

# Identification of bacterial leaf blight resistance genes in Malaysian local rice varieties

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**ABSTRACT.** *Xanthomonas oryzae pv oryzae* (*Xoo*) is a bacterial pathovar that causes a serious bacterial leaf blight disease of rice. This disease poses significant constraint on food security in Asia, as it causes yield loss in rice. There is an urgent need to control bacterial blight disease through resistance cultivars. However, the genetic potential of Malaysian rice cultivars has not been explored. We screened 10 cultivated Malaysian varieties with high yield performance for resistance genes using three simple sequence repeat and two sequence tagged sites markers coupled with phenotypic screening. All 10 rice genotypes were found to carry *xa* recessive gene. Four genotypes had two resistance genes tightly linked with the specific markers; Mahsuri Mutant carried the dominant resistance genes, *xa4* and *xa2* whereas NMR152 and the Tongkat Ali mutant had the dominant genes, *xa21* and *xa2*. However, *xa13* and *xa5* resistance genes were not detected in this Malaysia rice germplasm group. In a greenhouse assessment, genotypes carrying more than a single resistance gene were found to be resistant against *Xoo*

MXO 1410 isolates. These cultivars have potential as genetic materials for rice quality breeding programs.

**Key words:** Malaysian rice; SSR marker; STS marker; *Xanthomonas oryzae pv. oryzae*; *xa* gene

## INTRODUCTION

Rice (*Oryza sativa*) is a primary food source for 3.5 billion people in the world, and Asia is the top rice consuming and producing continent (Herman et al., 2015). Rice is classified as a principal crop with regards to cultivation area and production in Malaysia (Wahab, 2018). The rapid growth of the human population in Asia has led to an estimation of about 70% rice production increase needed to meet future demands (Rajamoorthy et al., 2015).

Annually, biotic stresses (weeds, insect pests and pathogens) contribute to the world's rice crop losses (Patra et al., 2016). Among these, *Xanthomonas oryzae pv. oryzae* (*Xoo*), the causative agent of bacterial leaf blight disease, considered the oldest and most important disease of rice (Chukwu et al., 2019). In 1884, farmers in Japan first recognized this disease (Tagami and Mizukami, 1962). In Malaysia, the infection was first noticed on a small scale in the early 80s rice fields of Peninsular Malaysia (MOA, 2018). Previously, this disease had spread more vigorously where 10–20% crop losses were observed under moderate prevailing conditions, whereas under conducive conditions, up to 50% crop losses were recorded. In recent years, an increase in bacterial leaf blight attack was recorded in 12,080 hectare rice-growing areas in Peninsular Malaysia including Selangor (5,945 ha), Kedah (4,415 ha), Pulau Pinang (620 ha), Terengganu (440 ha), Negeri Sembilan (291 ha), Perak (174 ha), Pahang (46 ha), Perlis (141 ha), Johor (5 ha), Kelantan (1 ha), and Melaka (less than 1 ha) (DOA, 2019).

Various chemical and cultural approaches have been used for the management of bacterial leaf blight. However, this use is limited because the technique is relatively time consuming and laborious. Host resistance is the most favored approach to manage the bacterial pathogen, *Xoo* by developing durable BLB-resistant cultivars (Vikal and Bhatia, 2017). However, the durability of plant resistance has often been limited and some varieties was broken down within several years. Therefore, resistance breeding requires continuous efforts to evaluate the diversity within the germplasm for details of genes. Artificial inoculation method to screen the resistant variety are always recommended and ideal because disease symptom develop quickly.

Advances in DNA marker technology have sped up the identification of resistant cultivars carrying resistance gene(s) (Nor'Aishah et al., 2013). Till now, about 34 genes (23 dominants and 16 recessive genes) conferring resistance to bacterial leaf blight have been identified (Zhou, 2019). By using DNA markers, numerous commercial rice varieties have been introgressed with race-specific resistance genes including *xa4*, *xa5*, *xa7*, *xa13*, and *xa21* and improved their resistance against *Xoo* (Bharani et al., 2010). SSR markers have been commonly used as they are highly informative, easy to use and cost effective (Nadeem et al., 2018). New advances in molecular genetic field such as application of DNA markers in plant selection, quantitative trait locus mapping, and genetic transformation have provided insight for breeders to produce new rice varieties with durable bacterial leaf blight

disease resistance. We screened the *xa21*, *xa13*, *xa5*, *xa4*, and *xa2* resistance genes in 10 local Malaysian rice varieties based on high agronomic performance and to help provide fundamental information for the development of new durable resistant varieties that can withstand bacterial leaf blight using the gene pyramiding approach.

