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DETECTION OF GENETIC VARIABILITY AND PATHOGENICITY OF *Xanthomonas oryzae* pv. *oryzae*: CAUSAL ORGANISM OF BACTERIAL LEAF BLIGHT IN RICE

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ABSTRACT

Bacterial Leaf Blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most destructive diseases of rice in Sri Lanka. Understanding the genetic and pathogenic variability is important in developing resistant rice varieties and recommending varieties for different locations. Randomly Amplified Polymorphic DNA based – Polymerase Chain Reaction (RAPD-PCR) was carried out on seven isolates collected from different locations using three primers (OPD7, OPD20 and OPK16) to detect the genetic variability of the pathogen. Screening of differentials with single gene and gene combinations (IRBB4, IRBB5, IRBB13, IRBB21 and IRBB60) was carried out to detect the pathogenicity of each isolate. Pooling all the genetic information, pathogen isolates were identified as six groups (0.6 average distance, SAS version 8.0). Information obtained with OPD7 primer depicted that two isolates from the same agro–ecological zone had no differences at the genetic level. Differentials with a single resistant gene showed resistance, moderate resistance, susceptibility and moderate susceptibility (standard scale:-<2cm: highly resistant, 2-3cm:- moderately resistant, 3-4cm:- moderately susceptible and> 4cm:- highly susceptible) to each individual isolate. IRBB60 was highly resistant to all the isolates tested. Potential to find relationships between DNA types and geographical locations was evident.

KEYWORDS: Bacterial leaf blight, Pathogenicity, *Xanthomonas oryzae* pv. *oryzae*, Polymerase chain reaction, Randomly amplified polymorphic DNA.

INTRODUCTION

Bacterial Leaf Blight (BLB), caused by *Xanthomonas oryzae* pv. *Oryzae* (Xoo) is one of the most destructive diseases of rice in Sri Lanka. Pathogenicity of the pathogen varies depending on the location (Noda *et al.*, 1995) and therefore, a rice variety resistant in one location may be susceptible elsewhere. This causes a problem in recommending rice varieties to be cultivated in different locations and resistant variety development. Moreover, resistant genes become susceptible with long-term cultivation and when cultivated on a large scale. In many countries including, Indonesia, India, China and the Philippines, rice varieties with only *Xa4* resistant gene have become susceptible to the pathogen (Huang *et al.*, 1997). In Sri Lanka too, such observations have been reported (Rajapakse, 2001). This is probably due to evolution of the pathogen or lack of horizontal resistance. Therefore, studying the genetic and pathogenic variability of the contemporary pathogen populations is important to understand the genetic relationships with degree of virulence and geographical location of the pathogen, develop resistant rice varieties and recommend varieties for different agro-climatic regions. Therefore, the objective of this study is to detect the genetic variability and pathogenicity of seven isolates of BLB causal organism.

MATERIALS AND METHODS

Pathogen

Bacterial Leaf Blight infected leaf samples were collected from seven random locations in five agro-ecological zones (Table 1). Pure cultures of each isolate were obtained and authenticity of the causal organism was proven by Koch's postulates by inoculating the highly susceptible variety TN-1. Pathogen cultures were maintained on slants of modified Wakimoto medium (Ou, 1985) at 28°C.

Table 1. Pathogen isolates and their location.

Isolate	*Location*	*Agro-ecological zone*
X_{wat}	Watapuluwa	WM_2
X_{gan}	Gannoruwa	WM_2
X_{ala}	Alawwa	IL_1
X_{bg}	Bathalagoda	IL_2
X_{lb}	Labuduwa	WL_4
X_{mat}	Ranna	WL_4
X_{at}	Ambalantota	DL_1

(W-Wet Zone, I-Intermediate Zone, D- Dry Zone, M-Mid Country, L-Low Country)

Genomic DNA extraction

Genomic DNA of the pathogen was extracted using lysosyme and Rnase-free protocol. A single colony of pure bacteria was suspended in 15ml agar-free Wakimoto's medium and grown overnight in an incubator-shaker at 28°C, at a speed of 60 rpm. Then, bacterial suspensions were kept in polypropylene tubes and centrifuged at 12,000 rpm for 10 min at room temperature. Resulting mass of bacterial cells was re-suspended by vortexing in 2ml TE (tris-ethelenediaminetetraaciticacid) buffer. 250µl of 10% sodium dodisyl sulphate and 50µl of proteinase K (10mg/ml) was then added and incubated at 37°C for 1hr. To this, 450µl of 5M NaCl and 400µl of 10% cetyltrimethylammoniumbromide (CTAB) in 0.7M NaCl were added.

