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DEVELOPMENT OF BLAST-RESISTANT RICE LINES THROUGH MARKER-ASSISTED SELECTION

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Abstract

Rice blast (*Pyricularia oryzae)* is a major constraint for rice productivity in Bangladesh and many other countries. An experiment was conducted from November 2022 to April 2023 in the Biotechnology Division of the Bangladesh Institute of Nuclear Agriculture (BINA) aimed at developing blast-resistant rice varieties using marker-assisted selection. Three rice varieties were used: BRRI dhan48, BRRI dhan58, and IRBL9-W. SSR markers were employed for F_1 confirmation, selecting 14 plants for backcrossing. PCR analysis and gel electrophoresis confirmed F_1 plants through polymorphic markers. A total of 97 BC_1F_1 seeds were produced. The study contributes to developing blast-resistant rice lines, crucial for mitigating yield loss caused by *Pyricularia oryzae*, a significant threat to rice production in Bangladesh.

Keywords: Backcrossing, Marker-assisted selection, *Pyricularia oryzae*, SSR Marker

Introduction

In Bangladesh, rice production is significantly harmed by blast disease. Blast is one of the most devastating diseases in rice-growing regions worldwide, responsible for 11-15% yield loss annually (Jabeen *et al.*, 2012). Rice is cultivated in both paddy and upland conditions. The relationship between lower water conditions and increased incidence of rice diseases, such as those caused by *Pyricularia oryzae*, has been extensively documented (Khan *et al.*, 2021). Environmental factors, including climatic changes leading to water scarcity, have been found to create conditions favorable for the proliferation of rice pathogens (Ramona *et al.*, 2021). Specifically, reduced water availability can stress rice plants, compromising their natural defenses and making them more susceptible to diseases (Zampieri *et al.*, 2023). Therefore, investigating production technologies aimed at cultivating rice under lower water conditions is crucial for understanding and mitigating the increased incidence of rice diseases in such environments. The pathogen might infect at any of the growth stages of the rice plant, from seedling to the pre-maturity stage of the crop (Yaduraju, 2013). Rice blast (*Pyricularia oryzae*) poses a vicious threat to the country's economy (Ganesh *et al.*, 2012).

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It is estimated that each year enough rice is destroyed by rice blast alone to feed 60 million people over three years. Out of the total yield loss due to diseases in rice, 35% is attributed to blast, 25% to sheath blight, 20% to BLB, 10% to tungro, and the remaining 10% to other diseases (Prasanna *et al.*, 2013). The disease causes yield losses ranging from 1-100% in Japan, 70% in China, 21-37% in Bali Indonesia, and 30-50% in South America and Southeast Asia (Kato, 2001). In 2017, Bangladesh experienced significant yield losses attributed to an epiphytotic outbreak of blast diseases caused by *Pyricularia oryzae*, affecting various regions including Dinajpur, Rangpur, Thakurgaon, Panchghar, Kushtia, Jashore, Pabna, Barishal, Mymensingh, Munshigonj, Chuadanga, among others. This outbreak led to substantial reductions in the production of Boro rice and transplanted Aman, with recorded disease severities of 21.19% and 11.98%, respectively. The most affected Boro rice varieties were BRRI dhan28 (29.6% disease severity), followed by BRRI dhan29 (25.9% disease severity) and BRRI dhan61 (21.9% disease severity), while the most affected T. Aman rice was BRRI dhan34 (22.9% disease severity) (Hossain and Ali, 2017). The major symptoms of this disease are found on leaves, with brownish spots having a grey center, a sunken lesion on the node, and a brown or black lesion found on the neck of the panicle. Among them, panicle blast is the most devastating and sometimes causes 80-100% yield loss (Pagliaccia *et al.*, 2018). The occurrence of this disease increased the market value of rice, posing a threat to national food security and the economy.