## MATERIAL AND METHODS

### Plant material and research design

The study consists of Malaysia's five breeding lines (MR84, MR303, MR307, MR297, MR219), four mutant lines (NMR151, NMR152, Mahsuri Mutant, Tongkat Ali), and one landrace cultivar (Pongsu Seribu 2) obtained from GeneBank of Malaysian Agricultural Research and Development Institute (MARDI). Towuti and Tetep varieties act as a positive check (resistant) while MR284 was used as negative check (susceptible) in this study. Seed germination was examined by incubating the seeds at 28°C in the dark for 4 days. Plastic trays (36 cm x 23 cm x 10 cm) were used to transfer the germinated seeds and each seed was sown with ten replicates in ten-centimeter rows per tray. Replication was carried out three times in a completely randomized design. The plants were grown at 25-30°C for 45 days (active tillering phase) supplemented with standard amount of fertilizer in a greenhouse following the method of Fillipi and Prabu (2001).

### DNA extraction

Fresh and young leaves of three weeks old were collected from the 13 transplanted local rice varieties. Leaves were swabbed with 70% ethanol to avoid any contamination from foreign DNA or spores. 1 mL of pre-heated CTAB (Cetyltrimethylammonium bromide) buffer were transferred in 1.2 mL collection microtubes containing 3-mm tungsten beads. Forty mg of leaves were disrupted and homogenized into a fine powder at 30Hz for 4 min using QIAGEN TissueLyser (QIAGEN TissueLyser II, QIAGEN, USA). Extraction of genomic DNA were then isolate using fine powdered leaf sample following method by Doyle and Doyle (1990). Nano-drop spectrophotometry (PerkinElmer, Singapore) was used to quantify the DNA and 1.0% agarose gel was used to visualize the DNA quality.

### PCR amplification with SSR markers

SSR and STS markers that were reported to be previously linked with *xa* gene were employed to identify the presence of BLB resistance gene in local rice varieties. Amplification of Polymerase Chain Reaction (PCR) was performed following the method mentioned by McCouch et al. (2002). A 25 µL volume of mixture PCR consisting of 5 µL of 5× PCR buffer, 5 µL of 10 mmol l<sup>-1</sup> dNTPs (Promega, Madison, WI, USA), 0.1 µL of 1.5 units of Taq DNA polymerase (Promega, Madison, WI, USA), 10.4 µL of sterile ultrapure deionized water, 2 µL of 50 ng DNA template, and 4.0 µL of 10 pmolµL of forward and reverse primers was prepared. Markers Xa13, RM122 (STS), RM317, RM224, pTA-248 (STS) were used to identify *xa* gene (Table 1). Sterile deionized water acted as a negative-DNA control. PCR amplification was performed following a program of 30 cycles of 94°C for 1 min at denaturation, 1 min at 55°C for annealing, 72°C for 2 min for

polymerization, 72°C for 7 min for final elongation and allow to fast cooling at 4°C prior to analysis. Molecular Imager® (GelDoc™ XR, Bio-Rad) was used to visualize the PCR product. Alpha Ease Fc5.0 software was used to calculate the molecular weights of different alleles.

### Pathogenicity test

The culture of *Xoo* (strain MXO 1410) was obtained from the pathology laboratory, MARDI Seberang Perai, Malaysia and was sub cultured on peptone sucrose agar medium containing 2% sucrose (w/v), 2.5% peptone (w/v), 0.05% K<sub>2</sub>PO<sub>4</sub> (w/v), and 0.025% MgSO<sub>4</sub>·7H<sub>2</sub>O (w/v) and maintained it at pH 7.0 (Fany and Presley, 1983). The bacterial culture was then adjusted to an OD<sub>600</sub> of 1.0 in sterile water. Rice seedlings (fully developed leaves approximately 45 days old after transplanting) were inoculated with isolate MXO 1410 strain of *Xoo* by leaf clipping method. Sterilized scissors dipped in the 10<sup>8</sup> cfu/mL bacterial suspension were used to cut 2-3cm of leaves at the top (Kauffman et al., 1973). Phenotypic evaluation based on disease scoring were done after 21 days post inoculation following the Standard Evaluation System (SES) of the International Rice Research Institute (IRRI, 2013).

