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Improvement of rice cultivar for bacterial blight disease through marker assisted breeding approach

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Abstract
The present investigation was undertaken with the objective to develop high yielding, fine grain rice varieties possessing broad spectrum durable resistance by transferring bacterial leaf blight (BLB) resistant genes *viz., xa13* and *Xa21* from Improved Pusa Basmati 1. A popular high yielding and fine grain rice variety, HUBR 2-1 (Malviya Dhan 2-1), susceptible to BLB and Improved Pusa Basmati 1 carrying resistant genes for BLB (*xa13* & *Xa21* genes) were selected as parents for crossing. Improved Pusa Basmati 1 was verified for the presence of target genes by using gene linked primers *viz., xa13 promotor* and *pTA 248*. The cross *viz.,* HUBR 2-1 x Improved Pusa Basmati 1 was undertaken during K*harif* 2011 and F_1 progenies were confirmed during r*abi* 2011. The F_1 plants confirmed as true hybrids for both the genes were advanced to the F_2 generation and foreground selection was done using gene linked markers. Genetic analysis in the F_2 population confirmed that the genes (*xa13* & *Xa21*) governing BLB resistance followed Mendelian inheritance. The phenotypic data analysis revealed that the plants carrying two resistance gene combinations (*xa13xa13 Xa21Xa21, xa13xa13Xa21xa21*) showed BLB resistance (0-3 scale), while the gene combinations *viz., Xa13Xa13Xa21Xa21, Xa13Xa13Xa21xa21* and *Xa13xa13Xa21Xa21, Xa13xa13Xa21xa21* showed BLB resistance (5-30).

Key words
Bacterial leaf blight; F_2 population; marker assisted selection; rice

INTRODUCTION

Rice is the important staple food for more than half of the world's population, but rice production is limited by various biotic and abiotic factors; bacterial leaf blight (BLB) being one of the major diseases. Host plant resistance (HPR) has been considered the most economical and eco-friendly strategy for the management of biotic stresses (Hulbert *et al.,* 2001). Molecular markers are widely applied in agriculture, and their application in rice improvement has been recently reviewed (Mackill and McNally 2004; Jordan *et al.,* 2004; Xu *et al.,* 2004; Toojinda *et al.,* 2005; Liu *et al.,* 2006; Mackill, 2007; Soumya and Sindhumole 2016, Vennisa *et al.,* 2018). Kalaichelvan (2009) used 78 SSR's for varietal identification and also assessed the genetic relationship among the elite rice cultivars using morphological and molecular markers. The marker used in the selection must have a tight linkage with the target gene in order to have a relatively high selection efficiency (Yunbi, 2010).

MAS has also been employed for moving genes from pyramided lines into new plant type (Sanchez *et al.,* 2000) as well as into improved varieties grown in India (Singh *et al.,* 2001). Development of broad spectrum durable resistance through gene pyramiding or gene

stacking for biotic stress resistance can be accelerated through the process of marker assisted selection (Joshi and Nayak, 2010). BLB is caused by *Xanthomonas oryzae pv. oryzae* and is one of the most devastating diseases of rice causing yield losses ranging from 74 to 81% (Srinivasan and Gnanamanickam, 2005) in severe conditions.

Till date, 34 BLB genes (Chen *et al.,* 2011) have been identified in rice and a number of them have been deployed into breeding lines but disease breakdown has resulted due to a significant shift in pathogen race frequency (Mew *et al.,* 1992). Such breakdown can be delayed by marker assisted gene pyramiding. The *xa13* gene is fully recessive, conferring resistance only in the homozygous status (Khush and Angeles, 1999). Perumalsamy *et al.* (2010) introgressed three BLB resistance genes *xa5*, *xa13* and *Xa21* into two high yielding BLB susceptible *indica* rice cultivars, 'ADT 43' and 'ASD 16' from isoline IRBB60 and F_2 populations that were screened for the presence of all the three resistance genes by using functional markers. These pyramided genotypes with two or three resistance genes exhibited high levels of resistance against two predominant *Xanthomonas oryzae* isolates of South India.

