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RESEARCH ARTICLE

MARKER-ASSISTED BREEDING FOR RESISTANCE TO BACTERIAL LEAF BLIGHT IN POPULAR FINE GRAIN VARIETY JAGITIAL SANNALU

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ABSTRACT

The present study focus on developing new Bacterial Blight resistant rice lines, using markers assisted backcrossing (MABC). A total of 536 SSR markers covering all / distributed throughout the 12 rice chromosomes were screened between the two parents for polymorphism, out of which 81 markers were found to polymorphic and all the 81 markers were screened in the breeding population. For each backcross generation of JGL1798/B95-1, the confirmed plants were screened with 81 polymorphic markers distributed on 12 chromosomes. From BC1F1 generation, two plants P28 and P44 with highest recipient alleles were chosen for the further backcrossing. From BC2F1 plants with the recipient alleles were selfed to produce BC2F2 and In BC2F2, fifty two plants were analyzed through phenotypic assays with different BLB cultures at two locations west Godavari and ARI Hyderabad. Five BC2F2 progenies possessing two BLB resistance genes in homozygous condition (xa13xa13Xa21Xa21) showed BLB resistance along with Jagitial Sannalu Characteristics. These five plants were chosen as the breeding lines for result of Bacterial Blight introgression.

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INTRODUCTION

Rice is the most important food source for half of the world's population and also the main staple food for most of the country's 86 million people. But the rice production is limited by various biotic and abiotic factors; Bacterial Leaf Blight (BLB) being the major disease. Host plant resistance (HPR) has been considered as the most economical and eco-friendly strategy for management of biotic stresses (Hulbert et al., 2001). Pyramiding of two or more resistance genes through Marker Assisted Selection (MAS) has been a successful approach to ensure multiple and durable host plant resistance against a wide spectrum of biotic stresses (Yoshimura *et al.*, 1995). Bacterial Blight (BB), caused by Xanthomonas oryzae pv. oryzae (*Xoo)*, is one of the serious diseases of rice across countries and can cause yield losses as high as 74 to 81% (Srinivasan and Gnanamanickam, 2005).

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Till date, at least 38 BB resistance (R) genes have been identified (Sundaram *et al.*, 2014), of which 19 genes have been tagged and mapped with closely linked markers. Using MAS breeding approaches three or more BB genes, like xa5, xa13,Xa21 have been successfully pyramided in diverse elite rice varieties like IR64, PR106, Pusa Basmati 1, Lalat, Tapaswini, Swarna, IR64 and Samba Mahsuri (Sundaram *et al.*, 2014). One of these cultivars called Improved Samba Mahsuri (ISM) has been released for commercial cultivation and several breeding lines in the genetic background of Samba Mahsuri possessing different resistance genes. Here we report pyramiding of two major BB resistance genes (Xa21 and xa13 for BB resistance in the genetic background of Sannalu (JGL 1798) through marker assisted selection.

MATERIALS AND METHODS

Plant material

Improved Samba Mahsuri (i.e. B95-1) possessing xa5*, xa13, Xa21* genes in homozygous condition was used as the donor

parent as the source of resistance against BLB. JGL1798 was used as the recurrent parent. Jagitial Sannalu (JGL1798), was released by Acharya N G Ranga Agricultural University (ANGRAU), Rajendranagar, Hyderabad. JGL1798 variety is being widely grown in Northern Telangana region during both *kharif* and *Rabi* seasons. Samba Mahsuri is one of the parents [(Samba Mahsuri (BPT 5204) × Kavya (WGL 48684)] of this variety and is susceptible to bacterial leaf blight.

DNA extraction

The DNA was isolated following the modified CTAB (Cetyl Tri Methyl Ammonium Bromide) method (Murray *et al.,* 1980) The quality and quantity of DNA was estimated in 0.8% agarose gel using 500ug/ml lamda (λ) *Hind III* DNA (New England Biolabs) as reference standard. Fresh young rice leaves were taken into the 2mL eppendorf tube, adding liquid nitrogen to grind into powder and rapidly adding 600μl extract buffer incubating at $65^{\mathbf{o}}$**c** water bath for 30~40 min, then adding 600μl mixture of chloroform and isoamylalcohol with 24 to1 and mixing at room temperature for standing 30 min. After centrifuging at 10000 r/min for 15 min, the supernatant was transferred to another centrifuge tube, then adding an equal volume of chilled isopropanol and keep 15min at -20°c for DNA precipitation, After centrifugation at 10000 r/min for 15 min, while precipitation was washed with 70% ethanol 2 times, then adding 600μl sterile water for fully dissolving after naturally dried, placed in refrigerator at 4°c for ready to use.

