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Research Article

Genotypic and phenotypic analysis of backcross inbred lines for brown plant hopper resistance in rice

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Abstract
The most devastating insect pest of rice, Brown plant hopper (BPH) (*Nilaparvata lugens* Stal.) feeds on the rice plant and results in huge yield loss. Breeding of resistant cultivars is more economical and eco-friendlier than use of chemical pesticides. Rice cultivars with improved resistance to disease and pest are developed with pyramiding techniques to incorporate the stronger resistance genes in single plant. In this study, CO51, a popular elite variety of Tamil Nadu, improved with introgression of bacterial blight and blast resistance genes was used as a recurrent parent and BPH resistance genes were introgressed in it using marker-assisted selection (MAS) method of breeding. The lines having multiple resistance genes were evaluated using foreground and agronomic trait selection and forwarded toBC_2F_4 generation. The bioassay of selected twenty-seven lines showed either single or both gene introgression enhanced resistance than the recurrent parent.

Keywords: Rice, Brown plant hopper resistance, Gene introgression, marker assisted selection, phenotypic screening.

INTRODUCTION

Diseases and insect pests have consistently posed significant challenges in agriculture, leading to substantial reduction in crop yield and a decline in grain quality. Rice, being a crucial cereal crop in the Asia-Pacific region, serves as a host to a diverse range of insects that feed on it. Among these pests, the Brown Plant Hopper (BPH), scientifically known as *Nilaparvata lugens* Stal., is particularly detrimental to rice production, causing yield losses ranging from 20 to 80% and the estimated economic damage caused by BPH alone is approximately $300 million per year in Asia(Satturu *et al.*, 2020). BPH feeds on the phloem sap of rice plants, resulting in symptoms known as “hopper burn” and ultimately leading to the senescence of the entire plant (Dale, 1994).It is also known to transmit various viral diseases such as grassy stunt and ragged stunt virus in rice plant (Jena *et al.*, 2006). The application of chemical pesticides like imidacloprid is the primary strategy used to control BPH attack in plants. However, this strategy is not only expensive but also dangerous for environment and human health. Additionally, it inadvertently eliminates natural predators and promotes the growth of BPH biotypes that are resistant to insecticides(Tanaka *et al.*, 2000). Therefore, using host-plant resistance approach to manage insects and increase yield is the most cost-effective, efficient and ecologically friendly (Ramalingam *et al.*, 2020b).

The investigation of genetic control in developing BPH resistant rice varieties led to the discovery of a large number of the BPH resistance genes/ loci. Among these, IR26, released during 1973 carried *Bph1* gene, was the

first BPH resistant gene variety to successfully manage BPH in large cultivated areas. Unfortunately, because of the introduction of BPH biotype2, some BPH population became adapted to IR26 within a short period of time, thereby making it susceptible (Jena and Kim, 2010). Till date, the number of BPH resistant genes identified in rice is more than 40, of which most of the genes are present in 1,3,4,6 and 12 chromosome (Pannak *et al.*, 2023).

Nine genes, *Bph3/Bph17, Bph9, Bph14, Bph15, Bph18, Bph26, Bph29*and *Bph32* have been successfully cloned and characterised (Muduli *et al.,* 2021; Liu *et al.,*2015; Tamura *et al.,*2014; Wang *et al.,*2015; Ji *et al.,*2016; Zhao *et al.,*2016; Ren *et al.,*2016).Through the introduction of several BPH resistant 'R' genes through marker breeding, a sustainable and environmentally friendly method called "gene pyramiding" is being employed to create resilient resistant cultivars against BPH(Vignesh *et al.*, 2023). Successful striking of multiple genes for biotic stress resistance has been reported for several crops (Ramalingam *et al.*, 2017), (Chithrameenal *et al.*, 2018), (Ramalingam *et al.*, 2020a) (Ramalingam *et al.*, 2020b).This strategy seeks to develop BPH resistance that will ensure long lasting resistance in combating pest. The BPH resistant genes, *bph2*(Jena *et al.*, 2017)and *Bph32*(Ren *et al.*, 2016)was introduced into elite variety of Tamil Nadu, CO51, along with introgressing bacterial blight and blast resistance genes.

