Indian Journal of Agricultural Sciences **85** (4): 519–24, April 2015/Article

Detection of *Xanthomonas oryzae* pv *oryzae* from seeds and leaves of rice (*Oryza sativa*) using *hrp* gene based BIO-PCR marker

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Received: 11 June 2014; Revised accepted: 3 February 2015

ABSTRACT

Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is a serious disease of rice. Detection of pathogen from infected plant parts and seeds is important to eliminate pathogen from disease cycle. For detection of *X. oryzae* pv. *oryzae* from natural infected leaves and stored seeds, a polymerase chain reaction technique using *hrp* gene (hypersensitive response and pathogenicity) sequence of *X. oryzae* pv. *oryzae* based primers DXoo_hrp1F (5'-GGACCCTCCGGTTCTGAG-3') and DXoo_hrp1R (5'-CTGCAGGCATTGCTTAAAGA-3') were used to amplify at 384 bp DNA fragment. The specificity of the primer was tested with *X. oryzae* pv. *oryzae* along with other *Xanthomonas* species such as *X. campestris* pv. *campestris, X. axonopodis* pv. *punicae* and *Ralstonia solanacearum.* It amplified only DNA fragment of *X. oryzae* pv. *oryzae* at 384 bp. The developed set of primer was highly sensitive and detected 2.6×10^2 cfu/ ml of bacteria. The *X. oryzae* pv. *oryzae* was detected from naturally infected leaves of rice collected from the bacterial ooze as DNA template through conventional – PCR. To detect *X. oryzae* pv. *oryzae* from seeds, the bacterial culture was artificially inoculated on rice cv. Pusa Basmati 1 plant at flowering time and the matured seeds were harvested and stored for 8 months under ambient conditions. The bacterium was detected from seeds after 8 months of storage even 2 seeds/ml, when DNA of bacterium was extracted by short cut methods adding 95% ethanol through BIO-PCR. This method is reliable, more sensitive than conventional–PCR and may detect X. *oryzae* pv *oryzae* within 2 days from the seeds and other planting materials.

Key words: BIO-PCR, Detection, *hrp* gene, Rice, Seed, *Xanthomonas oryzae* pv. *oryzae*

Rice (*Oryza sativa*) is the primary food grain consumed by almost half of the world's population, making it the most important food crop currently produced. Rice production in both temperate and tropical rice growing regions is reduced due to bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. The disease is epidemic potential and cause yield losses up to 20% or more in severely infected field (Mew *et al.* 1993). *X. oryzae* pv *oryzae* is reported to survive on seed (Sakthivel *et al.* 2001), plant debris (Schaad *et al.* 1995), wild rice (Valluvaparadedasan and Mriappan 1989) and weeds and they may serve as primary source of inoculums to cause bacterial leaf blight disease in rice. *X. oryzae* pv *oryzae* is a plant quarantine pest (EPPO A1) and is subjected to phytosanitory regulations. The diverse communities of non-pathogenic microbes also found on the seed surface along with bacterial pathogens (Cottyn *et al.* 2001). Hence isolation of *X. oryzae* pv *oryzae* from seeds by conventional methods is often difficult (Sakthivel *et al.* 2001) due to slow growing nature of this bacterium and other fast growing saprophytic microorganisms on the surface of culture media. Selective medium used in isolation of *X. oryzae* pv *oryzae* is not able to eliminate fast growing microbial contaminants (Di *et al.* 1991) and mask the surface of the culture medium. Other techniques such as fatty acid and metabolic, biochemical tests and serological methods have been used to detect *X. oryzae* pv *oryzae* from seeds by various workers (Schaad *et al.* 1995, Sakthivel *et al.* 2001, Adachi and Oku 2009). The PCR based technique is currently used for detection of phytopathogenic bacteria from seeds and planting materials (Berg *et al.* 2005). For development of specific primer different genes such as insertion sequence (Sakthivel *et al.* 2001), ITS region of rDNA (Marefat *et al.* 2006) and *hrp* gene (Berg *et al.* 2005) have been used for detection and identification of bacteria. Among them *hrp* gene (hypersensitive response and pathogenicity gene) has potential for specific amplification of the targeted bacteria, which was previously reported for the detection and identification of *X. axonopodis* pv. *vesicatoria* in tomatoes and capsicum (Leit *et al.* 1994) and *X. campestris* pathovar in crucifers (Berg *et al.* 2005, Singh and Shri Dhar 2011). To improve sensitivity level of primers and study the viability of bacterial cells on the seeds, combined biological and

