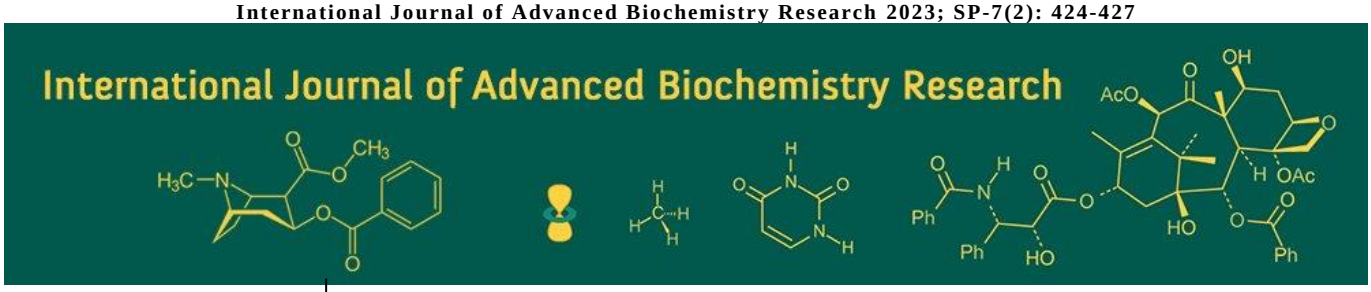


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# Molecular marker assisted selection of introgressed four bacterial blight resistance genes (*Xa*4, *Xa*7, *Xa*13 and *Xa*21) in the genetic background of Mahamaya

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**Abstract**
Bacterial leaf blight (BLB) of rice is one of the important constrains in rice production. BLB is caused by gram negative bacterium *Xanthomonas oryzae* pv *oryzae*. Host plant resistant are being used to develop improves varieties of rice by incorporating BB- resistant genes in the genetic background of mega rice cultivars. Present study was carried out to incorporate four bacterial blight resistant genes (*Xa4*, *Xa7*, *xa13* and *Xa21*) in the genetic background Mahamaya: An important variety of Chhattisgarh region. Segregating populations were screened against seven isolates of *Xoo* collected from Chhattisgarh region. Foreground selection was carried out for the target resistance genes using gene-specific PCR-based markers. Eleven different gene combinations found in selected lines in segregating population. *Xoo* isolates were also checked for their pathogenic diversity to know best combination of resistance genes for breeding program.

**Keywords:** Bacterial blight of rice, molecular markers, gene pyramiding and resistance

## Introduction
Rice is one of the chief grains of India. Moreover, this country has the largest area under rice cultivation, as it is one of the principal food crops. India is one of the leading producers of this crop. Chhattisgarh is among top ten highest rice producing states of India along with West Bengal and Madhya Pradesh. (India Today). It is also known as "Rice Bowl of India". To meet the demand of increasing population, Rice production should be increased in current years. Rice production is affected by many biotic and abiotic constrains. Bacterial bight (BB) of rice is one of the important diseases and it can cause 30 to 50% yield loss (Reddy, 1989, Adhikari *et al*. 1994) [19, 1]. BB is caused by a Gram negative Bacteria *Xanthomonas oryzae* pv o*ryzae*. Bacteria enter through hydathodes in the rice plant and cause leaf blightening, which is characterized by water soaked lesions on leaves, gradually turns straw in colour. Wilting of plants occur when plants become infected at seedling stage. Disease has increased in both intensity and geographical distribution in India, as exemplified by several reports of BB occurrence in recent years in epidemic form. Chemical control against this disease has not been very successful (Laha *et al*, 2009) [13]. Therefore, major emphasis is placed on the development and deployment of BB-resistant rice varieties. However, the pathogen is highly diverse in nature, particularly in India (Yugander *et al*, 2017) [23], and several cases of breaking down of resistance with single BB genes have been reported (Yugander *et al*, 2017, Chen *et al*, 2000, Mandal *et al*, 2014) [23, 4, 15]. Therefore, pyramiding of multiple BB-resistant genes in the genetic background of rice varieties through marker-assisted selection is the most effective approach to develop durable BB resistance (Pradhan *et al*, 2015) [17]. More than 42 BB resistance (R) genes, designated from *Xa*1 to *Xa43*, conferring resistance against various strains of *Xoo*, have been identified from cultivated, mutant population, and wild rice species (Cheema *et al*. 2008, Kim *et al.* 2015, Busungu *et al*., 2016, Kim *et al*., 2019) [3, 12, 2, 11]. These are now being incorporated into mega rice cultivars all over the world.
Mahamaya is important rice variety of Chhattisgarh region. Mahamaya is suitable for '*Poha*' (flaked rice) industry. This is also resistant to gall midge and tolerance to brown spot and sheath rot, susceptible to BB. Here, we have tried to incorporate four BB resistance genes (*Xa4*, *Xa7*, *Xa*13 and *Xa21*) in the background of Mahamaya. IRBB65 was used as donor parent and crossed with Mahamaya.