To identify blast-resistant F_1 genotypes, the marker-assisted selection technique was performed in the laboratory. Simple Sequence Repeat (SSR) markers are widely used as they are highly polymorphic in nature. They are powerful genetic markers due to their abundance, genetic co-dominance (identification of loci in either homozygous or heterozygous condition), dispersal throughout the genome, multi-allelic variation, and high reproducibility (Gao *et al*., 2005; Zhang *et al*., 2007; Thomson *et al*., 2009). In the present study, SSR markers were used to identify blast-resistant gene-containing varieties using Marker-Assisted Backcrossing (MABC).

Materials and Methods

Plant materials and crossing scheme

This experiment was conducted from November 2022 to April 2023 at the research field and laboratory of the Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh. Three different rice varieties were obtained from the Bangladesh Rice Research Institute (BRRI) and the Bangladesh Institute of Nuclear Agriculture (BINA). Among them, the blast-resistant and high-yielding breeding line IRBL9-W was used as the donor parent. On the other hand, two popular high-yielding and blast-susceptible varieties, BRRI dhan48 and BRRI dhan58, were used as recipient parents (Nihad *et al*., 2022). Seeds of all these parental lines were sown in the experimental field at BINA. Subsequently, a primer survey was conducted among the parental lines to identify polymorphic primers, and conventional breeding was carried out to produce F_1 seeds. Two months later, F_1 seeds of two cross varieties, BRRI dhan48×IRBL9-W and BRRI dhan58×IRBL9-W, were soaked in petri plates for 48 hours to break dormancy. Seeds of each variety were placed in distinct, tagged petri plates. The

bottom of each petri dish was filled with sterilized tissue paper, and a certain amount of water was added to each plate. Once the seeds started sprouting, they were removed from the water and air-dried for germination. After germination, the seeds were dispersed in the main seedbed. At 30 days of age, seedlings were transplanted into the research field at BINA, and the plants were labeled with stick labels.

PCR analysis for SSR markers

Vigorous 21-day-old leaves were carefully cut with sterilized scissors and washed in 70% ethanol and distilled water. The collected leaf samples were then placed in polythene bags with proper labeling and stored in an icebox to prevent damage to the leaf tissues during transportation to the laboratory. Subsequently, the samples were stored in a -80°C freezer. DNA was extracted from the leaves of each genotype using the DNA extraction miniprep method. The simplified mini-scale methodology of DNA extraction for PCR analysis recommended by IRRI was followed. The quality of the isolated DNA was confirmed to be satisfactory for PCR analysis (Zhang *et al*., 2009). For quantification of DNA concentration, a NanoDrop 2000c Spectrophotometer was used (Desjardins and Conklin, 2010). The total volume of PCR master mix for the study was 10 µl per sample, containing 1 µl of DNA. The components of the PCR cocktail preparation included Go Taq Green master mix (5.0µl), ddH_2O (3.0 µl), Primer Forward (0.5µl), Primer Reverse (0.5 µl), and DNA (100ng/ µl) (1.0 µl). The thermal profile of PCR cycles consisted of an initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, repeated for 35 cycles. The final extension was performed at 72°C for 5 minutes. After PCR, the products were mixed with 3 µl of 2X gel loading dye. The reagents used for Polyacrylamide Gel included 40% Acrylamide, 10% APS, 10X TBE Buffer, and TEMED (N,N,N',N'- tetramethylethane-1,2-diamine). For the preparation of an 8% Polyacrylamide Gel, premix (40% Acrylamide, 10X TBE Buffer, dH2O), APS (10%), and TEMED (N,N,N',N'- tetramethylethane-1,2-diamine) were mixed in the appropriate proportions. Approximately 2.5 µl of each PCR product was loaded into each well, and a 25bp DNA ladder was used for size determination. Electrophoresis was run for approximately 1.5 hours at 70 volts for each loaded gel. Subsequently, the gel was soaked in ethidium bromide (10mg/mL) solution for 20-30 minutes and placed on a high-performance ultraviolet light box (UV trans-illuminator) of a gel doc for analysis of the DNA bands. The DNA bands were observed using Alpha Easefc 4.0 software and the records were saved (Nadim *et al.*, 2022).

