African Journal of Microbiology Research Vol. 6(30), pp. 5950-5956, 9 August, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.543
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

The reliable and rapid polymerase chain reaction (PCR) diagnosis for *Xanthomonas axonopodis* pv. *punicae* in pomegranate

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Accepted 23 April, 2012

Bacterial blight is a major disease in pomegranate (*Punica granatum*) cultivation in India threatening the export potential of this important fruit crop. The disease is caused by a yellow pigmented, Gram negative, rod shaped bacterium, *Xanthomonas axonopodis* pv. *punicae*. We developed a polymerase chain reaction (PCR) based detection technique for this blight pathogen using primers designed from *gyrB* gene. A primer set KKM5 and KKM6 was synthesized based on sequence alignment of 530 nucleotides of C-terminus region in the *gyrB* genes from 15 different bacterial strains. The primer set was validated for amplification of 491 bp of *gyrB* gene. No amplification was observed in other phytopathogenic Xanthomonads including *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas axonopodis* pv. *mangiferaeindicae*, *Xanthomonas campestris* pv. *manihotis, Xanthomonas oryzae* pv. *oryzae, Xanthomonas oryzae* pv. *oryzicola, Xanthomonas axonopodis* pv. *axonopodis* and *Pantoea agglomerans.* The developed technique could detect the pathogen in infected pomegranate plant samples including leaf, fruit and stem within 3 h, at a detection limit 0.1 ng µl^{-1} template DNA.

Key words: *Xanthomonas axonopodis* pv. *punicae,* bacterial blight, pomegranate, polymerase chain reaction (PCR) detection.

INTRODUCTION

Pomegranate (*Punica granatum*) is an important fruit crop of India with export potential. Bacterial blight caused by *Xanthomonas axonopodis pv. punicae* (Xap) (Figure 1a) is a major threat to pomegranate cultivation (Hingorani and Mehta, 1952; Chand and Kishun, 1991; Akhtar and Bhatti, 1992; Mondal and Singh, 2008, 2009; Petersen et al., 2010; Mondal and Mani, 2011). The disease alone causes 60 to 80% losses in pomegranate production in India (Mondal and Mani, 2009). On the leaf symptom appears as small (2 to 5 mm), irregular, prominent water soaked spots (Figure 1d), which later become necrotic with light to dark brown centre surrounded by prominent water soaked margins. At the advance stage of the disease the individual spots coalesce giving an eventual blighted appearance. Lesions on the stem are dark brown to black that very often leads to cracking and the breaking of branches (Figure 1c). The lesions on fruit appears as irregular, shining brown to black spots with Y or L shaped cracking or splitting of pericarp, reducing the marketability of the

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fruits (Figure 1b).

Management of the disease has been the challenge before plant protectionist (Kumar et al., 2009, Mondal and Sharma, 2009); however, the major hurdle in containing the disease is mainly due to its unrestricted spread throughout the countries. The disease is spread from infected orchard to healthy one through cutting of pomegranate. During last 4 to 5 years the disease appeared in epiphytotic form in major pomegranate growing states of South West India, including Maharashtra and Karnataka. Subsequently, the disease spread to Rajasthan, Gujarat, parts of Haryana and UP, mainly due the bulk movement of planting materials (cuttings) of most popular cultivar, *Bhagwa* (but highly susceptible to bacterial blight) from Maharashtra to these regions. Therefore, to restrict the spread of the pathogen disease-free planting materials is essential. In order to ensure the presence of pathogen in suspected planting materials (or cutting) we need a very robust and reliable PCR based detection technique. *gyrB* gene has shown to be a good candidate to differentiate pathovars of bacterial plant pathogens including pathovars of *Xanthomonas axonopodis* (Motoshima et al., 2007; Parkinson et al., 2009). Here we develop a very rapid and robust PCR based diagnostics for this blight pathogen, which will detect the presence of blight pathogen in the pomegranate plant tissues.

