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Research Article

Development of biotic stress resistant version of CO 51 Rice cultivar through Marker Assisted Introgression of major genes, *Pi9* and *Xa21*

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Abstract

Rice, is a staple crop for the global population and faces significant challenges from diseases viz., blast and bacterial blight. To combat these biotic stresses, Marker-assisted selection is employed for the development of resistant breeding lines against the concerned stresses. This study successfully demonstrated the stacking/pyramiding of *Pi9* and *Pi54* genes for blast resistance (*Magnaporthe oryzae*) and *Xa21* gene for bacterial blight resistance (*Xanthomonas oryzae* p.v *oryzae*) and stringent phenotypic screening in the early generation of CO 51 X 562-4. Recurrent parent, CO 51 scored 5.0 for blast as a moderately resistant and susceptible reaction to blight screening. Among the five breeding lines of F4, blast resistance ranged from 1.5 to 3.8, while blight disease ranged from 0.6 to 0.7 cm as a resistant reaction. Progeny #6-28-3 recorded resistant reactions to blast and blight disease followed by Progeny #6-28-4.

Keywords: Rice, Blast, Bacterial blight disease, broad spectrum genes and gene stacking

INTRODUCTION

The world population is expected to increase by 8.5 billion by 2030 and will reach 9.7 billion by 2050 (UN, 2022). In a world where the number of people who cannot afford healthy food has increased from 112 million to about 3.1 billion, this reflects the impact of rising food costs on consumers during the pandemic (FAO, 2022). According to the findings presented by Godfray in 2010 and Parker in 2011 in their reports titled “Feeding the Future” and “The 9 Billion People Question (9BPQ),” they both tackled the issues concerning food security and the challenges associated with providing sustenance to a global population of 9 billion (Parker, 2011). Geneticists, biotechnologists, and plant breeders aim to generate sufficient food resources to support an additional 0.7

billion people by 2050 and 2.4 billion people by 2080. Rice plays an important role in human nutrition, and accounts for 20 % of daily calories, hunger, and The 9 Billion People Question (9BPQ) (Timmer *et al*., 2010; Jacquemin *et al*., 2013). Rice production needs to increase to 160 million tonnes to meet the food demand in 2050 (WFP and UNICEF, 2022). However, rice production is threatened by several biological pressures, including fungal and bacterial diseases, which affect rice productivity across South Asia and Asian countries, including India (Yugander *et al*., 2017). One of the most important biological diseases affecting rice is the fungal plague (*Magnaporthe oryzae*), which can lead to yield reductions of up to 10-30 % worldwide (Ramalingam *et al*., 2020). In India, rice blast causes 74-100 % yield losses under favorable conditions (Ramalingam *et al*., 2020). Bacterial blight (BB), another important biological disease, is a devastating disease caused *by Xanthomonas oryzae* pv *oryzae* (*X. o.* pv*., oryzae*), leading to 50 % of yield losses in the initial stage and up to 40% of yield losses at the tillering stage (Yasmin *et al*., 2017).

To date, 102 resistance genes have been identified against rice blast disease caused by *Magnaporthe Oryzae* (Mi *et al*., 2018). Among them, 38 R genes have been mapped and characterized at the molecular level (**Table 1**). Among the R genes, eight genes, namely *Pi9, Pi54, pi21, Pi50, Pi7, Pi57*, *Pigm* and *Ptr,* have been reported as broad-spectrum resistance genes against blast disease (Devanna *et al*., 2022). For BB resistance, 45 R genes have been identified so far and only a few R genes are identified as tightly linked with molecular markers (**Table 1**) (Ullah *et al*., 2023) and only a few R genes, namely *Xa1, Xa4 , xa5, Xa7, xa8, xa13, Xa21,* and *Xa27* are identified as tightly linked with molecular marker. Some of the R genes are dominant genes: *Xa1, Xa4, Xa7, Xa21, Xa23, Xa26* and *Xa27* whereas s *xa5, xa8 and xa13* are *recessive genes against X .o. pv., oryzae* (Hari *et al*., 2013). The major dominant and recessive R genes, *Xa21* and *xa13* confer broad-spectrum resistance to many virulent isolates in India (Chu *et al*., 2006).

