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Molecular and phenotypic evaluation of restorer lines for bacterial blight and blast resistance for developing heterotic hybrids in rice (*Oryza sativa* L.)

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Abstract
The present study was carried out with an objective to identify restorer lines harbouring fertility restoration genes and resistance to the biotic stresses, Bacterial Blight (BB) and blast diseases. Seventy-one breeding lines were screened for the presence of fertility restorer genes *viz., Rf4* and *Rf3* along with target disease resistance genes, *Pi54* for blast and *Xa21, xa13* and *xa5* for BB. Phenotypic evaluation against BB and blast diseases was done by artificial inoculation and raising the plants in disease screening nursery. Simultaneously, selected parental lines along with previously identified R lines were crossed with two CMS lines *viz.,* APMS6A and CRMS32A for developing experimental 176 F_1 rice hybrids. The rice hybrids along with seven checks were evaluated in Augmented RCBD for 15 yield and yield contributing traits. Based on the results of pollen/spikelet fertility analyses of the F_1 hybrids, parental lines were identified as restorers, partial restorers or partial maintainers. The identified highly heterotic rice hybrids will be subjected to large scale multi-location testing. The potential restorers identified in this study will be highly useful for three-line hybrid rice breeding for developing biotic stress-resistant rice hybrids with higher grain yield.

Keywords: Hybrid Rice, Bacterial leaf blight, Blast, Fertility Restoration, Heterosis

INTRODUCTION

Hybrid rice technology is a promising, sustainable and eco-friendly option to unlock the present yield limits and to achieve higher yield in rice (Virmani, 1996). India has made a commendable progress in hybrid rice technology and a total of 152 rice hybrids have been released so far, including 105 hybrids through Central Variety Release Committee (CVRC) and 47 through State Variety Release Committee (SVRC). These hybrids are cultivated in 3.5 million hectares in India (59th ARGM draft proceedings, 2024). Currently, the predominant method utilized for the commercial development of rice hybrids involves the three-line system, comprising a cytoplasmic male sterile (CMS) line, a maintainer line, and a restorer line. Screening of breeding lines for fertility restoration involves test crossing with a set of CMS lines and evaluation of F_1 for pollen and spikelet fertility, which is laborious and time consuming. However, with the use of molecular markers linked to *Rf* genes, one can identify restorer lines within a short period (Revathi et al., 2013). Till date a total of 18 genes (from *Rf*1 to *Rf*18) for fertility restoration have been identified in rice, all of which are dominant, except, *Rf*17 (Liu *et al.,* 2023). Among these, two major genes,

viz., *Rf*3 and *Rf*4, are known to control fertility restoration of Wild Abortive (WA) cytoplasm (Zhang *et al*. 1997 and Yao *et al*. 1997).

In India, irrespective of the progress in hybrid rice technology, adoption of rice hybrids has been much slower than anticipated, mainly because of unacceptable grain quality, higher seed cost and their susceptibility to pests and diseases. In order to ensure wider adoption of hybrids, identification of restorer lines having resistance to major pests and/or diseases is essential, along with enhanced level of heterosis. Among the biotic stresses affecting rice, bacterial blight (BB) and blast are two major diseases that cause severe yield losses. In the present study, breeding lines were evaluated for BB and blast resistance, pollen fertility and grain yield heterosis and potential restorers were identified that can be used in future for three-line hybrid rice breeding for developing biotic stress resistant rice hybrids with higher grain yield heterosis.

MATERIALS AND METHODS

This experiment was carried out at ICAR-Indian Institute of Rice Research, Rajendranagar. Seventy one breeding lines along with the checks *viz*., BPT 5204, Tetep, Improved Samba Mahsuri and HR 12 were raised in Randomized Block Design with two replications during Kharif, 2022.

DNA Isolation and Molecular Screening: The DNA extraction was carried out by the CTAB method (Murray and Thompson, 1980). The PCR amplification for molecular screening for the presence of the target genes *viz*., *Xa21*, *xa13*, *xa5*, *Pi54*, *Rf4* and *Rf3* was carried out as reported by earlier studies (**Table 1)**.

