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Pathotyping and Virulence Analysis of *Xanthomonas oryzae* pv. *oryzae* Causing Bacterial Blight of Rice in Tamil Nadu

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ABSTRACT

Background: Bacterial leaf blight (BB) caused by *Xanthomonas oryzae pv. oryzae (Xoo*) is one of the most destructive diseases affecting rice production worldwide. Resistance breeding is considered a durable, effective and eco-friendly approach to control BB. Several BB resistance (R) genes have been identified that confer resistance against various strains of *Xoo.* The dynamic nature of this pathogen needs proper knowledge of pathotype composition and its virulence among the diverse *Xoo* strains which is imperative for designing a resistance breeding program.

Methods: In this study, we conducted an extensive survey to collect BB infected samples from diverse rice-growing regions of Tamil Nadu. Further, the samples were identified as *Xoo* through morphological and molecular identification methods. The pathotyping and virulence profiling of *Xoo* isolates was carried out on a set of rice differentials consisting of 22 near-isogenic lines (NILs) of IR24 possessing different BB resistance genes under glasshouse conditions during *Kharif* 2021-2022.

Result: Based on the disease reaction in 22 rice BB differentials (IRBB lines), the *Xoo* isolates were grouped into six pathotypes (I II, III, IV, V and VI). Further, the virulence analysis revealed that the near-isogenic lines possessing single R gene and gene pyramids were susceptible to about 20-88% and 4-20% respectively to *Xoo* isolates. R genes and their pyramids such as (*Xa7, Xa21, Xa4+xa5+Xa21, Xa4+xa5+xa13, Xa4+xa13+Xa21, xa5+xa13+Xa21 and Xa4+Xa21+xa5+Xa21*) were found to be effective against all the pathotypes in Tamil Nadu. The pathotype III was predominantly distributed (40%) over the Tamil Nadu. The isolates found in the pathotypes I and II are more virulent and in the pathotypes V, VI are less virulent. The current study identified the virulence nature of diverse *Xoo* isolates and the effectiveness of resistance (R) genes against the *Xoo* isolates. Therefore, the effective R genes identified in this study could be an effective source for resistance breeding program against BB in Tamil Nadu.

Key words: Differentials, Pathogenicity, Resistance breeding, *Xa genes*.

INTRODUCTION

Bacterial blight (BB), caused by *Xanthomonas oryzae pv. oryzae (Xoo)*, is one of the most widely distributed and most devastating rice disease in Asia. It occurs in both temperate and tropical regions, but outbreaks are found to be more frequent in irrigated and rain fed lowland areas (Gautam *et al*., 2015). Reports suggested that the disease led to 20-80% yield losses depending on the plant growth stage at the time of infection, weather conditions, varieties and extent of nitrogenous fertilizers used (Kim *et al*., 2016). BB can be managed by the application of synthetic chemicals, but due to their residual effects their application is not advisable (Liu *et al*., 2007). The use of biocontrol agents has gained importance but their inconsistent field efficiency and commercialization have resulted in their slow adoption among farmers (Ahmed *et al*., 2020). Therefore, the deployment of resistant varieties emerges as an economically viable, environmentally safe, durable and promising strategy to manage BLB (Pinta *et al*., 2013). The resistance confered by single R genes are lost quickly due to the outbreak of *Xoo* races (Biswas *et al*., 2021). Pyramiding different types of resistant genes is more likely to achieve durable resistance against diverse *Xoo* races (Pradhan *et al*., 2022). Near isogenic rice lines (NIL's) with single resistance genes have provided unique tools for the identification of races/pathotypes and studying the mechanism of their resistance (Ogawa *et al*., 1991). Several successful attempts were made to document the pathotypic variation among the *Xoo* in the major rice growing countries of Asia and about 30 races have been reported in several countries (Liu *et al*., 2007; Mannan *et al*., 2009; Tekete *et al*., 2020). In India, several studies have been conducted to assess the pathotype of *Xoo* based on the interaction of rice cultivars with different resistance genes (Mishra *et al*., 2013; Amin *et al*., 2023). However, the study focussed on

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the *Xoo* population structure and its virulence in Tamil Nadu is limited. In this context, the present investigation focussed to analyse the pathotypic distribution and evaluate the pathogenicity patterns of various *Xoo* isolates from diverse rice growing regions of Tamil Nadu.

