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Development of Broad Spectrum and Durable Bacterial Blight Resistant Variety through Pyramiding of Four Resistance Genes in Rice

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Abstract: Bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* is a major biotic constraint on obtaining higher grain yields in rice. Marker-assisted backcross breeding (MABB) was performed by the pyramiding of *Xa4*, *xa5*, *xa13* and *Xa21* resistance genes in the popular variety, Ranidhan. A foreground selection in BC_1F_1, BC_2F_1, and BC_3F_1 progenies detected all the target genes in 12, 7 and 16 progenies by using the closely linked markers from a population size of 426, 410, and 530, respectively. The BB-positive progenies carrying the target genes with a maximal similarity to the recipient parent was backcrossed in each backcross generation. A total of 1784 BC_3F_2 seeds were obtained from the best BC_3F_1 progeny. The screening of the BC_3F_2 progenies for the four target genes resulted in eight plants carrying all the four target genes. A bioassay of the pyramided lines conferred very high levels of resistance to the predominant isolates of bacterial blight disease. In addition, these pyramided lines were similar to Ranidhan in 16 morpho-quality traits, namely, plant height, filled grains/panicle, panicles/plant, grain length, grain breadth, grain weight, milling, head rice recovery, kernel length after cooking, water uptake, the volume expansion ratio, gel consistency, alkali-spreading value, and the amylose content.

Keywords: bacterial blight disease; gene pyramiding; resistance genes; foreground selection; background selection; marker-assisted selection; *Xanthomonas oryzae* pv. *Oryzae*

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1. Introduction

Rice is sometimes referred to as the queen among the cereal grains. This crop is a livelihood for millions of people around the world. Rice grains serve as a staple food for more than half of the world's population. In India, the crop is cultivated in the diverse agro-ecology of the country, such as at high elevations to below sea level and from upland to waterlogged ecologies, including the favorable and lowland ecologies. Globally, the crop is cultivated in about 163.2 million hectares of land and 45% of these areas are under rainfed ecology with low productivity [1,2]. Many biotic and abiotic stresses are limiting the higher production from this rainfed ecology. In India, rainfed lowland rice occupies about 16 million ha of which 92% is in the eastern region of the country. The average productivity of rice in this region is low due to major biotic and abiotic stresses. Improved varieties that combine a high grain yield with in-built resistance to major diseases and insect pests are needed for this region.

Bacterial leaf blight (BB) disease is a major rice yield reducing factor for achieving higher production in this region of the country. This disease is widespread in India and causes considerable damage worldwide. BB is a destructive disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Losses due to attacks from this disease are very high and have been estimated in the range of 20–80% depending on the location, season, and resistance in the varieties [2–5]. A total of 45 resistance genes for the disease have been reported to date [6]. Gene-based and closely linked molecular markers are available for the majority of these reported BB resistance genes [2,6,7]. The future target for staple food grain demand is increasing in the country. India requires an additional rice production of about 2 million tons per year to meet the targeted demand by 2050 [1,5]. Again, this increase needs be obtained from less land, less water, less labor, and fewer chemicals, due to the constant battle against new emerging pathogens and pests and the possible adverse effects from climate change [2,8]. In addition, the country has varied agro-climatic zones for rice cultivation. Hence, breeding for broad spectrum and durable disease resistance is the most economical, effective, and environment friendly manner to manage this disease.

The large acreage cultivation of the BB-susceptible rice variety Ranidhan is being taken up in India. Therefore, it is urgently necessary to develop a broad spectrum and durable BB disease-resistant Ranidhan variety for the eastern Indian states, particularly for the Odisha state of the country. In addition, the deployment of a single resistant gene for the development of host plant resistance may lead to the breakdown of the resistance due to long term cultivation and the presence of diverse climatic zones in the country. Thus, this situation necessitates the development of a BB-durable version of the variety in the country. Therefore, we attempted the gene pyramiding of four resistance genes in the popular variety 'Ranidhan' through marker-assisted breeding to check the resistance breakdown in the variety. The incorporation of multiple resistance genes into a popular variety through the conventional breeding method is difficult and time consuming. The BB resistance breakdown is much lower in the four-resistance gene combination of *Xa21*, *xa13*, *xa5*, and *Xa4* in a single genetic background [9–11]. BB resistance genes have been pyramided successfully in the high yielding but BB-susceptible rice varieties for broad spectrum resistance to the disease through marker-assisted breeding in the country [2,5–7]. Several research reports are available for tightly linked molecular markers for BB resistance genes that are used in many marker-assisted breeding programs [12–20]. We report herein the successful development of four BB resistance gene-pyramided lines carrying *Xa21*, *xa13*, *xa5*, and *Xa4* in the popular variety 'Ranidhan' through a marker-assisted breeding approach.

2. Materials and Methods

2.1. Plant Materials

Ranidhan is a popular variety from Odisha state but is highly susceptible to BB disease. The rice variety CR Dhan 800 carries four BB resistance genes, viz., *Xa21*, *xa13*, *xa5*, and *Xa4*, used as the donor parent in the resistance gene pyramiding program, as described in the schematic diagram (Figure 1). The donor parent, CR Dhan 800, was hybridized with Ranidhan to produce the F_1 seeds. Hybridity in F_1 plants was checked using *Xa21* marker and the true F_1 plant was backcrossed with recipient parent, Ranidhan. All the BC_1F_1 seeds were raised and the foreground positive progenies for *Xa4*, *xa5*, *xa13*, and *Xa21* resistance genes were selected using the closely linked markers (Table 1). Phenotypic selection was performed among the foreground positive plants in BC_1F_1 generation to select the type most similar to Ranidhan parent. Best identified plant in the BC_1F_1 4 resistance gene-carrying plant was crossed with recurrent parent Ranidhan, and BC_2F_1 seeds were produced. All BC_2F_1 plants were raised and the foreground positive plant with maximum similarity to the recurrent parent was again hybridized to produce BC_3F_1 seeds. Foreground selections were continued in BC_3F_1 generation progenies to select four target-gene-carrying plants from the segregating population. A phenotypically maximally similar plant with recipient parent was selfed to select homozygous lines for target gene combinations in BC_3F_2 generation. Seeds of the plant carrying homozygous target genes were increased

during dry season, 2020, for bioassay and evaluation trials. Evaluation and bioassay trials for the BB-pyramided and parental lines were conducted during the wet seasons, 2020 and 2021.