Phenotypic evaluation against Bacterial Blight and Blast diseases: All the 71 parental lines along with the resistant check, Improved Samba Mahsuri and the susceptible check, BPT 5204 were screened against a hyper virulent BB isolate DXO22 collected from ICAR-IIRR. The culture density of 109 cells/ml was utilized for BB screening following leaf clipping method at maximum tillering stage as described by Kauffman *et al*., 1973. The disease reaction was scored by measuring the lesion length after 14 days of inoculation (Chen et al., 2000). For blast screening, the parental lines along with checks *viz*., Tetep (resistant) and HR-12 (check) were raised at Uniform Blast Nursery (UBN) (Soujanya *et al*., 2023). The blast disease reaction was recorded 15 days post-inoculation as per Standard Evaluation System (SES) (IRRI, 2013).

Developing Experimental Rice Hybrids: Based on both molecular and phenotypic screening, best restorer lines with BB and blast resistance along with previously identified restorers were crossed with two CMS lines *viz*., APMS6A and CRMS32A, during Rabi 2022-23 and 176 experimental rice hybrids along with seven checks were developed and evaluated in Augmented RCBD in six blocks. These hybrids were evaluated for yield and yield-attributing on five randomly selected plants to record the observations of twelve characters *viz*., plant height (cm), flag leaf length (cm), panicle length (cm), number of productive tillers per plant, days to 50% flowering, pollen fertility (%), spikelet fertility (%), thousand grain weight (g), single plant yield (g), total number of grains per panicle, grain length-breadth ratio and per day productivity. The mean data of observations were analysed using the statistical package, R (Augmented RCBD). The genotypic and phenotypic coefficient of variation were estimated from the corresponding variance parameters.

Standard Heterosis estimation: To identify highly heterotic rice hybrids, standard heterosis with hybrid and varietal check were estimated. The Standard heterosis for single plant yield over high-yielding hybrid and varietal checks *viz*., US314, 27P63, HRI-174, Gontrabidhan, BPT 5204, respectively was estimated as per Liang *et al*., (1971) and expressed in percentage. The mean grain yield of each hybrid and standard checks were taken for estimating standard heterosis as follows

$$\text{Standard heterosis} = \frac{F_1 - SC}{F_1} \times 100$$

where,

F_1 = Average performance of the hybrid

SC = Average performance of standard checks (US 314, BPT 5204)

The best restorer lines carrying resistance to BB and/or blast and with higher grain yield heterosis were identified for future hybrid rice breeding.

