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Genotypic and phenotypic dissection of *Xanthomonas oryzae pv. oryzae* on Indica rice cultivars for bacterial blight resistance

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Abstract

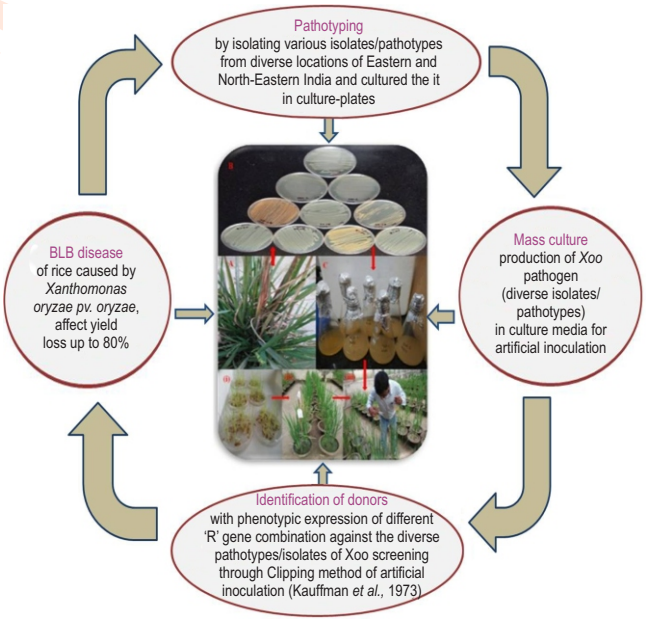
Aim: To evaluate the level of virulence of different *Xoo* isolates/ pathotypes of Eastern and North-eastern India and to identify the suitable donors in rice cultivars having various R-gene combination against virulent *Xoo* races of Bacterial Blight disease of rice.

Methodology: Thirty six *Xoo* isolates were collected from different places of Eastern and North-eastern India and genetic diversity/ similarity was examined by genotyping of pathotypes using JEL1/JEL2 markers. The 34 Indica rice cultivars carrying different R-gene combination were selected and grown in net house and inoculated artificially with *Xoo* inoculants from these races/ isolates bacterial of blight disease.

Results: The selected 36 *Xoo* isolates of Eastern and North-eastern India were grouped into seven different isolates/ races based on their genetic diversity using JEL1/JEL2 markers. Among 34 *Indica* rice cultivars, three or more R-gene combination (*xa5 + xa13 + Xa21 and/or Xa4 + xa5 + xa13 + Xa21*) cultivars exhibited highly resistant as compared to cultivars with single and double gene combination cultivars against most of the *Xoo* isolates/ races.

Interpretation: The cultivars may determine different level of resistance due to complementary effect of inheritance of suitable R-gene combination. Identified donors may be used for rice resistance breeding programme for Eastern and North-eastern India.

Key words: Bacterial blight, Genetic diversity, Indica rice, Pathotyping, Resistant gene



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Introduction

Rice belongs to genus *Oryza*, of the tribe Oryzeae, and the family Poaceae or Gramineae is an important staple food crop and provide calories as a primary source for more than half of the world's population. Rice production is considerably affected by various biotic factors such fungal, bacterial and viral diseases as well as abiotic factors, *i.e*., as submergence, drought, salinity, heat, cold etc. Bacterial blight disease of rice is one of the most devastated diseases among all biotic stresses in Asian countries (Wang *et al.,* 1996). It is caused by Gram-negative rod shaped bacterium *Xanthomonas oryzae pv. oryzae* (*Xoo*) which affects the yield typically ranging from 20-50% (Khush *et al.,* 1989), but in severe cases, it can cause 80% yield reduction (Singh *et al.,* 1977; Singh *et al.,* 2014). Bacterial blight disease was first reported by Japanese farmers in 1884 Fukuoka, in Japan (Ou, 1985; Singh *et al*., 2020). In order to feed exponentially growing population throughout the globe, farmers are cultivating high yield varieties for cultivation. These high yielding varieties require higher amount of nitrogenous fertilizers.

Excessive use of nitrogenous fertilizers, especially in inorganic forms to maintain the yield of the high yielding varieties is one of the main cause of bacterial blight disease severity (Onasanya *et al.,* 2009). During seedling stage, the disease is termed as '*Kresek*' which leads to wilting as well as death of seedlings. If the symptom appears during late tillering stage or at reproductive stage, then it is termed as 'Blight'. It leads to more number of sterile and immature grains. Chemical control of bacterial blight disease in rice is not much effective. Transfer of naturally occurring bacterial blight resistance (R) genes in rice plant is the most effective way of disease control; however, several high yielding rice varieties lack these R- genes. The varietal improvement using R-gene is effective, economical and eco-friendly control strategy for the managing of this disease (Yamamoto *et al.,* 1977; Singh *et al.*, 2020). To date, more than 44 R-genes including some major and some minor which conferring host resistance to various strains/races of *Xoo* have been identified in different wild rice and landraces and some have been characterized (Bhasin *et al.,* 2012; Natrajkumar *et al.,* 2012; Suh *et al.,* 2013; Kim *et al.,* 2015; Kim *et al.*, 2019; Singh *et al.*, 2020). Transferring these R-genes using conventional breeding technique is not only a time taking process as well as confirmation of transferred genes in progeny plants may also not be accurate.

Sometimes seasonal dependence for some specific traits is also a barrier. Pyramiding these R-genes in susceptible high yielding rice varieties via marker assisted backcross breeding (MABB) provides a durable and broad-spectrum Bacterial Blight resistance. In India, particularly, Eastern and North-Eastern regions, the household food and nutritional security predominantly depend on rice. However, its production in these areas is drastically reduced by Bacterial Blight disease incidence which is severe due to high humidity and low light intensity. Although a lot of research work has been done on pathotyping and genetic diversity of *Xoo* races (Shanti *et al.,* 2001; Mondal *et al.,* 2014; Bharath kumar *et al.,* 2014), the effect of different R-gene combination were assessed on different races/ strains of *Xoo* (Khan *et al.,* 2012; Singh *et al.,* 2014; Singh *et al.,* 2018; Kumar *et al.,* 2019). For improving of locally adapted high yielding but Bacterial Blight disease susceptible cultivars, selection of donor parents with effective R-gene combination against various Xoo races of eastern and north eastern India are mandatory for developing of durable and broad-spectrum Bacterial Blight disease resistant cultivars. Therefore, screening of resistant genes (either dominant or recessive) in combination with more than two resistance genes for Bacterial Blight disease is essential against indigenous *Xoo* isolates of the respective regions. In view of the above, the present study was undertaken to detect virulent strain/ races among *Xoo* isolates of the eastern and north eastern India using pathotyping and to analyze genetic diversity/ similarity though genotyping. Further, in this study, suitable donors/ genotypes with effective R-gene combination against different *Xoo* isolates/ pathotypes of BB disease present in eastern and north eastern India for Bacterial Blight resistance breeding were also identified.