Segregating population were phenotyped with seven isolates of *Xoo* collected from different region of Chhattisgarh. PCR based DNA markers were used for selective genotyping of lines having gene(s) in combination.

## Materials

Plant material comprises of population derived from cross Mahamaya (Recipient Parent) **X** IRBB65 (Donor Parent). Plants are grown during wet season of 2018 at Indira Gandhi Agriculture University, Raipur, Chhattisgarh. Twenty four Near Isogenic Lines were grown to evaluate pathogenic diversity of *Xoo* isolates.

Infected leaf samples collected from seven different regions of Chhattisgarh: Dhamtari (XO-DMT), Balrampur (XO-BLRP), Durg (XO-DUR), Raipur (XO-RPR), Mahasamud (XO-MHS), Surajpur (XO-SRP) and Bahatapara (XO-BTP). Bacteria were isolated using procedure described by Kotasthane (2003). Isolates were purified and maintained in wakimoto's medium. These seven isolates were used for Phenotyping of plant material.

## Phenotyping of plant population

During the wet season 2018, the experimental plant material were grown in field and inoculated at maximum tillering stage with bacterial culture following the clip inoculation technique (Kauffman *et al.*, 1973) [9]. After 21 days of inoculation, Phenotyping of segregating population were done by scoring following Standard Evaluation System of Rice developed by IRRI, Philippines. Based on the scores four discrete classes (Highly Resistant, Resistance, Moderately Resistant and Susceptible) were created from derived population which will form the basis of MAS activity.

## DNA extraction and PCR amplification

The plants with high resistant reaction were selected for selective genotyping. Modified CTAB protocol was followed for DNA extraction from Rice seedlings (Keb-Llanes *et al.*, 2002) [10]. Molecular markers commercially available were used for tracing presence of genes in selected lines. Molecular Markers linked to *Xa4:- Xa4* (Xa4-MP), *Xa4* (RM224), *Xa7*:- Xa7-M5, Xa7GD, *xa13:-* xa13promotor *Xa21*:-PTA248, RM21, Xa21. Reaction mixture for PCR contained autoclaved distilled water 5.25 µl, *Taq* buffer 1 µl, dNTPs 1 µl, primers 1 µl, 0.25 µl *Taq* polymerase and 1.5 µl DNA templates for 10 µl volume. Template DNA was initially denatured at 94 °C for 5 min followed by 30 cycles of PCR amplification with the following parameters: a 30-s denaturation at 94 °C, a 30-s primer annealing at 55 °C and 1 min of primer extension at 72 °C. The amplified product was electro-phoretically resolved on a 5% PAGE gel in 1× TBE buffer. Initially, 5 µl of PCR product was used for gel electrophoresis and visualized under UV light after staining with ethidium bromide.

## Results

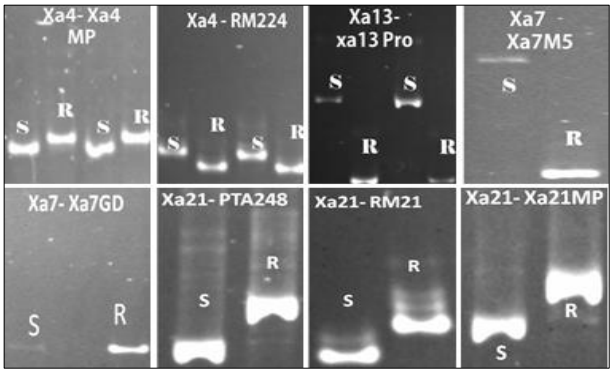
Mahamaya (MM) was crossed with IRBB65 (donor of four BB resistance genes: *Xa4* + *Xa7* + *xa13* + *Xa21*). Population of the cross was phenotyped against seven *Xoo* isolates collected from Chhattisgarh. Total population of cross MM X IRBB65consists total 3565 plant, in which 2289 lines were showing resistance reaction, 350, 566 and 360 plants were observed to be highly resistant(HR), moderately resistant (MR) and susceptible (S) respectively. Highest proportion of susceptible plants recorded in case of XO-BTP followed by XO-BLRP and XO-RPR. Table # showing number plants in each category inoculated with seven isolates.

