Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(5): 2460-2464
Received: 19-07-2018
Accepted: 20-08-2018

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Screening and identification of rice cultivars for BLB resistance at vegetative stage employing gene linked markers

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Abstract
Bacterial leaf blight (BLB) is one of the most devastating diseases in rice accounting for a considerable yield loss globally, posing an untamable threat to food security in near future. To tackle this issue, breeding efforts need to be focused around development of durable rice cultivars that are high yielding as well as pest resilient. The present study was conducted to investigate the potential of 21 rice genotypes including resistant and susceptible checks, against Bacterial leaf blight (BLB) at both genotypic and phenotypic level. BLB resistance genes (*Xa4*, *xa5* and *xa13*), linked markers were employed to determine the allelic status amongst the genotypes. The results showed the frequency of *Xa4* resistant allele (78.95%) and *xa5* alleles (15.79%) in the test rice genotypes but *xa13* resistant allele (00.00%) were not detectable in any of them. At phenotypic level, based on disease severity data, except for the resistant check (IRRBB56) none of the test genotypes were recorded resistant against *Xoo*, although 2 of the genotypes showed moderate resistance due to the presence of two BLB resistant alleles in their genetic background as reflected in their genotypic screening, justifying the fact that a combination of two or more BLB resistant gene is effective against the evolving *Xoo* races. The results of the study could serve as useful information to the breeders for developing BLB resistant cultivars.

Keywords: Bacterial leaf blight, Rice, Gene-linked markers, Disease severity, *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*)

1. Introduction
Rice is one of the major cereal crops that serve as a rich source of carbohydrate for more than half of the world population, next to wheat. It is cultivated in diverse agro-climatic ecosystems, occupying nearly one-fifth of the total arable land area under cereal cultivation (Chakravarthi and Naravaneni, 2006) [4]. However, in recent years, there is a dramatic shift in rice production due to several biotic and abiotic stress factors, those not only reduce yield but also have a negative impact on the livelihood of farmers. One such major biotic threat to rice cultivation is bacterial leaf blight (BLB) or bacterial blight (BB), caused by a gram-negative bacteria *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*). It accounts for typical yield loss ranging from 20-30%, to as much upto 80% in cases of severe infestation based on the stages of crop growth, susceptibility of cultivar, geographic suitability and environmental conditions (Ou, 1985; Akhtar *et al*., 2004; Srinivasan and Gnanamanickam, 2005; Perumalsamy *et al*., 2010) [16, 1, 24, 17].

BB causes potential yield loss in rainfed lowland areas that constitutes around 16 million hectares of rice growing states in India, of which a greater fragment falls under the eastern region of the country, mostly accounts for lower productivity (Ismail *et al*., 2013) [10]. To address this problem, several attempts have been made to identify and characterize BB resistance genes. Till date, 40 bacterial blight resistance genes that confer resistance against various *Xoo* biotypes have been identified (Kim *et al*., 2015) [12]. Most of them successfully tagged with linked DNA markers (Gu *et al*., 2008; Rao *et al*., 2002; Sonti 1998; Yoshimura *et al*., 1995) [8, 20, 25, 28].Though, several management practices such as use of chemicals, dosage of nitrogen and potash administration etc, are also feasible to control BB disease but are not considered 100% effective. So identification and use of resistant cultivars would be most competent, reasonable and environmentally safe alternative for effective management of BB disease (Sidhu *et al*., 1978; Khush *et al*., 1989) [22, 13]. However, long term cultivation of genotypes carrying single resistance gene proves to be ineffective due to mutation in prevalent strains of pathogens. Therefore, pyramiding of more than one major resistance genes into the genetic background of high yielding cultivars seems to be a plausible strategy to combat against the evolving *Xoo* races (Rajpurohit *et al*., 2010) [18]. But achieving the objective of

