Detection of blast resistance gene(s) in some rice genotypes using molecular markers and pathogenicity assessment

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Abstract

Blast is one of the most devastating rice diseases in Bangladesh and the pathogen of blast is Magnaporthe oryzae. In this work, employing four molecular markers, namely RM276, RM403, RM 302 and RM 155, an effort was made to identify seventy-nine rice genotypes for four important blast-resistant genes, Pi9, Pita, Pish and Pita-2. Screening was done by the Plant Pathology Division, BINA and the Department of Plant Pathology, BAU. Findings indicated that just three genotypes contained the rare Pita-2 gene, while 54 genotypes carried the Pi9 gene, 44 Pita gene, 23 Pish gene and so on. Pi9 was the most common resistance gene, with genetic frequencies ranging from 6.12% to 77.5%. One genotype was resistant, sixteen were somewhat susceptible, eleven were somewhat resistant, sixteen were susceptible and three were highly susceptible according to phenotypic screening. One genotype was resistant, sixteen were relatively susceptible, eleven were moderately resistant, sixteen were susceptible and three were highly susceptible, according to phenotypic screening.

When compared to genotypes with a single gene, the advanced line BN-P-102, which possessed all four resistance genes, demonstrated increased resistance. The blast disease propagated quickly, according to the area under the disease progress curve (AUDPC) approach, with 7.67% of plants afflicting 7 days after inoculation (DAI) and 11.92% by 21 DAI. According to the research, BN-P-102 and Sete Pajam-2 show promise as blast-resistant rice cultivars. Furthermore, the results of the AUDPC highlight how crucial early disease control is in the field.

Keywords: Rice leaf Blast, Molecular test, Pathogenicity test, Resistant gene, Gene-specific markers.

Introduction

Rice (*Oryza sativa* L.) belonging to the family Gramineae is the staple food crop for more than 50% of the world's population[25]. In Bangladesh, rice cultivation plays a crucial role in both food security and economic growth, as evidenced by its substantial contribution to the nation's GDP[6]. Although Bangladesh is the third most rice-producing country in the world, its limited arable land makes it difficult to meet rising demand[21].

Blast disease, caused by the fungus *Pyricularia oryzae* (Telemorph: *Magnaporthe oryzae*)[18], poses a major threat to rice production in Bangladesh. Contemporary outbreaks have caused considerable yield reductions as high as 98% during epidemics, severely affecting more than half of the rice production, particularly in irrigated lowland areas[1,13].

Cultural practices such as planting resistant varieties, applying fungicides and adjusting farming techniques are common management strategies for effectively managing blast[11]. Determining the genes that confer resistance is a critical step in developing rice varieties that are resistant to blast. Over 100 blast resistance genes have been identified in rice with 31 molecularly characterized including *Pi9, Pish, Pikh, Pi-1, Pi9, Pi20, Pi27, Pi39, Pi40 and Pita* and R genes that provide broad spectrum resistance against blast[4,20].

Molecular marker technologies are indispensable in this realm, enhancing traditional breeding efforts and facilitating the precise identification of desirable germplasms. These sophisticated tools play a vital role in the development of robust rice cultivars with superior resistance to blast disease[19].

In summary, overcoming the hurdles of blast in Bangladesh's rice production requires innovative approaches, with molecular technologies acting as crucial tools for developing resistant rice varieties[24]. These developments support the objectives of programs like "Rice Vision 2050," which aim to guarantee a robust rice system in Bangladesh[10].

Material and Methods

Collection of germplasm: A total of 79 germplasms (Table 1) along with four blast-resistant *R* gene containing monogenic lines viz. IRBL9-W (*Pi9*), IRBLsh-B (*Pish*), IRBLta-CP1 (*Pita*), IRBLta2-Re (*Pita-2*) and US-2 as a susceptible line were collected from the International Rice Research Institute (IRRI), Philippines.

Inoculum preparation: The MoO isolate was collected from the Plant Pathology Division, BINA and cultured on PSA plates at 26°C for 15 days. A purified culture was developed through repeated reculturing confirmed by colony morphology and pear-shaped conidia. After incubation, the pure culture was scraped off with a sterilized toothbrush. For robust sporulation, plates were exposed to continuous light for 4-5 days. Conidia were collected into distilled water with 0.01% Twenty, filtered through gauze to remove debris and the spore concentration was adjusted to 10^5 conidia/mL using a hemocytometer.

