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Morpho-Molecular Screening for Bacterial Leaf Blight Resistance in Some Rice Lines and Varieties

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Abstract: Bacterial leaf blight (BLB) is one of the major and oldest diseases of rice. The best cost effective management of this disease is the development of resistant cultivars. Morphological and molecular screening are widely used approach in the recent scenario for identifying BLB resistant cultivar. In present study, two experiments were carried out to identify the BLB resistant rice lines and varieties. An active strain of *Xanthomonas oryzae pv. oryaze (Xoo.)* BXO-09 was used for the inoculation experiments. Then, morphological rating was done to find out the resistant rice lines and varieties whereas three RFLP primers (RG136, RG556 and pTA248) were used for identifying the BLB resistant genes. Through morphological analysis, the rice line RC251 was recorded as susceptible where the rest of the rice lines resulted moderately susceptible or moderately resistant. Among the rice varieties, BR-26, BRRI Dhan31, IRBB5, IRBB21, IRBB60, IRBB65, and Kumragur were recorded as resistant where BR-11, Binadhan-8, and Binadhan-10 were found highly susceptible to BLB. In case of RFLP marker based analysis, a specific type of banding pattern was observed for IRBB5 for RG556 primer which indicated the presence of *xa5* gene. For RG136 primer 8 kb single band was formed for different lines and varieties whereas only IRBB60 showed 2 fragments of 8 kb and 5 kb which also indicated the presence of *xa13* gene. Finally, DNA amplification of 27 rice lines and varieties using pTA248 primer showed the band at 4 kb position for the BLB resistant lines and varieties and 3.7 kb position for the susceptible lines and varieties. The findings of morpho-molecular characterization information could be useful to the breeders for further planning of developing BLB resistant cultivars.

Keywords: Bacterial Leaf Blight (BLB), Restriction Fragment Length Polymorphism (RFLP), Resistance

1. Introduction

Rice bacterial leaf blight caused by *Xanthomonas oryzae pv. oryzae (Xoo.)* is one of the oldest as well as major diseases of rice. It is observed almost in every rice field of the world specially where there is irrigated and rainfed lowland [1]. Affecting panicle emergence and grain filling, this disease can reduce grain production by 20-50% and yield by 25% [2]. Eventually, for susceptible variety, this severity of yield loss can go up to 70% [3].

Due to its high damage to the rice field, a lot of studies and observations has already been done for controlling this disease but effective control measures are yet to be found. So, besides cultural practices, chemical control, biological control, disease forecasting, use of resistant cultivar is the best cost-effective approach for controlling this disease [4, 5]. Moreover, pyramided lines carrying two, three or four bacterial blight resistance genes showed broad spectrum and higher resistance than the lines with single resistance gene [6-8]. A total of 40 BLB resistance genes (R genes) has been

identified in cultivated rice and the wild relatives [9].

Molecular markers can be used to identify and pyramid favorable and multiple alleles for biotic and abiotic stress resistance in a collection of diverse genotypes [10-13]. Among different types of molecular markers Restriction Fragment Length Polymorphism (RFLP) markers are used in broad spectrum for detecting bacterial leaf blight resistant gene. RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. This type of molecular marker is closely linked to resistance genes present in BLB resistant variety and detecting BLB resistance genes present in different rice cultivars this marker is widely used [14-16].

In the present study, with the aim of finding out the resistant rice lines and varieties ten IRRI advanced rice lines and seventeen rice varieties were inoculated using an active strain of *Xoo.* (BXO-09). Morphological rating was carried out for finding out the level of resistance among these rice lines and varieties whereas DNA amplification was done by three RFLP primers (RG136, RG556 and pTA248) for identifying the BLB resistance genes. These results can contribute significantly towards developing new BLB resistant rice varieties and can be useful for breeders for further marker-assisted selection and backcrossing.

2. Materials and Methods

2.1. Morphological Screening for BLB Resistance Rice Lines and Varieties

2.1.1. Experimental Site

The Experiment was conducted at the experimental field and Laboratory of Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA).

2.1.2. Collection of Test Materials

Ten International Rice Research Institute (IRRI) advanced lines and seventeen varieties of rice were collected from BINA, BRRI, and Gazipur district of Bangladesh (Table 1). The variety IRBB21 and BR-11 were used as respectively the resistant and susceptible cultivars for check [8, 14, 17].