Materials and Methods

Experimental setup: Experiment was conducted at ICAR-National Rice Research Institute, Cuttack, India during wet season 2014 to 2016. A set of 34 rice genotypes/ near isogenic lines (NILs) carrying different Bacterial Blight resistance genes (R-genes) along with susceptible high yielding varieties (SHYV's) were collected from eastern and north eastern India (Table1). Seeds were thoroughly washed with sterile double distilled water, followed by washing with 70% ethanol for 30 sec and 0.5% sodium hypo-chloride for 1min to minimize the chance of any seed born contamination. Seeds were sown in sterilized petri plates under plant growth chamber with normal photoperiodic condition to maintain optimum moisture level. Ten-day-old mature healthy seedlings were planted in pots in net house. Finely crushed decayed farmyard manure was spread over the pots and pots were irrigated continuously to maintain proper moisture level. Further, pots were fertilized with urea (N 65 kg ha^{-1}) in two doses, *i.e.,* 50% at vegetative stage and 50% at reproductive stage.

***Xoo* isolation, maintenance, culture and subculture:** Thirty six different Bacterial Blight infected leaf samples (Table2) were collected in separate sterilized 50 ml falcon tubes from 6 states of India, *i.e.,* Manipur, Mizoram, Meghalaya, Sikkim, Tripura and Odisha and stored at -80°C.These samples were collected randomly from distant location and were distinct from each other. Bacterial Blight infected leaf samples were aseptically separated by cutting into 1-2 cm pieces using sterilized scissors and dipped in sterilized double distilled water for 1hr to collect *Xanthomonas* oozes in aqueous condition. These aqueous solutions were then used for culturing *Xanthomonas* colonies into modified ATCC (American type culture collection) medium and the pH of media was adjusted to 7.00. After autoclaving at 15psi, for 20 min at 121°C, the media was allowed to cool at room temperature for a while and poured into pre-sterilized Petri plates and culture

tubes. Plating of aqueous solution of *Xanthomonas* in media was done under laminar air flow using aseptic condition followed by incubating the cultures at 25°C under normal light condition for growth and multiplication of bacterial colonies. For transportation/long term storage, nutrient broth media or 5% glycerol was prepared and bacterial colonies were added aseptically to it and stored at –20°C. Stored cultures of *Xoo* sometimes loose its virulence, therefore to maintain its virulence and pathogenicity, all Xoo cultures were inoculated into TN-1 (susceptible check).

Genotyping of *Xoo* samples and analysis of genetic diversity: Stored *Xanthomonas* cultures were revived by sub-culturing on modified ATCC and colonies of pure culture were taken for DNA isolation using modified CTAB method. PCR reaction of bacterial (*Xoo*) DNA was carried out (George *et al.,* 1995) in a 25µl reaction mixture. The reaction mixture was prepared with 50 picomole of seach primers (IDT) JEL1 and JEL2 (Table 3) complementary to each end of IS 1112, 20ng of genomic DNA of *Xoo*, 185 µMd NTPs mix (Genetix), 2.5 units of Taq polymerase (Kappa Bio-system),1X PCR buffer-B (Kappa Bio-system), 0.1X DMSO (dimethylsulfoxide) (vol/vol), 7.5 µl of Tris-HCl (pH 9.5) and 1 drop of mineral oil. After PCR amplification, 15 µl of each PCR products were loaded in a 0.5% agarose + 0.75% synergel (Sigma)+0.1ug ethidium bromide + 0.1X Tris-borate-EDTA buffer and 1kb DNA ladder (thermo Fisher Sc.) was used as reference marker in first lane of gel. Amplified products were resolved by electrophoresis in 0.1X Tris-borate EDTA buffer for 5-6hr at 100 Volt, followed by capturing Gel images on Gel Doc (Syngene-ingenious). The banding pattern was scored using binary system of each band in 0 to 1 score, 0 for absence and 1 for presence. For analysis of genetic diversity/similarity, NTSY Spc version 2.02i (Exeter Software Setauket, New York) were used to determine genetic diversity/ similarity between and among *Xoo* samples. Binary scoring of *Xanthomonas* JEL1/JEL2 PCR banding pattern were used to import data into *NTSYSpc* software and to

Table 1: Rice genotypes/ lines used in the study which consists of near isogenic lines(NILs) and rice differentials containing different R-gene combination of bacterial leaf blight resistance

Genotypes	Feature/gene reported	Sources
IRBB3	*Xa3*	
IRBB4	*Xa4*	
IRBB5	*xa5*	
IRBB7	*Xa7*	
IRBB8	*xa8*	
IRBB10	*Xa10*	
IRBB13	*xa13*	
IRBB14	*Xa14*	
IRBB21	*Xa21*	
IRBB50	*Xa4+xa5*	International Rice Research Institute (IRRI), Philippines
IRBB51 (IR72912.15-1-5)	*Xa4+ xa13*	
IRBB52 (IR72913-52-1-4)	*Xa4+Xa21*	
IRBB53 (IR72914-21-1-3)	*xa5+ Xa13*	
IRBB54(IR72915-17-2-4)	*xa5+Xa21*	
IRBB55 (IR72916-51-1-3)	*xa13+Xa21*	
IRBB56 (IR72918-37-1-1)	*Xa4+xa5+xa13*	
IRBB57 (IR72919-10-1-3)	*Xa4+xa5+Xa21*	
IRBB58(IR72920-1-99-3)	*Xa4+xa13+Xa21*	
IRBB59 (IR72920-1-2-40)	*xa5+xa13+Xa21*	
IRBB60 (IR72920-1-44-4)	*Xa4+xa5+xa13+Xa21*	
Improved PR114	*Xa38*	
Improved Lalat		
Improved Tapaswini	Improved high yielding local variety with *xa5+xa13+Xa21*	ICAR-National Rice Research Institute, (NRRI), Cuttack, Odisha, India
Improved Ir64		
Improved Swarna		
Naveen	Susceptible high yielding local variety of Eastern India	
Pooja		
CAU- R1		Central Agricultural University, Imphal, Manipur, India
Shahsarang		
Lampnah	Susceptible high yielding variety of north eastern India	
Ranjit		
PD-10		
VL-82		
TN-1	Negative control for BB Disease	ICAR-National Rice Research Institute, (NRRI), Cuttack, Odisha, India

Table 2: *Xoo* isolates collected from different regions of Eastern and North-eastern India

