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RESEARCH ARTICLE

Bio-PCR based diagnosis of *Xanthomonas campestris* pathovars in black rot infected leaves of crucifers

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ABSTRACT: *Xanthomonas campestris* pv *campestris* caused black rot disease of crucifers occurs worldwide and causes substantial losses under favorable conditions. A bio-PCR based method has been standardized for the rapid and reliable diagnosis of the black rot disease in crucifers. Primers were used specifically to amplify a 619bp fragment of the *hrp*F gene from *X. campestris*. To test specificity of primers, DNA of *X. campestris* pv. *campestris*, and other species of *Xanthomonas* such as *X. oryzae* pv. *oryzae*, *X. citri* pv. *citri, X. axonopodis* pv. *punicae, Erwinia carotovora* subsp. *carotovora, Pseudomonas fluorescens* and *P. syringae pv. phaseolicola* were also used as template for amplification at 619 bp. For diagnosis, samples of suspected black rot infected leaves of mustard, sarson sag, cauliflower, cabbage, broccoli and radish were collected and isolated bacteria on nutrient agar medium. The colonies of bacteria grown on the medium were used as template for PCR and the same colonies were inoculated to their respective hosts for pathogenicity test. Amplification at 619 bp was visible in all isolates of *X. campestris* pv. *campestris* and no amplification was noted with other species of *Xanthomonas, Erwinia* and *Pseudomonas*. The colonies amplified at 619 bp were able to produce the symptoms on their respective hosts. There was no amplification in radish sample. It shows that the bio- PCR is confirmatory to the pathogen *X. campestris* pv. *campestris,* which rapidly diagnose the disease within 3 - 4 days with great accuracy.

Key words: Bio-PCR, *Brassica* species, crucifers, diagnostic, *Xanthomonas campestris* pv. *campestris*

Xanthomonas campestris pv. *campestris* infects a large numbers of cruciferous plants including cole crops, oilseeds and flowers and causes black rot disease which significantly reduce the yield of crops all over the world (Williams, 1980). As per reclassification of this species based on DNA- DNA hybridization studies, pathovars of *X. campestris* being amended from over 140 to just six (Vauterin *et al*., 1995). This comprises only those pathovars that cause diseases on crucifers such as *X. campestris* pv. *aberrans* (*Brassica oleracea*), *X. campestris* pv. *armoraciae* (*Armoracia* spp.), *X. campestris* pv. *barbarae (Barbarea vulgaris), X. campestris* pv. *campestris* (*Brassica* spp., *Cheiranthus, Raphanus sativus*), *X. campestris* pv. *incanae* (*Mathiola* spp.) and *X. campestris* pv. *raphani* (*R. sativus*). Zhao *et al*. (2000) described black rot and leaf spot diseases in Brassicas, which have been attributed to pathovars *campestris, armoraciae, aberrans* and *raphani,* and further divided into races on the basis of interactions with differential Brassica cultivars (Vicente *et al.,* 2001). Pathovar *campestris* typically produces V- shaped lesions at margin of leaves and *aberrans*, *armoraciae* or *raphani* produce leaf spots. However, black rot isolates can cause leaf spots when spray inoculated on to plants for pathogenicity test (Zhao *et al*., 2000), and plants inoculated with leaf spot isolates have been known develop lesions indistinguishable from black rot (Moffett *et al*., 1976), whereas Alvarez *et al.* (1994) differentiated the leaf spotting from black rot- inducing isolates in pathogenicity tests. Identification of bacterial isolates had been confirmed by classical bacteriological tests such as colony characteristics, hydrolysis of starch, esculin and casein, growth at 35^{0}C, acid from glucose, arabinose, and mannose (Schaad *et al*., 2001) and inoculation of susceptible brassica seedlings (Roberts and Koenraadt, 2003). These methods are time consuming (more than 10 days) and labourious, which are not suited for rapid and high throughput screening of plant samples. Molecular DNA based techniques especially those based on PCR are very powerful in plant disease diagnosis (Vincelli and Tisserat, 2008; Massimo *et al.,* 2007; Berg *et al*., 2005). Walton (1997) described that the hypersensitive response and pathogenicity (*hrp*) gene clusters are crucial for the interaction between phytopathogenic bacteria and their hosts, which produce diseases in susceptible plants or the hypersensitive reaction in resistant plants. It has been reported that *hrp* gene clusters are largely conserved among plant pathogenic bacteria, where they encode type III secretion systems, which deliver pathogenicity factors, elicitors and avirulence proteins to the plant cell (Hueck, 1998). The potential for specific amplification of the *hrp* genes has previously been investigated for the detection and identification of *X. axonopodis* pv. *vesicatoria* in tomato and capsicum (Leite *et al.,* 1994) and *X. campestris* pathovars in crucifers (Berg *et al.,* 2005). Diagnosis of diseases is an important step in plant disease management and it can be done either by on the basis of conventional methods such as symptoms, colony characters, and biochemical tests or some tests advanced techniques like serological tests, and nucleic acid based techniques (Janse, 2006). However, correct diagnosis of diseases can be done only by pathogenicity test, but it takes more than 10 days to confirm the disease and the pathogen. Hence, the diagnosis of black rot disease of crucifers was done through PCR technique *i.e.* Polymerase chain reaction (PCR) to reduce the time and labour. This paper describes the methods of PCR that