Table 1. *List of lines and varieties of rice used for the study.*

Type	Name of the test material	Source of Collection
Line	RC191, RC192, RC193, RC217, RC221, RC222, RC225, RC229, RC251, RC249	BINA
Variety	BR-10, BR-11, BR-14, BR-16, BR-26, BRRI Dhan31, BRRI Dhan32, IRBB 5, IRBB 21, IRBB 60, IRBB 65	BRRI
Variety	Binadhan-7, Binadhan-8, Binadhan-10, Binadhan-11, Binadhan-12	BINA
Variety	Kumragur	Gazipur

BINA= Bangladesh Institute of Nuclear Agriculture; BRRI = Bangladesh Rice Research Institute.

2.1.3. Preparation of Bacterial Inocula

The strain responsible for BLB disease BXO-09 collected from BRRI is regarded as the most virulent strain among all 41 types of isolates in Bangladesh [18]. This isolate was maintained in slants containing peptone sucrose agar (PSA) medium. After growing of *Xoo.* on PSA media for 72 hours at 30°C the inoculum was prepared by mixing the cultured bacteria with 10 ml sterile distilled water in a slant. The concentration of bacterial suspension was adjusted to 108 CFU/ml prior to inoculation using sterile distilled water.

2.1.4. Inoculation of Rice Plant by Active Strain of BXO-09

Inoculation of rice seedlings by active strain of *Xoo.* done under induced epiphytotic condition by following clipping method [19]. To prepare the rice plant for inoculation, they were measured to see if they have reached five inches in length. The instrument used to inoculate the rice plant with the bacteria inocula was scissor and before using the scissor it was sterilized using 70% ethanol. The scissor was dipped into the bacterial suspension and used to cut the leaf of rice plant (Fig. 1). After 21 days, data was taken for measurement of BLB.

Figure 1. *Inoculation of rice plant by active strain of BXO-09.*

2.1.5. Morphological Rating

Percent diseased leaf area for each inoculated seedlings was measured by 1-9 scale and was scored to rate the resistance capacity of rice plants to bacterial leaf blight according to the IRRI BLB scoring method mentioned in Table 2.

Table 2. *Scale for rating BLB resistant lines and varieties.*

Scale	Diseased Leaf Area (%)	Description
1	1-5	Resistance (R)
3	6-12	Moderately Resistance (MR)
5	13-25	Moderately Susceptible (MS)
7	26-50	Susceptible (S)
9	>50	Highly Susceptible (HS)

2.2. Molecular Screening for BLB Resistance Rice Lines and Varieties

2.2.1. STS Marker Analysis for xa5 and xa13 Gene Identification

The sequence tagged site (STS) primers RG556 and RG136 (Table 3) were used to detect *xa5* and *xa13* gene, respectively. After DNA extraction and PCR amplification, 10 µl of each amplified products were resolved by electrophoresis on a 1.5% agarose gel in 0.5x TBE buffer to determine whether PCR amplification was successful. When the combinations of said primers were used to amplify the DNA of the lines and varieties, PCR products were monomorphic. Therefore restriction enzymes were used to generate SAP fragment following the method described earlier by Williams [20].

The reaction mixtures consisted of 3.2 µl sterile distilled water, 1.5 µl restriction buffer (10x), 0.3 µl restriction enzyme (10unit/µl) and 10 µl of PCR product. *Dral* restriction enzyme was used for restriction digestion of RG556 and *hinf*1 was used for RG136. The reaction mixture was incubated for 6 h at 37°C. The DNA fragment produced by restriction digestion were separated by gel electrophoresis (1.5% agarose) and visualized under UV light after staining with ethidium bromide.

Table 3. *Primers used for marker assisted selection for BLB.*

Primer Name	Primer sequence		Linked Gene	Reference
RG136	For	TCCCAGAAAGCTACTACAGC	*xa13*	[14]
	Rev	GCAGACTCCAGTTTGACTTC		
RG556	For	TAGCTGCTGCCGTGCTGTGC	*xa5*	[21]
	Rev	AATATTTCAGTGTGCATCTC		
pTA248	For	AGACGCGGAAGGGTGGTTCCCGGA	*Xa21*	[14]
	Rev	AGACGCGGTAATCGAAAGATGAAA		

2.2.2. PTA248 Primer Analysis for Xa21 Gene Identification

Xa21 forward and reverse primers were used for the detection of *Xa21* gene. Each PCR reaction mixture and electrophoresis condition was followed the same method as RG556 and RG136 primers. The amplification profile consisted of an initial denaturation for 5 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec and primer extension for 1min at 72°C. A final 5 min extension at 72°C was allowed for primer extension. The PCR products were mixed with 5 µl of 10x loading dye (bromophenol) and run in a 1.5% agarose gel in 0.5x TBE buffer of check for amplification and polymorphism and visualized under UV light after staining with ethidium bromide.

