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ORIGINAL PAPER

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Detection of *Xanthomonas oryzae* pv. *oryzae* in artificially inoculated and naturally infected rice seeds and plants by molecular techniques

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Abstract A polymerase chain reaction (PCR) technique was developed for detecting the presence of *Xanthomonas oryzae* pv. *oryzae*, the bacterial leaf blight (BLB) pathogen in rice seed and for studying the transmission of this bacterium from seed to plant. Primers TXT and TXT4R from an insertion sequence (IS1113) of the pathogen were used to amplify a 964-bp DNA fragment. A combined biological and enzymatic amplification (BIO-PCR) technique was used to detect the pathogen in naturally infected seed. The level of detection of TXT and TXT4R primers was 55 fg DNA of *X. o.* pv. *oryzae*, which is roughly the equivalent of seven cells (and four cells in pure culture suspension) of *X. o.* pv. *oryzae*. Hybridization of IS1113 with the amplified DNA fragment in Southern blot analysis confirmed that the 964-bp DNA fragment was amplified from *X. o.* pv. *oryzae*. The presence of the IS1113 element in strains of *X. o.* pv. *oryzae* from 16 rice-growing countries was confirmed by DNA dot blot analysis. *X. o.* pv. *oryzae* was detected from the seed washes and DNA extracted from the seed washes of naturally infected seeds of cvs Jaya and TN1. When stored at 4 °C, the pathogen was recovered up to 4 months and 9 months from naturally infected seeds of cvs Jaya and TN1, respectively. The BLB bacterium was also detected in seedlings, mature plants and seeds collected from plants raised from naturally infected seeds.

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Introduction

Xanthomonas oryzae pv. *oryzae* is a major pathogen of rice and is a threat to rice production in both temperate and tropical rice-growing regions, due to its high epidemic potential (Mew 1987). Yield losses of 10–20% are common and losses of 50–70% have been recorded in severely infected fields (Raina et al. 1982; Mew 1987; Mew et al. 1993). The spread of bacterial leaf blight (BLB) occurs through plant debris (Goto et al. 1953; Guo et al. 1980), wild rice (Aldrick et al. 1973), weeds (Goto et al. 1953; Valluvaparadesasan and Mariappan 1989) and water (Singh 1971; Srivastava 1972). The presence of *X. o.* pv. *oryzae* in infected seeds (Fang et al. 1956; Srivastava and Rao 1964; Chakravarthi and Rangarajan 1967; Mew et al. 1993) and disease transmission from seeds have been demonstrated (Srivastava and Rao 1964; Chattopadhyoy and Mukerjee 1971; Reddy 1983). However, other scientists report controversy about its transmission (Goto et al. 1988; Unnamalai et al. 1988), probably due to the limited usefulness and accuracy of techniques used in detecting low numbers of viable cells of the pathogen (Singh and Rao 1977). The BLB bacterium is considered as a quarantine organism and is subject to phytosanitary regulations in many countries. Isolation of *X. o.* pv. *oryzae* from rice plant and seed by conventional techniques is often difficult, usually due to the masking effect of fast-growing, yellow-pigmented bacteria. Agar media used in the isolation of the BLB pathogen are not selective enough to eliminate fast-growing contaminants (Di et al. 1991). Biochemical tests (Vera Cruz et al. 1984), serological assays (Benedict et al. 1989), fatty acids and metabolic profiling (Chase et al. 1992; Jones et al. 1993) have been used in the identification of the pathogen. These assays however, have shortcomings including lack of sensitivity and specificity.

