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Detection of *Xanthomonas oryzae* pv. *oryzae* in Rice Seeds by Molecular Techniques

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ABSTRACT

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most important bacterial diseases on rice and is known to cause heavy economic loss. In the present study, 30 rice seed samples from affected regions were screened for the presence of *X. oryzae.* pv. *oryzae* and 20 isolates were identified based on morphological, physiological, biochemical tests and hypersensitive reaction (HR) on tobacco. Molecular detection using a 16S rRNA primer revealed DNA amplification in all 20 isolates, confirmed the pathogen as *X. oryzae* pv. *oryzae*. These amplified isolates were further confirmed by using a specific primer synthesized for the hypothetical protein gene. A 331-bp PCR product was obtained in 16 isolates. The application of these molecular tools in the characterization of *X. oryzae* pv. *oryzae* is discussed.

Keywords: specific primer, hypersensitive, bacterial blight, PCR. *X. oryzae* pv. *oryzae*

INTRODUCTION

Rice cultivation is the principle activity and source of income for millions of households around the globe and in several countries of Asia and Africa. The world's major rice growing countries are China and India. Rice constitutes 52% of the total food grain production and 60% of the total cereal production in India (Anon. 2010). India is the second leading producer of rice in the entire world, next to China with an annual rice production is 147.0 million tones in 42.24 million ha of cultivation (FAO 2011). The major rice growing states of India are West Bengal, Uttar Pradesh, Andhra Pradesh, Punjab, Tamilnadu, Bihar, Orissa, Assam, and Karnataka. More than 50% of the total rice seeds production comes from the first four states (FCI 2010). Presently, cultivation of rice is being affected due to many diseases and pests such as bacterial, fungal, viral, insect and nematodes. Several diseases cause high yield losses ranging from 5-90% (FCI 2010). Diseases are the most important limiting factors that affect rice production. Among the many bacterial diseases, 'Bacterial Leaf Blight' (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Mizukami Ishiyama) is a wide spread disease in Asia (Adhikari and Mew 1994). It has also been reported from Vietnam, Philippines and Thailand, with the first report from Japan in 1884. In India BLB was noticed for the first time in Kolaba district of Maharashtra (Bhapkar *et al.* 1960). Studies have shown that the disease is present in most of the rice growing states of India (Mizukami and Wakimoto 1970). The disease is more prevalent in both rain fed and irrigated lands in the wet season. In temperate countries, it is common during the rainy season. BLB occurs at all stages of rice plant growth and depends on the environmental conditions. In India it is a severe problem during the monsoon season. In this season the pathogen spreads rapidly and loss may be up to 6-84% in tropical Asia, depending on habitat, climate and variety or cultivar used. According to Liu (2006), loss at the tillering stage by BLB is 50% and yield reduction increases from 10-20% (Thind and Bala 2002).

Fig. 1 Tobacco plant inoculated with *Xanthomonas oryzae* pv. *oryzae* showing necrotic lesion.

Rapid detection and accurate identification of pathogens are critical steps to guide responses for containment or elimination of pathogens. However, robust and inexpensive diagnostic tools are not available for identification and classification of many plant pathogens (Mew 1987; Lopez *et al*. 2011). Historically, the primary hurdle in developing highly specific, easily used diagnostic tools for any pathogen has been the difficulty in finding unique features, apart from the importance of accurate identification and distinction of pathogen for efficient control (Lang *et al*. 2010).

The polymerase chain reaction (PCR) is a powerful technique that has widespread application in molecular boilogy. This technique is used to amplify a specific nucleic acid fragment that lies between two regions of known nucleotide sequence, and often from an extremely small amount of target nucleic acid in biologically complex samples (Lee *et al.* 2004). Amplified fragments can then be further characterized by size fractionation on agarose gels, restriction enzyme digestion and hybridization with probes or by DNA sequencing. The specificity of the PCR is based

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on the use of oligonucleotide primers that are complementary to the regions flanking the fragment to be amplified. Because of the sensitivity and specificity of the PCR technique, the procedure is likely, to have widespread applications in the detection of plant pathogens, and can be used for the identification of pathogens, populations and variability (Potter *et al.* 2007). In the present study with the help of PCR we tried to identify 16 isolates of *X. oryzae* pv. *oryzae* causing BLB disease of rice in major rice-growing regions of Karnataka, India.