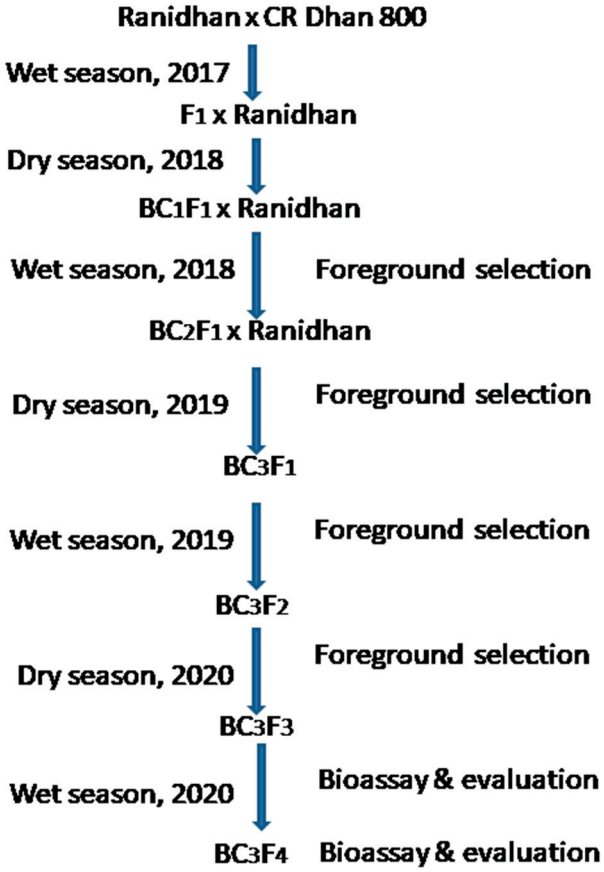

Figure 1. Schematic diagram for introgression of four BB resistance genes, *Xa4*, *xa5*, *xa13*, and *Xa21*, into popular rice variety, Ranidhan, through marker-assisted backcross breeding.

Table 1. Markers used for foreground selection of four bacterial blight resistance genes in marker-assisted backcross breeding.

Resistance Gene	Chromosome Number	Marker	Primer Sequences Used for Gene Detection		Expected Size (bp)	Band Type	References
			Forward (5′-3′)	Reverse (5′-3′)			
xa5	5	RM122	GAGTCGATGTAAT GTCATCAGTGC	GAAGGAGGTATCG CTTTGTTGGAC	260 bp	SSR	[21,22]
		xa5S (Multiplex)	GTCTGGAATTTGCT CGCGTTCG	TGGTAAAGTAGATA CCTTATCAAACTGGA	160 bp	STS	[10]
		xa5SR/R (Multiplex)	AGCTCGCCATTCAA GTTCTTGAG	TGACTTGGTTC TCCAAGGCTT			
xa13	8	Xa-13 prom	TCCCAGAAAGCTA CTACAGC	GCAGACTCCA GTTTGACTTC	500 bp	STS	[23]
Xa21	11	pTA248	AGACGCGGAAGG GTGGTTCCCGGA	AGACGCGGTAATC GAAGATGAAA	1000 bp	STS	[24]
Xa4	11	MP-Nbp-131	ATCGATCGATCTT CACGAGG	TCGTATAAAAG GCATTCGGG-	160 bp	STS	[25]

2.2. DNA Isolation and PCR Amplification

A mini scale DNA preparation was isolated following the procedure of standard protocol [26]. The PCR reaction mixture was prepared containing 30 ng templates DNA, 200 µM dNTPs, 5 picomoles of each of the primers, 1X PCR buffer (10 mM Tris–HCl, pH

8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.01 mg/mL gelatin), and 0.6 units of Taq DNA polymerase in a volume of 20µL. PCR amplification of the target sequences were performed as per earlier reports (Table 1). Gel electrophoresis was performed to separate the PCR products and gel images were captured by documentation system (SynGene, Germany) and further analyzed. The steps for polymerase chain reaction, electrophoresis, and gel documentation were performed following the protocols as per earlier publications [27–29].

2.3. Marker Analysis

The molecular markers (gene specific and linked), available publicly for the four target genes, were used in the backcross-segregating populations for foreground selection (Table 1). Data analysis was performed, and similarity matrix was constructed from the binary data using the Jaccard's coefficients. The dendrogram was generated using the unweighted pair group method arithmetic average (UPGMA) algorithm and applying DARwin 6 software (Montpellier cedex, France) [30]. The marker data analysis, construction of similarity matrix from the binary data using Jaccard's coefficients, generation of the dendrogram, and principal component analyses were performed following the earlier publications [31–33].

2.4. Bioassay against BB Resistance

Seedlings of the pyramided and parental lines at 45 days were inoculated with eight virulent *Xoo* isolates. The eight highly virulent strains of BB pathogen maintained at ICAR-National Rice Research Institute, Cuttack, Odisha, India, were used for inoculation in the tested materials. These eight isolates were identified based on their reaction against the near isogenic line differentials carrying resistance genes *Xa3, Xa4, xa5, Xa7, Xa10, xa13,* and *Xa21*. Procedure of Kauffman et al. [34] was followed for preparing the *Xanthomonas* strains' suspension in sterile water to obtain approximately 10^9 cells/ml *Xoo* strains in the prepared solution. Scissors were dipped into the bacterial suspension and clip inoculation treatment was applied to five leaves of five different plants from each entry at the maximum tillering stage. The lesion lengths (LL) were recorded after 15 days of inoculation. The pyramided lines or parents were categorized as resistant (R, LL $\leq$ 3.0 cm), moderately resistant (MR, 3.0 cm < LL $\leq$ 6.0 cm), moderately susceptible (MS, 6.0 cm < LL $\leq$ 9.0 cm), or susceptible (S, LL > 9.0 cm), as in the previous publications [11,35].

2.5. Characterization for Morphologic, Quality and Yield Traits

Twenty-five-day-old seedlings of pyramided lines carrying 4 resistance genes and single gene along with the parents were transplanted in plots with a size of 9.6 m^2 at 15 $\times$ 20 cm spacing for each test genotype. Planting was adopted using randomized complete block design (RBD) with three replications at forty plants per row with eight rows per entry in the research farm of ICAR-National Rice Research Institute (NRRI), Cuttack, during wet seasons, 2020 and 2021. Data were recorded for 16 morpho-quality traits, namely, plant height (cm), number of filled grains/panicle, panicles/plant, grain length (mm), grain breadth (mm),1000-grain weight (g), milling (%), head rice recovery (%), kernel length after cooking (mm), water uptake (ml), volume expansion ratio, gel consistency, alkali-spreading value, and amylose content (%), recorded from 10 plants of each entry and replication, while days to 50% flowering and plot yield were based on the whole plot. Standard method described by Tan et al. [36] was used for calculation of head rice recovery. Gel consistency (GC) was estimated as per the standard procedure of Cagampang et al. [37]. The procedure of Little et al. [38] was used to estimate the alkali-spreading value of the samples. Cooking qualities were determined by analyzing 25 grains in a test tube. For this, 20 min soaking of the grains in 20 ml distilled water was applied and then the test tubes were kept in boiling water for 10 min. After cooling, the length and breadth of 10 cooked kernels were measured and average value was estimated. For estimation of amylose content of the test genotypes, standard protocol of Juliano [39] was used. Analysis of variance for

the recorded morphologic and quality traits and principal component analysis (PCA) were performed using cropstat software as in the previous publications [40–43].

