Journal of
Plant Pathology & Microbiology

Roohie and Umesha, J Plant Pathol Microb 2012, 3:4
DOI: 10.4172/2157-7471.1000127

Research Article Open Access

Development of Multiplex PCR for the Specific Detection of *Xanthomonas campestris* pv. *campestris* in Cabbage and Correlation with Disease Incidence

RK Roohie and S Umesha*

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore, 570006, Karnataka, India

Abstract

Black rot, the most serious disease of crucifers especially *Brassica oleracea var. capitata* (Cabbage) causes huge yield losses. Black rot is a seed-borne disease and the assessment of seeds for the pathogen is very crucial. The cabbage seeds were tested for *X. campestris pv. campestris* infection by biochemical and PCR analysis. The seed samples were subjected to PCR analysis with three primers, out of which one of the pairs of primers specific for hrpF gene could detect the black rot pathogen. The current study is the first of its kind wherein the locally available cabbage cultivars were assessed using molecular assays and the widely grown cultivars in the study area were checked for the correlation between laboratory and field performances. The aim of the study was to use the available specific and sensitive PCR-based procedures for routine detection of *X. campestris pv. campestris* in cabbage seeds. Multiplex PCR was standardized for the simultaneous detection of pathogen and *Brassica* species, internal transcribed spacer (ITS) in infected seed and leaf material. The multiplex PCR fingerprints were obtained which amplified a 619 bp fragment of the hrpF gene from *X. campestris pv. campestris* and a 360 bp section of the internal transcribed spacer region from *Brassica sp*. The assay also readily detected *X. campestris pv. campestris* infections in diseased plants and from bacterial colonies isolated on selective media, and was more sensitive and specific than traditional plating methods. Thus by the use of multiplex PCR, determination of the threshold level for bacterial detection in cabbage seeds was possible.

Keywords: Black rot; hrpF; 16S rRNA; Cabbage; X*anthomonas campestris pv. campestris*; estA

Introduction

Cabbage (*Brassica oleracea* var. *capitata*) is an important vegetable of cole group, which is a rich source of vitamin A, B, C and minerals. *B. oleracea* is a diploid species with CC genome type and nine haploid chromosomes. Estimated genome size varies from 599-868 Mb, depending on the species. Various subspecies of *B. oleracea* show different morphological characteristics like enlarged terminal bud in cabbage. It covers about 4% of total area under vegetables. India comes next to China in cabbage production. It is now grown throughout the year. Orissa, West Bengal, Bihar, Karnataka, Maharashtra, Gujarat and Punjab are major cabbage-growing states. In Karnataka it is grown in all the seasons over an area of 0.0089 Mkm^2 with an annual production of 0.18 Mt having an average productivity of 20.22 t/ha [1]. Commonly grown cabbage in India is white. There are number of cabbage cultivars available in the Indian market but only few of them are tolerant to the most important bacterial disease known as Black rot of cabbage. There is a considerable decrease in the production of cabbage from past two years that can be attributed to the prevalence of disease in the area.

In India, the disease was first reported on cabbage from Bombay [2]. It was later reported from Katrain (Kullu district) area of Himachal Pradesh [3]. Since then the occurrence of the disease has also been reported from other parts of India. Black rot of cabbage is a very destructive disease and causes losses by premature defoliation and also affects the quality of heads in cabbage. Crop loss upto 50% in cabbage has been recorded. 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 [4]. 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 [5]. *X. campestris* pv. *campestris* is a small, rod shaped, aerobic gram-negative, nonspore-forming bacterium [6]. 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 [5,6]. 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 [7,8] and seeds, in which *X. campestris* pv. *campestris* cells can survive up to three years [9]. 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 [10], transmission by a wide range of host plants including cruciferous weeds [11] and by an epiphytic survival also on non-cruciferous crops acting as a shelter for the pathogen in the field [8].

***Corresponding author:** S. Umesha, Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore, 570006, Karnataka, India, Tel: 91-08212419884, India; E-mail: umeshgroup@yahoo.co.in; pmumesh@gmail.com

Received July 24, 2012; **Accepted** August 18, 2012; **Published** August 24, 2012

Citation: Roohie RK, Umesha S (2012) Development of Multiplex PCR for the Specific Detection of *Xanthomonas campestris* pv. *campestris* in Cabbage and Correlation with Disease Incidence. J Plant Pathol Microb 3:127. doi:10.4172/2157-7471.1000127

Copyright: © 2012 Roohie RK, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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.

Our objective 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. But it 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 identify 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 [12] 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 host. 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.