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distinguish *X. campestris* from other groups of Xanthomonads as well as other phytopathogenic bacteria and standardize the protocols for detection of *X. campestris* pathovar.

MATERIALS AND METHODS

Bacterial strain

Eighteen isolates of *Xanthomonas campestris* were collected from various hosts of *Brassica* spp. and *Raphanus sativus* (radish) mentioned in Table 1. The isolates of *X. campestris* and other plant pathogenic bacteria were grown on nutrient agar medium to prepare the inoculum for further study.

Genomic DNA extraction from bacteria

The isolates of *X. campestris pv. campestris* (Xcc) and other test bacteria *X. oryzae* pv. *oryzae, Pseudomonas fluorescens, P. syringae pv. phaseolicola, Erwinia carotovora* subsp. *carotovora, X. axonopodis* pv. *punicae and X. axonopodis* pv. *citri* were grown in nutrient broth at 28^{0}C for 24 h. Total DNA from bacteria was extracted with guanidium thiocyanate as described by Pitcher *et al*. (1989). Eluted DNA was diluted by adding 100 µl 1X TE buffer for the use in PCR and stored at -20^{0}C.

Primers and PCR condition

Primer pair DLH 120 forward 5'-CCGTAGCACTTAG TGCAATG-3' and DLH 125 reverse: 5'-GCATTTCCATCGG TCACGATTG-3' with a predicted PCR product size 619 bp amplified the 3' end of *hrp*F and DLH 109 (5'-ATGTCGCTCAACACGCTTTC-3') and DLH 112 (5'-GTTTTGCGTGTAGCCCTTTGC-3') with PCR product size 1.4kb were used to amplified the 5' repeat sequence within *hrp*F (Berg *et al.,* 2005). PCR amplification was performed in Perkin Elmer thermocycler. The amplifications were carried out in a final volume of 25 µl containing 1.5mM $MgCl_2$, 200µM dNTPs (Promega), 1X PCR buffer, 1 unit taq polymerase, 500nM each *hrp* F primer and 1 µl 20ng DNA template. Each PCR experiment included a negative control without DNA template. Reactions were run for 40 cycles and each consisting of 40s at 95°C, 40s at 63°C, 40s at 72°C with initial denaturation of 3 min at 95°C and final extension of 5 min at 72°C. A 15 µl aliquot of each amplified PCR product was fractionated on a 1.0% agarose gel in 0.5% TBE buffer at 80v for 1.5 hours, stained with ethidium bromide and visualized under a UV transilluminator, and calculation was done by using Alfaimager software.

Specificity and validity of primers

DNA from 18 isolates of *X. campestris* pv. *campestris* and 2 isolates of *X. oryzae* pv. *oryzae,* 2 isolates of *P. fluorescens* and one isolate each of *P. syringae* pv. *phaseolicola, E. carotovora* subsp. *carotovora, X. axonopodis* pv. *punicae* and *X. axonopodis* pv. *citri* was used to evaluate validity and specificity of the primer DLH 120 and DLH 125. Total DNA of bacteria was used as template for PCR reaction.