3. Results

3.1. Selections in the Backcross Progenies during the Forward Breeding

A resistance breeding program for the improvement of BB resistance in the popular variety Ranidhan was undertaken with gene-specific and linked molecular markers for the pyramiding of four BB resistance genes, namely, *Xa4*, *xa5*, *xa13,* and *Xa21*. The gene specific and linked markers were integrated in each backcross generation to select the four target genes carrying progenies. The molecular markers used for screening the progenies carrying the target genes in the derived plants were first validated in the parental lines (Table 1).The quality of true hybridity in the F_1 generation plants was checked with the *Xa21* marker. One of the true F_1 plants was crossed with a recipient parent to produce BC_1F_1 seeds. A total of 446 BC_1F_1 seeds were produced and grown in the next season. All the BC_1F_1 progenies were screened in a step-wise manner using the foreground markers. The foreground selections for the *Xa21* gene using the marker pTA248 in the 426 BC_1F_1 plants detected the presence of 213 progenies carrying *Xa21*-resistance-gene-specific bands (1000 bp). The plants carrying *Xa21* genes were further screened for the presence of the *Xa4* resistance gene using the marker MP-Nbp-131. A total of 105 plants showed the presence of the *Xa4* specific band (160bp) among the *Xa21* positive plants. All those positive plants were screened for the presence of the *xa5* resistance gene using the markers RM122 and the multiple marker (xa5S and xa5SR/R). These 105 plants were screened for the presence of *xa5* resistance gene specific bands (260bp) and 51 plants were positive. All those positive 51 plants carrying *Xa21* + *Xa4* + *xa5* were screened for the presence of the *xa13* gene. The foreground selection detected 12 plants with *xa13* specific bands (500bp). All these 12 plants were carrying the gene combination of *Xa21* + *Xa4* + *xa5* + *xa13* (Figure 2). Based on the phenotypic selection, the most similar plant among these 12 plants, SBMAS 2232-203, a BC_1F_1 progeny, was selected for next backcrossing.

The best identified plant in BC_1F_1, SBMAS 2232-203, carrying four resistance genes, was crossed with a recipient parent 'Ranidhan' and 410 BC_2F_1 seeds were produced. All the BC_2F_1 plants were screened using the four target gene foreground markers. The results of the foreground selection revealed the presence of 187 progenies carrying *Xa21*-resistance genes. All the plants that were positive for the *Xa21* gene were screened for the presence of the *Xa4* resistance gene. The genotyping data analysis revealed 78 BC_2F_1 plants carrying *Xa21* + *Xa4* genes. Those 78 positive plants were screened for the presence of the *xa5* resistance genes. The results indicated the presence of *xa5* specific band in 31 plants. These progenies carrying *Xa21* + *Xa4* + *xa5* resistance genes were genotyped using the xa13 markers. Finally, seven plants were observed to produce *xa13* gene specific band. Therefore, when counted for the presence of all four BB resistance genes, seven BC_2F_1 derived lines were found to carry these genes (Figure 3). Among these seven plants, SBMAS 2232-203-32, a BC_2F_1 progeny, was observed to possess maximum similarity with the recipient parent, Ranidhan.

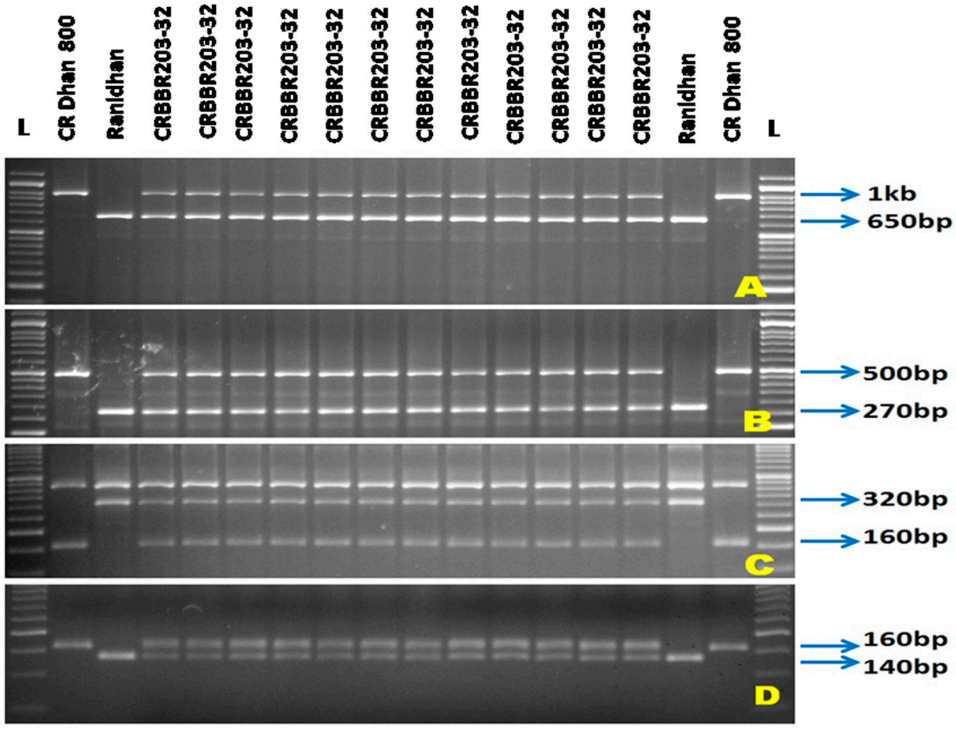

Figure 2. Electropherogram of BC_1F_1 derivatives of rice variety Ranidhan for four BB resistance genes *Xa21*, *xa13*, *xa5*, and *Xa4* using markers. (**A**) pTA248; (**B**) Xa-13 prom; (**C**) xa5S/R (Multiplex); (**D**) MP-Nbp-131. Lane 1, 18: 50 bp ladder; Lane 2, 17: CR Dhan 800; Lane 3, 16: Ranidhan; Lane 4–15: BC_1F_1 derivatives of rice variety, Ranidhan.