RESULTS AND DISCUSSION

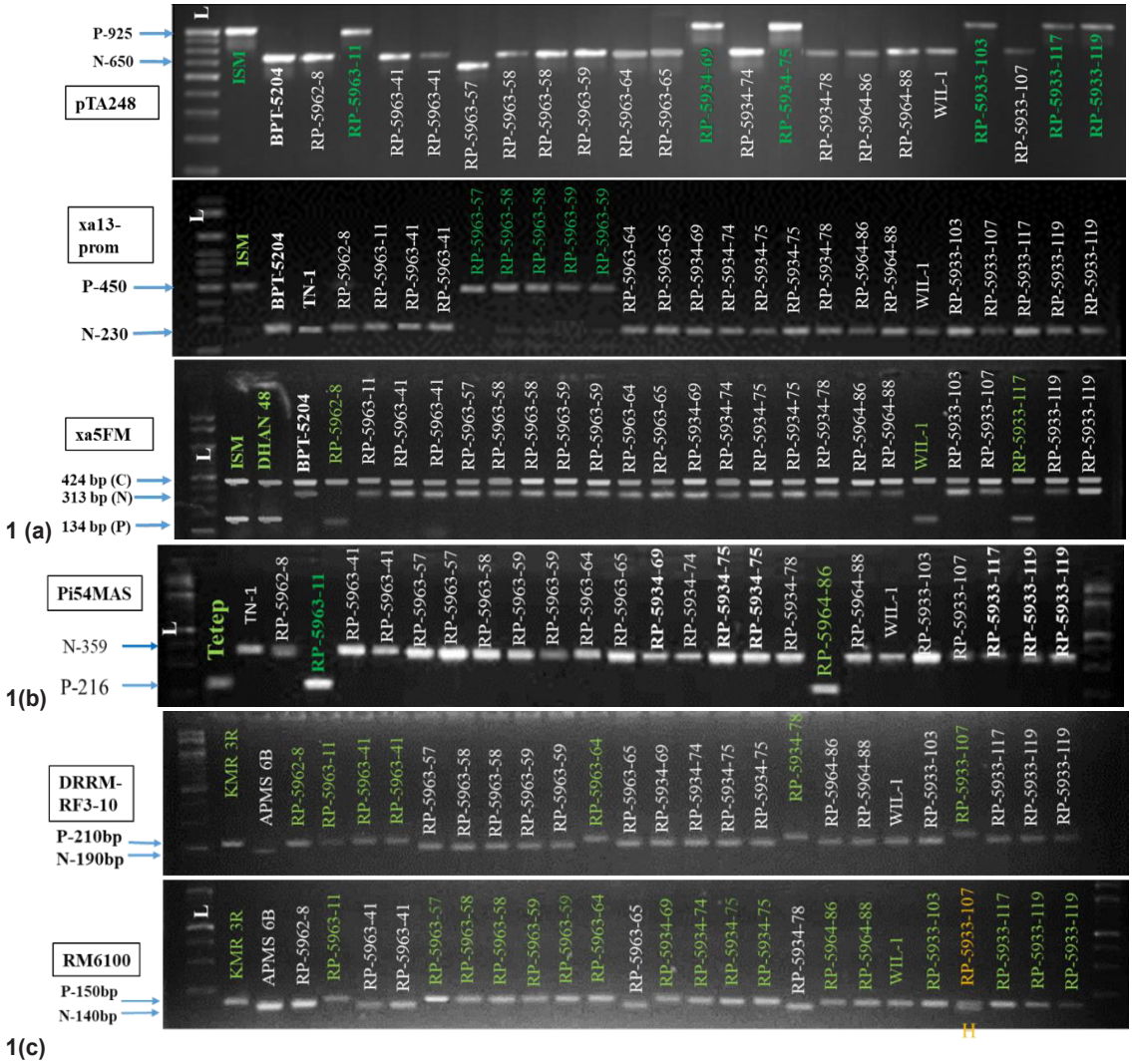
A total of 71 breeding lines were phenotyped as well as genotyped for resistance to BB and Blast as well as restorability to identify the best resistant restorer lines. These lines were screened for the presence of target genes i.e., *Xa21*, *xa13*, and *xa5*, for BB, *Pi54,* for blast, *Rf3* and *Rf4* restorer genes, by utilizing SSR markers either gene based or linked molecular markers (**Table 1**). The list of genotypes that were found possessing target genes are presented in **Table 2 and Fig. 1a, 1b, 1c.**

Phenotypic screening for BB disease identified 14 breeding lines with resistance and 12 lines with moderate resistance, while the remaining lines fell into the susceptible class. Molecular screening identified 12 lines with *xa5* gene, five lines carrying *xa13* gene, and 14 lines with *Xa21* (**Table 2**). Notably, one line RP 6469-117 exhibited a triple combination of resistance genes (*xa5*+*xa13*+*Xa21*), while lines with double gene combination were RP 5933-117, RP5933-137 and WIL-53 (*xa5*+*Xa21*), and also RP 5933-142 (*xa13*+*Xa21*). All these lines carrying the genes/gene combinations also showed field resistance to BB. Based on the results of phenotypic and genotypic screening, it was found that

Table 1. List of SSR markers utilized for molecular screening

Gene	SSR Marker	Primer Sequence (5' to 3')	Amplicon size (bp)		Reference
			Positive	Negative	
xa5	xa5FM-S	F: 5 GTCTGGAATTTGCTCGCGTTCG R: GGTAAAGTAGATACCTTATCAAACTGGA	134	313	Sundaram *et al*., 2008
	xa5FM-R	F: AGCTCGCCATTCAAGTTCTTGAG R: TGACTTGGTTCTCCAAGGCTT			
xa13	xa13prom	F : T C C C A G A A A G C T A C T A C A G C R:GCAGACTCCAGTTTGACTTC	500	250	
Xa21	pTA248	F : A G A C G C G G G A A G G G T G G T T C C C G G A R:AGACGCGGGTAATCGAAAGATGAAA	925	650	
Pi54	Pi54MAS	F: TACCTGATGGTTCTTTAAAATTGGG R: CATAAGCTAGACCTTGAAGGATGTC	216	359	Ramkumar *et al.,* 2011
Rf3	D R R M - RF3-10	F: TCACCTCTTCCTGCTTCGAC R: CTCCACCAGTGCAGGTTTTT	210	190	Revathi et al.,2013, Singh *et al*., 2005, Prasanna et al., 2022
Rf4	RM6100	F: TCCTCTACCAGTACCGCACC R: GCTGGATCACAGATCATTGC	150	140	