MATERIALS AND METHODS

Collection of *Xoo* infected samples

The plants showing typical bacterial blight symptoms were collected during 2021-2022 from various rice-growing regions of Tamil Nadu. The roving survey was made to collect the symptomatic leaves from the field and the samples were packed in sterile paper envelopes. The samples brought to laboratory and stored at 4°C until further processing.

Isolation and maintenance

Under gnotobiotic conditions, the diseased samples were cleaned with tap water and cut into small pieces about 5-7 cm and surface sterilized with 70% alcohol for 30 s followed by washing twice with sterilized double distilled water. These pieces were chopped finely and transferred into the sterile 1.5 mL microfuge tubes containing 1 ml of sterilized distilled water and allowed for about 5-10 min for the bacterial ooze to come out from the leaf tissues. Using the sterilized loop, the bacterial suspension was streaked onto Petri dishes containing peptone sucrose agar and allowed it for incubation at 28±2°C for 3 days. After that, pinhead-sized yellow colonies were picked up and purified further. The pure cultures of *Xoo* isolates were maintained on PSA slants at 4 °C for short term storage and in 15% glycerol at -70°C for long-term storage (Mondal *et al*., 2014). Further, the isolates were molecularly characterized by using *Xoo*-specific primers (Lang *et al*., 2010).

Determination of pathotypes and virulence analysis of *Xoo* isolates

Plant materials

Seeds of rice differentials (IRBB lines) and checks *viz*., TN1 (susceptible check) and improved Samba Mahsuri (resistant check) were obtained from the All India Coordinated Rice Improvement Programme (AICRIP), Department of Rice, Tamil Nadu Agricultural University, India. The seeds were sown in earthen pots (13×10×6 cm) with three replicates (3 plants/ pots) maintained for each differential lines. All pots were filled up with a mixture of soil and farm yard manure (3:1). The fertilizer application was carried out for each pots as per the recommended dosage and the plants were irrigated regularly. The entire experiment was conducted under glasshouse conditions at Paddy Breeding Station, Department of Rice, Tamil Nadu Agricultural University, Coimbatore during *Kharif* 2021-2022.

Artificial inoculation

A total of 25 *Xoo* isolates were taken for pathotyping and virulence profiling assay. The inoculum of each isolate was prepared by resuspending the 3-day old fresh culture with 10 ml distilled water and adjusting the concentration of inoculum to OD_{600} =1 (3.3×10^8 CFU/ml) (Rashid *et al*., 2021). The plants (40-45 days old) were artificially inoculated at the maximum tillering stage with individual *Xoo* isolates following a leaf clipping method (Kauffman *et al*., 1973). For each differential, 5-7 fully expanded leaves were clip-inoculated with sterilized scissors dipped in a bacterial suspension and the optimum temperature of 28-30°C with high relative humidity (~90%) was maintained for proper development of disease symptoms.

Data assessment

The disease reaction on differentials was recorded at 14 days post-inoculation (DPI) by measuring the lesion length. Three measurements were taken from three plants maintained for each isolate in all differentials along with the checks and then, the average lesion length was calculated. The severity of disease was evaluated as per the standard evaluation system (SES) scale of IRRI, 2013. Pathotype grouping and virulence analysis were done based on the disease reaction pattern on the differentials. The average lesion lengths were then converted into different reaction categories *viz*., R (up to 5 cm), MR (5-10 cm), MS (10-15 cm) and S (>15 cm). The isolates were then categorized into different pathotypes according to Lore *et al*., (2011).

RESULTS AND DISCUSSION

Host plant resistance is the most effective, durable and eco-friendly sustainable approach to combat bacterial blight disease in rice (Gautam *et al*., 2015). However, the development of resistant cultivars requires a clear knowledge about the population structure and virulence distribution of the target pathogen. Several attempts made by the researchers to study the virulence diversity of *Xoo* in India (Mondal *et al*., 2014; Yugander *et al*., 2017; Amin *et al*., 2023).

In the present study, we conducted a field survey in different rice ecosystems of Tamil Nadu and collected 40 infected samples showing the typical BB symptoms. Further, we isolated the *Xoo* pathogen and identified its morphological characteristics as described by Amin *et al*. (2023). Out of 40 samples, only 25 samples showed smooth, yellow, mucoid and round individual colonies that were selected and sub-cultured on PSA medium. The isolates were maintained as a pure culture through the single colony method. The molecular characterization was also done by using a set of two *Xoo* specific primers, it showed the expected amplicon of 331 bp and 162 bp according to Lang *et al*. (2010) in all the 25 isolates and confirmed their identity as *Xoo* (data not shown).