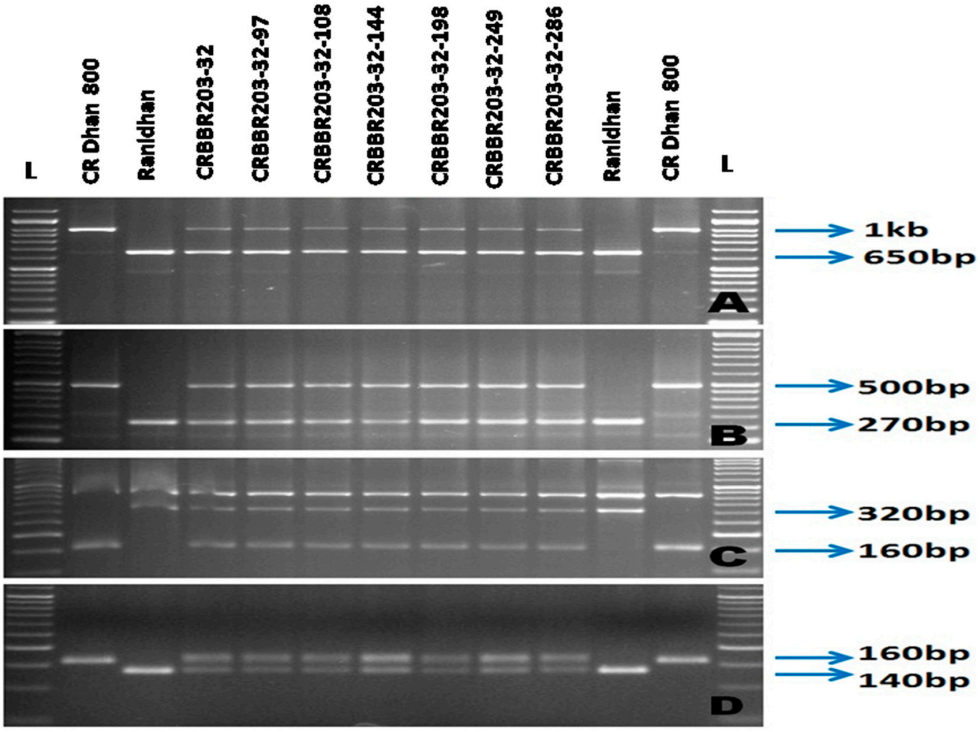

Figure 3. Electropherogram of BC_2F_1 derivatives of rice variety, Ranidhan for four BB resistance genes *Xa21*, *xa13*, *xa5* and *Xa4* using markers (**A**) pTA248; (**B**) Xa-13 prom; (**C**) xa5S/R (Multiplex); (**D**) MP-Nbp-131. Lane 1, 13: 50 bp ladder; Lane 2, 12: CR Dhan 800; Lane 3, 11: Ranidhan; Lane 4–10: BC_2F_1 derivatives of rice variety, Ranidhan.

The BC_2F_1 line, SBMAS 2232-203-32, was crossed with the recipient parent 'Ranidhan' and 530 BC_3F_1 seeds were produced. All those BC_3F_1 plants were screened with four foreground markers. The banding analysis of the BC_3F_1 plants showed a total of 234 plants positive for *Xa21.* Those 234 BC_3F_1 progenies were screened for the presence of the *Xa4* resistance gene using the foreground marker MP-Nbp-131. A total of 98 progenies were detected to carry the target gene, *Xa4.* The 98 progenies carrying the *Xa21* and xa4 genes in combination were screened for the presence of the *xa5* resistance gene. The foreground selection detected 42 progenies carrying the target gene, *xa5*. Finally, all those 42 progenies carrying the resistance gene combination of *Xa21* + *Xa4* + *xa5* were genotyped using the marker of the resistance gene *xa13*. Among those progenies, 16 plants were observed to carry all the four target genes (Figure 4).

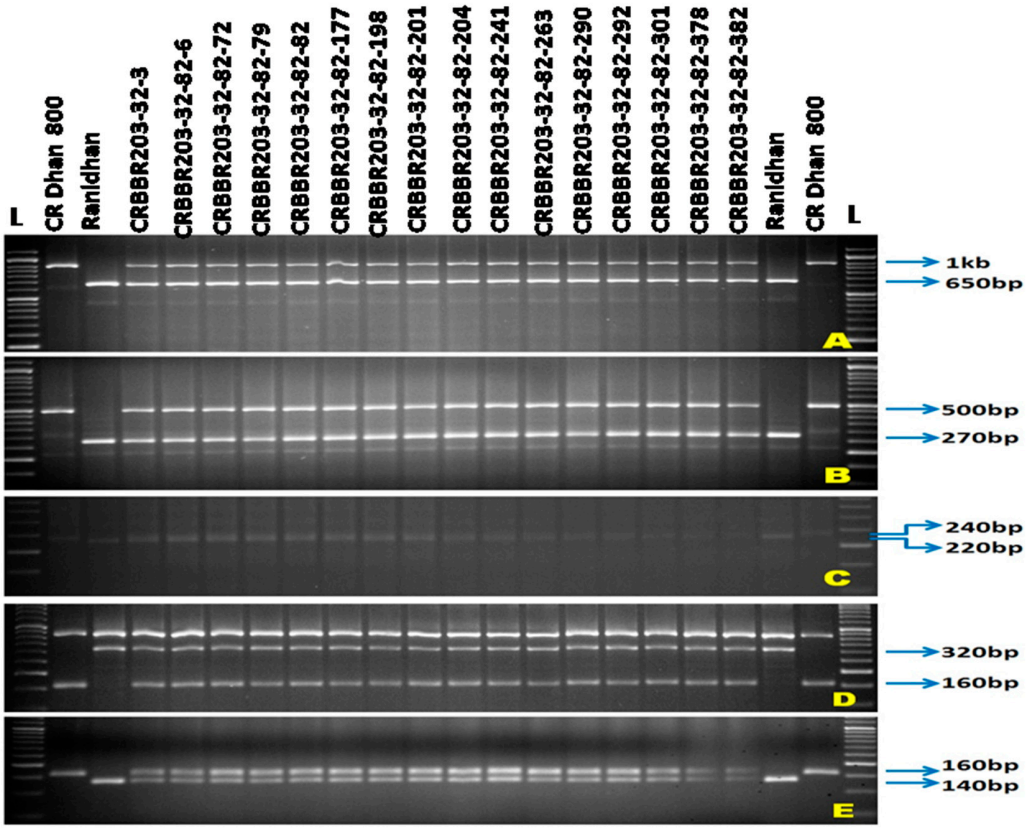

Figure 4. Electropherogram of BC_3F_1 derivatives of rice variety, Ranidhan for four BB resistance genes *Xa21, xa13*, *xa5* and *Xa4* using markers (**A**) pTA248 for *Xa21*; (**B**) Xa-13 prom for *xa13*; (**C**) RM122 & (**D**) xa5S/R (Multiplex) for *xa5*; (**E**) MP-Nbp-131 for *Xa4*. Lane 1, 22: 50 bp ladder; Lane 2, 21: CR Dhan 800; Lane 3, 20: Ranidhan; Lane 4–19: $BC3F_1$ derivatives of rice variety, Ranidhan.