Confirmation strategy of F1 plants through MAS

Markers were evaluated based on the intensity of bands, consistency within the individual, presence of smearing, and potential for population discrimination. In this experiment, 96 random SSR markers were screened for two rice genotypes to evaluate their suitability for amplifying DNA sequences that could be accurately scored. The list of markers is provided in Table 1.

Table 1. List of SSR Markers

Sl. No.	Primer Name	Sl. No.	Primer Name	Sl. No.	Primer Name
1	RM271	37	RM34	73	RM103
2	RM515	38	RM101	74	RM86
3	RM316	39	RM112	75	RM47
4	RM408	40	RM39	76	RM3330
5	RM205	41	RM40	77	RM7023
6	RM17	42	RM41	78	RM115
7	RM171	43	RM132	79	RM7102
8	RM5493	44	RM109	80	RM1359
9	RM544	45	RM55	81	RM48
10	RM407	46	RM136	82	RM519
11	RM258	47	RM100	83	RM206
12	RM292	48	RM52	84	RM158
13	RM11	49	RM5501	85	RM1178
14	RM123	50	RM83	86	RM23818
15	RM18877	51	RM84	87	RM26063
16	RM23679	52	RM116	88	RM300
17	RM283	53	RM70	89	RM528
18	RM5953	54	RM3843	90	RM26416
19	RM166	55	RM107	91	RM445
20	RM131	56	RM80	92	RM27694
21	RM7	57	RM1377	93	RM7643
22	RM4	58	RM106	94	RM5404
23	RM49	59	RM6836	95	RM556
24	RM8	60	RM1361	96	RM7175
25	RM9	61	RM88		
26	RM50	62	RM53		
27	RM13	63	RM54		
28	RM14	64	RM87		
29	RM16	65	RM108		
30	RM19	66	RM5473		
31	RM126	67	RM8225		
32	RM22	68	RM117		
33	RM23	69	RM5961		
34	RM27	70	RM113		
35	RM29	71	RM82		
36	RM2	72	RM114		

DNA samples from 54 F_1 plants (39 plants of BRRI dhan48 × IRBL9-W and 15 plants of BRRI dhan58 × IRBL9-W) were collected for extraction. Using representative polymorphic markers as shown in Table 1, Polymerase Chain Reaction (PCR) was performed. Each F_1 genomic DNA was compared with its respective parental DNA. F_1 plants containing sharp parental DNA bands (both donor and recipient) were confirmed as true F_1 plants and subjected to subsequent background selection. The selected 28 F_1 plants were transferred to plastic pots with proper labeling and kept under shed for emasculation.

Backcrossing process for BC1F1 seeds production

For producing BC_1F_1 seeds, F_1 plants were kept in labeled pots. Two sets of BRRI dhan48 and BRRI dhan58 were seeded to synchronize flowering with F_1 plants for producing BC_1F_1 seeds. F_1 plants were emasculated, and labeled pots were kept in a glasshouse. The next morning, pollination was performed by dusting male flowers from the donor line onto the female stigma of F_1 plants and bagging them with glassine plastic bags with proper tagging. After a few days, when the seeds became mature, they were carefully collected and stored with proper tagging.

Statistical analysis

While traditional statistical tests were not the focus of this study, molecular marker analysis and selection processes played a critical role in achieving the study's goal of developing blast-resistant rice varieties through marker-assisted breeding. These genetic analyses are fundamental in plant breeding programs to select and propagate desirable traits efficiently. However, only the bar graph demonstrating comparison among the number of survived F_1 plants, selected F_1 plants for backcross breeding and BC_1F_1 seeds was constructed through Microsoft Excel 2019 software.

