Vietnam Journal of Agricultural Sciences

ISSN 2588-1299

VJAS 2018; 1(3): 240-248
https://doi.org/10.31817/vjas.2018.1.3.05

Evaluation of Local Black Glutinous Rice Germplasm of Vietnam for Resistance to Bacterial Leaf Blight Disease

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Abstract

Most rice growing areas frequently encounter the bacterial leaf blight, *Xanthomonas oryzae pv oryzae* (*Xoo*). To prevent the disease, development of resistant varieties is considered to be the most economical and environmentally safe solution. In this study, three PCR-based markers, Npb181, RM122, and P3, were used for the identification of the genes *Xa4, xa5, and Xa7*, respectively, from 56 local black glutinous rice accessions of Vietnam. Phenotypic screening of the accessions for resistance to 10 *Xoo* strains of North Vietnam, along with IRBB4, IRBB5, and IRBB7 as resistant controls and IR24 as a susceptible control were carried out in the 2016 Autumn season. 19 accessions containing the resistant genes were found, of these, 6 accessions carried *Xa4* gene, 6 accessions carried *xa5* gene, and 11 accessions carried *Xa7* gene. Three accessions carried two resistance genes, viz. Nep do (*Xa4* and *Xa7*), Pau cam (*xa5* and *Xa7*), and Pe lon cam (*Xa4* and *xa5*). Accessions with *xa5* and *Xa7* alone or with a combination of two genes (*Xa4* and *xa5*, *Xa4* and *Xa7*, or *xa5* and *Xa7*) were resistantto 8-9 *Xoo* strains (8-9R/0M/1-2S). Accessions containing *Xa4* showed resistance to 5-6 strains of *Xoo* (5-6R/0M/4-5S). *Xoo* strain No1 (HUA01043) showed the lowest virulence, infecting only 14 accessions (42R/4M/14S). Strains No3 (HUA 0020131-2), No4 (HUA202361), No5 (HUA20212), and No8 (HUA 020083) showed highest virulence, and they each infected more than 40 accessions with 19R/0M/41S, 20R/0M/40S, 16R/4M/40S, and 20R/0M/40S, respectively. These strains can even infect some accessions containing effective resistant genes (*Xa4* or *Xa7*).

Keywords

Vietnamese local black glutinous rice, bacterial leaf blight *(Xanthomonas oryzae* pv. *Oryzae)*, effective resistant genes (*Xa4*, *xa5*, *Xa7*).

Received: April 2, 2018
Accepted: September 11, 2018

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Introduction

Rice is an important staple food crop for many countries. Currently, most rice production areas in the world frequently encounter *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the cause of bacterial leaf blight (BLB). Bacterial leaf blight disease prevention can be achieved by several ways such as reduction or balance of nitrogen fertilizer application, use of resistant varieties, and chemical control. The use of host plant resistance is considered to be the most effective, economical, and environmentally safe option for the management of the disease (Khush *et al*., 1989). The diversity of *Xoo* was characterized across Asia, and the research revealed five distinct clusters composing of seven pathotypes. Pathotype 1 was widespread in Malaysia, the Philippines, and Korea. *Xoo* populations in Indonesia mainly belonged to pathotype 3, while pathotypes 4 and 6 were common in Nepal, India, and the Philippines (Adhikari *et al*., 1995). According to Ton and Thuy (2003), 10 *Xoo* strains were identified from the rice growing regions of Northern Vietnam. In a more recent study, about 40 genes conferring resistance against various strains of *Xoo* were identified in both cultivated rice and wild relatives of rice, including the genes derived from artificial mutation induction (Zhang *et al*., 2014). Several resistance genes have been used in breeding resistant cultivars, and many useful cultivars have been released (Chen *et al.*, 2002).