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polymerase chain reaction techniques are used to detect bacteria from seeds and planting materials (Schaad *et al.* 1995, Sakthivel *et al.* 2001, Singh and Shri Dhar 2011). However, until now, there is very few validated PCR protocol has been reported for detection of bacteria from seeds and plant parts. The objective of present investigation was to develop *hrp* gene based marker and standardize BIO-PCR based protocol for detection of *X. oryzae* pv *oryzae* from seeds of rice even in low sample size.

MATERIALS AND METHODS

Nine strains of *Xanthomonas oryzae* pv *oryzae,* one strains each of *X. campestris* pv *campestris,* and *X. axonopodis* pv *punicae* and *Ralstonia solanacearum* were obtained from Division of Plant Pathology, IARI, New Delhi, collected from different parts of India and used under this study (Table 1) These bacteria were grown on nutrient sucrose agar medium to prepare the inoculum for further study (Schaad *et al.* 2001).

The bacteria were grown in nutrient broth at 30^0C for 24hr. Total DNA from bacteria was extracted with guanidium thiocyanate as described by Pitcher *et al.* (1989). A pair of primers DXoo_hrp1F (GGACCCTCCGGTTCTGAG), and DXoo_hrp1R (CTGCAGGCATTGCTTAAAGA) was design from cluster of *hrp* gene between 1298 to 1681 bp of product size 384 bp of total 12940 bp nucleotide sequences of *X. oryzae* pv. *oryzae* strain PXO99A (Accession No. AY536514.1) from NCBI website (http://blast.ncbi.nlm.nih.gov.). The primers were designed using Primer 3 program (www.frodo.wi.nit.edu) and checked for specificity in silico by using website www.insilico.ehu.es. The primer was BLAST to see the specificity of primer with *X. oryzae* pv *oryzae*. The reaction was performed by using primer pairs. To study the specificity and validity of the primer, 1 µl of 50ng DNA of *X. oryzae* pv *oryzae*, *X. campestris* pv *campestris*, *R. solanacearum* and *X. citri* susbsp. *citri* was used as template for PCR. The PCR product of *hrp* gene amplified at 384 bp of strain BB1 of *X. oryzae* pv *oryzae* was purified using (Bangalore Genei) kit in accordance to the protocol described by manufacturer and directly used for sequencing. Sequencing was performed by Applied Biosystem Machine-3130, Chromas Biotech, Bangalore, India. The evolutionary history was inferred using the Neighbour-joining Method.

To determine the detection threshold of primer, bacterial suspension of *X. oryzae* pv *oryzae* strain BB1 was prepared by making a serial dilution from a 48 hr old liquid culture. 5 µl of bacterial culture was directly used as DNA template for PCR. To determine the population of bacteria, 100 µl of bacterial suspension from dilution 10^{-4} to 10^{-8} was inoculated on the nutrient sucrose agar medium in the Petri plates and incubated for 72 hr at 28 ± 1°C. PCR assays were performed