Materials and Methods

Isolation of pathogen from leaf material and seeds

Recovery of *X. campestris pv. campestris* from plant material: The leaf material was collected from different fields in and around Mysore viz., three fields in Srirampura out of which 10 infected samples were obtained. Similarly out of 12 fields visited in Jaypura village, 15 samples which showed black rot symptoms were collected. The number of villages visited is listed in Table 1. 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 suspected to be caused by *X. campestris pv. campestris* were transferred to a drop of sterile distilled water and viewed under 100×magnification for the presence of typical bacterial ooze. The suspension was aseptically recovered from the slide and plated onto Nutrient Agar (NA) medium. The isolated pathogens were subjected to biochemical and molecular characterization.

Name of taluk	Village	Village	Cultivars grown
Srirampura	Parasayyana Hundi	1	Indam Krishna
	Mahadevapura	2	Unnati
Jayapura	Madahalli	3	Gaurav
	Kergalli	2	Indam Krishna
	Daripura	4	Unnati
	Danagalli	3	Indam saina

Table 1: Cabbage fields visited in Mysore dist.

Small pieces of the infected leaves were cut aseptically. The leaf bits were 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 100 µl of the diluted bacterial suspension was poured onto nutrient agar plates. The inoculated plates were kept at 28°C for 72 h. Observations were made for the development of light yellow, convex small bacterial colonies on nutrient agar medium.

Genomic DNA isolation from cabbage leaves: DNA extraction from greenhouse-grown cabbage leaves, infected leaf material and seed samples was performed by grinding tissue in liquid Nitrogen and 100 mg of the ground tissue was transferred to a 1.5 ml microfuge tube. The leaf tissue was homogenized in 100 µl DNA 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. The samples were vortexed for 60 s and incubated. The samples were incubated at 65°C for 20 min for cell lysis. Then 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 water. The aliquots were placed in -20°C. The DNA was quantified using spectrophotometer (Beckman, USA).

Detection of *Xanthomonas campestris* pv. *campestris* in cabbage seeds: All the 20 varieties of cabbage seeds (Round head) available in the study area were collected from the commercial dealers in Mysore. The seeds were subjected to various seed 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&S), growing-on test (GOT) and biochemical tests followed by the PCR based detection methods.

Direct plating method: Seed assays were performed on all seed varieties by plating out seeds on nutrient agar (NA) and Star's minimal (SX) agar (a semi-selective medium), as described by [13]. 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. Fifteen sterilized seeds were plated in each petri plate. 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.

The grind and soak method: From each seed cultivar 15 randomly selected seeds per working sample were sterilized in 1% sodium hypochlorite for 30 s, rinsed in SDW for 2-3 minutes and then dried

on sterile filter paper. The dried seeds were ground, and then soaked in 5 ml of phosphate buffer saline (PBS), pH 7.4, for three hours. The resultant supernatant collected after filtering was used to prepare serial dilutions. These dilutions were plated onto NA and SX and incubated at 28°C for two days.

The "growing-on" test: A glasshouse pot experiment was conducted to determine the presence of *X. campestris* pv *campestris* in seed lots 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 lots 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: 0- Immune (I) with no visible infection, 1- Resistant (R), 2- Moderately Resistant (MR), 3- Moderately Susceptible (MS), 4-Susceptible (S), 5-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.

Conventional characterization of *X. campestris pv. campestris:* The physiological and biochemical characters of the isolates was studied for colony morphology on yeast dextrose calcium carbonate agar (YDCA) medium and the biochemical characters like gram's staining, starch hydrolysis, oxidase test, catalase test, growth on aspargine medium, presence of xanthomonadin pigment. The tests were conducted as per the procedure described by Bradbury [14] and Schaad [13].

Gram staining reaction: The Gram-reaction of each isolate was determined following the staining procedure in Schaad [13]. Isolates that appeared pink were considered as gram negative bacteria and were subjected for further tests.

Starch hydrolysis: The isolates were streaked on starch agar medium (starch soluble, 20 g L^{-1}; Peptone, 5 g L^{-1}; Beef extract, 3 g L^{-1}; agar, 15 g L^{-1} in distilled water with pH 7 and autoclaved at 121°C for 15 min) to evaluate their ability to hydrolyze starch (amylase production). The plates were incubated at 28°C and for 2-3 days starch hydrolysis was observed by flooding the plates with Gram's iodine solution for 30 seconds. The appearance of clear zone around the line of growth of each isolate indicated starch hydrolysis.

Oxidase Test: A small loopful of each bacterial isolate was placed on a filter paper with drops of 1x (w/v) aqueous N, N, N, N`- tetra methyl-p-Phenylenediamine dihydrochloride solution. Isolates which developed purple color within 10 s were taken as positive, purple color in 10-30 s were taken as slow positive and those with no color for more than 30 s taken as negative to oxidase test.