MATERIALS AND METHODS

Plant materials: The rice variety, CO51 is renowned for its short duration (105-110 days), semi dwarf, high yield potential and fine grain quality popularly grown in South India. To improve its resistance, bacterial blight resistant genes *xa5, xa13*, and *Xa21* and blast resistant gene *Pi54* were introgressed previously (Ramalingam *et al.,* 2020). As the recurrent parent, the improved CO51 was crossed with the donor parent PTB33, which carries the brown plant hopper resistant genes *bph2*(Jena *et al.*, 2017) and *Bph32*(Ren *et al.*, 2016), to further enhance its resistance for brown plant hopper.

In the previous study, F_1, BC_1F_1, BC_2F_1 and BC_2F_2 populations were developed using marker assisted backcross breeding(Gokulakrishnan *et al.*, 2022). The current study was aimed to select the genotypes with prominent BPH genes,*bph2* and *Bph32*along with bacterial blight and blast resistant genes in the background of CO51 using marker assisted selection in BC_2F_3 and BC_2F_4 generation. BC_2F_3 population was analysed using functional/ linked markers and agronomic traits. Further, the selected homozygous lines were screened for BPH resistance, against the biotypes present in Thanjavur and Coimbatore region of Tamil Nadu.

Molecular marker analysis and evaluation of agronomic traits:The foreground selection was carried out using SSR markers*BPH18-ind2, PASH 6, xa5-1, xa13-prom,pTA248,Pi54-MAS* for *bph2, Bph32, xa5, xa13, Xa21* and *Pi54* genes (**Table 1**). ADT55, variety developed with the help of functional markers using marker assisted selection by Tamil Nadu Agricultural University, Coimbatore, officially released to Tamil farmers during 2022 (Sairachana *et al.*, 2023) (Sakthivel *et al.*, 2017), was used as a positive check for the confirmation of bacterial blight genes. Tetep, donor for *Pi54* was used as a check for blast resistance. The BC_2F_3homozygous lines with BPH resistant genes along with either bacterial blight and blast gene combinations were selected and analysed for agronomic traits *viz*., plant height (cm), number of panicles per plant, grain yield per plant (g). The plants with superior genotypic and phenotypic traits similar to CO51 were selected. Seeds of selected lines were further grown and analysed for BPH bioassay with parents, Improved CO51 and PTB33 along with Taichung native1 (TN1) as a susceptible check in the next BC_2F_4 generation.

Genomic DNA of selected 530 plant of BC_2F_3 was extracted using modified CTAB method(Dellaporta *et al.*, 1983). Concentration of the extracted DNA was estimated with the help of Genova Jenway Nanodrop spectrophotometer. The genomic DNA was further diluted to 50-100ng/µl for using in PCR amplification.

For PCR, 10µl of the reaction was prepared containing 1µl of the template DNA, 0.5µl of forward and reverse primer each, 4µl of Emerald Takara Master Mix, 4µl of nuclease free water. The PCR profile was used for 35 cycles at 94˚C for 5 min. initial denaturation, 94˚C for 1 min. denaturation followed by primer annealing at 56˚C for BPH18-ind2, xa5-1, Pi54-MAS, 57˚C for PASH6, 59˚C for xa13-prom, 65˚C for pTA248, followed by extension at 72˚C for 1 min., final extension at 72˚C for 7 min. and infinite hold at 4˚C.The PCR products were resolved in 3% agarose gel in which Ethidium Bromide was added for band visualization in gel documentation unit (BIO Rad Gel Doc EZ Imager) under UV.