Table 1 List of bacterial strains used under this study

Isolates of Bacteria	Name of bacteria	Host	Location	Collection year
PNA	*Xanthomonas oryzae* pv *oryzae*	Rice	G B Pant University of Agriculture and Technology, Pantanagar, Uttarakhand	2009
PNB	*X. oryzae* pv. *oryzae*	Rice	G B Pant University of Agriculture and Technology, Pant nagar, Uttarakhand	2010
BB49	*X. oryzae* pv. *oryzae*	Rice	Punjab Agriculture University, Ludhiana, Punjab	2010
BB50	*X. oryzae* pv. *oryzae*	Rice	Punjab Agriculture University, Kapurthala, Punjab	2010
BB7	*X. oryzae* pv. *oryzae*	Rice	Baswada, Rajshtana	2012
BB1	*X. oryzae* pv. *oryzae*	Rice	HAU regional station, Kaul, Karnal, Haryana	2006
BB2	*X. oryzae* pv. *oryzae*	Rice	Mathura, Uttar Pradesh	2008
BB40	*X. oryzae* pv. *oryzae*	Rice	Central Rice Research Institute, Cuttak, Orissa	2008
IARI	*X. oryzae* pv. *oryzae*	Rice	Indian Agricultural Research Institute, New Delhi, Delhi	2009
MAFF311018	*X. oryzae* pv. *oryzae*	Rice	Japan	
KACC10331	*X. oryzae* pv. *oryzae*	Rice	Korea	
PXOA	*X. oryzae* pv. *oryzae*	Rice	USA	
Xoo clone sequence	*X. oryzae* pv. *oryzae*	Rice	Korea	
Xoo *hrp* clone	*X. oryzae* pv. *oryzae*	Rice	China	
B156Hpa 2	*X. oryzae* pv. *oryzicola*	Rice	China	
Xcc-C1	*X. campestris* pv. *campestris*	Cauliflower	Indian Agricultural Research Institute, New Delhi, Delhi	2007
UTT-25	*Ralstonia solanacearum*	Tomato	Chorgaliya, Nainital , Uttarakhand	2009
	X. axonopodis pv. *punicae*	Pomegranate	Indian Agricultural Research Institute, New Delhi, Delhi	2010
Hrp gene	*X. ampestris* pv. *vesicatoria*	Pepper	Island (France)	
	X. campestris pv. *vesicatoria*	Pepper	Germany	
Type III effector	*X. campestris* pv. *vesicatoria*	Pepper	USA	

with a Gradient thermocycler (Model: C 1000TM BIO-RAD). The amplifications were carried out in a final volume of 20 µm containing 1.5 µl of 25mM $MgCl_2$, 0.4 µl of 10mM dNTPs, 2.0 µl of 10X PCR buffer, 1.5 unit Taq Polymerase, 0.4 µl of 20 µM Primer F, 0.4 µl of 20 µM Primer R, and 1 µl of 50 ng DNA template or 5 µl of bacterial suspension. Each PCR experiment included a control without DNA. Reactions were run for 35 cycles each consisting of 1 min at 94°C, 30 seconds at 56 °C, 1 min at 72°C with initial denaturation of 2 min at 95°C and final extension of 10 min at 72°C. In case, bacterial suspension used as DNA template, initial denaturation was done at 96°C for 2 min. A 10 µl aliquot of each amplified PCR product was fractionated on a electrophoresis on 1.0% agarose gel (80 V) for 1 h and visualized on gel documentation system (Gel DocTM XR^+ BIO-RAD), under UV light (300nm) and photographed using Image Labs version 2.0.1 software (BIO-RAD) for gel analysis.

Seedling of rice cv Pusa Basmati 1 was raised in pots in month of August 2012. *X. oryzae* pv. *oryzae* strain BB1 was grown in the Petri plates containing nutrients sucrose agar medium at 28 ± 1°C for 48 hr. The bacterial culture was harvested from Petri plates and made suspension into sterilized distilled water and maintain the population 10^9cfu/ml. The bacterial suspension was sprayed on the inflorescence of the rice plants when grain setting was started and after 45 days of inoculation, the matured seeds of rice were harvested. The uninoculated (healthy) and inoculated seeds were stored at ambient condition (temperature: 16–36°C and relative humidity: 30-90%) from December 2012 to July 2013. The seed samples were taken out at 60 days intervals from lots. The 2, 5 and 10 seeds were taken from each samples and dipped into 1 ml of 0.85% NaCl solution (brine solution) in 2 ml of Eppendorf tube and kept for 2 hr on the shaker at 100 rpm. In second method as called BIO-PCR, 100 µl bacterial suspensions was taken out from each samples and inoculated into 900 µl of nutrient broth in Eppendorf tube and incubated at 28 ± 1°C for 24 hr at 100 rpm on a shaker. Then either 2 µl of bacterial culture was directly used as DNA template for PCR or 200 µl of bacterial culture was taken and extracted bacterial DNA using short cut method as described by George *et al*. [10]; then 2 µl of DNA was used for PCR in a final volume of 20 µm master mixture as described earlier. The PCR conditions were slightly modified as a denaturation step at 96°C for 3 min followed by 40 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and then one cycle of 72°C for 10 min. Amplified PCR products were separated by electrophoresis on 1.0% agarose gel (80 V) for 1 h and visualized UV light (300nm) after ethidium bromide staining under as described previously.