**Table 1:** Identified lines with highly Resistance (HR), Resistance (R), moderately resistance (MR) and Susceptible (S) reactions against seven *Xoo* isolates.

| Isolates /Crosses | Mahamaya X IRBB 65 | | | |
|---|---|---|---|---|
| | HR | R | MR | S |
| XO-BTP | 51 | 317 | 68 | 70 |
| XO-DMT | 60 | 341 | 71 | 34 |
| XO-DURG | 64 | 344 | 66 | 42 |
| XO-BLRP | 47 | 293 | 107 | 65 |
| XO-MHS | 55 | 340 | 73 | 44 |
| XO-RPR | 38 | 328 | 89 | 55 |
| XO-SRP | 35 | 326 | 92 | 50 |
| Total | 350 | 2289 | 566 | 360 |
| Total Population | 3565 | | | |

## Marker assisted selection

Eighty highly resistant lines were selected from a cross between Mahamaya and IRBB65 for selective genotyping with gene specific molecular markers. First we have screened 10 SSR markers (*Xa4*: Xa4MP, RM224; *Xa7*: Xa7GD and Xa7M5, *xa13*: xa13 Promoter, SR6 and SR11, *Xa21*: pTA248, Xa21MP and RM21) for parental polymorphism. Markers which have resolved polymorphism between recipient (susceptible) and donor (resistant) parent were selected for genotyping and tracing the presence of gene(s) in combination. Table # is showing segregating patter of markers as per phenotype.




**Picture 1:** Parental polymorphism resolved by existing tightly linked molecular markers to gene of interest.

**Table 2:** Segregating pattern of gene linked markers as per phenotype in selected lines.

| | *Xa4* | | *Xa7M5* | | *xa13* | *Xa21* | | |
|---|---|---|---|---|---|---|---|---|
| | RM224 | XA4MP | Xa7M5 | Xa7GD | xa13 | RM21 | Xa21 | PT248 |
| R | 37 | 35 | 48 | 0 | 41 | 24 | 25 | 23 |
| H | 10 | 16 | 0 | 0 | 19 | 18 | 4 | 24 |
| S | 33 | 29 | 32 | 0 | 20 | 38 | 51 | 33 |

R: recombination from phenotype recorded, H: Heterozygous, showing banding pattern of both susceptible and resistant type.

*Xa4* gene was tagged using RM224 and Xa4MP markers. Fifteen lines were found to be positive for presence of *Xa4* gene in homozygous condition. Similarly nineteen and fifteen lines were recorded to have *Xa7* gene based on markers Xa7M5 and Xa7GD. Presence of *xa13* was traced using marker xa13-promoter and 16 lines were found to be positive. Gene *Xa21* was tagged with the help of three markers RM21, Xa21MP and pTA258 in 7, 12 and 10 lines respectively.

**Identified gene pyramids in selected lines**

Selective genotyping of 80 resistance lines derived from cross Mahamaya X IRBB65 revealed eleven types of gene combinations based on SSR markers linked to gene of interest. BB resistance gene *Xa4*, *xa13* and *Xa7* present in 8 (#76, 49, 68, 71, 72, 19, 32 and 12), 3 (#34, 66 and 74) and 2 (#23 and 35) lines respectively. Six combinations of two genes (*Xa7 + Xa21*, *xa13 + Xa21*, *Xa7 + xa13*, *Xa4 + Xa21*, *Xa4 + Xa7* and *Xa4 + xa13)* were speculated in 4(#13, 16, 17, 57), 4 (#39, 40, 15, 62), 6 (#6, 7, 11, 18, 26, 30), 7 (#56, 53, 25, 20, 55, 78, 33), 9 (#1, 21, 45, 47,48, 51, 52, 69, 70) and 3 (#31, 42, 50) lines respectively. Three gene pyramids in four combinations (*Xa4+ xa13 + Xa21*, *Xa4 + Xa7 + Xa21*, *Xa7 + xa13+ Xa21* and *Xa4 + xa13 + Xa7)* were traced in 4 (#29,61, 63, 80), 8 (#22, 27, 36, 46, 58, 59, 60, 79), 6 (#3, 4, 5, 8, 14, 28), and 9 (#9, 10, 37, 38, 43, 44, 64, 65, 73) lines respectively. It was observed that all four incorporated genes (*Xa4 + Xa7 + xa13 + Xa21*) present in 6 lines (#2, 24, 41, 54, 67 and 75).