Location	Region	Isolates	Total isolate
Manipur, India		MaI	1
(Andro, Patsoi, Lamphel, NambolIroisemba,		MaAn-1, MaAn-2, MaAn-3	3
Saitor, masa- Saitor)		MaLa	1
		MaNa-1, MaNa-2, MaNa-3	3
		MaPa-1, MaPa-2, MaPa-3, MaPa-4	4
		MaIr	1
		MaUc	1
		MaSa	1
Tripura, India	*North-eastern India	TrUd-1, TrUd-2	2
(Udaipur, Gomti, lembucherra, Checksemite,		TrCh	1
Belaria, Sipahijala)		TrGo	1
		TrLe	1
		TrBe	1
		TrSi	1
Mizoram, India		MiCh-1, MiCh-2, MiCh-3, MiCh-4	4
(ChampaiNaobe, Khwatal)		Mi Kh	1
Sikkim, India		SiRa	1
Ranipool		SiNa	1
Meghalaya, India		MeKh	1
ICAR Barapaniumiam, Soro Weat,		MeMu	1
Khliehumstum, Ri-Bhoi, Mynriumsuing,		MeLi	1
Ri-Bhoi, Liarsluid, Ri-Bhoi		MeUm	1
		MeSw	1
Odisha, India	Eastern India	NRRI 1	1
(ICAR-NRRI, Cuttack)		NRRI 2	1
	Total		36

Table 3: Details of molecular markers and their sequence used for genotyping

Genes	Primers	Sequence 5'-3'	Annealing temperature	References
Xa4	Npb181-F	ATCGATCGATCTTCACGAGG	55	Yoshimura *et al.* (1995)
	Npb181-R	GTGCTATAAAAGGCATTCGGG		
xa5	RM122- F	GAGTCGATGTAATGTCATCAGTGC	55	Blair, (1997)
	RM122-R	GAAGGAGGTATGCCTTTGTTGGAC		
xa13	*xa13-prom-F*	GGCCATGGCTCAGTGTTTAT	55	Sundaram *et al.* (2011)
	xa13-prom-R	GAGCTCCAGCTCTCCAAATG		
Xa21	pTA248-F	AGACGCGGAAGGGTGGTTCCCGGA	55	Ronald *et al.* (1992)
	pTA248-R	AGACGCGGTAATCGAAAGATGAAA		
Xa38	Oso4g53050-1 F	TCTTCTATTGCTAACATTGGTG	55	Bhasin *et al.* (2012)
	Oso4g53050-1R	TCGCATTCATTTTCAGAG		
Xoo genetic diversity	JEL1	CTCAGGTCAGGTCGCC3	55	Leach *et al.* (1992); George *et al.* (1995)
	JEL2	GCTCTACAATCGTCCGC		

obtaine dendrogram with similarity coefficient ranging between 0.00 to 1.00. However, for dendrogram prediction, Sequential, Hierarchical, Agglomerative, and Nested Clustering (SAHN) using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering program (Sneath and Sokal, 1973) with Jaccard (J) similarity coefficient were used (Sneath *et al.,* 1973).

Isolation of plant genomic DNA, genotyping and disease assessment: Genomic DNA was isolated from healthy leaves of 34 rice genotypes/ NILs (Table1) following the modified CTAB protocol (Doyle and Doyle, 1987). PCR (Eppendorfvapo-protect) reaction was performed to assess the presence of Bacterial Blight resistant genes using primers Npb181-*Xa4*, RM122- *xa5*, *xa13*-prom-*xa13* gene, pTA248-*Xa21* gene and Oso4g53050-1-*Xa38* (Table 3) in all the lines. PCR reaction mixture of 20 µl was consisting of 2 µl of 50 ng genomic DNA, 1 µl each of 10 picomole

Table 4: PCR amplification results of near isogenic lines (NILs) and other BB resistant/ susceptible high yielding varieties/ lines of eastern and North-eastern India

Genotypes	R-genes				
	Xa4	*xa5*	*xa13*	*Xa21*	*Xa38*
IRBB3	-	-	-	-	-
IRBB4	+	-	-	-	-
IRBB5	-	+	-	-	-
IRBB7	-	-	-	-	-
IRBB8	-	-	-	-	-
IRBB10	-	-	-	-	-
IRBB13	-	-	+	-	-
IRBB14	-	-	-	-	-
IRBB21	-	-	-	+	-
IRBB50	+	+	-	-	-
IRBB51 (IR72912.15-1-5)	+	-	+	-	-
IRBB52 (IR72913-52-1-4)	+	-	-	+	-
IRBB53 (IR72914-21-1-3)	-	+	+	-	-
IRBB54(IR72915-17-2-4)	-	+	-	+	-
IRBB55 (IR72916-51-1-3)	-	-	+	+	-
IRBB56 (IR72918-37-1-1)	+	+	+	-	-
IRBB57 (IR72919-10-1-3)	+	+		+	-
IRBB58(IR72920-1-99-3)	+		+	+	-
IRBB59 (IR72920-1-2-40)	-	+	+	+	-
IRBB60 (IR72920-1-44-4)	+	+	+	+	-
Improved PR114	-	-	-	-	+
Improved Lalat	-	+	+	+	-
Improved Tapaswini	-	+	+	+	-
Improved IR64	-	+	+	+	-
Improved Swarna	-	+	+	+	-
Naveen	-	-	-	-	-
Pooja	-	-	-	-	-
CAU- R1	-	-	-	-	-
Shahsarang	-	-	-	-	-
Lampnah	-	-	-	-	-
Ranjit	-	-	-	-	-
PD-10	-	-	-	-	-
VL-82	-	-	-	-	-
TN-1	-	-	-	-	-

forward and reverse primers (IDT), 2 µl 10X PCR buffer ($MgCl_2$ added), 2 µl 10 mM dNTPs mix (Genetix), 0.2 µl 5U μl^{-1} *Taq* DNA polymerase (Kappa Bio-system) and nuclease free 11.8µl MilliQ water. After PCR amplification, 15 µl of each PCR products were loaded on 2.5 to 3.5% agarose gel + 10µl ethidium bromide + 0.1X Tris-borate-EDTA buffer. Amplified products were resolved by electrophoresis in 0.1X Tris-borate-EDTA buffer for 4hr at 100 V and gel images were captured using gel documentation system (Syngene- ingenious). Scoring of banding pattern (0-1) was done using 1kb (Pure-gene) DNA ladder as reference size marker.

Different isolates of *Xoo* were grouped into different clusters and from each cluster, one representative isolate was taken to make aqueous suspension of bacterial cells. Forty eight hour old cultures were used to prepare inoculums in distilled water and the concentration of bacterial suspension was maintained at 10^8 CFU ml^{-1} (1OD). For artificial inoculation, clipping method (Kauffman *et al.,* 1973) was used for inoculating *Xoo* inoculums in three replications of each genotype (NILs and SHYV's) after 45 days of transplanting or at maximum tillering stage during *Kharif* season. However, TN-1 was used as negative control for all isolate groups to check the efficiency of *Xoo* inoculants. The BB disease infestation was recorded by measuring leaf lesion length after 15 days of inoculation and scored for pathotyping. Bacterial Blight disease response for each genotype was classified as resistant, if the mean leaf lesion length was between 0-5cm, moderately resistant if >5-10cm, moderately susceptible if >10-15cm and susceptible if >15cm. . The statistical analysis of data generated from molecular markers banding pattern were analyzed by following the procedure of Verma *et al.*, 2017 and Pandey *et al.*, 2020.