Results and Discussion

The primary aim of this study was to develop blast-resistant characteristics in a blast-susceptible rice variety through marker-assisted breeding. The selected plants were subsequently backcrossed to retain all desirable features. Two crossed rice genotypes were utilized for this experiment, namely F_1 genotypes of BR48× IRBL9-W and BR58× IRBL9-W, for the improvement of advanced blast-resistant lines. Initially, 96 primers were employed for the primer survey. Out of these, a total of 30 and 34 primers exhibited polymorphism in the two crosses, respectively. Ultimately, 5 primers showing highly polymorphic bands were selected and utilized for F_1 confirmation (Table 2). One of the primary challenges in rice production in Bangladesh is the prevalence of various diseases. In recent years, rice blast caused by *Pyricularia oryzae* has emerged in epidemic proportions, resulting in significant yield losses and posing a serious threat to our nation's food security and economy (Jabeen *et al*., 2012). To ensure food security, it is imperative to take necessary measures to manage this disease. Consequently, enhancing rice blast resistance has become one of the most crucial breeding objectives. This study aimed to develop blast-resistant rice varieties with the goal of producing blast-resistant advanced rice lines in the BC_1F_1 generation.

Primer survey

In this study, a total of 96 SSR primers were surveyed on both parental DNA to identify polymorphic markers. Primers containing heterozygous alleles (in comparison to one donor and one recipient parent) in the banding pattern of sample DNA were utilized for the selection process. Each number in the gel represented two parental DNA bands for one SSR primer. Both corners (right and left) of the gel contained various DNA ladders (20 bp and 100 bp) as indicators. DNA samples were isolated from both donor and recipient parental plants, and PCR was conducted using 11 polymorphic SSR markers. PCR bands from all parental plants were scored for heterozygous alleles for the donor and recipient parents.

Survey analysis

The survey was conducted with one recipient and one donor parent each, such as BRRI dhan48 and IRBL9-W, BRRI dhan58 and IRBL9-W, for each marker. A total of 48 primers were used for survey 1, and initially, 16 primers were selected, which exhibited a "Circular marking" in the gel. The primarily selected polymorphic primers from survey 1 were RM271, RM408, RM205, RM5493, RM258, RM11, RM123, RM23679, RM7, RM4, RM9, RM13, RM16, RM19, RM23, and RM55. Similarly, 14 primers were initially chosen from survey 2, with selected polymorphic primers including RM1377, RM1361, RM5473, RM8225, RM5961, RM3330, RM7102, RM519, RM206, RM26063, RM300, RM528, RM26416, and RM7175. Additionally, 20 primers were initially selected in the gel, with chosen primers from survey 3 being RM271, RM408, RM205, RM5493, RM407, RM258, RM11, RM23679, RM166, RM7, RM4, RM9, RM13, RM16, RM19, RM22, RM23, RM29, RM101, and RM55. Out of the 48 primers, 15 were primarily chosen, with primers from survey 4 being RM5501, RM107, RM1361, RM87, RM8225, RM5961, RM82, RM7102, RM519, RM206, RM1178, RM26063, RM528, RM27694, and RM7175.

Marker-Assisted Selection (MAS) is a molecular breeding process whereby a molecular marker, based on DNA variation, is used for the indirect selection of an agronomic trait of interest. MAS is not influenced by environments and can be conducted at any plant growth stage. It is particularly useful for the selection of recessive genes and biochemical traits in heterozygous plants. Undesirable genotypes can be quickly eliminated by MAS. This feature is particularly important and useful for some breeding schemes such as backcrossing and recurrent selection, which require crossing with or between selected individuals (Luo *et al*., 2014).

Highly polymorphic primer selection

Following primary selection, highly heterozygous band-showing primers were selected to identify true F_1 plants. Among these primers, five highly polymorphic markers were selected based on their sharp banding pattern and heterozygosity (Table 2).