Due to the problems encountered in conventional methods, polymerase chain reaction (PCR) technology has found wide application in detecting plant pathogenic bacteria (Blakemore et al. 1992; Goodwin and Nassuth 1993; Prosen et al. 1993; Seal et al. 1993; Lopes and Damann 1994; Schaad et al. 1995). A combined biologi-

cal and enzymatic amplification (BIO-PCR) technique was developed to detect *Pseudomonas syringae* pv. *phaseolicola* from bean seeds (Schaad et al. 1995). Although PCR assays (Cottyn et al. 1994; Gnanamanickam et al. 1995) and ligation-mediated PCR tests (DNA fingerprint assays; George et al. 1994) for *X. o.* pv. *oryzae* have been reported, available PCR techniques have not been successful in the detection of this pathogen from artificially inoculated, symptomatic and symptomless tissue and naturally infected rice seeds. Seed transmission of the closely related organism, *X. o.* pv. *oryzicola*, the bacterial leaf streak pathogen, has already been established. The present study focuses on the development of a BIO-PCR assay for the rapid detection of *X. o.* pv. *oryzae* in rice seeds and leaves and investigates its transmission to rice plants.

Materials and methods

Bacteria

Bacterial strains were obtained from the culture collection of the Danish Government Institute of Seed Pathology for Developing Countries, Denmark and also from the culture collection of the Department of Plant Pathology, Kansas State University. All strains were grown in peptone/sucrose broth (PSB; Tsuchiya et al. 1982) for preparation of inoculum, or were stored long-term in 15% glycerol at –70 °C. Reference strains of *X. o.* pv. *oryzae* used in this work and their origin were: CAMXO1 (Africa), AU6 (Australia), B13 (Bangladesh), KL1 (Malaysia), BU1 (Myanmar), C1 (China), CIAT1186 (Colombia), NCPPB1936 (India), IG12 (Indonesia), JXOT7133 (Japan), KOX1 (Korea), NXO1 (Nepal), PXO99 (Philippines), CL6 (Sri Lanka) and TL12 (Thailand); and other bacteria, such as *X. o.* pv. *oryzicola* BLS175, *Acidovorax avenae* subsp. *avenae* (*Pseudomonas avenae*) NCPPB1011, *P. fuscovaginae* SR532, *P. fluorescens* PFCP1, *P. aeruginosa* PFAR, *P. s.* pv. *syringae* PSS1, *Burkholderia glumae* (*P. glumae*) NCPPB2981, *X. campestris* pv. *holcicola* XCH429, *X. c.* pv. *campestris* A249, *X. c.* pv. *glycines* 712, *X. c.* pv. *pisi* KS, *X. c.* pv. *begoniae* A915, *X. c.* pv. *phaseoli* A584, *X. c.* pv. *vesicatoria* A1782 and *Clavibacter michiganensis* subsp. *sepedonicus* RR2 were used in this study (Benedict et al. 1989; Leach et al. 1990; Prosen et al. 1993; Cottyn et al. 1994; Gnanamanickam et al. 1995).

Germplasm

Healthy rice seeds of cv. IR 64 and naturally infected seeds of cv. TN1 obtained from Hyderabad, Andra Pradesh, and naturally infected seeds of cv. Jaya obtained from Ludhiana, Punjab, were used in this study.

Genomic DNA extraction from bacteria and plants

Total DNA from bacteria was extracted as described by Leach et al. (1992) and the C-TAB method was followed to extract plant DNA (Murray and Thompson 1980). DNA from the Gram-positive bacterium, *C. m.* subsp. *sepedonicus* was extracted using the genomic DNA extraction kit supplied by Promega Biotech (Madison, Wis.).

