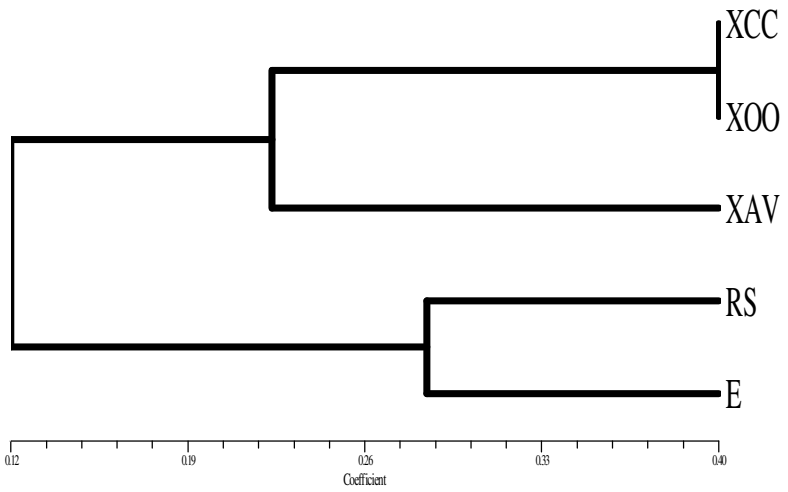

Figure 5.4. Dendrogram based on the RFLP analysis with *AlwI* restriction enzyme. The tree was obtained by UPGMA cluster analysis. '*XCC'* indicates *X. campestris* pv. *campestris*, '*XAV'* indicates *X. axonopodis* pv. *vesicatoria*, '*RS'* indicates *R. solanacearum*, '*XOO'* indicates *X. oryzae* pv. *oryzae*, *E. coli* indicates *Escherichia coli.*

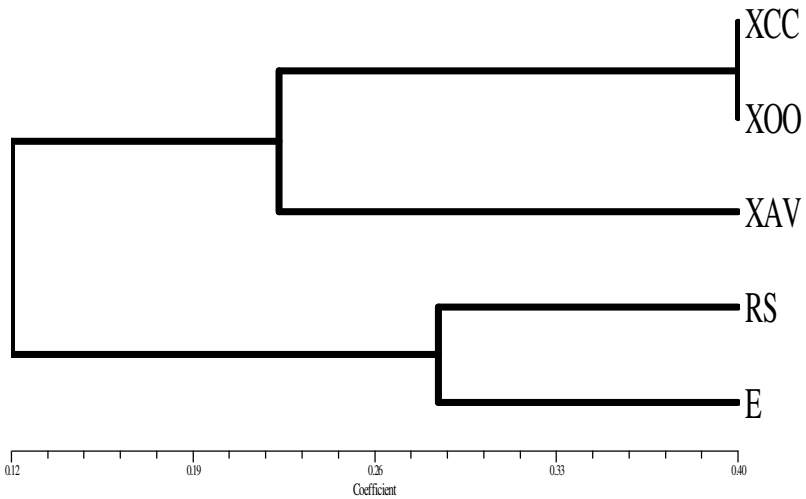

Figure 5.5. Dendrogram based on the RFLP analysis with *FokI* restriction enzyme. The tree was obtained by UPGMA cluster analysis. '*XCC'* indicates *X. campestris* pv. *campestris*, '*XAV'* indicates *X. axonopodis* pv. *vesicatoria*, '*RS'* indicates *R. solanacearum*, '*XOO'* indicates *X. oryzae* pv. *oryzae*, *E. coli* indicates *Escherichia coli.*

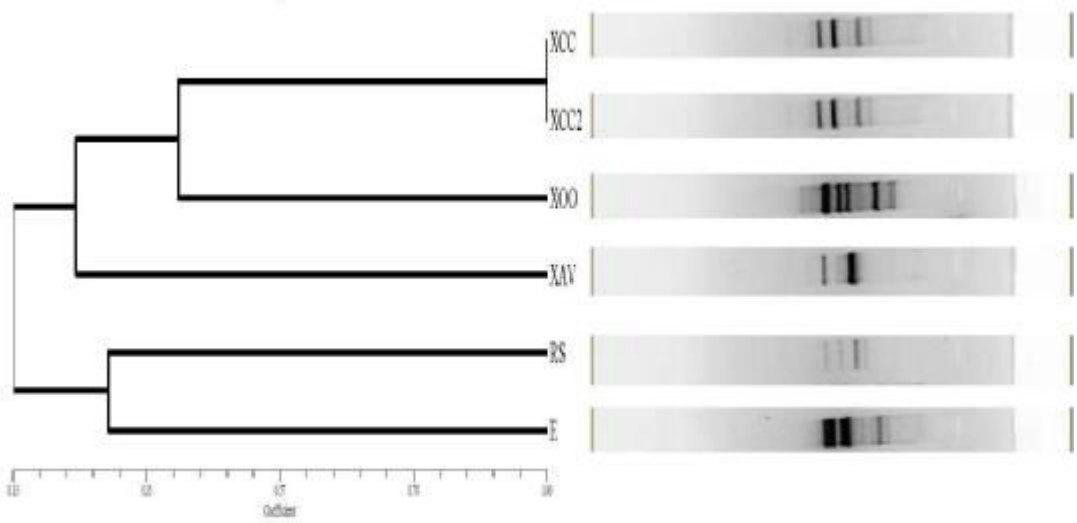

Figure 5.6. Dendrogram based on the RAPD-M13 profile analysis. The tree was obtained by UPGMA cluster analysis of the RAPD-M13 of various phytopathogens. '*XCC'* and '*XCC2'* indicates *X. campestris* pv. *campestris*, '*XAV'* indicates *X. axonopodis* pv. *vesicatoria*, '*RS'* indicates *R. solanacearum*, '*XOO'* indicates *X. oryzae* pv. *oryzae*, *E. coli* indicates *Escherichia coli.*. The left part of the figure indicates the dendrogram and the right part the RAPD-M13 banding pattern.