Among these 16 BC_3F_1 progenies, SBMAS 2232-203-32-82 was observed to possess the maximum similarity with the recipient parent Ranidhan. The best progeny, SBMAS 2232-203-32-82, which was carrying the *Xa21* + *Xa4* + *xa5* + *xa13* gene combination, was self-pollinated in the next generation. A total of 1784 BC_3F_2 seeds were obtained from SBMAS 2232-203-32-82's progeny and were raised during the 2018 wet season. All the BC_3F_2 progenies were screened using the markers of the resistance genes *Xa4*, *xa5*, *xa13*, and *Xa21*. Out of the total 1784 BC_3F_2 progenies, 442 plants showed the presence of the *Xa4* resistance gene. All those positive plants were genotyped using the *Xa21* resistance gene marker, pTA248, and 108 were detected with its specific band. All the positive progenies were genotyped using RM122 and the multiplex marker for the detection of the *xa5* resistance gene. A total of 26 plants were homozygous for the *xa5* resistance gene. Finally, all those plants carrying the *Xa4* + *Xa21* + *xa5* gene combination in homozygous

condition were genotyped for detection of *xa13* resistance gene. The genotyping analysis showed only eight progenies to be homozygous for the target genes *Xa4*, *Xa21, xa5*, and *xa13* (Figure 5). The number of seeds of these eight pyramided lines increased in the next season for conducting bioassay and evaluation trials. The dendrogram obtained by using the SSR data classified the eight pyramided lines carrying four-gene combinations into two major clusters (Figure 6). Cluster I contained the recipient parent Ranidhan with eight pyramided lines while the donor parent was observed in cluster II.

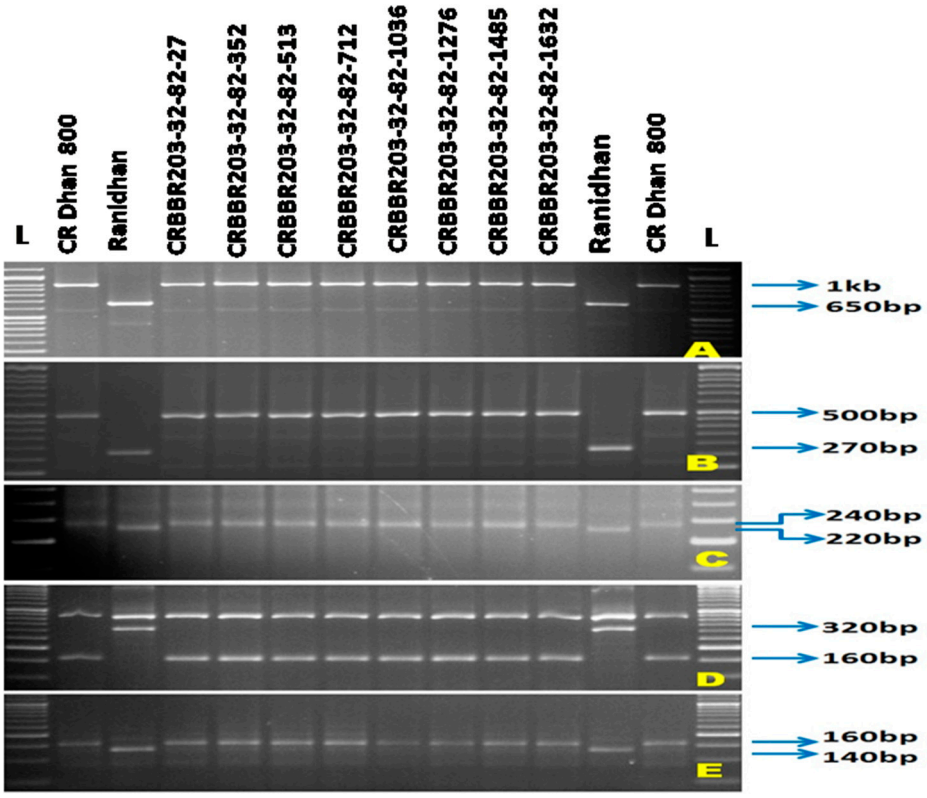

Figure 5. Electropherogram of BC_3F_2 derivatives of rice variety Ranidhan for four BB resistance genes, *Xa21, xa13*, *xa5* and *Xa4,* using markers (**A**) pTA248 for *Xa21*; (**B**) Xa-13 prom for *xa13*; (**C**) RM122 & (**D**) xa5S/R (Multiplex) for *xa5*; (**E**) MP-Nbp-131 for *Xa4*. Lane 1, 14: 50 bp ladder; Lane 2, 13: CR Dhan 800; Lane 3, 12: Ranidhan; Lane 4–11: BC_3F_2 derivatives of rice variety, Ranidhan.

3.2. Bioassays against BB Disease Pathogens

The eight BC_3F_3 and BC_3F_4 pyramided lines along with the parents (CR Dhan 800 and Ranidhan) were evaluated for their resistance and susceptibility to the BB pathogen using eight virulent *Xoo* strains (Table 2). The donor parent that contributed four BB resistance genes, CR Dhan 800, showed a resistance reaction to the pathogen exhibiting a mean lesion length of 2.55 cm (2.1–3.1 cm), while the recurrent parent Ranidhan was highly susceptible, exhibiting an average lesion length of 12.7 cm (10.6–14.1cm) (Table 2). The measured mean lesion lengths of the pyramided lines carrying the resistance gene combination *Xa21* + *xa13* + *xa5* + *Xa4* ranged from 2.49 to 2.89 cm. The pyramided line SBMAS 2232-203-32-82-85 showed a maximum resistance with a lesion length of 2.49 cm, while the donor parent CR Dhan 800 exhibited a similar length of 2.55 cm.

3.3. Grain Yield and Morpho-Quality Traits of the Converted Lines Carrying Four BB Resistance Genes

Eight new versions of the pyramided lines derived from Ranidhan in the BC_3F_3 and BC_3F_4 generations were evaluated during the wet seasons in 2020 and 2021, respectively. The recipient parent, Ranidhan, produced a grain yield of 5.865t/ha. All the pyramided

lines carrying four target genes produced greater grain yields than the recipient parent, Ranidhan (Table 3). All the pyramided lines were similar to the recipient parent, Ranidhan, based on 16 recorded major morpho-quality traits (Table 3). The dendrogram obtained based on the 16 morpho-quality traits recorded from 8 pyramided and 2 parental lines showed two clusters and all the pyramided lines were observed in a single cluster (cluster II) along with the recipient parent, Ranidhan (Figure 6). Cluster I was a mono-genotypic cluster accommodating only the donor parent, CR Dhan 800 (Table 3; Figure 6). The biplot diagram obtained for the genotype-by-trait using the 16 morphologic and quality traits of the 8 pyramided lines along with the parents also clearly distributed the pyramided and parental lines in the quadrants based on the similarity of the pyramided lines to the recipient parent (Figure 7). The majority of the new converted lines were in the first quadrant along with the recipient parent Ranidhan. The PCA1 explained 63.905% of the total variation while the second component exhibited 22.278% of the entire variation. Amongst the 16 studied agro-morphologic and quality traits, panicles/plant and water use contributed the maximum amount to the diversity (Figure 7).