Experimental design and pathogenicity test: To evaluate blast resistance, experiments were conducted for *MoO* strains. Seeds of all germplasm along with the US2 were sown in a seedling nursery. Twenty-one days old seedlings were transplanted to three experimental fields at Plant Pathology Division, BINA, Mymensingh (Longitude: 24.7232° N, Latitude: 90.4316° E) following randomized complete block design (RCBD) during Boro 2024. Rice plants were inoculated at the maximum tillering stage by following the spraying method[8]. After inoculation, plants were monitored at every 7 days' interval to note disease appearance. The disease severity data (percentage) were recorded at 21 days after inoculation from 20 leaves of each entry.

Based on disease severity, entries were classified as highly resistant >1% leaf area infected (Score 0), resistant 1% (Score 1), moderately resistant 1-5% (Score 2), moderately susceptible 5–25 % (Score 3), susceptible 26-50% (Score 4) and highly susceptible <50% (Score 5)[7]. The percentage of disease severity covering the whole infected region of the leaf was measured with a scale.

DNA extraction and preparation of working DNA: New immature leaves were collected and stored in 50 mL falcon tubes at -20°C. For the extraction of genomic DNA from leaf samples, the modified Cetyltrimethylammonium bromide (CTAB) method was used in this study[2]. DNA quality and concentration were checked using a Nanodrop spectrophotometer (Jenova Nano, UK). Finally, the working DNA solution was prepared by diluting the stock solution to 100 ng/µL DNA concentration using 1X TE buffer stored at 4°C. Polymerase chain reactions (PCR) were performed to identify resistance gene(s) among the selected germplasms. For the detection of blast-resistant gene(s), four SSR markers tightly linked to *Pi9, Pish, Pita and Pita-2* genes were used (Table 2).

PCR amplification: The PCR reaction was prepared to analyze the markers. Preparation of PCR reaction included 2µL of 100 ng DNA template, 7.5 µL PCR master mix (GoTaq® G2 Green Master Mix which contains green buffer, dNTPs and 4 mM $MgCl_2$ from Promega Company), 1µL of primer, 3.5 µL nuclease free water for making 15µL PCR reactions mixture. In the next step, PCR was run. Touch Down protocol was used in this experiment for running the PCR machine. This protocol contained in 3 phases. Before the first phase, the temperature was adjusted to 94°C for 5 minutes. Then, the denaturation temperature was set at 94°C for 45 seconds, annealing at Tm of each primer for 45 secs and the elongation temperature was set at 72°C for 90 seconds. The procedure was continued for up to 35 cycles[12].

Table 1
List of rice genotypes used in molecular screening for the detection of blast-resistant gene (s)

S.N.	Name	S.N.	Name	S.N.	Name	S.N.	Name
1	Cheodhan	21	Topa Boro	41	BNDR-48	61	IRBBN-L-6
2	Koshihikari	22	Pajam	42	BNDR-55	62	IRBBN-L-11
3	Ati Tajhat	23	Saita	43	B-32-3-4	63	IRBBN-L-12
4	Bihari	24	Rajshahi	44	BNRM-9-4	64	IRBBN-L-14
5	Ratna	25	Bolega	45	B/M/2	65	IRBBN-L-17
6	Awned	26	Tora Boro	46	B/M/3	66	IRBBN-L-18
7	Mota Pajam	27	Boro Digha	47	B/M/4	67	IRBBN-L-25
8	Sonali Boro-1	28	Agu Sarsori	48	B17/M6/P-13-(2)	68	IRBBN-L-26
9	Tepi Boro	29	Ful Badami	49	BPH-P-043	69	IRBBN-L-28
10	Kali Boro	30	Rahaman Dhan	50	BPH-P-065	70	IRBBN-L-36
11	Sete Pajam-2	31	BN-P-102	51	B-32-2-3	71	IRBBN-L-43
12	Jagli Boro	32	BN-P-110	52	IZSD-10	72	MEF-27
13	Rata	33	BN-P-114	53	IZSD-26	73	N4/M6/P-3-4-1
14	Khiani Boro	34	BN-P-115	54	IZSD-44	74	N4/M6/P-10(2)
15	Kamra	35	BN-P-120	55	IRBN-2	75	N4/M6/P-5-(1)-1
16	Fijar	36	BN-P-310	56	IRBN-6	76	N/M/2
17	Boro	37	BN -P-317	57	IRBN-11	77	BPH-P-034
18	Jaguli	38	BN-P-318	58	IRBN-16	78	B17/M6/P-5-4
19	Tora Boro	39	BNDR-09	59	IRBBN-L-4	79	B/M/1
20	Shata	40	BNDR-26	60	IRBBN-L-5		

