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# Genetic Enhancement of Blast and Bacterial Leaf Blight Resistance in Rice Variety CO 51 through Marker-Assisted Selection

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**Abstract:** The increased use of chemicals in rice farming poses significant issues regarding the emergence of pesticide/fungicide resistance and environmental sustainability concerns. This study was aimed at the genetic improvement of blast, bacterial leaf blight (BB) and gall midge resistance in a popular rice variety CO 51 which already harbours a blast resistance gene *Pi54*. Efforts were made to pyramid an additional blast resistance gene *Pi9* along with two BB resistance genes (*xa13* and *Xa21*) and two gall midge resistance genes (*Gm1* and *Gm4*) into an elite rice variety CO 51 to enhance the resistance level to biotic stresses. The superior lines were selected using functional markers conferring resistance to blast (NBS4 and Pi54MAS linked to *Pi9* and *Pi54* genes, respectively) and BB [(*xa13Prom* (*xa13*) and pTA248 (*Xa21*)] and SSR markers linked to *Gm1* (RM1328) and *Gm4* (RM22550) for phenotypic screening and agronomic evaluation. The genotyping and phenotyping of $F_6$ and $BC_2F_6$ progenies of CO 51 X 562-4, for agronomic traits and resistance to BB and blast, identified ten superior progenies in $F_6$ and five superior progenies in $BC_2F_6$. The breeding lines harbouring both *xa13+Xa21* exhibited high levels of resistance to BB (score $\leq$ 1 cm) and *Pi9+Pi54* exhibited strong resistance to blast (score $\leq$ 2). Identified lines can be evaluated further for varietal improvement or utilised as genetic stocks in breeding programs.

**Keywords:** rice; gene pyramiding; stacking; blast; bacterial leaf blight; gall midge



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## 1. Introduction

Nearly half of India's population and one-third of the world's population rely on rice for calorie and carbohydrate intake [1]. Despite enormous production and resolving global hunger, a 30% increase in rice production is required by 2030 from the present level, and rice production needs to be boosted by 160 million tonnes [2] and should increase by 70% in 2050 [3] to ensure global food security and nutritional security. Due to modern civilisation, there is a reduction in cultivatable land and water resources for irrigation [4]. Continuous changes in the ecosystem, and pests and pathogens have evolved for their survival in an adverse environment. Due to several biotic and abiotic stresses, rice production can be affected by unfavourable climate changes. The productivity of rice continues to be under threat by biotic stresses, viz., blast and bacterial leaf blight (BB), which cause significant

losses in rice production [5]. Among fungal diseases affecting rice crops, blast disease ranked first among the top 10 diseases [6,7]. Rice yield losses are severe in the southern state of India due to blast and BB [5,8,9]. Blast, a fungal disease caused by the ascomycete fungus *Magnaporthe grisea* Barr., poses a significant threat to global rice production, resulting in yield losses of 70–80% [10,11]. Bacterial blight, caused by *X. o. pv. oryzae*, is considered the oldest and most destructive disease affecting rice [12]. It can lead to significant crop yield losses, up to 80% [13,14]. In India, during the *Kharif* season, the gall midge leads to a substantial yield loss of approximately USD 80 million, while the estimated loss on the Asian continent amounts to USD 550 million [15,16].

By way of releasing high-yielding varieties, food demand can be mitigated to meet the demand of the growing population [17]. In the modern era of genetics and biotechnology, various techniques are available to increase rice production and productivity by enhancing the host plant resistance against biotic stresses, through the pyramiding/stacking of multiple resistance genes/alleles into elite cultivars which provides strong and broad-spectrum resistance against many pathogens. So far, 102 resistance (*R)* genes [18,19] and nearly 500 QTLs [20] have been identified for rice blast disease. Among them, 38 R genes have been characterised at a molecular level and eight genes, namely *Pi9*, *Pi54*, *pi21*, *Pi50*, *Pi7*, *Pi57*, *Pigm*, and *Ptr*, have been reported as broad-spectrum resistance genes against blast disease [21]. The R genes were distributed to all 12 chromosomes in the rice genome except chromosome 3 [22]. In rice, *Pi-b* is the first R gene that was identified in the japonica variety [23]. *Pi9* is a major resistance gene, isolated from the wild species *Oryza minuta* [24,25], and *pi-kh*, which was renamed to *Pi54* [26] and isolated from the Tetep cultivar mapped in chromosome 11L [27], has resistance to major isolates of *M. oryzea* [28].

Another important and destructive disease is bacterial leaf blight (BB) caused by *(X.o* pv., *oryzae)*, which resulted in up to 40% of yield losses at the tillering stage and up to 50% of yield losses in the initial stage [29]. So far, 46 R genes have been identified for BB resistance and 13R genes, viz., *Xa1*, *Xa3/Xa26*, *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa10*, *xa13*, *Xa21*, *Xa23*, *xa25*(*t*), *Xa27*(*t*), and *xa41*(*t*), have been cloned and characterised at the molecular level [30]. In total, 29 genes are dominant resistant, viz., *Xa1*, *Xa3/Xa26*, *Xa4*, *xa5*, *Xa10*, *xa13*, *Xa21*, *Xa23*, *xa25*(*t*), *Xa27*(*t*), and *xa41*(*t*), whereas 17 genes are recessive resistant, viz., *xa5*, *xa8*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa25*(*t*), *xa26*(*t*), *xa28*(*t*), *xa31*(*t*), *xa32*(*t*), *xa34*(*t*), *xa41*(*t*), *xa42*, *xa44*(*t*), and *xa45*(*t*) [30]. A major dominant R gene, '*Xa21*', confers broad-spectrum resistance against many virulent isolates in India and was initially identified in wild species, *O. longistaminata* [31]. Another R gene *xa13* also confers broad-spectrum resistance against many virulent isolates in India and possesses mutation in the promoter region and is homologous to nodulin [5,32].

In 1978, Nelson proposed the concept of integrating specific QTLs/genes for resistance to abiotic and biotic stresses into crop varieties via gene pyramiding or introgression. Gene pyramiding involves combining two or more genes from different donor parents, each controlling multiple traits, into the genetic background of a desired variety, known as multi-trait introgression. This can be accomplished through three methods described by Singh [33]. 1. Separate backcross programs: Each donor parent is used as a pollen parent and crossed with the recurrent parent as a seed parent. The goal is to introgress the target gene from each donor parent into the genetic background of the recurrent parent. The resulting F1 lines are then crossed with the recurrent parent to produce Backcross Inbred Lines (BILs) with homozygous or heterozygous conditions for the targeted QTLs/genes [33]. Subsequently, the BILs carrying the targeted QTLs/genes are intercrossed to combine all desired QTLs/genes into a single genetic background. 2. Single backcross: Symmetrical mating- Each donor parent serves as a pollen parent and is crossed with the recurrent parent as a seed parent. The resulting F1 lines, genotyped with targeted QTLs/genes, are selected and intercrossed to combine all desired QTLs/genes into a single genetic background [33]. These intercrossed progenies carrying targeted QTLs/genes are then backcrossed with the recurrent parent to the Recover Recurrent Genome (RRG) and develop BILs. 3. Single backcross: Tandem mating. In this approach, the recurrent parent is first introgressed

with targeted QTLs/genes by crossing with one donor parent (improved recurrent parent). Then, the improved recurrent parent, now containing targeted QTLs/genes, is crossed with a second donor parent (also called the improved recurrent parent) to further introgress targeted QTLs/genes [33]. These methods allow for the development of crop varieties with improved stress tolerance and resistance by combining multiple beneficial traits from different donor parents into a single genetic background.

Das and Rao [34] developed an Improved Lalat variety that is tolerant to multiple stresses by incorporating four bacterial blight resistance genes (*Xa4*, *Xa21*, *xa13*, and *xa5*), as well as QTLs/genes for blast, gall midge, salinity, and submergence. In another study, Das et al. [35] successfully combined several QTLs/genes into a cultivar named enhanced Tapaswini (*xa13* and *Xa21*), which exhibits resistance to submergence (*Sub1*), salinity (*Saltol*), blast (*Pi9*, *Pi54*), and gall midge (*Gm1*, *Gm4*). This groundbreaking research involved the stacking of ten distinct genes/QTLs (six previously identified and four newly introduced) and their expression at desired levels in a new genetic background, showcasing the potential for a new era of molecular plant breeding. Dixit et al. [36] achieved the pyramiding of genes for blast (*Pi9*), bacterial leaf blight (BLB) (*Xa4*, *xa5*, *xa13*, and *Xa21*), brown plant hopper (BPH) (*Bph3*, *Bph17*), gall midge (*Gm4* and *Gm8*), and QTLs for drought tolerance (*qDTY1.1* and *qDTY3.1*) in the Swarna variety, combining a total of 11 genes with existing traits.

Through the utilisation of MAS, researchers introduced five biotic stress resistance genes (*Pi40*, *Xa4*, *Xa5*, *Xa21*, and *Bph18*) into a Korean japonica rice variety Jinbubyeoa, resulting in the creation of gene-pyramided breeding lines specifically targeting bacterial blight, blast, and brown plant-hopper [37]. These gene-pyramided lines exhibited impressive resilience against biotic stresses. SSR graphical mapping indicated that gene-pyramided lines retained 93% of the recurrent parent Jinbubyeo's genome. The researchers evaluated the impact of these QTLs/genes in a novel genotype using phenotypic screening methods, demonstrating enhanced levels of resistance/tolerance against the targeted stresses. Furthermore, through marker-assisted backcrossing, two significant QTLs (*Sub1* and *Qbph12*), associated with abiotic and biotic tolerance/resistance, were introduced into traditional jasmine rice cultivar KDML105. Positive progenies carrying both QTLs exhibited tolerance to both abiotic and biotic stresses, showcasing notable differences in Days to Flowering, Plant Height, and grain yield [38].