Detection of pathogen in symptomatic leaves of *Brassica* and *Raphanus* species by PCR

Infected leaves of *Brassica* species (*B. oleracea* var. *botrytis*, *B. oleracea* var. *capitata*, *B. oleracea* var. *italica* and *B. carinata)* and *Raphanus sativus* showing symptoms of black rot were collected from farmer's field near Yamuna river, in Laxminagar, Delhi. The infected leaves were cut into 10 very thin (about 1mm) cross sections. Leaf sections were soaked in 200 µl of sterile distilled water for at least 1 h. Aliquot of bacterial ooze was streaked on to the nutrient sucrose agar medium and incubated for 72 h at 28^{0}C. Two types of bacterial colonies appeared on the medium *i.e.* light yellow, mucoid and dark yellow colonies. The same colony was used for pathogenicity and also used as DNA template for PCR.

Table 1. Host and identification number of bacterial isolates used in this study

Species/ strain	Host plant (English name)
Xcc- C1, Xcc- C2, Xcc- C3	*Brassica oleracea* var. *botrytis* (cauliflower)
Xcc- C4, Xcc- C9	*B. oleracea* var. *italica* (broccoli)
Xcc- C5, Xcc- C6, Xcc- C7, Xcc- C8	*B. oleracea* var. *capitata* (cabbage)
Xcc- C10, Xcc- C11	*Raphanus sativus* (radish)
Xcc- C12, Xcc- C13	*B. juncea* (Indian mustard)
Xcc- C14, Xcc- C15	*B. oleracea* var. gongylodes (knol khol)
Xcc- C16, Xcc- C17	*B. juncea* (leafy sarson sag)
Xcc- C18, Xcc- C19	*B. rapa* (turnip)
Xcc- C20, Xcc- C21, Xcc- C22	*B. campestris var. brown sarson* (brown sarson)
X. oryzae pv. o*ryzae* (Xoo-4)	*Oryza sativa* (rice)
X. citri pv. *citri*	*Citrus* sp. (lemon)
X. axonopodis pv. *punicae*	Pomegranate
Pseudomonas syringae pv. *phaseolicola*	*Vigna radiata* (mungbean)
P. fluorescens	Phyllosphere of rice
P. fluorescens	Phyllosphere of cauliflower
Erwinia carotovora subsp. *carotovora*	*Dacus carrota* (carrot)

For pathogenicity test, 48 h old cultures of bacteria were inoculated to the respective 35 days old host plants by leaf cutting method (Vicente *et al.*, 2001). The disease symptom was recorded after 15 days of inoculation.

RESULTS AND DISCUSSION

Two pairs of oligonucleotide primers were evaluated for amplification of fragments from total genomic DNA of *X. campestris* containing *hrp* F region. These two primers DLH 109 & DLH 112 and DLH 120 & DLH 125 were used for DNA sequence from strains of *X. campestris* pv. *campestris* isolated from host cauliflower and cabbage amplified at 1.4 kb and 619 bp respectively (Fig. 1). The nucleotide sequences of the *hrp* gene cluster were compared for the *Xanthomonas* species such as *X. campestris* pv. *campestris*, *X. citri* pv. *citri, X. axonopodis* pv. *punicae*, *X. oryzae* pv. *oryzae*. It appears that the organization and sizes of the majority of the *hrp* genes are highly conserved within the genus as predicted from southern hybridization analysis by Bonas *et al.,* 1991. Selective amplification of DNA from X*. campestris* required a candidate gene that is divergent from its homologous encoded by other *Xanthomonas* species. A collection of 25 isolates of *Xanthomonas* and 3 isolates of *Pseudomonas* and one *Erwinia* was screened by using primer DLH 120 & DLH 125, which were designed to amplify a fragment encompassing the least 580bp of *hrp* F and 39bp of down stream intergenic sequence as reported by Berg *et al.*, 2005.