Developing cultivars resistant to Blast and BB diseases is expected to increase resistance against pathogens and improve rice yields. The host plant resistance is a powerful tool for combating to biotic stress such as blast and BB disease. In rice breeding, marker-assisted selection helps to introgress or stacking of genes into different genetic background from donor parents. The blast-resistant R gene and the BB-resistant R gene were effectively introgressed by foreground selection (FGS) from the donor parent, and a definitive improvement was achieved (Hari *et al*., 2013). Therefore, current study focuses on the introgression of blast and BB resistance genes into the mega variety CO 51.

MATERIALS AND METHODS

Plant material: Rice variety CO 51 is a short duration (105-115 days) medium slender variety, notified for 14 states of India for cultivation in all three rice growing seasons of Tamil Nadu (Thulasinathan *et al*., 2020; Viswabharathy *et al*., 2023). The presence of the *Pi54* allele in CO51 makes moderate resistance to blast and susceptible to BB disease. This study focused on the *Pi9* and *Xa21* genes stacking/pyramiding in CO 51 background to enhance strong, stable and sustained resistance to blast and BB pathogens. The donor parent 562-4 developed from a near-isogenic line of CO 43 x VRP1 and has the genes of *Pi9*, *xa13*, *Xa21 Gm1* and *Gm4*. The donor parent 562-4 was used as genetic stock in the breeding programme aim at developing resistant against blast and BB disease.

Breeding strategies for introgression of *Pi9 and Xa21* into *CO 51+Pi54:* Advanced breeding lines (Progeny #8-13 and Progeny #6-37) of CO 51 X 562-4 have been

Table 1. List of blast and BB genes was characterized at molecular level

S. No	Blast genes	S. No	Blast genes	S. No	Blast genes	S. No	BB genes
1	Pish	14	Pizh,	27	Pikm	1	Xa1
2	Pi35	15	Pigm	28	Pike	2	Xa4
3	Pi37	16	Pi-d2	29	Pik-h	3	xa5
4	Pi64	17	Pi-d3	30	Pi1	4	Xa7
5	Pit	18	Pi25	31	Pi54	5	xa8
6	Pi-b	19	Pid3-A4	32	Pi54rh	6	xa13
7	pi21	20	Pi36	33	Pi54of	7	Xa21
8	Pi36	21	Pi5	34	Pia	8	Xa27
9	PiPR1	22	Pii	35	Pi-CO39		
10	Pi9	23	Pi56	36	Pita		
11	Pi2	24	Pb1	37	Pi65		
12	Piz-t	25	Pik	38	Ptr		
13	Pi50	26	Pik-p				