3. Results

3.1. Morphological Screening for BLB Resistance of Test Materials

Morphological screening for the bacterial leaf blight resistance of ten IRRI advanced rice lines and seventeen rice varieties was done at seedling stage of the inoculated plant using an active strain of *Xoo.* (BXO-09) following clipping method. There was no rice line recorded as resistant to BLB. The lines RC191, RC192 and RC225 were recorded as moderately resistant (Fig. 2). Only the line RC251 was recorded as susceptible and rest of the lines resulted moderately susceptibility (Table 4).

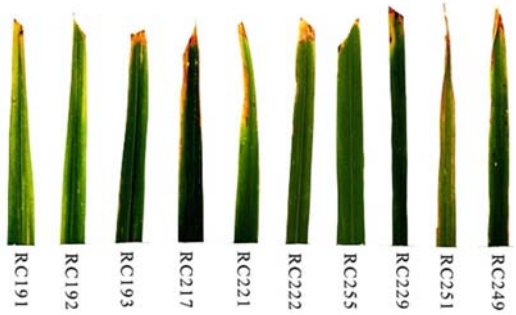

Figure 2. *Morphological screening for BLB resistance of ten IRRI advanced rice lines.*

Table 4. *Morphological screening for BLB resistance of ten IRRI advanced rice lines.*

Line	% diseased leaf area	Remarks
RC191	9.667 ef	MR
RC192	7.667 f	MR
RC193	15.00 cd	MS
RC217	19.33 b	MS
RC221	16.33 bcd	MS
RC222	16.67 bcd	MS
RC225	6.333 f	MR
RC229	13.33 de	MS
RC251	32.33 a	S
RC249	17.67 bc	MS
LSD(0.05)	3.81	-
Level of significance	**	-

Among the rice varieties BR-16, BR-26, IRBB5, IRBB21, IRBB60, IRBB65 and Kumragur were recorded as resistant (Fig 3). The varieties BRRI Dhan32 and BR-14 were found as moderately resistant to BLB whereas BR-10, BR-16, Binadhan-7, Binadhan-11 and Binadhan-12 were found as moderately susceptible to BLB (Table 5). Among all the rice varieties BR-11, Binadhan-8 and Binadhan-10 were recorded as highly susceptible to BLB.

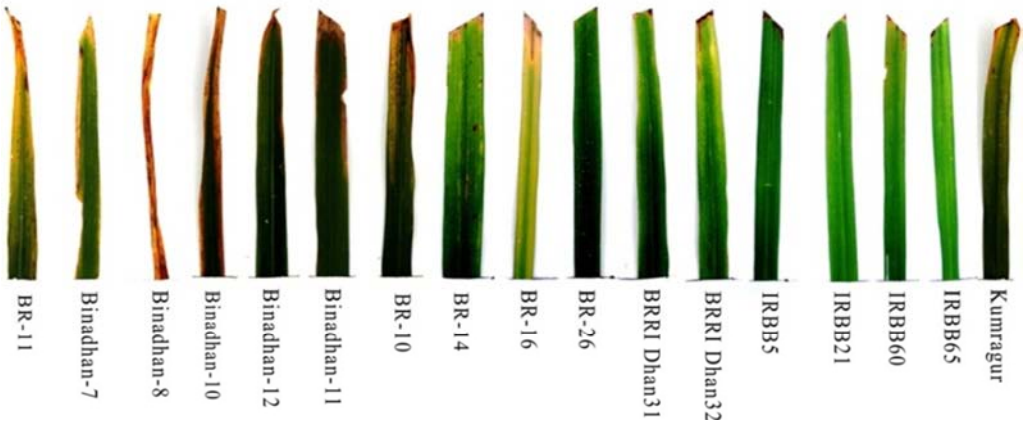

Figure 3. *Morphological screening for BLB resistance of seventeen rice varieties.*

Table 5. *Morphological screening for BLB resistance of seventeen rice varieties.*