To test the validity of primer DLH 120 & DLH 125, 18 isolates of *Xanthomonas campestris* were tested. DNA fragments delineated by the primers were consistently amplified from strains of *Xanthomonas campestris* (Fig. 2), which were isolated from different host of *Brassica* spp. and *Raphanus sativa* (Table 1). All strains of *Xanthomonas campestris* produced high yields in the amplification of the 619bp fragment. To study the specificity of the primers, total DNA of 8 isolates of *X. campestris*, 2 isolates of *X. oryzae* pv. *oryzae*, 2 isolates of *P. fluorescens* and one isolate each of *X. citri* pv. *citri*, *X. axonopodis* pv *punicae*, *E. carotovora* subsp. *carotovora* and *P. syringae* pv. *phaseolicola* was evaluated. All isolates of *X. campestris* isolated from different hosts of brassicas and *Raphanus* amplified at 619bp after gel electrophoresis. No amplification product were observed in other species of *Xanthomonas*, *P. fluorescens* and *P. syringae* pv. *phaseolicola* and *E. carotovora subsp. carotovora*. It indicates that primers DLH 120 & DLH 125 were specific to *X. campestris* pathovars, which was also reported by Berg *et al.,* 2005. It has been proved that *hrp* F gene is a useful target for the PCR based detection of *X. campestris* pathovars. This gene is highly conserved to enable differentiation of the pathovars, primers targeting the 3' end of the *hrp* successfully amplified a 619 bp product only from *X. campestris*. The product was neither amplified from DNA extracted from other bacterial genera of phytopathogenic bacteria nor from *Xanthomonas* species isolated from non cruciferous plants.

Detection of pathogen in symptomatic leaves of *Brassica* and *Raphanus* species by PCR

Detection of *X. campestris* through PCR, 72 h old cultures of bacteria isolated from leaves of cauliflower, cabbage,

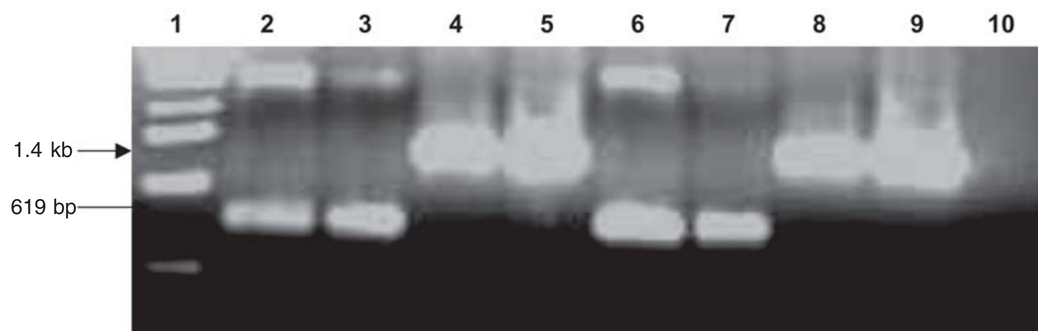

Fig. 1. *hrp* F PCR products separated on an agarose gel showing the 1.4 kb (Primer: DLH 109/ DLH 112) and 619 bp PCR (Primer : DLH 120/ DLH 125) products amplified from two isolates of *X. campestris* pv. *campestris*. Lane 1: 1 Kb DNA ladder, lane 2 - 5: Xcc- C1 and lane 6-9: Xcc- C5, lane10: -ve control

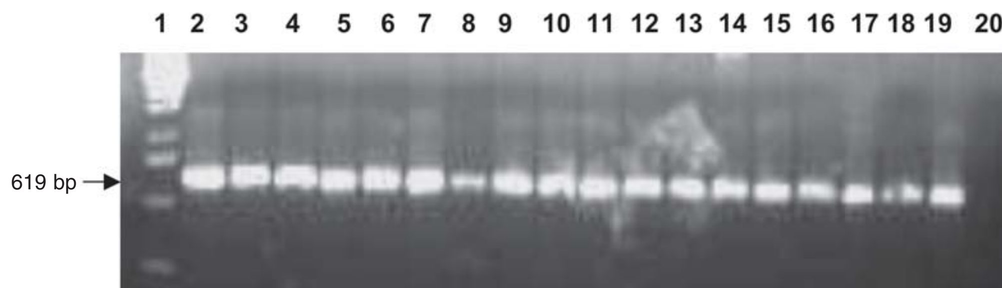

Fig. 2. *hrp* F PCR products separated on an agarose gel showing the 619 bp PCR product amplified from different isolates of *X. campestris* pv. *campestris.* Lane 1: 1 Kb DNA ladder, lane 2 - 9: *X. campestris* pv. *campestris* isolates collected from different hosts of crucifers ; lane 2 & 3: cauliflower (Xcc- C1 and Xcc- C2, lane 4 & 5: broccoli (Xcc- C4 & Xcc- C9), lane 6,7 & 8: cabbage (Xcc- C5, Xcc- C6 & Xcc- C7), lane 9 & 10: radish (Xcc- C10 & Xcc- C11), lane12 & 13: Indian mustard (Xcc- C12 & Xcc- C13), lane 14 & 15: knol- khol (Xcc- C14 & Xcc- C15), lane 16: Leafy sarson sag (Xcc- C16), lane 17: turnip (Xcc- C19), lane 18 & 19: mustard Xcc- C20 & Xcc- C22), lane 20: -ve control

Table 2. Pathogenicity test of suspected colonies of *X. campestris* pv. *campestris* isolated from different crucifers.