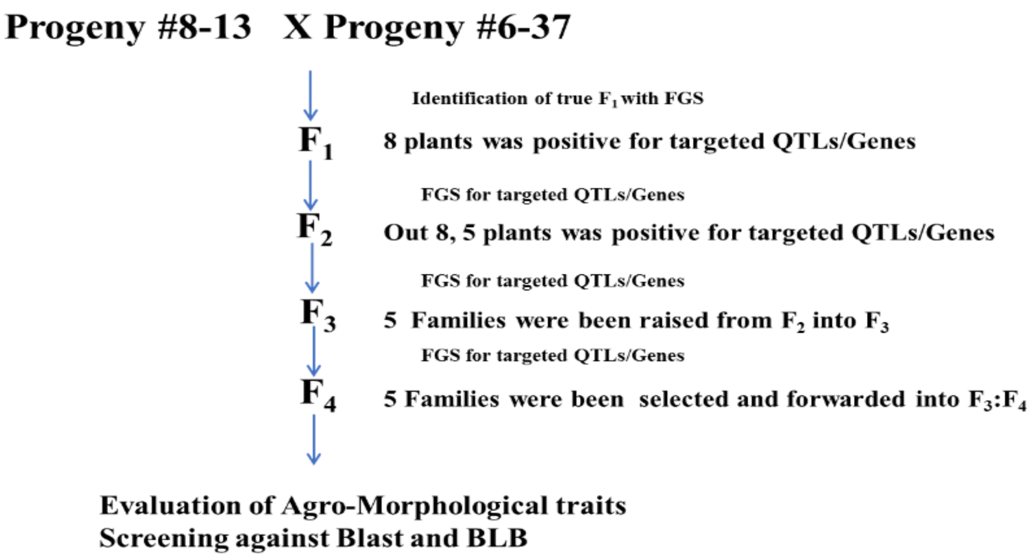


Fig. 1. Schematic representation of development of F_4 population

developed in the Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore, Tamil Nadu (Thiyagarajan, 2021). Progeny #8-13 from CO 51 X 562-4 has been stacked with *Pi9* and *Pi54,* broad-spectrum resistant genes against blast disease whereas whereas Progeny #6-37 has been introgressed with *Xa21* against bacterial blight disease. To bring both three genes together into CO 51 genetic background, Progeny #6-37 was used as male crossed with Progeny #8-13 as female. Schematic representations of the development of the breeding population are mentioned in **Fig. 1.**

Genomic DNA isolation and genotyping: Marker Assisted Breeding (MAB) involved collecting leaf samples three weeks after transplanting, which were then preserved at -80°C until genomic DNA extraction. Genomic DNA (gDNA) was extracted using the CTAB method as described in Ausubel *et al.* (1992). Molecular markers NBS4, Pi54MAS, and pTA248 were employed for foreground selection, as they are closely associated with *Pi9, Pi54* and *Xa21*, respectively **(Table 2)**.

Phenotypic screening against Blast and BB pathogen: The five selected F_4 families were sown in the Uniform Bed Nursery (UBN) at the Hybrid Rice Evaluation Center (HREC), Gudalur, Nilgiris district. Progenies were raised along with the recurrent and donor parents. To ensure a continuous supply of inoculum, the susceptible check variety CO 39 was planted on both sides of UBN, with one row for every five rows of breeding lines of CO 51 X 562-4.

The disease infection was measured twice at intervals of 30 days and 45 days after sowing for test entries. The scores 0–3 were considered as resistant (R), 4–5 as moderately resistant (MR) and 6–9 as susceptible (S) (Janaki Ramayya *et al.*, 2021). To conduct the BB screening, 21-day-old seedlings from five selected F_4 families were transplanted into the main field. The parents, CO 51, 562-4 and susceptible checks, TN1 were also included in this planting. A virulent bacterial blight pathogen *X. o. pv oryzae* (CFU 10^8-10^9) was inoculated in ten leaves per plant at the maximum tillering stage (40 to 45 days) using a clip inoculation method with a sterilized scissor. Ten plants and approximately 5-6 upper leaves/plants were inoculated. The lesion lengths were measured in each entry after 14- and 21 days post-inoculation when the lesion was stable. Based on lesion length the plant was classified as resistant (R) where length was 0-3 cm, moderately resistant (MR) where length was more than 3-6 cm, moderately susceptible (MS) where length was more than 6-9 cm and susceptible (S) where length was more than 9 cm (Dixit *et al.*, 2020).

Table 2. List of foreground markers and primer sequence

Marker Name	Forward sequence	Reverse sequence
NBS4	5'ACTTTGTTGTGCTTGATAAC3'	5'ATGGTGAACGGTATCTGTAT3'
Pi54MAS	5'CAATCTCCAAAGTTTTCAGG3'	5'GCTTCAATCACTGCTAGACC3'
pTA248	5'AGACGCGGAAGGGTGGTTCCCGGA3'	5'AGACGCGGTAATCGAAGATGAAA3'

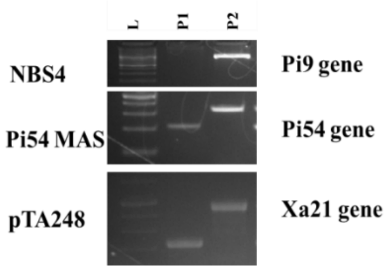
Agro-morphological performance of F_4 and CO 51 parent: The progeny of selected F_4 including CO 51 were raised in a nursery bed and 21 days old seedlings were transplanted into the main field with two replications to evaluate the superiority of selected F_4. The biometric parameters *viz*., plant height, number of productive tillers per plant, flag leaf length and width, panicle length, grain length and width, 1000 grain weight and single plant yield have been observed in all the progenies and parents.