This present study focused on the development of a durable resistant cultivar against gall midge, blast, and BB pathogens through the stacking of six R genes. The closely linked markers, viz., xa13Prom for the *xa13* gene, pTA248 for *Xa21*, NBS4 for the *Pi9* gene, Pi54MAS for *Pi54* genes, RM1328 for *Gm1*, and RM22550 for *Gm4*, were used to confirm the presence of the target genes against blast, BB, and gall midge in rice cultivar CO 51.

## 2. Materials and Methods

### *2.1. Genotypes Used*

A popular rice variety CO 51 was used as a recurrent parent in this study. It is a short-duration (110–115 days), fine-grain, and high-yielding rice genotype cultivated in 14 states in India [39,40]. CO 51 is moderately resistant to blast due to the presence of the *Pi54* allele and is severely susceptible to BB disease. An intermittent genetic stock #562-4 derived between CO 43 X VRP 1 harbouring *Pi9*, *xa13*, *Xa21*, *Gm1*, and *Gm4* was used as a donor and was developed in the Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu India (Table 1. The details of markers listed in Supplementary Table S1) [41].

### *2.2. Development of Pyramided Lines to Enhance Biotic Stress Tolerance of CO 51*

For marker-assisted selection (MAS), leaf samples from all progenies and parent lines were collected 3 weeks after transplanting. DNA extraction was performed with the CTAB method [42]. Molecular markers NBS4, Pi54MAS, xa13Prom, pTA248, RM1321, and RM22550 were used for foreground selection which are closely linked with *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4*, respectively.

**Table 1.** Target region from donor parent and position.

| Target Traits | Target QTLs/Genes | Donor Parents | Chromosome | Position in Mb | References |
|---|---|---|---|---|---|
| Blast resistance | *Pi9* | 562-4 | 6 | 10.38 | [26,36,42] |
| | *Pi54* | CO 51 | 11 | 24.2 | [43,44] |
| BB resistance | *xa13* | 562-4 | 8 | 26.0 | [44–46] |
| | *Xa21* | 562-4 | 11 | 20.5 | [44–46] |
| Gall midge resistance | *Gm1* | 562-4 | 9 | 9.20 | [35] |
| | *Gm4* | 562-4 | 8 | 5.45 | [35] |

QTLs: Quantitative Trait Loci, BB: bacterial leaf blight.

The rice cultivar CO 51 was used as a female parent and the donor parent 562-4 was used as a male parent to develop $F_1$. The identification of true $F_1$ was performed using closely linked markers for the target genes, viz., NBS4, Pi54MAS, xa13Prom, pTA248, RM1328, and RM22550. Then, these true $F_1$s were crossed with recurrent parent CO 51 to generate $BC_1F_1$, and selfing was allowed for generating $F_2$. The positive $BC_1F_1$ plants were identified with foreground markers and crossed with CO 51 to generate $BC_2F_1$. Then, $BC_2F_1$ was allowed to $BC_2F_6$ and each generation was genotyped with foreground markers.

Then, simultaneously, the selected $F_2$ was allowed to generate $F_3$ and genotyped with linked markers. Superior $F_3$ progenies were selected based on grain type and advanced to $F_4$ in the field conditions. In $F_{4:5}$, selections were based on grain type and high yield over the recurrent parent CO 51. Selected progenies in $F_{5:6}$ and $BC_2F_6$ were used for blast screening in natural hotspot areas and BB screening in field conditions.

### *2.3. Screening of Selected RILs and BILs against Blast Pathogen*

Selected progenies of $F_{5:6}$ and $BC_2F_6$ were raised in the Uniform Bed Nursery (UBN) in the Hybrid Rice Evaluation Centre, Gudalur, Tamil Nadu, India. Plants were raised along with recurrent and donor parents. The susceptible check CO 39 was sown on both sides of UBN and one in every five rows to ensure a continuous supply of blast inoculum. The disease infection was measured at an interval of 15 days up to 45 Days After Sowing (DAS) in all the test entries. Scores 0–3 were considered resistant (R), 4–5 moderately resistant (MR), and 6–9 susceptible (S) [1].

### *2.4. Screening of Selected RILs and BILs against BB Pathogen*

Twenty-one day-old seedlings of selected progenies of $F_{5:6}$ and $BC_2F_6$ were transplanted in the main field along with parents and the susceptible check TN 1. TN 1 was transplanted once every five rows to ensure a continuous supply of BB pathogen. A virulent bacterial blight pathogen *X. o* p.v. *oryzae* was collected from the Department of Plant Pathology, Tamil Nadu Agricultural University (TNAU), Coimbatore. The pathogen *X. o* p.v. *oryzae* strain was grown in peptone sucrose agar media for the production of inoculum. The bacterium was scraped from all plates and suspended in sterilised double-distilled water. The maximum tillering stage (40–45 days) of rice was inoculated with BB pathogen *X.* p.v., *oryzae* using the clip inoculation method with sterilised scissors [47]. A total of ten plants were inoculated and approximately ten upper leaves/plants were measured for lesion length. The lesion lengths were measured 14 and 21 days post inoculation when the lesion was stable. The average lesion length was calculated from 10 maximum lesion lengths per entry. Based on lesion length, plants were classified as resistant (R) when the length was 0–3 cm, moderately resistant (MR) when the length was more than 3–6 cm, moderately susceptible (MS) when the length was more than 6–9 cm, and susceptible (S) when the length was more than 9 cm [36,48].

### *2.5. Agronomic Performance and Grain Quality Parameters of RILs and BILs*

Selected superior progenies in $F_{5:6}$ and $BC_2F_6$ populations were raised under field conditions. Twenty-one day-old seedlings were transplanted in the main field. Those

selected RILs were grown in 3 rows of 2 m in length along with recurrent and donor parents with two randomised replication plots. The row-to-row distance was 0.2 m and the plant-to-plant distance was 0.15 m. Standard agronomic practices were followed. Phenotypic traits including Plant Height (PH), Number of Tillers (NT), Number of Productive Tillers (NPT), Days to First Flowering (DFF), Days to 50% Flowering (D50%F), Flag Leaf Length (FLL), Flag Leaf width (FLW), Panicle length (PL), and Grain Length and width (GL and GW) were recorded.

## 3. Results

### *3.1. Introgression of Pi9, xa13, Xa21, Gm1, and Gm4 into CO 51 Harbouring Pi54*

Marker-assisted breeding was followed for the stacking of genes in the background of CO 51 (Figure 1). The cultivar CO 51 already possesses the *Pi54* gene, giving broad-spectrum resistance against blast pathogens. This study focused on the stacking/pyramiding of *Pi9*, *xa13*, *Xa21*, *Gm1*, and *Gm4* genes in the CO 51 background to enhance strong and stable durable resistance against blast pathogens, BB pathogens, and the gall midge biotype in the southern part of India.

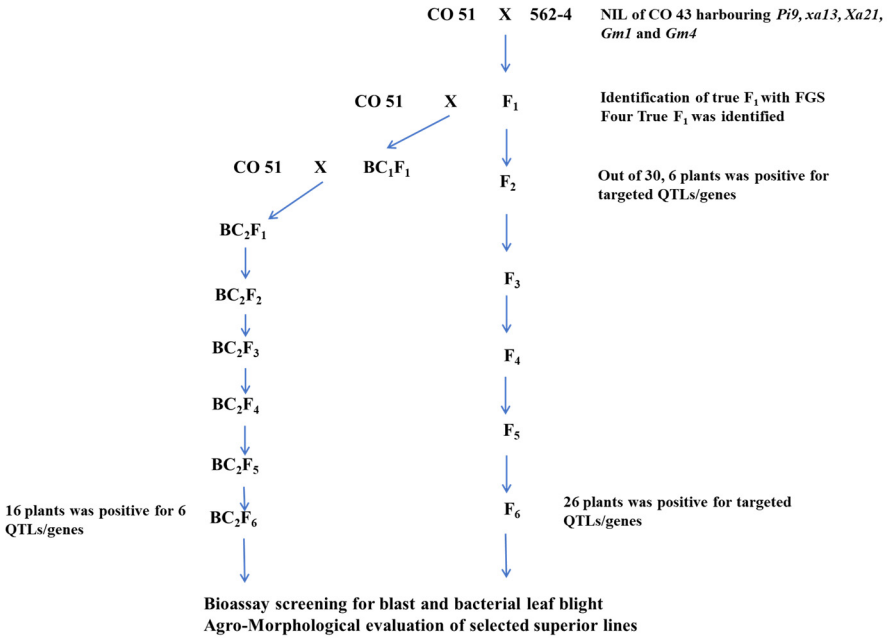



**Figure 1.** Flowchart depicting the breeding method for the development of RILs of CO 51 harbouring blast and BB-resistant genes.

Initially, donor parent 562-4 was crossed with CO 51 to generate $F_1$ plants. The markers which were closely linked to genes *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4* were used to identify true $F_1$ plants. A total of 17 plants were raised in a pot in greenhouse conditions and four were identified as true $F_1$ plants, viz., #2, #7, #9, and #14. The true $F_1$ was identified using the foreground markers linked with the target genes (NBS4, Pi54MAS, xa13Prom, pTA248, RM1328, and RM22550 for *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4*, respectively). The $F_1$ progeny (#9) was forwarded into $F_2$, and six progenies out of thirty in $F_2$ were found to harbour all four genes, viz., *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4* (Supplementary Table S2). Those six selected progenies of $F_2$ were harvested and raised as $F_3$ in a greenhouse. Those $F_3$ progenies were genotyped with linked markers of *Pi9*, *Pi54*, *xa13*, *xa21*, *Gm1*, and *Gm4*. A total of 26 $F_3$ were selected based on the grain type and forwarded into $F_4$. In the segregating population of $F_{4:5}$, five plants/progeny were tagged in all 26 families and genotyped with foreground markers for *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4*. Out of five plants per progeny, one plant was selected that harboured the maximum QTLs/genes. In $F_{5:6}$, 26 progenies were selected with different QTL/gene combinations of *Pi9*, *Pi54*, *xa13*,

*Xa21*, *Gm1*, and *Gm4* with different zygotic statuses of target QTLs/genes (Figure 2 and Table 2).