RESULTS AND DISCUSSION

PCR standardization

The primer pair DXoo_hrp1F and DXoo_hrp1R amplified at 384 bp DNA fragment from strains of *X. oryzae* pv *oryzae* isolated from India, when 50 ng DNA was used as the template under optimized condition (20 µl PCR reaction, containing 25mM $MgCl_2$, 10 mM dNTPs, 10 µM of each primer, 2 unit of Taq polymerase enzyme (Promega, Finnzyme, Bioline, USA) and 10X PCR buffer. Taq polymerase enzymes supplied by Promega and Bangalore Genie manufacturers produced similar PCR results. PCR techniques based on DXoo_hrp1 primers can be used in detecting strains of *X. oryzae* pv *oryzae* by differentiating other group of bacteria (Fig 1). Based on our results, the protocol including a set of primer DXoo_hrp1 has been standardized. PCR technique based on *hrp* gene primers DXoo_hrp1F and DXoo_hrp1R amplified at 384 bp DNA fragment can be used in detecting strains of *X. oryzae* pv *oryzae* from different rice producing states of India. PCR conditions such as primers, DNA template, $MgCl_2$ (Bassam *et al.* 1992) thermocycler and thermostable polymerase origin have been shown to affect amplifications. In this study, all these parameters were optimized in a gradient thermocyler C 1000TM PCR (Make: BIO RAD) to avoid such artifacts and to ensure the reproducibility of amplification. The consistent results of amplification of a 384 bp fragment from *X. oryzae* pv *oryzae* by primers were also obtained by using various PCR machines and different Taq polymerase enzymes supplied by various agencies. Although the protocol for detection of seeds and planting materials has been standardized but it may be required some time slight modification in PCR conditions using thermo cycler by various laboratories.

Specificity and sensitivity of primers

To test the specificity of developed primers, DNA of *X.*

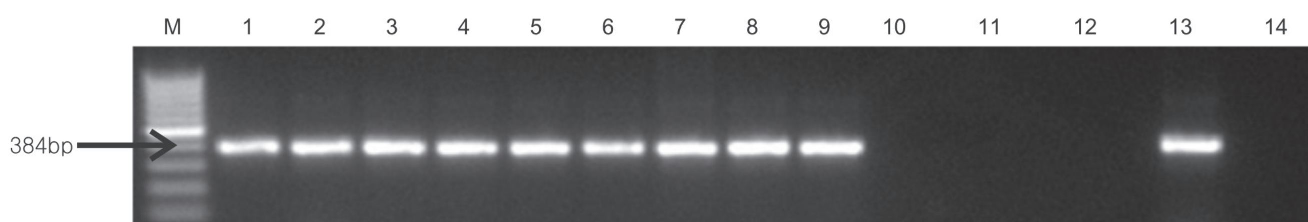

Fig 1 DNA bands of 9 strains of *X. oryzae*. pv *oryzae* isolated from different locations of India after PCR amplification by using *hrp* gene based primer at 384 bp. Lanes M: 1 kb DNA ladder, lanes 1-9 and 9 strains, viz. PNA, PNB, BB49, BB50, BB7, BB1, BB2, BB40 and IARI of *X. oryzae* pv *oryzae,* 10: *X. campestris* pv. *campestris*, 11: *X. axonopodis* pv. *punicae,* 12: *R. solanacearum*, 13: +ve control, 14: -ve