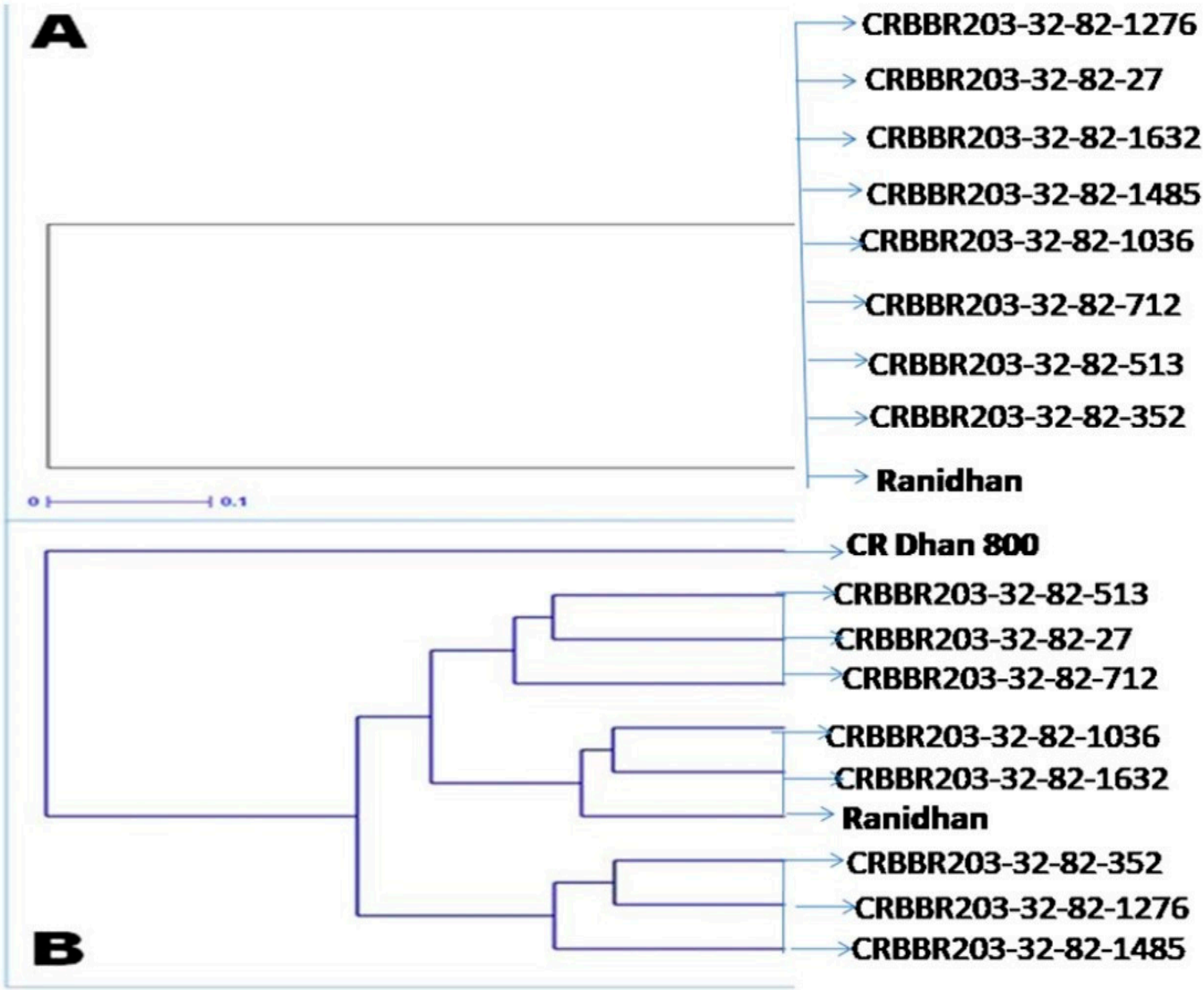

Figure 6. Dendrogram showing the relationship based on the phenotyping of 16 studied traits among (**A**) the 8 pyramided and recipient parent, Ranidhan and (**B**) the 8 pyramided and two parental lines.

Table 2. Bioassay of BC_3F_3 and BC_3F_4 pyramided lines using eight *Xoo*-inoculated strains. R—Resistant; MR—Moderately resistant; S—Susceptible; MLL—Mean lesion length in cm.

Sl. No.	Pyramided Lines	Gene Combination	Mean Lesion Length (MLL) in cm (Mean ± Standard Error)									
			Xoo Strains Inoculated									Disease Reaction
			Xa-17	Xa-7	xa-2	xb-7	xc-4	xd-1	xa-1	xa-5	MLL	
1	CRBBR203-32-82-27	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	2.81 ± 0.83	2.53 ± 0.72	3.14 ± 0.84	2.44 ± 0.68	2.73 ± 0.93	2.83 ± 0.76	2.23 ± 0.95	2.34 ± 0.85	2.63 ± 0.38	R
2	CRBBR203-32-82-352	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	2.53 ± 0.63	2.68 ± 0.64	2.42 ± 0.62	3.32 ± 0.96	2.92 ± 0.93	2.62 ± 0.94	2.71 ± 77	2.41 ± 0.63	2.70 ± 0.37	R
3	CRBBR203-32-82-513	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	2.12 ± 0.65	2.41 ± 0.62	2.53 ± 0.77	2.62 ± 0.72	2.71 ± 0.85	2.59 ± 0.52	2.92 ± 0.68	2.68 ± 0.85	2.57 ± 0.4	R
4	CRBBR203-32-82-712	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	3.39 ± 0.90	2.53 ± 0.56	2.59 ± 0.72	2.72 ± 0.82	2.83 ± 0.91	2.34 ± 0.53	3.24 ± 0.91	2.59 ± 0.57	2.69 ± 0,38	R
5	CRBBR203-32-82-1036	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	2.72 ± 0.85	2.24 ± 0.64	2.32 ± 0.56	2.43 ± 0.81	2.74 ± 0.84	2.13 ± 0.78	2.81 ± 0.82	2.71 ± 0.89	2.51 ± 0.4	R
6	CRBBR203-32-82-1276	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	3.38 ± 0.97	2.57 ± 0.61	2.91 ± 0.73	3.14 ± 0.85	2.94 ± 0.55	2.58 ± 0.59	2.53 ± 0.66	3.13 ± 0.93	2.89 ± 0.36	R
7	CRBBR203-32-82-1485	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	3.13 ± 0.83	2.51 ± 0.47	3.24 ± 0.92	2.83 ± 0.58	2.88 ± 0.55	2.61 ± 0.46	2.52 ± 0.73	2.92 ± 0.78	2.83 ± 0.35	R
8	CRBBR203-32-82-1632	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	2.62 ± 0.74	2.72 ± 0.58	2.62 ± 0.73	2.71 ± 0.92	3.23 ± 0.96	3.14 ± 0.93	2.54 ± 0.56	2.84 ± 0.75	2.80 ± 0.35	R
9	CR Dhan 800 (donor)	*Xa*21 + *xa*13 + *xa*5 + *Xa*4	2.41 ± 0.74	2.13 ± 0.48	2.34 ± 0.52	2.63 ± 0.67	3.14 ± 0.72	2.79 ± 0.91	2.61 ± 0.76	2.52 ± 0.69	2.57 ± 0.36	R
10	Ranidhan (recipient)	-	11.78 ± 1.25	13.18 ± 1.63	13.83 ± 1.52	11.71 ± 1.12	10.63 ± 1.25	14.14 ± 1.23	12.32 ± 0.92	14.13 ± 1.13	12.72 ± 1.26	S