Catalase Test: Few drops of 3% hydrogen peroxide were added on the surface of 48 h old culture of each isolate on NA medium and bubble formation was recorded as positive for catalase activity.

Growth on Asparagine medium: All Gram-negative isolates were plated on Asparagine medium (Asparagine, 0.5 g L^{-1}; KH_2PO_4, 0.1 g L^{-1}; $MgSO_4.7H_2O$, 0.2 g L^{-1}; KNO_3, 0.5 g L^{-1}; $CaCl_2$, 0.1 g L^{-1}; NaCl, 0.1 L^{-1}g and agar, 12-15 g L^{-1} (for plates) with pH 7 and autoclaved at 121°C for 15 min) at 28°C for 48-72 h without any other carbon and nitrogen sources. This is used as a diagnostic test for *Xanthomonas* because they are not able to grow on it while others like yellow *Enterobacteriaceae* and many *Pseudomonas* can grow on it. The growth of the bacteria on Asparagine agar plates was recorded and those isolates that were unable to grow on the medium were taken for further tests.

Presence of Xanthomonadin pigment: Each isolate was streaked on nutrient agar and incubated at 28°C for 48 h. About 2-3 loopful colonies of each bacterial isolate were transferred to 3 ml of spectrophotometry grade methanol in test tubes and were placed in boiling water bath until the pigment was removed. The suspension was then centrifuged at 13,000 rpm for 15 min to remove cell debris. The supernatant was decanted and the methanol was allowed to evaporate by keeping the methanol extract in 50-60°C-water bath until the optical density of the pigment extract reached 0.4 at 443 nm. 5 µl of each extract was spotted on a precoated, silica gel plates and a total 25 µl was spotted. The silica gel plates were developed in methanol solvent. The solvent was allowed to move approximately 10 cm and the yellow spots were marked with a pencil while wet. A yellow spot with an average Rf value of 0.45 was taken as positive for the presence of the pigment [13]. A scan using absorption spectrum analysis from 530-350 nm was also performed. A peak at 445 nm with shoulders at 467 and 420 nm indicates the presence of xanthomonadin pigment.

Hypersensitivity test: Forty-eight hours old cultures of each isolate was suspended in sterilized distilled water and adjusted to 0.45 absorbance at 460 nm (10^7-10^8 cfu/ml bacterial cell concentration) using spectrophotometer (Schimadzu, Japan). An aliquot of 2 ml of each bacterial culture suspension was injected using a sterilized hypodermic syringe into the intercellular spaces of expanded leaves of a one-month tobacco plant (*Nicotiana tabacum*). Injection of sterilized distilled water was used as negative control. All the tobacco plants were kept in green house at 25-30°C and 15-18°C day and night temperature until symptom developed and a complete collapse of tissues occurred with yellow chlorosis to brown necrosis around the injection point was taken as positive for the test [15].

Pathogenicity test: Cabbage seedlings were raised in sterilized coir pith in small polythene bags. Thirty to forty day's old seedlings were transplanted to plastic pots for the purpose of inoculation of the bacterial isolate. Isolates that were preserved at 4°C were grown on YDCA medium at 28°C for 48 h, harvested, suspended in sterilized water and adjusted to an absorbance at 460 nm (10^7-10^8 cfu/ml bacterial cell concentration with an OD value of 0.2-0.3) using spectrophotometer. The six to eight weeks old cabbage plants were preincubated at 28°C for 72 h. An aliquot of 1 ml of the bacterial suspension were inoculated using a hypodermic sterile syringe to the second innermost leaf petiole with three replications. Sterilized distilled water was also inoculated as negative control. The inoculated plants were kept in glasshouse. Observations were made for the development of symptoms of black rot. The bacterium was re-isolated from the infected seedlings and compared with the pure culture by studying the biochemical characteristics. The disease symptoms of the plants were observed in every week for 3 months.

Molecular characterization of *Xanthomonas campestris pv campestris*

Genomic DNA extraction of *Xanthomonas campestris pv campestris* colonies: Ten 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 [16]. 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 1 ml buffer (50 mM ethylene diamine tetra acetate (EDTA), 0.15M 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 50°C for 1 h. Equal volume of Chloroform-Phenol-Isoamyl alcohol (24:25:1) buffered with 10 mM 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, 3 M Sodium acetate and 1 vol of Iso-propanol was added and mixed by vortexing. The precipitate 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. [17] and hrpF according to Berg et al. [18].