### Disease scoring

For each variety, 10 plants per genotype and five leaves per plant from each replication were randomly chosen to record the disease severity. Disease reaction were adjusted based on the mean lesion length following a disease index (IRRI, 1996): highly resistant (<1 cm), resistant (1-3 cm), moderately resistant (3-6 cm), moderately susceptible (6-10 cm), and susceptible (>10 cm). Lesion length were calculated in the infected region of the leaf from one end to another.

**Table 1.** Information of polymorphic microsatellite markers linked with *xa* for *Oryza sativa* used in this study.

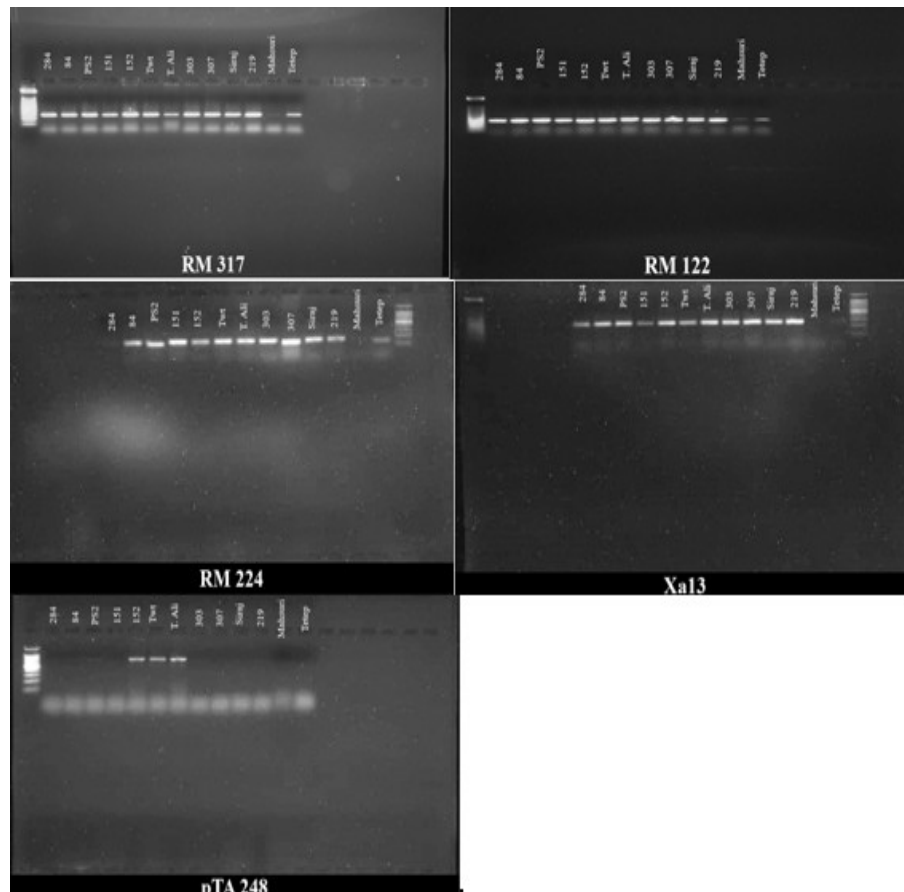
Primer	Chromosome	Forward	Reverse	PCR product	Linked gene
Xa13	8	F: GGCCATGGCTCAGTGTTAT	R: GAGCTCCAGCTCTCCAAATG	498bp	<i>xa13</i>
RM122 (STS)	5	F: GAGTCGATGTAATGTCATCAGTGC	R: GAAGGAGGTATCGCTTTGTTGGA	240-230bp	<i>xa15</i>
RM317	4	F: CATACTTACCAGTTCACCGCC	R: CTGGAGAGTGTGCTAGTTGA	154bp	<i>xa2</i>
RM224	11	F: ATCGATCGATCTTCACGAGG	R: TGCTATAAAAGGCATTCGGG	160bp	<i>xa4</i>
pTA-248 (STS)	11	F: AGACGCGGAAGGGTGGTCCCGGA	R: AGACGCGTAATCGAAGATGAAA	1100/925bp	<i>xa21</i>

## RESULTS

### Genotype screening for bacterial leaf blight resistance

Microsatellites and sequence tag sites markers (Xa13, RM122, RM317, RM224, and pTA-248) were used to examine 10 local Malaysian rice varieties for resistance genes