Table 2. Highly polymorphic SSR markers with their representative parental genotypes

Parental genotypes	Highly polymorphic loci
BRRI dhan48 and IRBL9-W	RM23679, RM26063
BRRI dhan58 and IRBL9-W	RM13, RM9, RM101

A similar study was conducted by Xiao *et al*. (2019), in which they developed seven improved lines, comprising three monogenic lines, three two-gene pyramids, and one three-gene pyramid, by introgressing R gene(s) into a common genetic background using marker-assisted backcross breeding (MABB). They screened 302 SSR markers. All the improved lines conferred a wider resistance spectrum compared with their current parents. However, the three monogenic lines did not perform well under the field conditions of the two nurseries. Given their similar performances on the main agronomic traits as the recurrent parent, the two-gene pyramids achieved the breeding goals of a broad resistance spectrum and effective panicle blast resistance.

Another similar study was conducted by Singh *et al*. (2015). In that study, molecular screening and genetic diversities of major rice blast resistance genes were determined in 192 rice germplasm accessions using simple sequence repeat (SSR) markers. Two parents were screened for parental polymorphism using 96 SSR markers, of which 5 markers exhibited high polymorphism (Table 2). These markers were: RM23679= Chr. 9; RM26063= Chr. 11; RM101= Chr. 12; RM9= Chr. 1; RM13= Chr. 5. These loci were selected based on the polymorphic markers found in this study, which were used to confirm the F_1 plants.

F_1 plants generation

F_1 seeds of two crossed rice genotypes were sown in the experimental field of BINA with proper spacing and line maintenance. The number of F_1 seedlings that survived is presented in Fig. 3.

After the primer survey, five highly polymorphic primers were selected and tested for those F_1 genotypes (BRRI dhan48 and IRBL9-W= RM23679, RM26063; BRRI dhan58 and IRBL9-W= RM13, RM9, RM101). F_1 confirmation was conducted by analyzing DNA bands. F_1 plants carrying both parental DNA were confirmed as true F_1. Besides the selected F_1 plants, residual F_1 plants were removed. Out of 54 plants, only 14 F_1 plants were confirmed (BRRI dhan48 and IRBL9-W= 11 plants, BRRI dhan58 and IRBL9-W= 3 plants) (Fig. 3). Hasan *et al*. (2015) conducted a similar study. Leaf blast resistance line D521, neck blast resistance line D524, and BB resistance were developed through the introgression of leaf resistance gene *Pi1*, neck blast resistance gene *Pi2* derived from donor BL122, and bacterial resistance gene *Xa23* derived from donor CBB23 into an elite, early maturing maintainer line of hybrid rice susceptible to both blast and blight, Ronfeng B hybrid rice through marker-assisted backcross breeding programs. By using three SSR markers (MRG4766, AP22, and RM206), *Pi1*, *Pi2*, and *Xa23* were identified and used to test the recovery of the genetic background for the improved lines by using 131 polymorphic markers. Primer survey plays an important role, which is necessary for successful foreground, recombinant, and background

selection. Polymorphic markers are essential for a marker-assisted selection process. They are required for confirming F_1 genotypes and improving advanced rice lines.

Confirmation of F_1 plants through polymorphic markers

DNA samples were collected from 54 F_1 plants. PCR was carried out using 5 polymorphic SSR markers. The following figures show the gel picture of F_1 confirmation. A particular SSR marker was used to compare alleles of each F_1 plant DNA with their parental DNA. Among all the F_1 plants, only a few were confirmed as true F_1. For F_1 confirmation of BRRI dhan48 × IRBL9-W cross generation using the RM23679 marker: P_1 and P_2 stand for BRRI dhan48 and IRBL9-W, respectively, and both corners of the gel contain 1kb DNA ladder (L) (Fig. 1). A total of 39 F_1 plants were compared with their parental DNA using the RM23679 marker, and out of these, 19 F_1 plants (plant no. 1, 8, 9, 10, 11, 12, 14, 15, 16, 19, 24, 26, 27, 28, 29, 31, 33, 35, and 39) were selected (Fig.1).