Results and Discussion

Presently, more than 44 R-genes, including major and some minor genes which confer host resistance to various strains/races of *Xoo* were identified and some were characterized (Chen *et al.,* 2011; Bhasin *et al.,* 2012; Natrajkumar *et al.,* 2012; Suh *et al.,* 2013; Kim *et al.,* 2015; Singh *et al.,* 2020). In order to confirm the presence of reported genes before artificial inoculation of *Xoo* isolates, PCR reaction was performed for *Xa4, xa5, xa13, Xa21* and *Xa38* genes for all rice genotypes *viz.,* BB NILs, susceptible and resistance checks, SHYV's and improved resistant varieties. Gel electrophoresis results confirmed the product/ band size of 130bp to 150bp for PCR product of npb181-*Xa4* gene, 227bp to 239bp for RM122-*xa5* gene, 250bp to 500bp for xa13-prom.-*xa13* gene, 750bp to 1000bp for pTA248-*Xa21* gene and 250bp to 300bp for Oso4g53050-1 of *Xa38* gene in IRBB60 (resistant check) and TN-1 (susceptible checks), respectively (Fig. 1.I). Accordingly, PCR screening was performed in all the 34 genotypes and PCR product confirmed the presence of resistant gene *Xa4* in IRBB4, *xa5* in IRBB5, *xa13* in IRBB13, *Xa21* in IRBB21 and *Xa38* in Improved PR114 (Table 4).

All the five genes (*Xa4, xa5, xa13, Xa21* and *Xa38*) were found absent in high yielding varieties *i.e.,* Naveen and Pooja of eastern India and CAU-R1, Shahsarang, Lampnah, Ranjit, PD-10 and VL-82 of North-Eastern India and presence of four Bacterial Blight genes was confirmed in IRBB60 *i.e., Xa4* + *xa5* + *xa13* + *Xa21* gene. However, the presence of 3 Bacterial Blight resistant gene combination, *i.e., xa5* + *xa13*+ *Xa21* was confirmed in improved high yielding rice varieties *i.e.,* Imp. Lalat, Imp. Tapaswini, Imp. IR64 and Imp. Swarna and IRBB59. Similarly, the presence of other 3 Bacterial Blight resistant gene combination *i.e., Xa4* + *xa5* + *xa13* in IRBB56, *Xa4* + *xa5* + *Xa21* in IRBB57, *Xa4* + *xa13* + *Xa21* in IRBB58 were observed. Other two Bacterial Blight resistant gene combinations were present, *i.e., Xa4*+*xa5* in IRBB50, *Xa4*+ *xa13* in IRBB51, *Xa4*+ *Xa21* in IRBB52, *xa5*+ *xa13* in

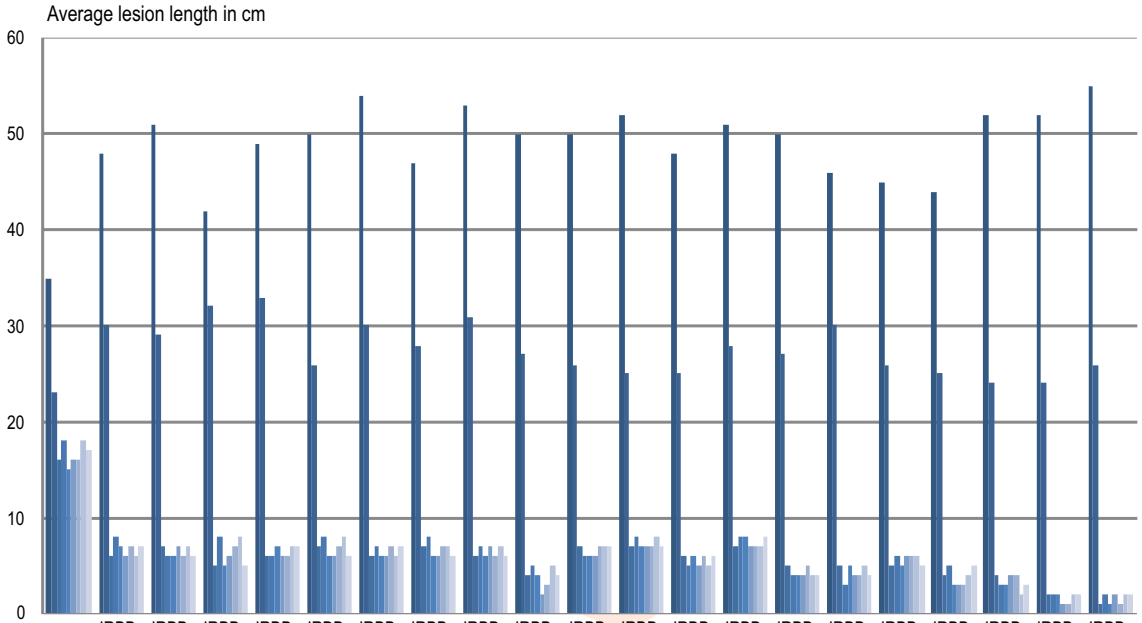
Table 5: Distribution of *Xoo* isolates in Eastern state Odisha and North-eastern states Manipur, Tripura, Mizoram, Sikkim and Meghalaya

Location	Isolates	Pathotype distribution						
		Isolate-I	Isolate-II	Isolate-III	Isolate-IV	Isolate-V	Isolate-VI	Isolate-VII
Manipur, India	MaI				IV			
	MaAn-1			III				
	MaAn-2	I						
	MaAn-3				IV			
	MaLa			III				
	MaNa-1				IV			
	MaNa-2			III				
	MaNa-3	I						
	MaPa-1				IV			
	MaPa-2			III				
	MaPa-3				IV			
	MaPa-4	I						
	MaIr				IV			
	MaUc	I						
	MaSa	I						
Tripura, India	TrUd-1	I						
	TrUd-2	I						
	TrCh	I						
	TrGo				IV			
	TrLe			III				
	TrBe	I						
	TrSi	I						
Mizoram, India	MiCh-1				IV			
	MiCh-2				IV			
	MiCh-3			III				
	MiCh-4			III				
	Mi Kh			III				
Sikkim, India	SiRa							VII
	SiNa				IV	V		
Meghalaya, India	MeKh							VII
	MeMu					V		
	MeLi							VII
	MeUm							VII
	MeSw					V		
Odisha, India	NRRI 1						VI	
	NRRI 2		II					