The broad spectrum BLB resistance gene *Xa21* is expressed in dominant condition and was introgressed from a wild species *O. longistaminata* into *O. sativa* chromosome 11 through conventional breeding (Khush *et al.,* 1989). Basavaraj *et al.* (2010) also used markers *RG 136* and *pTA 248* linked to BLB resistance genes *xa13* and *Xa21* respectively, for foreground selection to improve Pusa 6A by using improved Pusa 6B as a donor for *xa13* and *Xa21*.

The present study was undertaken to develop a high yielding, fine grain, short duration rice variety resistant to BLB by introgression of two BLB resistance genes *viz., xa13* and *Xa21* from Improved Pusa Basmati 1 into the genetic background of HUBR 2-1. The gene linked markers *viz., xa13 promotor* and *pTA 248* were validated in resistant parent and parental polymorphism was studied between susceptible and resistant parent. The genotypic and phenotypic segregation was analyzed to determine the inheritance pattern of these genes in the single hybrid derived F_2 population.

MATERIAL AND METHODS

The variety HUBR 2-1 (Malviya Dhan 2-1) is a short duration, high yielding, fine grain, blast resistant rice variety released from the Institute of Agricultural Sciences, Banaras Hindu University in 2004, was used in the present investigation as a susceptible parent for BLB. Improved Pusa Basmati 1 (PB 1460), was used as a resistant parent as it possesses BLB resistance genes *viz., xa13* and *Xa21* and released from the Indian Agricultural Research Institute, New Delhi.

Genomic DNA was isolated from parents (HUBR 2-1 and Improved Pusa Basmati 1), F_1, F_2 and their Backcross progenies (BC_1F_1, BC_2F_1 and BC_2F_2) following the mini preparation procedure (modified method of Zheng *et al*., 1991). Quantification of DNA samples was done by using 0.8% agarose gel electrophoresis with diluted uncut DNA ladder as standard and spectrophotometer (Thermo electronic corporation UV1) as per the procedure described by Sambrook and Russell *et al.* (2001).

The 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 (Genei, Bangalore, India) on Applied Biosystems verity 96 well thermal cycler. The template DNA was amplified in PCR profile with an 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 min, and cooling at 4^{0}C for ∞. These steps were repeated for 35 cycles for amplification of DNA. The amplified products were then mixed with bromophenol blue and resolved electrophoretically in 2% agarose gel along with the marker 50 bp DNA ladder (Biolabs) for an hour in 1X Tris Acetic acid EDTA (TAE) buffer. The resolved PCR bands were documented using BioRad Gel Doc XR System.

The F_1 crosses were affected during *Kharif* 2011 *viz.,* HUBR 2-1 × Improved Pusa Basmati 1. F_1 seeds were raised in the main field by planting a single seedling per hill at a spacing of 20 × 20 cm during r*abi* 2011. DNA isolated from all the F_1 plants were used for genotyping of target genes. The seeds harvested from a single hybrid plant carrying both *xa13* and *Xa21* genes (HUBR 2-1 x Improved Pusa Basmati 1) were selfed and advanced to the F_2 generation during k*harif* 2012. These segregating populations were screened by using gene linked SSR markers for the resistance genes *viz., xa13* and *Xa21.*

A total of 200 F_2 plants from HUBR 2-1 × Improved Pusa Basmati 1 along with parents were genotyped to determine the inheritance of target genes. The inheritance of BLB resistant genes *viz., xa13* and *Xa21* were studied with the help of gene linked SSR markers *viz., xa13 promotor* and *pTA 248* respectively. Alleles at the SSR loci were detected on 2% or 3% agarose gel and 50 bp or 100 bp DNA ladder was added with the first load to confirm the allele sizes observed in the parental survey. Scoring of alleles was done to identify the plants carrying different genotypic combinations. The F_2 plants that showed a pattern similar to the susceptible parent alleles were scored as '9' and those with a banding pattern similar to the resistant parent alleles were scored as '0' and the plants with the heterozygous allelic pattern were scored as '3'.