16S rRNA amplification: For determination of the identities of the colonies, a PCR assay using 16S-F3 (5'-CCAGACTCCTACGGGAGG-CAGC-3') and 16S-R1 (5'-GCTGACGACAGCCAT GCAGCACC-3') as primers. The PCR assay was carried out using the primer set for 16S rRNA which were custom synthesized by Sigma (Sigma Aldrich, 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, 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 (Bangalore Genei, 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) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

PCR analysis with estA primer pairs: The characteristic colonies of the bacterium isolated were suspended in 50 µl of sterile distilled water. The bacterial suspension was incubated for 10 min at 100°C for cell lysis and was maintained at 4°C before use. The PCR assay was carried out using the primer set for estA. 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, 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 (Bangalore Genei, 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) 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. [18] 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 extract 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, 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, USA). The BE3 and BE4 primers were used to amplify the ITS region of the ten cultivars used in the present study.

Multiplex PCR: 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, USA). The BE3 and BE4 primers were used to amplify the ITS region of the ten cultivars used in the present study. A multiplex PCR was performed using the hrpf (BE1& BE2) and ITS (BE3 & BE4) primers for the simultaneous detection of pathogen in the infected leaf material collected 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.

Detection of Amplified DNA: 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.

Results

Isolation of pathogen from leaf material and seeds

Cabbage leaf samples collected from different cabbage growing regions in Mysore were analysed for the presence of *X. campestris* pv. *campestris*. Out of ten leaf materials collected from Srirampura, Mysore dist, only two samples were found to be infected with *X. campestris* pv. *campestris*. Out of 15 samples from Jayapura village, only three were found to be positive, one from Salundi village and one from Daripura village. The isolates were designated as Xcc -01 to Xcc - 05. The isolates, which showed highly mucoid type of growth with yellow colony color as compared with their growth on YDCA and there were also isolates with less mucoid growth and yellow color. The *X. campestris pv. campestris*

Sl No	Cultivars	Results		
		DPM	G & 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	+	+	+

Table 2: Results of the Direct Plating method, Grind and soak and growing on test. '+' indicates presence of pathogen detected; '-' indicates pathogen not detected DPM: Direct plating method; G & S: Grind and soak method; GOT: growing on test.

isolates recorded typical symptoms viz., highly mucoid, glistening and convex. The genomic DNA from the infected material was quantified, good quality DNA was obtained and was used for PCR assays.

The cabbage seeds which were collected from local traders subjected to preliminary tests. The ten selected cultivars were as follows: Indam Krishna, Unnati, F1 Bhima, Golden acre, Kiran, Gaurav, Indam saina, Quisto, Ganesh Gol and NBH-Boss. The ISTA guidelines, which included direct plating, grind and soak method and growing on test respectively resulted in the development of colonies. The colonies were assessed based on the colony morphology and the colonies. The results of the DPM, G&S and GOT are summarized in Table 2.

In direct plating method 10 suspected colonies which showed highly mucoid type of growth with yellow colony color YDCA were designated as Xcc- 06 to Xcc 16. The isolates from cultivars F1 Bhima, Golden Acre, NBH-Boss and Kiran are the ones which were found to be highly infected with the pathogen.

In grind and soak method, out of 10 cultivars, none of the cultivars were free from bacterial infection. The method yielded some xanthomonad species which resembled *X. campestris pv. campestris* based on colony colour and shape on NA and SX. On SX, colonies of *X. campestris pv. campestris* were pale yellow, convex and mucoid, and were surrounded by a zone of starch hydrolysis. On NA, they were yellow. The 10 colonies were designated as Xcc-17 to Xcc- 27.

In growing-on test, noticeable symptoms of black rot were observed in some of the cultivars, four cultivars viz., F1 Bhima, Golden Acre, Kiran and NBH-Boss showed the typical symptoms of black rot of cabbage. From all the four cultivars which were recorded as positive for *X. campestris pv. campestris* symptoms, the leaf samples were collected and the pathogen was reisolated from the leaf samples. Such *X. campestris pv. campestris* isolates were designated as Xcc 28 to Xcc- 36.

Characterization of *X. campestris pv. campestris*

All the isolated cultures were subjected for characterization tests viz., Gram's staining, starch hydrolysis, catalase test, oxidase test, growth on aspargine medium and presence of xanthomonadin pigment, hypersensitivity and pathogenecity tests. Out of the 36 isolates subjected for Gram's staining, all of them appeared pink colored under the compound microscope indicating all of them to be gram negative bacteria.

When the isolates were subjected to starch hydrolysis, all the isolates showed a clear zone of around the line of growth of each isolate after flooding with Lugol's Iodine. Hence all the isolates were found to degrade starch and considered as positive for starch hydrolysis.

