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Pheno-Genotypic Screening of Medium Slender Rice Genotypes for Bacterial Leaf Blight (BLB) Disease Resistance

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ABSTRACT

The bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv. oryzae (*Xoo*) is an economically important and one of the most destructive disease of rice in both irrigated and rainfed environments in Asia. In particular, in recent years, the occurrence of abnormal climate and warming phenomena has produced a good environment for bacterial leaf blight. The rice yield continues to decrease, causing 30 to 50% yield loss. Since bacterial pathogen is difficult to manage by other means effectively, developing host plant resistance is the most effective way to control this disease. So, the study was carried out during rain-fed season (July–November 2019) at Agricultural Research Station (ARS) Gangavati, Karnataka, India to identify the bacterial leaf blight resistance reaction among 22 medium slender genotypes of rice by both phenotyping and genotyping by using molecular markers linked with resistance governing bacterial leaf blight genes. Alongside TN-1 was used as susceptible check and screening was done by artificial clip inoculation method. The disease reactions were recorded one week after inoculation, with Standard Evaluation Scale (SES) for BLB ranging from 0–9, when the susceptible check (TN-1) was completely killed. None of the genotypes showed a resistant reaction, but three genotypes *viz.* IET-27904, IET-25520 and Rp Bio-226 showed resistant reaction against BLB. The six major BLB resistant genes genetic frequencies varied from 42.85% to 14.28% in the molecular evaluation of promising genotypes for major BLB resistant genes using three Simple Sequence Repeats (SSR) and Sequence Tagged Sites (STS) markers respectively.

KEYWORDS: Bacterial leaf blight, rice, inoculation, markers, resistance, susceptibility

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Rice is a central staple food crop of most of the world's population and is among the three most important food crops, with maize and wheat being the other two. More than 3.5 billion people use rice as their staple food, which translates to at least half of the people worldwide (Gnanamanickam, 2009; Udemezue, 2018; Rajkumar et al., 2022). Due to climatic changes, the increasing temperature increases rice's susceptibility to *Xoo* and also provides favorable conditions for the growth of other pathogens, hence creating considerable challenges for BLB management (Coakley and Scherm, 1999; Garrett et al., 2006; Webb et al., 2010; Yasmin et al., 2017). A previous report showed that rice was severely affected by BLB due to heavy rainfall in tropical areas and particularly in Asia (Banito et al., 2010; Shimono et al., 2011; Yasmin et al., 2017).

Bacterial leaf blight (BLB) caused by *Xoo* is one of the key devastating diseases in rice farming, mostly in tropical Asia (Mizukami et al., 1969). *Xoo* is a vascular pathogen that enters rice leaves through wounds and hydathodes. It enters into xylem vessels after initial multiplication in the epithem, and it further multiplies in xylem tissue, spreads all throughout the leaves, and blocks water transport (Horino et al., 1989). BLB was reported for the first time in Japan during 1884–1885, which then spread and was reported in other rice-growing countries (Gnanamanickam, 2009). Xoo, being the causative agent of BLB, causes a severe loss of rice yield, and the disease it brings is broadly prevalent among diverse genotypes of rice worldwide (Singh et al., 2015).

The *Xanthomonas* genus causes serious bacterial leaf blight in many crops such as cassava and rice via a gram-negative bacterium (Park et al., 2020). In rice, it causes annual yield losses conservatively estimated at 50% (Broman et al., 2005). Bacterial leaf blight (BLB) is a vascular disease that causes a white-yellow or tannish-grey discoloration in the rice crop along the veins, leaf margins, and leaf blades, and these lesions may extend to the sheath (Tanvi et al., 2018; Barakat et al., 2021). Infection at the tillering stage can engender total crop losses (Mew et al., 1993; Busungu, 2017). Developing resistant cultivars is generally regarded as the most effective and economical means of controlling this disease (Mew et al., 1993; Guo et al., 2005; Khush, 2013).