Primers and PCR conditions

Primers TXT (5′-GTCAAGCCAACTGTGTA-3′) and TXT4R (5′-CGTTCGCGCCACAGTTG-3′) were made from an insertion sequence element, IS1113 of *X. o.* pv. *oryzae* (N. Sakthivel and J. E. Leach, unpublished data), with a predicted PCR product of 964 bp. PCR assays were performed with a Perkin Elmer 2400 thermocycler. Mg^{++} concentration and other conditions were optimized to test the DNA template and the bacterial cells in PCR. All amplifications were carried out in a final volume of 50 µl containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 M of each primer, 1 unit of *Taq* polymerase enzyme (Promega), 5 µl of thermo-DNA buffer (Promega) and 200 ng of DNA or 35 µl of suspension of bacteria (seed washing). PCR-water (Sigma) was used in the mixtures and the preparation of PCR reactions was carried out in a laminar-flow hood. Each PCR experiment included a control without DNA. Reactions were run for 30 cycles, each consisting of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, with initial denaturation of 5 min at 95 °C and final extension of 10 min at 72 °C. A 5-µl aliquot of each amplified PCR product was fractionated on a 0.7% agarose gel, stained with ethidium bromide and visualized under a UV transilluminator. PCR machines such as the Perkin Elmer 2400 (Perkin Elmer International, Rotkreuz), an Eppendorf mastercycler gradient (Eppendorf, Hamburg), MJ thermocycler (MJ Research, Watertown, Mass.) and PCR express thermal cycler (Hybaid US, Mass.) and different *Taq* polymerase enzymes supplied by their manufacturers (Promega, Finnzyme, Bioline USA and Bangalore Geni, Bangalore) were used to test the reproducibility of amplification. The results obtained using the Perkin Elmer 2400 PCR machine and *Taq* polymerase (Promega) are presented in this study.

Sensitivity of PCR

To determine the sensitivity threshold of PCR detection in the pure culture suspension, strain *X. o.* pv. *oryzae* NCPPB 1936 was grown in PSB, cells were pelleted and washed three times in sterile distilled water. A suspension of 10^7 CFU/ml was diluted eight times, in a ten-fold series. Aliquots of 2 µl of each dilution were directly used in PCR for amplification (Ausubel et al. 1987). Dilutions of genomic DNA of *X. o.* pv. *oryzae* NCPPB 1936 (50–550 fg) were also used to detect the sensitivity level of PCR with TXT and TXT4R primers. Single colonies (1–5 weeks old) of *X. o.* pv. *oryzae* NCPPB 1936 on PS agar were also directly tested in PCR to study whether the age of cultures affected amplification.

Specificity of PCR

To evaluate the specificity of PCR, DNA from a rice plant (cv. IR 64), reference strains of *X. o.* pv. *oryzae* and other strains (*X. o.* pv. *oryzicola* BLS175, *A. a.* subsp. *avenae* NCPPB1011, *P. fuscovaginae* SR532, *P. fluorescens* PFCP1, *P. aeruginosa* PFAR, *P. s.* pv. *syringae* PSS1, *B. glumae* NCPPB2981, *X. c.* pv. *holcicola* XCH429, *X. c.* pv. *campestris* A249, *X. c.* pv. *glycines* 712, *X. c.* pv. *pisi* KS, *X. c.* pv. *begoniae* A915, *X. c.* pv. *phaseoli* A584, *X. c.* pv. *vesicatoria* A1782 and *C. m.* subsp. *sepedonicus* RR2) and saprophytic yellow bacteria isolated from rice leaves and seeds were tested by PCR. To study whether primers detected *X. o.* pv. *oryzae* from seeds with the existing population of background microflora, rice seeds of cultivar IR 64 (10^7 CFU/g of background microflora) were inoculated with 10^8 CFU/ml of *X. o.* pv. *oryzae* NCPPB 1936. Infested seeds were incubated in a laminar-flow hood for 2 h and also at 4 °C for 2 h to permit absorption of bacteria. A total of 50 seeds/plate were plated onto PSA and incubated for 2 days at 29 °C. Bacterial growth appearing around the plated seeds was washed three times with 1 ml of sterile distilled water, pooled and stored at –20 °C. A 35-µl aliquot of plate washings was tested by PCR.