Pathogenicity Test	Cauliflower	Cabbage	Broccoli	Radish	*B. carinata*	Sarson sag
Colony No.1	-	+	-	-	-	+
Colony No 2	-	-	+	-	-	+

+ = Pathogenicity test positive; - = Pathogenicity test negative

Fig. 3. *hrp* F PCR products separated on an agarose gel showing the 619 bp PCR products amplified from different isolates of *X. campestirs* pv. *campestris*. Lane 1: 1kb DNA ladder, lane 2, 3, 9, 14, 15, 16, 17, 18: *X. campestris* pv. *campestris*, lane 5 & 6: *X. oryzae* pv. *oryzae,* lane 11 &12: *P. fluorescens,* lane 7: *P. syringae pv. phaseolicola,* lane 8: *Erwinia carotovora* subsp. c*arotovora,* lane 4: *X. axonopodis* pv. *punicae,* lane 10: *X. citri* pv *citri,* lane 13: -ve control

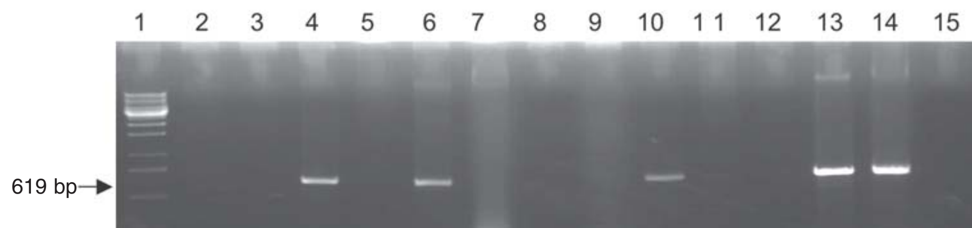

Fig. 4. Amplification at 619 bp separated on an agarose gel in which bacterial colonies isolated from different crucifers infected plants used as DNA template in PCR cocktail. Lane 1: 1 kb DNA ladder, lane 2 & 3: cauliflower; lane 4 & 5: cabbage, lane 6 & 7: broccoli, lane 8 & 9: *Raphanus sativus* (radish), lane 10: positive control (Xcc), lane 11 & 12: *Brassica carinata,* lane 13 & 14: leafy sarson sag, lane 15: -ve control

broccoli, radish, and leafy sarson sag. Two types of colonies were observed on the nutrient sucrose agar medium i.e. first one was light yellow, fluidal, mucoid and raised colony and second type was dark yellow, non- fluidal, small and flat. Second type of colonies did not cause the disease in their respective host as mentioned in Table 2. It means that such type of colonies are non pathogenic. It was further confirmed by using bio- PCR technique. The colonies amplified at 619 bp produced the black rot symptom on their respective hosts and however, no amplification was recorded in second type of colony isolated from cauliflower and radish samples.

Xanthomonas species isolated from brassicas, which were non pathogenic and they produced dark yellow, non fluidal colonies on nutrient agar medium and shown to lack of *hrp* F gene as indicated by the failure to amplify any portion of the gene using a primer combination. These strains, although morphologically indistinguishable from disease causing *X. campestris* appeared to carry other *hrp* sequences, especially, they had *hrp* G amplification product typically of *X. campestris*, but variable, a typical *hrp* W products. The proximity to another *hrp* F and *hrp* W on *X. campestris* chromosome (=2kb; de Silva *et al*., 2002) suggests that genetic rearrangements affecting both these genes may have occurred in non pathogenic isolates.

The PCR is confirmatory to the pathogen *X. campestris* pv. *campestris,* which can distinguish pathogenic and non pathogenic Xanthomonads and diagnose the black rot disease rapidly within 3 - 4 days and accurately.

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