IRBB53, *xa5+Xa21* in IRBB54, *xa13+Xa21* in IRBB55. Molecular tools enable us to identify resistant genes/ alleles for different abiotic and biotic traits from germplasm. This modern approach has been utilized to develop resistant crop through breeding programs (Manju *et al.,* 2018; Kim *et al*., 2019; Singh *et al*., 2020). In the present study, 36 isolates of *Xoo*, collected from 5 states of North-East (Manipur, Mizoram, Meghalaya, Sikkim, Tripura) and 1 Eastern state (Odisha) were characterized through PCR based DNA fingerprinting using JEL1/JEL2 marker and virulence analysis. Based on their genetic similarity and diversity, grouping of 36 *Xoo* isolates were performed through PCR reaction, which showed multiple alleles (Fig. 1.II) and alleles were scored as per binary scoring system. Genetic diversity prediction and dendrogram was made using software NTSYSpc version 2.02i with SAHN-UPGMA clustering and Jaccard similarity coefficient. It exhibited genetic diversity/similarity between and within *Xoo* isolates with similarity coefficient ranging from 0.01 to 1.00 (Fig. 2). Similarity coefficient between two clusters was 0.35, which means all 36 *Xoo* isolates were genetically conserved by 35% and were divided into two main clusters. However, similarity coefficient of 0.44 represented, two clusters divided in four sub-clusters and 0.50 similarity coefficient represented formation of 6 sub clusters and finally, 0.80 similarity coefficient exhibited, that seven groups were obtained.

Based on the results of genetic diversity/ similarity with similarity coefficient of 0.80, all 36 *Xoo* isolates were grouped into seven isolate groups (haplotype) ranging from Isolate I to VII (Table 5). Molecular pathotyping enable us for rapid, efficient and precise analysis of virulence by PCR fingerprinting of pathogen

Table 6A: Graph showing mean length of leaf lesion in several NILs of IR 24, represented as IRBB NILs along with negative checks, *i.e.,* TN-1

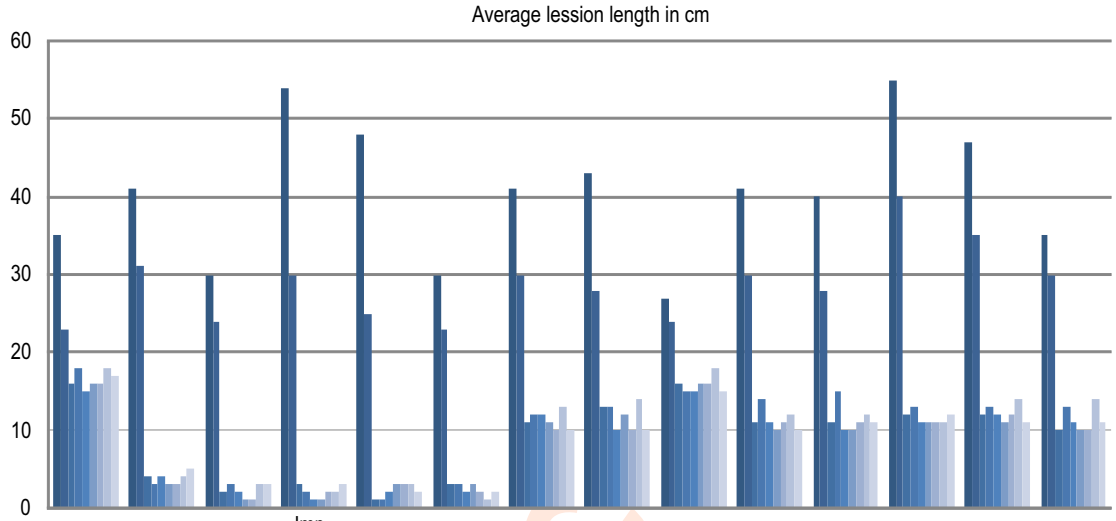


	TN-1	IRBB 3	IRBB 4	IRBB 5	IRBB 7	IRBB 8	IRBB 10	IRBB 13	IRBB 14	IRBB 21	IRBB 50	IRBB 51	IRBB 52	IRBB 53	IRBB 54	IRBB 55	IRBB 56	IRBB 57	IRBB 58	IRBB 59	IRBB 60
Main leaf length (cm)	35	48	51	42	49	50	54	47	53	50	50	52	48	51	50	46	45	44	52	52	55
Flag leaf length (cm)	23	30	29	32	33	26	30	28	31	27	26	25	25	28	27	30	26	25	24	24	26
Isolate -1	16	6	7	5	6	7	6	7	6	4	7	7	6	7	5	5	5	4	4	2	1
Isolate -2	18	8	6	8	6	8	7	8	7	5	6	8	5	8	4	3	6	5	3	2	2
Isolate -3	15	7	6	5	7	6	6	6	6	4	6	7	6	8	4	5	5	3	3	2	1
Isolate -4	16	6	7	6	6	6	6	6	7	2	6	7	5	7	4	4	6	3	4	1	2
Isolate -5	16	7	6	7	6	7	7	7	6	3	7	7	6	7	5	4	6	3	4	1	1
Isolate -6	18	6	7	8	7	8	6	7	7	5	7	8	5	7	4	5	6	4	2	2	2
Isolate -7	17	7	6	5	7	6	7	6	6	4	7	7	6	8	4	4	5	5	3	2	2

population, by finally limiting the number of isolates for handling. Shanti *et al.* (2001) reported that, a strong association between RFLP and *Xoo* isolate diversity, as well as a relationship among lineages and pathotypes in eastern India. In the present study, similar observation was found in the relationship among lineages and pathotypes in eastern and north-eastern India. The presence *Xoo* isolates in different states of India were further classified as these isolate were present in particular region such as Isolate I, III and IV was present in Manipur and Tripura region, Isolate III and IV in Mizoram, Isolate IV, V and VII in Sikkim and Isolate V and VII in Meghalaya and Isolate II and Isolate VI in Odisha region. It means several Isolates of *Xoo* were found present in north-eastern region of India, except Isolate II and VI and some isolate types were found in several regions. However, North-eastern states of India are rich source of genetic diversity in rice germplasm which may lead to genetic diversity in pathogens causing variation in level of disease virulence and resistance. Adhikari *et al.* (1999) reported that differences in host cultivar have significant role in *Xoo* pathogen diversity. *Xoo* strains distributed in several ecosystems which plays a significant role in the differentiation of pathogen population.