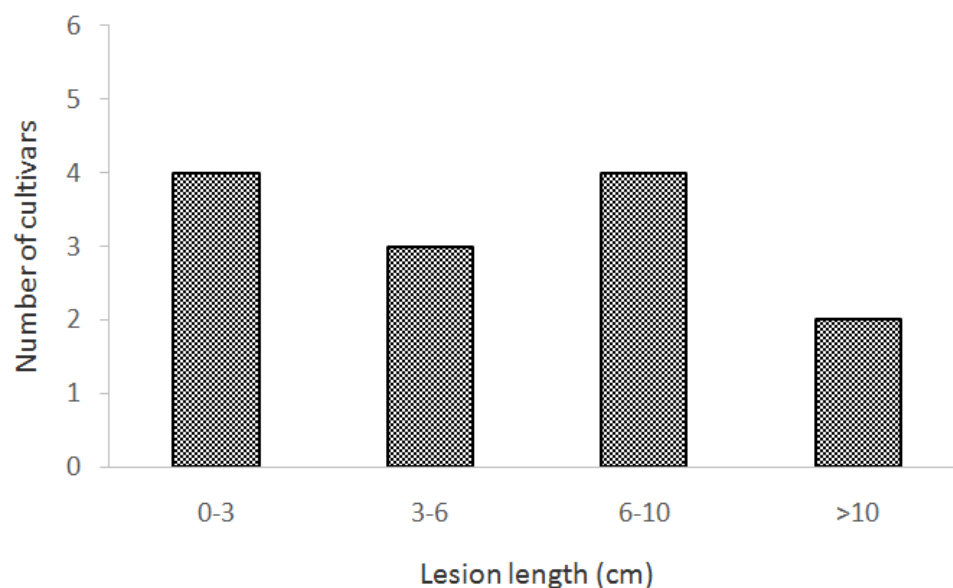
viz. *xa13*, *xa15*, *xa2*, *xa4*, and *xa21*. These bacterial leaf blight resistance genes were identified by visualization of amplicons from PCR results near 498 bp, 240-230 bp, 154 bp, 160 bp, and 1100/925 bp, respectively. The resistant, Tetep and Towuti, and susceptible, MR284 controls were included as a checking mechanism for the respective genes. Result of genotypic screening of ten local varieties are presented in Table 2 while Figure 1 shows the banding patterns of DNA markers for BLB resistance genes. Presence of *xa2* gene were successfully identified in 13 rice lines including Tetep and Towuti with positive fragment amplified at 154 bp. STS marker pTA-248 and SSR marker RM224 are utilized to amplify dominant resistance genes, however, only Mahsuri Mutant rice line amplified 925 bp fragments. Meanwhile, NMR152 and Tongkat Ali rice lines amplified 160 bp fragment along with the control resistant variety, Towuti, indicating the presence of *xa21* and *xa4* genes. None of the amplicons specific to *xa13* and *xa5* alleles were identified, suggested all the local rice accession did not carrying this resistance gene.



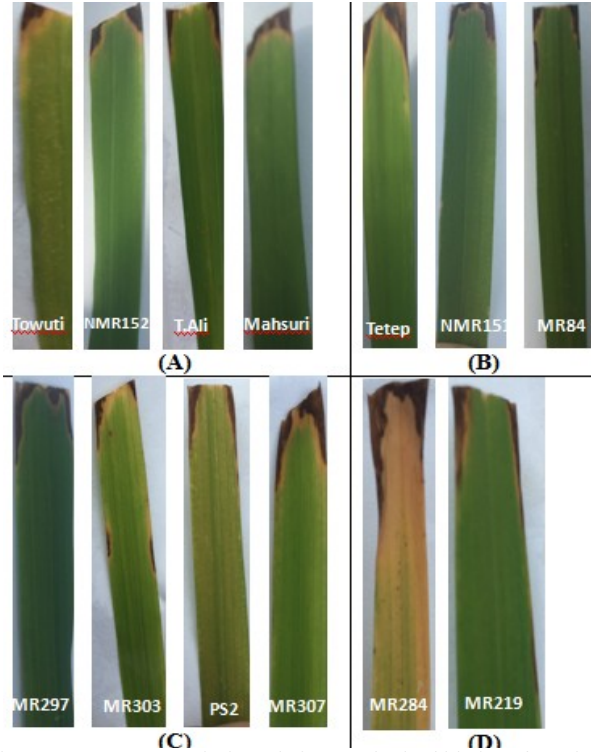
**Figure 1.** Polymorphism for markers RM317, RM122, RM224, XA13 and pTA-248, of 10 local varieties of rice on 1% agarose gel stained with gel star (M=100 bp ladder); Tetep and Towuti act as positive check (resistant), MR284 act as negative check (susceptible).