Black glutinous rice is an alternative source of bacterial leaf blight disease resistance. In order to develop rice varieties with strong and durable resistance to BLB disease, it is important to determine the components, distribution, and virulence of *Xoo* pathotypes, and effectively identify resistance genes to the virulent strains from different genetic resources. Therefore, identification of the resistant genes in the local black glutinous rice germplasm of Vietnam is necessary and will provide opportunities to further develop the BLB resistance breeding program. Seven BLB resistance loci *(Xa3, Xa4, xa5, Xa7, Xa10, Xa13,* and *Xa14)* originated from local rice germplasm of Vietnam. Three BLB resistance genes (*Xa4*, *xa5*, and *Xa7*) were effectively resistant to almost all of the 10 *Xoo* strains in Vietnam (Ton and Thuy, 2004). These genes are currently being widely used in many rice breeding programs in Vietnam.

In this study, three PCR-based markers, Npb181, RM122, and P3, were used for the identification of the genes *Xa4*, *xa5,* and *Xa7*, respectively, from 56 local black glutinous rice accessions of Vietnam.

Materials and Methods

Materials

The experimental materials were comprised of 56 local black glutinous rice varieties in Vietnam along with the resistant lines IRBB4 (*Xa4*), IRBB5 (*xa5*), and IRBB7 (*Xa7*), and the susceptible line IR24 (Table 1). Controls were received from the Center for Conservation and Development of Crop Genetic Resources - Vietnam National University of Agriculture. Single 20 day old seedlings of each genotype were transplanted to 2.0 x 3.0 m plots and were spaced 30 x 20 cm apart in the experimental field of the Center for Conservation and Development of Crop Genetic Resources - Vietnam National University of Agriculture during the 2016 Autumn season.

In this study, 10 strains of *Xoo* isolated from Northern Vietnam were used for pathogenicity testing. These were No1 (HUA 01043), No2 (HUA 0020131-1), No3 (HUA 0020131-2), No4 (HUA 020361), No5 (HUA 02012), No6 (HUA 010081), No7 (HUA 020020-2), No8 (HUA 020083), No9 (HUA 020020-1), and No10 (HUA 020131-3) (Ton *et al*., 2005).

Strain revival and pathogenicity test

The cultures of the 10 *Xoo* strains were obtained from the Department of Molecular Biology and Applied Biotechnology, Faculty of Biotechnology, Vietnam National University of Agriculture. The BLB strains were subcultured on peptone sucrose agar medium (1000 mL distilled water, 15 g sucrose, 300 g potatoes, 0.5 g $Ca(NO3)_2.H_2O$, 2.0 g $Na_2HPO_4.H_2O$, 5.0 g pepton, and 17 g agar) and maintained at pH 7.0 (Wakimoto, 1955).

A clipping method was used to inoculate the rice plants with the 10 *Xoo* strains. The test was conducted on fully developed leaves at 40 days after transplanting. The top 2.0-3.0 cm of completely developed leaves were clipped off one by one with sterilized scissors dipped in a bacterial suspension containing 10^8-10^9 cfu mL^{-1}. Following inoculation, the disease symptoms were recorded after 18 days and 10 infected leaves per plant in each accession were observed. The disease lesion lengths were measured with a ruler from the top of the leaf to the end covering the whole infected region of the leaf. All the accessions were classified as resistant (R: lesion length < 8 cm), moderately resistant (M: 8.1-11.9 cm), or susceptible (S: > 12 cm) using the disease index of Furuya *et al.* (2002).

DNA extraction

Young leaves were collected from the 56 transplanted local black glutinous rice accessions at 30 days old during the 2016 Autumn season. 0.5 g samples of leaves were cut into small pieces with sterilized scissors and placed in sterilized ceramic bowls. The isolation of the rice DNA genomes used the methods of Zheng and La (2003) briefly described as follows: Tissues were disrupted and homogenized in 400 µL of DNA extraction buffer (50 mM Tris HCl pH 8.0, 0.25 mM EDTA, 300 mM NaCl, SDS: 1%) by crushing until the solution turned blue, which indicated the breaking down of the rice cells. 400 µL of the solution was transferred into an Eppendorf tube and 700 µL of a chloroform: phenol: isoalcohol (25:24:1) solution was added. The tubes were centrifuged for 5 min, at 13000 rpm, 4ºC. Then, the upper solution was transferred into a new Eppendorf tube and 600 µL of a phenol: isoalcohol (24:1) solution was added and centrifuged at 13000 rpm, 4ºC. The top solution layer was removed to get the DNA precipitate below. The samples were washed with 70% ethanol and naturally dried by placing the test tubes on absorbent paper. The DNA precipitate was dissolved in 50 µL TE (Tris HCl: 10 mM, EDTA: 1 mM) and preserved at -20ºC. The DNA was spectrophotometrically quantified by measuring the samples at A260/280 nm and the DNA quality was checked by electrophoresis in 1% agarose gel.