5.4. Discussion

The currently used protocol for the detection of the pathogens present in seeds uses Fieldhouse-Sasser and mCS20ABN media (Koenraadt *et al.,* 2005). The morphology of cultures is checked in subcultures on media, such as yeast dextrose calcium carbonate (YDCC). Classical bacteriological tests, carbon source metabolic fingerprinting (Biolog, Hayward, CA, USA) (Poplawsky and Chun, 1995), fatty acid analysis (MIDI, Newark, DE, USA) (Massomo *et al.,* 2003) and serological tests using polyclonal or monoclonal antibodies (Alvarez *et al.,* 1994) have been used for the identification of the organisms. All of these methods are database dependent based on the results obtained with representative isolates of different species and pathovars. However, frequent problems with the standard isolates used (*e.g.,* misidentification) can complicate the interpretation of new results. The inoculation of susceptible brassica seedlings is still the most reliable method as it provides the ultimate confirmation of the identified pathovar. However, all of these methods are time consuming and inadequate for high-throughput screening (HTS). The identification of *X. campsteris* pv. *campestris* at the pathovar level is based on the isolation of the pathogen using semi-selective media.

16S rRNA gene demonstrate conserved sequence regions ideal for prior targeting. Since each bacterial species has a unique 16S rRNA sequence, all organisms can be differentiated from each other using PCR-SSCP (Turenne *et al.,* 2000). For instance, 16S rDNA fragments with different sequences may co-migrate, thus generating bands at the same position in the gel. The study of microbial communities have become common in microbial ecology, with the 16S rRNA gene being the most frequently used phylogenetic marker.

In the present study, 730 bp amplicon was obtained by 16S rRNA primer, which gave a single conformer in all the bacteria studied except a band-shift in one of them. In our study, the SSCP could not yield multiple conformers and hence, we decided to accomplish the restriction digestion of the 730 bp amplicon. SSCP has been reported to lose sensitivity for mutation detection when the fragment size is above 300 bp. RFLP can be considered as another alternative because the restriction digestion of the amplicon in RFLP profiles yielded a distinct fingerprint for each of the bacteria. We developed a molecular method for directly determining the identity of the bacteria based on the principle of SSCP of a PCR-amplified DNA fragment. The target DNA sequence was the 730 bp fragment of the 16S rRNA sequence obtained by using conserved primers. We made an attempt to detect DNA sequence among *Xanthomonas* sp. using PCR-based DNA fingerprinting method including both RAPD and SSCP. Overlapping patterns (in this case, PCR-RFLP and SSCP) between different species should not be observed. The size of PCR amplicon obtained in our study was 730 bp which is large for being detected by SSCP. Hence we decided to perform RFLP and RAPD-M13. Techniques that use enzyme digests such as amplified rDNA restriction analysis (ARDRA), restriction fragment length polymorphism (RFLP), and single-stranded conformation polymorphism (SSCP) produce multiple bands for single species, making patterns difficult to evaluate further. Hence we combined SSCP-RFLP and RAPD-M13 to overcome these drawbacks. Our main focus was to use two molecular typing tools to generate DNA profiles that could help distinguish the *X. campestris* pv. *campestris* from other *Xanthomonas* species. We used microsatellite typing, which involves the amplification of satellite sequences, short (usually <ten bp) tandem repetitive DNA sequences dispersed throughout the genome (Perez *et al.,* 2001). The method relies on the significant level of polymorphism in the lengths of the microsatellite loci and has

been previously reported generating distinguishing profiles in yeast. Amplification of DNA with a single 10 base-long primer of arbitrary DNA sequence is a PCR-based technique, which yields a series of discrete fragments. This method reveals a large number of polymorphisms, which can be used as genetic markers in research involving species' diagnostics, population differentiation and genetic fingerprinting. It is known that the region is highly conserved at the intra-species level, but very variable at inter-species level and has proved to be a useful tool for the diagnosis of closely related species.

The analyses indicated that they had, in general, a higher number of exact matches to the 16S rRNA gene sequences from members of the target group of bacteria. The enzyme restriction mapping disclosed the apparent sequence difference of 16S rRNA gene among them. This study also indicated that RFLP-SSCP would be more reliable comparing to SSCP when the amplicon size is >600 bp. The cluster analyses obtained for both of the restriction enzymes used in the study *viz.,* and *Alw*I and *Fok*I gave a similar clustering pattern with the RAPD-M13 primer (Giraffa *et al.,* 2000; Poplawsky and Chan, 1995). PCR amplification with M13 core sequence proved to be a useful method for discrimination at the species' level of *Xanthomonas* genus. We confirmed the applicability of M13 primer for the identification of *Xanthomonas* sp. The technique, which is rapid and easy to perform, could be an alternative to the conventional approach. Furthermore, in order to design new diagnostic primers, more effective in genetic discrimination among genotypes, discriminate bands could be cloned and sequenced. The alternative methods to study the genomic diversity as DNA microarrays and DNA hybridization are accurate in low-diversity systems and expensive (Turrene *et al.,* 2000). The DGGE (Denaturing and temperature gradient gel electrophoresis) results in a pattern of bands in which each band may represent more than one species because of comigration. Compared to these methods the combination of SSCP and RAPD-M13 core sequence prove to be an efficient method to detect diversity.