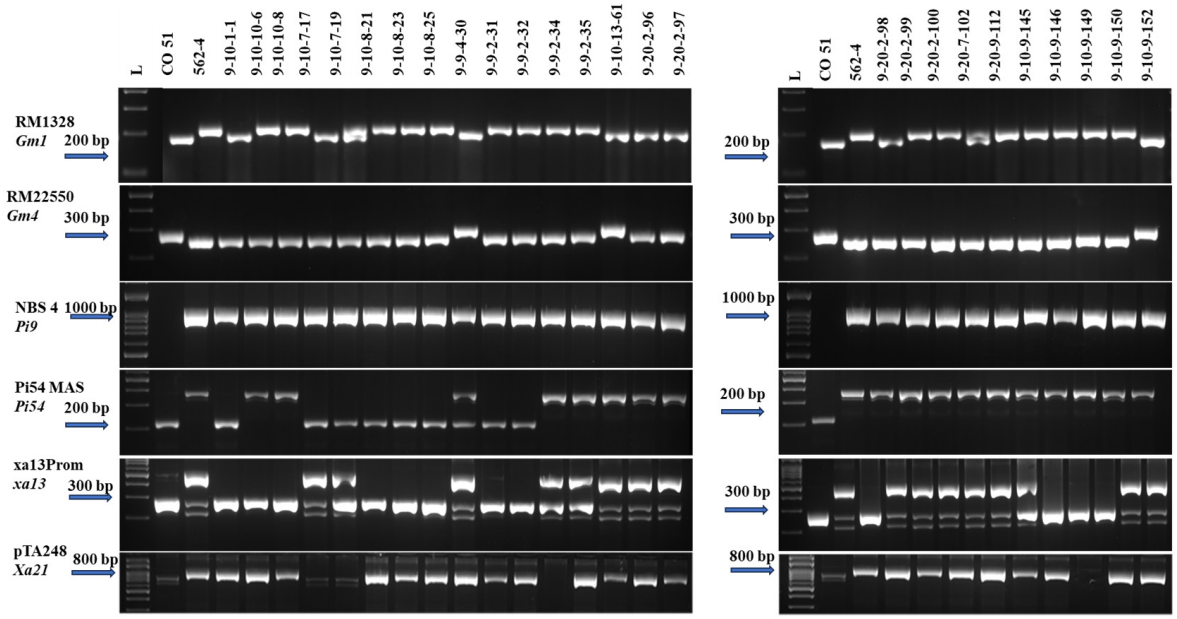



**Figure 2.** Progenies of CO 51 X 562-4 ($F_6$) genotyped with foreground markers.

**Table 2.** Genotyping of 26 $F_6$ progenies using markers linked to BB, blast, and gall midge resistance.

| S. No | RILs | *Gm1* (RM138) | *Gm4* (RM22550) | *Pi9* (NBS4) | *Pi54* (Pi54MAS) | *xa13* (xa13Prom) | *Xa21* (pTA248) |
|---|---|---|---|---|---|---|---|
| 1 | RIL #9-10-1-1 | - | + | + | + | - | + |
| 2 | RIL #9-10-10-6 | + | + | + | - | - | + |
| 3 | RIL #9-10-10-8 | + | + | + | - | - | + |
| 4 | RIL #9-10-7-17 | - | + | + | + | + | - |
| 5 | RIL #9-10-7-19 | H | + | + | + | H | - |
| 6 | RIL #9-10-8-21 | + | + | + | + | - | + |
| 7 | RIL #9-10-8-23 | + | + | + | + | - | + |
| 8 | RIL #9-10-8-25 | + | + | + | + | - | + |
| 9 | RIL #9-9-4-30 | - | - | + | H | + | + |
| 10 | RIL #9-9-2-31 | + | + | + | + | - | + |
| 11 | RIL #9-9-2-32 | + | + | + | + | - | + |
| 12 | RIL #9-9-2-34 | + | + | + | - | H | + |
| 13 | RIL #9-9-2-35 | + | + | + | - | H | + |
| 14 | RIL #9-9-13-61 | H | - | + | - | + | + |
| 15 | RIL #9-20-2-96 | H | + | + | - | + | + |
| 16 | RIL #9-20-2-97 | H | + | + | - | + | + |
| 17 | RIL #9-20-2-98 | H | + | + | - | + | + |
| 18 | RIL #9-20-2-99 | + | + | + | - | + | + |
| 19 | RIL #9-20-2-100 | + | + | + | - | + | + |

**Table 2.** *Cont.*

| S. No | RILs | *Gm1* (RM138) | *Gm4* (RM22550) | *Pi9* (NBS4) | *Pi54* (Pi54MAS) | *xa13* (xa13Prom) | *Xa21* (pTA248) |
|---|---|---|---|---|---|---|---|
| 20 | RIL #9-20-7-102 | H | + | + | - | + | + |
| 21 | RIL #9-20-9-112 | + | + | + | - | H | + |
| 22 | RIL #9-10-9-145 | + | + | + | - | - | + |
| 23 | RIL #9-10-9-146 | + | + | + | - | - | + |
| 24 | RIL #9-10-9-149 | + | + | + | - | - | + |
| 25 | RIL #9-10-9-150 | + | + | + | - | + | + |
| 26 | RIL #9-10-9-152 | - | - | + | - | + | + |

RIL: Recombinant Inbred Line, +: presence of QTLs/genes, -: absence of QTLs/genes, H: heterozygosity of concerned QTL/gene.

In $F_1$, progeny (#9) was used in backcrossing, and the evaluation of seven $BC_1F_1$ progenies identified one $BC_1F_1$ progeny, namely Plant# 9-2 possessing all six target genes, namely, *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4.* Then, $BC_2F_1$ was generated from crosses between #9-2 of $BC_1F_1$ and CO 51. A true $BC_2F_1$ plant was identified with foreground selection and $BC_2F_1$ was forwarded into $BC_2F_5$. Seventy-seven progenies were selected in $BC_2F_5$. The presence of target genes, viz., *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4*, was confirmed with SSR markers and functional markers. Out of 77 progenies, 16 progenies harbouring all six genes with a homozygous condition were selected for phenotyping for blast and BB screening (Figure 3 and Table 3).

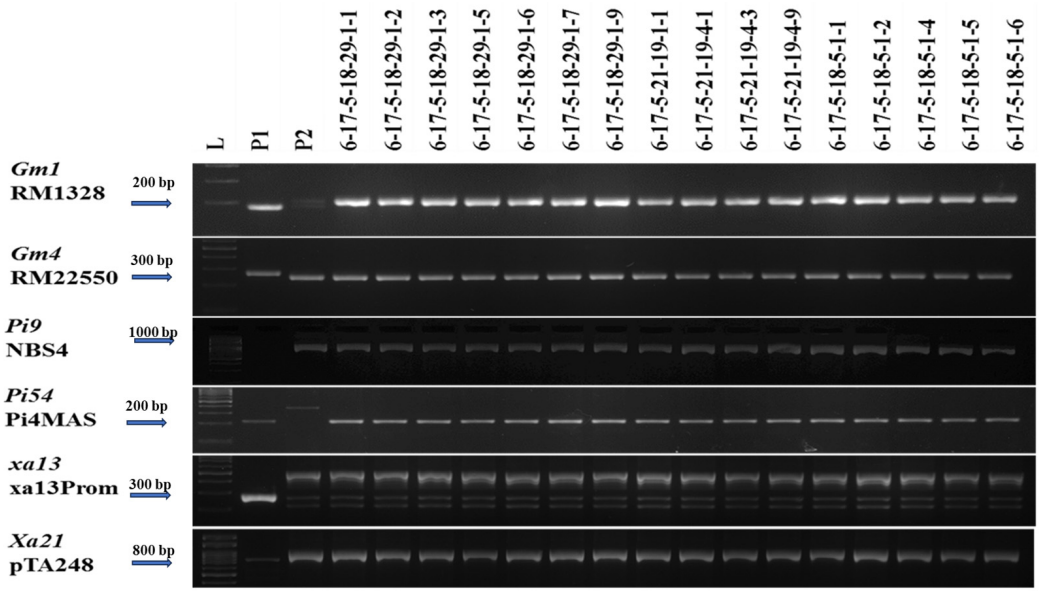



**Figure 3.** Foreground selection of selected BILs of $BC_2F_6$ harbouring *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4*.

**Table 3.** Selected progenies of $BC_2F_5$ of CO 51 harbouring six QTLs/genes.

| S. No. | $BC_2F_5$ | *Gm1* | *Gm4* | *Pi9* | *Pi54* | *xa13* | *Xa21* | No. of QTLs/Genes |
|---|---|---|---|---|---|---|---|---|
| 1 | BIL #6-17-5-18-29-1-1 | + | + | + | + | + | + | 6 |
| 2 | BIL #6-17-5-18-29-1-2 | + | + | + | + | + | + | 6 |
| 3 | BIL #6-17-5-18-29-1-3 | + | + | + | + | + | + | 6 |

**Table 3.** *Cont.*

| S. No. | $BC_2F_5$ | *Gm1* | *Gm4* | *Pi9* | *Pi54* | *xa13* | *Xa21* | No. of QTLs/Genes |
|---|---|---|---|---|---|---|---|---|
| 4 | BIL #6-17-5-18-29-1-5 | + | + | + | + | + | + | 6 |
| 5 | BIL #6-17-5-18-29-1-6 | + | + | + | + | + | + | 6 |
| 6 | BIL #6-17-5-18-29-1-7 | + | + | + | + | + | + | 6 |
| 7 | BIL #6-17-5-18-29-1-9 | + | + | + | + | + | + | 6 |
| 8 | BIL #6-17-5-21-19-1-1 | + | + | + | + | + | + | 6 |
| 9 | BIL #6-17-5-21-19-4-1 | + | + | + | + | + | + | 6 |
| 10 | BIL #6-17-5-21-19-4-3 | + | + | + | + | + | + | 6 |
| 11 | BIL #6-17-5-21-19-4-9 | + | + | + | + | + | + | 6 |
| 12 | BIL #6-17-5-18-5-1-1 | + | + | + | + | + | + | 6 |
| 13 | BIL #6-17-5-18-5-1-2 | + | + | + | + | + | + | 6 |
| 14 | BIL #6-17-5-18-5-1-4 | + | + | + | + | + | + | 6 |
| 15 | BIL #6-17-5-18-5-1-5 | + | + | + | + | + | + | 6 |
| 16 | BIL #6-17-5-18-5-1-6 | + | + | + | + | + | + | 6 |

BIL: Backcross Inbred Line, QTL: Quantitative Trait Loci, +: positive allele.