MATERIALS AND METHODS

Bacterial strain and media

Nine isolates of *Xanthomonas axonopodis* pv. *punicae* (Xap) were used for this study. Out of nine isolates, 5 from Delhi, 3 from Solapur, Maharashtra state, one was strain NCPPB 466 of Xap. Single colony of each isolate was stored in 50% glycerol at -70°C. The isolates were revived from stored culture by streaking on yeast peptone (YP) (yeast extract 5 g, peptone 10 g, agar 20 g and water 1 L, pH 7.0) agar plate and incubated at 28°C for 72 to 96 h. Well separated fluidal colony of Xap isolates were selected for genomic DNA extraction after growing them at 48 h at 28°C at 200 rpm in YP broth.

Primers design and specificity

A primer set KKM 5 & 6 was synthesized based on sequence alignment of 530 nucleotides of C-terminus region in the *gyrB* genes from 15 different bacterial strains including strain NCPPB 466 of *Xanthomonas axonopodis* pv. *punicae* (EU285148.1), strains 306 (AE008923.1) and ICMP 10022 (EU499045.1) of *Xanthomonas axonopodis* pv. *citri*, strains ICMP 9280 (EU499036.1) and 5739 (EU499005.1) of *X. axonopodis* pv. *malvacearum*, strain ICMP 12325 (EU499051.1) of *Xanthomonas axonopodis* pv. *phaseoli*, strain NCPPB 490 (EU285214.1) of *Xanthomonas axonopodis* pv. *mangiferaeindicae*, strain NCPPB1834 (EU285133.1) of *Xanthomonas campestris* pv. *manihotis*, strains MAFF311018 (84365597), PXO99A (CP000967.1), KACC10331 (AE013598.1) of *Xanthomonas oryzae* pv. *oryzae,* strain NCPPB 1585 (EU285219.1) of *Xanthomonas oryzae* pv. *oryzicola,* strain LMG2565 (EF988814.1), LMG 1286 (FJ617401.1) of *Pantoea agglomerans* and strain ICMP 8681 (EU499031.1) of *Xanthomonas axonopodis* pv. *axonopodis* using BioEdit sequence alignment Editor- Clustal W Mutiple alignment. The synthesized primers were checked for specificity using primer 3 programmes. An amplicon size of 491 bp was verified *in silico* with the synthesized primers KKM 5 and 6. For further confirmation, the amplified region was cloned into pGEMT vector following standard cloning technique and was sequenced.

Extraction of total genomic DNA from bacteria

The extraction of total genomic DNA was carried out following a modified protocol as described by Adachi and Takashi (2002). 200 µl YP broth grown culture was centrifuged for 5 min at 10,000 rpm and the bacterial cells were resuspended in 50 µl of proteinase K solution (50 µg ml^{-1} in 10 mMTris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and kept at 56°C for 15 min, then at 80°Cfor 15 min to denature the proteinase, then placed on ice to cool the samples for 5 min. After centrifugation for 5 min at 13000 rpm, the supernatant was transferred to a new eppendorf tube. The supernatant was treated with RNase A (100µg ml^{-1}) for 30 min at 37°C followed by extraction with phenol : chloroform : isoamylalcohol (25:24:1) and precipitation by adding 0.1 volume of ammonium acetate and 2 volumes of chilled ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 50 µl sterile water. The DNA samples were quantified using Nano-drop Spectrophotometer. For PCR amplification the concentration of DNA used was of 100 ngμl^{-1}.