Detection of pathogen in symptomatic or symptomless leaves of inoculated rice plants

To obtain the kresek symptom of BLB, rice seeds (inoculated with *X. o.* pv. *oryzae* NCPPB 1936, as described earlier) were planted

in pots (100 seeds/pot) containing sterile soil mixture and were grown in 12 h dark/light cycles at 30–35 °C. Rice plants of cv. IR 64 (45 days old) were inoculated by clipping the tips of expanded leaves with sterile scissors that had been dipped in a suspension (10^8 CFU/ml) of *X. o.* pv. *oryzae* NCPPB 1936. Two weeks after inoculation, plants were monitored for BLB symptoms. One gram of symptomatic or symptomless upper leaves was sampled, DNA was extracted and tested in PCR. Uninoculated plants or seeds were used as controls. All experiments were conducted twice.

Detection of pathogen by BIO-PCR in naturally infected rice seeds

To detect *X. o.* pv. *oryzae* in naturally infected seeds of cvs TN1 and Jaya, 500 g of seeds were soaked overnight in 750 ml of 0.01% Tween 20 at 4 °C (Schaad et al. 1995). Samples of 100 µl of seed extract were plated onto PSA in duplicates and incubated for 2 days at 29 °C. Plates were then washed three times with 1 ml of sterile distilled water and a 35-µl sample of the washings was used in PCR assays. To study the survival of the pathogen in naturally infected seeds, the seeds were stored at 4 °C and subjected to a PCR test every 2 weeks.

Transmission studies

To test the transmission of *X. o.* pv. *oryzae* from naturally infected seeds to seedlings, 500 infected seeds of cv. TN1 were germinated and 50 seeds were planted into 10 pots, each containing sterile soil mixture. The pots were maintained at 30–35 °C with 12 h dark/light cycles. Seedlings were observed for BLB symptoms. Leaf samples were collected after 45 days. At maturity, seeds were collected and subjected to a BIO-PCR test as described.

DNA dot blot and Southern blot hybridization studies

To confirm whether the 964-bp PCR DNA fragment was amplified from the IS1113 of *X. o.* pv. *oryzae*, hybridization studies were conducted, following standard methods (Mass 1983; Leach et al. 1990). The probes for hybridization studies were based on a DNA insertion element IS1113, which was cloned in plasmid pBSTnX1 (N. Sakthivel and J. E. Leach, unpublished data) and further subcloned in plasmid pGEMTPUDBT (A. Kumar and N. Sakthivel, unpublished data).

DNA dot blot analysis was carried out to confirm whether IS1113 element was present in all reference strains of *X. o.* pv. *oryzae* from different rice-growing countries. Samples of 10 ng of genomic DNA isolated from cells of pure cultures of *X. o.* pv. *oryzae* strains CAMXO1(Africa), B13 (Bangladesh), KL1 (Malaysia), BU1 (Myanmar), C1 (China), CIAT1186 (Colombia), NCPPB1936 (India), IG12 (Indonesia), JXOT7133 (Japan), KOX1 (Korea), NXO1 (Nepal), PXO99 (Philippines), CL6 (Sri Lanka) and TL12 (Thailand) were spotted onto nylon membrane. They were UV cross-linked to bind the DNA onto the membrane and hybridized with labelled IS1113. DNA samples from the rice seed-associated, yellow saprophytic bacterial mixture and *P. fuscovaginae* SR532 were used as a control. Hybridization and washing conditions (Leach et al. 1992) were at high stringency. Hybridization was performed at 65 °C in a mixture of 0.1% SDS, 50 mM sodium phosphate buffer (pH 7.0), 1.0 M NaCl, and 300 mg of denatured salmon sperm DNA/ml for 18 h. After hybridization, the blots were washed three times (20 min each) at 65 °C in 2×SSC (1×SSC is 0.15 M NaCl plus 0.015 sodium citrate) containing 0.1% SDS and 5 mM sodium phosphate buffer (pH 7.0) and then washed three times in 0.5×SSC containing 0.1% SDS and 3 mM sodium phosphate buffer.