	Biochemical reaction					
Isolates	Gram's reaction	Starch Hydrolysis	Oxidase test	Catalase test	Aspargine medium	Xanthomonadin pigment
1	-	+	+	+	+	-
2*	-	+	+	+	-[a]	+[b]
3*	-	+	+	+	-[a]	+[b]
4*	-	+	+	+	-[a]	+[b]
5*	-	+	+	+	-[a]	+[b]
6	-	+	+	+	+	-
7	-	+	+	+	+	-
8	-	+	+	+	+	-
9	-	+	+	+	+	-
10*	-	+	+	+	-[a]	+[b]
11	-	+	+	+	+	-
12	-	+	+	+	+	-
13	-	+	+	+	+	-
14*	-	+	+	+	-[a]	+[b]
15	-	+	+	+	+	-
16	-	+	+	+	+	-
17	-	+	+	+	+	-
18	-	+	+	+	+	-
19	-	+	+	+	+	-
20*	-	+	+	+	-[a]	+[b]
21	-	+	+	+	+	-
22	-	+	+	+	+	-
23	-	+	+	+	+	-
24	-	+	+	+	+	-
25*	-	+	+	+	-[a]	+[b]
26	-	+	+	+	+	-
27	-	+	+	+	+	-
28*	-	+	+	+	-[a]	+[b]
29	-	+	+	+	+	-
30	-	+	+	+	+	-
31	-	+	+	+	+	-
32	-	+	+	+	+	-
33*	-	+	+	+	-[a]	+[b]
34	-	+	+	+	+	-
35	-	+	+	+	+	-
36	-	+	+	+	+	-

'+' indicates positive reaction; '-' indicates negative reaction: [a] indicates isolates failed to grow on Asparagine medium; -[b] indicates presence of xanthomonadin pigment. '*' indicates typical *X. campestris pv. campestris* characteristics

Table 3: Identification of isolates *X. campestris pv. campestris* according to biochemical characteristics.

The oxidase test showed that most of the isolates which developed purple color within 10 seconds were taken as positive, purple color in 10-60 seconds were taken as slow positive and those with no color for more than 60 seconds taken as negative to oxidase test.

When few drops of 3% hydrogen peroxide was added on the surface of 48 h old culture of each isolate on YDCA medium, bubble formation was recorded in all the 36 isolates. The formation of bubbles indicated that the isolates are positive for catalase activity.

The growth of the bacteria on Asparagine agar plates was recorded and only ten isolates out of the total 36 isolates did not grow on the aspargine medium, the isolates are viz., isolate 2, 3, 4, 5, 10, 14, 20, 25, 28, and 33. All the isolates which didn't grow were considered and only those were taken for further tests. In all cases, uninoculated medium was taken as negative control.

All the ten isolates which didn't grow on aspargine medium were

Biochemical Tests	Results
Gram Reaction	-
Starch hydrolysis	+
Catalase test	+
Growth on aspargine medium	+
Presence of xanthomonadin pigment	+
Tobacco hypersensitivity test	+
Pathogenicity test	+

All the tests were conducted in three replicates and repeated thrice; '+' indicates positive reaction; '-' indicates negative reaction

Table 4: Summary of Biochemical tests.

S. No.	Isolates	Source	Colony Shape	Colony Edge	Color	Appearance of colony
1	Xcc 02	Leaf material (Daripura)	Circular	Entire	Yellow	Opaque Glistening Convex
2	Xcc 03	Leaf material (Salundi)	Circular	Entire	Light yellow	Opaque Glistening Convex
3	Xcc 04	Leaf material (Jaypura)	Circular	Entire	Dark yellow	Opaque Glistening Convex Opaque Glistening Convex
4	Xcc 05	Leaf material (Daripura)	Circular	Entire	Yellow	Opaque Glistening Convex
5	Xcc 10	Golden acre	Circular	Entire	Light Yellow	Opaque Glistening Convex
6	Xcc 14	Kiran	Circular	Entire	Light Yellow	Opaque Glistening Convex
7	Xcc 20	Golden acre	Circular	Entire	Dark yellow	Opaque Glistening Convex
8	Xcc 25	Kiran	Circular	Entire	Dark yellow	Opaque Glistening Convex
9	Xcc 28	Golden acre	Circular	Entire	Dark yellow	Opaque Glistening Convex
10	Xcc 33	Kiran	Circular	Entire	Dark yellow	Opaque Glistening Convex

Table 5: List of positive isolates of *X. campestris pv. campestris* and their colony characteristics and source.

tested for the presence of xanthomonadin pigment. The pigment isolated was run on silica gel plate, yellow spots were observed and marked with a pencil while wet. A yellow spot with an average Rf value of 0.45 was taken as positive for the presence of the pigment. A peak at 445 nm with shoulders at 467 and 420nm was observed spectophotometrically which indicated the presence of xanthomonadin pigment. The reaction of all the 36 isolates is summarised in Table 3.