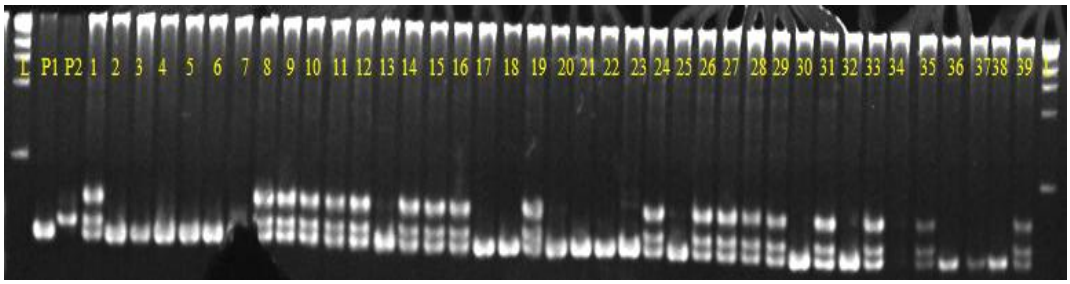

Fig. 1. F_1 confirmation using RM23679 marker for BRRI dhan48 × IRBL9-W cross generations

F_1 confirmation for the BRRI dhan48 × IRBL9-W cross generation using the RM26063 marker: BRRI dhan48 × IRBL9-W is marked as P_1 and P_2, respectively, and both corners of the gel contain a 1kb DNA ladder (L). The RM26063 marker was used to compare among 39 F_1 plants with their parental DNA, and out of these, 11 F_1 plants (plant no. 1, 9, 10, 11, 14, 15, 16, 19, 26, 28, and 31) were selected (Fig. 2).

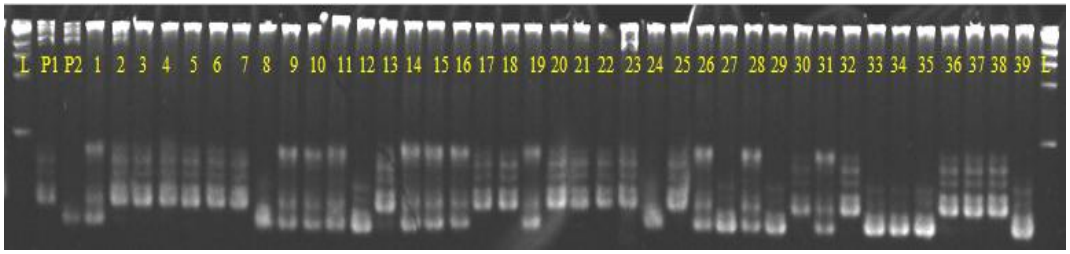

Fig. 2. F_1 confirmation using RM26063 marker for BRRI dhan48×IRBL9-W cross-generation

After analyzing the data from two gel docs (Fig. 1 and 2), 11 F1 plants were confirmed for the BRRI dhan48 × IRBL9-W genotype. True F_1 plants were confirmed based on the presence of double bands, each corresponding to the parental bands shown by the polymorphic primers. The confirmed F_1 plants for BRRI dhan48 × IRBL9-W were 1, 9, 10, 11, 14, 15, 16, 19, 26, 28, and 31.

For F_1 confirmation of the BRRI dhan58 × IRBL9-W cross generation using the RM13 marker: P_1 and P_2 represent BRRI dhan58 and IRBL9-W, respectively, and both corners of the gel contain a 1kb DNA ladder (L) (Fig. 2). A total of 15 F_1 plants were compared with their parental DNA using the RM13 marker, and out of these, 5 F_1 plants were selected.

Similarly, for F_1 confirmation of the BRRI dhan58 × IRBL9-W cross generation using the RM9 marker: A total of 15 F_1 plants were compared with their parental DNA using the RM9 marker, and out of these, 4 F_1 plants were selected.

For F_1 confirmation of the BRRI dhan58 × IRBL9-W cross generation using the RM101 marker: A total of 15 F_1 plants were compared with their parental DNA using the RM101 marker, and out of these, 3 F_1 plants were selected. After analyzing the data from three gel docs, 3 F_1 plants were confirmed for the BRRI dhan58 × IRBL9-W genotype. True F_1 plants were confirmed based on the presence of double bands, each corresponding to the parental bands shown by the polymorphic primers. The confirmed F_1 plants for BRRI dhan58 × IRBL9-W were 5, 6, and 10.