To determine the segregation patterns of BLB resistance genes, F_2 seedlings were inoculated with a hyper-virulent isolate (DX-066) of *Xanthomonas oryzae* pv. *oryzae* collected from DRR, Rajendranagar. The F_2 population was inoculated with the bacterial culture at maximum tillering stages by using the leaf clipping method described by Kauffman *et al.* (1973). The inoculum was prepared by suspending bacteria, grown on Hayward's agar media for 2 to 3 days at 28^0C, in sterile distilled water at a final concentration of approximately 108 cfu/ml. Inoculum density was adjusted to 107-108 (cfu/ml) and plant inoculation was carried out by clipping the tip (about 1 to 2 cm) of the fully expanded uppermost leaf with scissors that had been dipped into the inoculum. Disease scoring was done 15 days after inoculation. Five leaves per plant were taken for scoring and the plant reaction was rated on a 0-9 scale according to lesion length scores as given in **Table 1**.

For the inheritance studies of BLB resistance in the segregating population, the goodness of fit of expected genetic ratios was tested by χ^2 test (Singh and Chaudhary *et al*., 1977). The chi-square analysis for genotypic and phenotypic ratios was calculated by using the following formula: $\chi^2 = \Sigma (O-E)^2/E$, where, O is the observed value, E is the expected value and Σ stands for summation.

RESULTS AND DISCUSSION

Rice is one of the most important cereal crops for global food security. Resistance breeding with MAS has been employed to develop broad spectrum durable BLB resistance in rice. A clear marker trait association was established for BLB. Hence, it is possible to monitor the transmission of trait genes *viz., xa13* and *Xa21 via* closely linked makers, (*xa13 promotor* and *pTA 248*). The primer pairs *viz., pTA 248* (Huang *et al.,* 1997) and *xa13 promotor* (Sundaram *et al.,* 2008) were used as gene sequence

Table 1. Infection per cent, score and host response

INFECTION %	SCORE	HOST RESPONSE
0%	0	Highly resistant (HR)
>1-10%	1	Resistant (R)
>10-30%	3	Moderately resistant(MR)
>30-50%	5	Moderately Susceptible (MS)
>50-75%	7	Susceptible (S)
>75-100%	9	Highly susceptible (HS)

based marker for BLB resistance genes *viz., Xa21* and *xa13* in marker assisted selection. Similarly, Mc Couch *et al.* (1997) and Olufowote *et al. (*1997) also used SSR's to study the polymorphism in rice varieties.

The present investigation clearly indicated that two resistance genes *viz., xa13* and *Xa21* for BLB were present in Improved Pusa Basmati 1. The susceptible parent, HUBR 2-1 was carrying both corresponding susceptible alleles (**Table 2**). Since, the polymorphism was very clear among the parents for both the target genes, these markers were selected for foreground selection in the segregating generations. The F_1 crosses were made during k*harif* 2011 *viz.,* HUBR 2-1 × Improved Pusa Basmati 1 and F_1 plants were raised in the field during r*abi* 2011. The primer pair *xa13 promotor* and *pTA 248* were used to confirm the hybridity of 100 F_1 plants from cross HUBR 2-1 × Improved Pusa Basmati 1 out of which 78 plants were confirmed as true hybrids (*Xa13xa13Xa21xa21*) for both BLB resistant genes *viz., xa13* and *Xa21* as shown in **Fig.1& 2**.

The F_2 population was subjected to gene linked markers to study the co-segregation of the disease resistant genes *viz., xa13* and *Xa21*. Two hundred F_2 plants developed from cross HUBR 2-1 × Improved Pusa Basmati 1 was analyzed with *xa13 promotor* (**Fig. 3**). The result revealed that 49 F_2 plants were identical to the susceptible parent (250 bp) while, 47 F_2 plants were identical to the resistant parent (500 bp) and 104 F_2 plants exhibited heterozygous nature for both the alleles. The χ^2 analysis indicated that *xa13* gene segregated in a genotypic ratio 1*Xa13Xa13*: 2*Xa13xa13*: 1*xa13xa13* and exhibited a good fit to the expected segregation ratio for single gene model with χ^2 square value 0.36 at $p < 0.05$.