There is some research directed at understanding the principles underlying the interaction between the pathogen and its host, leading to either a compatible or an incompatible disease reaction. When rice is infected by *Xanthomonas oryzae* pv. *oryzae* (Xoo), although the symptoms of the disease may be observed at the tillering stage, the disease may continue to increase as the plant grows (Park et al., 2020). It was observed that rice plants at less than 21 days old are more susceptible to the disease and that the bacteria may favor temperatures at 28–34°C for growth (Li et al., 2014). The improvement of host resistance and the application of chemical and biological measures have been used for the control of BLB (Chen et al., 2012).

It is important to screen rice for bacterial leaf blight resistance in order to assess the diversity within the germplasm and to provide information about resistance/susceptibility for further use in breeding practice (Van et al., 2006). Moreover, screening for varietal resistance based on artificial inoculation may always be conclusive because of to the presence of adequate inoculation initiating the disease (Liang et al., 2017). In the present study, 22 medium slender rice cultivars were observed for their reaction to virulent *Xoo* race.

2. MATERIALS AND METHODS

2.1. Plant materials

A set of 22 medium slender rice genotypes and susceptible check (TN–1) were obtained from AICRIP- Rice Breeding, Agricultural Research Station, Gangavati, Karnataka and were evaluated phenotypically for BLB disease reaction during kharif 2019 in BLB evaluation nursery at Agricultural Research Station (ARS), Gangavati located at latitude of 150 43' N and longitude 760 53' E and an altitude of 406 meters above mean sea level (MSL) and comes under the Northern Dry Zone of Karnataka with the annual rainfall of 523 mm.

2.2. Phenotypic screening for BLB resistance reaction

The Standard layout was followed as per IRRI guidelines using TN-1 as a susceptible check and each entry was planted in two rows of 2m length following standard IRRI method for BLB evaluation. Laboratory grown virulent strain of *X. oryzae* pv. *oryzae* was inoculated by leaf clipping method (Kauffman et al., 1973) to all twenty two entries after thirty days of post transplanting and BLB disease reaction was recorded using 0-9 SES scale (IRRI,2013).

2.3. Disease scoring for BLB

After 25–30 DAS (1 week after inoculation) the test entries were scored based on BLB severity following SES scale (Table 1). Based on the BLB severity, the reactions of the lines are categorized into different categories of resistance and susceptibility (Table 3).

2.4. DNA isolation

Leaf samples were collected from 20–25 days old seedlings and were stored immediately at -20°C till DNA was isolated. Genomic DNA was isolated from fresh, healthy and young leaves from 7 promising rice genotypes following CTAB (Cetyl-Tri Methyl Ammonium Bromide) method (Murray and Thompson, 1980). The quality and quantity of DNA

Table 1: Scoring system for BLB disease resistance (IRRI, 2013)

Grade	Rating	Leaf area infection
1	Highly resistant	15%
3	Resistant	612%
5	Moderately resistant	13-25%
7	susceptible	26-50%
9	Highly susceptible	51-100%

was also analyzed by running the genomic DNA samples on 0.8% agarose gels. This additional step would give us an idea on the extent of DNA shearing.

2.5. PCR and marker analysis

Three SSR and three STS markers each were used for molecular validation of 7 MS rice genotypes for BLB resistance reaction (Table 2). The primer sequences were obtained from www.graminae.org and other previously published research work on BLB resistance genes with their associated markers. The primer sequences were used and the oligos were synthesized from commercial facility (Eurofins, Bengaluru, India). Each polymerase chain reaction (PCR) was conducted in 10 µL reaction volume. The following temperature profiles and cycles were maintained by using a thermal cycler from Applied Biosystems. 1 cycle at 95°C for 5 min (initial denaturation), followed by 34 cycles of at 95°C for 30 sec (Denaturation), annealing at 55-64°C (depending on primers) for 30 sec, extension at 72°C for 1 min, 1 cycle of final extension at 72°C for 10 min, and storage at 4°C. After completion of PCR, products were run on 3% agarose gel, prepared using 1X TE buffer and ethidium bromide. After the completion of the electrophoresis, the DNA profile was documented using gel documentation unit (Essential V6, USA). Gel pictures were scored on the basis of expected bp for resistant allele, as 1 for presence and 0 for absence of resistant allele.