F: Forward; R: Reverse



P- Positive; N-Negative; H –Heterozygote L-Ladder (100 bp)

Fig. 1. Molecular screening for the target genes (a) *Xa21*, *xa13*, *xa5*, (b) *Pi54*, and (c) *Rf3, Rf4*

lines that carried *Xa21* gene alone, or in combination with other genes, showed complete resistance, whereas those with *xa13*, *xa5* showed moderate resistance. Since our goal is to improve the restorer lines for hybrid development, incorporating the dominant *Xa21* gene into restorer (**Table 2**) would be beneficial for enhancing disease resistance in the resulting rice hybrids. Govintharaj *et al*., (2020), introgressed *Xa21*, *xa13,* and *xa5* into CB 174 R, a restorer line, using marker-assisted breeding and demonstrated high level of BB resistance. Similar to the present study, they also reported that Xa*21* was most effective as nine lines possessing this gene showed a mean lesion length of 2.24 cm. Balachiranjeevi *et al*., (2018) also reported that DRR17B improved lines carrying *Xa21,* showed lesion length of 2-2.8cm, for BB resistance.

Phenotypic screening for blast disease revealed 42 breeding lines with resistance and 18 lines with moderate resistance. Molecular screening identified that 12 lines carried the *Pi54* blast disease resistant gene (**Table 2**) and it was observed that all these 12 breeding lines also expressed blast resistance reaction phenotypically. However, 30 lines showed resistance/ moderate resistance to blast, though they did not carry *Pi54* gene. It is possible that these lines carry some other resistance gene(s) that contribute to field resistance for blast. Further studies would be needed to identify the genes involved in blast resistance reaction. Ramalingam *et al*. (2020) improved the blast-susceptible restorer lines CB 87 R and CB 174 R by introducing the *Pi54* gene through marker-assisted backcross breeding (MABB), resulting in enhanced blast resistance and fertility restoration. Kumar *et al*.,(2018) introgressed *Pi54* gene into popular mega variety Samba Mahsuri through MABB approach. Singh *et al*. (2023) improved maintainer line DRR 9B with a *Pi2* gene for developing blast resistance rice hybrids. The dominant genes are preferred for restorer line improvement, as hybrid development results in heterozygous progeny, where recessive genes are typically masked and do not express.

Based on BB, Blast resistance and presence of *Rf* gene (**Table 2**) selected breeding lines along with previously identified R lines were crossed with two CMS lines *viz.,* APMS6A and CRMS32A for developing experimental rice hybrids. A total 183 entries which comprising of 120 F_1 hybrids along with parents and seven checks, including

Table 2. List of genotypes possessing bacterial blight (BB) and blast resistance genes

Genotypes	Pedigree	BB R gene	Rf gene(s)	Genotypes	Pedigree	Blast R gene	Rf gene(s)
RP 5963-11	Improved Samba Mahsuri *1 x KMR 3R	*Xa21+xa13+xa5*	*Rf4 & Rf3*	RP 5933-11	Improved Samba Mahsuri *1 X KMR 3R	*Pi54*	*Rf4 & Rf3*
RP 5934-69	Swarna *1 x KMR 3R	*Xa21*	*Rf4*	RP5964-86	KMR 3R x Swarna*1	*Pi54*	*Rf4*
RP 5934-75	Swarna *1 x KMR 3R	*Xa21*	*Rf4*	WIL -55	Wild Introgression lines	*Pi54*	*Rf4*
RP 5933-103	Swarna*1 x IBL	*Xa21*	*Rf4*	RP 5933-214	Swarna*1 x IBL 57	*Pi54*	*Rf4*
RP 5933-117	Swarna*1 x IBL 57	*Xa21 +xa5*	*Rf4*	RP 6469-162	IR 36 Genetic male sterile restorer population improvement derived line	*Pi54*	*Rf4*
RP 5933-119	Swarna*1 x IBL 57	*Xa21*	*Rf4*	RP 6469-163	IR 36 Genetic male sterile restorer population improvement derived line	*Pi54*	*Rf4*
RP 5933-137	Swarna*1 x IBL 57	*Xa21+xa5*	*Rf4*	SERB-42-3	Wild Introgression lines	*Pi54*	*Rf4*
RP 5933-142	Swarna*1 x IBL 57	*Xa21+xa13*	*Rf4*	RP 6619- 4	RP 5933-1-19-2 R x *O. minuta, Tetep*	*Pi54*	*Rf4*
RP 5933-149	Swarna*1 x IBL 57	*Xa21*	*Rf4*	RP 6619- 20	RP 5933-1-19-2 R x *O. minuta, Tetep*	*Pi54*	*Rf4*
RP 5933-175	Swarna*1 x IBL 57	*Xa21*	*Rf4*	RP 6619- 26	RP 5933-1-19-2 R x *O. minuta, Tetep*	*Pi54*	*Rf4*
WIL-3	Wild Introgression lines	*Xa21*	*Rf4*	RP 6619- 46	RP 5933-1-19-2 R x *O. minuta, Tetep*	*Pi54*	*Rf4*
WIL-53	Wild Introgression lines	*Xa21+xa5*	*Rf4*	RP 6469-117	IR 36 Genetic male sterile restorer population improvement derived line	*Pi54*	-
WIL-65	Wild Introgression lines	*Xa21*	*Rf4*				
RP 6469-117	IR 36 Genetic male sterile restorer population improvement derived line	*Xa21+xa13+xa5*	*Rf3*				