Grain parameters **:** The process involved pre-soaking ten whole milled grains in 25 ml of distilled water. Afterward, they were placed in a water bath at a temperature of 98°C for 10 minutes. The cooked kernels were then transferred into a petri dish, and various traits such as kernel length and breadth after cooking (KLAC and KBAC), were measured (Muthu *et al*., 2020).

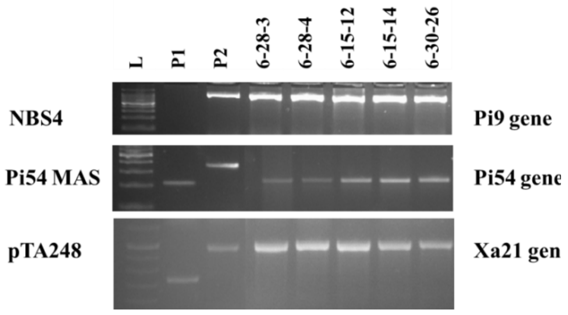
RESULTS AND DISCUSSION

Introgression of *Pi9, Xa21* in CO51 plus *Pi54* gene**:** The Marker Assisted Breeding (MAB) has been followed for stacking of genes in the background of CO 51. Initially, Progeny #6-37 (harboring *Pi9* and *Pi54*) was crossed with Progeny #8-13 (harboring *Xa21*) to generate F_1 plants. Schematic representations of the development of the breeding population are mentioned in **Fig. 1**. **and 2** displays the polymorphisms observed between the recurrent parent, CO 51, and the donor parent, 562-4. The markers which were closely linked markers *viz*., NBS4 for the *Pi9* gene, Pi54MAS for *Pi54* gene and pTA248 for *Xa21* gene were used to identify true F_1 plants. The true F_1 was identified with a linked marker and forwarded to F_2. In F_2, all progenies were screened with linked markers for targeted traits and identified five homozygous plants harboring all three target genes and forwarded to F_3. The selected progenies of the F_3 population were raised in the field and evaluated for agronomic traits in F_4. Genotyping of targeted genes and selected F_4 have been shown in **Fig. 3 and Table 3**.

Evaluation of RILs against Blast and BB pathogen: Recombinant inbred line of CO 51 in F_4, were stacked *Pi9* along with *Pi54* which confer blast resistance and *Xa21* has been introgressed for BB resistance. For validation of *Pi9+Pi54* into CO 51, Blast (*Magnaporthe grisea*), screening was done in five progenies of F_4 along with donor and recurrent parents in a hotspot area, at Hybrid Rice Evaluation Centre (HREC), Guddalur. The blast scoring was recorded first after the 30th and 45th days after sowing in UBN and the second scoring was recorded after the 15th day's interval. The average of each score was used to categorize immune reactions to blast pathogens as per SES, IRRI (SES, 2013). The donor parent 562-4 score of 1.8 showed its R reaction whereas recurrent parent CO 51 score of 5 showed its MR reaction to blast pathogen. All five progenies of CO 51 RILs harbor *Pi9* and *Pi54* genes, and recorded average scores ranging from 1.5 to 3.8, which confirms its enhanced resistance reaction as compared with recurrent parent CO 51 (**Table 4**). The progeny #6-28-4 showed an average score of 1.5 whereas progeny #6-28-3 showed an average score of 1.6 conferring strong resistance. Other RIL of CO 51, progenies #6-15-12, #6-15-14 and #6-30-26 showed an average score of 3.2, 3.4 and 3.8 and were recorded as resistant to blast pathogen respectively (**Fig. 4**). The susceptible check, CO 39 recorded an average score of 7.4 as a susceptible reaction to pathogen. To address major biotic stresses (Blast and BB) in rice production, three major gene combinations, *Pi9* and *Pi54* for rice blast and *Xa21* for BB were selected for introgression. In this study, the *Pi9* gene was stacked with *Pi54* in the background of the CO 51 gene, and the improved lines harbor both genes (*Pi9* and *Pi54*). Introgression of broad-spectrum genes into elite cultivar against blast pathogen in endemic areas increased resistance against blast pathogen and over the cultivars with only one resistance gene (Mew *et al*., 1992). Therefore, pyramiding of multiple resistance genes has been proposed to develop resistance against pathogens (Shanti and Shenoy, 2005; Sundaram *et al*., 2008). *Pi9* and *Pi54* major resistance genes were introduced into 07GY31, and improved NIL harboring *Pi9+Pi54* recorded resistance response against blast pathogen (Xiao *et al*., 2017). The broad-spectrum genes *Pi54* and *Pi1*, *Pi33*, and *Pigm* resulted in enhanced resistance to leaf and panicle blasts (Wu *et al*., 2019). Recurrent parent CO 51 harboring *Pi54* and exhibited an