MATERIALS AND METHODS

A field survey was undertaken in the major rice-growing regions of Karnataka, India, during June to November 2009 and February to June 2010. In the course of the field survey, the plants were inspected during the nursery stage, after transplanting, and at the flowering stage. BLB incidence was recorded, among the randomly selected subplots of 1 m^2 each (10 subplots/ha). The diseased plants were considered as infected on the basis of typical symptoms of BLB, which included yellow water soaked lesions at the leaf blade margin, the lesions run parallel along the leaf with bacterial discharge appearing on young lesions that look like a milky-dew drop in the early morning. As the disease progress the leaf dries up with white lesions and the leaf blade has wavy margin (EPPO 2007). The suspected diseased plant parts were collected separately from the field, labelled and brought to the laboratory for further studies.

Seeds of 30 different rice cultivars were collected from farmers' fields in different agro-climatic conditions, and also from public and private seed agencies in Karnataka. The collected seed samples were subjected to different seed health testing methods such as direct plating, liquid assay and seedling symptom test to determine the seed-borne *X. oryzae* pv. *oryzae* infection (Mew *et al.* 1993).

The seed samples were surface sterilized with 1% sodium hypochlorite solution for 3 min, followed by repeated washing with distilled water (3 times) and blot-dried, then plated directly (25 seeds/plate) on to yeast dextrose calcium carbonate agar medium (YDC; yeast extract – 10 g, calcium carbonate – 20 g, agar – 20 g in 950 ml distilled water and dextrose 20 g in 50 ml distilled water, the two solutions were autoclaved separately and mixed well when temperature of the medium was 50°C (ISTA 1999). Plated seeds were incubated at 28 ± 2°C for 24-72 h and observed for the presence of bacterial colonies based on the morphological characters such as shiny, raised, mucoid, pale yellow at first, straw yellow later. The suspected colonies were subjected to different physiological, biochemical, hypersensitive reaction and pathogenicity tests for confirmation of *X. oryzae* pv. *oryzae*. The experiment was carried out in four replicates of 100 seeds each and repeated twice.

Three layers of filter papers were placed in each Petri dish and it was soaked in 0.15% of carbendazim solution and plates were sterilized at 121°C for 20 min. The kernels from 100 seeds were removed and 25 kernels were placed on filter paper, the plates were incubated at 28 ± 2°C for 24 h. Then plates were transferred into deep freezer for 12-18 h, later incubated for 72 h and observed for the development of yellow mucoid bacterial colonies on kernel.

Four hundred seeds of each sample were ground to coarse powder and suspended in 200 ml of sterile saline (0.85% sodium chloride) and kept for 2 h on a rotary shaker at 150 rpm. The samples were serially diluted in 4 × 1:10 concentrations and streaked 50 µl of undiluted and diluted suspension on growth factor medium plates. The plates were incubated at 26 ± 2°C for 2 to 4 days and observed for the presence of small shiny yellow colonies. Number of colonies were counted and recorded, the experiments were repeated twice (Mortensen 1994; Razak *et al.* 2009).

Seeds were soaked in water for a few minutes, four replicates of 50 seeds were put on the paper towel in equal distance and incubated at 30 ± 2°C for 9 days. After 9 days seedlings were examined for typical symptoms of BLB disease (ISTA 2005). Bacteria were isolated directly from the seeds and from infected plant materials. Test for bacterial ooze from the suspected portion of the plants was carried out by immersing the freshly cut portion of the leaf on glass slide containing a drop of water followed by compound microscopic observation. Leaf parts from where ooze was observed, were selected for further isolation of the bacteria. Leaf sections were surface sterilized with 1% (w/v) sodium hypochlorite for 3 min followed by repeated washing with distilled water; blot dried and plated on to YDC and NA media. The plates were incubated at 28 ± 2°C for 24-72 h, and after 48 h plates were observed for the presence of bacterial colonies, and they were pure cultured on YDC slants. All bacterial isolates were maintained at 4°C for short term storage and for long term the bacteria were stored in 40% glycerol at -80°C (Ghasemie *et al.* 2008).

Identification and characterization of the bacterial blight pathogen was carried out by subjecting the bacterial isolates to various biochemical tests, such as Gram`s staining, potassium hydroxide (KOH) solubility test, Kovac's oxidase test (Hilderbrand and Schroth 1972) starch hydrolysis, lipase activity and Arginin dehydrogenase test (Lelliot and Stead 1987), gelatin hydrolysis, oxidative/fermentative metabolism of glucose and catalase tests. The strains were also subjected to hypersensitive reaction in tobacco (*Nicotiana tabacum var. xanthi*) plants (Carlton *et al.* 1998) and pathogenicity test (**Fig. 1**) (Kauffman and Rao 1972). Each test was conducted with four replicates and repeated twice.