Table 2
List of gene-based molecular markers, resistant genes and their details

Resistant gene	Chr.	Primer name	Primer sequences (5'–3')	Annealing Temp	Resistant band (bp)	Susceptible band (bp)	Type of marker
Pish	1	RM302- F	TCATGTCATCTAC CATCACAC	55˚C	130	150	Gene Specific[22]
		RM302- R	ATGGAGAAGATG GAATACTTGC				
Pi9	6	RM276-F	CTCAACGTTGAC ACCTCGTG	55˚C	150	120	Gene Specific[14]
		RM276-R	TCCTCCATCGA GCAGTATCA				
Pita	12	RM403- F	CAATGCCGAGTG TGCAAAGG	55˚C	400	350	Gene Specific[3]
		RM403- R	TCAGGTTGAAGA TGCATAGC				
Pita-2	12	RM155- F	GAGATGGCCCCC TCCGTGATGG	55˚C	250	100	Gene Specific[12,26]
		RM155- R	TGCCCTCAATCG GCCACACCTC				

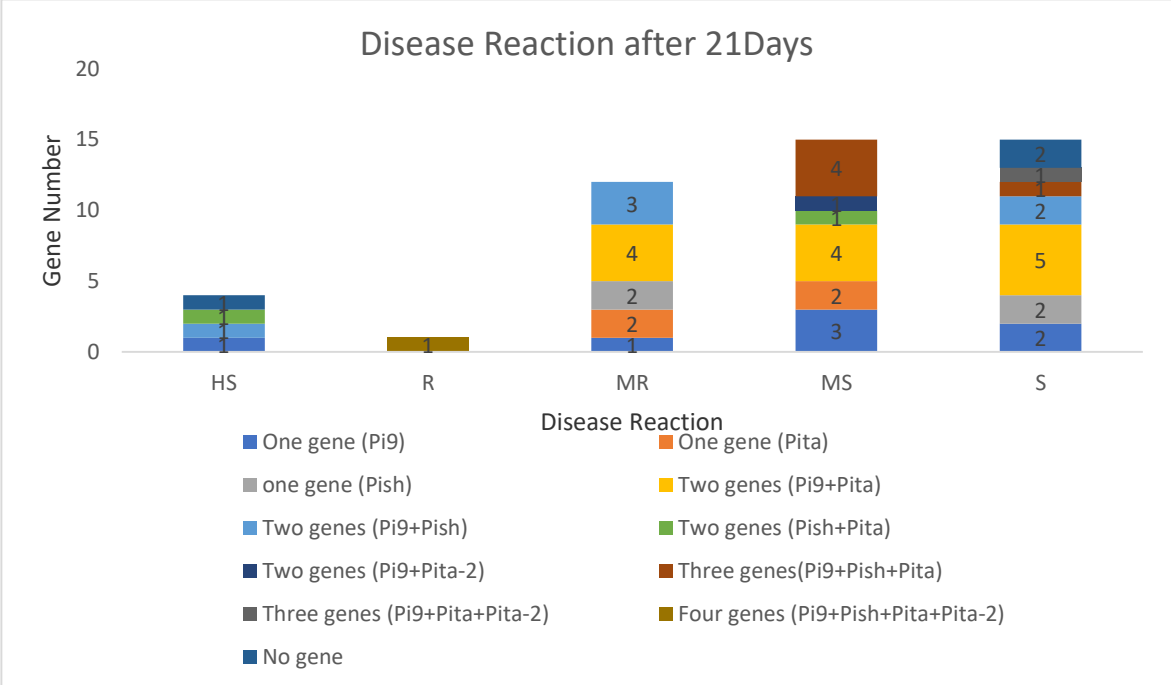


Figure 1: Disease reaction after 21 days.

The PCR products were stored at 4ºC for further use. The PCR products were resolved in 1.5% agarose gel using 1X TBE buffer at 70 V-60 min. The monogenic resistant line of the respective gene was used as a resistant check and US-2 was used as a susceptible check for identification of the resistant genes. The gels were visualized under a transilluminator (Bio-Rad, Hercules, CA, USA).

Area under Disease Progress Curve (AUDPC): AUDPC was computed based on the severity of the condition as per formula[15]:

$$AUDPC = \sum_{i=1}^{n-1}\left(\frac{y_i+y_{i+1}}{2}\right)(t_{i+1}-t_i)$$

where y_i is assessment of the disease at the ith observation, t_i is time (in days, hours, etc.) at the i^{th} observation and n is total number of observations.

Data analysis: All statistical analyses were performed using Statistix statistical software (version 10).