BC_1F_1 seeds production

To produce BC_1F_1 seeds, F_1 plants were kept in labeled pots. Two sets of IRBL9-W were seeded for synchronization of flowering with F_1 plants to produce BC_1F_1 seeds. Subsequently, F_1 plants were emasculated, and then pots were placed in the greenhouse. The next morning, pollination was performed by dusting male flowers onto the female stigma and then bagged with a glassine plastic bag with proper tagging. After a few days, unpolished seeds matured. A total of 97 BC_1F_1 seeds were produced from 14 F_1 plants (Fig. 3).

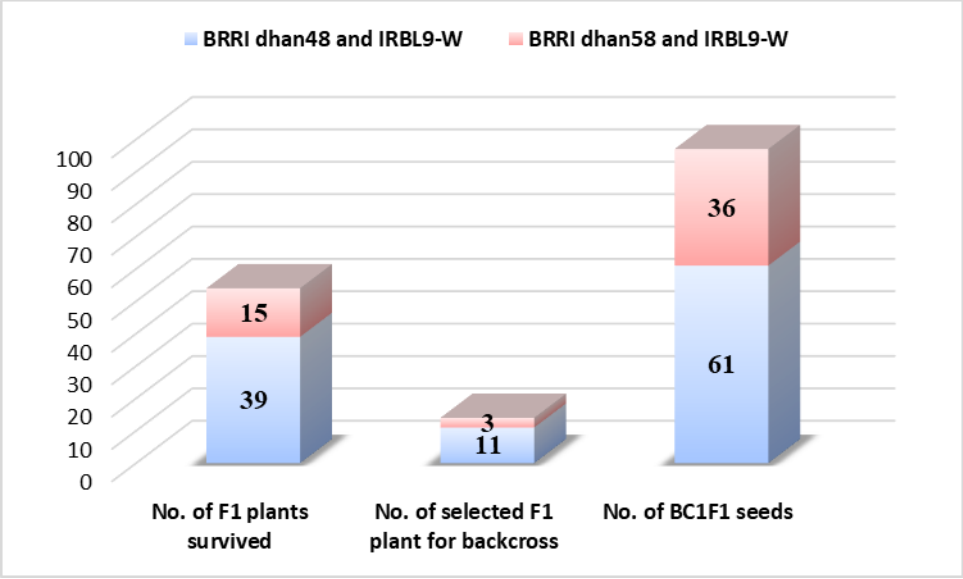

Fig. 3. Number of survived F_1 plants, selected F_1 plants for backcross breeding and BC_1F_1 seeds produced from BRRI dhan48 × IRBL9-W & BRRI dhan58 × IRBL9-W cross

The confirmed 14 F_1 plants were individually planted in plastic pots with appropriate labeling. Artificial emasculation was performed on the selected F_1 plants, which were then backcrossed with the recurrent parents (BRRI dhan48 and BRRI dhan58). Upon reaching maturity, BC_1F_1 hybrid seeds were collected, dried, and stored in paper bags with proper labeling. A total of 97 BC_1F_1 seeds were obtained from the selected cross combinations during the reporting period. The outcomes of the experiment mirror those of previously conducted studies aimed at developing blast-resistant varieties through marker-assisted selection processes. This study may offer unique opportunities for researchers to enhance blast-resistant advanced rice lines using various approaches.

Conclusion

The study involved primer surveys, PCR analysis for SSR markers, confirmation of F_1 plants, and backcrossing for BC_1F_1 seed production. Through meticulous selection processes and molecular marker analysis, 14 true F_1 plants were confirmed, leading to the production of 97 BC_1F_1 seeds. This approach holds promise for improving blast-resistant rice lines, crucial for ensuring food security amid disease threats.

Conflicts of Interest

The authors declare no conflicts of interest regarding publication of this manuscript.

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