### *3.2. Introgression of Pi9 to Enhance the Resistance to Blast in CO 51*

A total of 26 RILs and 16 BILs were pyramided with *Pi9*, *Pi54*, *xa13*, *Xa21*, *Gm1*, and *Gm4* and were subjected to screening against blast and BB. The blast screening was carried out in the Hybrid Rice Evaluation Centre, Gudalur, Tamil Nadu, India. Blast scoring was performed on the 30th and 45th Days After Sowing in UBN at 15-day intervals. The susceptible check CO 39 had scales of 6.8 and 9.9 in the first and second scores with an average of 8.35 showing a susceptible reaction to pathogens (Figure 4a and Supplementary Table S3). CO 39 acted as an inoculum source and the blast pathogen spore reproduced in CO 39, which ensures a continuous supply of spores in UBN. The donor parent (562-4) recorded scales of 1.3 and 2.3 in the first and second scores with an average score of 1.8 showing resistance, whereas the recurrent parent CO 51 exhibited scales of 4.4 and 5.9 in the first and second scores with an average of 5.15, showing moderate resistance to blast pathogens.

All 26 progenies of CO 51 RILs harbouring *Pi9* and *Pi54* genes recorded scores of blast ranging from 1.2 to 3.6 with an average of 2.24 in the $F_6$ population, and they exhibited a resistance reaction to blast pathogens. RIL #9-10-8-23 recorded 1.8 and 1.8 in scores I and II, respectively, with constant resistance against blast pathogens. RIL # 9-10-8-25 recorded scores of 2.2 and 1.4 in scores I and II with an average of 1.8. RILs #9-20-2-99 and #9-20-2-100 recorded scores of 1.0 and 2.2 in scores I and II with an average of 1.6. RIL #9-20-7-102 recorded scores of 1.0 and 2.6 in scores I and II with an average of 1.8, whereas RIL #9-20-9-112 recorded scores of 0.6 and 1.8 in scores I and II with an average of 1.2. All the RIL populations of CO 51 showed better resistance than the recurrent parent against blast disease.

Sixteen $BC_2F_6$ progenies of CO 51 X 562-4 harbouring *Pi9* and *Pi54* ranged from 1.3 to 2.8 with an average resistance score of 2.0 to blast pathogens. BILs #6-17-5-18-29-1-1, #6-17-5-18-29-1-2, #6-17-5-21-19-4-1, and #6-17-5-18-5-1-5 all recorded scores of 1.4 and 1.8 in scores I and II, respectively, with an average of 1.6 as strong resistance. BIL #6-17-5-18-5-1-6 recorded scores of 1.2 and 1.4 in scores I and II, respectively, with an average of 1.3 as strong resistance. The BILs harbouring *Pi9* and *Pi54* recorded scores of $0 < 2$ against blast (Figure 4b and Table 4).

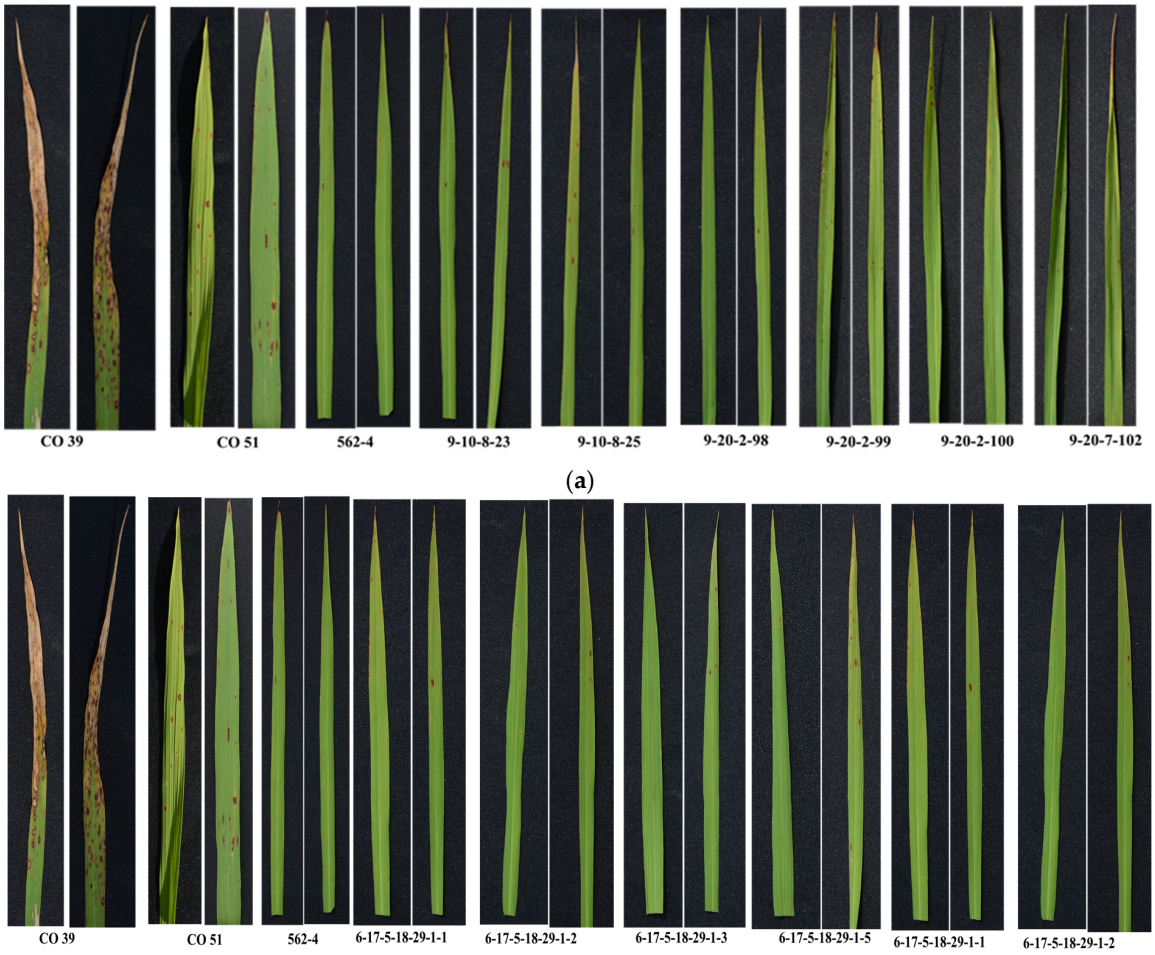



**Figure 4.** (**a**) Blast screening of $F_6$ harbouring *Pi9*- and *Pi54*-gene-stacked lines. (**b**) Blast screening of $BC_2F_6$ harbouring *Pi9*- and *Pi54*-gene-stacked lines.

**Table 4.** Blast and BB scores of 16 progenies of $BC_2F_6$.

| | Blast Score | | | BLB Score (in cm) | | |
|---|---|---|---|---|---|---|
| **$BC_2F_6$** | **Score I** | **Score II** | **Average** | **Score I** | **Score II** | **Average** |
| CO 51 | 4.4 | 5.9 | 5.2 (MR) | 7.49 | 12.32 | 9.91 (S) |
| BIL #6-17-5-18-29-1-1 | 1.4 | 1.8 | 1.6 (R) | 0.28 | 0.45 | 0.37 (R) |
| BIL #6-17-5-18-29-1-2 | 1.4 | 1.8 | 1.6 (R) | 0.32 | 0.53 | 0.43 (R) |
| BIL #6-17-5-18-29-1-3 | 1.6 | 1.8 | 1.7 (R) | 0.25 | 0.52 | 0.39 (R) |
| BIL #6-17-5-18-29-1-5 | 1.8 | 2.2 | 2.0 (R) | 0.28 | 0.32 | 0.30 (R) |
| BIL #6-17-5-18-29-1-6 | 1.6 | 2.0 | 1.8 (R) | 0.27 | 0.44 | 0.36 (R) |
| BIL #6-17-5-18-29-1-7 | 1.8 | 2.6 | 2.2 (R) | 0.33 | 0.48 | 0.41 (R) |
| BIL #6-17-5-18-29-1-9 | 1.5 | 2.4 | 2.0 (R) | 0.32 | 0.56 | 0.44 (R) |
| BIL #6-17-5-21-19-1-1 | 1.8 | 2.2 | 2.0 (R) | 0.28 | 0.41 | 0.35 (R) |
| BIL #6-17-5-21-19-4-1 | 1.4 | 1.8 | 1.6 (R) | 0.24 | 0.39 | 0.32 (R) |
| BIL #6-17-5-21-19-4-3 | 1.4 | 3.0 | 2.2 (R) | 0.21 | 0.45 | 0.33 (R) |
| BIL #6-17-5-21-19-4-9 | 2.0 | 2.8 | 2.4 (R) | 0.48 | 0.52 | 0.50 (R) |
| BIL #6-17-5-18-5-1-1 | 2.4 | 3.0 | 2.7 (R) | 0.31 | 0.38 | 0.35 (R) |
| BIL #6-17-5-18-5-1-2 | 2.4 | 3.2 | 2.8 (R) | 0.30 | 0.44 | 0.37 (R) |
| BIL #6-17-5-18-5-1-4 | 1.6 | 2.4 | 2.0 (R) | 0.35 | 0.46 | 0.41 (R) |
| BIL #6-17-5-18-5-1-5 | 1.4 | 1.8 | 1.6 (R) | 0.24 | 0.38 | 0.31 (R) |
| BIL #6-17-5-18-5-1-6 | 1.2 | 1.4 | 1.3 (R) | 0.23 | 0.38 | 0.31 (R) |