Variety	% diseased leaf area	Remarks
BR-11	59.67 b	HS
Binadhan-7	20.67 c	MS
Binadhan-8	71.67 a	HS
Binadhan-10	65.00 ab	HS
Binadhan-12	14.33 cd	MS
Binadhan-11	20.67 c	MS
BR-10	20.00 c	MS
BR-14	6.333 e	MR
BR-16	19.67 c	MS
BR-26	2.333 e	R
BRRI Dhan31	2.667 e	R
BRRI Dhan32	8.00 de	MR
IRBB5	4.00 e	R
IRBB21	1.667 e	R
IRBB60	3.667 e	R
IRBB65	4.00 e	R
Kumragur	2.667 e	R
LSD(0.05)	7.28	-
Level of significance	**	-

The regression studies between affectivity of the pathogen (virulence) and infection reaction level leaf area diseased (level of resistance) for ten rice lines are shown in Fig. 4. It is revealed that there is highly significant relationship between them with the regression equation, y = 5.4901x – 9.8216 with the significance value of r = 0.942**. The results indicate that affectivity of virulence of the pathogen was as much as on the lines RC191, RC192, and RC225 which conferred moderately resistance as their better genetic potentiality than that of other lines showing less genetic potentiality of resistance and less virulence potentiality of the pathogen.

For the varieties, the regression studies between affectivity of the pathogen (virulence) and infection reaction level of leaf area diseased (level of resistance) is shown in Fig. 5. It is revealed that there is highly significant relationship between them with the regression equation, y = 7.3192x – 8.7491 with the significance value of r = 0.946**. The results indicate that affectivity of virulence of the pathogen was as much as on the varieties IRBB21, IRBB65, and Kumragur which conferred resistance as their better genetic potentiality than that of other varieties showing less genetic potentiality of resistance and less virulence potentiality of the pathogen.

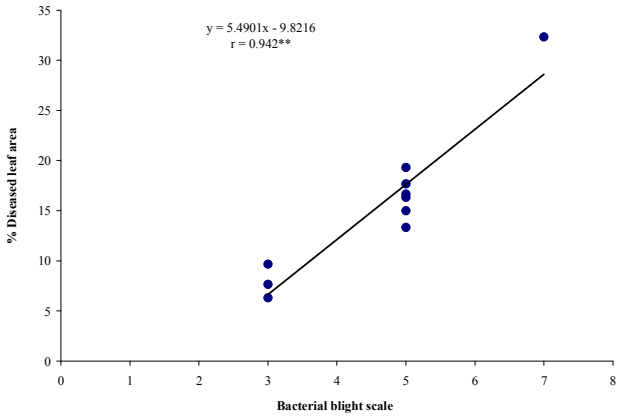

Figure 4. *Regression between virulence of the pathogen and level of resistance in ten rice lines.*

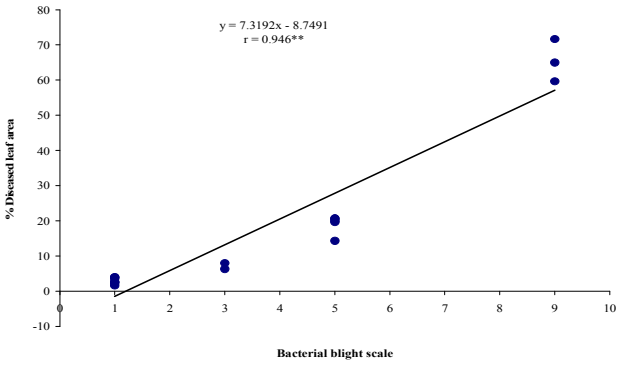

Figure 5. *Regression between virulence of the pathogen and level of resistance in rice varieties.*

3.2. Molecular Screening for BLB Resistance of Test Materials

3.2.1. Xa5 and xa13 Linked Markers Analysis for BLB Resistant Line Detection

RG556 and RG136 STS markers generated from RFLP markers are closely linked to *xa5* and *xa13*, respectively [14]. BLB resistance along with the presence of *xa5* and *xa13* gene in the following rice lines and varieties were determined through PCR analysis.

PCR markers designed for RG556 is linked to *xa5* BLB resistant gene. Amplification of genomic DNA from ten rice lines and seventeen rice varieties with RG556 did not produce any polymorphism. The PCR products were therefore digested with *DraI* endonuclease restriction enzyme. Four distinct fragments (8 kb, 6 kb, 5 kb and 3 kb) were produced after digestion (Fig. 6). Exclude Binadhan-7, all the varieties and lines produced fragments at 8 kb and 6 kb position.