Results

Identification of blast-resistant germplasm: Based on the disease, reaction patterns of 38 germplasm against the fungal infections were shown in table 3 at 7, 14 and 21 DAI (days after inoculation). At 21 DAI, only 1 germplasm was found resistant (score 1), 10 germplasms were found moderately resistant (score 2), 13 germplasms were found moderately

susceptible (score 3), 11 germplasm were found susceptible (score 4) and 3 germplasm were found highly susceptible (score 5) against active isolates of *MoO* (Figure 1). BN-P-102 showed resistant reactions (score 0) while susceptible check line US-2 and advance line IRBBN-L-25, BN-P-110 showed a highly susceptible reaction (score 5).

Table 3
Disease incidence, Disease severity and disease reaction of *MoO* inoculated rice germplasm

S.N.	Genotypes	7 DAI			14 DAI			21 DAI		
		DI (%)	DS (%)	DR	DI (%)	DS (%)	DR	DI (%)	DS (%)	DR
1	BN-P-317	48.33	1.6667	MR	48.33	1.78	MR	41.667	1.78	MR
2	BNDR-9	44.333	2.2233	MS	44.333	2.2233	MS	40	2.2233	MR
3	BN-P-114	43	2.4433	MS	43	2.5	MS	31	2.5	MS
4	B/M/2	40	3.22	S	40	3.33	S	40	3.33	S
5	B/M/3	40	3.33	S	40	3.33	S	36.667	3.33	S
6	N/M/4	40	2.5	MS	40	2.5	MS	35.667	2.5	MR
7	US-2	40	1.6133	MS	55.667	1.5567	S	86.667	1.6667	HS
8	B/M/1	38.667	2	MR	38.667	2	MR	38.867	2	MR
9	BPH-P-034	38	3.4467	S	37	3.5567	S	35.467	3.5567	S
10	N/M/1	36.667	1.67	MR	36.667	1.67	MR	36.667	1.68	MR
11	N/M/2	35.333	2.5	MS	33.333	2.5	MS	33.333	2.5	MS
12	BNDR-26	32.333	1.9433	MR	34.667	1.6133	MR	34.667	1.6133	MR
13	BN-P-318	31.667	2.8333	MS	31.667	2.8333	MS	27.333	2.8333	MS
14	IRBBN-L-43	31.667	2.0567	MS	26.667	2.6133	MS	35	2.0567	MS
15	IZSD-10	31.667	1.78	MR	40.667	1.78	MR	31	1.78	MR
16	MEF-27	31	2.333	MS	31	2.32	MS	35.8	2.32	MS
17	IRBBN-L-6	30.667	2.2233	MS	37.667	1.8333	MR	34.333	1.78	MR
18	BN-P-120	29.333	2.6667	MS	29.333	2.6667	MS	27.167	2.6667	MS
19	BPH-P-065	29	3.3333	S	56.333	3.3333	S	61	3.3333	S
20	IRBN-6	29	3.61	S	26	3.61	S	37.667	3.3333	S
21	BNRM-9-4	28.333	2.61	MS	43.333	2.61	MS	51.667	2.5667	MS
22	IZSD-44	28.333	2.0567	MS	44	2.0567	MS	44	2.0567	MS
23	B-32-3-4	26.333	2.7233	MS	46.667	2.8367	MS	50	2.8367	MS
24	BNDR-48	25	1.5567	MR	59	1.1667	MR	59	1.1667	MR
25	IRBBN-L-18	25	2.7233	MS	49.333	2.6667	MS	55	2.6667	MS
26	IRBN-11	24.333	3.22	S	30.667	3.22	S	34.33	3.22	S
27	IRBN-2	24	3.4467	S	37.667	2.89	MS	49.333	3.5	S
28	IZSD-26	24	2.9433	MS	35.333	2.9433	MS	38.667	2.9433	MS
29	BN-P-102	23.333	2	MR	30.333	2	MR	16.8	2	R
30	IRBBN-L-5	22.333	3.89	S	37	3.0567	S	41.667	3.1133	S
31	IRBN-16	22.333	2.5	MS	28.667	2.5	MS	34.333	2.5	MS
32	BN-P-310	21.667	3.1667	S	33	3.1667	S	33	3.11	S
33	N4/M6/P-3-4-1	20	1.9467	MR	29	1.9467	MR	26.667	1.9467	MR
34	BN-P-115	19.667	3.1133	S	32.333	3.2233	S	35.333	3.2233	S
35	BN-P-110	19	5.83	HS	29.333	6.11	HS	22	6.11	HS
36	IRBBN-L-25	19	4.7233	S	20	4.7233	S	13	5.1667	HS
37	B/M/4	17.33	2.3333	MS	30	2.0533	MS	37.667	2.0533	MS
38	B-32-2-3	12	5.5	S	25.333	5.8333	S	26.333	5.8333	S
39	Koshihikari	46.667	3.0533	S	37.667	2.9433	MS	32.6	2.7767	MS
40	Sonali Boro	38.667	4.4433	S	57.667	4.4433	S	61	4.4433	S
41	Khiani Boro	30	1.7233	MR	43.333	1.7233	MR	43.333	1.7233	MR
42	Bolega	27.667	2.6667	MS	34.333	2.6667	MS	43.333	2.6667	MS
43	Rata	26	2.0567	MS	43	2.0567	MS	30	2.0567	MS
44	Tora Boro	24.667	4.1633	S	34.667	4.1633	S	34.667	4.1633	S
45	Sete Pajam-2	24.333	2	MR	34.333	2	MR	24.333	2.2233	MR
46	Agu Sarsori	21	3.61	S	39.333	4.0567	S	31	3.7233	S