Phenotypic screening for BPH resistance: For BPH screening, BPH adults were collected from the field and mass cultured in controlled condition. The seeds of 27 lines of BC_2F_4were pre-soaked a day prior to sowing and seeds were sown in protrays including TN1 on both the extreme corners and CO51 and PTB33 in the middle, for checking the performance of BC_2F_4 generation plants. The protrays are made up of polythene sheets and a size of 51x28cm accommodating 50 cells. A total of 10-15 seedlings were maintained in each cell. Two replications were maintained for each entry. The protrays were maintained in wire mesh cages to prevent entry of other insects. After seven days of sowing, the seedlings were infested with second and third instar BPH nymphs. Plants were observed on daily basis. Nearly five days after infestation 'hopper burn' symptoms were observed. Scoring was done when TN1 seedlings of both the sides dried completely. Scoring was done individually based on the scoring system of International Rice Research Institute (Rice, 1996).

Table 1.List of Linked/ functional markers used for foreground Selection

Gene	Chromosome	Marker		Primer sequence	AT (°C)	Size (bp)	Reference
bph2	12	BPH18-ind2	F	TGGGCTGACAAATGGGTCC	56°C	257	Ji et *al.* (2016)
			R	CCTTGTCGGGTGTAGCCAA			
Bph32	6	PASH 6	F	CCGACAACAAGACCTCCAAT	57°C	193	Jena *et al.* (2017)
			R	CTGAACTGCACCTGGGTTTT			
xa5	5	xa5-1	F	CGGATAGCAGCATTTCCAAGAG	56°C	299	Iyer-Pascuzzi and McCouch, (2007)
			R	GATTCCTTTAGCAAGGTGTG			
xa13	8	xa13-prom	F	GAGCTCCAGCTCTCCAAG	59°C	500	Chu *et al.*(2006)
			R	GGCCATGGCTCAGTGTTTAT			
Xa21	11	pTA248	F	ATAGCTAGTTCATAGAGG	65°C	925	Song*et al.*(1995)
			R	ACATCCGTCACTCTGCCA			
Pi54	11	Pi54-MAS	F	CAATCTCCAAAGTTTTCAGG	56°C	216	Ramkumar *et al.*(2011)
			R	GCTTCAATCACTGCTAGACC			

RESULTS AND DISCUSSION

Introgression of *bph2* **and** *Bph32* gene in Improved CO51 variety: CO51 is a semi dwarf with fine rice grain type and short duration variety widely cultivated in Tamil Nadu. CO51 was improved by introgression of bacterial blight (*xa5, xa13, Xa21*) and blast (*Pi54*) resistance gene. In order to provide BPH resistance along with bacterial blight and blast in CO51, improved CO51 was crossed with the donor PTB33 harbouring *bph2* and *Bph32* resistance genes and backcross inbred lines (BILs) were developed. In each generation, the plants heterozygous to all the above six genes were selected and forwarded to next generation. In BC_2F_1, the plants with heterozygous alleles were selected and successively forwarded to produce a large segregating population in BC_2F_2 and BC_2F_3 generation respectively. Breeding scheme was followed as shown in **Fig.1**.

Selection methods adopted for the production of homozygous lines: Foreground selection in BC_2F_3 generation was carried out for six genes *bph2*, *Bph32*, *xa5*, *xa13*, *Xa21* and *Pi54* using gene specific/linked markers to specific gene that were*BPH18-ind2, PASH6, xa5-1, xa13-prom, pTA248* and *Pi54-MAS* respectively (**Fig. 2**).A total of 530 plants evaluated in BC_2F_3 generation in which multiple gene combinations were observed comprising of 6 (*bph2, Bph32, xa5, xa13, Xa21, Pi54*), 23 (*bph2, Bph32, xa5, xa13, Xa21),* 247 (*bph2, xa5, xa13, xa21*), 33 (*bph2, xa5, xa13, xa21, Pi54),* 4 (*bph2, Bph32, Pi54),* 194 (*bph2, Pi54)*, 23 (*bph2, xa13, Xa21, Pi54)*. Upon phenotypic selection and agronomic trait selection, the twenty-seven homozygous plants showing similar to CO51 along with multiple gene combinations were selected. Observations were taken on the following agronomic trait *viz*., plant height (cm), number of panicles and grain