P1; CO 51 and P2; 562-4
Fig. 2. Polymorphisms between recurrent parent and donor parent



P1; CO 51 and P2; 562-4
Fig. 3. Genotyping details of selected F_4

Table 3. List of selected progenies in F_4

S. No	F_4	*Pi9* (NBS4)	*Pi54* (Pi54MAS)	*Xa21* (pTA248)	Number of genes
1	Progeny #6-28-3	+	+	+	3
2	Progeny #6-28-4	+	+	+	3
3	Progeny #6-15-12	+	+	+	3
4	Progeny #6-15-14	+	+	+	3
5	Progeny #6-30-26	+	+	+	3

Table 4. Mean SES scoring for blast and BB for breeding lines

	Blast Scoring			BB Scoring in cm		
F_4	Score I	Score II	Mean	Score I	Score II	Mean
CO 51	4.2	5.8	5.0	7.2	12.5	9.9
Progeny #6-28-3	1.4	1.8	1.6	0.5	0.7	0.6
Progeny #6-28-4	1.2	1.8	1.5	0.4	1.0	0.7
Progeny #6-15-12	4.6	1.8	3.2	0.4	1.1	0.7
Progeny #6-15-14	2.6	4.2	3.4	0.4	0.8	0.6
Progeny #6-30-26	4.2	3.4	3.8	0.3	0.9	0.6
562-4 (as Donor)	1.4	2.2	1.8	0.3	0.4	0.3
CO 39 (as Check	6.6	8.2	7.4	-	-	-
TN 1 (as Check)	-	-	-	15.1	19.6	17.3

SES; Standard Evaluation System, BB; Bacterial leaf Blight, CO 39 used as check for blast screening; TN 1 used as check for BB screening, S; Susceptible, R; Resistance and MR; Moderately Resistance