Genotype analysis

Three PCR based markers, Npb181, RM122, and P3, were used to detect the BLB resistance genes. Their primer sequences were published by Yoshimura *et al*. (1992), Blair and McCouch (1997), and Taura *et al*. (2004), respectively.

The Npb181 marker was used to identify the *Xa4* gene with the primer pair:

5': ATCGATCGATCTTCACGAGG and 3': GTGCTATAAAAGGCATTCGGG.

The RM122 marker was used for identifying the *xa5* gene with the primer pair:

5': GAGCGATGTAATGTCATCAGTGC and 3': GGAAGGAGGTATCCGCTTTGTTGGAC.

The P3 marker was used to detect the resistance gene *Xa7* with the primer pair:

5': CAGCAATTCACTGGAGTAGTGGTT and 3': CATCACGGTCACCACCATATCGGA. Amplification was carried out in a reaction mixture of 20 µL containing: 1.0 µL DNA, 10 µL PCR master mix (2X), 1 µL forward primer, 1 µL reverse primer, sample, and 7.0 µL nuclease-free water.

The thermal cycling program for detection of the *Xa4* and *Xa7* genotypes was performed as follows: an initial denaturation at 94°C for 4 minutes, followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and primer extension at 72°C for 2 minutes, followed by a final extension at 72°C for 8 minutes. PCR detection of the *xa5* gene was performed with an initial denaturation at 94ºC for 4 minutes, 34 cycles: 94ºC for 1 minute, 55ºC for 1 minute, 72º for 1 minute 50 seconds, and a final step at 72ºC for 7 minutes. The amplified PCR products, alongside a 100 bp DNA marker ladder, were sized fractioned by electrophoresis in 2% agarose gel prepared in TAE buffer, visualized by staining with ethidium bromide (0.5 µg mL^{-1}), and photographed under UV light.