**Table 4.** *Cont.*

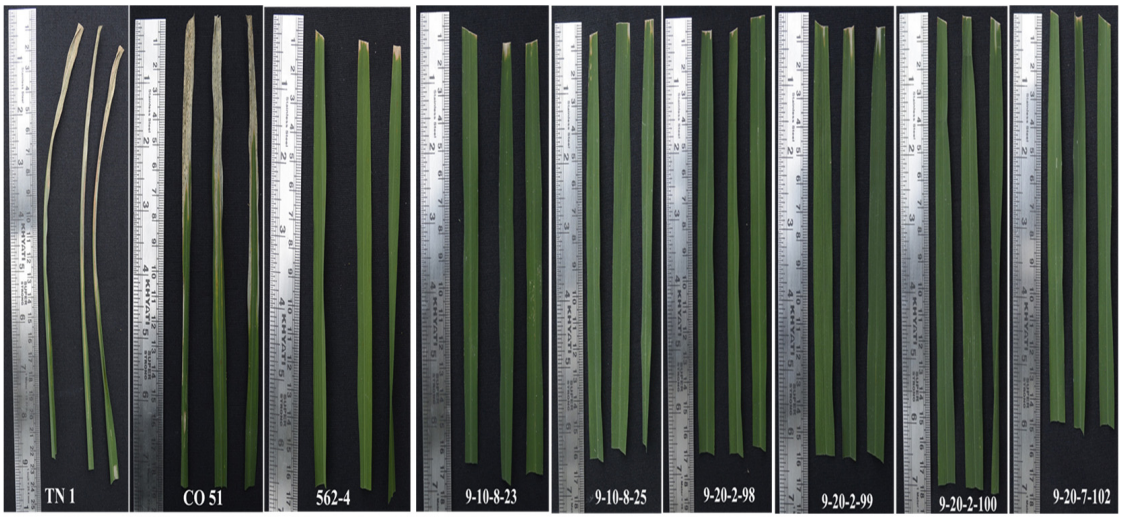
| | Blast Score | | | BLB Score (in cm) | | |
|---|---|---|---|---|---|---|
| **$BC_2F_6$** | **Score I** | **Score II** | **Average** | **Score I** | **Score II** | **Average** |
| Mean of BILs | 1.7 | 2.2 | 2.0 (R) | 0.29 | 0.44 | 0.37 (R) |
| 562-4 | 1.3 | 2.3 | 1.8 (R) | 0.23 | 0.34 | 0.29 (R) |
| CO 39 | 6.8 | 9.9 | 8.4 (S) | - | - | - |
| TN1 | - | - | - | 15.00 | 19.82 | 17.41 (S) |
| SD | 1.5 | 1.7 | 1.6 | 3.93 | 4.99 | 4.46 |
| SE | 0.3 | 0.4 | 0.4 | 0.90 | 1.15 | 1.02 |
| CD (at 5%) | 1.24 | 1.15 | 1.19 | 1.19 | 1.21 | 1.20 |

BIL: Backcross Inbred Line; BB: bacterial leaf blight; CO 39 and TN 1 were used as checks in blast and BB screening, respectively; SD: Standard Deviation; SE: Standard Error; CD: Critical Difference; R: resistant; MR: moderately resistant; S: susceptible.

### *3.3. RILs Harbouring Xa13 and Xa21 Exhibited Enhanced Resistance to BB*

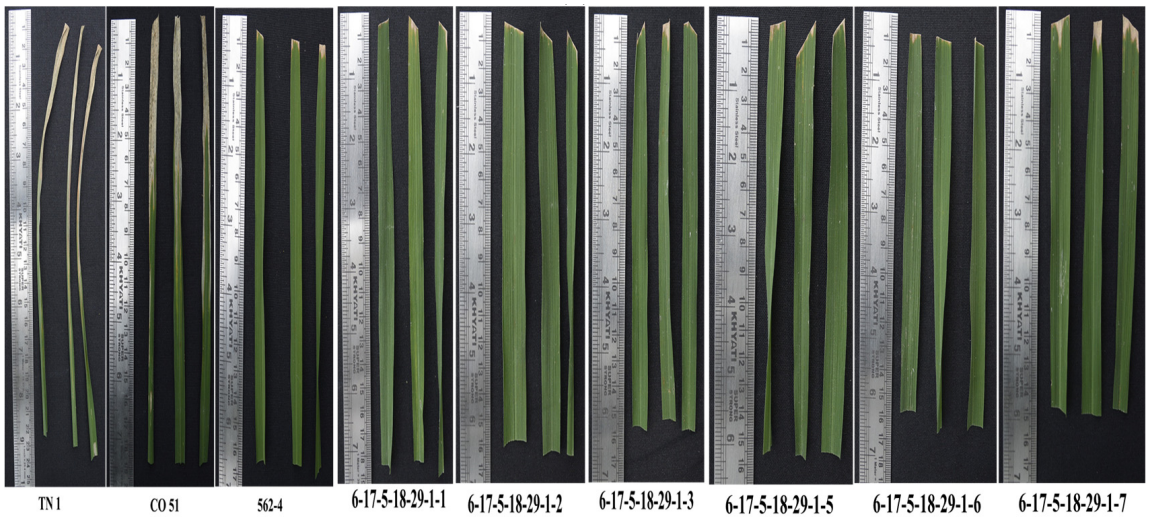
A total of 26 RILs and 16 BILs of CO 51 X 562-4 along with recurrent parent CO 51, donor parent 562-4, and TN 1 (susceptible check) were sown in the nursery and then transplanted into the main field. TN 1 recorded 15 cm in score I and 19.82 cm in score II with an average of 17.41 cm. The recurrent parent CO 51 recorded 7.49 cm in score I and 12.32 cm in score II with an average of 9.91 cm, whereas donor parent 562-4 recorded 0.23 cm in score I and 0.34 cm in score II with an average of 0.29 cm (Figure 5a).

The RILs of the population ranged from 0.24 cm to 0.43 cm in score I with a mean of 0.31 cm and 0.36 cm to 0.79 cm with a mean of 0.54 cm in score II (Supplementary Table S3). The grant means ranged from 0.29 cm to 0.58 cm with an average of 0.42 cm. RIL #9-10-8-23 recorded 0.32 cm and 0.42 cm in scores I and II with a mean of 0.37 cm. RIL #9-10-8-25 recorded scores of 0.37 cm and 0.79 cm in scores I and II with an average of 0.58 cm. RILs #9-20-2-99 and #9-20-2-100 recorded scores of 0.28 cm and 0.29 cm in score I and scores of 0.38 cm and 0.39 cm in score II with an average of 0.33 cm and 0.34, respectively. RIL #9-20-7-102 recorded scores of 0.24 cm and 0.34 cm in scores I and II with an average of 0.29 cm, whereas RIL #9-20-9-112 recorded scores of 0.26 cm and 0.36 cm in scores I and II with an average of 0.31 cm. All the RIL populations of CO 51 pyramided with resistance genes showed better resistance over the recurrent parent in BB disease. The graphical representation for the blast and BB scoring of CO 51 and 562-4 and the 10 selected superior progenies is given in Figure 6 and Table 5.




(**a**)

**Figure 5.** *Cont.*




(**b**)

**Figure 5.** (**a**) BB screening in $F_6$ stacked with xa13 and Xa21 genes. (**b**) BB screening in $BC_2F_6$ stacked with xa13 and Xa21 genes.