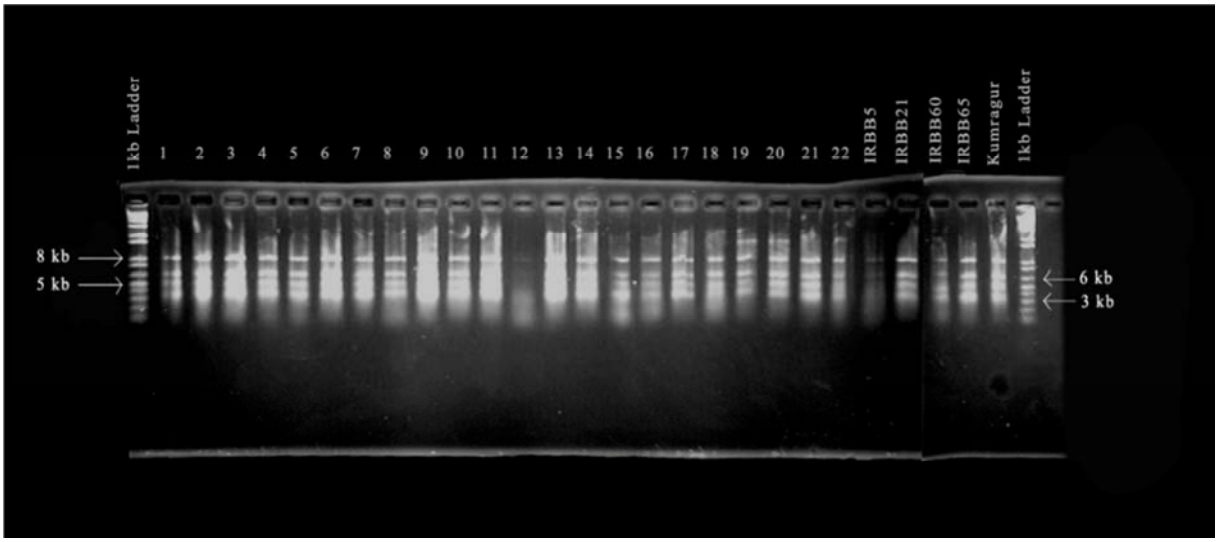

Figure 6. *Marker-Assisted Selection for bacterial blight resistance rice by RG556. Here, 1-RC191, 2-RC192, 3-RC193, 4-RC217, 5-RC221, 6-RC222, 7-RC225, 8-RC229, 9-RC251, 10-RC249, 11-BR11, 12- Binadhan-7, 13- Binadhan-8, 14- Binadhan-10, 15- Binadhan-12, 16- Binadhan- 11, 17-BR-10, 18-BR-14, 19-BR-16, 20-BR-26, 21-BRRI Dhan31 and 22-BRRI Dhan32.*

STS marker RG136 generated from RFLP marker linked to the recessive resistant BLB gene *xa13* amplified a monomorphic product of 1100 bp in all the genotypes studied. Digestion of the PCR product with the restriction enzyme *Hinf*I generated two distinct bands. 8 kb single band was formed for different lines and varieties (Fig. 7) whereas IRBB60 showed 2 fragments of 8 kb and 5 kb.