Thereafter, it was mixed and incubated at 65°C for 20min. An equal volume of Chloroform:Isoamyl alcohol (24:1) was added and samples were inverted several times and centrifuged at 12,000 rpm for 15 min. To the separated upper aqueous layer, 0.6vol of cold Iso-propanol was added and resulting DNA was spooled using a glass hook and washed in 1ml of 70% ethanol two to three times, air dried and dissolved in 100µl of TE buffer. Extracted DNA was quantified using the spectrophotometer.

RAPD-PCR

Randomly amplified polymormpic DNA (RAPD) based polymerase chain reaction (PCR) was performed using three random primers OPD7 (5′- TTGGCACGGG-3′), OPK16 (5′-GAGCGTCGAA-3′) and OPD20 (5′-ACCCGGTCAC-3′) in 25µl reaction volume. PCR mixture consisted of genomic DNA (25ng/µl), PCR buffer (final reaction concentration: 10mM Tris-HCl, pH8.3, 50mM KCl, 2.0mM $MgCl_2$), 2.5mM of each dNTP, 0.2µM random primer and 0.5 units Taq polymerase (Takara). Reaction mixture was overlaid with a drop of mineral oil. DNA amplification was performed using a PCR program with 40 cycles (1 min at 94°C, 1 min at 36°C and 2 min at 72°C) and with 2 min at 94°C initial denaturation and 7min at 72°C final elongation steps in a Perkin-Elmer thermocycler. Amplified PCR products were separated in agarose (1.4%) using TBE electrode buffer. Agarose gel was stained with ethidium bromide and visualized under UV light.

Banding patterns that resulted on the agarose gel were recorded in binary form, "1" representing the presence and "0" the absence of a band. Scored data were analyzed using average linkage cluster analysis program in Statistical Analysis System (SAS), version 8.

Plant materials

In order to detect the pathogen reaction to resistance gene (s), seeds of near isogenic lines with single resistant gene(s) (NILs), IRBB4, IRBB5, IRBB21 and IRBB60 were obtained from the gene bank of the Plant Genetic Resources Center (PGRC), Gannoruwa. Seeds were treated with 1% Captan solution and germinated in the seed germinator. Four to five-day old plants were transferred to pots. Water level was maintained at 3-4cm. Application of fertilizer and control of pests were done as recommended by the Department of Agriculture.

Inoculation

A single colony of *Xanthomonas oryzae* pv. *oryzae* was streaked on modified Wakimoto's medium [20g/l sucrose, 5g/l peptone, 0.5g/l $Ca(NO_3)_2 \cdot 4H_2O$, 1.8g/l $Na_2HPO_4 \cdot 7\ H_2O$, 0.05g/l $FeSO4 \cdot 7\ H_2O$, 18g/l agar). Three to four 72 hr-old cultures were dissolved in sterilized distilled water and concentration of the cell suspension was adjusted to 10^8 CFU/ml. The top three to four leaves of four five-day old plants were prick inoculated.

The lengths of lesions on leaves were recorded fourteen days after inoculation. Resistance/susceptibility was evaluated according to the following standard.

Length < 2 cm: highly resistant
2-3 cm: moderately resistant
3-4 cm: moderately susceptible and
> 4 cm: highly susceptible.

RESULTS AND DISCUSSION

Genetic variability

Spectrophotometer readings (OD_{260} / OD_{280}) for genomic DNA of *Xanthomonas oryzae* pv. *oryzae* were between 1.7 and1.8 for all the DNA samples except one. Therefore, genomic DNA extracted was of high purity.