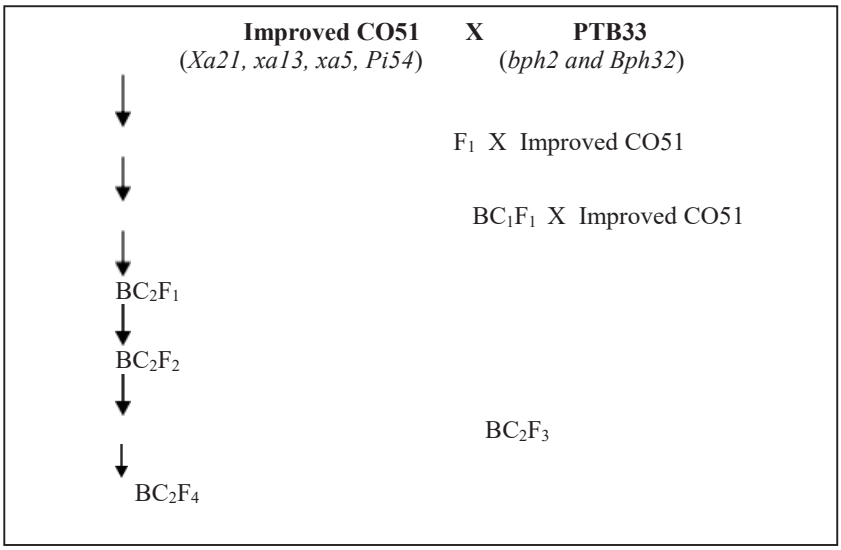

Fig. 1. Development scheme of backcross inbred lines with the help of marker assisted backcross breeding