Here, DI = Disease incidence, DS = Disease severity, DR = Disease reaction, R = Resistance, MR = Moderately resistance, MS = Moderately susceptible, S = Susceptible, HS = Highly susceptible.

Identification of the blast-resistant genes: Among the 79 rice lines, out of thirty naturally occurring rice cultivars, four (13.33%) carried the *Pish* gene, eight (26.67%) the Pi9 gene and ten (33.33%) the Pita gene. Furthermore, one cultivar had both the *Pi9* and *Pish* genes, whereas six cultivars carried both the *Pita* and *Pi9* genes. Regarding the Pita-2 gene, no cultivars were found. The most common genes were *Pita, Pi9 and Pish*, in order of distribution. Four carried the Pi-9 gene, three carried Pish and seven carried Pita among the 49 advanced rice lines provided by IRRI. Furthermore, multiple resistance genes were present in 22 lines: 16 lines carried *Pi9* and *Pita*, 1 line carried *Pish* and *Pita* and 2 lines carried *Pi9* and *Pish*.

Triple resistance genes (*Pi9, Pish and Pita*) were found in eight lines: BPH-P-043, IRBBN-L-17, IRBBN-L-18, N4/M6/P-3-4-1, BN-P-114, BN-P-120, BN-P-310, IRBN-6 and IRBN-16. *Pita, Pita-2, Pi9 and Pish* were the four genes present in BN-P-102, while *Pita, Pita-2 and Pi9* were the three genes present in BN-P-310. The lines IRBBN-L-25 and BPH-P-034 did not contain any resistance genes. *Pi9* was the most prevalent gene, present in 77.5% of the lines. *Pita* (67.3%), *Pish* (38.7%) and *Pita-2* (6.12%) were the next most common genes (Figure 2 and Figure 3). Most of the resistant germplasm was discovered to carry numerous genes in various combinations, which is interesting.

18 of the germplasms under study contained two genes in different combinations, whereas one germplasm had four genes. The pathogenicity test revealed that out of the 46 germplasm samples, only BN-P-102 (including *Pi9, Pish, Pita and Pita-2*) exhibited resistance. Notable results from the pathogenicity test included the finding that germplasms with two to three genes were resistant and germplasms with a single R gene were determined to be moderately resistant to being susceptible.

Area under Disease Progress Curve (AUDPC): The progress of blast disease was scored using the percent disease index (PDI) and area under the disease progress curve (AUDPC) in 46 rice genotypes over three weeks after inoculation (Figure 4). PDI and AUDPC were 0 at 0 days after inoculation (DAI) because there was no visible symptom. PDI had increased to 7.67% by 7 DAI with an AUDPC of 26.8 (recorded early onset disease). PDI increased to 11.75% by the end of 14 DAI and AUDPC reached 67.9 during this period, indicating a higher level of disease severity.

However, it appeared to stop increasing and reached 11.92% by the time of plots rated with PDI (21 DAI), indicating that this is the accumulated effect of the disease when AUDPC was also elevated up to as high a value as 82.9. After this, the disease reached a plateau phase till day 17.

Discussion

This study highlights the importance of the *Pi9* gene, as it was found that 58.7% of rice germplasm (46 out of 79) contained this dominantly resistant gene; of these, the native rice cultivars contained about 26.67% (8 out of 30) of the *Pi9* gene, while advanced lines contained about 77.5% (38 out of 49) of the Pi9 gene. Similarly, the high occurrence of the Pi9 gene among the genotypes was also reported in previous studies[5,14].