The Near-isogenic rice lines possessing different major genes for resistance to *Xoo* were developed by IRRI and were used for the identification of *Xoo* races. Variability of pathogens needs to be unveiled to find the effective resistant gene (s) and to develop race-specific resistant variety (Adhikari *et al*., 1999). In the present investigation, we conducted the pathotypic analysis of 25 *Xoo* isolates in 22

near-isogenic lines were grouped into six pathotypes/races and designated as I, II, III, IV, V and VI. We chronologically arranged the pathotypes based on their virulence in differentials as shown in Table S1. Similarly, the 22 pathotypes were detected using nine NILs lines in India (Yugander *et al*., 2017); 21 pathotypes were found in Malaysia (Koogethavani *et al*., 2021); 12 pathotypes were identified based on their virulence patterns on the NILs in Bangladesh (Rashid *et al*., 2021) and 7 pathotypes were identified among the Jammu districts in India (Amin *et al*., 2023). Among the pathotypes, pathotype III was identified as most prevalent in the major rice growing regions of Tamil Nadu and the distribution of each pathotype are given in Fig 1. Similarly, the report of Mishra *et al*., (2013) revealed that, the pathotype III was the most frequent pathotype distributed all over of India and accounted for 40.7% of the isolates. Likewise, three pathotypes namely; II, V and VII, were considered as major pathotypes distributed widely in the Jammu district (Amin *et al*., 2023). Our results are line with the results of Ochiai *et al*. (2000), they found pathotype I showed compatibility to all the major resistance genes in Sri Lanka. We also identified, the pathotype I was virulent to most of the single gene NILs except IRBB 7 and IRBB 21, the isolate found in pathotype was collected from the Coimbatore district of Tamil Nadu and the isolates found in the pathotype VI showed incompatible reaction (Resistant) on all the differentials, it could be noted as less virulent. The other pathotypes such as II, III, IV and V were scattered in Thanjavur, Villupuram, Erode, Thoothukkudi, Thirunelveli and Ramanathapuram districts of Tamil Nadu.

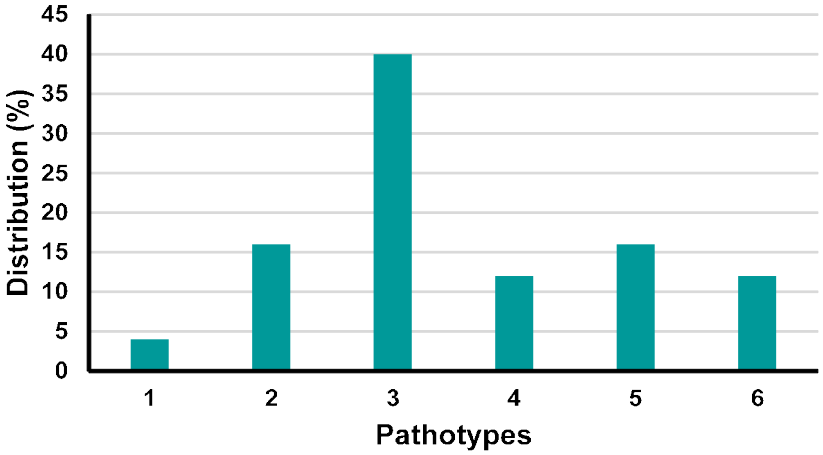
The variability in virulence of *Xoo* pathotypes has been well documented in many rice-growing regions of India (Mishra *et al*. 2013; Yugander *et al*., 2017). In this study, we conducted the virulence analysis of *Xoo* isolates in rice differentials (IRBB lines). From that, we found all the 25 *Xoo* isolates produced characteristics BB disease symptoms on susceptible check TN1. The isolate *Xoo*12 exhibited compatible (susceptible) reaction in the nine single gene and six two gene pyramids such as IRBB 1, IRBB3, IRBB 4, IRBB5, IRBB8, IRBB 10, IRBB 11, IRBB 13, IRBB 14, IRBB 50, IRBB 51, IRBB 52, IRBB 53, IRBB 54 and IRBB 55. None of the isolates showed the compatible reaction in the effective single gene NILs *viz*., IRBB 5 (*xa5*) and IRBB 13 (*xa13*) except *Xoo*12 and it was identified as highly virulent compared to other isolates. Meanwhile, the isolates such as *Xoo*10, *Xoo*11 and *Xoo* 15 from Dindigul, Salem and

Table S1: Pathotyping of *Xanthomonas oryzae* pv. *oryzae* isolates on rice differentials.