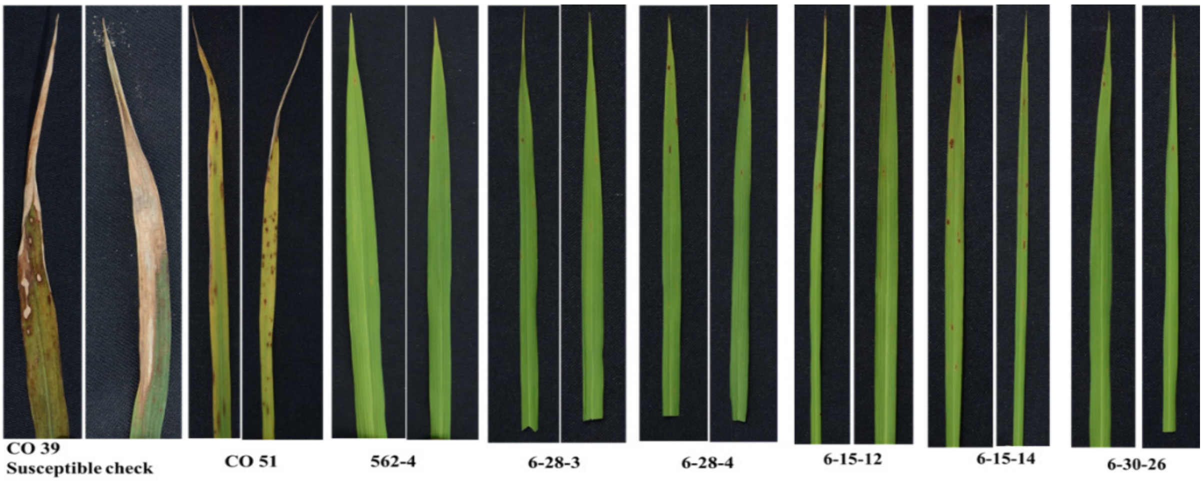


Fig. 4. Screening of MAB Lines along with parents and CO 39 as susceptible check against Blast

MR (SES score 5.0). On the other hand, improved CO 51 containing *Pi9* together with *Pi54* showed enhanced resistance responses to blast pathogens (SES- Score 1.5 to 3.8).

Similarly, resistant reaction to *X. o.* pv. *oryzae* was recorded by lesion length. The lesion lengths were measured in each entry in the 14th and 21st days post-inoculation when the lesion was stable. The recurrent parent CO 51 scored 9.9 cm (susceptibility response) whereas the donor parent, 562-4 secured an average score of 0.3 cm and was regarded as a highly resistant reaction to BB pathogen. All five progenies of CO 51 RILs harbor the *Xa21* gene and showed an average score ranging from 0.6 to 0.7 cm (as resistance) as compared with recurrent parent CO 51 (**Table 4**). The progeny

#6-28-4 showed an average score of 0.7 cm whereas progeny #6-28-3 showed an average score of 0.6 cm with strong resistance. The rest of the progenies #6-15-12, #6-15-14 and #6-30-26 showed an average score of 0.7 cm, 0.6 cm and 0.6 cm respectively (**Fig. 5 and Table 4).** The susceptible check, TN1 recorded an average score of 17.3 cm (susceptible). Recurrent parental CO 51 showed 9.9 cm of BB lesion length and the improved line 0.6 cm to 0.7 cm (as a resistance response). However, the improved line retained only *Xa21* in the CO 51 background, whereas the donor parent 562-4 contained *xa13* and *Xa21*, with a disease score of 0.3 cm with a high resistance to *X. o.* p.v*. oryzae*. It indicated that the *Xa21* gene alone in F_4 RIL progenies shows significant immune reaction and enhanced resistance over recurrent parent CO 51. The major resistant genes *viz*., *xa13* and *Xa21* have been successfully introgressed into popular rice varieties through MAS such as Improved Pusa Basmati 1 (Gopalakrishnan *et al.*, 2008), Tellahamsa (Jamaloddin *et al.*, 2020) Pusa basmati 1728 and Pusa basmati 1718 (Singh *et al.*, 2018). A similar strategy has been followed to develop a resistance against BB pathogen by introgression of *Xa21* into CO 51 genetic background.

Agronomic evaluation of F_4 progenies along with parents: The plant height of recurrent parent CO 51 was recorded as 88.55 cm and in RILs lines, it ranges between 87.94 and 88.80 cm with an average of 87.89 cm (**Table 5**). The number of productive tillers per hill in CO 51 has been recorded at 23.21 whereas in RILs, ranges between from 21.79 to 23.05. The mean grain yield of CO 51 was recorded as 37.01 g whereas in RILs, it ranges between 35.95 g and 38.64 g. The 1000-grain weight of RILs ranged from 19.30 g to 20.83 g

Grain parameters and cooking quality of RILs and recurrent parent: Grain quality of CO 51 and pyramided RILs were measured before and after cooking, for ten grains in each entry. The KLBC and KBBC of CO 51 were recorded as 5.5 mm and 1.9 mm respectively whereas the KLAC and KBAC were 8.9 mm and 2.3 mm respectively (**Fig. 6 and Table 6**). In pyramided RIL lines, KLBC varied from 5.5 to 5.7 mm with an average of 5.6 mm whereas KBBC ranged from 1.8 to 2.0 mm with an average of 1.9 mm. KLAC in pyramided RIL lines was observed to range from 8.5 to 9.0 mm whereas KBBC ranged from 2.3 to 2.6 mm.