Table 2: Details of markers used for detection of respective R genes for BLB disease in PCR

Gene	Primer name	Marker type	Annealing temperature	Forward (5'-3')	Reverse (3'-5')
Xa4	RM224	SSR	55°C	ATCGATCGATCTTCAC-GAGG	TGCTATAAAAGGCATTCGGG
xa5	xa5S	STS	60°C	AGCTCGC-CATTCAAGTTCTTGAG	TGACTTGGTTCTCCAAGGCTT
Xa7	RM251	SSR	64°C	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC
Xa8	RM263	SSR	57°C	CCCAGGCTAGCTCAT-GAACC	ATGCGGGTTCAAGATTCGATC
Xa13	RG136	STS	60°C	TCCCAGAAAGCTACTA-CAGC	GCAGACTCCAGTTTGACTTC
Xa21	pTA248	STS	62°C	AGACGCGGAAGGGTG-GTTCCCGGA	AGACGCGGTAATCGAAAGAT-GAAA

3. RESULTS AND DISCUSSION

The results of the evaluation are presented in table 3. The Standard layout was followed as per IRRI guidelines using TN-1 as a susceptible check and each entry was planted in two rows of 2m length following standard IRRI method for BLB evaluation. Laboratory grown virulent strain of BLB pathogen *Xoo* was clip inoculated to all twenty-two entries after thirty days of post transplanting as shown in figure1 and BLB disease reaction was evaluated using 0–9 SES scale as per (IRRI, 2013), when the susceptible spreader TN 1 was completely killed.

Among the 22 medium slender genotypes, 3 (17%) were resistant (with a score 3), 3 (14%) were found to be moderately resistant (score of 5), while 11 (50%) were susceptible (score of 7) and 5 (23%) were highly susceptible as shown in figure 2. The observations also note that the range of disease severity varied from lesions infecting 6-12 % (resistant) to more than 51–100% (highly susceptible) of leaf area affected. Similar method was followed by Banito et al. (2012) for screening of twenty-one rice varieties and near isogenic lines against BLB under green house and observed that 12 lines, IRBB1, IRBB2, IRBB3, IRBB4, IRBB7, IRBB8, IRBB10, IRBB11, IRBB13, IRBB14, IRBB21 and IR24, were resistant while four lines, NERICA4, NERICA8, NERICA14 and Giganté, were moderately resistant. The susceptible group also had five genotypes, TGR203, IR841, NERICA19, TOG5681 and IRBB5.

In the present investigation, validation of BLB resistant genes was carried out by using reported molecular markers and the set of markers for the present study were carefully

Table 3: Phenotypic scoring of medium slender genotypes for BLB disease resistance reaction

Sl. No.	Genotypes	Phenotypic score	Disease reaction	Sl. No.	Genotypes	Phenotypic score	Disease reaction
1.	GNV-1905	7	S	12	BPT mutant 1804	7	S
2.	GNV-1906	7	S	13	BPT mutant 1805	9	HS
3.	GNV-1907	5	MR	14	BPT mutant 1806	9	HS
4.	IET-27904	3	R	15	BPT mutant 1809	9	HS
5.	IET-27416	7	S	16	BPT mutant 1811	7	S
6.	IET-27870	7	S	17	RNR - 15048	7	S
7.	IET-26241	5	MR	18	Gangavati sanna	7	S
8.	IET-27438	9	MS	19	Rp-Bio 226	3	R
9.	IET-25520	3	R	20	GNV 10-89	5	MR
10.	BPT mutant 1801	7	S	21	GGV-05-01	7	S
11.	BPT mutant 1802	9	HS	22	BPT-5204	7	S
Susceptible check					TN-1	9	HS

selected after thorough review of literature and experience of their previous usage. These genotypes were screened with six known gene linked markers for BLB resistance genes and the list of the markers for this study are given in table 2.