The Hypersensitivity test was performed on *Nicotiana tobaccum cultivar. xanthi.* The pure culture of *X. campestris pv. campestris* injected into the intercellular spaces of tobacco leaves produced characteristic water soaked lesions within 16 h of inoculation and after 24 h post inoculation light yellow area started collapsing and formed a light brown necrotic area.

The pathogenecity test which was performed for all the ten isolates showed typical black rot symptoms. The leaves showed light yellow to dark brown necrosis around the inoculated area of the leaves within 7 days and no necrosis was observed in control inoculated with sterilized water. Most of the *X. campestris pv. campestris* strains in this study caused systemic black rot symptoms in cabbage. The observed symptoms from some *X. campestris pv. campestris* strains, was characterised by necrosis and sudden collapse of large areas of mesophyll in advance of blackening of veins. The summary of the biochemical tests is given in Table 4.

Out of 36 isolates from different sources, the five isolates were obtained from leaf material, only four were proven to be *X. campestris pv. campestris*, viz., isolate Xcc-02, 3, 4 and 5 isolated from the leaf material collected from Daripura, Salundi and Jaypura , Mysore dist respectively. The seed samples which were screened for the presence of *X. campestris* pv. campestris, out of 31 isolates from seed samples only six were proven to be *X. campestris pv. Campestris*. They are Xcc - 9, Xcc-10 from the direct plating method, Xcc -20 and Xcc -25 by grind and soak method, Xcc -28 and Xcc -33 from growing- on test. All the six isolates from seed samples were from two cultivars viz., Golden acre and Kiran. The summarized list of the ten positive isolates of *X. campestris pv. campestris* with their source and colony characteristics are listed in Table 5.

The ten cultivars were checked for their response upon infection with *X. campestris pv. campestris*, which revealed that only four of the cultivars viz., Indam Krishna, Unnati, Gaurav and Indam Saina were moderately resistant to the disease, whereas the other six cultivars were either susceptible or highly susceptible. No cultivar was found to be resistant to black rot of cabbage. The results obtained in the greenhouse were in terms with the field response of the cultivars. The response of the ten cultivars is summarized in Table 6.

PCR assay

The DNA was isolated from all the positive ten isolates and good quality DNA was observed. The isolated DNA was quantified using Spectrophotometer (Beckman Coultier, USA). The DNA was later used for amplification using three different primers (Figure 1).

The isolates were later subjected to molecular characterization by PCR using 16S rRNA, estA gene and hrpF genes.

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 730 bp region (Figure 2).

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).

PCR analysis hrpF primer: The hrpF primer amplifies a region of

Response of cabbage varieties		
Varieties	**Greenhouse Response**	**Field Response**
Indam Krishna	MR[1]	MR
Unnati	MR	MR
F1 Bhima	HS[2]	-
Golden Acre	S[3]	-
Kiran	HS	-
Gaurav	MR	MR
Indam Saina	MR	MR
Quisto	S[3]	-
NBH-Boss	HS	-
Ganesh gol	HS	

'MR' - Moderately resistant; 'HS' - Susceptible; 'S' - Highly Susceptible

Table 6: Response of cabbage varieties against *X. campestris pv. campestris.*

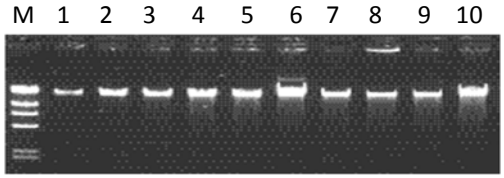

Figure 1: Agarose gel electrophoresis (1%) of DNA isolated from ten isolates of *X. campestris* pv. *campestris*. Lane 1- 10: Isolates of *X. campestris* pv. *campestris* (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively) M: 1kb DNA ladder(Bangalore Genei).

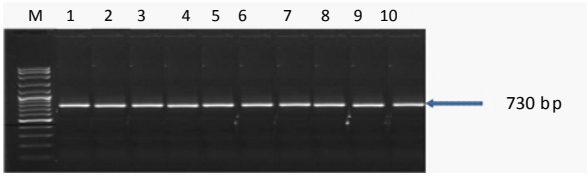

Figure 2: Agarose gel electrophoresis (2%) of PCR products amplified with the 16S rRNA primer set. Lanes: 1-10, *X. campestris* pv. *campestris* isolates (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively) The arrow indicates 730bp amplification. Lane M: molecular size markers (100bp DNA ladder, Bangalore Genei).