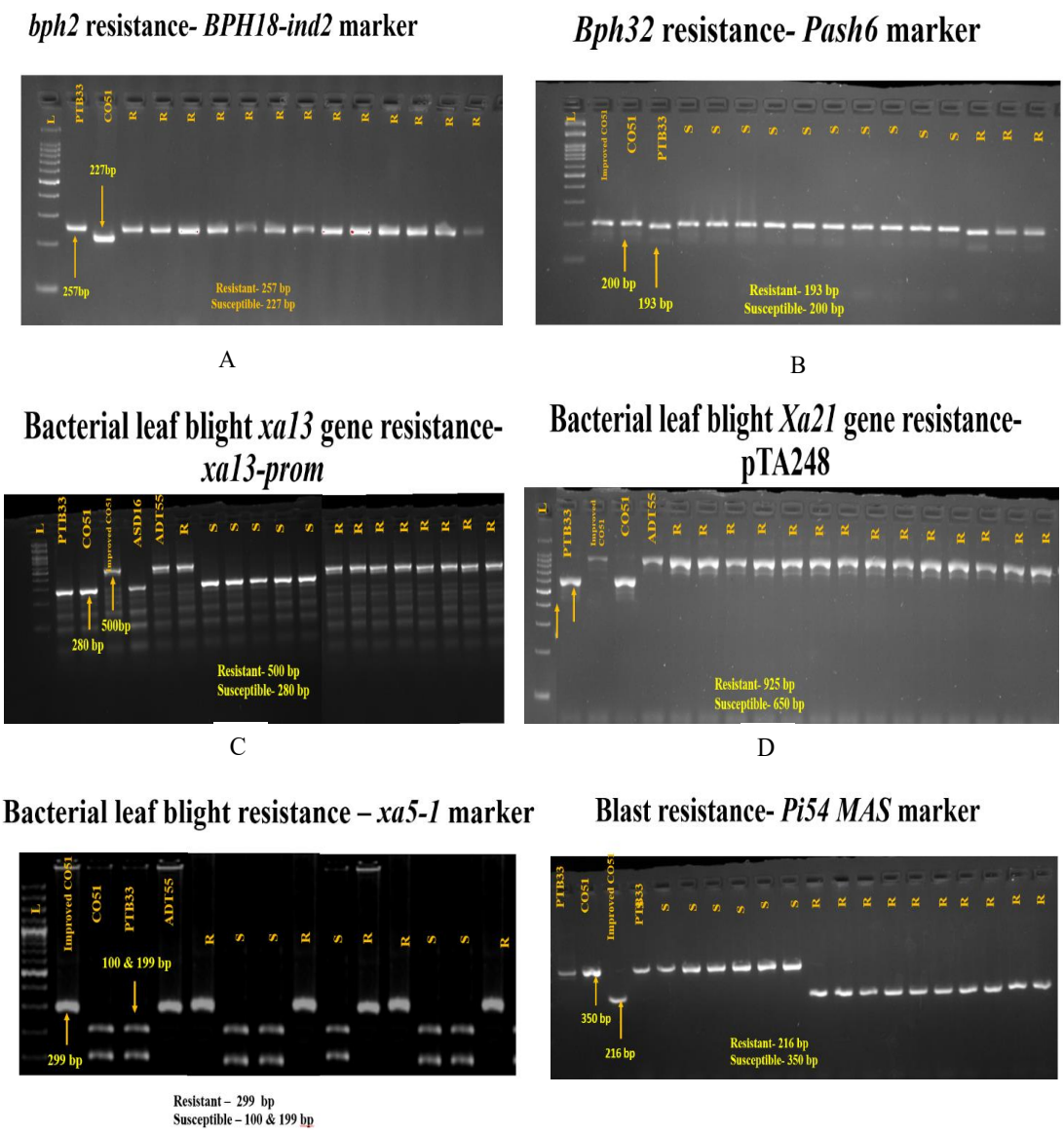

Fig. 2.PCR amplified products representing (A) marker *BPH18-ind2* for *bph2* alleles, (B) *Pash-6* marker for *Bph32* alleles, (**C**) Pi51-MAS marker for *Pi54* alleles (**D**) xa13-prom marker for *xa13* alleles, (**E)** xa5-1 marker for *xa5* alleles (**F**) pTA248 marker for *Xa21* alleles, in ImprovedCo51xPtb33 cross at BC_2F_3generation. **L** – 100 bp ladder; **R**– Resistant allele and **S** –Susceptible allele.

yield per plant (g),which showed high grain yield per plant ranging from 23.03g to 49.02g signifying superiority to the recurrent parent having grain yield 22.05g. Twenty-seven plants with recurrent parent characteristics(plant height, grain size, grain type)were obtained having multiple gene combination as follows -6 (*bph2, Bph32, xa5, xa13, Xa21),* 10 (*bph2, xa5, xa13, xa21*), 3 (*bph2, xa5, xa13, xa21, Pi54),*2 (*bph2, Bph32, Pi54),* 5 (*bph2, Pi54)*1 (*bph2, xa13, Xa21, Pi54)*(**Table 2**). Overall, the yield of improved lines was higher and more or less similar to that of CO51, under natural field conditions. Further subjected to phenotypic screening for BPH bioassay.

Protray screening test (PST) for the evaluation of backcross inbred lines for BPH resistance: To evaluate the effect of BPH resistant genes alone and in combination, the selected progenies of BC_2F_3 generation were sown as BC_2F_4 generation and bioassay was done at seedling (seven days old) stage using protray screening test (**Fig. 3**). Significant difference was observed in the resistance level to BPH among the selected progenies after seven days of BPH infestation. Progenies and parents were scored after TN1 was completely dried. CO51 recorded 9 score. Whereas PTB33 harbouring both*bph2*and *Bph32* scored 3. The lines containing