Scoring of genotypic data was done by counting the numbers of intense bands appeared in the marker banding pattern of test genotypes for different markers associated with specific BLB resistance genes as shown in figure 2. Scoring of genotypic data on the basis of presence or absence of band with BLB specific primers is presented in the table 4.

The rice BLB R gene *Xa* 4 was amplified using the RM224 primer and was visualized by a product of 160-bp and was only detected in one rice variety. The rice BLB R gene *xa*5 yielded a 120-bp fragment when amplified with the *xa*5S primer and was detected in two genotypes. *Xa*7 gene was amplified using the RM251 primer and visualized as an amplicon of 120-bp and was found in three genotypes. *Xa*8 gene was amplified using the RM263 primer and visualized as an amplicon of 170-bp. Three genotypes contained the BLB R gene *Xa8*. *Xa13* was detected with marker RG136 produced amplicon of 530-bp and was detected in three rice genotypes. PCR-based screening of *Xa*21 showed that three genotypes produced band of 1000-bp when amplified with pTA248 primer.

From comparative analysis of genotypic and nursery screening for BLB resistance, it was found that IET-27904, IET25520 and Rp Bio 226 gave resistant reaction in phenotypic screening and has three resistant genes in molecular profiling. Similarly, GNV 10-89 and IET-26241 has one and two resistant genes respectively for BLB

Table 4: Scores of promising genotypes for the presence of BLB resistance genes following genotypic evaluation with markers

Genotypes	Marker, gene name with expected base pair size (bp) for resistance allele						Number of R genes present
	RM224	xa5S	RM251	RM263	RG136	pTA248	
	Xa4	xa5	Xa7	Xa8	Xa13	Xa21	
	160	180	120	170	530	1000	
IET 27904	0	1	1	0	1	0	3
IET 26241	1	0	0	1	0	0	2
GNV 10-89	0	0	1	0	0	0	1
IET 25520	0	0	1	1	1	0	3
Rp Bio 226	0	1	0	0	1	1	3
BPT 5204	0	0	0	1	0	0	1
GNV1907	0	0	0	0	0	0	0
Genetic frequency (%)	14.28	28.57	42.85	28.57	42.85	14.28	

resistance but both showed moderately resistance reaction in the phenotypic scoring. BPT-5204 and GNV-1907 has one and zero resistant genes respectively, both showed susceptible reaction in phenotypic reaction. Similar results were previously reported by Acharya et al. (2018) for sixty genotypes of rice, which were screened at glass house for BLB resistance, among them twenty four genotypes were detected for *Xa4* gene respectively. Twenty-five with *Xa5* gene and fourteen genotypes with *Xa*7 gene and eleven genotypes did not showed presence of any gene.

Avirulent gene in bacteria exhibits the specificity for resistance gene in the rice plant. Some resistance genes are effective only in adult plants, while others are effective at all stages of growth. Some genes confer resistance to a broad spectrum of *Xanthomonas* races, whereas others do so against only one or a few races. This observation could be influenced by particular geographical locations. Disease resistant system is controlled by the developmental control which has been observed in other plant-pathogen systems. Some rice resistance genes are expressed at the adult stage at highest level. *Xa*7 gene shows broad resistance in adult stages of plant while *Xa*21 mediated resistance increases progressively from susceptible juvenile stage to full resistance at the later adult stage (Khush et al., 1977). However, none of the genotypes under this investigation were highly resistant to BLB disease. One of the reasons for these results could be quantitative nature of the disease resistance, which is controlled by many genes. More than 40 R genes were already reported for BLB resistance. Therefore, indicating the possible role of some other R genes.