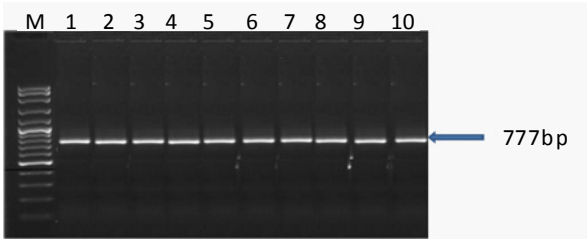

Figure 3: Agarose gel electrophoresis (2%) of PCR products amplified with estA primer set. Lanes: 1-10, *X. campestris* pv. *campestris* isolates (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively) The arrow indicates 777 bp amplification. Lane M: molecular size marker (100bp DNA ladder, Bangalore Genei).

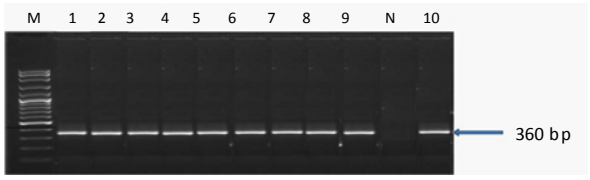

Figure 4: Agarose gel electrophoresis (2%) of PCR products amplified with hrpF primer set. Lanes: 1-10, *X. campestris* pv. *campestris* isolates (Xcc-2, 3, 4, 5,10, 14, 20, 25, 28 and 33 respectively) The arrow indicates 619 bp amplification. Lane M: molecular size marker (100bp DNA ladder, Bangalore Genei).

the hrpF gene. All the ten isolates showed a 619 bp amplification which is specific for *X. campestris pv. campestris* as no other *xanthomonas sp* could amplify the hrp F gene (Figure 4).

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 5).

Multiplex PCR: A multiplex PCR was performed in which an ITS primer specific to *brassica sp* was used along with the hrpF gene. When the infected material and the pathogen DNA were isolated and subjected to multiplex assay, a 360 bp 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 by DPM, G&S, GOT methods also showed a typical 619 bp amplification which shows that the seed samples carry the infection (Figure 6).

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. The hrpF primers amplify the hypersensitivity and pathogenecity genes which are highly conserved and are very pathogen specific [18]. 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 our 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 [18]. The laboratory experiments conducted showed that only two cultivars viz., Indam Krishna and Unnati proved to be relatively tolerant to other cultivars taken up in the study. This study has given an insight into taking up two more cultivars viz., Gaurav and Indam Saina for production at large scale.

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*. Initially, it was a disease of minor importance, has become a major

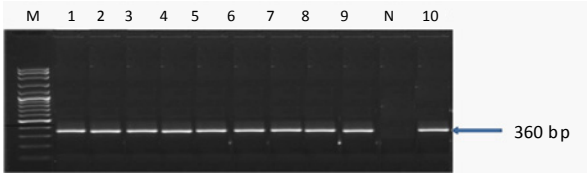

Figure 5: Agarose gel electrophoresis (2%) of PCR products amplified with the ITS primer set. Lanes: 1-10 the ten cvs viz., Indam Krishna, Unnati, Golden Acre, Kiran, Gaurav, Indam Saina, Quisto and NBH- Boss. Lane N: Non brassica sp. The arrow indicates 360bp amplification. Lane M: molecular size markers (100bp DNA ladder, Bangalore Genei).

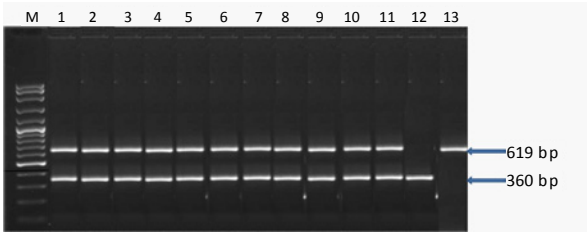

Figure 6: Agarose gel electrophoresis (2%) of PCR products amplified with hrpF and ITS primer set. Lanes: 1-4 infected leaf samples, Lane 5- 10 infected seed samples, lane 12: uninfected leaf sample. Lane 13: Black rot pathogenThe arrows indicate 619 and 360bp amplification. Lane M: molecular size markers (100bp DNA ladder, Bangalore Genei).

disease among the vegetable crops in recent years. In India, the disease was first reported on cabbage from Bombay on cauliflower in 1949 [19], 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 [11], 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 [5,6,20,21]. The data obtained in the present study reveals the 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 and 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.

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 by PCR is 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*. PCR based seed assays are 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.

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* was developed. 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 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 to be a useful target for the PCR-based detection of *X. campestris* pathovars. The black rot bacteria may be carried within the seed, often attached to the funiculus (infection), or simply associated with the surface [20]. 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 confirmed 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 [22], 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). 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 [23]. 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.