oryzae pv *oryzae*, *X. campestris* pv. *campestris* isolated from cauliflower, *R. solanacearum* (tomato), and *X. axonopodis* pv *punicae* (pomegranate) was used as DNA template for PCR. The amplification was found in all the strains of *X. oryzae* pv *oryzae* only, which was collected from different parts of India and no amplification was recorded in other group of bacteria other species of *Xanthomonas* and *R. solanacearum* (Fig 1). The sequence *hrp* gene of *X. oryzae* pv *oryzae* strain BB1 was BLST at NCBI website and the evolutionary history was inferred using Neighbor–joining method using MEGA 5 software and also did *in silico* sequence analysis. The *hrp* sequence of our strains of *X. oryzae* pv *oryzae* matched only with *X. oryzae* pv *oryzae hrp* gene cluster and as this part of the gene showed lower homology to sequences from *hrp* gene of *X. campestris* pv. *vesicatoria* and *X. oryzae* pv *oryzicola* (Fig 2) and did not match with other species of *Xanthomonas*. Sensitivity of the PCR detection assay was evaluated for bacterial suspension containing 10^9cfu/ml and further serially diluted. It was possible to detect *X. oryzae* pv *oryzae* in pure culture suspensions using the specific primer for PCR. Minimum number of bacterial cells detected was 2.6×10^2 cfu/ml (Fig 3). Based on the serial dilution, the PCR reaction contained 1-2 per 5 µl of suspension. Bacterial population

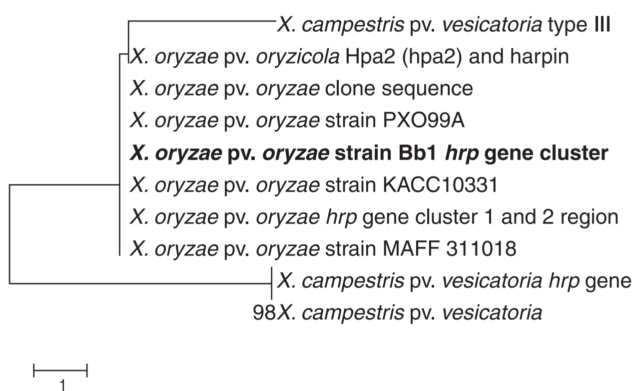

Fig 2 Phylogenetic analysis based on hrp gene sequences of *X. oryzae* pv. *oryzae* showing the position of with respect to related species available from NCBI database. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 292 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 5.

higher showed bright band at 384 bp and the bands appeared as faint or did not appear by reducing the bacterial population. The sensitivity level of these primers was 2.6×10^2 cfu/ ml, which were less than 2 cells in each PCR reaction. Primers developed in this study did not amplify DNA from other pathogenic and saprophytic bacteria. However, we did not test *X. oryzae* pv *oryzicola* in this study, which is one of the bacterial pathogen to cause bacterial leaf streak in rice. However we tested *in silico* and found that *X. oryzae* pv *oryzicola* and *X. campestris* pv *vesicatoria* have less than 92.0% maximum similarity index.

Detection of X. oryzae pv. oryzae from seeds

X. oryzae pv *oryzae* was artificially inoculated on rice cv. Pusa Basmati 1 plants at flowering stage of plant and matured seeds were harvested to store for 8 months under ambient conditions. *X. oryzae* pv *oryzae* was detected by PCR from artificially inoculated seeds through bio-PCR from 8 months stored rice seeds cv. Pusa Basmati 1 under ambient conditions when DNA was extracted by short cut methods by adding 95% ethanol through BIO- PCR. The *X. oryzae* pv *oryzae* was even detected in 2 seeds/ml after 8 months of storage under ambient conditions and no differences in banding pattern of amplification was observed by using BIO-PCR (Fig 4a,b,c and d). When seed extracted suspension of bacteria grown in broth culture was used directly as DNA template for PCR, *X. oryzae* pv *oryzae* was detected from seeds immediately after harvest and thereafter no amplification was obtained. Moreover, when the DNA template was used as suspension of bacteria in 0.85% brine solution extracted from seeds for PCR, no amplification was found even 10 seeds/ml.

The PCR method is unable to distinguish living from dead bacteria, so if either is used to screen seed batches that have undergone treatment for bacterial leaf blight, the potential contribution of dead *X. oryzae* pv *oryzae* cells needs to be considered. This could be overcome by plating samples and testing the resultant colonies by PCR (Schaad *et al.* 2003). The technique was employed in this study to improve the sensitivity of primers to detect bacteria and also demonstrate the viable cells of bacteria, that was earlier used in detection of *X. campestris* pv *campestris* (Berg *et al.* 2005, Singh and Shri Dhar 2011) and *X. axonopodis* pv *phaseoli* (Osdaghi *et al.* 2010) from seeds and planting materials. It has also advantage of detecting the pathogen without the need for pure cultures. Method of DNA extraction has affected the amplification of PCR product. The extraction of DNA of *Xanthomonas* by using 95% ethanol is a simple