Direct detection from plant samples

In order to detect the Xap in the plant tissue, the inoculated pomegranate samples including leaves, fruits and stem (100 mg) were homogenized in 1 to 2 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 3.5% CTAB, 0.2% β-mercaptoethanol) in sterile mortar and pastel. Then the extract was incubated at 60°C for 30 min and vortexed for 5 min. The suspension was mixed with 1 volume of chloroform/isoamyl alcohol (24:1) and centrifuged for 10 min at 6000 rpm. The aqueous phases were transferred into fresh tube and add 0.8 to equal volume of isopropanol and centrifuge 20 min at 13000 rpm. The supernatant was discarded and the pellet was washed twice with 70% ethanol by spinning at 10,000 rpm for 2 min and dry for 5 to 10 min at room temperature.The pellet was suspended in 20 to 30 µl sterile distilled water and 1µl was used for PCR amplification. 5 µl of PCR product was loaded on agarose gel (1%) with 0.5 µg ml^{-1} of ethidium bromide and run in 1XTAE buffer at 100 volt for 30 min.

PCR and amplification conditions

The PCR condition followed consisted of initial denaturation at 94°C for 5', then 30 cycles comprising denaturation at 94°C for 30 Sec , annealing at 55°C for 1', extension at 72°C for 1' followed by anfinal extension cycle at 72°C for 3'and final shock at 4°C. A 25 µl PCR reaction volume was made by mixing 1µl template (100 ng μl^{-1}), 2.5 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 5µl 5X buffer, 1 µl forward primer (10 pmol μl^{-1}), 1µl reverse primer (10 pmol μl^{-1}), 0.25 µl TaqDNA polymerase (5 units μl^{-1}) (Fermentus Co.), and 14.75 µl nuclease free sterile water.

Sensitivity of the detection technique

The sensitivity of the detection technique was judged by serially diluting the template DNA (100 ngμl^{-1}) from bacteria like 100, 10,

0.1, 0.01, 0.001 ng μl^{-1} and each diluted DNA samples were subjected to PCR amplification. The PCR was performed following the conditions as stated earlier. The presence of visible band of desired size was indicative of sensitivity.

Plant inoculation

The pomegranate plants (cv. Bhagwa) were grown in the pots in glasshouse. The inoculation was carried out a three different methods, namely sprays, pinpricked, and syringe infiltration. The inoculum was adjusted at 0.1 OD (600 nm) by suspending cells of Xap in sterile distilled water. The leaf samples from inoculated plants were collected for PCR detection after 5 days of inoculation. The PCR was replicated thrice in each case three independent leaf samples. Total DNA from water inoculated leaves and Xap were kept as negative and positive control respectively.

Validation of PCR detection technique using field samples

In order to validate the optimized template DNA preparation as well as PCR amplification protocols, 86 pomegranate samples representing 51 leaf samples, 20 fruit samples and 15 stem samples (from 14 different villages covering states, Maharshtra, Karnataka and Delhi) were subjected to PCR amplification. Besides, 10 leaf samples from tissue-culturally raised saplings were also subjected for PCR amplification. The template DNA preparations and subsequent PCR amplification using primer set KKM5 and 6 was performed following the procedure as described earlier. For positive control bacterial DNA from Xap as well as DNA from artificially inoculated symptomatic leaves was used, while for negative control DNA from non-symptomatic healthy leaves were kept.

RESULTS

Pathogenecity of Xap isolates

The pure colonies Xap isolates used for this study appeared on YP agar as smooth, circular, glossy, convex, 1.5 to 2 mm at 72 h, pale to light yellow in colour. The identity of the Xap isolates were confirmed through 16S rRNA gene based universal primers (Forward 5'-AGAGTTTGATCCTGGCTAG-3'and reverse 5'-AGGAGGTGATCCAGCCGCA-3'). The pathogenecity of the Xap isolates were confirmed following Koch's postulate. The 16S rRNA sequence of Xap isolates were deposited at Bankit (Accession no. of 5 Delhi isolates are JN036620, JN036621, JN036622, JN036623, JN036624, and 3 Solapur isolates are JN109171, JN109172, and JN109173).