gene pyramiding through conventional breeding approach is difficult, particularly in cases of recessively inherited resistance genes such as *xa5* and *xa13*.These constraints could be surpassed, with the scientific progress achieved in the field of DNA marker technology that allows rapid and precise identification of plants with multiple BLB resistance genes, that not only confers wide spectrum durable resistance to plants but also minimizes the chances of mutated pathogens to overcome the host resistance ability of cultivars carrying multiple resistance genes. Many a times Polymerase Chain Reaction (PCR) based gene linked and gene specific DNA markers have been employed for determining the allelic status of resistance genes in rice cultivars (Blair and McCouch, 1997) [3], in order to utilize the available information for development of rice cultivars with multiple resistance genes imparting durable resistance. (Perumalsamy *et al*., 2010; Rajpurohit *et al*., 2010) [17, 18]. In the present study, 21 rice genotypes including resistant and susceptible checks were fingerprinted using three gene linked markers (STSs and SSRs) to identify and tag the BLB resistance. The information generated in this study supposed to be useful for future rice improvement programmes.

2. Materials & Methods

2.1 Seed Materials and DNA Extraction

Seeds of twenty one rice genotypes mostly adapted to lowland ecology, including resistant (IRBB56) and susceptible (TN1) checks were obtained from National Hybridization Nursery (NHN) facility at National Rice Research Institute (NRRI), Cuttack, Odisha, India (Table 1). The seeds were sown manually in nursery beds to raise seedlings of each cultivar for further experimental studies. Total genomic DNA of each genotype was isolated from young leaves of 21 days old seedlings using a modified DNA isolation protocol (Dellaporta *et al*., 1983) [5]. The quality of genomic DNA was analyzed by resolving the DNA samples in a 0.8% agarose gel while their concentration was quantified in a spectrophotometer (Eppendorf Make) by measuring A260/A280. The total genomic DNA was further diluted as per suitability with autoclaved double distilled water and stored at 4ºC for PCR purpose.

Table 1: List of genotypes used in the study.

Sl.	Genotype	Parentage	Region of cultivation	Sl.	Genotype	Parentage	Region of cultivation
1	IRBB56	IR24 x BLB resistance gene donor	-	12	Golak	Jhigasail x CN-644	Assam
2	TN1	Dwarf Chow-wu-gen x Tsai-Yuan-Chunj	Punjab	13	Kshira	CR-94-1512-6 x Vijaya	All India
3	Jaya	T(N)1 x T-141	All India	14	Lalat	Obs.677 x IR-207 x Vikram	All India
4	Khitish	BU-1 x CR-115	West Bengal	15	Kanchana	Jajati x Mehsuri	Orissa
5	Tapaswini	Jagannath x Mahsuri	Orissa	16	Jitendra	Selection from land races	West Bengal and Uttar Pradesh
6	Satabdi	CR-10-114 x CR-10115	West Bengal	17	Sonasali	RP-1015-348-85-1 x Sona x Manoharsali	All India
7	Padmini	Mutant selection from the CR-1014 variety	All India	18	Sarala (CR-260-77)	CR-151 x CR-1014	Orissa
8	Sudhir	FR-13A x CNM-539	West Bengal, Assam, Bihar and Uttar Pradesh	19	Konark	Lalat x OR 135-3-4	Orissa
9	Cottondora Sannalu (MTU-1010)	Krishnaveni x IR-64	Andhra Pradesh	20	Khandagiri	Parijat x IR-13429-94-3-2-2	Orissa
10	Pooja	Vijaya x T.141	Andhra Pradesh and Madhya Pradesh	21	Gayatri	Pankaj x Jagannath	All India
11	Samalei	Leuand-152 x IR-8	All India				

2.2 PCR Amplification and Marker Analysis

Amplification of isolated DNA fragments was carried out using previously reported gene linked STS and SSR markers to determine the status of BLB resistance genes in 21 rice cultivars including resistant and susceptible checks. The detailed sequence information of linked markers used in the study is listed (Table 2). DNA amplification was carried out in 15µL PCR reaction mixture containing 30 ng of genomic DNA, 1X PCR buffer, 0.2mM each of dATP, dCTP, dGTP and dTTP (Thermo Scientific; Waltham, MA, USA), 5 pmol of each primer, 2 mM MgCl2 and 1 U of Taq DNA polymerase (Gene aid; USA). The thermal cycler profile involved an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 2-3 °C below the calculated T_m of the respective primers for 1 min, and primer extension at 72 °C for 1 min 30s, followed by a final extension at 72 °C for 7 min.