PCR amplification with random primers resulted in distinct (polymorphic) banding patterns (Figure 1). The three primers OPD7, OPK16 and OPD20 generated 15, 18 and 12 bands respectively. Three separate dendograms were obtained for data generated for the three primers and one dendogram that combined all the data. In the dendogram of OPD7 primer all the isolates were clustered into three groups at an average distance of 0.85. Isolates from Gannoruwa and Watapuluwa were found in one cluster with no average distance between them. Therefore, information revealed from OPD7 primer does not depict genetic difference between the two isolates (Xgan and Xwat) from the same agro-ecological zone (WM_2). Pathogen isolates from Matara (Xmat) and Labuduwa (Xlb) from the WL_4 agro-ecological zone, were also found in one group at an average distance of 0.81 (Figure 2). Therefore, it is evident that information generated from OPD7 random primer is useful to determine possible relationships between Xoo isolates and their geographical locations.

In the dendogram constructed with information generated by OPK16 primer, Xgan and Xwat from WM_2 agro-ecological zone were clustered into one group at an average distance of 0.81, but Xmat and Xlb from WL_4 were found in two distinct groups (data not shown). Dendogram obtained from the genomic information revealed by OPD20 primer did not show any relationship between Xoo isolates and the agro-ecological zones they were isolated from (data not shown). Analysis of data using different probes showed that Xoo population structure is dependent on the probes used. Not all probes have been equally informative in revealing genetic variation among strains (Raymundo *et al.,* 1990). Likewise, random primers could also give different results. Three main clusters were observed at an approximate average distance of 0.95 in the dendogram constructed by combining the data of all three primers. Again Xgan and Xwat were found in one cluster at an average distance of 0.60 but Xmat and Xlb were found in two distinct groups (Figure 3). Moreover, Xlb was found in a separate branch in dendograms constructed with information generated by OPD20 and combining information of all three primers. Therefore, not all primers are useful in revealing genomic information. Hence, depending on the objective, proper primers or probes should be identified in advance. It is quite clear that only OPD7 primer is promising when compared to the other two primers used alone or when all three primers were used together, in revealing genomic information that has a relationship with the geographic location of the isolates.

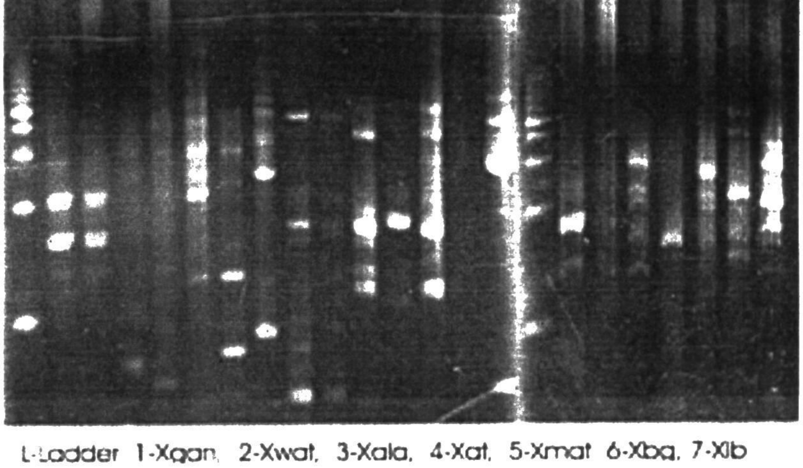

Figure 1. PCR products of OPD7, OPK6 and OPD20 random primers.