Table 3. Analysis of morphologic and grain quality parameters of BC_3F_3 and BC_3F_4-pyramided and parental rice lines evaluated during wet seasons, 2020 and 2021.

Serial Number	Pyramided Lines	PH (cm)	DFF (Days)	PN	SF (%)	TW (g)	KL (mm)	KB (mm)	Milling (%)	HRR	VER	WU (ml)	KLAC (mm)	ASV	GC	AC (%)	PY (t/ha)
1	CRBBR203-32-82-27	108	115	294	87.2	19.75	5.45	2.28	68.7	63.2	4.4	162.5	8.4	6.0	59	23.25	6.325
2	CRBBR203-32-82-352	107	114	308	87.3	19.23	5.55	2.34	68.5	64.5	5.0	165.0	8.5	5.5	63	24.75	6.075
3	CRBBR203-32-82-513	109	115	296	88.1	19.65	5.58	2.35	69.1	61.7	4.7	170.0	8.3	6.0	57	24.55	6.385
4	CRBBR203-32-82-712	106	110	294	86.5	20.15	5.65	2.35	68.9	64.2	4.7	168.5	8.6	5.0	63	24.75	6.250
5	CRBBR203-32-82-1036	103	116	290	88.3	20.25	5.75	2.26	69.2	65.1	4.8	155.5	8.4	5.0	59	23.18	6.580
6	CRBBR203-32-82-1276	105	112	306	86.7	19.65	5.46	2.16	67.3	66.2	4.4	161.5	8.3	5.5	61	25.25	6.210
7	CRBBR203-32-82-1485	110	113	310	86.2	19.52	5.82	2.25	66.8	65.1	4.4	158.0	9.1	5.5	57	25.05	6.385
8	CRBBR203-32-82-1632	104	112	292	87.5	20.35	5.48	2.15	66.7	65.5	4.8	159.5	8.3	6.0	59	24.35	6.165
9	CR Dhan 800 (donor)	108	115	312	89.2	20.65	6.36	2.14	71.1	58.1	4.7	176.5	9.9	4.5	67	22.36	6.115
10	Ranidhan (recipient)	105	116	288	83.1	19.3	5.44	2.30	69.5	64.8	4.7	158.5	8.1	6.0	57	25.12	5.865
	$LSD_{5\%}$	5.46	4.96	31.36	9.32	2.16	0.74	0.164	7.324	7.456	-	16.38	0.754	-	-	2.724	0.348
	CV%	3.86	1.24	10.78	5.18	4.84	7.35	8.146	6.685	9.328	-	6.284	7.36	-	-	6.742	10.36

PH—Plant height (cm); DFF—Days to 50% flowering; PN—number of panicles/m^2; SF—spikelet fertility (%); TW—1000-seed weight; KL—kernel length; KB—Kernel breadth; M—Milling (%); HRR—Head rice recovery (%); KLAC—Kernel length after cooking (mm); VER—Volume expansion ratio; WU—Water uptake (mL; ASV—Alkali-spreading value; GC—Gel consistency; AC—Amylose content (%); PY—Plot yield (t/ha).

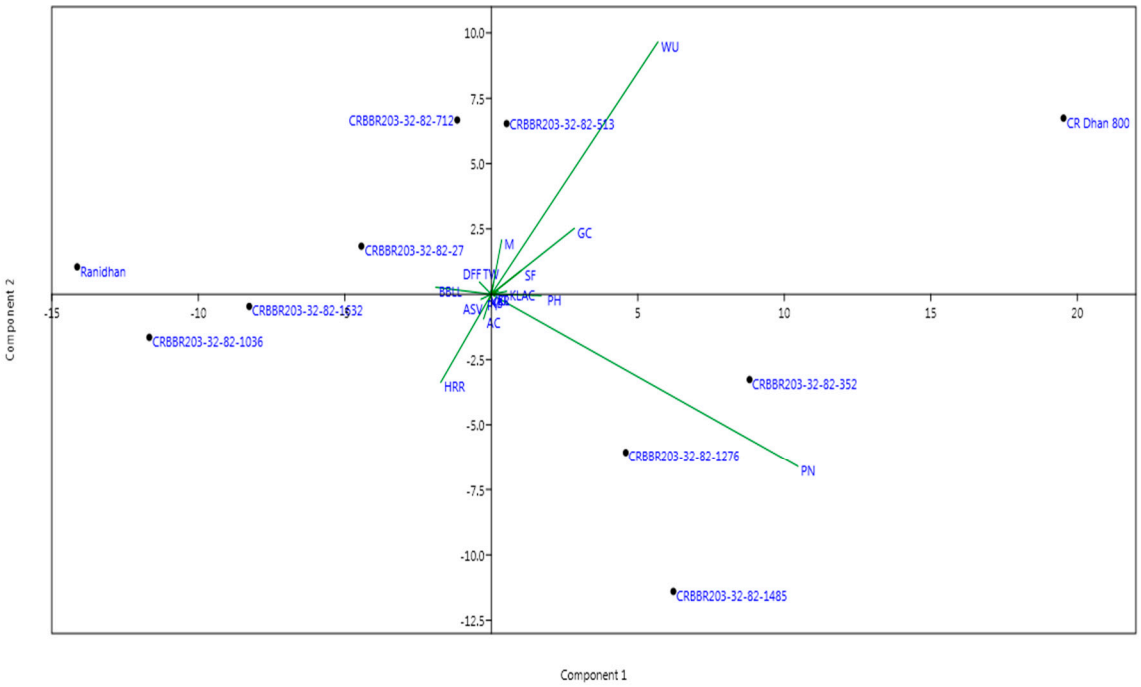

Figure 7. Biplot diagram of the 8 pyramided and parental lines of rice for the first two principal components: PH—Plant height (cm); DFF—Days to 50% flowering; PN—Panicles/plant; SF–Spikelet fertility TW—1000-grain weight (g); GL—Grain length (mm); GB—Grain breadth (mm); M—Milling (%); HRR—Head rice recovery (%); KE—Kernel length after cooking (mm); VER—volume expansion ratio; WU—water uptake (mL) ASV—Alkali-spreading value; GC—gel consistency; AC—Amylose content (%); BB MLL—Bacterial blight lesion length (cm); YLD—plot yield (t/ha).