Primer design and specificity

A primer set, KKM-5 Forward 5'GTTGATGCTGTT CACCAGCG3' and KKM-6 Reverse 5'CATTCATTT CGCCCAAGCCC3' were designed from the 530 nt C-terminus region of *gyrB* genes from 14 strains after alignment analysis (the details of alignments are provided as supplementary Figure 1). The primer set gave product size of 491 bp, which is specific to only Xap (Figure 2). No amplification was observed in other phytopathogenic Xanthomonads including *X. axonopodis* pv. *citri*, *X. campestris* pv. *campestris*, *X*. *oryza*e pv. *oryzae*, *X. campestris mangiferae indicae*, *Pantoea agglomerans* except *Xanthomonas axonopodis* pv. *malvacearum* (Xam). However, Xam being a specific pathogen for cotton would not interfere with PCR detection protocol for pomegranate pathogen.

Direct detection from plant samples

A very simplified less than 3 h protocol for direct detection from the infected leaf extract has been optimized, which could successfully detect the presence of bacteria in the infected samples both experimental inoculated leaf (Figure 3), and naturally infected samples (Figure 4). Briefly, the protocol includes a 50 min step for template preparation from plant tissues, 90 min for PCR amplification and 30 min for gel electrophoresis. No amplification was detected in healthy pomegranate leaf (Figures 3 and 4). The sequencing of the amplified 491 bp from infected plant DNAs as well as from bacterial DNA revealed the similar nt sequence.

Validation of PCR detection technique using field samples

Among the 128 infected samples (leaf-66, fruits-32 and stem-30) chosen for PCR amplification using primer sets KKM 5 and 6, 121 samples were PCR positive indicating the suitability of the developed protocol for detection of blight pathogens. The details of location, types of samples and PCR amplification result are provided in Table 1. All the 10 leaf samples from tissue culturally raised saplings were found to be PCR negative, indicating that the samples were free of blight bacteria. In control sets, the desired amplification was found in positive control and no amplification was detected in negative control.

Sensitivity of the detection technique

The sensitivity of the detection was determined using different titers of template DNAs from bacterial cells. It was found that the minimum concentration of DNA required for the detection is 0.1 ng μl^{-1} (Figure 5).

DISCUSSION

DNA *gyrB* gene has been demonstrated to offer greater resolution in discriminating pathovars of bacterial species including the genus *Xanthomonas* (Motoshima et al, 2007; Parkinson et al., 2009). Recently, *gyrB* gene based

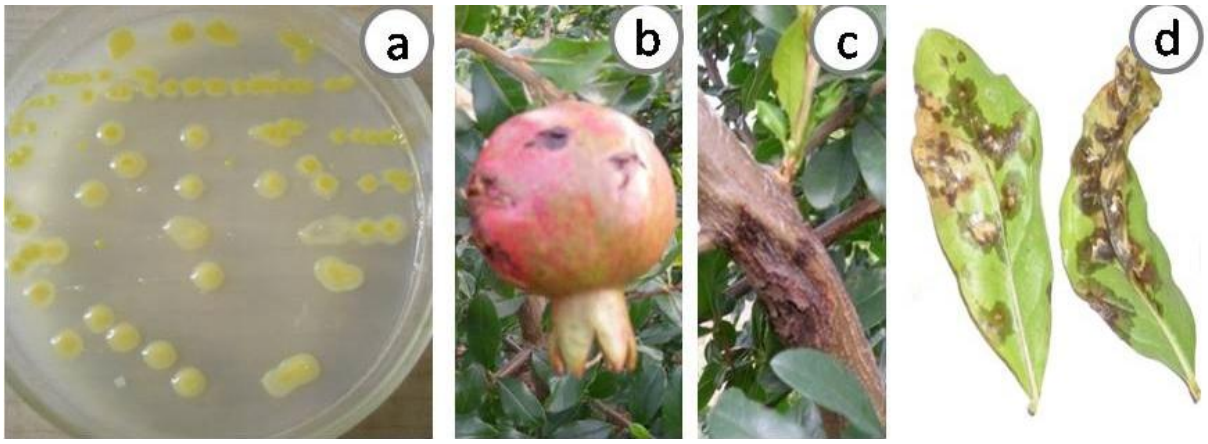

Figure 1. *Xanthomonas axonopodis* pv. *punicae* colonies (a) and the symptom produced by it on fruit (b), on stem (c) and on leaves (d).