Table 3. Mean squares from ANOVA for augmented randomized complete block design for 12 traits measured in 183 entries

Source	df	PH	PL	NPT	FL	SPY	LBR	PF	SPF	DFF	TGW	PDP	TGPP
Block	5	399.75**	149.28**	14.82*	10.32^{ns}	46.78**	0.3**	384.65**	743.75**	28.93**	62.51**	0**	7260.9**
Treatment	182	139.56**	214.03**	13.91**	18.89**	98.26**	0.09**	570.07**	470.06**	81.7**	58.39**	0**	5011.78**
Treatment: Check	6	258.16**	17.03*	6.94 ^{ns}	29.12**	14.91^{ns}	0.33**	193.36**	212.27**	227.67**	17.32**	0.01**	4973.84**
Treatment: Test and Test *vs.* Check	176	135.52**	220.75**	14.15**	18.54**	101.1**	0.08**	582.91**	478.85**	76.72**	15.92**	0.01**	4972.54**
Residuals	45	46.7	6.1	5.63	4.86	7.42	0	15.4	32.54	0.91	3.43	0	987.6
Mean		95.72	23.31	10.46	28.45	17.23	2.80	66.04	63.09	100.80	20.22	0.13	195.82
CV%		7.12	10.65	21.7	7.83	14.26	1.71	5.52	8.56	0.96	9.2	13.82	16.61
SE		0.82	1.03	0.26	0.3	0.67	0.02	1.56	1.47	0.59	0.28	0.01	4.9

^{ns} *P > 0.05; * P <= 0.05 (significant at 5% probability); ** P <= 0.01 (Significant at 1% probability)*

PH-Plant height, PL-Panicle length, NPT-Number of productive tillers, FL-Flag leaf length, SPY-Single plant yield, LBR-Length breadth ratio, PF-Pollen fertility, SPF-Spikelet fertility, TGW-Thousand grain weight, PDP-Per day productivity, TGPP- Total grains per panicle