Results and Discussion

Genotypic screening for BLB resistance

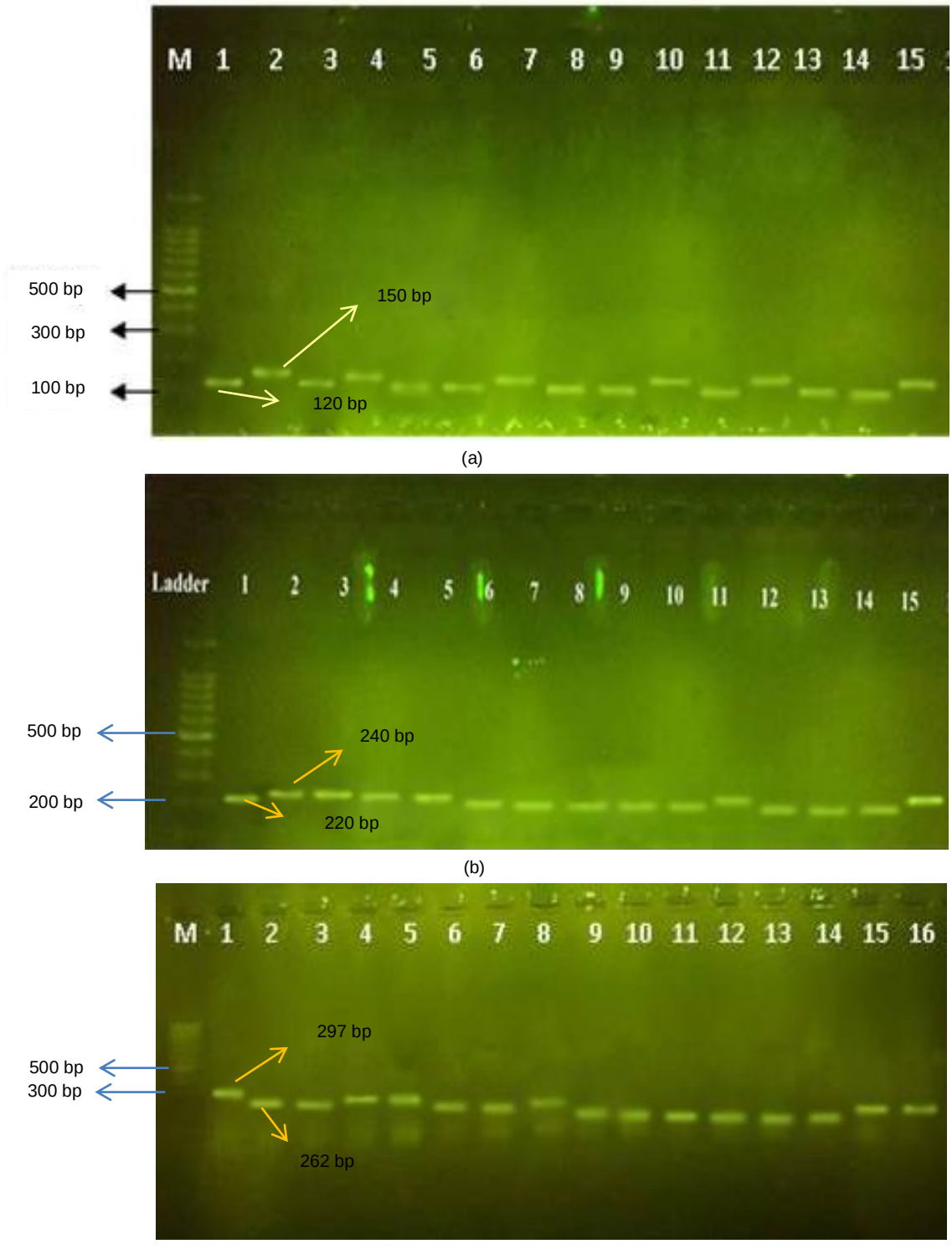
Fifty-six black glutinous rice accessions were screened for the presence/absence of three

effective resistance genes, *Xa4*, *xa5*, and *Xa7*, using the PCR-based markers Nbp181, RM122, and P3, respectively linked to these genes. Resistant (IRBB4, IRBB5, and IRBB7) and susceptible (IR24) controls were included as gene differential lines. The BLB resistance genes were determined by visualization of the amplicons near 150/120 bp, 240/220 bp, and 297/262 bp of positive fragments for *Xa4*, *xa5*, and *Xa7* presence/absence, respectively. Results of the genotypic screening are presented in Table 1, and the electrophoresis patterns of the PCR-based markers are shown in Figure 1. A total of 19 accessions were found containing resistance genes. For *Xa4* detection, six accessions, along with IRBB4, amplified the 150 bp fragment indicating the presence of *Xa4*, and 50 accessions, along with IR24, amplified the 120 bp fragment indicating the absence of the *Xa4* gene. Five accessions (Nep hac, Cam deo, Pe lon cam, Nep den, and Pau cam), along with IRBB5, amplified the 240 bp fragment, indicating the presence of *xa5* and 51 other accessions amplified the 220 bp fragment, along with IR24, for the absence of *xa5*. Eleven accessions amplified the 297 bp fragment, along with the IRBB7 line, indicating the presence of the *Xa7* gene, while 45 others, along with IR24, amplified the 262 bp fragment indicating the absence of the *Xa7* gene. Three accessions were found containing two genes, namely Nep do (*Xa4* and *Xa7*), Pau cam (*xa5* and *Xa4*), and Pe lon cam (*Xa7* and *xa5*).