The PCR products of *Xa4* and *xa5* gene linked markers were resolved in a 3.5% agarose gel using a 50bp DNA marker ladder (BRBLS, India), while *xa13* amplified products were electrophoresed in a 2% agarose gel using 1kb+ DNA marker ladder (BRBLS, India) and visualized by staining with ethidium bromide (0.5 µg/ml) in a gel documentation system (Syngene G:BOX, USA). The PCR products of all DNA samples were analyzed against the resistant and susceptible checks. The DNA bands were then scored for the presence and absence of *Xa4*, *xa5* and *xa13* linked DNA fragments in sample populations.

Table 2: List of Bacterial Leaf Blight (BLB) resistance gene-linked markers used in this study.

Sl.	Gene	Chromosome	Linked Marker	Primer Sequence	Band Size (bp)	Reference
1	Xa4	11	Npb 181 (STS)	F: ATCGATCGATCTTCACGAGG	160	Yoshimura *et al*.(1995) [28]
				R: GTGCTATAAAAGGCATTCGGG		
2	xa5	5	RM 122 (SSR)	F:GAGTCGATGTAATGTCATCAGTGC	240	Mc Couch *et al.* (1996) [14]
				R:GAAGGAGGTATCGCTTTGTTGGAC		
3	xa13	8	13 Prom (STS)	F: GGCCATGGCTCAGTGTTTAT	500	S. K. Hajira *et al.* (2016) [9]
				R: GAGCTCCAGCTCTCCAAATG		

2.3 Bioassay

The *Xoo* isolate used for inoculating the rice cultivars was obtained from Crop Protection Division, NRRI, Cuttack. The bacteria were first sub-cultured on a peptone sucrose agar medium at a temperature of 30ºC maintained for nearly 72 hrs. A bacterial suspension was prepared (distill water: 1 L, sucrose: 20 g, peptone: 5 g, K2HPO4: 0.5 g, MgSO4.7H2O: 0.25 g, agar: 15 g) by mixing the cultured bacteria with 10 ml sterile distill water in a slant, maintained at a pH 7.2–7.4 (Fahy and Persley, 1983) [6], adjusting to a final concentration of 10^8 colony forming units (CFU)/mL (optical density = 0.2 at 600 nm).

Clip inoculation method (Jennings *et al*., 1979) [11] was used to inoculate bacterial suspension into the leaves of 45 days old rice plants, under induced epiphytotic condition. Five plants of each cultivar at maximum tillering stage were inoculated with *Xoo* by cutting their leaves using scissors dipped in the bacterial inoculum. Fourteen days post inoculation, the disease severity was recorded as resistant (R, LL ≤ 3.0 cm), moderately resistant (MR, LL 3.0 cm ≤ 6.0 cm), moderately susceptible (MS, LL 6.0 cm ≤ 9.0 cm), or susceptible(S, LL > 9.0 cm) (Amante-Bordeos *et al*. 1992) [2], by measuring the bacterial lesions formed on the leaf surface. Percentage disease incidence was calculated with the help of the formula given by Gnanamanickam *et al*. (1999) [7]. Microsoft excel software was used for estimation of the standard error for lesion length measurement of all cultivars.

3. Results & Discussion

3.1 Genotyping for BLB Resistance

Twenty one rice genotypes including the resistant (IRBB56) and susceptible (TN1) checks as gene differential lines were screened to determine the allelic status of BLB resistance genes *Xa4, xa5* and *xa13*, using previously reported PCR based gene-linked STS and SSR markers viz., Npb 181, RM 122 and 13-prom, respectively.The PCR products of *Xa4, xa5* and *xa13* gene-linked markers upon electrophoresis revealed amplicons of sizes 160bp, 240bp and 498bp DNA fragments in resistant check (positive). The genotyping data of 21 rice genotypes are shown in Table 3, while their electrophoregram for BLB resistance gene-linked markers are presented in Figure 1. The PCR products of *Xa4* (Npb 181) and *xa5* (RM 122) markers upon electrophoresis in 3.5% agarose gel showed DNA amplicons (bands) basically of size 160bp for Npb 181 and 240bp for RM 122 that coexists with resistant check. For Npb 181, fifteen genotypes (78.95%) displayed 160bp homologous band equivalents to resistant check (IRBB56), while four of them (21.05%) along with susceptible check (TN1) showed a 140bp homologous fragment. While in case of RM 122 marker, the electrophoregram displayed homology of approximately 240bp in three genotypes (15.79%), identical to resistant check while sixteen genotypes (84.21%) displayed 220bp homologous fragments similar to susceptible check. Likewise, gene linked STS markers for *xa13* (13-prom) was used to analyze for presence of either resistant or susceptible alleles in 21 genotypes. After PCR amplification, the electrophoretic pattern of 13- prom marker, revealed 280bp homologous bands in all the 19 test genotypes (100%), identical to susceptible (TN1) check, whereas the resistant check (IRBB56) carried a 498bp homologous fragment (approximately 500bp), which was missing in all cultivars studied.