The above findings enable us to select representative isolates for identification of suitable R-genes for breeding program of concerning region using artificial inoculation. Artificial inoculation method was applied to determine the response of 34 rice genotypes against seven Isolate groups, representing 36 *Xoo* Isolates of Eastern and North-Eastern India. The significance Bacterial Blight disease reaction was observed on 34 rice genotypes after inoculating cultures from seven groups of isolates (Table 6A, 6B; Fig. 3). The highest lesion length of 18 cm was recorded for isolate II and VI, however, the remaining five isolates showed 02-17 cm lesion length against total main and flag leaf length of 35-55 cm and 23-33 cm, respectively. As compared to the performance of NILs on main leaf / flag leaf length ratio, IRBB59 (52cm/ 24cm) and IRBB60 (55cm/ 26cm) showed only 1-2 cm lesion of Bacterial leaf Blight on all seven isolates. Similarly, 2-5 cm long lesion was recorded in IRBB57 and IRBB58, however, 5 cm long lesion was observed in IRBB56 (45cm/26cm) for isolate I, III and VII and 6 cm long lesion was observed in Isolate II, IV, V and VI. The other NILs of IR 24 such as IRBB52, IRBB54 and IRBB55 showed a lesion length of 5-7 cm in all seven *Xoo* isolates whereas IRBB4,

Table 6B: Graph showing average length of leaf lesion in rice bacterial blight differentials, improved varieties, high yielding susceptible varieties of Eastern and North-Eastern India along with TN-1 (negative checks).



	TN-1	Imp. PR114	Imp. Lalat	Imp. Tapaswini	Imp. IR64	Imp. Swarna	Naveen	Pooja	CAU-R1	Shahsarang	Lampnah	Ranjit	PD-10	VL-82
Main leaf length (cm)	35	41	30	54	48	30	41	43	27	41	40	55	47	35
Flag leaf length (cm)	23	31	24	30	25	23	30	28	24	30	28	40	35	30
Isolate-1	16	4	2	3	1	3	11	13	16	11	11	12	12	10
Isolate-2	18	3	3	2	1	3	12	13	15	14	15	13	13	13
Isolate-3	15	4	2	1	2	2	12	10	15	11	10	11	12	11
Isolate-4	16	3	1	1	3	3	11	12	16	10	10	11	11	10
Isolate-5	16	3	1	2	3	2	10	10	16	11	11	11	12	10
Isolate-6	18	4	3	2	3	1	13	14	18	12	12	11	14	14
Isolate-7	17	5	3	3	2	2	10	10	15	10	11	12	11	11

IRBB13, IRBB3, IRBB7, IRBB8, IRBB10 and IRBB14 recorded 6-8 cm long lesion against all the isolates. The main leaf length/flag leaf length of IRBB21 and Imp. PR114 was 50cm/20cm and 48cm/ 25cm. Naveen, a high yielding variety of Odisha showed a lesion length of 11-13cm in isolate I, II, III, IV and VI and 5 cm in Isolate V and VII, respectively, however, other variety Pooja showed 12-14cm long lesion in isolate I, II, IV and VI and 10 cm in isolate III and IV. The improved HYV's of eastern India *viz.,* Imp. Tapaswini, Imp. Lalat, Imp. Swarna and Imp. IR64 exhibited the lesion length of 1-4 cm in all isolates whereas high yielding varieties of North-east *viz.,* CAU- R1 (27/24cm), Shahsarang (41cm/ 30cm), Lampnah (40cm/ 28cm), Ranjit (55cm/ 40cm), PD-10 (47cm/ 35cm) and VL-82 (48cm/ 31cm) showed different response to seven different isolates and ranged the lesion length from with reference to 10 to 18 cm.

Diversity in level of virulence of *Xoo* Isolates, isolate II and VI were found more virulent in terms of leaf lesion length as compared to other isolates and the maximum lesion length of 18cm was observed in isolate II and VI. The improved HYV's of Eastern India, *i.e.,* Imp. Tapaswini, Imp. Lalat, Imp. Swarna and Imp. IR64 introgressed with *xa5, xa13* and *Xa21* genes were found resistant to all isolates. The HYV's of Eastern and North-eastern India *i.e.,* Naveen, Pooja, CAU- R1, Shahsarang, Lampnah, Ranjit, PD-10 and VL-82 exhibited diverse behavior against different isolates of *Xoo* with moderately susceptible to susceptible reaction. While, the near isogenic lines (NILs) of IR24, IRBB60 (*Xa4, xa5, xa13* and *Xa21*) and IRBB59 (*xa5, xa13* and *Xa21*) showed resistance reaction to all the 7 isolates of Xoo, however, the susceptible check, TN-1 was found susceptible to all the isolate groups, except Isolate III, which showed moderate resistance (Table 7). NILs with two gene combinations, *i.e.,* IRBB54 and IRBB55 were resistant and IRBB50, IRBB51, IRBB52 and IRBB53 showed moderate resistance. However, NILs with single gene such as IRBB4 (*Xa4*), IRBB13 (*xa13*), IRBB3 (*xa3*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB8 (*xa8*), IRBB10 (*Xa10*) and IRBB14 (*Xa14*) was moderately resistant to all isolates. Single gene cannot confer resistance to all the *Xoo* isolates (Shanti *et al.,* 2001) and similar data was recorded in IRBB5 (*xa5*). As it confers resistance to isolate I, III, VII and moderately resistant to isolate II, IV, V, VII. Surprisingly, IRBB21 (*Xa21*) and Imp. PR114 (*Xa38*) was found resistant to all *Xoo* isolates due to *Xa21*, a dominant gene is located on chromosome 11 of rice genome, confer

Table 7: Bacterial blight disease reaction on near isogenic lines of Indica rice cultivars and distribution of pathotypes

	Pathotype and their prevailing location/states						
Genotypes/ races	**Isolate-I**	**Isolate-II**	**Isolate-III**	**Isolate-IV**	**Isolate-V**	**Isolate-VI**	**Isolate-VII**
TN-1 (Negative check)	S	S	MS	S	S	S	S
IRBB3	MR	MR	MR	MR	MR	MR	MR
IRBB4	MR	MR	MR	MR	MR	MR	MR
IRBB5	R	MR	R	MR	MR	MR	R
IRBB7	MR	MR	MR	MR	MR	MR	MR
IRBB8	MR	MR	MR	MR	MR	MR	MR
IRBB 10	MR	MR	MR	MR	MR	MR	MR
IRBB 13	MR	MR	MR	MR	MR	MR	MR
IRBB 14	MR	MR	MR	MR	MR	MR	MR
IRBB 21	R	R	R	R	R	R	R
IRBB 50	MR	MR	MR	MR	MR	MR	MR
IRBB 51	MR	MR	MR	MR	MR	MR	MR
IRBB 52	MR	R	MR	R	MR	R	MR
IRBB 53	MR	MR	MR	MR	MR	MR	MR
IRBB 54	R	R	R	R	R	R	R
IRBB 55	R	R	R	R	R	R	R
IRBB 56	R	MR	R	MR	MR	MR	R
IRBB 57	R	R	R	R	R	R	R
IRBB 58	R	R	R	R	R	R	R
IRBB 59	R	R	R	R	R	R	R
IRBB 60	R	R	R	R	R	R	R
Improved PR114	R	R	R	R	R	R	R
ImprovedLalat	R	R	R	R	R	R	R
Imp.Tapaswani	R	R	R	R	R	R	R
Improved IR64	R	R	R	R	R	R	R
Improved Swarna	R	R	R	R	R	R	R
Naveen	MS	MS	MS	MS	MR	MS	MR
Pooja	MS	MS	MR	MS	MR	MS	MR
CAU-R1	S	MS	MS	S	S	S	MS
Shahsarang	MS	MS	MS	MR	MS	MS	MR
Lampnah	MS	MS	MR	MR	MS	MS	MS
Ranjit	MS	MS	MS	MS	MS	MS	MS
PD-10	MS	MS	MS	MS	MS	MS	MS
VL-82	MR	MS	MS	MR	MR	MS	MS