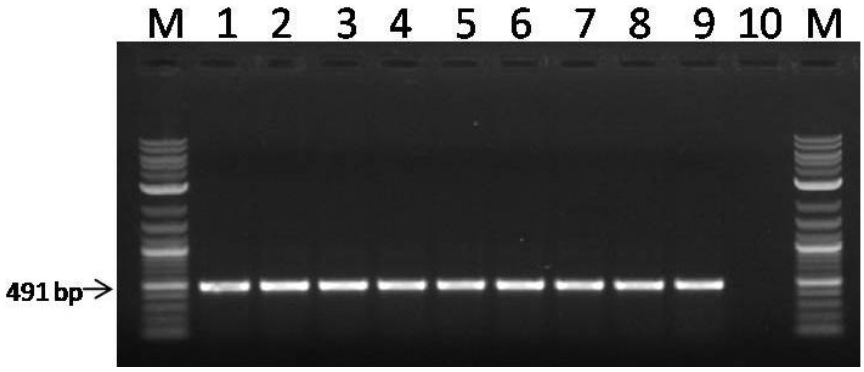

Figure 2. Amplification of Xap using primer set KKM 5 and 6 from bacterial DNA. M = 100 bp marker, 1 to 5 = Delhi Xap isolates, 6 to 8 Solapur Xap isolates; 9 and 10 = Xap strain 466 and strain *Pantoea agglomerans* LMG 1286, respectively.

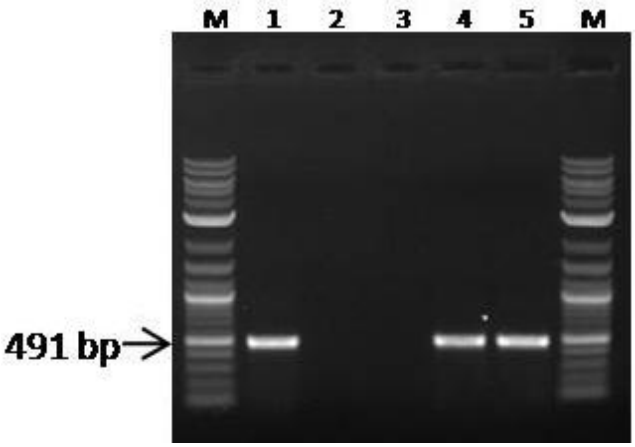

Figure 3. Specific amplification of Xap from artificially inoculated leaves using primer set KKM 5 and 6. M = 100 bp ladder, 1 = Bacterial DNA (+ve control); 2, 3 = Plant from healthy leaves sprayed and infiltrated with water, respectively; 4, 5 = Plant DNAs from leaves artificially inoculated with Xap by spray and infiltration method respectively.

Table 1. Validation of PCR-based detection system with pomegranate affected field samples from different locations in India.