Gene *xa5* acts as a recessive gene and is considered to be the strongest resistance gene to the BLB strains of the Northern region of Vietnam as it can resist 9 of the 10 existing strains (Furuya *et al*., 2012). The *Xa7* gene, on the other hand, is a dominant gene, which is also very strongly resistant to the bacterial pathotypes in Northern Vietnam. This gene is located on chromosome 6, according to Taura *et al*. (2004). The recessive *xa5* gene encoding the gamma subunit of transcription factor IIA is the only gene to be linked with resistance to strain 9 and furthermore confers broad-spectrum resistance to Philippine strains 1, 2, 3, 5, 7, 8, and 10 (Iyer and McCouch, 2004). According to Gu *et al*. (2009), *Xa7* is a dominantly inherited TAL effector R gene that recognizes TAL effector proteins and confers resistance to strain 1 (PXO61), strain 2 (PXO86), and strain 3 (PXO79) in India. According to Ton *et al*. (2003) and confirmed by Furuya *et al.* (2012), there are currently 10 strains of BLB in Northern Vietnam, and four genes (*Xa4*, *Xa5*, *Xa7*, and *Xa21*) are effectively resistant against almost all the strains found in North of Vietnam.

Phenotypic screening for BLB resistance

To evaluate resistance to bacterial leaf blight disease of rice, 56 black glutenous rice accessions along with resistant (IRBB4, IRBB5 and IRBB7) and susceptible (IR24) controls were screened against 10 *Xoo* strains under epiphytotic conditions during the 2016 Autumn season. The HUA 0020131-1 and HUA 0020131-2 *Xoo* strains are predominant in almost all the rice growing regions in Northern Vietnam and are the most aggressive and highly virulent strains. These two strains are mostly used to screen rice germplasm in Vietnam and give diverse responses with the host (Furuya *et al*., 2012). The results of the phenotypic screening are also presented in Table 1. Of the lesion lengths measured 18 days after being inoculated with the 10 *Xoo* strains, 18 accessions (Cam rau, Nep do, Cam lun, Em lua, Nep hac, Cam deo, Blau cam, Pe lon cam, P. Lenh do, Nep khau mau, Khau bai, Nu lung, Nep den, Pau cam, and Nep cam rau) plus IRBB5 and IRBB7 were resistant against 8-9 of the 10 *Xoo* strains isolated in Northern Vietnam (8-9R/0M/1S). Almost all of these accessions contained at least one or both of the resistance genes *xa5* and *Xa7*. Five accessions, Cam tim, Khau cam, Khau cam pung, Khau doc du, and IRBB4, contained only the *Xa4* gene and showed moderate resistance (6R/0M/4S). Thirty-three accessions were susceptible, including IR24, (0R/0M/10S) without any genes resistant to 8 or more of the *Xoo* strains, with the exceptions of Raymay do, Cam doi, Nat cam, Cam nuong, Ble hua, P khoa, No cam, and Nep cam nuong (4-5R/0M/5-6S). Similar results with a wide range of responses of the tested genotypes containing *Xa4*, *xa5*, and *Xa7* were reported by Ton and Thuy (2003).



Note: PCR based-markers: (a) Nbp181 for Xa4: Lane 2 is the resistant control IRBB4 amplified 150 bp DNA fragment containing Xa4. Lanes 4, 7, 10, 12, and 15 amplified similar sized DNA fragments containing the Xa4 gene; (b) RM122 for xa5: Lane 2 is the IRBB5 resistant control amplified 240 bp DNA fragment containing the xa5 gene; and (c) P3 for Xa7: Lane 1 is the 100 bp DNA marker, lane 2 is IRBB7, lanes 4, 5, 8, 15, and 16 are accessions amplifying the 297 bp fragment containing Xa7 gene. Other lanes of accessions amplified 262 bp fragments that do not contain the Xa7 gene. Other numbers are black glutenous rice accessions indicating the presence (+) or absence (-) of the genes Xa4, xa5, and Xa7 as described in Table 1.