S: Susceptible; MS: Moderately susceptible; R: Resistant and MR: Moderately resistant

significant resistant solely or in combination with other *Xa* gene against a range of isolates; however, some isolates showed virulence and overcame *Xa21* gene (Shanti *et al.,* 2001). Resistance break of *Xa21* gene has been reported by some highly virulent strains of India, Japan, Korea, Nepal and Sri Lanka (Goel *et al.,* 1998; Adhikari *et al.,* 1999). A newly identified Bacterial Blight resistance gene was identified from *Oryza nivara* acc. IRGC 81825, and mapped on chromosome 4L in a 38.4-kb region (Bhasin *et al.,* 2012). As it confers significant level of resistance to a range of *Xoo* isolates, *Xa38* gene was introgressed in Indian basmati rice variety 'PB1121'from donor parent PR114- *Xa38* using a modified marker-assisted backcross (MABC) breeding scheme (Ellur *et al.,* 2016).

To confer broad spectrum and durable resistance in rice cultivar, it is pre-requisite to select the best strategy for deployment of resistance gene and suitable donor. In the present study, *Xa21* gene showed important component of resistance as a single gene as well as combination of two or three or four genes. This may be due to the effect of complementary interaction between different R- genes. Synergetic effect of other genes with *Xa21* enhanced the resistance level with strong effect as low resistance was compensated by complementary R-gene that was alone moderately resistant or defeated by some virulent isolates. However, the *xa13* gene is recessive in nature and present on chromosome 8. As a single gene, *xa13* confer resistance to *PXO99A* a highly virulent isolate of Philippines and seven highly virulent isolate of India, Vietnam and Srilanka (Loan *et al.,* 2006;

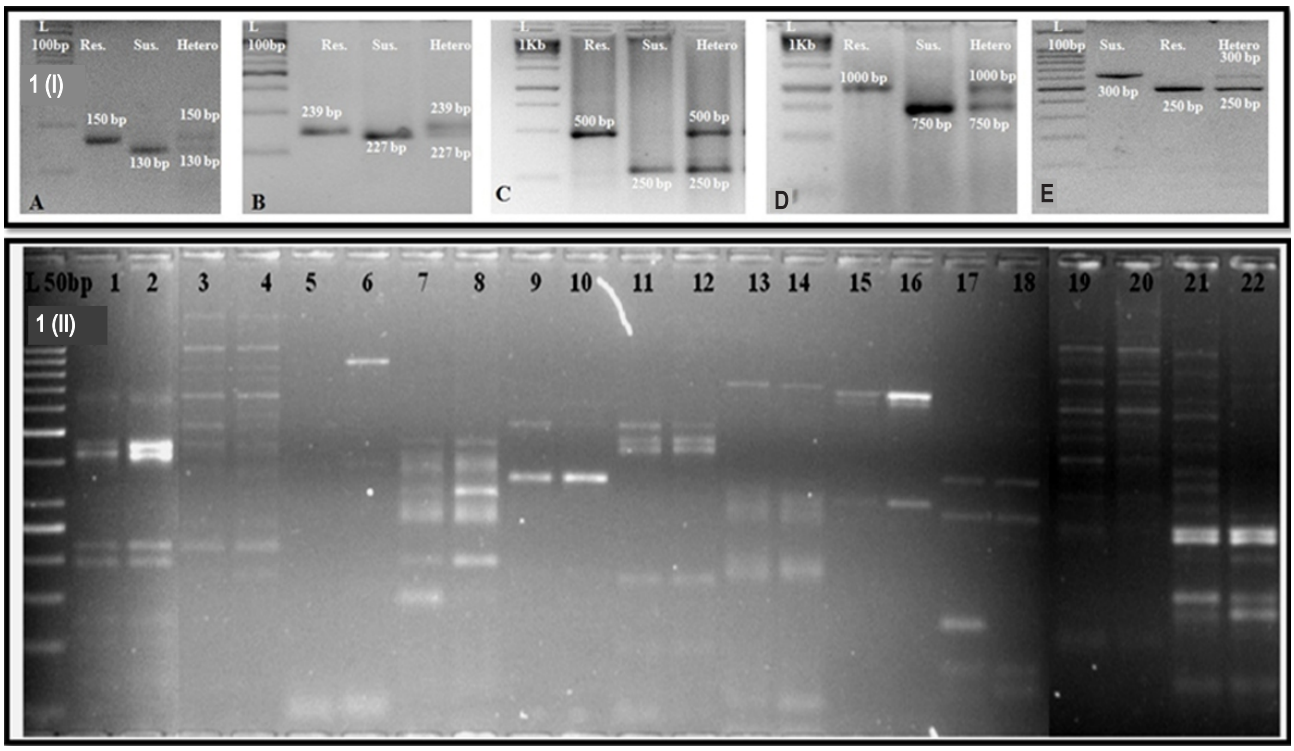


Fig. 1(I): Banding pattern of PCR product using (A) Npb181(*Xa4* gene), (B) RM122 (*xa5* gene), (C) *xa13*-Prom (*xa13* gene), (D) pTA248 (*Xa21* gene), and (E) Os04g53050-1(*Xa38* gene). **(II)** Banding pattern of JEL1/JEL2 primer, used for grouping of isolated based on genetic diversity in *Xoo*.