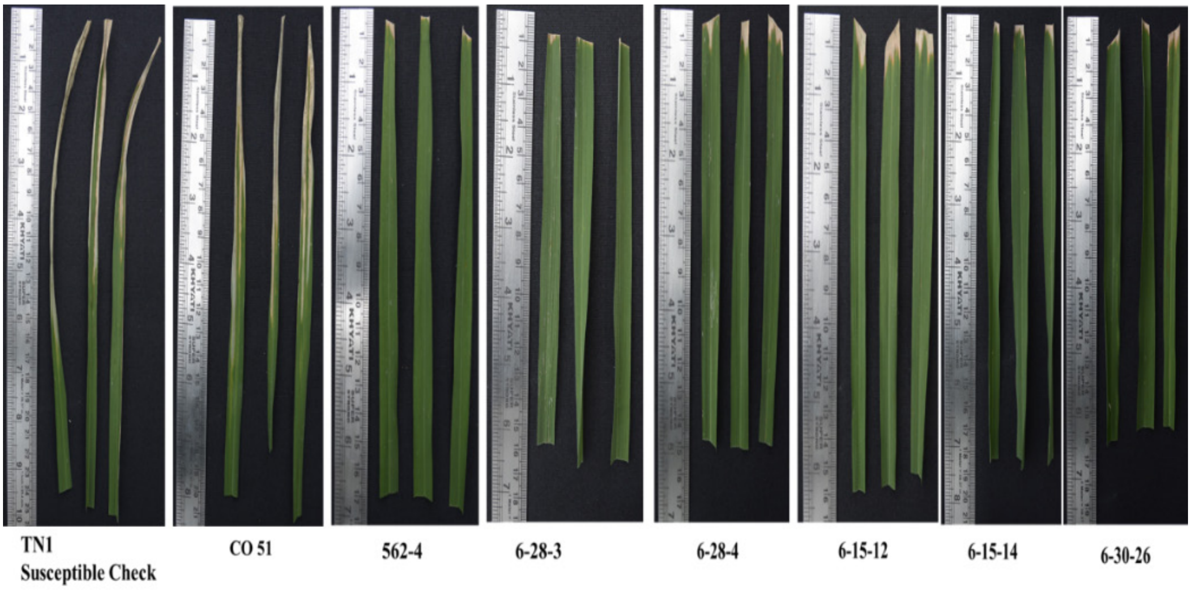


Fig. 5. Screening of MAB Lines along with parents and TN 1 as susceptible check against BB

The success of MAB always depends on the genotypic selection in early generation and stringent selection of progenies based on morphology traits like the recurrent parent. When creating improved varieties of Pusa Basmati-1, Samba Mahsuri, KMR-3R and Improved White Ponni, the targeted trait was selected based on marker-based selection for grain type (Muthu *et al.*, 2020). Similar methods were used in the current MAB, stringent selection based on morphology traits of grain character *viz*., kernel length and breadth similar with CO 51 recurrent parent. In $F_{3:4}$, five homozygous plants were selected based on grain characters and homozygous for targeted genes for blast and BB pathogens. During the analysis of cooking parameters for CO 51 and the advanced breeding lines derived from CO 51 X 562-4, it was observed that these advanced breeding lines exhibited characteristics such as KLBC and KBBC, which were similar to the medium slender grain type found in recurrent parent CO 51.