Genetic analysis of *xa13 promoter* clearly exhibited goodness of fit to the expected segregation ratio of 1*Xa13Xa13*: 2*Xa13xa13*: 1*xa13xa13* for single gene model for *xa13* gene. Phenotypically, it is not possible to differentiate all the genotypes. However, it is possible to make the selection of plants carrying desirable gene combination *i.e., xa13xa13* by marker assisted selection.

Table 2. Polymorphism between resistant and susceptible alleles

Trait	Gene	Primer	Resistant allele	Susceptible allele
Bacterial Leaf Blight	*xa13*	*xa13 promoter*	500bp	250bp
Resistance	*Xa21*	*pTA248*	925bp	730bp

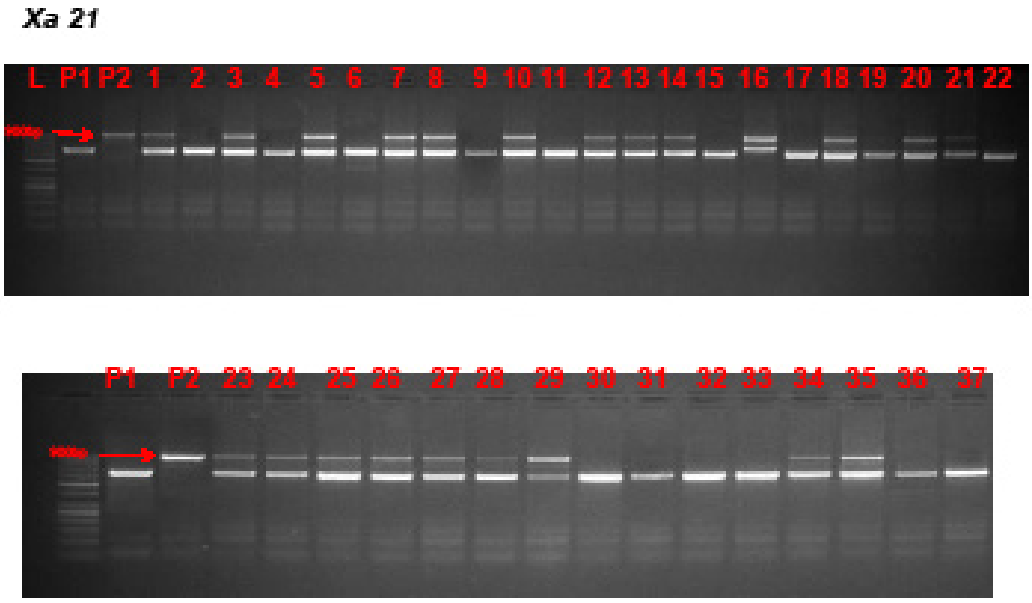

Fig 1. Confirmation of F_1 plants for *Xa21* gene by using *pTA248 promoter* primer