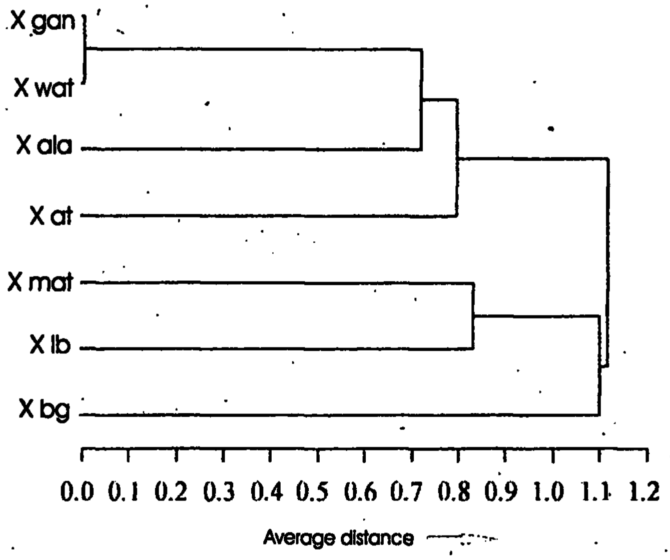

Figure 2. **Dendogram obtained with OPD7 primer.**

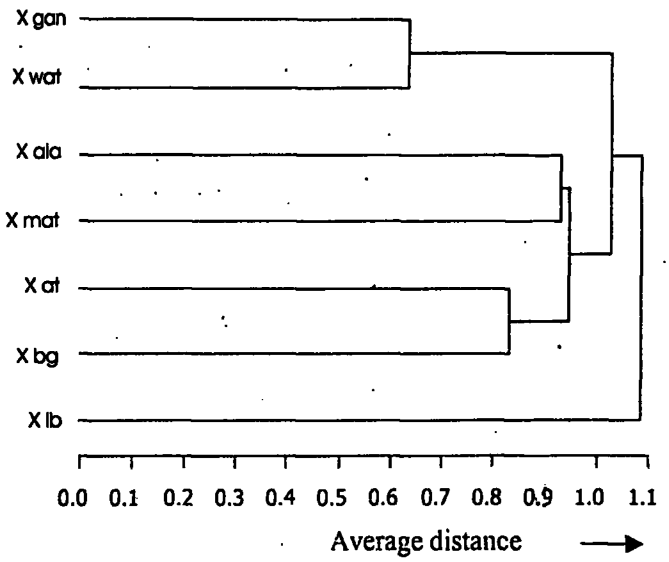

Figure 3. **Dendogram obtained with all three Primers (OPD7, OPK16, OPD20).**

Pathogenicity

An average lesion length of susceptible check TN-1 was over 4cm for each isolate tested. Near Isogenic Lines developed lesions of different lengths upon inoculation. Lengths of the lesions ranged from 0.1cm to over 14cm. Some lesions showed quick development while some were slow. Some lesions did not show development beyond 0.1cm. Each isolate showed different reactions to differentials. Lines with single resistant gene showed resistance (R), moderate resistance (MR), susceptibility (S) and moderate susceptibility (MS) to different isolates. IRBB21 with *Xa21* gene showed vertical resistance comparative to other lines with a single resistant gene. Isolates from IL_2, WL_4 and DL_1 agro-ecological zones were comparatively more virulent than isolates from other agro-ecological zones (Table 2). In a research carried out to study the virulence of Xoo on rice lines containing single resistant genes and gene combinations, all the rice lines with a single gene for resistance were susceptible to all the isolates tested (Adhikari *et al.*, 1998). It has also been reported that genes *xa5*, *xa13*, *Xa21* were resistant to all the isolates in another study (Huang *et al.*, 1997). Therefore, isolates from different locations show high variation in pathogenicity for different resistant genes. IRBB60 with all four resistant genes (*Xa4*, X*a13*, *Xa5* and *Xa21*) showed resistance to all the isolates tested. Therefore, four resistant genes in one host show broad spectrum of resistance.

Table 2. Average lesion length and reaction to host differentials.

	Xgan	*Xal*	*Xwat*	*Xat*	*Xmat*	*Xbg*	*Xlb*
TN-1	S 10.5	S 11.2	S 13.4	S 7.8	S 4.9	S 10.1	S 5.6
IRBB4	R 0.1	MR 2.7	R 0.9	S 4.3	S 4.1	R 0.4	MR 2.4
IRBB5	R 0.8	MS 3.0	MS 3.3	MR 2.4	R 1.3	S 4.6	MS 3.6
IRBB13	R 1.2	MS 3.4	R 1.5	S 5.6	MR 2.6	S 4.2	S 4.5
IRBB21	R 0.1	R 0.1	R 0.1	S 4.1	MS 3.1	MS	S 4.9
IRBB60	R 0.1	R 0.2	R 0.1	R 0.1	R 0.1	R 0.1	R 0.1

CONCLUSIONS

There is high variation of the Bacterial Leaf Blight causal organism at DNA level. The OPD7 primer was promising in revealing genomic information that had a relationship with geographical location of the BLB pathogen. Pathogen reactions on differentials were highly variable. Four resistant genes in one host plant conferred a broad spectrum of resistance to BLB in rice.

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