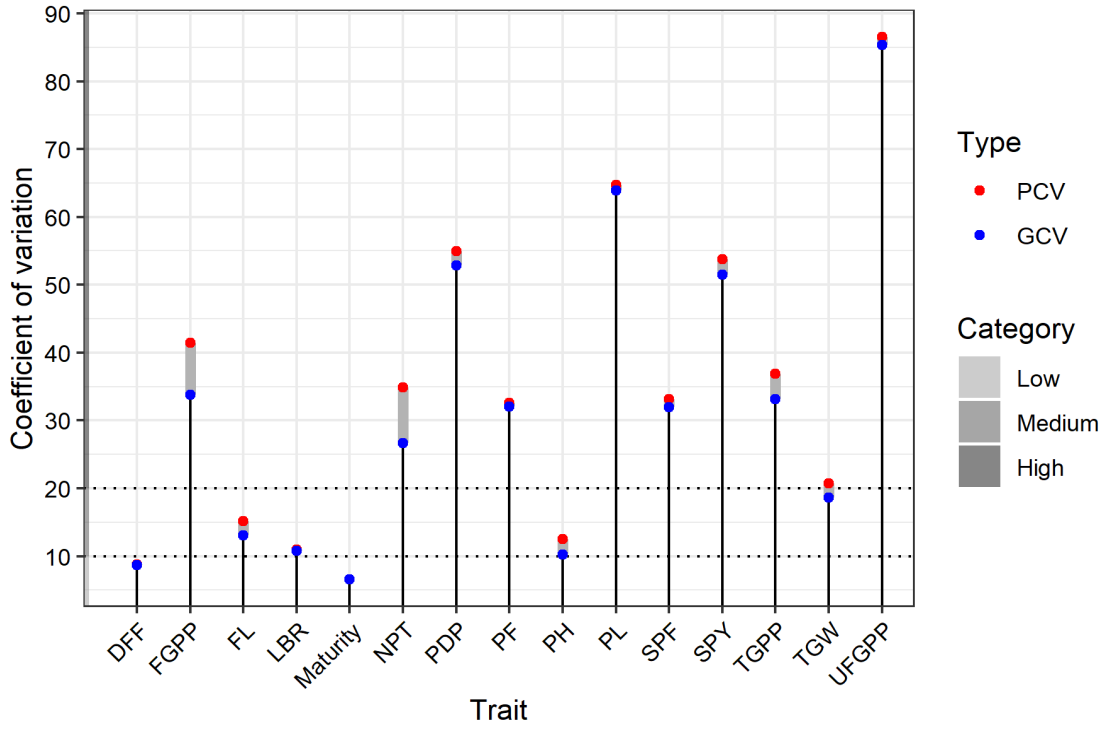


Fig. 2. Phenotypic and Genotypic Coefficient of Variability

PH-Plant height, PL-Panicle length, NPT-Number of productive tillers, FL-Flag leaf length, SPY-Single plant yield, LBR-Length breadth ratio, PF-Pollen fertility, SPF-Spikelet fertility, TGW-Thousand grain weight, PDP-Per day productivity, TGPP- Total grains per panicle, FGPP-Filled grains per panicle, UFGPP-Unfilled grains per panicle

Table 4. Mean performance of top 25 entries for grain yield and standard heterosis for grain yield

S. No	Entry	F_1 hybrid/Genotype	Mean Single plant yield (gms)	SH over US 314 %	SH over Gontrabidhan %	SH over BPT 5204 %	SH over 27P63 %	SH over HRI-174 %
1	PRTC-61	CRMS 32A X WIL 57	48.4	69.3	78.2	90.3	74.7	88.0
2	PRTC-103	APMS 6A X RP 5933- 211	47.9	67.6	76.4	88.4	73.0	86.1
3	PRTC-113	APMS 6A X RP 6964- 417	47.7	66.7	75.5	87.4	72.1	85.1
4	PRTC-81	APMS 6AX RP 6619-4	43.0	50.5	58.4	69.2	55.3	67.1
5	PRTC-22	APMS 6AX RP 5933- 103	41.0	43.3	50.8	61.1	47.9	59.1
6	PRTC-101	CRMS 32A X RP 5962-1	40.3	41.1	48.5	58.6	45.6	56.6
7	PRTC-41	CRMS 32A X RP 5933- 170	39.4	37.7	44.9	54.8	42.1	52.9
8	PRTC-77	APMS 6A X RP6964- 469	35.4	23.9	30.5	39.3	27.9	37.6
9	PRTC-83	APMS 6A X RP 6619- 26	33.8	18.1	24.3	32.7	21.9	31.1
10	PRTC-15	APMS 6A X RP5934-74	33.2	16.1	22.2	30.5	19.8	28.9
11	PRTC-38	CRMS 32A X RP 6964- 80	31.5	10.2	16.0	23.9	13.7	22.3
12	PRTP-84	WIL-18	30.4	6.2	11.8	19.4	9.6	17.9
13	PRTP-19	RP 5964- 86	30.2	5.6	11.2	18.7	9.0	17.2
14	PRTC-82	CRMS 32 AX RP6619-20	29.7	3.9	9.4	16.8	7.3	15.4
15	PRTC-30	APMS 6A X RP 5933- 146	29.7	3.8	9.2	16.7	7.1	15.2
16	PRTC-18	APMS 6A X RP 5934- 78	29.5	3.1	8.6	16.0	6.5	14.5
17	PRTP-42	RP5933- 175	28.8	0.6	5.9	13.1	3.8	11.7
18	US-314	US 314	28.6	0.0	5.3	12.5	3.2	11.1
19	PRTC-25	APMS 6A X RP 5933- 119	28.1	-1.7	3.5	10.6	1.5	9.2
20	KRH-4	KRH -4	28.1	-1.8	3.4	10.4	1.3	9.0
21	27P63	27P 63	27.7	-3.1	2.0	8.9	0.0	7.6
22	KMR 3R	KMR 3R	27.4	-4.2	0.9	7.7	-1.1	6.4
23	HRI-174	HRI 74	27.4	-4.3	0.7	7.6	-1.2	6.2
24	Gontrabidhan	Gontrabidhan	27.2	-5.0	0.0	6.8	-1.9	5.5
25	BPT 5204	BPT 5204	25.4	-11.0	-6.4	0.0	-8.2	-1.2