Figure 1. Agarose gel electrophoretic pattern of some presentative accessions generated by using PCR based-markers

Table 1. Responses of the black glutinous rice accessions to 10 *Xoo* strains

No	Accessions	*Xoo strains*										*Ratio*	*Gene detection*		
		1	2	3	4	5	6	7	8	9	10	R/M/S	*Xa4*	*xa5*	*Xa7*
1	Cam vo do	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
2	Cam rau	R	R	R	S	S	R	R	R	R	R	8/0/2	-	-	+
3	Raymay do	S	S	S	S	S	S	R	S	R	R	4/0/6	-	-	-
4	Doan ket do	S	R	S	S	S	M	S	S	S	S	1/1/8	-	-	-
5	Nep cam vo trang	M	S	S	S	M	S	S	S	S	S	0/2/8	-	-	-
6	Cam doi	R	S	S	S	S	S	S	R	R	R	4/0/6	-	-	-
7	Nat Cam	R	S	R	S	S	S	R	S	R	R	5/0/5	-	-	-
8	Cam meo	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
9	Nep do	R	R	R	R	S	R	R	R	R	R	9/0/1	+	-	+
10	Nep nuong do	M	S	S	S	M	S	S	S	S	S	0/2/8	-	-	-
11	Y den cam	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
12	Cam tim	S	R	S	S	R	R	R	S	R	R	6/0/4	+	-	-
13	Cam nuong	R	S	S	S	S	S	S	R	R	R	4/0/6	-	-	-
14	Cam trang	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
15	Cam mong	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
16	Cam lun	R	R	R	S	S	R	R	R	R	R	8/0/2	-	-	+
17	Em lua	R	R	R	S	S	R	R	R	R	R	8/0/2	-	-	+
18	Nep tim	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
19	Nep hac	R	R	R	R	S	R	R	R	R	R	9/0/1	-	+	-
20	Nep hac hat tron	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
21	Ble choi	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
22	Ble hua	R	S	S	S	S	R	S	S	R	R	4/0/6	-	-	-
23	P.kho a	R	S	S	S	S	R	S	S	R	R	4/0/6	-	-	-
24	Nep rong	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
25	Khau cam	S	R	S	S	R	R	R	S	R	R	6/0/4	+	-	-
26	Khau cam pi	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
27	Cam roc	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
28	Cam deo	R	R	R	R	S	R	R	R	R	R	9/0/1	-	+	-
29	Cam thai	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
30	Cam hat to	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
31	Khau cam pung	S	R	S	S	R	R	R	S	R	R	6/0/4	+	-	-
32	Blau cam	R	R	R	S	S	R	R	R	R	R	8/0/2	-	-	+
33	Pe lon cam	R	R	R	R	S	R	R	R	R	R	9/0/1	-	+	+

No	Accessions	*Xoo strains*										*Ratio* R/M/S	*Gene detection*		
		1	2	3	4	5	6	7	8	9	10		*Xa4*	*xa5*	*Xa7*
34	P lenh cam	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
35	Khau cang	R	R	R	R	S	R	R	R	R	R	9/0/1	-	-	+
36	Nep chan den	S	R	S	S	S	M	S	S	S	S	1/1/8	-	-	-
37	Lo to keng	S	R	S	S	S	M	S	S	S	S	1/1/8	-	-	-
38	Koi san	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
39	Khau lech	R	R	R	R	S	R	R	R	R	R	9/0/1	-	-	+
40	Cam vang	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
41	Dau mung	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
42	Khau pai	R	R	R	R	S	R	R	R	R	R	9/0/1	-	-	+
43	Nu lung	R	R	R	R	S	R	R	R	R	R	9/0/1	-	-	+
44	Nep den	R	R	R	R	S	R	R	R	R	R	9/0/1	-	+	-
45	Khau nua deng	M	S	S	S	M	S	S	S	S	S	0/2/8	-	-	-
46	Khau nay deng	R	S	S	R	S	M	M	S	R	S	3/2/5	-	-	-
47	Khau nua khao	R	S	S	R	S	M	M	S	M	S	2/3/5	-	-	-
48	Khau nua dam	M	S	S	S	M	S	S	S	S	S	0/2/8	-	-	-
49	Pau cam	R	R	R	R	S	R	R	R	R	R	9/0/1	+	+	-
50	Cam den lun	R	S	S	S	R	S	M	S	M	S	2/2/6	-	-	-
51	Nep cam moc	S	R	S	S	S	M	S	S	S	S	1/1/8	-	-	-
52	Nep hai	S	R	S	S	S	M	S	S	S	S	1/1/8	-	-	-
53	Nep cam rau	R	R	R	S	S	R	R	R	R	R	8/0/2	-	-	+
54	Lo cam	S	S	R	S	S	S	S	R	R	R	4/0/6	-	-	-
55	Nep cam nuong	S	R	S	S	S	S	R	S	R	R	4/0/6	-	-	-
56	Khau doc du	S	R	S	S	R	R	R	S	R	R	6/0/4	+	-	-
C	IRBB4	S	R	S	S	R	R	R	S	R	R	6/0/4	+	-	-
	IRBB5	R	R	R	R	S	R	R	R	R	R	9/0/1	-	+	-
	IRBB7	R	R	R	S	S	R	R	R	R	R	8/0/2	-	-	+
	IR24	S	S	S	S	S	S	S	S	S	S	0/0/10			
	Ratio (R/M/S)	42/4/14	28/0/32	19/0/41	20/0/40	16/4/40	24/14/22	25/20/15	20/0/40	38/12/10	30/0/30				