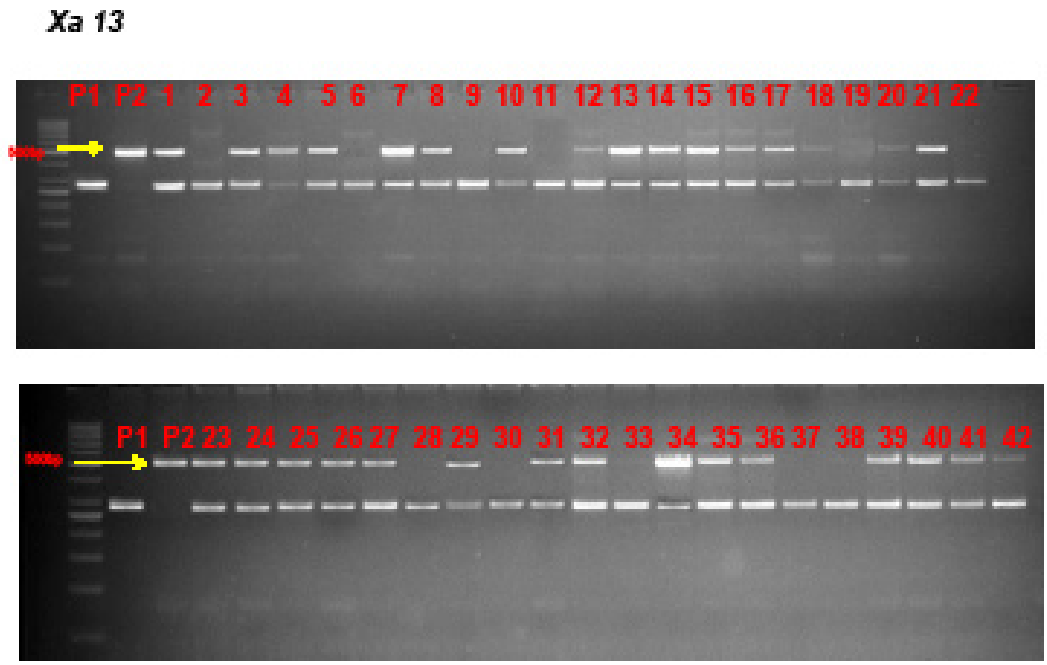

Fig 2. Confirmation of F_1 plants for *xa13* gene by using *xa13 promoter* primer

xa 13

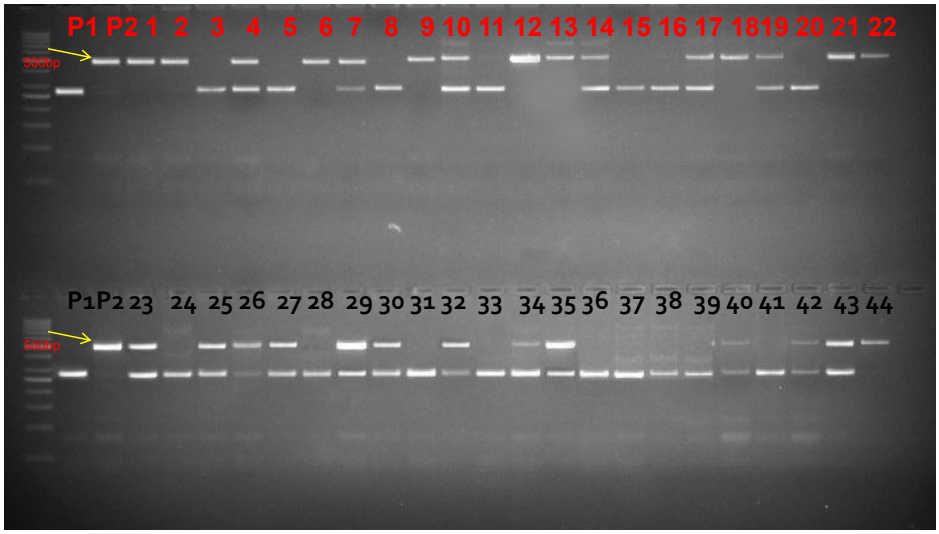

Fig. 3. Segregation of F_2 individuals derived from cross HUBR 2-1 × Improved Pusa Basmati 1 for *xa13 gene*. P_1 is HUBR 2-1 is the susceptible parent and P_2 is Improved Pusa Basmati 1 is the resistant parent