SH- Standard Heterosis

both hybrid and varietal checks, were evaluated in augmented RCBD for yield and yield contributing traits. The results of analysis of variance showed significant block effects and treatment effects for most of the traits evaluated (**Table 3**). Santhiya *et al*.,(2024) also studied combining ability effects on grain yield and grain quality traits in rice and observed significant variation. The largest variation was observed in number of productive tillers with CV of 21.7% followed by total grains per panicle (16.61%) and single plant yield (14.26%). The genotypic and phenotypic coefficient of variation results revealed that phenotypic coefficient of variation was higher than the genotypic coefficient of variation for the traits studied (**Fig 2**). The heritability and genetic advance estimation explained that heritability values ranged from 58.7 % (Productive tillers) to 98.81% (LBR). Genetic advance as percent mean values ranged from 17.37% (PH) to 101.64% (Single plant yield).

Among 120 hybrids, desirable low plant height was observed for cross, CRMS 32A x SM GN-4-1 (73.0 cm), panicle length with high mean value was observed for CRMS 32A x RP 5933-119 (25.7 cm), highest number of productive tiller per plant was observed for CRMS 32A x WIL-65 (21), minimum number of days to 50% flowering was observed in APMS6A x RP 5963-11 (95 days), highest pollen fertility percentage was observed for CRMS 32A x RP 5934-64 (96.4%), highest spikelet fertility was recorded for CRMS 32A x RP 5934-64 (91.9%), highest number of filled grains per panicle was observed for APMS6A x RP 5934-75 (228), maximum 1000 grain weight was recorded by CRMS 32A x RP 5933-41 (32.0g). Highest grain yield per plant was observed in the following hybrids viz., CRMS 32A x WIL-57 (48.4g), APMS 6A X RP 5933-211 (47.9g), APMS 6A X PRP 417 (47.7g), APMS 6A X RP6619-4 (43.0g), APMS 6A X RP 5933-103(41.0g), CRMS 32A X RP 5962-1(40.3g), CRMS 32A X RP 5933-170 (39.4g), APMS 6A X RP 6619-26 (33.75g), APMS 6A X RP5934-74 (33.2g), and CRMS 32A X RP 6964- 80 (31.5g), these hybrids exhibited higher standard heterosis over hybrid checks (US 314, 27P63, HRI 174) and varietal checks (BPT 5204, Gontrabidhan), respectively

(**Table 4**). The rice hybrids developed using improved restorers, particularly RP-5933-103 with *Xa 21* BB resistant gene, RP 6619-4 and RP 6619-26 with *Pi54* and *Pi9* gene were outperformed high yielding hybrids and varietal checks (**Table 4**). The identified best BB and/or blast resistant breeding lines are potential restorers for developing heterotic biotic stress resistance rice hybrids. The best hybrids identified with biotic stress resistance would be subjected to large scale hybrid seed production for multi-location testing followed by commercial release.

These identified parental lines and hybrid combinations could be valuable resources for future three-line hybrid rice breeding. The development of climate-smart rice hybrids with enhanced tolerance to multiple biotic and abiotic stresses will play a crucial role in expanding the area under hybrid rice cultivation. This, in turn, will contribute significantly to increasing rice production and productivity, thereby strengthening global food security in the face of climate change and emerging biotic stress challenges.

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