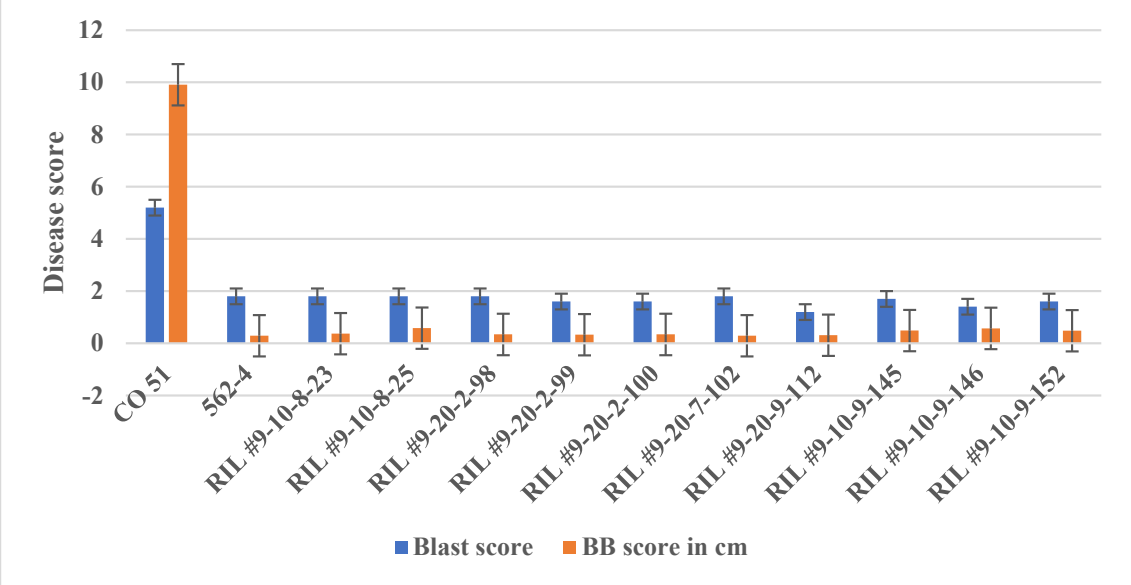



**Figure 6.** Disease reaction of $F_6$ of CO 51 X 562-4 and progenies against blast and BB screening.

**Table 5.** Blast and BB scoring of selected superior progenies of the $F_6$ population.

| | Blast Score | | | BB Score in cm | | |
|---|---|---|---|---|---|---|
| **RILs** | **Score I** | **Score II** | **Average** | **Score I** | **Score II** | **Average** |
| RIL #9-10-8-23 | 1.8 | 1.8 | 1.8 (R) | 0.32 | 0.42 | 0.37 (R) |
| RIL #9-10-8-25 | 2.2 | 1.4 | 1.8 (R) | 0.37 | 0.79 | 0.58 (R) |
| RIL #9-20-2-98 | 1.4 | 2.2 | 1.8 (R) | 0.29 | 0.39 | 0.34 (R) |
| RIL #9-20-2-99 | 1.0 | 2.2 | 1.6 (R) | 0.28 | 0.38 | 0.33 (R) |
| RIL #9-20-2-100 | 1.0 | 2.2 | 1.6 (R) | 0.29 | 0.39 | 0.34 (R) |
| RIL #9-20-7-102 | 1.0 | 2.6 | 1.8 (R) | 0.24 | 0.34 | 0.29 (R) |
| RIL #9-20-9-112 | 0.6 | 1.8 | 1.2 (R) | 0.26 | 0.36 | 0.31 (R) |
| RIL #9-10-9-145 | 1.6 | 1.8 | 1.7 (R) | 0.29 | 0.68 | 0.49 (R) |
| RIL #9-10-9-146 | 1.0 | 1.8 | 1.4 (R) | 0.36 | 0.77 | 0.57 (R) |
| RIL #9-10-9-152 | 1.4 | 1.8 | 1.6 (R) | 0.43 | 0.53 | 0.48 (R) |

**Table 5.** *Cont.*

| | Blast Score | | | BB Score in cm | | |
|---|---|---|---|---|---|---|
| **RILs** | **Score I** | **Score II** | **Average** | **Score I** | **Score II** | **Average** |
| CO 51 | 4.4 | 5.9 | 5.2 (MR) | 7.49 | 12.32 | 9.91 (S) |
| 562-4 | 1.3 | 2.3 | 1.8 (R) | 0.23 | 0.34 | 0.29 (R) |
| CO 39 | 6.8 | 9.9 | 8.4 (S) | - | - | - |
| TN 1 | - | - | - | 15.00 | 19.82 | 17.41 (S) |
| Mean of RILs | 2.10 | 2.40 | 2.20 (R) | 0.30 | 0.50 | 0.40 (R) |
| SD | 1.737 | 2.384 | 2.051 | 4.385 | 6.050 | 5.211 |
| SE | 0.482 | 0.661 | 0.569 | 1.216 | 1.678 | 1.445 |
| CD (at 5%) | 1.38 | 1.24 | 1.31 | 0.49 | 1.12 | 0.80 |

RIL: Recombinant Inbred Line; BB: bacterial leaf blight; CO 39 and TN 1 were used in blast and BB screening, respectively; SD: Standard Deviation; SE: Standard Error; CD: Critical Difference; R: resistant; MR: moderately resistant; S: susceptible.

Sixteen $BC_2F_6$ progenies of CO 51 X 562-4 harbouring *xa13* and *Xa21* ranged from 0.30 to 0.50 cm with an average of 0.37 cm against blight pathogens (Figure 5b and Table 4). BIL #6-17-5-18-29-1-1 recorded scores of 0.28 and 0.45 cm in scores I and II with an average of 0.37 cm. BIL #6-17-5-18-29-1-2 recorded scores of 0.32 cm and 0.53 cm in scores I and score II with an average of 0.43 cm. BIL #6-17-5-21-19-4-1 recorded scores of 0.24 and 0.39 cm in scores I and II with an average of 0.32 cm, and BIL #6-17-5-18-5-1-5 recorded scores of 0.24 and 0.38 cm in scores I and II with an average of 0.31 cm. BIL #6-17-5-18-5-1-6 recorded scores of 0.23 cm and 0.38 cm in score I and score II with an average of 0.31 cm. The BILs harbouring *Pi9* and *Pi54* recorded a score of 0 < 0.5 against the BB pathogen (Figure 7).

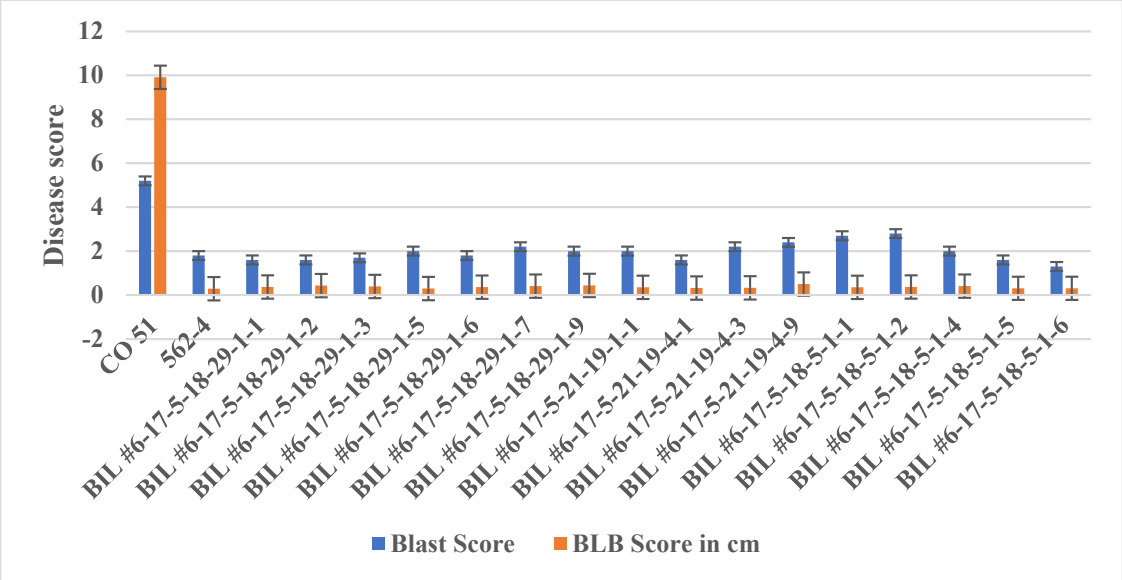



**Figure 7.** Performance of BILs of $BC_2F_6$ against blast and BLB pathogen.

### *3.4. Performance of RILs and BILs of CO 51 X 562-4 Lines for Important Traits*

The 10 superior progenies of $F_6$ of CO 51 X562-4 were selected based on resistance against blast and BB diseases. Those selected progenies were evaluated for their agronomic performance for yield and its attributing traits. CO 51 single plant yield (SPY) was recorded as 29.58 g, whereas those for RILs and $F_6$ ranged from 29.97 to 36.45 g with an average of 32.94 g. The 1000-grain weight was recorded as 19.02 g in CO 51, whereas those for RILs ranged from 15.50 g to 23.28 g with an average of 19.63 g (Supplementary Table S4). The yield in improved CO 51 lines ranged from 1.32 to 42.70% over CO 51 (Figure 8). Maximum

yields were recorded in RILs #9-10-9-146 (42.70%), #9-10-8-23 (26.27%), #9-20-2-98 (23.23%), and #9-10-8-25 (19.10%).