Polymerase Chain Reaction

PCR amplification was performed in 10 μl volume containing 50 ng of template DNA, 5 picomoles of each primer, 2 mM dNTPs, 10X PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM MgCl2 and 0.01 mg/ml gelatin) and 1U Taq DNA polymerase polymerase (Genei, Bangalore, India) on Applied Biosystems verity 96 well thermal cycler. The template DNA was amplified in PCR profile with initial denaturation at 94^{0}C for 5 min, denaturation at 94^{0}C for 45 sec, primer annealing at 55^{0}C (xa13 promotor) and at 58^{0}C (pTA 248) for 45 sec, extension at 72^{0}C for 1 min, final extension at 72^{0}C for 10, and cooling at 4^{0}C. These steps were repeated for 35 cycles for amplification of DNA. The amplified products were then mixed with bromophenol blue and resolved electrophoretically on 3% agarose gel along with the 1000ug/ml 50bp DNA ladder (New England Biolabs) for an hour in 1x Tris–Acetic acid–EDTA (TAE) buffer. The resolved PCR bands were documented using Bio-Rad Molecular Imager Gel Doc XR+ System.

Marker-assisted selection for BB

For targeted introgression of *xa13*, *Xa21genes* into JGL1798, a stepwise transfer marker-assisted backcross breeding programme was adopted (Fig-1). Backcrosses were carried out where in the BB resistance genes, *Xa21* + *xa13* were introgressed into JGL1798. The F_1 plants derived from the cross were confirmed for their hybridity (i.e. heterozygosity) using the *Xa21* gene-specific co-dominant marker pTA248 and xa13-promo for xa13 gene (Ronald *et al*., 1992; Sundaram *et al*., 2011); for the first cross, i.e. Improved Samba Mahsuri/ JGL1798) and backcrossed with JGL1798. At $BC_1F_{1,}$ backcross plants were screened with pTA248 and xa13-prom (Sundaram *et al*. 2011) to identify plants heterozygous for *Xa21* and *xa13*, respectively. The forward and reverse primer sequence information for these 2 markers were presented in (Table 1). The positive plants identified from of BC_1F_1 were then screened with a set of parental polymorphic SSR markers (81 markers used for background selection. Backcrossing was performed till BC_2 generation, and selfed to produce BC2F2 and In $BC_2F_{2,}$ fifty two plants were analyzed through phenotypic assays with different BLB cultures at two locations West Godavari and ARI Hyderabad. Five BC_2F_2 plants possessing two BLB resistance genes in homozygous condition (*xa13xa13Xa21Xa21*) showed BLB resistance along with Jagitial Sannalu Characteristics.

Screening of BC_2F_2 progenies for BB resistance

Highly virulent isolate of the Bacterial Blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) collected from BB hot-spot location in India, viz. DRR *Xanthomonas* collection-022 (DX-022) (Laha *et al*., 2009), were used to screen the donor and recurrent parents along with backcross-derived plants of BC_2F_2 for bacterial blight resistance under both glasshouse and field conditions. The *Xoo* strains were cultured and stored as described by Laha *et al* (2009). The rice plants were clip-inoculated with a bacterial suspension of 109 cfu/ml at maximum tillering stage (45–55 days after transplanting) through the methodology of Kauffman *et al* (1973). Approximately 10 leaves per plant were inoculated, and disease reaction was scored 14 days after inoculation. BB lesion length was measured and the disease score was calculated as per IRRI standard evaluation system (IRRI-SES) scale (IRRI, 1996). The results of the phenotypic screening against BB reaction of the BC_2F_2 progenies carried the *Xa21* and *xa13* genes with a back ground of the recurrent parent JGL1798 conferred highly resistance against Bacterial Blight pathogens at two locations West Godavari and ARI Hyderabad.