Table 2. Genotypic and phenotypic data of BC_2F_3 generation

Plant number		Genotypic screening data						BPH - scoring data*	Phenotypic data		
		BPH18-ind2	PASH6 and RM589	xa5-1	xa13-prom	pTA248	Pi54-MAS		Plant height (cm)	Number of panicles per plant	Grain yield/ plant (g)
1	13	+	-	+	+	+	+	6.46 (MS)	69	20	25.96
2	14	+	-	+	+	+	+	6.89 (MS)	69	17	28.05
3	17	+	-	-	-	-	+	7.19 (MS)	72	18	25.89
4	24	+	-	-	+	+	+	7.1 (MS)	71	24	37.90
5	35	+	-	+	+	+	+	6.33 (MS)	71	30	32.97
6	43	+	-	-	-	-	+	6.36 (MS)	72	24	25.93
7	57	+	-	+	+	+	-	4.9 (MR)	66	27	27.22
8	60	+	+	+	+	+	-	3.54 (R)	63	19	26.54
9	65	+	+	+	+	+	-	4.18 (MR)	65	17	23.42
10	66	+	+	+	+	+	-	4.14 (MR)	65	25	25.29
11	81	+	+	+	+	+	-	3.16 (R)	70	19	22.59
12	82	+	+	+	+	+	-	5.0 (MR)	70	18	28.16
13	88	+	-	-	-	-	+	7.5 (MS)	72	18	22.05
14	106	+	-	-	-	-	+	8.2 (MS)	71	24	28.34
15	132	+	+	+	+	+	-	4.8 (MR)	68	21	36.9
16	137	+	-	+	+	+	-	6.63 (MS)	60	15	22.24
17	144	+	-	+	+	+	-	7.44 (MS)	61	11	24.61
18	167	+	-	+	+	+	-	5.44 (MR)	64	20	26.37
19	189	+	-	+	+	+	-	5.63 (MR)	65	21	28.28
20	211	+	-	+	+	+	-	6 (MS)	65	23	33.14
21	214	+	-	+	+	+	-	7.6 (MS)	69	30	32.79
22	226	+	-	+	+	+	-	6.83 (MS)	66	25	44.32
23	227	+	-	+	+	+	-	6.77 (MS)	70	30	37.31
24	268	+	-	+	+	+	-	7.28 (MS)	62	18	26.52
25	371	+	+	-	-	-	+	4.55 (MR)	67	15	22.60
26	446	+	+	-	-	-	+	4.87 (MR)	72	20	49.02
27	498	+	-	-	-	-	+	6.80 (MS)	63	18	21.13
Parents	**Co51**	-	-	-	-	-	-	9 (S)	68	18	21.66
	Imp. Co51	-	-	+	+	+	+	9 (S)	69	20	22.05
	Ptb33	+	+	-	-	-	-	3 (R)	101	17	26.03

+ Resistant allele, -susceptible allele

*BPH phenotypic screening data of BC_2F_4 generation, as per IRRI standard evaluation – resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S).

both the BPH genes scored between 3 and 4, showing resistance to moderately resistance, respectively. Whereas the plant carrying single genes scored between 5 and 6 showing moderately resistance to moderately susceptibility, respectively. The lines carrying single or two BPH resistant genes showed greater resistant compared to recurrent parent (CO51). In conjugation with the negative effect of climate change and modernized rice production practices, the predominance of pests, notably BPH has become a serious threat to world food security (Liu *et al.,* 2015). Marker-assisted selection and backcross breeding together can resolve the weakness in rice cultivars that are particularly vulnerable. The technique has shown promise in developing varieties with improved resistance to pest and diseases including bacterial blight, sheath blight, blast and gal midge (Huang *et al.*, 1997); (Datta *et al.*, 2002); (Maruthasalam *et al.*, 2007);(Jiang *et al.*, 2012). The