Location	Sample type*	No. of samples	PCR amplification**
Gavali, Nasik, Maharashtra	Leaf	3	+ (3)
	Fruit	2	+ (1), -(1)
	Stem	2	+ (3)
Sangamner, Ahmadnagar, Maharashtra	Leaf	3	+ (3)
	Fruit	2	+ (2)
	Stem	2	+ (1)/-(1)
Vadgaon landge, Ahmadnagar, Maharashtra	Leaf	2	+ (2)
	Stem	2	+ (2)
Malegaon, Nasik, Maharashtra	Leaf	3	+ (3)
	Twig	2	+ (2)
Arakta, Nasik, Maharashtra	Fruit	2	+ (2)
	Leaf	3	+ (2)/-(1)
Beed, Nasik, Maharashtra	Fruit	2	+ (2)
Satana, Nasik, Maharashtra	Fruit	2	+ (2)
Satana, Nasik, Maharashtra	Leaf	3	+ (3)
Lakhmapur, Nasik, Maharashtra	Leaf	2	+ (2)
Talwade, Nasik,, Maharashtra	Fruit	2	+ (2)
Deola, Nasik, Maharashtra	Fruit	2	+ (2)
	Leaf	3	+ (2)
	Twig	2	+ (2)
Talwade, Malegaon, Nasik, Maharashtra	Fruit	2	+ (2)
	Leaf	3	+ (3)
Solapur, Maharashtra	Leaf	5	+ (4)/-(1)
	Stem	3	+ (2)/-(1)
	Fruit	4	+ (4)
	Apparently healthy leaves	4	+ (2)/-(2)
Dharwad, Karnataka	Stem	2	+ (2)
IARI, New Delhi	Leaf	14	+ (14)
Andhra Pradesh	Leaf	6	+ (6)
	Stem	6	+ (6)
	Fruit	6	+ (6)
Rahuri, MPKV	Leaf	9	+ (9)
	Stem	9	+ (9)
	Fruit	6	+ (6)
Total samples		**128**	**+(121)/-(7)**
Control sets	Xap-DNA	20	+ (20)
IARI, New Delhi	Artificially inoculated symptomatic leaves	20	+ (20)
Solapur, Maharashtra	Healthy nonsymptomatic leaf	3	-(3)
Solapur, Maharashtra	Healthy leaf from tissue culturally raised saplings	10	-(10)
% detection ability***			**94.53%**

*Samples were chosen for PCR amplification based on symptoms if otherwise not described. ** "+"denotes PCR product seen, while "- "PCR product not detected; in parenthesis the number of samples found to be positive or negative for PCR amplification. ***Percent detection ability was calculated based on following formula: no. of PCR +ve samples (121) divided by no. of tested samples (128) X 100.

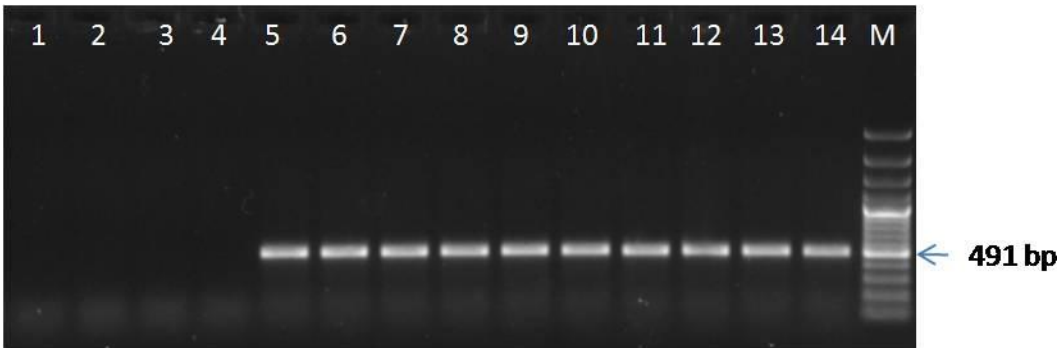

Figure 4. Amplification of Xap using primer set KKM 5 and 6 from naturally infected samples. 1 to 4 = DNA from healthy plant: leaf (1, 2), stem (3) and fruit (4) as control; 5 to 7 = plant DNAs from naturally infected leave samples; 8 to 10 = plant DNAs from naturally infected stem samples 11-13 = plant DNAs from naturally infected fruit samples; 14 = Bacterial DNA (+ve control) and M = 100bp ladder.