Table 3: Genotyping and Phenotyping data for BLB resistance in 21 genotypes.

Sl.	Genotypes	BLB Resistance Genes			MLL ± SD (Severity %)	Host Response
		Xa4	*xa5*	*xa13*	14DAI	
BLB Checks	IRBB56 (R)	R	R	R	3.39 ± 1.03	R
	TN1 (S)	S	S	S	16.59 ± 1.14	S
1	Jaya	S	S	S	13.93 ± 1.98	S
2	Khitish	R	S	S	12.67 ± 1.75	S
3	Tapaswini	R	R	S	5.99 ± 1.23	MR
4	Satabdi	R	S	S	10.44 ± 1.93	S
5	Padmini	S	S	S	14.16 ± 1.37	S
6	Sudhir	R	S	S	10.17 ± 1.20	S
7	Cottondora Sannalu (MTU-1010)	R	S	S	11.69 ± 1.58	S
8	Pooja	R	S	S	9.55 ± 1.74	S
9	Samalei	R	S	S	14.02 ± 2.83	S
10	Golak	R	S	S	9.89 ± 1.51	S
11	Kshira	R	S	S	10.33 ± 1.26	S
12	Lalat	R	S	S	6.76 ± 1.19	S
13	Kanchana	R	S	S	15.12 ± 1.62	S
14	Jitendra	R	S	S	9.81 ± 1.23	S
15	Sonasali	S	S	S	12.24 ± 1.34	S
16	Sarala	S	R	S	8.47± 1.29	S
17	Konark	R	R	S	5.86 ± 1.16	MR
18	Khandagiri	R	S	S	9.54 ± 1.05	S
19	Gayatri	R	S	S	11.87 ± 1.13	S

Frequency %	78.95	15.79	00.00	

R= allele equivalent to resistant parent allele, S = allele equivalent to susceptible parent allele.
MR= moderate resistance, S= susceptible.

The polymorphic survey of 21 genotypes revealed the existence of *Xa4* specific allele in majority of genotypes similar to the resistant (IRBB56) check, followed by *xa5* resistant allele that were detectable in fewer genotypes as compared to *Xa4* gene, which has widely been implicated in rice breeding programmes in many Asian countries and globally as well. This gene has been reported to have a durable resistance in many commercial rice cultivars (Mew *et al*., 1992; Sun *et al*., 2003) [15, 26]. However repeated cultivation of these cultivars having single gene resistance has recently led to breakdown of host resistance due to evolution of Xoo pathotypes. Similar findings are reported in our bioassay studies, where the cultivars carrying single *Xa4* specific allele failed to reflect host resistance against *Xoo* infestation. Furthermore, out of 21 cultivars, no amplicons specific to *xa13* alleles were detected, showing the absence of this gene in all them except resistant (IRBB56) check. Similar to our findings, Singh *et al.* (2012) [23] reported presence of *Xa4* specific allele in 29 landraces out of 42 surveyed, with none of them confirming the presence of *xa13.*