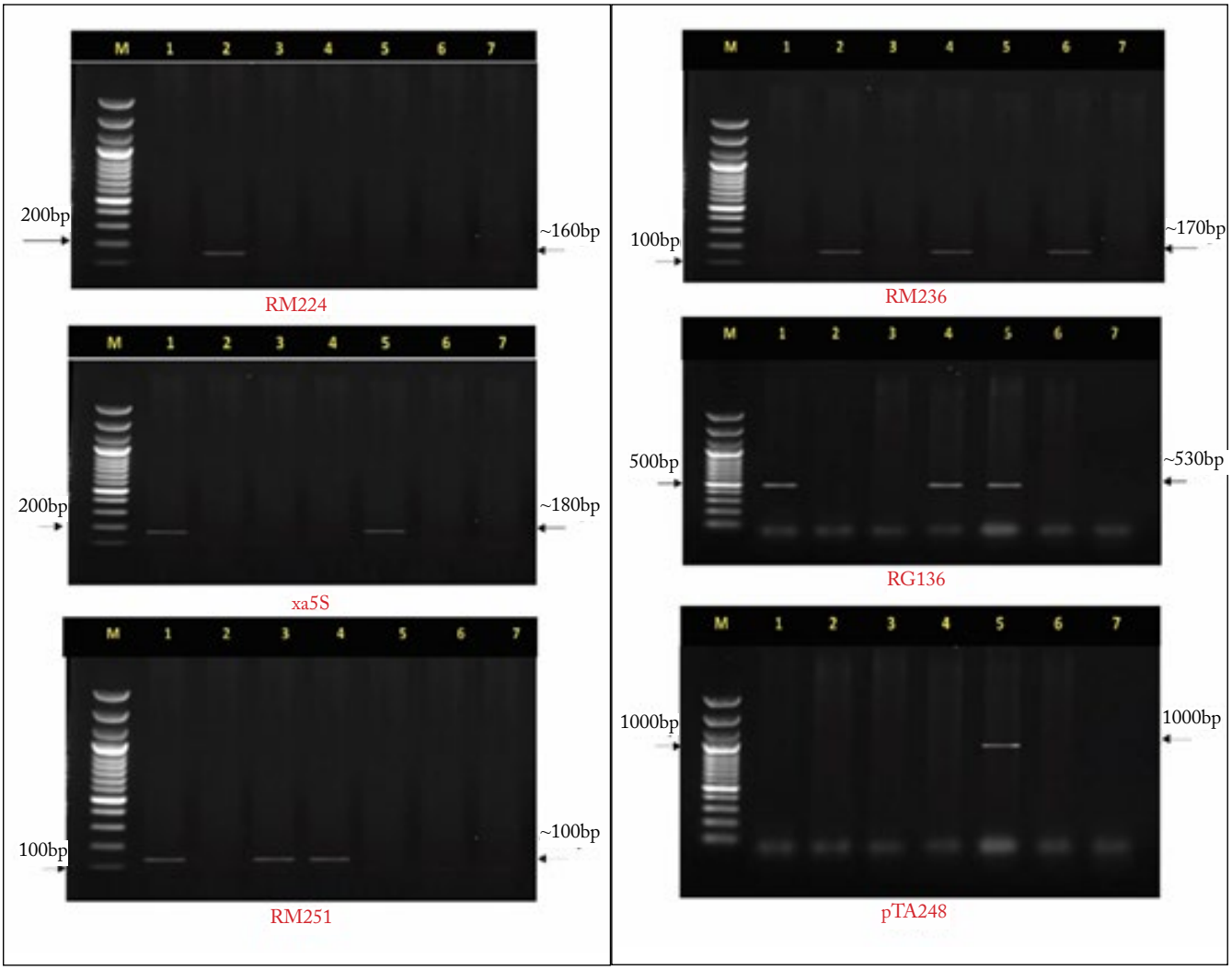

Figure 2: Molecular profiling of promising genotypes resistant to BLB; M-Ladder (100bp), 1-IET 26241, 2- IET27438, 3-GNV 10-89, 4-IET-25520, 5-Rp Bio 226, 6-GGV-05-01, 7-BPT5204

4. CONCLUSION

Rp Bio-226, IET-27904 and IET25520 (three resistant genes) were reported to be resistant, GNV 10-89 (one resistant gene) and IET-26241 (two resistant genes) were reported to be moderately resistant and BPT-5204 (one resistant gene) was reported to be susceptible.

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