Isolates were grown on NA medium for 24-48 h at 27 ± 2°C and bacterial cells were harvested and bacterial DNA was isolated using bacterial genomic DNA isolation kit, (Chromous Biotech, Bangalore, India) as per manufacturer's instructions. The 16SrRNA region was amplified by PCR using 16S rRNA primers: 16S forward primer (5'-TGGTAGTCCACGCCCTAAAC-3') and 16S reverse primer (5'- CTGGAAAGTTCCGTGGATGT-3'), which were designed using Primer3 software (Rozen and Skaletsky 2000). PCR was performed in a 0.2 ml PCR tube in volumes of 25 µl, containing 2.5 µl of genomic DNA (80-100 ng), 1 µl of 25 pmol of both primers, 1 µl of 100 mM dNTP (25 mM each), 2.5 µl of PCR buffer and 0.5 µl of 5 U *Taq* polymerase (Chromous Biotech, Bangalore, India). The PCR was performed in a Labmate gradient thermal cycler. The procedure followed was as follows initial denaturation at 95°C for 5 min; 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C and extension for 45 sec at 72°C; the final extension at 72°C for 2 min, followed by cooling to 4°C until the sample was recovered. For all the PCR protocols, sample containing without template DNA and non-infected host plant were used as negative control. These amplified isolates were confirmed by using specific primer which is custom synthesized (XO3756 forward 5'-CATCGTTAGGACTGCCAG AAG-3') and (XO3756 reverse 5'-GTGAGAACCACCGCCATCT-3' (Lang *et al.* 2010). PCR reaction mixture contained 0.5 µl of 10 mM dNTPs, 2.5 µl of 10X buffer, 2.0 µl of 25 mM $MgCl_2$, 0.02 U of *Taq* polymerase and 0.5 µl of each 10 µM primer in a total volume of 25 µl. The PCR protocol included an initial denaturing step at 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min 30 s; and a final extension at 72°C for 7 min (Lang *et al*. 2010). Products were analysed by separation in 2% agarose gels (1% Tris acetate-EDTA [TAE] buffer), gel was stained with ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

Among the different fields visited, most of them were infected with BLB with varying degrees of incidence. In the Koppala district of Gangvathi, the disease incidence was 13-20%, whereas in Madikeri it was only 1.5-6.5%, in Mandya district it was 4-34%. In Nanjanagudu, the disease incidence was 5-23%, whereas in the Davangere district it was as high as 13-37%.

Thirty seed samples were collected from the different districts surveyed (**Table 1**). Among these sample no cultivar was found free from *X. oryzae* pv. *oryzae* infection. 'Rathnachoodi' recorded a minimum of 4% of *X. oryzae* pv. *oryzae* infection and 'Jyothi' recorded the highest infection of 75% in the direct plating method. 'Pusa Basumati' recorded the lowest number (63×10^4 cfu/g) and 'Jyothi' recorded the highest number (310×10^4 cfu/g) in the liquid assay method. However, in the seedling symptom test, the highest incidence of 68% was recorded by 'Jyothi' and the lowest

Table 1 Farmer stored rice seed samples collected from different places in Karnataka.

Variety	Location
Gowrisanna	Ramanagara
IR 64	Hasana
1001	C R Nagara
Thanu	Malavalli
Jaya	S.R. Patna
Ankursona	Mandya
BR	S.R. Patna
Jyothi	Davanagere
Rasi	Tumakur
MR 1001	Pandavapura
Sonamasuri	Pandavapura
JGL	Maddur
1010	Gangavathi
Emergencey sona	Gangavathi
Doddi	Madikeri
BKB	Madikeri
Naga	Shivalli
Rajabhoga	Periyapatna
64 Sona	Koppala
B.T. Mallige	Koppala
Intana	Madikeri
Hemavathi	Madikeri
Tamilnadu sona	Koppala
Kanathumba	Shivalli
Dehradum Basumathi	Shivalli
Pusa Basumathi	Shivalli
Rathnachudi	Shivalli
MTU 1001	Haveri
Local variety	Madikeri
Kerala	Madikeri

Table 2 Biochemical test results of *Xanthomonas oryzae* pv. *oryzae*.

Biochemical test	Results
Gram's reaction	-
KOH test	+
Starch hydrolysis	-
Kovacs hydrolysis	-
Lipase activity	-
Gelatin hydrolysis	-
Arginine test	+
O/F test	+/-
Catalase	+
Hypersensitivity test	+
Pathogenicity test	+

+ ve =Positive; -ve = Negative.

incidence of 8% was recorded by 'Sonamahsuri' and 'Uma'. In the kernel plating method, the lowest incidence of 4% was recorded by 'Uma' and highest incidence was recorded by 'Jyothi'. The typical *X. oryzae* pv. *oryzae* colony showing yellow mucoid shining growth around the seed and kernel was observed for typical BLB disease symptoms. The isolates of *X. oryzae* pv. *oryzae* was purified and used for further studies.