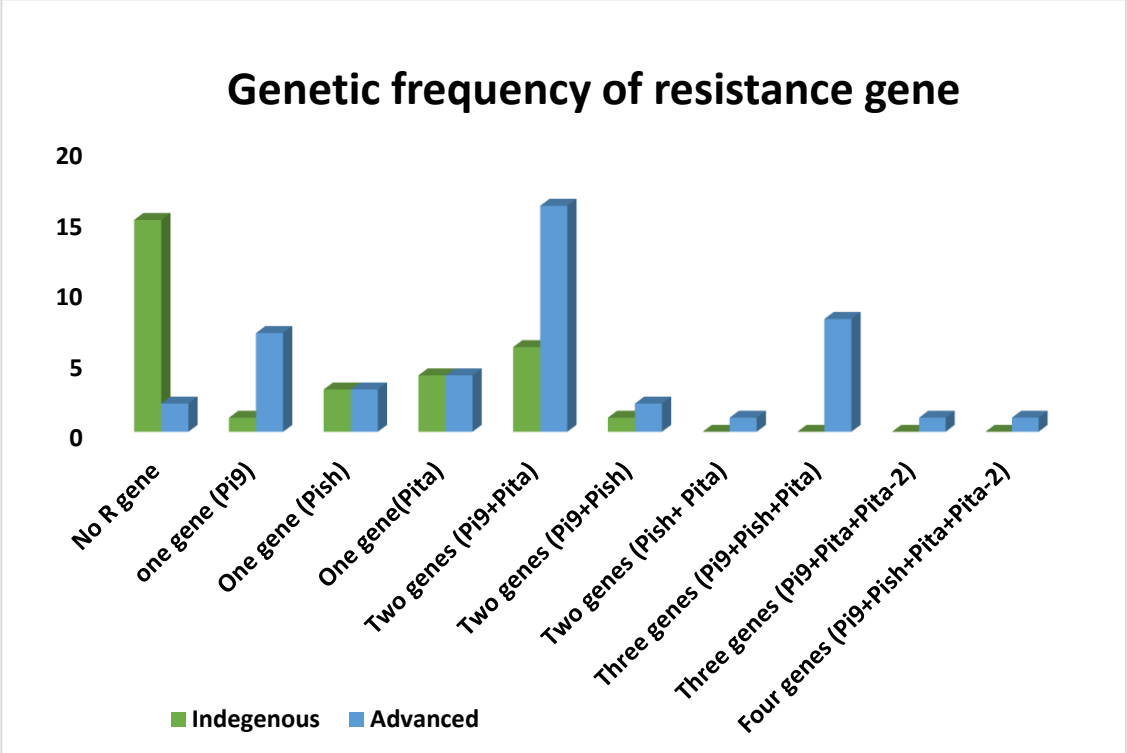
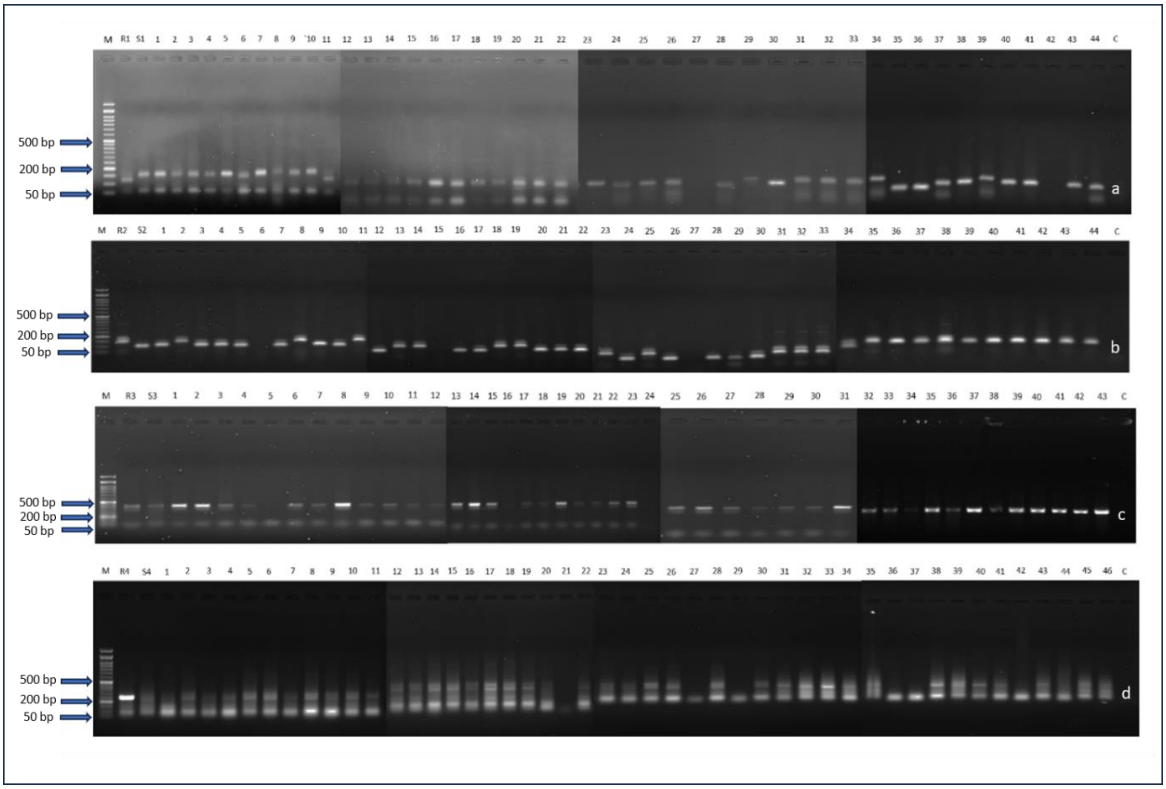