Xa 21

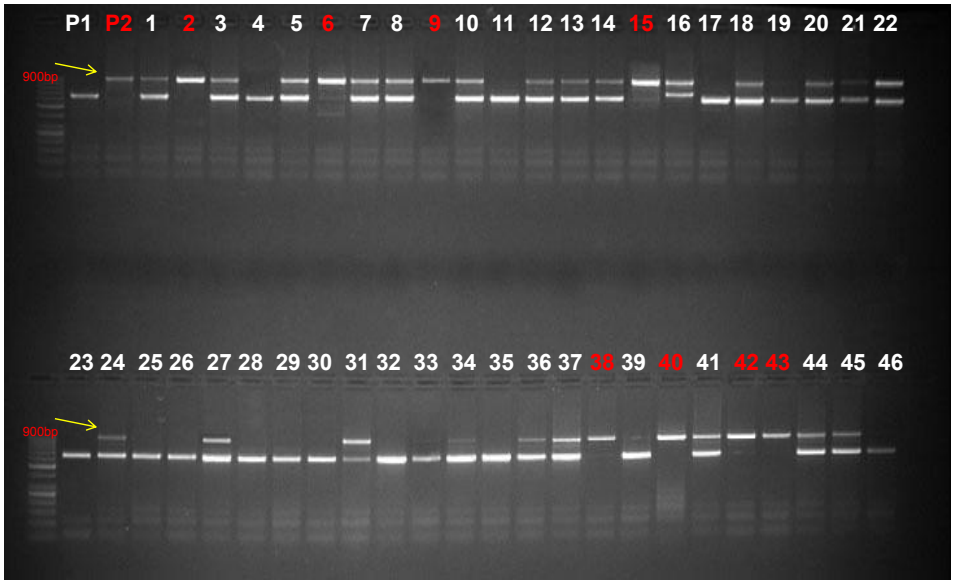

Fig. 4. Segregation of F_2 individuals derived from cross HUBR 2-1 × Improved Pusa Basmati 1 for *Xa21* gene. P_1 is HUBR 2-1 is the susceptible parent and P_2 is Improved Pusa Basmati 1 is the resistant parent

Similarly, *pTA 248* primer pair was used to study the co-segregation of the *Xa21* gene. Out of 200 F_2 plants, *pTA 248* primer pair amplified an allele of 730 bp in 50 F_2 plants identical to the susceptible parent, an allele of 925 bp in 54 F_2 plants identical to the resistant parent and 96 F_2 plants exhibited heterozygosity (**Fig 4**).

The χ^2 analysis indicated a good fit to the expected segregation ratio 1*Xa21Xa21*: 2*Xa21xa21*: 1*xa21xa21* for the single gene model. This result is in agreement with the results of Jiang *et al.* (2004) for the *Xa21* gene in the F_2 population of 'Minghui 63'. The *xa13* gene confers resistance only when it is present in the homozygous recessive condition whereas, *Xa21* is dominant in nature and can be expressed even in the heterozygous condition governing resistance to multiple races of *Xoo*.

The co-segregation analysis of the two BLB resistance genes *viz., xa13* and *Xa21* together showed the goodness of fit to the expected ratio 1:2:2:4:1:2:1:2:1, for two genes with a high degree of significance (**Table 3**). This result indicated that the two BLB resistant genes *viz., xa13* and *Xa21* followed Mendelian inheritance. Joseph *et al.* (2004) also reported that the two genes segregated into nine distinct classes as 1:2:2:4:1:2:1:2:1 out of which, the seven resistant genotypic classes *viz., xa13xa13Xa21Xa21, xa13xa13Xa21xa21, xa13xa13xa21xa21,Xa13xa13Xa21Xa21, Xa13xa13Xa21xa21, Xa13Xa13Xa21Xa21* and *Xa13Xa13Xa21xa21* are expected to segregate in the ratio of 1:2:1:2:4:1:2 for the two gene combinations.