The isolated bacterium showed a stain pink red and thin viscid mucoid strand indicating positive for KOH tests and Gram-negative nature (**Table 2**). A clear zone of hydrolysis was formed around the bacterial colonies when the plates were flooded with Lugol's iodine. Hence the bacterium indicated positive for the starch hydrolysis test (**Table 2**) and the inoculated Tween-80 agar plates showed white precipitates around the colonies of bacteria. Hence the bacterium was positive for lipase test (**Table 2**). After 2-3 days of incubation, test isolates showed liquefaction of the gelatin medium when compared to control. Hence the bacterium indicated positive for gelatin hydrolysis (**Table 2**). After the incubation period, O/F medium showed colour change from green to yellow indicating acid production from glucose. There was no change in colour of O/F medium when it was covered with white petroleum jelly (**Table 2**). Necrosis was observed on tobacco plant leaves within 24 h after infiltration with bacterial cells (**Fig. 1**), whereas sterile distilled water infiltrated leaf region did not show any change in leaf colour, which served as control (**Table 2**). Rice plants inoculated with suspected *X. oryzae* pv. *oryzae* isolates showed bacterial blight symptoms, whereas control plants did not show any symptoms.

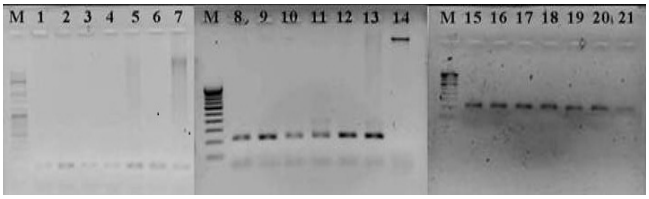

Fig. 2 PCR product of 20 *Xanthomonas oryzae* pv. *oryzae isolates* amplified by 16SrRNA primer. Lane M: 100 bp marker; 1-21: *Xanthomonas oryzae* pv. *oryzae* isolates; 22: negative control.

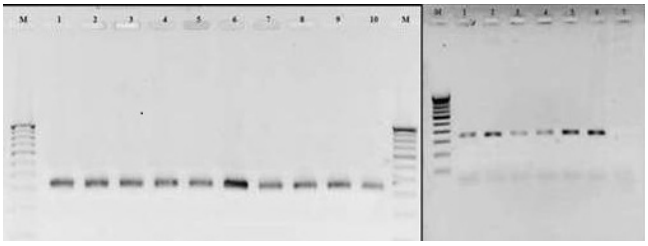

Fig. 3 PCR product of Xanthomonas *oryzae* pv. *oryzae* amplified with specific primer XO3756F and 3756R. Lane M: 100-bp marker; 1-16: *Xanthomonas oryzae* pv. *oryzae* isolates.

16S rRNA primers were used to amplify the tested bacterial DNA in all the respective positive isolates to confirm the pathogen. The PCR product of 210 bp was obtained in all the isolates (**Fig. 2**), while the negative control did not show any amplification. All 16 *X. oryzae* pv. *oryzae* isolates were identified by specific primers: XOF and XOR. On agarose gel electrophoresis isolates produced a characteristic band of 331-bp and the bands of isolates were similar to those of the reference (**Fig. 3**). Based on the phenotypic characteristics, biochemical test, pathogenicity test and PCR test all 16 isolates were classified as *X. oryzae* pv. *oryzae* (**Fig. 3**).

BLB of rice caused by *X. oryzae* pv. *oryzae* is a major threat to the world rice production and also in India. In Karnataka, the rice crop is suffering from BLB disease (Shivalingaiah and Umesha 2011). Earlier reports revealed the presence of BLB disease in almost all rice-growing states of India which are seriously disturbed by this disease (Nayak *et al.* 2008). Based on the morphological, biochemical and pathogenicity test and PCR reaction using specific primer we identified the causal organism of BLB in rice. All *X. oryzae* pv. *oryzae* isolates produced blight symptoms on rice. No significant differences were observed in the intensity of blight on inoculated plants. These results suggest that isolates obtained from different fields did not differ in their degree of virulence (Ochiai *et al.* 2000). Based on this work, we attempted to confirm the presence of *X. oryzae* pv. *oryzae* in rice seeds. In the present study, 16 isolates of *X. oryzae* pv. *oryzae* were identified from the different districts of Karnataka where rice is a major crop. These isolates were confirmed by biochemical, pathogenicity and hypersensitivity tests and further confirmed by PCR reaction using a specific primer (Lang *et al.* 2010). To our knowledge, the occurrence and incidence of this disease on rice in different geographic regions of Karnataka have not been studied using any molecular technique. This study will generate molecular information of *X. oryzae* pv. *oryzae* causing BLB of rice in Karnataka.

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