To conduct the Southern blot analysis, IS1113 was labelled with [^{32}P] dCTP by nick translation with DNA polymerase and used as a hybridization probe against the amplified DNA fragments (964 bp in size) obtained from PCR experiments using different strains of *X. o.* pv. *oryzae*. The PCR fragments were fractionated by gel electrophoresis (horizontal 0.7% agarose gel) in Tris-acetate buffer. A 1-kb ladder DNA (Bethesda Research laboratories, Md.) was included in the gel as a size standard. Southern transfer onto nylon membrane was done according to the manufacturer's instructions (GeneScreen Plus; Du Pont NEN Research Products, Mass.). Hybridization and washing of the blot was done as described earlier.

Autoradiography

Autoradiography was done at –70 °C with Cronex film, using Cronex Lightning-Plus intensifying screens (Du Pont NEN Research Products, Mass.).

Results

PCR optimization

The primer pair, TXT and TXT4R, amplified a 964-bp DNA fragment from strains of *X. o.* pv. *oryzae* from all rice-growing countries, when 25 µl suspensions of bacteria (10^8 CFU/ml) or 200 ng DNA were used as the template under optimized conditions (50 µl PCR reaction, containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of each primer, 1 unit of *Taq* polymerase enzyme, 5 µl of thermo DNA buffer). Similar conditions yielded reproducible results in the Perkin Elmer 2400 and in other PCR machines such as the Eppendorf mastercycler gradient, MJ thermocycler and Hybaid PCR express thermal cycler. *Taq* polymerase enzymes supplied by different manufacturers (Promega, Finnzyme, Bioline USA and Bangalore Geni) also yielded similar PCR results.

Sensitivity and specificity of PCR

It was possible to detect *X. o.* pv. *oryzae* in pure culture suspensions tested by PCR. The minimum number of cells detected was about 10^5 CFU/ml (Fig. 1). Based on the dilution series, the PCR reaction contained 4 cells per 2 µl of suspension. When genomic DNA of *X. o.* pv. *oryzae* was used as template, the PCR detection level was 55 fg. Primers did not amplify any fragments of DNA from the rice plant, or from *A. a.* subsp. *avenae* NCPPB 1011, *P. fuscovaginae* SR532, *P. s.* pv. *syringae* PSS1, *B. glumae* NCPPB2981, saprophytic yellow bacteria (Fig. 1), *X. c.* pv. *holcicola* XCH429, *X. c.* pv. *campestris* A249, *X. c.* pv. *glycines* 712, *X. c.* pv. *pisi* KS, *X. c.* pv. *begoniae* A915, *X. c.* pv. *phaseoli*A584, *X. c.* pv. *vesicatoria* A1782 or *C. m.* subsp. *sepedonicus* RR2, with the exception of *X. o.* pv. *oryzicola* BLS175 (data not shown). The presence of background bacterial microflora (10^7 CFU/g of seed) or the age of the *X. o.* pv. *oryzae* (NCPPB 1936) culture did not inhibit the amplification of target DNA. A single colony of *X. o.* pv. *oryzae* NCPPB 1936 was also directly amplified in PCR.