Note: C: control; "+" gene detected; "-" no gene detected; R: resistant; M: moderate; S: susceptible.

The virulence of the 10 *Xoo* strains were determined through the R/M/S ratio of each strain infecting the 56 black glutinous rice accessions and 4 isogenic lines as controls for resistance, moderate resistance, and susceptibility. The results are shown in Table 3. Among these *Xoo* strains, No1 showed the lowest virulence with a 42R/4M/14S ratio. The No3, No4, No5, and No8 strains showed the strongest virulence with 19R/0M/41S, 20R/0M/40S, 16R/4M/40S, and 20R/0M/40S ratios, respectively. These strains infected some of the accessions containing the resistance genes *Xa4* or *Xa7*. Strains No2 and No3 were reported by Phan Huu Ton and Bui Trong Thuy (2004) as predominant in almost all the rice growing regions of Northern Vietnam. The No3 strain showed the strongest virulence in this study. These results indicated that the different strains of *Xoo* have different levels of aggressiveness. *Xa4*, *xa5*, and *Xa7* are strong and effective resistance genes against *Xoo* strains of Vietnam and should be introduced to popular varieties for host plant resistance improvement. Though pyramiding of these known loci is also another promising approach for disease management, novel sources of resistance will be required to keep the upper hand in the continuous plant-pathogen arms race. In our previous study, some local Vietnamese rice landraces were detected for resistant genes of bacterial leaf blight (Thanh *et al.,* 2018). Thus, the search for new genes is crucial to reinforce host resistance breeding. Rice has a wide range of genetic diversity and global germplasm collections, including local Vietnamese black glutinous rice of Vietnam, serve as rich sources for all three effective resistance genes *Xa4*, *xa5*, and *Xa7*.

Conclusions

By using PCR-based markers, 19 accessions containing resistant genes were found. Of these, 6 accessions carried the *Xa4* gene, 6 accessions carried the *xa5* gene, and 11 accessions were identified with the *Xa7* gene out of the 56 local black glutinous rice germplasms of Vietnam tested. Three accessions were found containing two resistance genes, namely Nep do (*Xa4* and *Xa7*), Pau cam (*xa5* and *Xa7*), and Pe lon cam (*Xa4* and *xa5*). Different resistant genes showed resistance against particular bacterial leaf blight strains. Accessions with *xa5* and *Xa7* alone or with a combination of two genes (*Xa4* and *xa5*, *Xa4* and *Xa7*, and *xa5* and *Xa7*) could resist 8-9 *Xoo* strains (8-9R/0M/1-2S). Accessions containing *Xa4* showed resistance to 5-6 strains of *Xoo* (5-6R/0M/4-5S). *Xoo* strain No1 (HUA01043) showed the lowest virulence, infecting only 14 accessions (42R/4M/14S). Strains No3 (HUA 0020131-2), No4 (HUA 020361), No5 (HUA 02012), and No8 (HUA 020083) showed the highest virulence, and infected more than 40 accessions with 19R/0M/41S, 20R/0M/40S, 16R/4M/40S, and 20R/0M/40S ratios, respectively. These strains even infected several accessions containing effective resistant genes (*Xa4* or *Xa7*).

Acknowledgements

We thank the Faculty of Biotechnology, Vietnam National University of Agriculture for providing materials for this study.

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