Figure 2: Distribution of blast-resistant genes in different rice populations



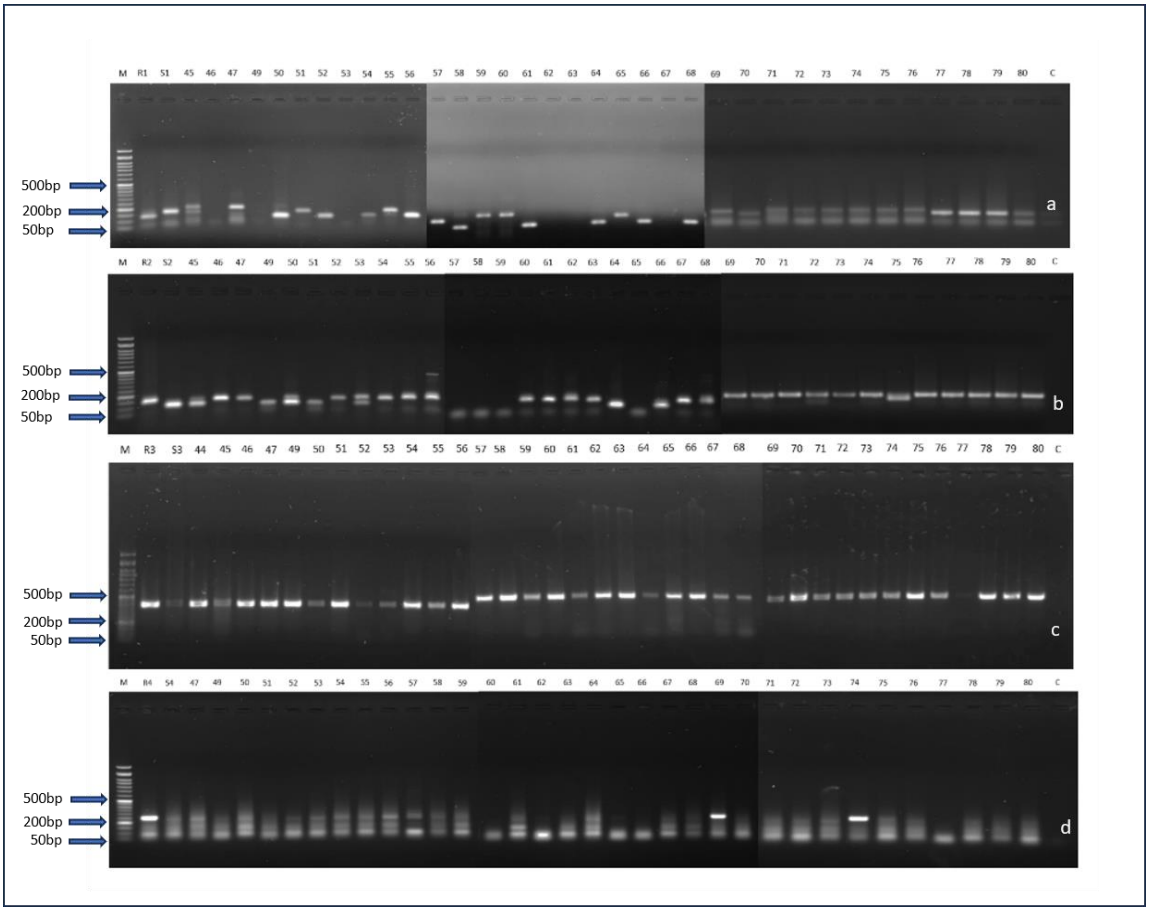


Figure 3: Representative Gel pictures showing amplification patterns generated by different SRS markers used in the study, a primer RM302 (Pish gene linked), b RM276 primer (Pi9 gene linked), c RM403 primer (Pita gene linked), d RM155 primer (Pita-2 gene linked), S1, S2, S3 and S4 all are indicating one susceptible check is US-2 and R1- IRBLsh-B, R2- IRBL9-W, R3-, IRBLta-CP1, R4- IRBLta2-Re. M corresponds to 50 bp DNA ladder respectively. 1 to 80 where 48 is missing so a total (79) represents the studied 79 germplasm mentioned in table 1.