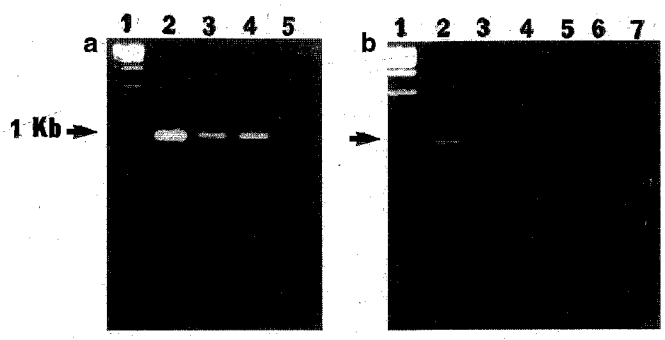

Fig. 1a, b Amplification of a 964-bp DNA fragment from a dilution series of *Xanthomonas oryzae* pv. *oryzae* NCPPB 1936. **a** Ethidium bromide-stained agarose gel showing the amplification of the 964 bp DNA fragment. *Lane 1* 1-kb DNA ladder (*arrow* indicates the 1-kb size fragment), *lane 2* 10^7 CFU, *lane 3* 10^6 CFU, *lane 4* 10^5 CFU and *lane 5* 10^4 CFU. **b** Specificity of primers TXT and TXT4R on the amplification of the 964 bp DNA fragment. *Lane 1* 1-kb ladder DNA (*arrow* indicates the 1-kb size fragment), *lane 2 X. o.* pv. *oryzae* NCPPB 1936, *lane 3 Acidovorax avenae* subsp. *avenae* (*Pseudomonas avenae*) NCPPB1011, *lane 4 P. fuscovaginae* SR532, *lane 5 P. s.* pv. *syringae* PSS1, *lane 6 Burkholderia glumae* (*P. glumae*) NCPPB2981 and *lane 7* saprophytic yellow bacteria

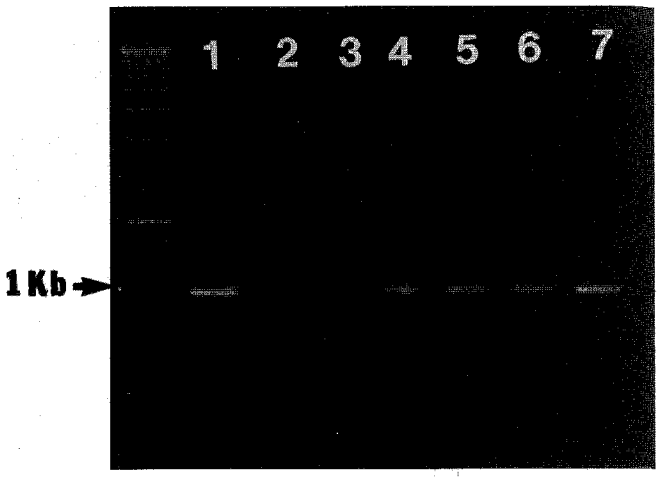

Fig. 2 Detection of *X. o.* pv. *oryzae* by PCR in naturally infected seeds and plants. *Arrow* indicates the 1-kb size fragment of the 1-kb ladder. *Lane 1 X. o.* pv. *oryzae* (NCPPB 1936), *lane 2* DNA of uninoculated rice plants, *lane 3* DNA of saprophytic bacteria isolated from uninoculated rice plants, *lane 4* agar plate-washings from naturally infected seeds (cv. TN1), *lane 5* agar plate-washings from naturally infected seeds (cv. Jaya), *lane 6* DNA of symptomatic leaves of cv. TN1, *lane 7* agar plate-washings from seeds collected from infected plants of cv. TN1

Detection of pathogen in symptomatic or asymptomatic leaves of inoculated rice plants

BLB lesions of 5–10 cm were observed 2 weeks after clip inoculation with *X. o.* pv. *oryzae* NCPPB 1936. When genomic DNA from inoculated leaves, upper symptomless leaves, or seedlings raised from artificially infested seeds were used as template, a 964-bp fragment of pathogen was detected in PCR assays. No amplification was observed from the DNA of uninoculated control plants, or seedlings raised from uninoculated seeds.

Detection of pathogen by BIO-PCR in naturally infected rice seeds

The BLB pathogen was detected by PCR in naturally infected seeds of cvs TN1 and Jaya (Fig. 2). When these naturally infected seeds were stored at 4 °C, *X. o.* pv. *oryzae* was detected up to 16 weeks from harvest in the seeds of cv. Jaya and up to 36 weeks in the seeds of cv. TN1.

Transmission studies

BLB symptoms were observed in the plants developed from naturally infected seeds of cv. TN1. *X. o.* pv. *oryzae* was detected by PCR in infected leaves and seeds at maturity (Fig. 2).