Marker assisted introgression of BB into JGL1798

The F_1 progeny generated from the cross, C_5 (JGL1798/B95-1) were screened for presence of the target resistance genes *Xa21* and *xa13* using the *Xa21*-specific co-dominant marker, pTA248 to identify the 'true' F_1s showing heterozygous amplification pattern. Out of 130 F_1 plants 101 were identified to be true heterozygous plants and these were then used as male parent and backcrossed with JGL1798 to generate BC_1F_1 plants. 289 BC_1F_1 plants, a total of 55 were identified to be positive for *Xa21*, 72 were positive for *xa13* and 22 were identified to be double positive for both *Xa21* and *xa13* genes. The twenty two BC_1F_1 plants (which were heterozygous for *Xa21, xa13* were then subjected for background selection using 81 parental polymorphic SSR markers, and 'positive' BC_1F_1 plants (C_5-BC_1F_1-28,44) possessing maximum recovery of recurrent parent genome were selected and then backcrossed with JGL1798 to generate BC_2F_1 plants. A similar marker-assisted selection procedure was followed for selection of BC_2F_1 plants and BC_2F_1 plant (C_5-BC_2F_1-18,21) possessing maximum recovery of recurrent parent genome was selected

on phenotypic and other grain characters and one BC_2F_1 plant was allowed for selfing and advanced as BC_2F_2 population for future study.

RESULTS

Confirmation of marker polymorphism for gene-specific markers and identification of parental polymorphic markers

DNA was isolated from the recurrent parent, JGL1798 (susceptible to BLB) and the donor parent, B95-1(resistant to BLB with *Xa21* and *xa13*) and amplified in a thermal cycler. The primer pair, pTA248 amplified fragments of size ~ 900 bp in the resistant parent (i.e. ISM), while that from the susceptible parent JGL1798 was ~ 650 bp as indicated in Ronald *et al*. (1992). With respect to the primer pair, xa13-prom, ISM amplified a ~500 bp fragment, while JGL1798 amplified at 250 bp as observed by Sundaram *et al*. (2011). Thus the results revealed that all the markers were able to distinguish resistant lines from susceptible ones in a co-dominant fashion.

Table 1. Sequence of Primer Pairs (i.e. markers) used for Foreground Selection

Gene	Marker	Chr No.		Sequence of the marker	References
xa13	*xa13* promoter	8	F	TCCCAGAAAGCTACTACAGC	Sundaram *et al.*
			R	GCAGACTCCAGTTTGACTTC	(2011)
Xa21	pTA 248	11	F	AGACGCGGGAAGGGTGGTTCCCGGA	Ronald *et al.*
			R	AGACGCGGGTAATCGAAAGATGAAA	(1992)

Note: F-Forward sequence, R-Reverse sequence

Table 2. Scoring of two gene BC_2F_2 plants for Bacterial Blight

S.No	Plant identity	Allelic status of *xa13, Xa21*	Disease scoring scale/ Range	Background Recovery in %
1	JGL1798	*Xa13Xa13, xa21xa21*	9	-
2	Improved Samba Mahsuri	*xa13xa13, Xa21Xa21*	1	-
3	BC_2F_2-4-112	*xa13xa13, Xa21Xa21*	1	92
4	BC_2F_2-4-374		1	82
5	BC_2F_2-4-390		2	82

Parental polymorphism survey was carried out using 536 SSR markers spread across all the 12 chromosomes of rice. Out of 536 SSR markers tested, 544 (84.88%) were found to be monomorphic; while a total of 81 (15.11 %) showed polymorphism between recurrent parent (JGL1798) and the donor parent (ISM). The F_1s generated from the cross, C_5 were screened for presence of the target resistance genes *Xa21* and *xa13* using the *Xa21*-specific co-dominant marker, pTA248 and xa13 promoter to identify the 'true' F_1s showing heterozygous amplification pattern. Of 130 F_1s, 101 were identified to be true heterozygous plants and these were then used as male parent and backcrossed with JGL1798 to generate BC_1F_1 plants. Genetic analysis of 289 BC_1F_1 plants for *Xa21* gene revealed 55 plants as heterozygous, while 72 plants showed heterozygocity for *xa13* gene. Thus a total of 22 BC_1F_1 plants showed heterozygocity for both genes on using both markers. The advanced back cross plants of BC_2F_1 were obtained from the cross made between selected BC_1F_1 and JGL1798. Foreground analysis showed that 144 plants as heterozygous of 365 BC_2F_1 (Fig 2 and 3) plants studied for *Xa21*gene, while 187 plants out of 365 plants were observed as heterozygous for *xa13* gene.