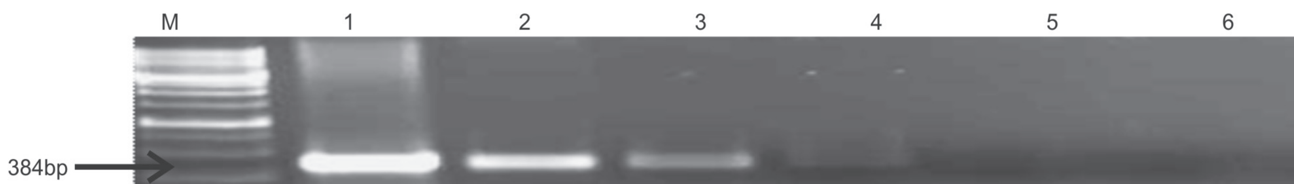

Fig 3 Amplification of *hrp* gene DNA fragment of *X. oryzae* pv *oryzae* at 384 bp in different inoculum load. Lane M: 1 kb DNA ladder, Lanes 1: 2.6×10^5 cfu/ml, 2: 2.6×10^4 cfu/ml, 3: 2.6×10^3 cfu/ml, 4: 2.6×10^2 cfu/ ml, lane 5: 2.6×10^1 cfu/ml, 6: -ve