Host differentials	Gene combinations	*Xanthomonas oryzae* pv. *oryzae* pathotypes					
		I	II	III	IV	V	VI
IRBB 1	*Xa1*	S	S	S	S	S	R
IRBB 3	*Xa3*	S	S	S	S	S	R
IRBB 4	*Xa4*	S	S	S	S	MR	R
IRBB 5	*xa5*	S	S	MR	MR	MR	R
IRBB 7	*Xa7*	MR	MR	MR	MR	MR	R
IRBB 8	*xa8*	S	MR	S	MR	MR	R
IRBB 10	*Xa10*	S	S	S	S	S	R
IRBB 11	*Xa11*	S	S	S	S	S	R
IRBB 13	*xa13*	S	S	MR	MR	MR	R
IRBB 14	*Xa14*	S	S	S	S	S	R
IRBB 21	*Xa21*	MR	MR	MR	MR	MR	R
IRBB 50	*Xa4+xa5*	S	S	MR	MR	R	R
IRBB 51	*Xa4+xa13*	S	S	MR	MR	R	R
IRBB 52	*Xa4+Xa21*	MS	MR	MR	R	R	R
IRBB 53	*xa5+xa13*	S	S	MR	R	R	R
IRBB 54	*xa5+Xa21*	MS	MR	MR	R	R	R
IRBB 55	*xa5+Xa21*	MS	MR	R	R	R	R
IRBB 56	*Xa4+xa5+xa13*	R	R	R	R	R	R
IRBB 57	*Xa4+xa5+Xa21*	R	R	R	R	R	R
IRBB 58	*Xa4+xa13+Xa21*	R	R	R	R	R	R
IRBB 59	*xa5+xa13+Xa21*	R	R	R	R	R	R
IRBB 60	*Xa4+xa5+xa13+Xa21*	R	R	R	R	R	R
ISM (Improved samba mahsuri)	Resistant check	R	R	R	R	R	R
TN1	Susceptible check	S	S	S	S	S	MR

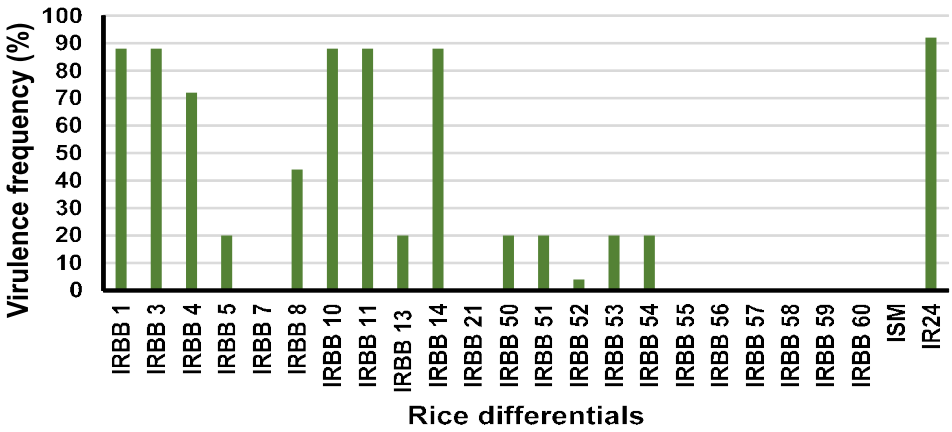
*Based on mean lesion lengths, the nature of disease responses was classified into susceptible (S) (above 15 cm), moderately resistant (MR) (between 5-10 cm), moderately susceptible (MS) (between 10-15) and resistant (R) (up to 5 cm).