Table 5. Agro-morphological performance of RILs and CO 51

F_4	Plant height (cm)	No of productive tiller per plant	Flag leaf length (cm)	Flag leaf width (cm)	Panicle length (cm)	Days to 50% flowing	Grain length (mm)	Grain width (mm)	1000 grain weight (g)	Single Plant Yield (g)
CO 51	88.55	23.21	28.56	1.15	24.48	95.4	5.47	1.89	18.73	37.01
Progeny #6-28-3	88.08	23.05	28.83	1.11	24.64	94.6	5.49	1.88	19.30	37.09
Progeny #6-28-4	88.17	22.99	28.52	1.09	24.70	94.0	5.48	1.88	19.73	36.41
Progeny #6-15-12	87.94	22.67	28.26	1.09	24.47	93.8	5.47	1.90	19.44	35.95
Progeny #6-15-14	86.49	21.79	28.51	1.09	24.39	93.8	5.46	1.90	20.20	38.26
Progeny #6-30-26	88.80	21.84	27.96	1.09	24.16	94.0	5.49	1.90	20.83	38.64
Mean of RIL	87.89	22.46	28.41	1.09	24.47	94.0	5.478	1.89	19.90	37.27

RIL; Recombinant Inbred Line, cm; centi meter, mm; milli meter and g; grams

Table 6. Kernel Length and Breadth before and after cooking

	KLBC (mm)	KBBC (mm)	KLBR	KLAC (mm)	KBAC (mm)	KLERC	Volume expansion
CO 51	5.5	1.9	2.9	8.9	2.3	1.6	3.4
Progeny #6-28-3	5.6	1.8	3.1	8.7	2.4	1.6	3.1
Progeny #6-28-4	5.7	1.9	3.0	8.6	2.5	1.5	2.9
Progeny #6-15-12	5.5	1.9	2.9	8.5	2.6	1.5	3.0
Progeny #6-15-14	5.6	1.9	2.9	8.7	2.3	1.6	3.1
Progeny #6-30-26	5.5	2.0	2.8	9.0	2.6	1.6	3.5
Mean of F_4	5.6	1.9	2.9	8.7	2.5	1.6	3.1

KLBC; Kernel Length Before Cooking, KBBC; Kernel Breadth Before Cooking, KLAC; Kernel Length After Cooking, KBAC; Kernel Breadth After Cooking, KLBR; length/breadth ratio, KLERC; Kernel Length Elongation Ratio on Cooking and mm; milli Meter.

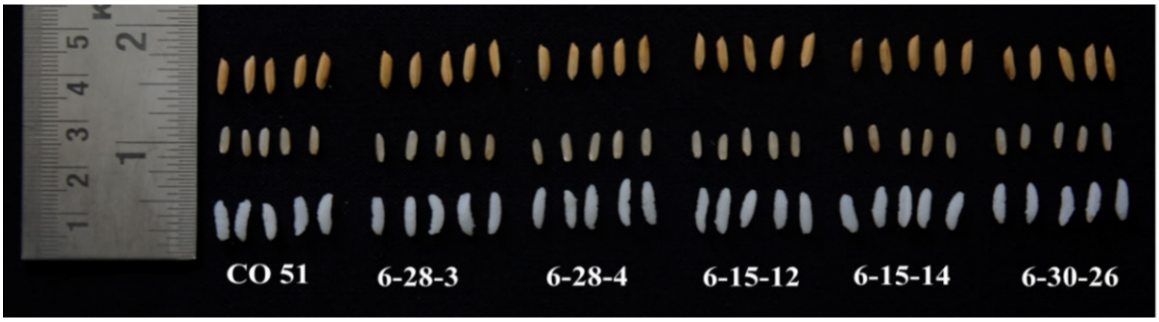


Fig. 6. Before and after cooking of grain parameter of CO 51 and Progenies of F_4

Among five RILs of CO 51 x 562-4, Progeny #6-28-3 recorded a blast score of 1.6 and a blight score of 0.6 cm with resistance reaction and with grain yield of 37.01 g. It was followed by Progeny #6-28-4 which recorded a blast score of 1.5 and blight score of 0.7 cm showing a resistance reaction with a grain yield of 36.41 g.

The present study aimed to stack or pyramid genes that are resistant to blast and BB disease into CO 51 background to enhance resistance against blast and BB pathogen. This goal was achieved by MAS which uses markers closely linked to the targeted traits and stringent selection in earlier generations. A phenotypic screening against BB and the blast pathogen demonstrated complete expression of the targeted *QTLs*/genes in the CO 51 genetic background.

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