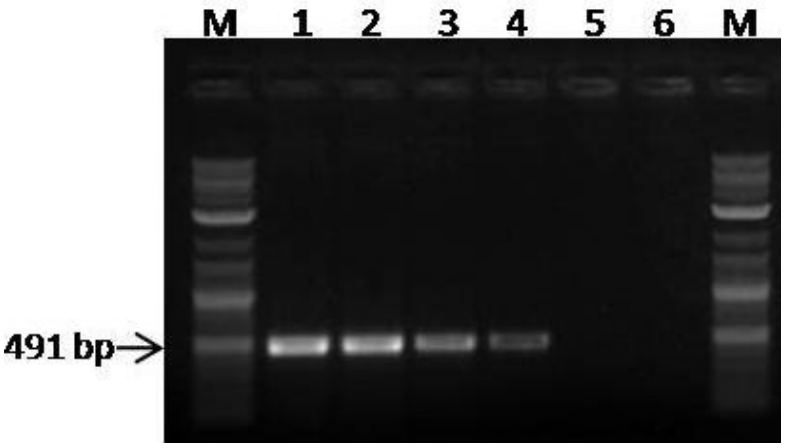

Figure 5. Agarose gel electrophoresis of PCR products from a dilution series of template DNA from Xap using primer set KKM 5 and 6. Lane 1-6 = PCR products from 100, 10, 1, 0.1, 0.01, and 0.001 of template DNAs, respectively; M = 100 bp ladder.

phylogeny resolved the distinctiveness of the species, *Xanthomonas citri* and *X. fuscans*, which were previously classified as pathovars of *X. axonopodis* (Schaad et al., 2007). The species designation of pomegranate blight bacterium has recently been changed from *X. campestris* pv. *punicae* to *X. axonopodis* pv. *punicae* based on *gyrB* sequence (Parkinson et al., 2009). Therefore, we targeted *gyrB* gene, particularly C-terminus variable region, while developing a PCR based detection technique for bacterial blight pathogen, Xap. We synthesized a primer set, namely KKM5 and 6 for PCR amplification of 491 bp from *gyrB* gene. Subsequently, a template DNA extraction protocol from plant tissue was optimized and validated. The developed technique could able to detect pathogenic bacteria in all types of the infected pomegranate samples including leaf, fruits and stems (Figure 4). Most notable, it takes less than 3 h to detect the presence of pathogen in the plant samples (50 min for cell extraction from plants, 90 min for PCR and 40 min for electrophoresis).

The validation data (Table 1) suggests that the developed PCR detection system could successfully able to detect the presence of blight bacterium in the affected samples with 94.5% success rate. We did not find any amplification in the leaf tissue from tissue culturally raised plants. However, among 4 apparently healthy leaf tissues tested 2 were found positive, indicating that the detection system could even work in asymptomatic (but suspected to be diseased) plant samples. However, the sampling of plant tissues to be tested for PCR detection need to be carried out very carefully as the blight bacterium, Xap is a mesophyll localized not systemic one.

The sensitivity of detection technique (Figure 5) was sufficiently high (0.1 ng μl^{-1}) compared to the methods reported for other bacteria using the same target gene, *gyrB* (Weller et al., 2007). To date no PCR based

detection technique is available for Xap. Therefore, the PCR technique described here using KKM-5 and KKM-6 would be very useful in screening planting materials for the presence of Xap, and thereby reducing the spread of Xap from infected orchard to healthy one. More importantly, now we routinely use the developed primer set as important marker for identification purpose of the bacterial blight pathogen of pomegranate.

ACKNOWLEDGEMENTS

We thank Director General and Secretary to Department of Agricultural Research and Extension, Indian Council of Agricultural Research, New Delhi, India for his keen interest to the programme. We duly acknowledge the financial support received to execute the programme from Director, Indian Agricultural Research Institute, New Delhi, India. We are also thankful to our Joint Director (Research) for providing necessary support during the programme.

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