Table 3. Co-segregation analysis of the two BLB resistance genes *viz., xa13* and *Xa21*

S. No.	Genotypes	Observed value	Expected ratio	Expected value	χ2 value
1.	*Xa13 Xa13 Xa21 Xa21*	12	1	12.5	0.02
2.	*Xa13 Xa13 Xa21 xa21*	27	2	25	0.16
3.	*Xa13 xa13 Xa21 Xa21*	24	2	25	0.04
4.	*Xa13 xa13 Xa21 xa21*	49	4	50	0.02
5.	*xa13 xa13 Xa21 Xa21*	14	1	12.5	0.18
6.	*xa13 xa13 Xa21 xa21*	24	2	25	0.04
7.	*Xa13 Xa13 xa21 xa21*	12	1	12.5	0.02
8.	*Xa13 xa13 xa21 xa21*	26	2	25	0.04
9.	*xa13 xa13 xa21 xa21*	12	1	12.5	0.02
Total		200	16	200	0.54**

The calculated χ^2 value, 0.54 less then tabulated value, 15.5 at df = 8 and P = 0.05 and 20.090 at P = 0.01

Table 4. Co-segregation of two genes in F_2 population from cross MTU 1010 × B95-1 against *Xoo* isolate DX-066

S. No.	Gene combinations	Disease Reaction/ Scale	Observed value	Expected ratio	Expected value	χ2 value
1.	*Xa13 Xa13 Xa21 Xa21*	Resistant (only due to *Xa21*) 0 to 2.0	111	9	112.5	0.02
2.	*Xa13 Xa13 Xa21 xa21*					
3.	*Xa13 xa13 Xa21 Xa21*					
4.	*Xa13 xa13 Xa21 xa21*					
5.	*xa13 xa13 Xa21 Xa21*	Resistant (due to both *xa13* & *Xa21*) 0.5 to 3.0	39	3	37.5	0.06
6.	*xa13 xa13 Xa21 xa21*					
7.	*Xa13 Xa13 xa21 xa21*	Susceptible (both genes in susceptible combination)	40	3	37.5	0.16
8.	*Xa13 xa13 xa21 xa21*					
9.	*xa13 xa13 xa21 xa21*	Moderately Resistant (only due to *xa13*)	10	1	12.5	0.5
Total			200	16	200	0.74**

The calculated χ2 value, 0.74 is less then tabulated value, 7.815 at df = 3 and P = 0.05 and 11.345 at P =0.01

The F_2 population from the cross HUBR 2-1 × Improved Pusa Basmati 1, showed segregation for resistance and susceptibility reactions for BLB with isolate, DX-066 (**Table 4**). The χ^2 analysis of the result exhibited a good fit for the Mendelian segregation ratio. This indicated that resistance to BLB is governed by both single genes independently. Higher levels of resistance in gene pyramid lines containing multiple BLB resistance genes as compared to lines having single (or fewer) resistance genes have been reported earlier (Yoshimura *et al.,* 1996).

In this study, the co-segregation analysis for the two gene combinations demonstrated a good fit to the phenotypic ratio of 9:3:3:1 indicating that the two genes segregated independently and revealed a simple dominant recessive relationship. The plants possessing *xa13* gene in homozygous condition along with *Xa21* gene in homozygous or homozygous condition showed BLB resistance (score ranged between 0 and 2), *Xa21* gene alone also showed resistance (0.5-3.0), *xa13* gene alone showed moderate resistance (2-4.5) and the plants with *Xa13* and *xa21* genes showed susceptible (>5-7) reaction.

Pandey *et al.* (2013) also improved traditional BB susceptible Basmati varieties (Taraori Basmati and Basmati 386) by introgressing two major BLB resistance genes, *Xa21* and *xa13*, coupled with phenotype based selection. They reported improved lines possessing a single resistance gene (*i.e.,* either *Xa21* or *xa13*) both in homozygous condition (*Xa21Xa21* or *xa13xa13*) displayed moderate resistance to BLB while, lines possessing both *Xa21* and *xa13* in homozygous condition (*Xa21Xa21xa13xa13*) showed significantly higher levels of resistance.

The present investigation indicated that the use of molecular markers that are closely linked to traits of interest in combination with the phenotype based selection resulted in effective selection of the desired combination of genotypes. Identification of desired genotypes possessing more than one gene is efficiently carried out when compared to the conventional breeding method (Dwivedi *et al.,* 2007). The results also further indicated that the selection based on genotypic data is reflecting at the phenotypic level.

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