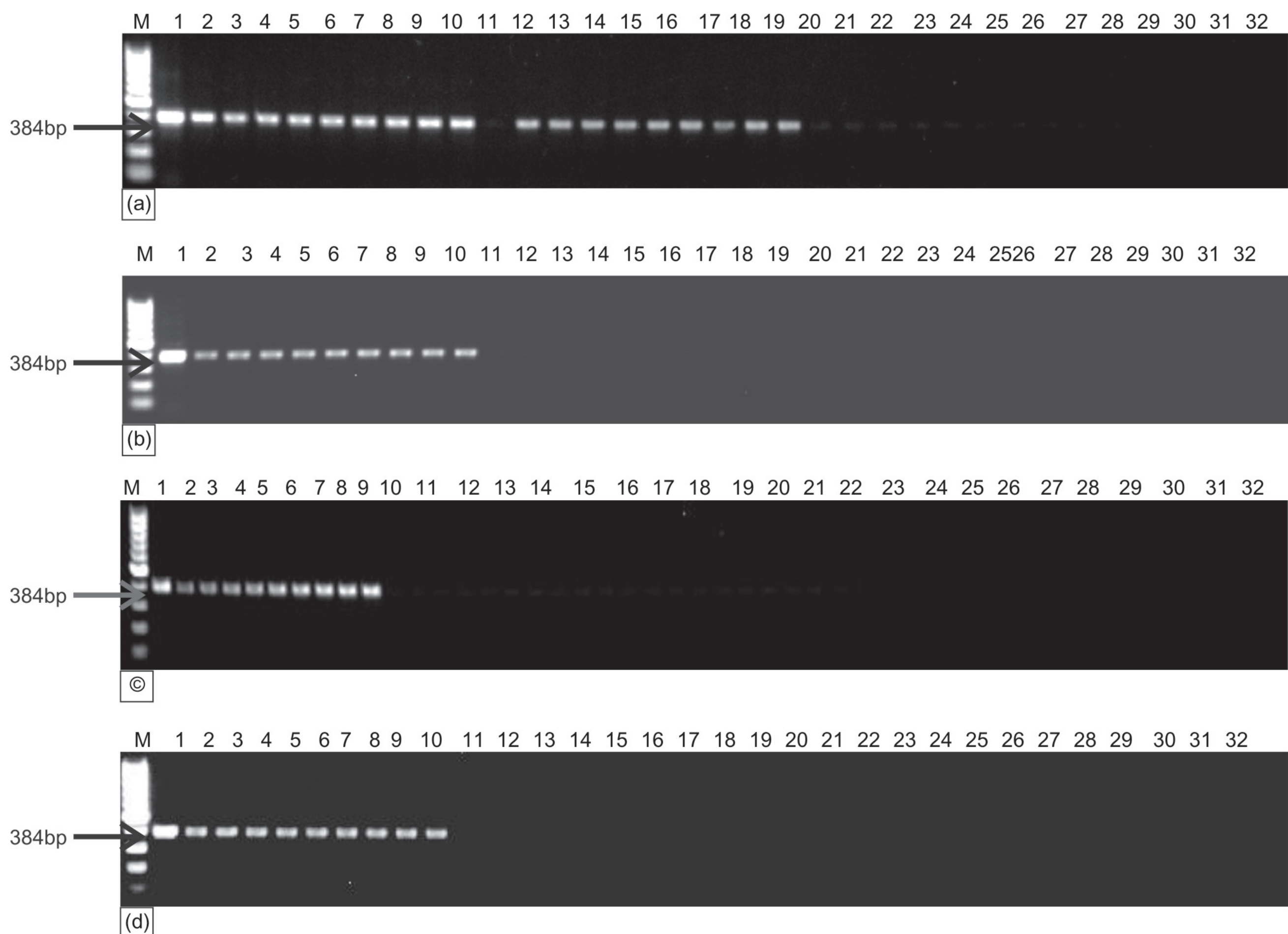

Fig 4 Amplification of *hrp* gene DNA fragment at 384bp of *X. oryzae* pv. *oryzae* detected from artificially inoculated rice plants subsequent stored seeds of rice up to 8 months under ambient conditions by using BIO-PCR. Seed samples used (a) immediate after harvest (b) after 8 months of storage for PCR. Lane M: 100 bp of DNA ladder, lane 2: +ve control (DNA of *X. oryzae* pv. *oryzae),* Lanes 2-11: DNA template used as bacteria extracted from seeds allow to grow in the nutrient broth, then DNA extracted using short cut method, Lanes 12-21: DNA template used for PCR as bacteria extracted from seeds and allow to grow in nutrient broth; Lanes 22-31: DNA template used as suspension of bacteria in brine solution extracted from seeds. Lanes 2, 5, 8, 12, 15, 18, 22, 25, 28 : 10 seeds/ ml, Lanes 3,6, 9, 13, 16, 19, 23, 26, 29: 5 seeds/ ml; Lanes 4, 7, 10, 14, 17, 20, 24, 27, 30: 2 seeds/ ml, Lanes 11, 21, 31: 10 healthy seeds/ ml, lane 32: -ve control.

and rapid method (George *et al.* 1996) and combination with a short of BIO- PCR in which using nutrient broth for multiplication to increase the population of bacteria (Schaad *et al.* 2003), which confirmed the viable cells of bacteria survive on the seeds. This short cut method of DNA of bacteria (George *et al.* 1996) may also be remove the PCR inhibitors present in broth medium and 0.85% brine solution during seed extraction. If high number of other microflora are encountered after enrichment, a DNA extraction step could be included to reduce possible inhibition PCR. Moreover, when DNA template used from 0.85% of brine solution for PCR, no amplification was obtained, it may be due to PCR inhibitor present in the samples extracted from seeds. In some samples, bands were not bright; this may be due to lesser population of bacteria or improper mixing of the bacterial cells. It indicates that the brightness of the bands depend on the population of bacteria, whether it is pure bacterial DNA or bacterial cells. The methods, which are able to detect *X. oryzae* pv *oryzae* in environment or seeds samples with an enrichment step definitely would be faster. However, there have been major obstacles to the development of such methods for assaying seeds including PCR inhibitors, particularly when small quantity seed samples mostly 2-10 seeds or even one seed, which has small number of target bacterial cells and large numbers of non-target seed borne bacteria. Although PCR inhibitor can also be reduced by simple dilution technique, but there is risk of understanding the population of *X. oryzae* pv *oryzae* in the samples.

Bio-PCR may be a valuable methods for detecting *X. oryzae* pv *oryzae* in seed lots even low sample size, naturally infected plant residues. The seed assay can be completed within 2-3 days, which is comparatively shorter than the 10-15 days required for traditional culturing and subsequently bacterial identification. This technique can be used to diagnose bacterial leaf blight of rice rapidly and reliably.

ACKNOWLEDGEMENT

The authors are thankful to the Head, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi for providing necessary facility to conduct the experiments.

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