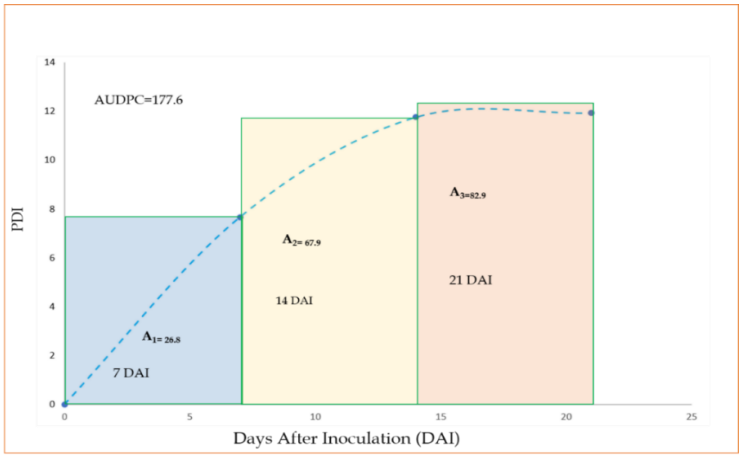


Figure 4: Rice leaf blast disease progression over time after inoculation (AUDPC)

In addition, the dominant R genes *Pita, Pish, Pita-2 and* Pish were examined. Of the rice genotypes (42 out of 79), approximately 53.2% carried the Pita gene. About 40% (10 out of 30) of the native rice cultivars carry the *Pita* gene, compared to approximately 65.3% (32 out of 49) of the advanced lines. In a similar vein, a high incidence of the Pita gene among the genotypes was shown.

Furthermore, *Pish* is present in 27.8% of rice genotypes, with 13.3% in indigenous cultivars and 36.7% in advanced lines. Further research shows that the *Pita-2* gene is present in 3.79% of rice genotypes, 0% in native rice cultivars and 6.12% in advanced lines. These findings are consistent with previous reports[9,23]. The study emphasizes the race-specific features of *Pita Pish* and *Pita-2*, particularly in the Indian subcontinent and highlights their varied prevalence in different regions. The absence of the *Pita-2* gene, known for broad-spectrum resistance, in the studied germplasm suggests its lower prevalence compared to other R genes, which is similar to the previous report[5]. The study evaluates the efficacy of single and multiple gene combinations in conferring resistance.

While the *Pi9* gene alone showed limited effectiveness, combinations like *Pi9+Pita* demonstrated moderate resistance. Particularly, the Pi9+Pita+Pish+Pita-2 combination, observed in BN-P-102, exhibited highly resistant reactions, highlighting the importance of multiple gene combinations for enhanced and durable resistance in rice breeding programs, aligned with the findings of previous reports[17,27]. In the present study, AUDPC tracked blast progression over 21 days. No symptoms were observed at 0 DAI. By 7 DAI, 7.67% of plants exhibited symptoms (PDI 7.67%), with an AUDPC of 26.8. At 14 DAI, approximately 12%, of the plants were affected (PDI 11.75%) with an AUDPC of 67.9%.

21 DAI slightly raised but stable in 12% of plants showing symptoms (PDI 11.92%) and an AUDPC of 82.9[7,16]. The data suggests rapid blast progression in the initial 14 days followed by a slowdown. This study contributes valuable insights into the findings of resistant varieties for blast, providing a foundation for future research and the development of bacterial blight-resistant rice varieties.

Conclusion

To screen the germplasms against *Magnaporthe oryzae* (MoO), a pathogenicity test was performed by exposing 46 samples of germplasm which represented highly resistant to susceptible. This molecular analysis was later followed by genotyping which identified one to four resistance (R) genes present in the germplasm samples. Based on blast disease reaction, one germplasm sample from each collector came to light as carrying high resistant reactions (BN-P-102 carried *Pi9, Pish, Pita* and *Pita2* genes) and noted its row is Sete pajam local variety (carrying both pi9 and pIsh). It can be recommended for farmers where moderately high resistance (in the case of set pajam) prevails among tested population data. Being strong resistance isolates, this germplasm is a good alternate source to be the most preferred inclusion in future breeding programs to develop superior rice varieties resistant to blast.

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