A total of 17 BC_2F_1 plants showed heterozygocity for both the genes. Selection was carried out in 17 genes positive BC_2F_1 plants based on phenotypic and other grain characters and two BC_2F_1 plants was allowed for selfing and advanced as BC_2F_2 population for future study.

Genetic analysis of BLB resistance genes (*Xa21 + xa13*) in BC_2F_2 population

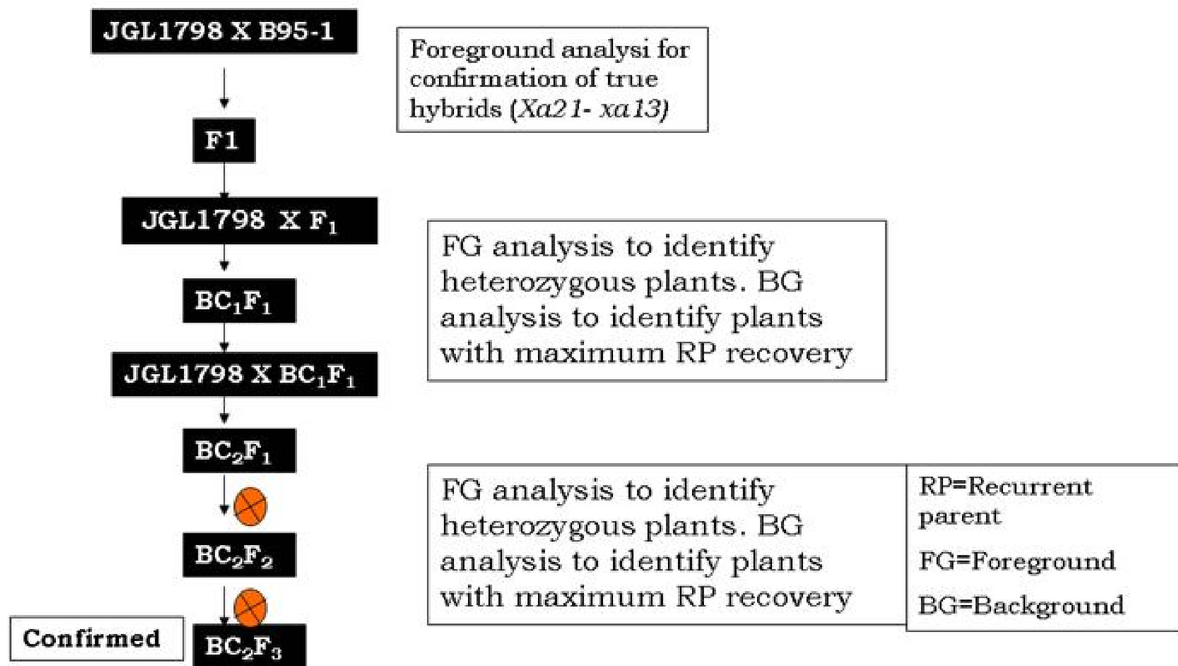
Genotyping was carried out in 884 BC_2F_2 plants were genotyped for identification of plants possessing the target resistance genes in homozygous condition (*xa13xa13 and Xa21Xa21)* . Out of 884 plants, 11 plants identified as a two gene positive pants in homozygous condition. Background analysis was carried in all these 11 plants having two gene combination using polymorphic SSR markers. Among eleven plants, BC_2F_2-4-112th (Table 2) plant (*xa13xa13Xa21Xa21*) scored the highest genome recovery (92%), while BC_2F_2-4-374 (*xa13xa13Xa21Xa21*) and BC_2F_2-4-390(*xa13xa13Xa21Xa21*) plants scored the lowest genome recovery (82%). the highest genome recovery plants were selfed and advanced to BC_2F_3 generation.