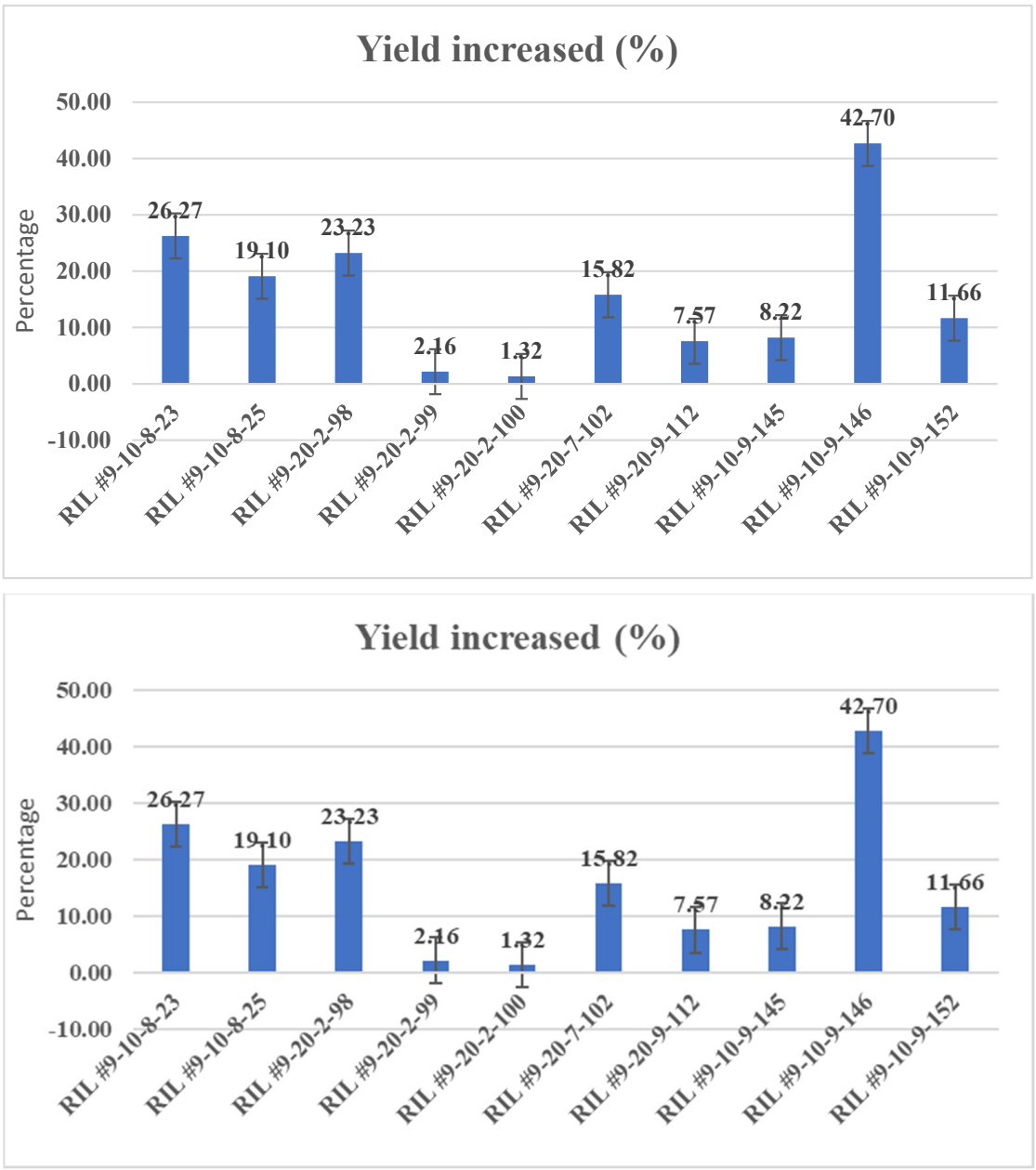



**Figure 8.** Graphical representation of RIL yield increased over CO 51.

The BILs of CO 51 X 562-4 recorded single plant yields of 33.29 to 43.74 g with an average of 37.64 g, whereas the 1000-grain weight recorded in RILs ranged from 18.50 to 23.92 g with an average of 21.16 g (Supplementary Table S4). The yield in improved CO 51 lines ranged from 12.54 to 47.87% compared to CO 51 (Figure 9). Maximum yields were recorded in BILs #6-17-5-18-5-1-5 (47.87%), #6-17-5-18-29-1-5 (45.17%), #6-17-5-18-5-1-4 (43.91%), and #6-17-5-18-29-1-6 (42.06%).

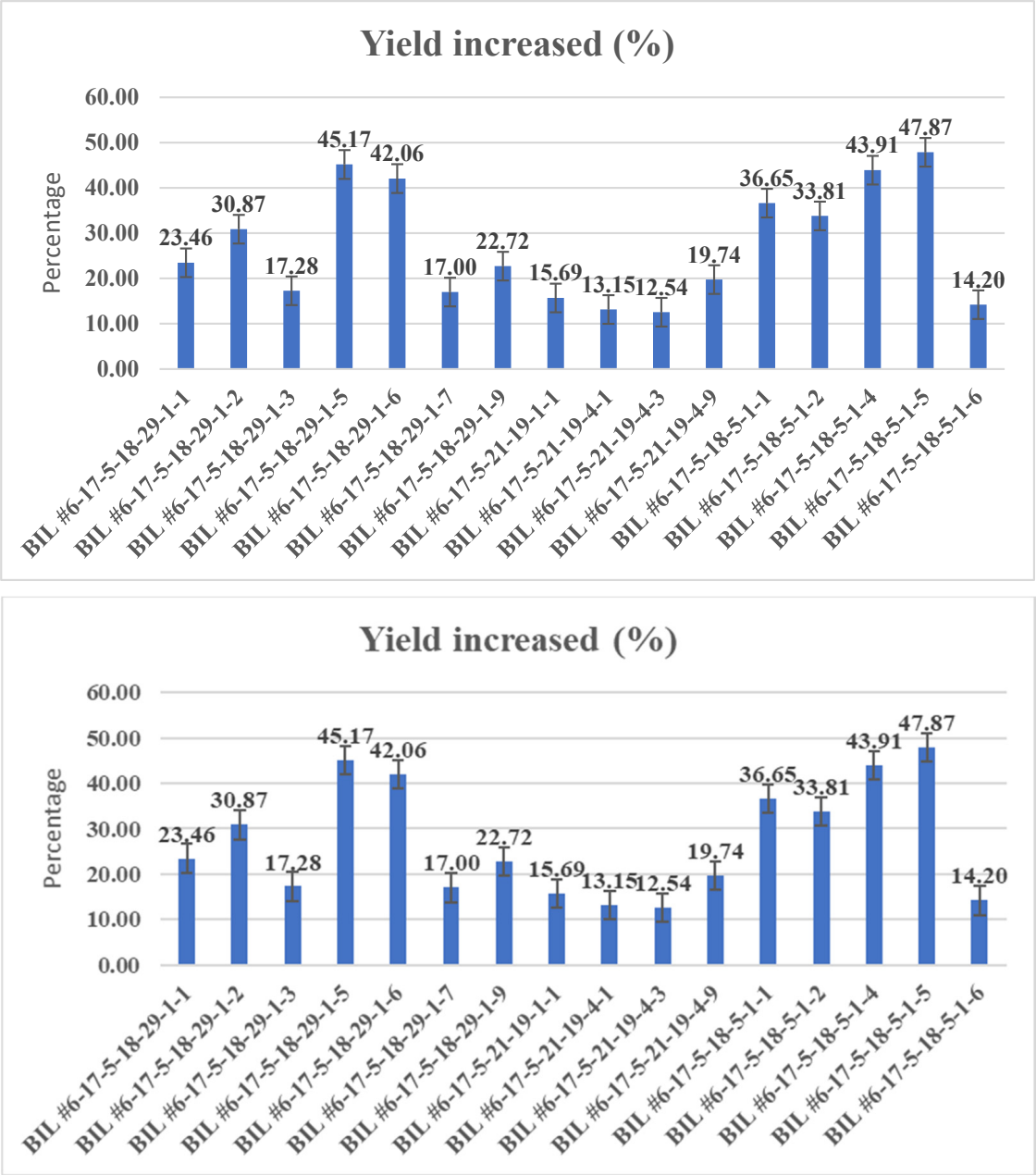



**Figure 9.** Graphical representation of increase in BIL yield compared to CO 51.

## 4. Discussion

The present study aimed to pyramid or stack genes against blast, BB, and gall midge disease into the CO 51 background to improve the existing cultivar, which can be quickly achieved using marker-assisted selection (MAS) and marker-assisted backcross breeding (MABB) through closely linked markers to targeted genes. The pyramiding of multiple genes/alleles in a single background gene-based marker or tightly linked markers paved the way for achieving the introgression of target QTLs/genes and saved time and resources [43–45]. In MAS, foreground selection, background selection, and recombination selection played a vital role in the selection of RILs harbouring pyramided QTLs/genes with a maximum of recurrent genomes and a minimum of donor segments. The recurrent parent, CO 51, was recorded as having an average yield of 29.58 g, a blast score of 5.2 as moderately resistant, and a BB score of 9.91 cm. RIL #9-10-9-146 recorded a single plant yield of 42.21 g, a blast resistance score of 1.4, and a BB resistance score of 0.57 cm. RIL #9-10-8-23 was recorded as having an average yield, average blast score, and average BB

score of 37.35 g, 1.8, and 0.37 cm, whereas RIL #9-20-2-98 recorded 36.45 g, 1.8, and 0.34 cm, respectively. RIL #9-10-8-25 recorded an average yield of 35.23 g, a blast score of 1.8, and a BB score of 0.58 cm. Transgressive segregation in the phenotypic traits of the RILs, viz., PH, NT, NPT, PL, total grain, FG, 1000-grain WT, and SPY, were recorded and compared to the recurrent parent, whereas DFF was recorded as earlier genotypes as compared with the recurrent parent [49–51]. BIL #6-17-5-18-5-1-5 recorded an SPY of 43.74 g, a blast score of 1.6 as a resistant reaction to blast pathogens, and a BB score of 0.31 cm as a resistant reaction to BB pathogens, and the SPY recorded in BIL #6-17-5-18-29-1-5 was 42.94 g, its blast score was 2.0 as a resistant reaction to blast pathogens, and its BB score was 0.30 cm as a resistant reaction to BB pathogens.