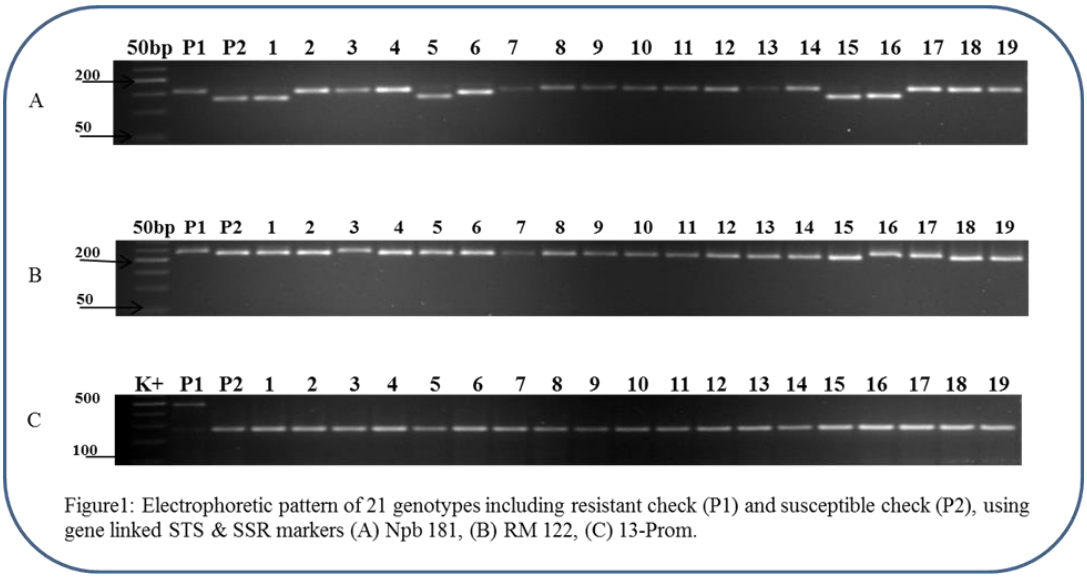

Figure1: Electrophoretic pattern of 21 genotypes including resistant check (P1) and susceptible check (P2), using gene linked STS & SSR markers (A) Npb 181, (B) RM 122, (C) 13-Prom.

3.2 Phenotyping for BLB Resistance

Twenty one genotypes including resistant and susceptible check were inoculated with an active strain of *Xoo* under artificial epiphytotic conditions to test for resistance and susceptible status of the cultivars at morphological level as well. Significant difference in disease severity was observed amongst all the cultivars. The results of bioassay are presented in Table 3. Based on the average mean lesion length recorded at 14 days after inoculation, two cultivars namely Tapaswini and Konark displayed moderate resistance, probably due to the presence of two resistant allele *Xa4* and *xa5*, the same as found in resistant genotype, 17 cultivars however were susceptibile against *Xoo* even though *Xa4* resistant allele was confirmed in their genetic background during molecular screening using *Xa4* gene linked marker. This further strengthens the fact that cultivars carrying single resistance gene are no longer a source of durable resistance against the evolving *Xo*o pathotypes. None of the cultivars under investigation, except for resistant check was found resistant, although majority of these cultivars had one or more BLB resistant genes either singly or a combination of two different genes. Similar findings of varying cultivar response with different resistant gene combination towards different *Xoo* strains have been reported (Ram *et al*., 2011; Thimmegowda *et al*., 2011; Sharma and Pandey, 2012) [19, 27, 21].

4. Conclusion

The present study revealed the presence of allelic fragments corresponding to BLB resistant genes viz., *Xa4* and *xa5*, present in different gene combinations in majority of rice cultivars studied, recording varying degree of host response from moderately resistant to susceptible, against *Xoo* isolate. Noticeably, Jaya and Padmini were the only cultivars whose phenotypic response (susceptible) against *Xoo* was in coherence with molecular data. The major concern for research community is to guarantee sufficient rice production in response to the growing world population employing efficient, environment friendly approach supposedly more climate and pest resilient. The results of the present study could be exploited as suggestive in future rice breeding programmes for development of durable rice cultivars in order to ensure future food security.

5. Acknowledgments

This research work was financial aided under Maulana azad national fellowship (MANF) for PhD funded by Ministry of Minority Affairs, co-ordinated by UGC. The authors express their gratitude to Director, ICAR-NRRI for providing the necessary field and laboratory facilities for carrying out the work and profoundly acknowledge Dr. M.K. Kar (Principal Scientist, Crop Improvement Division, NRRI) for providing seed materials for this study and also Dr. Arup Mukherjee (Principal Scientist, Crop Protection Division, NRRI) for providing bacterial inoculums.

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