DISCUSSION

Integration of molecular markers to the backcross breeding was highly effective for transfer of two bacterial blight resistance genes. Phenotypic selections in two backcrossing and selfing generation coupled with SSR based background selection was sufficient to transfer of **Xa21** and **xa13** genes into popular variety JGL1798. Jagtial Sannalu is a popular high yielding short duration and fine grain rice variety, susceptible to BLB. For effective use of MAS or MABB, polymorphic markers between the parents are highly useful to exercise background selection. Background selection is highly useful to identify the plants carrying desirable genome of interest. Among the 536 hyper variable primers 81primer pairs exhibited polymorphism between recurrent parent JGL1798 and donor parent B95-1. Several bacterial blight (BB) resistance genes have been associated with tightly linked DNA markers, and some of them have been cloned (*Xa1, xa5, xa13, Xa21, Xa26, Xa27*) and used for breeding BB-resistant rice cultivars. Because of the availability of DNA markers derived from the resistance genes, it is now possible to pyramid several genes, into susceptible elite rice cultivars.

The resistance genes *xa5, xa13, and Xa21* have been pyramided in to an indica rice cultivar (PR 106) using MAS that expressed strong resistance to BB races of India (Singh *et al*., 2001). It is the most effective way of transferring specific gene(s) to an elite susceptible cultivar. In rice, the feasibility of marker assisted back cross breeding (MABB) to pyramid BB resistance genes has been well demonstrated (Sundaram *et al*., 2008). Bacterial leaf blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a devastating disease in the rice growing countries of Asia.

To date, at least 38 BB resistance genes conferring host resistance against various strains of *Xoo* have been identified (Suh *et al*., 2013). Of the 38 R genes, six are physically mapped (*Xa2*, *Xa4*, *Xa7*, *Xa30*, *Xa33* and *Xa38*) and six are cloned (*Xa1*, *xa5*, *xa13*, *Xa21*, *Xa26*, *Xa3* and *Xa27*) (Bhasin *et al*., 2012 & Natrajkumar *et al*., 2012) and their corresponding avirulence (*avr*) genes have also been isolated (White and Yang 2009). Resistance breeding is considered as the most economical and eco-friendly strategy for management of the disease and achieving yield stability.