Among several R genes against diseases, the *Pi9* gene conferring resistance was reported in previous studies [9,46,52] and is resistant against a broad-spectrum range of Indian blast isolates [43]. Among the R gene, the *Pi54* gene confers resistance against *M. oryzea* [28]; *Pi1*, *Pi2*, and *Pi9* are the most effective fungus races; and *Pi9* is a major resistance gene, isolated from wild species *Oryza minuta* [24,25] which shows broad-spectrum resistance against a vast isolate of *M. oryzea*. In this study, *Pi9* was stacked along with the *Pi54* gene. The resistance level of CO 51 (plus *Pi54* gene) showed a mean of 5.2 as moderate resistance, and donor parent 562-4 (plus *Pi9* gene) showed 1.8 as a high-resistance reaction to blast pathogens (Tables 4 and 5). The RILs harbouring *Pi9* and *Pi54* genes together, viz., RIL #9-10-8-23, #9-10-8-25, #9-20-2-98, #9-20-7-102, #9-20-9-112, #9-10-9-145, #9-10-9-146, and #9-10-9-152, and all BILs harbouring *Pi9* and *Pi54* genes together showed high resistance to blast pathogens in UBN (Tables 4 and 5). The stacking of more than one R gene into the recurrent parent also revealed a strong and durable resistance being imparted to diverse isolates of blast pathogens [43]. The improved lines with R genes combinations of *Pi54+Pi1+Pita*, *Pib+Pi9+Pi5*, and *Pi2+Pib+Pi5* were shown to impart a high level of resistance to a wider range of isolates of blast pathogens [43,53]. The RIL lines, viz., RIL #9-10-8-23 and RIL #9-10-8-25, harbouring both R genes *Pi9* and *Pi54* were recorded as highly resistant to blast pathogens. The RIL line harbouring *Pi9* alone, viz., RIL #9-20-2-98, RIL #9-20-7-102, RIL #9-20-9-112, RIL #9-10-9-145, RIL #9-10-9-146, and RIL #9-10-9-152, also imparted high levels of resistance to blast pathogens over the recurrent parent CO 51. RILs harbouring *Pi9* monogenic genes and exhibiting high resistance to blast than the RIL and BILs harbouring *Pi9* and *Pi54* genes were identified [54,55]. The *Pi2* and *Pi54* broad-spectrum resistance genes were stacked in Pusa Basmati 1509 against blast disease and can confer high resistance to various isolates of pathogens [45]. The genes *Pi9* and *Pi54* trigger an effector-induced immune response to pathogen infection through nuclear binding site leucine-rich repeats (NBS-LRR) and have a synergistic effect on the pathogen, to enhance resistance for a wider range of pathogen isolates [54]. In this study, *Pi9* and *Pi54* together conferred a highly resistant reaction to leaf blast and panicle blast and the additive effect was recorded in a leaf blast-pyramided line that harboured either *Pizt* or *Pi9* and *Pi54* [54]. However, together, these genes exhibited lower resistance likely due to the presence of incompatible reactions of the pathogen for *Pi9* and *Pi54* genes [56].

A challenge that appears in stacking a gene against BB is the distinct virulence of *X.o* pv., *oryzae* strains in different geographical regions [57]. Hence, $F_6$ line with high resistance to BB strain, stacking more than one R genes, in contrast to single R genes to overcome the pathogenicity of *X.o* pv., *oryzae* strains that become virulent to stacked R genes [58]. In this study, *xa13* and *Xa21* genes were stacked in the RIL lines. The recurrent parent recorded a mean of 9.91 cm for BB symptoms, whereas donor parent 562-4 (harbouring *xa13* and *Xa21*) recorded a mean of 0.29 cm for BB symptoms against *X.o* pv., *oryzae*. The RILs harbouring monogenic *Xa21* alone recorded a mean of 0.37 cm to 0.79 cm for BB symptoms, whereas RILs harbouring monogenic *xa13* alone recorded 0.37 cm for BB symptoms (Table 3). The RILs harbouring digenic *xa13* and *Xa21* together recorded BB symptoms ranging from 0.29 cm to 0.48 cm. Also, BIL $BC_2F_6$ harbouring digenic *xa13* and *Xa21* recorded 0.30 to 0.50 cm with an average of 0.37 cm against blight pathogens. RIL #9-20-7-102 showed symptoms of 0.29 cm on par with donor parent 562-4 (Supplementary Table S3). The R genes,

viz., *xa13* and *Xa21* digenic or together, are resistant to broad-spectrum strains and impart resistance against the *X.o* pv., *oryzae* races in the Basmati growing region of India [44,45,59]. This study confirmed that, together, the R genes, viz., *xa13* and *Xa21*, revealed the genetic potential of stacked genes at a phenotypic level in the southern part of India. Those selected R genes *xa13* and *Xa21* have been used widely to improve popular rice varieties such as Improved Pusa Basmati 1 [59], Pusa Basmati 1728, and Pusa Basmati 1718 [60]. The R-gene-stacked plant is invaded by BB strains *X.o* pv., *oryzae*, which possesses a gene *pthXo1* for its virulence; gene *xa13* is a recessive allele of gene *Os8N3*, a Nodulin family gene; and the transcript of *xa13* is unresponsive to *pthXo1*, which reslts in resistance [61]. The other genes, *Xa21*, activated by *RaxX* protein, and tyrosine-sulphated protein from *X.o* pv., *oryzae* [62] encode receptor-like protein kinase [62,63]. Tyrosine-sulphated protein from *X.o* pv., *oryzae* triggers the immune defence responses in rice [64]. In plant immune response, WRKY transcriptional factors act as key regulators [65] and WRKY comprises a superfamily of mostly plant-specific transcriptional factors and a highly conserved WRKYGQK sequence at their N-termini [66,67]. A total of over 80 *WRKY* gene families have been identified in the rice genome [68] and have a region of approximately 60 amino acids containing a conserved WRKY amino acid sequence adjacent to a zinc-finger-like motif [66]. The RIL lines have been stacked with broad-spectrum resistance genes, viz., *Pi9*, *Pi54*, *xa13*, and *X21*, and have shown strong resistance to blast and BB pathogens. When plants are invaded by *M. oryzae* and *X.o* pv., *oryzae*, around 45 *OsWRKYs* are induced against early responsive genes to confer resistance reaction [69,70]. The resistance level of RILs could overexpress *OsWRKY* genes due to an immune response triggered by pathogens. Different studies revealed that the overexpression of *OsWRKY71* and *OsWRKY13* showed enhanced resistance to the *X.o* pv., *oryzae* pathogen [71,72], whereas a reduced expression of *OsWRKY45* compromised resistance to the *X.o* pv., *oryzae* pathogen [73].

The superior RIL lines harbouring *Pi9+xa13+Xa21* genes together (RIL #9-9-4-30, #9-20-2-98, #9-20-2-99, and #9-20-7-102) and BIL lines harbouring *Pi9+Pi54+xa13+Xa21in* (BIL #6-17-5-18-29-1-1, #6-17-5-18-29-1-2, #6-17-5-21-19-4-1, #6-17-5-18-5-1-5, and #6-17-5-18-5-1-6) imparted resistance (Supplementary Table S3 and Table 4) against biotic stress (*M. oryzae* and *X.o* pv., *oryzae*), which is probably a complete expression of *OsWRKY* genes in pyramided/stacked lines and was an additive effect of stacked genes. On the other hand, RIL lines, viz., RILs #9-10-10-6, #9-10-10-8, #9-10-9-145, #9-10-9-146, and #9-10-9-149, harbouring *Pi9+Xa21* and #9-10-7-17 harbouring *Pi9+Pi54+xa13* showed moderate resistance to *M. oryzae* and *X.o pv.*, *oryzae*. It may have a reduced expression of *OsWRKY* genes in pyramid/stacked lines. The R gene *Pi9+Pi54*-pyramided lines, viz., RILs #9-10-7-17, #9-10-7-19, #9-10-8-21, #9-9-2-31, and #9-9-2-32, showed blast resistance scores of 2.8, 2.2, 4.0, 2.4, and 2.6, whereas RILs harbouring *Pi9* alone, viz., RILs #9-20-2-98, #95-2-99, #9-20-2-99, #9-20-2-100, #9-20-7-102, and #9-10-9-145, recorded 1.8, 1.6, 1.6, 1.8, 1.2, and 1.7, respectively. RILs harbour *Pi9* monogenic genes and exhibit high resistance to blast than the RILs harbouring *Pi9* and *Pi54* digenic genes [54].

The RILs and parental lines were screened against blast, bacterial leaf blight, and agro-morphological performance. The RILs were recorded as superior progeny compared to their parent. As compared with agronomic performance and phenotypic screening, 10 promising progenies in $F_6$ (viz., RILs #9-10-8-23, #9-10-8-25, #9-20-2-98, #9-20-2-99, #9-20-2-100, #9-20-7-102, #9-20-9-112, #9-20-9-145, #9-10-9-146, and #9-10-9-152) and 5 promising progenies of $BC_2F_6$ (viz., BIL #6-17-5-18-5-1-5, #6-17-5-18-29-1-5, #6-17-5-18-29-1-6, #6-17-5-18-5-1-4, and #6-17-5-18-5-1-1) were selected as superior RILs and BILs as compared with their recurrent parent CO 51 (Supplementary Tables S2 and S3, Figure 6). In this study, RILs harbouring both genes, *Pi9+Pi54* (RIL#9-10-7-17, #9-10-7-19, and #9-10-8-21) and *xa13+Xa21* (RIL#9-20-2-96), displayed a moderate level of resistance to blast and BB, respectively. This may be due to the recombination between genes and markers that were used in this study [74], the interaction among QTLs/genes, and the possibility of antagonistic or synergistic interaction among QTLs/genes in the recurrent plant [1,35,75,76]. Because of climate change, different variants of pathogens still evolve across the environment and

appear active, and immediate attention should be given to new biotypes in developing varieties with resistance to concerned biotic stress. From the successful introgression of *Pi9*, *xa13*, *Xa21*, *Gm1*, and Gm4 into CO 51+*Pi54* through MAS in early generation and stringent phenotypic selection in advanced generation, pyramided lines impart resistance to blast and BB pathogens.

## 5. Conclusions

The present study was successful in stacking broad-spectrum resistance and durable genes against blast (*M. oryzae*), BB (*X.o* pv., *oryzae)*, and gall midge (*Orseolia oryzae*) through marker-assisted selection in early generation. From the phenotypic selection of blast and BB in advanced breeding lines, the genes with broad-spectrum and durable resistance can reveal the complete expression of the gene in the CO 51 elite cultivar. The selected superior improved CO 51 can be used as a genetic stock in future breeding programs.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture14050693/s1, Table S1: List of foreground markers and primer sequences; Table S2: Genotyping of F2 of CO 51 X 562-4 with FGS; Table S3: Phenotypic scoring of RILs against blast and BLB disease; Table S4: Agro-morphological performance of RILs population.

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**Data Availability Statement:** The details of primer sequences, genotypic, phenotypic scoring data of Blast and BLB resistance and Agro-morphological performance of RILs population is given as Data Availability Statement.

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