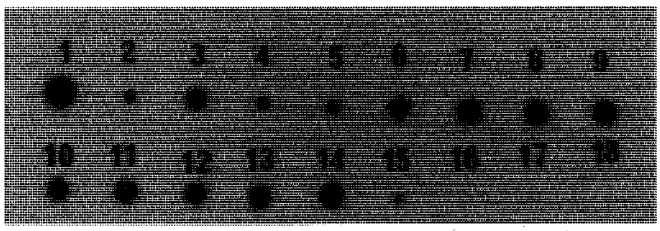

Fig. 3 DNA dot-blot hybridization of IS1113 with strains of *X. o.* pv. *oryzae* representing different countries of the world. *Lane 1* CAMXO1 (Africa), *lane 2* AU6 (Australia), *lane 3* B13 (Bangladesh), *lane 4* KL1 (Malaysia), *lane 5* BU1 (Myanmar), *lane 6* C1 (China), *lane 7* CIAT1186 (Colombia), *lane 8* NCPPB1936 (India), *lane 9* IG12 (Indonesia), *lane 10* JXOT7133 (Japan), *lane 11* KOX1 (Korea), *lane 12* NXO1 (Nepal), *lane 13* PXO99 (Philippines), *lane 14* CL6 (Sri Lanka) and *lane 15*TL12 (Thailand). *Lane 16* rice DNA, *lane 17* yellow saprophytic bacteria, *lane 18 P. fuscovaginae* SR532

DNA dot blot and Southern blot hybridization experiments

The presence of the IS1113 element in all strains of *X. o.* pv. *oryzae* representing rice-growing countries all over the world was confirmed by hybridization in a DNA dot blot; and the negative results in hybridization indicated that this element was not present in rice DNA, rice seed-associated, yellow saprophytic bacteria, or *P. fuscovaginae* (Fig. 3). The labelled probe DNA of IS1113 was hybridized with the 964 bp of PCR fragments amplified from all strains of *X. o.* pv. *oryzae*, representing rice-growing countries all over the world (Fig. 4).

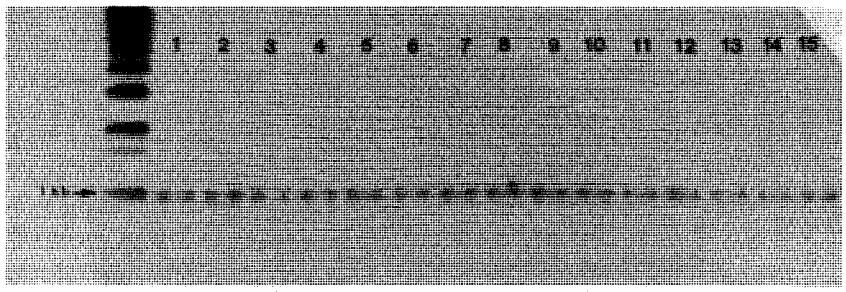

Fig. 4 Southern blot hybridization of IS1113 with PCR-amplified fragment (964 bp) from *X. o.* pv. *oryzae* strains. *Arrow* indicates the 1-kb size fragment of 1-kb DNA ladder. *Lane 1* CAMXO1 (Africa), *lane 2* AU6 (Australia), *lane 3* B13 (Bangladesh), *lane 4* KL1 (Malaysia), *lane 5* BU1 (Myanmar), *lane 6* C1 (China), *lane 7* CIAT1186 (Colombia), *lane 8* NCPPB1936 (India), *lane 9* IG12 (Indonesia), *lane 10* JXOT7133 (Japan), *lane 11* KOX1 (Korea), *lane 12* NXO1 (Nepal), *lane 13* PXO99 (Philippines), *lane 14* CL6 (Sri Lanka) and *lane 15*TL12 (Thailand)