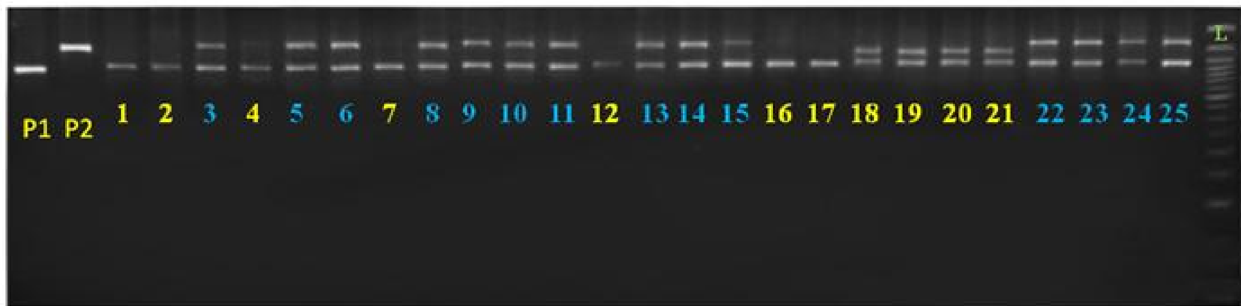


Figure 2. Confirmation of JGL1798 X B95-1 BC_2F_1 plants by pTA248 primer

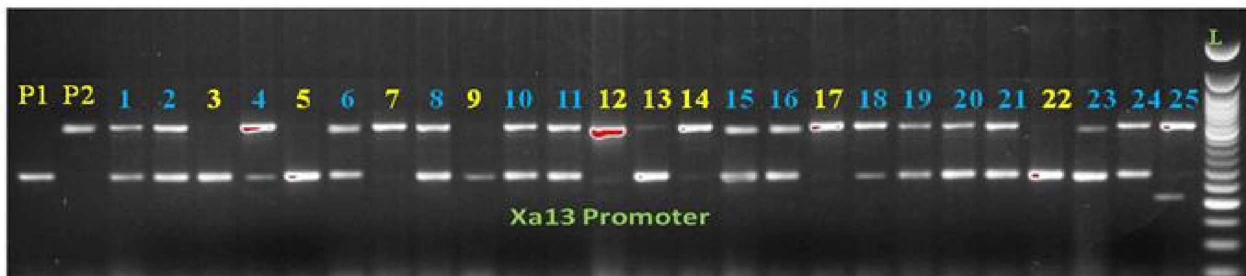


Figure 3. Confirmation of JGL1798 X B95-1 BC_2F_1 plants by xa13 primer

Pyramiding resistance genes is difficult to accomplish using conventional breeding strategy due to epistatic effects of genes controlling resistance and due to non-availability of screening facilities for multiple biotic stresses in addition to screening restricted only to specific seasons. Molecular markers can accelerate resistance breeding efforts (Sundaram *et al*., 2008), as segregating plants can be selected on the basis of molecular marker alleles instead of its phenotype and introgression of multiple resistance genes can be tracked easily in a population. Gene pyramiding is difficult using conventional breeding methods due to the dominance and epistasis effects of genes governing disease resistance. Moreover, genes with similar reactions to two or more races and dominant and recessive genes transfer at a time are difficult to identify and transfer through conventional approaches (Joseph *et al*., 2004; Rajpurohit *et al*., 2011; Sundaram *et al*., 2009).

However, the availability of molecular markers closely linked to each of the resistance genes makes the easy pyramiding of the genes and identification of plants with two and three genes possible by marker assisted selection (MAS) (Shanti *et al*., 2010; Singh *et al*., 2001; Sundaram *et al*., 2008). Among the BB resistance genes identified so far, the dominant resistance gene, *Xa21* is one of the major genes that confer resistance to many virulent isolates of the *Xoo* in India and mapped on the long arm of rice chromosome 11. The *xa13* gene was first discovered in the rice variety, BJ1 and mapped on the long arm of rice chromosome 8 (Ogawa *et al*., 1987; Zhang *et al*., 1996; Sanchez *et al*, 1999). It interacts strongly with other R genes such as *xa5*, *Xa4* and *Xa21* (Li *et al*., 2001) and gives resistance in the homozygous recessive status (Khush and Angeles 1999). Sundaram *et al*. 2011 developed *xa13* promoter as a functional marker, designed from promoter region of the *xa13* gene, used in the introgression of present study.

The two PCR-based markers used in the present study (i.e. *xa13*-promo, and pTA248) (Sundaram *et al*., 2011; Ronald *et al*., 1992) were able to identify the triple-positive (*xa13,* and *Xa21*) plants, precisely without any false positives at any stage of MABB. Polymorphic microsatellite markers could be utilized for background selection in order to recover the recurrent parent genome in the shortest number of backcross generations (Hospital and Charcosset 1997) to estimate the amount of recurrent parent genome contribution. Sundaram *et al.* (2008) in their study of introgression of bacterial blight resistance in Samba Mahsuri through marker-assisted backcross breeding MAS is particularly useful for identification of heterozygous individuals for recessive genes like *xa13*. In the absence of marker, identifying backcross plants that have this type of recessive genes would require progeny testing, which is an addition of one more generation and cumbersome too (Sundaram *et al*., 2008). The primers viz., *xa13*-promo, pTA248, used in the present study are located very near to/within *xa13*, *Xa21* and genes (Sundaram *et al*., 2011; Ronald *et al*., 1992). Hence these markers can be used to complement classical breeding techniques in order to select segregating plants at early stage based on the DNA marker genotype rather waiting to observe the phenotypic disease screening (i.e. rice bacterial blight). The present study was carried out with the objective to improve BB resistance of JGL1798 through marker-assisted backcross breeding coupled with phenotypic selection for agro-morphological traits. Earlier, Sundaram *et al*. (2008), Sundaram *et al*. (2009), Gopalakrishnan *et al*. (2008), Basavaraj *et al*. (2010) and Hari *et al*. (2011) Balachiranjeevi *et al.* (2015) developed improved, BB resistant versions of elite varieties Samba Mahsuri, Triguna, Pusa Basmati 1, the hybrid PusaRH10 and the maintainer line, KMR-3R, respectively, for BB resistance. By comparing the above results, the yield levels of the two gene pyramided lines were not significantly different from that of the parent JGL1798 indicating that there is no yield penalty associated with the presence of the resistance genes.Similar observation was noticed by Shanti *et al*. (2010), when worked with parental lines of hybrid rice for BB resistance.

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