4. Discussion

Plant breeding that integrates robust molecular markers for the target traits in the breeding increases the precision of introgressed target genes into the recipient variety. Marker-based breeding also reduces the time for the development of a variety compared to the breeding duration through conventional breeding. In this molecular breeding program, we could incorporate four BB resistance genes into the popular variety, Ranidhan, by integrating molecular markers with the phenotypic selections during the backcross generations. The BB-pyramided lines were developed in three backcrosses followed by one selfing to achieve the new version of Ranidhan carrying four resistance genes with the intact main traits of the recipient parent (Figure 1). Controlling the bacterial blight disease using the host plant resistance approach is a much cheaper technique from a farmer's perspective than the chemical control approach. In addition, this approach is also environment friendly. Therefore, the development of Ranidhan-pyramided lines carrying four BB resistance genes is an important improvement in late-maturing rainfed rice suitable for the BB-endemic areas. Success in the development and release of varieties through marker-assisted breeding has previously been reported, showing a shorter duration and more precision breeding in the transfer of the desired trait into the recipient parent [2,4,6–8,11–15,19,20,43,44]. However, this study's development, which uses precision breeding to achieve broad-spectrum resistance using four BB resistance genes in the popular 'Ranidhan' variety, is an important achievement for the late-duration rice ecology of Odisha state. The pyramided version of Ranidhan will be a good substitute for Ranidhan in Odisha state for its lowland rice ecology.

The developed pyramided plants carrying four target genes (*Xa21*, *xa13*, *xa5*, and *Xa4*) in the popular recurrent parent background were very similar to the recipient parent, Ranidhan, based on 16 agro-morphologic and quality traits. The improved donor line used in this breeding program may contribute fewer undesirable effects than using a landrace or wild type donor source for BB resistance, as has been performed in a few BB resistance

breeding programs. Previous publications using improved sources of resistance suggest that the donor lines may contribute less undesirable drag compared to the wild or landraces as a donor [5,10,11]. The evaluation results indicated that all the eight pyramided lines were higher yielding than the recipient variety and were highly similar to the recipient parent 'Ranidhan' (Table 3). The higher yield of the pyramided lines may be due to their greater BB resistance compared to the recipient parent. In addition, the pyramided lines are similar to the recipient variety with respect to the important agro-morphologic and quality traits that have already been adopted by farmers. Hence, the disease-resistant version of the popular variety is expected to be popular among farmers. Similar results were also reported by earlier researchers [1,11,19,21,36,44].

The biplot diagram for the genotype-trait plot placed the pyramided lines together while the BB donor line was in a separate quadrant. This clearly indicated that the pyramided lines were closer to Ranidhan in phenotypic terms (Figure 7). However, the recipient parent was placed closer to the pyramided lines and in the same quadrant. The pyramided lines located near the origin are more stable compared to the distant ones. The evaluation results of the eight pyramided lines showed that almost all the lines were similar to the recipient parent, and a few were even better in yield than the recipient parent (Figure 7; Table 3). In addition, it was revealed from the results that NILs carrying four BB resistance genes (*Xa4*, *xa5*, *xa13* and *Xa21*) together in a single variety background did not show antagonistic effects or penalties for yield and other traits. Similar findings were also reported by other researchers in their gene-pyramiding work [11,12,20,30,35,36].

The eight pyramided lines produced greater yields than the recipient parent, as revealed from the evaluation trial. The higher yield obtained might have been due to a higher level of resistance in the pyramided line for the BB disease and an absence of a yield penalty due to the pyramiding of BB resistance genes. Similar results were also reported earlier in many publications [8,11,36]. Thus, the deployment of four resistance genes in a popular variety such as Ranidhan will provide broad spectrum and durable resistance suitable for bacterial blight disease in endemic-lowland-rice areas in the region. The breeding work supports the use of marker-assisted breeding for conferring a higher resistance to BB stress, which is required in India due to the availability of varied agro-climatic zones in the country.

Resistance breeding using a single resistance gene is risky, as there are chances of resistance break-drown. The pyramiding work of BB resistance in Ranidhan using four resistance genes, namely, *Xa21*, *xa13*, *xa5*, and *Xa4,* and successfully increasing the level of resistance in the Ranidhan background, is an important achievement. The pyramided lines will be highly useful for Odisha state and will provide a solution in the BB disease-endemic areas towards eastern Indian lowland rice ecology. The grain quality and its cooking traits, namely, the milling %, head rice recovery %, kernel breadth, kernel length (mm), kernel length after cooking, water uptake, volume expansion ratio, alkali-spreading value, gel consistency, and amylose content (%), are almost retained in the pyramided lines to a degree similar to the recipient parent, Ranidhan. In addition, the pyramided lines were high yielding as in the recipient and the bacterial blight resistance has already been reported to be durable in the case of this four-resistance-gene combination. India has many agro-climatic zones that offer a wide scope for the creation of many virulent *Xoo* strains. Therefore, these BB-pyramided lines carrying *Xa21*, *xa13*, *xa5*, and *Xa4* and that are similar to the Ranidhan variety are expected to be adopted by the Ranidhan growers in the targeted region of the country.

5. Conclusions

The deployment of a single resistant gene is risky, as there are chances of resistance breakdown. The BB-pyramided lines carrying four resistance genes—*Xa21*, *xa13*, *xa5*, and *Xa4*—in the Ranidhan variety background exhibit a broad resistance against the virulent strains of the pathogen. The pyramided lines are suitable against multiple pathogen variation, which is a concern under the recent climate change and varied agro-climatic conditions.

The gene pyramiding work in the popular variety background through molecular breeding will provide a solution in the bacterial-blight-endemic areas such as the eastern Indian lowland rice ecosystems. The grain and its cooking quality characteristics such as the milling %, head rice recovery %, kernel length (mm), kernel breadth (mm), kernel length after cooking (mm), alkali-spreading value, gel consistency, and amylose content (%) are almost the same in the pyramided lines as in the recipient parent. The quality features of the popular variety are retained along with the high grain yield and durable bacterial blight resistance in the selected pyramided lines. India has many agro-climatic zones that offer a wide scope for the creation of many virulent *Xoo* strains. Therefore, these BB-pyramided lines carrying *Xa21*, *xa13*, *xa5*, and *Xa4* are expected to provide a good substitute to the existing susceptible varieties used by the farmers in the targeted region of the country.

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