Discussion

Our results showed that PCR, BIO-PCR and DNA hybridization techniques could be used to detect *X. o.* pv. *oryzae* in rice seeds and plants. Successful detection based on PCR techniques depends upon the specificity and sensitivity of primers. Based on the dilution series of cells, the detection level of TXT and TXT4R primers was 4 cells in a 50 µl reaction. The sensitivity level of these primers was 55 fg of *X. o.* pv. *oryzae* DNA. On the basis of the assumption that the genome size of *X. o.* pv. *oryzae* is approximately equivalent to the genome size of *P. fluorescens* (7.4×10^3 kb; Bak et al. 1970), the 55 fg DNA represented approximately 7 cells. Primers (TXT and TXT4R) used in this study did not amplify DNA from other pathogenic or saprophytic bacteria of rice, with the exception of *X. o.* pv. *oryzicola*, since IS1113 is also present in *X. o.* pv. *oryzicola* (George et al. 1994). PCR techniques with these primers can be applicable to detect both pathogens; and pathovars of *X. oryzae* can be differentiated by ligation-mediated PCR (George et al. 1994). Hybridization studies suggest that the 964-bp fragments were amplified from the IS1113 element of strains of *X. o.* pv. *oryzae* from different rice-producing countries. Therefore, PCR techniques based on TXT and TXT4R primers can be used in detecting strains of *X. o.* pv. *oryzae* with different geographical origins. PCR conditions such as primers, template, Mg^{++} (Caetano-Anolles et al. 1991; Bassam et al. 1992), thermocyclers and thermostable polymerase origin (Meunier and Grimount 1993; Schierwater and Ender 1993) have been shown to affect amplification. In this study, all these parameters were optimized in a Perkin Elmer 2400 thermocycler to avoid such artifacts and to insure reproducibility of amplification. Consistent results of amplification of a 964-bp fragment from *X. o.* pv. *oryzae* by TXT and TXT4R were also obtained by using various PCR machines and different *Taq* polymerase enzymes supplied by various manufacturers.

A PCR technique was successfully used to detect viable cells of *X. o.* pv. *oryzae* in naturally infected seeds (cv. TN1) up to 8 months from the date of harvest. Transmission of *X. o.* pv. *oryzae* from naturally infected seeds (cvs TN1 and Jaya) to seedlings was demonstrated. Although PCR is a highly sensitive technique, it cannot differentiate dead cells from live cells. This limitation is of major concern and it affects PCR application in quarantine laboratories. This problem was solved in an earlier investigation (Schaad et al. 1995) and in this study, by employing a BIO-PCR technique of plating seed or seed extract onto agar prior to the application of PCR and using plate-washing (bacteria) as the template. This method should eliminate the problem of false-negative results due to PCR-inhibitors in the plant or seed extracts, if any, and should also avoid false-positive results due to dead cells. BIO-PCR could be employed without the need for time-consuming DNA extraction and isolation of *X. o.* pv. *oryzae* from seeds.

The presence of plant pathogenic bacteria in symptomless plants (latent infection) and the transmission of bacteria from seed to seed has been documented (Thomas and Graham 1952; Tabei 1967). Based on our results, the primers TXT and TXT4R can be used to detect the infection of *X. o.* pv. *oryzae* in symptomless plants. Detection of pathogen in non-symptomatic seedlings is of importance in certification programs, for both domestic and international quarantine, because a latent population can lead to serious epidemics under favourable conditions. Early detection of diseases, using rapid methods, is important for assessing the health status of a rice nursery before the transplantation of seedlings to fields. In India, general recommendations given to farmers include the use of seed treatment and prevention measures, including cultural practices and the production of disease-free nursery plants (Reddy 1995).

Since PCR has the advantage of detecting the pathogen without the need for pure cultures, this technique should be employed for the rapid monitoring of pathogenic bacteria in seed lots from commercial seed consignments and for germplasm- and seed-testing in quarantine laboratories. Considering the lack of specificity and the amount of work involved in other methods, PCR is a quick, sensitive technique and should have wide application to detect and study the survival and transmission of plant pathogenic bacteria. Data generated in this study demonstrate the seed-borne nature and transmission of *X. o.* pv. *oryzae*, the causal agent of the BLB disease of rice.

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