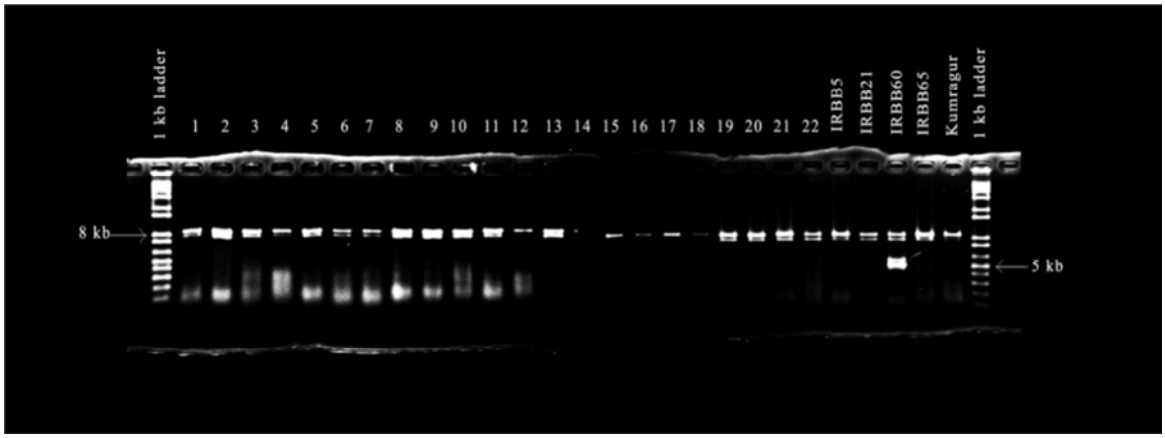

Figure 7. *Marker-Assisted Selection for bacterial blight resistance rice by RG136. Here, 1-RC191, 2-RC192, 3-RC193, 4-RC217, 5-RC221, 6-RC222, 7-RC225, 8-RC229, 9-RC251, 10-RC249, 11-BR-11, 12- Binadhan-7, 13- Binadhan-8, 14- Binadhan-10, 15- Binadhan-12, 16- Binadhan-11, 17-BR-10, 18-BR-14, 19-BR-16, 20-BR-26, 21-BRRI Dhan31, and 22-BRRI Dhan32.*

3.2.2. Xa21 Linked Marker Analysis for BLB Resistant Line Detection

Xa21 is the 1st resistant gene in rice that was cloned in 1996 [22] . DNA amplification of 27 rice lines and varieties was done using pTA248 primer linked to BLB resistant gene *Xa21*. The amplified product gave band at 4 kb position for BLB resistant lines and varieties and 3.7 kb position for the susceptible line and varieties. Along with IRBB21, IRBB60 and IRBB65 gave band at 4 kb position (Fig. 8).

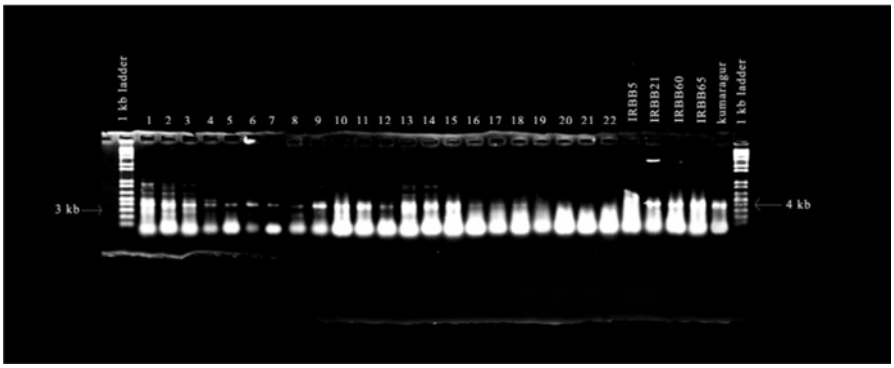

Figure 8. *Marker-Assisted Selection for bacterial blight resistance rice by pTA248. Here, 1-RC191, 2-RC192, 3-RC193, 4-RC217, 5-RC221, 6-RC222, 7-RC225, 8-RC229, 9-RC251, 10-RC249, 11-BR-11, 12- Binadhan-7, 13- Binadhan-8, 14- Binadhan-10, 15- Binadhan-12, 16- Binadhan-11, 17-BR-10, 18-BR-14, 19-BR-16, 20-BR-26, 21-BRRI Dhan31 and 22-BRRI Dhan32.*

4. Discussion

The morphological screening of ten IRRI advanced rice lines and seventeen rice varieties against the bacterial leaf blight pathogen as regards to the genetic potentiality of resistance has been determined. There was no rice line recorded as resistant to BLB pathogen in the tested lines. Statistically the lines RC191, RC192, and RC225 were recorded as moderately resistant. Only the line RC251 was recorded as susceptible and rest of the lines resulted moderately susceptible.

Again, morphological screening of seventeen rice varieties against the bacterial leaf blight pathogen as regards to the genetic potentiality of resistance performed highly significant result. BR-16, BR-26, IRBB5, IRBB21, IRBB60, IRBB65, and kumragur were recorded as resistant while the varieties BRRI Dhan32 and BR-14 were found as moderately resistant to BLB. BR-10, BR-16, Binadhan-7, Binadhan-11 and Binadhan-12 were found as moderately susceptible to BLB while BR-11, Binadhan-8 and Binadhan-10 were recorded as highly susceptible to BLB.