## Phenotypic screening for bacterial leaf blight resistance

Preliminary screening on bacterial leaf blight was evaluated on thirteen rice accessions along with resistant (Towuti and Tetep) and susceptible (MR219) controls against *Xoo* MXO 1410 isolate under greenhouse condition during 2019. At 21 days after inoculation, 3 genotypes showed resistant disease symptoms, 2 genotypes were moderately resistant, 4 genotypes were moderately susceptible, and 1 genotype was susceptible (Figure 2). Among these rice accessions, NMR152, Tongkat Ali, and Mahsuri Mutant showed the lowest disease severity while MR219 showed the highest. This symptom first visible in accession MR219 five days followed by accessions MR303 and MR297 after eight days after inoculation as compared with resistant controls (Towuti), which demonstrated the symptoms 11 days after inoculation while susceptible control MR284 displayed the symptoms after three days. Figure 3 represent the phenotypes of the three most resistant (i.e., NMR152, Mahsuri Mutant and Tongkat Ali) and the most susceptible cultivars (i.e., MR219) from the greenhouse inoculation. Lesions commonly appeared on leaf blades or leaf as yellow-orange stripes tips or water-soaked. On younger lesions, a milky dew drop was formed in the morning and turn into white lesions and mechanically injured parts of leaves as the disease progresses. Table 2 presents the results of phenotypic screening based on disease severity (diseased leaf area); the cultivars were evaluated and values were computed after inoculation with MXO 1410 strain of *Xoo*.



**Figure 2.** Distribution of bacterial blight lesion length (in cm) in 13 rice cultivars including two resistant controls (Towuti and Tetep) and one susceptible control (MR284) after *Xoo* MXO 1410 strain inoculation.



**Figure 3.** The effect of *Xoo* MXO 1410 strain inoculation on the leaf blades of 13 rice genotypes (Towuti and Tetep act as resistant controls; MR284 act as susceptible control): (A) resistant, 1-3cm; (B) moderate resistant, 3-6cm; (C) moderate susceptible, 6-10cm; (D) susceptible, >10cm. The progress in disease infestation as observed on the infected leaf blades was photographed 21 days post inoculation.

**Table 2.** Summary of the phenotypic and genotypic screening for bacterial leaf blight resistance in 13 rice accessions.

	Variety	Disease severity		Host response	Bacterial leaf blight resistance genes				
		14 DAI ± SD	21 DAI ± SD		<i>xa21</i>	<i>xa13</i>	<i>xa5</i>	<i>xa4</i>	<i>xa2</i>
Resistant	Tetep	3.08±0.405	4.3±0.340	MR	-	-	-	-	+
control	Towuti	1.28±0.263	2.03±0.287	R	+	-	-	-	+
Susceptible	MR284	8.91±0.557	12.41±1.038	S	-	-	-	-	+
control	MR219	6.43±0.709	10.97±1.38	S	-	-	-	-	+
1	MR84	2.83±0.361	5.21±0.487	MR	-	-	-	-	+
2	MR303	5.22±0.556	7.55±0.821	MS	-	-	-	-	+
3	MR307	5.91± 1.005	7.91±1.211	MS	-	-	-	-	+
4	MR297	5.11±0.446	7.6±0.804	MS	-	-	-	-	+
5	NMR151	3.26± 0.332	4.96±0.411	MR	-	-	-	-	+
6	NMR152	1.44± 0.325	2.26±0.272	R	+	-	-	-	+
7	Mahsuri	1.64± 0.239	2.51±0.305	R	-	-	-	+	+
8	Mutant								
9	Tongkat Ali	1.45±0.371	2.33±0.205	R	+	-	-	-	+
10	Pongsu Seribu	5.25±0.555	8.40±0.324	MS	-	-	-	-	+
	2				23.08	0	0	7.69	100

Scoring data for bacterial leaf blight resistance gene in rice, presence (+) or absence (-) of resistance genes linked to SSR and STS markers. DAI: Days after inoculation, SD: standard deviation, R: resistant, MR: moderately resistant, MS: moderately susceptible, S: Susceptible.