Theni showed incompatible reaction (resistant) to all the differentials. Therefore, these isolates were noted as less virulent (Table S2). This type of variation in virulence profile among the *Xoo* isolates from the same region has been reported earlier (Adhikari *et al*. 1999), who suggested that even the cultivar differences in a region exert strong selection pressure on the *Xoo* population resulting in variation in the virulence profile. In addition, we analysed the frequency of virulence and effectiveness of R genes in the differentials against *Xoo* isolates (Fig 2; Fig 3). We found that, the differentials with single gene were susceptible to 20-88 per cent of the isolates, except IRBB 7 and IRBB 21. On the other hand, NILs with the gene pyramids of two gene combinations were susceptible to only 4-20 % of the isolates and the NILs with three and four gene combinations exhibits 100 per cent resistance against all the *Xoo* isolates. To date, all the resistant *Xa* genes provides resistance against BB have been listed together with their source and country of origin (Khan *et al*., 2014). Mishra *et al*. (2013) suggested that the *Xa21* was found to be most effective gene towards the Indian *Xoo* strains followed by *xa13* and *xa8,* these genes appear to be good candidates to be deployed in Indian rice cultivars. Similarly, we also identified the resistance gene *Xa21*, was the most effective against all the *Xoo* isolates,



Six different pathotypes, designated as I to VI, were identified in this study. The x-axis indicates pathotype and the y-axis indicates % frequency amongst the 25 *Xoo* isolates that were collected and analysed.

Fig 1: Frequency of *X. oryzae* pv. *oryzae* pathotypes in Tamil Nadu.



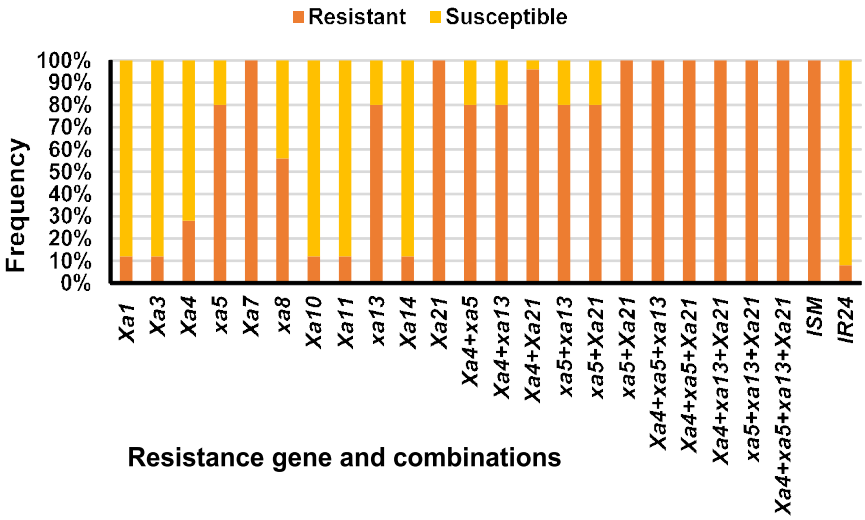
The *Xoo* isolates were inoculated at maximum tillering in differentials. After 14th day of inoculation virulence was measured based on diseases severity. The x-axis indicates BB differentials (IRBB lines) and the y-axis indicates virulence frequency (%) of 25 *Xoo* isolates collected from different districts of Tamil Nadu. Lesion lengths <5 cm is considered as resistant, 5 to 10 cm are considered as moderately resistant, 10 to 15 cm are considered as moderately susceptible and >15 cm is considered as susceptible.

Fig 2: Virulence frequency of *X. oryzae* pv. *oryzae* isolates collected from Tamil Nadu.

Table S2: Virulence profiling of *Xanthomonas oryzae pv. oryzae (Xoo)* isolates on rice differentials under glasshouse conditions during *kharif* 2021–2022.