Almost the similar findings have been reported earlier [17] where BR-14, BR-16, BRRI Dhan31, and BRRI Dhan32 was found as moderately resistant and BR-11 as highly susceptible BLB rice varieties. But the strain BXO-09 (*Xoo.*) responsible for bacterial leaf blight disease which was used for inoculation in the present study was proved as the most virulent strain among all 41 types of isolates in Bangladesh except IRBB21 [18]. IRBB21 performed as resistant cultivar against BLB inocula BXO-09. Also, IRBB21 was found as resistant cultivar against one hundred and fifty-three isolates of *Xoo.* collected from Guilan province in Iran [23].

Amplification of genomic DNA from ten rice lines and seventeen rice varieties with RG556 produced a specific type of banding pattern. The absence of band in Binadhan-7 represents its susceptibility to BLB. All other lines and varieties showed almost the same banding pattern as IRBB5 which represents resistance in response to RG556 for BLB. Four distinct bands for the STS marker RG556 for BLB resistant lines was reported [14] which represent similar result as the present study. To the contrary, a different finding was reported in 2012 [16] where amplification of DNA genomic was made from 166 local accessions and 25 parents of hybrid rice but no polymorphism for RG556 was obtained.

For STS marker 136, considering the fragment of 8 kb, Binadhan-7, Binadhan-8, Binadhan-10, Binadhan-12, Binadhan-11, BR-10, and BR-14 were identified as susceptible. RC191, RC217, RC222 and RC225 rice lines were resulted as moderately resistant and RC192, RC193, RC229, RC251, RC249, BR-16, BR-26, BRRI Dhan31 and BRRI Dhan32 were resistant. IRBB60 showed two fragments of 8 kb and 5 kb which were recorded to perform more resistance. 5 kb band also indicated the presence of resistant *xa13* gene in IRBB60 BLB resistant rice variety.

In 2012, 3 fragments of approximately 520, 450, and 130 bp in the line IRBB13 was found by a research group after digested the PCR product with the restriction enzyme *Hin*fI which indicated the presence of *xa13* gene [16]. Also, another research group reported three fragments of approximately, 520, 450 and 130 bp for resistant and two fragments of 970 bp and 130 bp for susceptible variety for BLB [15].

For *Xa21* linked marker analysis for BLB resistant line detection, amplification was made using marker pTA248. Along with IRBB21, IRBB60 and IRBB65 gave band at 4 kb position which indicated the presence of resistant *Xa21* gene, therefore was regarded as completely resistant variety. RC193, RC251, and RC249 lines gave band at 3.7 kb and were regarded as susceptible lines. BR- 11, Binadhan-8, Binadhan-10, and Binadhan-12 also gave band at 3.7 kb position, so these varieties were also predicted as susceptible to BLB. Other than this, RC191 and RC192 gave band near to the position of 4 kb and therefore, be concluded as moderately resistant lines while in 2010 another research group found the fragment for STS markers pTA248 amplified product for resistant varieties at 1,000 bp whereas the susceptible varieties generated band at 650 bp position [15].

5. Conclusion

From the above findings from both morphological and molecular, it may be concluded that among ten rice lines RC191, RC192, and RC225 were moderately resistant whereas RC193, RC217, RC221, RC222, RC229, RC 251, and RC249 were moderately susceptible to BLB. Among the rice varieties, BR-26, BRRI Dhan31, IRBB5, IRBB21, IRBB60, IRBB65, and Kumragur were resistant, whereas BR-14 and BRRI Dhan32 was moderately resistant. BR-10, BR-16, Binadhan-7, Binadhan-11, and Binadhan-12 were moderately susceptible and BR-11, Binadhan-8, and Binadhan-10 were found highly susceptible to Bacterial leaf blight disease. From the above summary of the study, the following recommendations can be made;

i. IRBB5, IRBB21, IRBB60, IRBB65, Kumragur, BR-16, and BR-26 could be utilized to develop BLB resistant rice varieties with desirable characters using marker-assisted selection and backcrossing.
ii. The rice lines RC191, RC192, and RC225 which were resulted moderately resistant could be tested further.
iii. To improve grain quality and bacterial leaf blight resistance, this molecular characterization information could be helpful to the breeders for further planning of rice breeding program

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