## DISCUSSION

Bacterial leaf blight disease is considered the most important disease causing yield loss in rice. To tackle this issue and avoid possible epidemics, several strategies have been proposed to constrain the evolution of pathogen populations. Host-plant resistance is considered as a significant control strategy. Therefore, information of varietal resistance is vital to develop cultivars with durable resistance. We examined 13 cultivars (including Towuti and Tetep for resistant check and MR284 for susceptible check) to screen for resistance to *Xoo* race MXO1410 strain, the causative agent of bacterial leaf blight. The MXO1410 strain of *Xoo* is the most aggressive and produces large lesions; it is widely used to screen rice cultivars in Malaysia as it provokes a wide range of host responses. Experiments were conducted in a greenhouse throughout November 2019.

Greenhouse evaluation revealed the different reactions of bacterial leaf blight expression of the rice genotypes due to inoculation. These reactions indicated the different characteristics of cultivars. None of the rice accessions were observed as being highly resistant. This might result in occurrence of aggressiveness and high virulence of the *Xoo* MXO1410 strain. Similarly, a wide range of responses of genotypes to *Xoo* have been observed and reported (Singh et al., 2015; Fred et al., 2016; Sombunjitt et al., 2017; Acharya et al., 2018). Plants are subjected to a wide range of environmental stresses during their growth and development stages. Plants being sessile in nature will encounter numerous defense mechanisms. Plant defense pathways involve a number of signaling compounds to protect themselves against attack by herbivorous insects and microbial pathogens (Koornneef and Pieterse, 2008).

In this study, rice cultivars Tongkat Ali, NMR152, and Mahsuri Mutant with dominant resistance genes, *xa21* and *xa4* which were combined with another recessive gene, *xa2* were resistant to the *Xoo* strain. These results from our analysis consistent with the reports made by Sabar et al. (2017) and Chukwu et al. (2019). According to Suh et al. (2013), incorporating the resistance genes *Xa4*, *xa21* conferred a durable resistance compare to the lines with one resistance gene. Pradan et al. (2015) reported that three-gene combinations found to be the most productive which *Xa21* contributes the durable resistance from susceptible juvenile stage to complete resistance at adult plant stage. Bacterial leaf blight resistance gene *xa21* commonly utilized resistance genes in many rice breeding programs and it confers durable resistance in many popular rice cultivars (Nguyen et al., 2018). Therefore, these rice cultivars potentially useful genetic resource for rice breeding programs. They will accelerate breeding efforts for the development of BLB resistant rice cultivars through pyramiding approach using marker assisted selection.

Genotypes identified with the presence of only *Xa2* gene by SSR marker (MR297, MR307, MR303 and PS2) were classified as moderately susceptible, whereas MR219 was susceptible. Tetep as resistant check also demonstrated moderately resistant phenotype against MXO1410 *Xoo* isolates. According to Panwar et al. (2018), avirulent genes in bacteria exhibit the specificity for resistance gene in the rice plants. Some resistance gene expressed and effective at different growth stage. Some genes confer a broad-spectrum disease resistance against two or more types of pathogen while other on different races of *Xoo*. These were observed in this study where eventhough the rice cultivars consist of resistance gene *xa2*, most of them were rendered susceptible against *Xoo* race MXO1410 strain; similar to the case of Jumli Marsh rice (Acharya et al., 2018).



Molecular characterization showed that none of our germplasm carried *xa13* and *xa5* genes in our findings. Result was aligned with Yugander et al. (2018) by using 38 of modern cultivated Malaysian varieties. Singh et al. (2015) also stated that none of the 25 landraces studied had *xa13* genes. According to Lore et al. (2011), the resistance gene *xa13*, was originally identified from a land race BJI from the Indian subcontinent, and it is exhibiting a wide spectrum of resistance individually or as combination with other resistance genes against multiple *Xoo* isolates. Therefore, it should be noted that we could not find *xa13* gene in this study among rice accession.

We report here a new approach that is based on the widespread conventional selection with the use of information from molecular markers, facilitating breeding programs through better combination of cost, time, precision, and durability. This study has revealed useful preliminary information for selection of genotypes with durable resistance to the disease in order to prevent farm fields from epidemics. However, further studies including trials under field conditions should be undertaken.

## CONCLUSIONS

We identified the *xa4* gene in Mahsuri mutant rice genotypes, *xa21* in NMR152 and Tongkat Ali mutant rice genotypes and the *xa2* gene in all genotypes tested. Among these, three genotypes were found to have a combination of dominant (*xa21* and *xa4*) and recessive (*xa2*) gene alleles. Genotypes carrying two bacterial leaf blight resistance genes were found resistant under greenhouse conditions. These findings provide fundamental information for breeders for developing broad spectrum bacterial leaf blight resistance in rice by using a marker assisted approach.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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