**Table 3:** Gene pyramids in selected 80 lines of cross MM X IRBB65

| Sr. No. | Pyramids | Number of Line(s) | Line number |
|---|---|---|---|
| 1 | *Xa4* | 8 | 76, 49, 68, 71, 72, 19, 32, 12 |
| 3 | *xa13* | 3 | 34, 66, 74 |
| 4 | *Xa21* | 2 | 23, 35 |
| 5 | *Xa7 + Xa21* | 4 | 13, 16, 17, 57 |
| 6 | *xa13 + Xa21* | 4 | 39, 40, 15, 62 |
| 7 | *Xa7 + xa13* | 6 | 6, 7, 11, 18, 26, 30 |
| 8 | *Xa4 + Xa21* | 7 | 56, 53, 25, 20, 55, 78, 33 |
| 9 | *Xa4 + Xa7* | 9 | 1, 21, 45, 47,48, 51, 52, 69, 70 |
| 10 | *Xa4 + xa13* | 3 | 31, 42, 50 |
| 11 | *Xa4+ xa13 + Xa21* | 4 | 29,61, 63, 80 |
| 12 | *Xa4 + Xa7 + Xa21* | 8 | 22, 27, 36, 46, 58, 59, 60, 79 |
| 13 | *Xa7 + xa13+ Xa21* | 6 | 3, 4, 5, 8, 14, 28 |
| 14 | *Xa4 + xa13 + Xa7* | 9 | 9, 10, 37, 38, 43, 44, 64, 65, 73 |
| 15 | *Xa4 + Xa7 + xa13 + Xa21* | 6 | 2, 24, 41, 54, 67, 75 |

**Discussion**

Improving of popular varieties by pyramiding BB resistance genes is one of the strategies to manage bacterial leaf blight of rice (Singh *et al*., 2001, Sundaram *et al*., 2008) [21, 22] as breakdown of resistance due to strong selection pressure has reported in cultivars with single BB resistance genes (Pink and Puddephat, 1999) [16]. Conventional backcross breeding has difficulty in confirming the several resistance genes combined in breeding lines using phenotypic selection with *Xoo* inoculation because of the dominance and epistasis effects of the genes controlling disease resistance (Rajpurohit *et al*. 2011; Shanti *et al*. 2010; Sundaram *et al*. 2008) [18, 20, 22]. Nevertheless, using the tools of biotechnology, it is possible to transfer or pyramid valuable genes of BB resistance into rice without linkage drag (Rajpurohit *et al*., 2011; Shanti *et al*., 2010; Singh *et al*., 2001; Sundaram *et al*., 2008) [18, 20, 21, 22]. Four bacterial blight resistance genes have been introgressed in the background of Mahamaya. We report here in successful transfer of four BB resistance gene into elite rice cultivar Mahamaya with the help of Molecular Markers linked to specific genes. We have found eleven types of gene combination in selected highly resistance lines from a cross between Mahamaya X IRBB65. Six combinations of two genes in 33 lines, four combinations of three genes in 27 lines, 6 lines were introgressed with all the four genes. These lines can be used as improved Mahamaya lines with bacterial blight resistance. Gene combination *xa13+Xa21* have been used by researchers to introgress into rice cultivars (Sundaram *et al*., 2008) [22]. Four lines have been identified in this investigation. It was reported that introgression of more genes results in lines with different gene combinations. Mixture of lines with different gene combinations might be used as multiliines in resistance breeding. It will create less selection pressure on pathogen population against particular resistance genes and gene combinations. The pyramided rice lines with multiple BB resistance genes (*xa5, xa13* and *Xa21*) provided a wide spectrum of resistance when combined in a single genotype (Huang *et al*., 1997, Singh *et al*., 2001, Joseph *et al*., 2004, Sundaram *et al*. 2008) [6, 21, 8, 22].

**Conclusion**

In conclusion, this study successfully employed Molecular Marker Assisted Selection to introgress four bacterial blight resistance genes (Xa4, Xa7, xa13, and Xa21) into the genetic background of the important rice variety Mahamaya. Through extensive phenotyping and selective genotyping, a diverse array of gene combinations was identified in the selected lines.

This approach holds great promise for developing durable bacterial blight resistance in rice varieties, as it overcomes challenges associated with the diversity and adaptability of the pathogen. The introgressed lines, especially those with multiple resistance genes, represent valuable genetic resources for future breeding programs aimed at enhancing the resistance of rice varieties to bacterial leaf blight. This work contributes to the ongoing efforts to increase rice production and address the challenges posed by biotic constraints in rice cultivation.

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