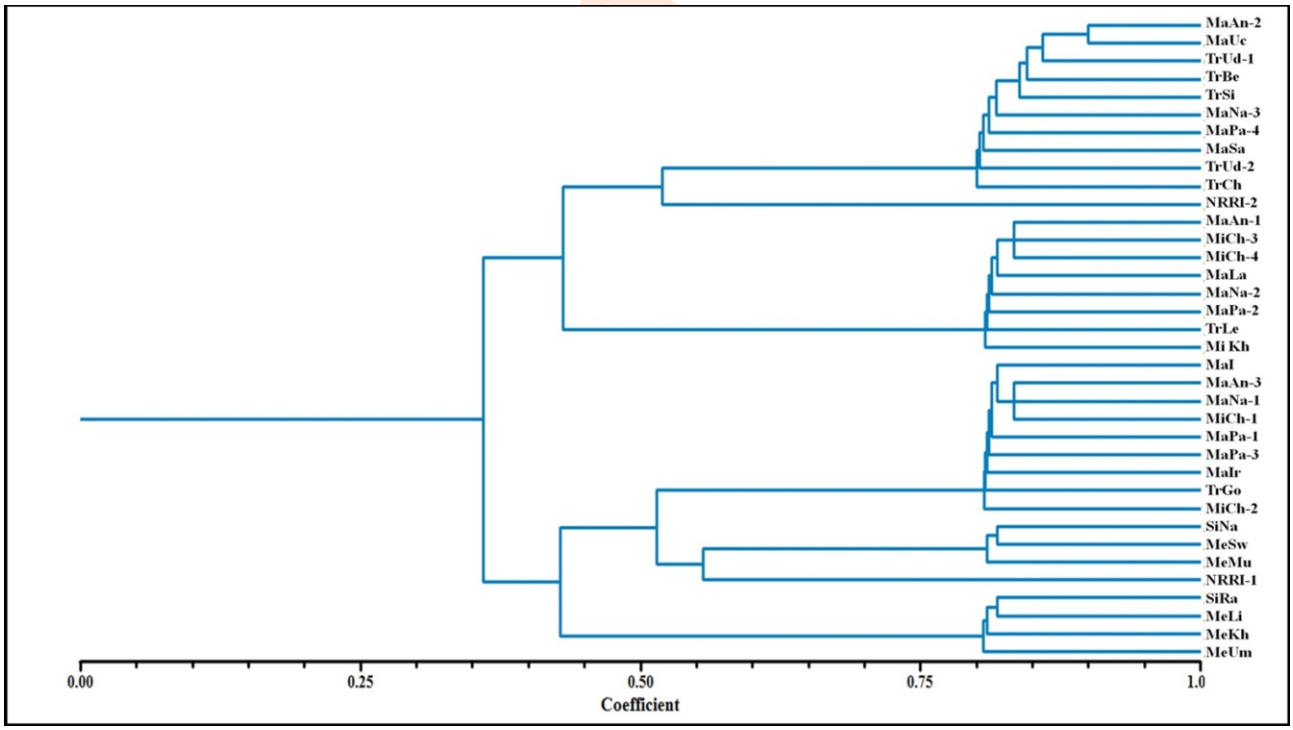


Fig. 2: Dendrogram showing genetic diversity and grouping of 36 *Xoo* isolates collected from Eastern and North-eastern India.

Fig. 3: BB disease Scoring; measurement of leaf lesion length in rice plant after 15 days of clip inoculation. All 34 genotypes were used for scoring, left side of each plates is *TN*-1 (negative check for BB) and right side of each plate is IRBB60 (positive check for BB).

Lore *et al.,* 2011). The *xa5* gene of rice is a recessive gene present on chromosome 5 and confers resistance to a range of Indian *Xoo* isolates. IRBB14 (*Xa14*) was susceptible to most of the *Xoo* isolates of Philippines and Punjab state of India (Lore *et al.,* 2011). Other Bacterial Blight resistant single genes were not found resistant to any of the isolates. Pyramiding of two or more R-gene in different combinations gives a durable and broad-spectrum resistance to a range of *Xoo* isolates than single R-gene due to synergetic and complementary effect between R-genes (Narayanan *et al.,* 2002; Singh *et al.,* 2014; Singh *et al*., 2020).

In the present study, genotypes with four R-gene combinations such as IRBB60 (*Xa4, xa5, xa13, Xa21*) showed resistance to all 7 *Xoo* isolates. Similarly, IRBB59, which contain three R-genes (*xa5, xa13, Xa21*) also showed resistance to all 7 isolates. The performance of these two genotypes against all 7 *Xoo* isolates was better as compared to other genotypes with different R-gene combination. However, improved rice varieties i.e. Tapaswini, Imp. Lalat, Imp. Swarna and Imp. IR64, which consisted similar R-gene combination were also found resistant as compared to IRBB60 and IRBB59, but the lesion length was higher than IRBB59 and IRBB60. Other NILs with three R-gene combination such as IRBB57 and IRBB58 showed resistance to all seven isolates but the BB lesion length was slightly higher compare to IRBB60 and IRBB59. However, IRBB56 was found resistant to isolate I, III, VII and moderately

resistant to Isolate II, IV, V and VI. Singh *et al.,* (2002) also reported IRBB54 provide broad spectrum and durable resistance against several isolates of Korea and India, respectively. Similarly, NILs with two R-gene combination *i.e.,* IRBB54 and IRBB55 were found resistant to all the isolates, however, IRBB50, IRBB51, IRBB52 and IRBB53 were moderately resistant to all isolates. *xa5* and *xa13* as best two recessive R-gene combination for effective resistance against all *Xoo* isolates of Punjab, India and Pakistan. (Goel *et al.,* 1998, Singh *et al.,* 2002; Khan *et al.,* 2012) Yoshimura *et al.,* 1995 also reported two genes combination for better resistance to *Xoo* Isolates of Philippine.

The outcome of the present study, provides significant implication for selecting suitable donors in rice having R-genes for durable and broad-spectrum Bacterial Blight disease resistance in Eastern and North-East Indian. Genetic diversity in *Xoo* isolates and their disease reaction against different Xa-gene combination confirmed the presence of diverse *Xoo* races/isolates in India. Although all 10 R-genes *i.e., Xa3, Xa4, xa5, Xa7, xa8, Xa10, xa13, Xa14, Xa21, Xa38* of BB were found useful against some specific isolates, except *Xa21* and *Xa38*, and none of R-gene alone were able to confer resistance against all isolates studied. Variation in gene sequence in *Xoo* pathogens determines the level of pathogenicity in different geographical regions, however, in rice plants the level of resistance is determined by the presence of different R gene combination and gene complementation.

These identified lines may be utilized as new identified potential donors for pyramiding of R-gene into locally adapted Bacterial Blight susceptible high yielding popular rice cultivars of different agro-ecological locations. After R- gene incorporation, these Bacterial Blight pyramided lines can reduce the yield penalty due to Bacterial Blight disease in a durable and broad-spectrum manner.

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Add-on Information

Authors' contribution: J. Kumar: Conducted experiments and wrote the MS; **A. Hussain:** Collection of leaf samples; **P. Singh:** Analyze the data and wrote the MS; **S.K.Y. Baksh:** Inoculated the culture in field; **M.K. Kar:** Provide the facility at NRRI, Cuttack; **A.K. Mukherjee:** Provide the Xoo-culture at NRRI; **N.R. Singh:** Collected the disease infected sample; **B. Sinha:** Provide the Xoo-culture at CAU, Imphal; **Pramesh Kh.:** Collected the samples from field; **N. Singh K.:** Provide the Xoo-culture at CAU, Imphal and checked the MS; **J.N. Reddy:** Designed experiment and overall guidance.

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