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 developed. 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 management or seed disinfection. The present results indicate the existence of different strains of *X. campestris pv. campestris* and 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.

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.

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.

Acknowledgement

RKR acknowledges Council of Scientific and Industrial Research (CSIR), New Delhi, India for financial support in the form of Senior Research Fellowship.

References

1. http://nhb.gov.in
2. Patwardhan GB (1928) Field, garden and orchard crops of the Bombay presidency. Bull of Department of Agriculture, Bombay.
3. Rao YP, Srivastava DN (1964) Epidemic of black rot disease of cauliflower at katrain and measures for its prevention. Indian J of Horticulture 21: 244-246.
4. Gay PA, Tuzun SA (2000) Temporal and spatial assessment of defense responses in resistant and susceptible cabbage varieties during infection with *Xanthomonas campestris pv. campestris*. Physiol Mol Plant Pathol 57: 201-210.
5. Williams PH (1980) Black rot: A continuing threat to world crucifers. Plant Disease 64: 736-742.
6. Onsando JM (1992) Black rot of Crucifers. New Jersey, USA: Prentice Hall, Englewood Cliffs 243–252.
7. Dane F, Shaw JJ (1994) Endophytic and epiphytic growth of *Xanthomonas campestris pv. campestris* on susceptible and resistant host plants in the field environment, detection by bioluminescence. Microb Releases 2: 223–229.
8. Arias RS, Nelson SC, Alvarez AM (2000) Effect of soil-matric potential and phylloplanes of rotation-crops on the survival of a bioluminescent *Xanthomonas campestris pv. campestris*. Eur J Plant Pathol 106: 109–116.
9. Clayton EE (1925) Second progress report of black-rot (*Pseudomonas campestris*) investigations on Long Island: seed infection and seasonal development. Phytopathology 15: 48-49.
10. Kocks CG, Zadoks JC, Ruissen MA (1999) Spatio-temporal development of black rot (*X. campestris* pv. campestris) in cabbage in relation to initial inoculum levels in field plots in the Netherlands. Plant Pathol 48: 176–188.
11. Schaad NW, Alvarez A (1993) *Xanthomonas campestris pv. campestris*: Cause of black rot of crucifers. Xanthomonas Chapman and Hall, London, United Kingdom.
12. Lindgren PB, Peet RC, Panopoulos NJ (1986) Gene cluster of Pseudomonas syringae pv. "phaseolicola" controls pathogenicity of bean plants and hypersensitivity of nonhost plants. J Bacteriol 168: 512-522.
13. Schaad NW (1988) Laboratory Guide for Identification of plant pathogenic bacteria. (2ndedn), The American Phytopathological Society, Minnesota, USA.
14. Bradbury JF (1984) Xanthomonas Dowson 1939. In: Bergey's manual of systematic Bacteriology. Williams and Wilkins, Balimore.
15. Quimio JA (1992) Annual report of the plant pathologist. EnsetTeam Support Project Sidamo GamoGofa. Peasants Agricultural Development Program-PADEPIII. Awasa Research Center (IAR). Awasa, Ethiopia.
16. Gabriel DW, De Feyter R (1992) RFLP analyses and gene tagging for bacterial identification and taxonomy. In: Molecular Plant Pathology. A Practical Approach. IRL Press, Oxford 1: 51-66.
17. Lee YA, Sung AN, Liu TF, Lee YS (2009) Combination of chromogenic differential medium and estA-specific PCR for isolation and detection of phytopathogenic *Xanthomonas spp*. Appl Environ Microbiol 75: 6831-6838.
18. Berg T, Tesoriero L, Hailstones DL (2006) A multiplex real-time PCR assay for detection of *Xanthomonas campestris* from brassicas. Lett Appl Microbiol 42: 624-630.
19. Patel MK, Abhyankar SG, Kulkarni YS (1949) Black rot of cabbage. Indian Journal of Phytopathol 2: 58-61.
20. Cook AA, Walker JC, Larson RH (1952) Studies on the disease cycle of black rot of crucifers. Phytopathology 42: 162–167.
21. Alvarez A (2000) Black rot of crucifers. Mechanisms of Resistance to Plant Diseases. Dordrecht, Kluwer Academic Publishers, USA.
22. Randhawa PS, Schaad NW (1984) Selective isolation of *Xanthomonas campestris pv. campestris* from crucifer seeds. Phytopathology 74: 268-272.
23. Johnson JR (2000) Development of polymerase chain reaction-based assays for bacterial gene detection. J Microbiol Methods 41: 201-209.