District of isolates	Isolates	Rice differentials lines (IRBB)																								
		IRBB 1	IRBB 3	IRBB 4	IRBB 5	IRBB 7	IRBB 8	IRBB 10	IRBB 11	IRBB 13	IRBB 14	IRBB 21	IRBB 50	IRBB 51	IRBB 52	IRBB 53	IRBB 54	IRBB 55	IRBB 56	IRBB 57	IRBB 58	IRBB 59	IRBB 60	ISM	TN1
Thoothukudi	*Xoo* 1	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
	Xoo 2	S	S	S	MR	MR	MR	S	S	MR	S	MR	MR	MR	R	R	R	R	R	R	R	R	R	R	S
	Xoo 3	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
Thirunelveli	*Xoo* 4	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
	Xoo 5	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
	Xoo 6	S	S	MR	MR	MR	MR	S	S	MR	S	MR	R	R	R	R	R	R	R	R	R	R	R	R	S
Madurai	*Xoo* 7	S	S	S	MR	MR	MR	S	S	MR	S	MR	MR	MR	R	R	R	R	R	R	R	R	R	R	S
Ramanathapuram	*Xoo* 8	S	S	S	S	MR	MR	S	S	S	S	MR	S	S	MR	S	MR	MR	R	R	R	R	R	R	S
	Xoo 9	S	S	MR	MR	MR	MR	S	S	MR	S	MR	R	R	R	R	R	R	R	R	R	R	R	R	S
Madurai	*Xoo* 10	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	MR
Theni	*Xoo* 11	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	MR
Coimbatore	*Xoo* 12	S	S	S	S	MR	S	S	S	S	S	MR	S	S	MS	S	MS	MS	R	R	R	R	R	R	S
Erode	*Xoo* 13	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
	Xoo 14	S	S	S	S	MR	MR	S	S	S	S	MR	S	S	MR	S	MR	MR	R	R	R	R	R	R	S
Salem	*Xoo* 15	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	MR
Nilgiris	*Xoo* 16	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
Pudukkottai	*Xoo* 17	S	S	MR	MR	MR	MR	S	S	MR	S	MR	R	R	R	R	R	R	R	R	R	R	R	R	S
Thiruchirapalli	*Xoo* 18	S	S	MR	MR	MR	MR	S	S	MR	S	MR	R	R	R	R	R	R	R	R	R	R	R	R	S
Thanjavur	*Xoo* 19	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
	Xoo 20	S	S	S	S	MR	MR	S	S	S	S	MR	S	S	MR	S	MR	MR	R	R	R	R	R	R	S
	Xoo 21	S	S	S	MR	MR	MR	S	S	MR	S	MR	MR	MR	R	R	R	R	R	R	R	R	R	R	S
Villupuram	*Xoo* 22	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
	Xoo 23	S	S	S	S	MR	MR	S	S	S	S	MR	S	S	MR	S	MR	MR	R	R	R	R	R	R	S
	Xoo 24	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S
Thiruvarur	*Xoo* 25	S	S	S	MR	MR	S	S	S	MR	S	MR	MR	MR	MR	MR	MR	R	R	R	R	R	R	R	S

*Based on mean lesion lengths, the nature of disease responses for *Xoo* isolates was classified into susceptible (S) (above 15 cm), moderately resistant (MR) (between 5-10 cm), moderately susceptible (MS) (between 10-15) and resistant (R) (up to 5 cm).



The x-axis indicates resistance gene and combinations and the y-axis indicates the frequency of isolates against which this gene confers resistance. Inoculation of rice leaves with isolates was done as described in Methods and lesion lengths were measured 14 days after inoculation.

Fig 3: Effectiveness of resistance (R) genes against *X. oryzae* pv. *oryzae* isolates.

with 100 % resistance efficiency, followed by *Xa5* and *xa13* genes (80%). In addition to these effective single R gene, the gene combinations such as *Xa4+xa13+Xa21, xa5+xa13+Xa21, Xa4+xa5+Xa21, Xa4+xa5+xa13* and *Xa4+Xa21+xa5+Xa2)* offered complete resistance against all the *Xoo* isolates of Tamil Nadu. Therefore, the effective R gene and its combinations identified in this study can be used in a resistant breeding programme to develop a bacterial blight resistant cultivars in rice breeding programs.

CONCLUSION

From this study, the *Xoo* population in different rice-growing regions of Tamil Nadu are highly diverse in nature and the stringent evaluation of different BB resistance genes is imperative before the initiation of resistance gene-pyramiding programs. Our results revealed that the most of single BB resistance genes became susceptible to all the *Xoo* isolates. But the lines with gene combinations such as *Xa4*, *xa5*, *xa13* and *Xa21* can provide broad-spectrum and durable resistance to BB. Moreover, this finding will be useful for better understanding the characteristics of *Xoo* isolates in Tamil Nadu for developing the BB-resistant variety and helpful to develop an efficient and sustainable management strategy to control bacterial blight disease of rice.

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Conflict of interest: None.

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