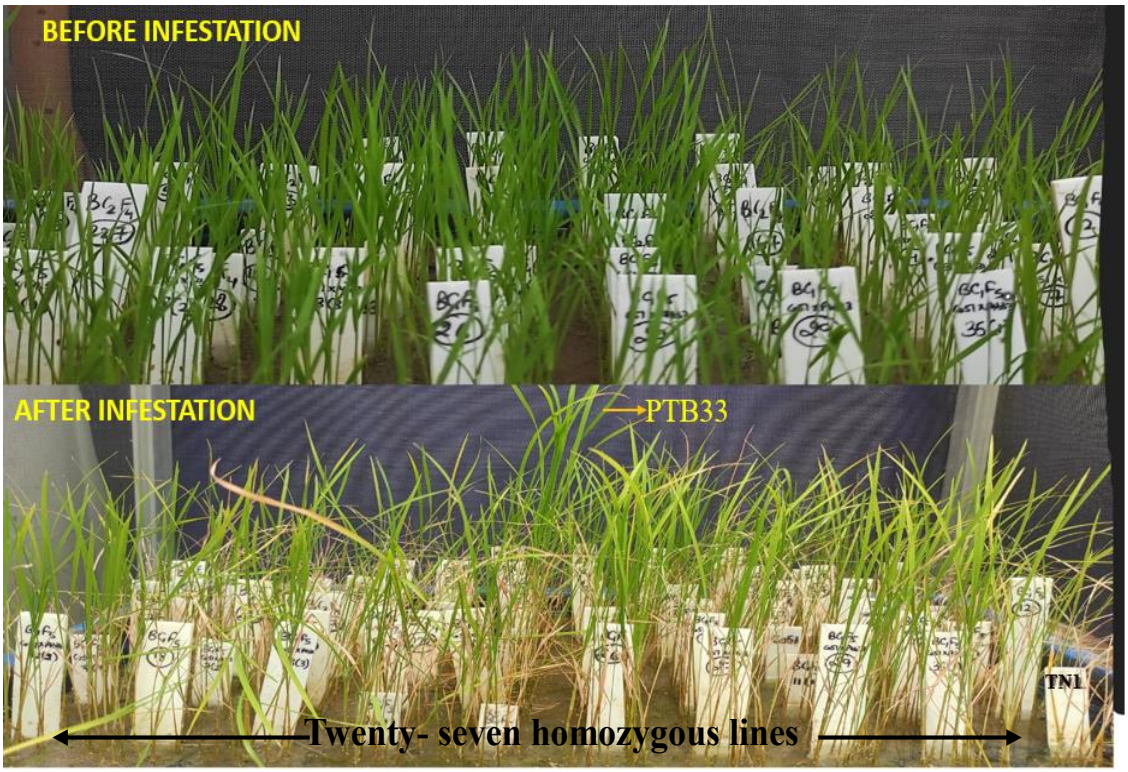

Fig. 3. Comparison of seven days seedlings before and after BPH infestation

effectiveness of resistant cultivars with single resistance against BPH is currently limited due to rapid breakdown of BPH resistance(Alam and Cohen, 1998). Therefore, there is a need to use stronger BPH resistance genes or to combine multiple genes through gene pyramiding in order to create rice cultivars with improved and long-lasting resistance. In the breeding programme, the majority of the segregating population can be screened using the protray screening method(Soundararajan and Jeyaprakash, 2019).Additionally, because the seeds are placed and planted in a circular pattern, insects can easily migrate through plants that are vulnerable, and hopper burn symptoms can appear sooner than they would in a field.

In the present study, two BPH- resistance genes were introgressed or pyramided into an important elite cultivar (CO51) of Tamil Nadu using marker assisted backcrossing and improved lines containing single or two BPH resistance genes along with bacterial blight or blast resistance genes were obtained. BPH bioassay showed that lines having single or two genes conferred different level of resistance at seedling stage. The lines (P-60, P-65, P-66, P-81, P-82, P-132, P-371, P-446) carrying both the resistance genes were more resistant that lines having single genes. Thus, to pursue a durable and broad- spectrum resistance, gene pyramiding will be highly effective, as it will restrict the rapid increase in insect population and thereby reduce the crop damage.

The present study demonstrated successful introgression of single or two BPH resistance genes into the improved elite variety CO51 using MAS. BPH resistance levels was improved significantly as a result of introgression of BPH resistance genes. The advancement of BPH resistance lines holds great promise in the molecular breeding of long lasting BPH-resistant rice cultivars.

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