ORIGINAL RESEARCH ARTICLE

Assessment of potentiality of known bacterial blight resistant genes against *Xanthomonas oryzae* pv. *oryzae* pathotypes exist in Bangladesh

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ABSTRACT

Bacterial blight (BB) caused by *X. oryzae* pv. *oryzae* is a destructive disease of rice and causes 30-50% losses to rice depending on the outbreak. Development BB resistant rice varieties have long been considered as one of the most effective approach to control the disease. However, the durability of host resistance is breaking down due to the change of pathotypes of *X. oryzae* pv. *oryzae* globally. Pathotypic analyses of 239 *X. oryzae* pv. *oryzae* Bangladeshi isolates on Near Isogenic Lines (NILs) containing resistance (*R*) gene (s) revealed the existence of eight pathotypes of *X. oryzae* pv. *oryzae*. Among eight pathotypes, pathotypes IV and V were considered as major comprising maximum number of isolates, (30.13% and 23.01%, respectively), whereas pathotype VIII considered as minor consisting only 2.51% of total isolates. Pathotype, I showed highest virulence or aggressiveness compatible with all NILs, whereas pathotype VIII exhibited lowest virulence to these NILs. Bacterial blight resistant genes viz. *Xa1* (75.00%), *Xa11* (62.50%) and *Xa21* (50.00%) showed resistance to most of the pathotypes while *Xa4* performed worst as compared to all others *R*-genes. In pyramid lines, IRBBB63 (*Xa5+Xa7+Xa13*) and IRBB57 (*Xa5+Xa7 +Xa21*) showed resistance reaction and IRBB61 (*Xa4+Xa5+Xa7*), IRBB60 (*Xa4+Xa5+Xa13+Xa21*), IRBB54 (*Xa5+Xa21*), and IRBB53 (*Xa4+Xa21*) showed susceptible reaction to *X. oryzae* pv. *oryzae* pathotypes. These results collectively indicated the deployment of *Xa1, Xa11, Xa4, Xa5, Xa7, Xa13* and *Xa21* either alone or in combination against BB would be a best choice for the development of BB resistant rice varieties in Bangladesh.

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INTRODUCTION

Bacterial blight (BB) of rice caused by the vascular pathogen *Xanthomonas oryzae* pv. *oryzae* (Ishiyama, 1922; Swings *et al.*, 1990) is one of the supreme devastating diseases of rice across the globe (Mew *et al.*, 1982). 20 to 40 % yield lessened due to having gigantic infection at the highest tillering stage, resulting in a yield reduction from 20 to 40% (Mew *et al.*, 1993). Highest 50% yield completely deteriorated by initial Bacterial blight infection (Ou *et al.*, 1985; Alam *et al.*, 2016). New races of this pathogens were detected in recent years in Bangladesh resulting considerable yield abatement (50-70%) mostly in irrigated hybrid varieties (Islam *et al.*, 2016). Anyway, several control methods were practicing all across the globe including cultural, chemical, biological, disease forecasting and crucially host genetic resistance (Bharani *et al.*, 2010) and Host plant resistance exhibited the highest elevation in controlling this disease compared to chemical control (Gautam *et al.*, 2015).

Therefore, breeding method could be one of the fruitful approaches to develop resistant cultivars containing *R* (resistance) genes mitigating BB. Worldwide above 38 *R* genes (*Xa/xa* have been exploited (Nino Liu *et al*., 2006; Cheema *et al*., 2008). Moreover, 42 *R*-genes also have been detected developing from *japonica* varieties, *Oryza sativa* sp. *indica* cultivars and its relevant wild species viz. *O. minuta, O. longistaminata, O. officinalis* and *O. rufipogon* (Brar and Khush, 1997; Lee *et al*., 2003, Busungu *et al*., 2018) and in rice molecular markers have been associated with BB resistance (*R*) genes with a number of 25 or more amount (Lee *et al*., 2003; Yang *et al*., 2003). Lately, *Xa1, Xa10, Xa21, Xa23, Xa3, Xa5, Xa13, Xa25, Xa27 and Xa26* genes are cloned and characterized which has been encoding several types of proteins and exhibiting numerous mechanisms of *R*-gene triggered *Xoo* resistance as well as three (*Xa4, Xa7,* and *Xa13)* have been mapped physically (Vikal *et al*., 2017). Depending on the availability of the several resistance genes, it is quite necessary to evaluate their effectiveness solely or in a combination for accurately introgression of these marker assisted genes into well known cultivars (Sundaram *et al*., 2008). Space and times are the crucial factor to check the long-lasting effectiveness of new races of this pathogen (Adhikari *et al*., 1999; Noda *et al*., 2001; Lore *et al*., 2011) who revealed *Xoo* is awfully variable and over 30 pathotypes/races found across the globe.

New races of the pathogens continue to emerge and overcome the deployed resistance genes (Mew, 1987), majority of them fluctuate depending on regions, areas, and including fields within an area (Nelson *et al*., 1994). Thus, a significant approach is needed to develop particular resistant cultivar with the help of predominate races in that specific geographical area through breeding approaches. To unveil the complex connection between genotypes and pathotypes of the BB pathogen (*X. oryzae* pv. *oryzae*) in Bangladesh the diversity of pathotypic and genetic were exploited (Islam *et al*., 2016). However, some genes provide resistance to a wide spectrum of *X. oryzae* pv. *oryzae* races (e.g., *Xa21, Xa23*), whereas others are effective against only one or a few races that may be limited to a particular geographical location (e.g., *Xa1*). Most *R*-genes to BB are dominant, while some are recessive (e.g., *xa5, Xa13*) and some display semi dominance (e.g., *Xa27*). Most *R*-genes to BB have been introgressed into the background of the susceptible *indica* cultivar IR24 to develop a set of near isogenic lines (NILs) and some have been pyramided, either through classical breeding and marker-assisted selection or through genetic engineering, to develop new plant types and NILs (Narayanan *et al*., 2002; Sanchez *et al*., 2000; Singh *et al*., 2001). Similar research was conducted by Chen *et al*., (2020)

Compared to parental NILs with only one *R*-genes pyramid lines manifested gigantic range of resistance to bacterial blight showing synergism as well as complementation within *R*-genes (Adhikari *et al*., 1999a; Huang *et al*., 1997). Testing of *X. oryzae* pv. *oryzae* isolates from each lineage or haplotype defined by DNA fingerprinting against near-isogenic lines (NILs) and pyramid lines may allow the identification of suitable resistance

genotypes and its deployment strategies as well as efficient characterization of the resistance spectra of the genes in relation to pathogen strains representing the population diversity of *X. oryzae* pv. *oryzae*. Mishra *et al*. (2013) showed numerous *Xoo* strains that exhibited compatible reaction with the *xa13 R*-gene and by pyramiding *Xa4*, *Xa21* and *xa5* genes were auspicious against strains from Eastern part of India. In total 224 *Xoo* strains coalesced between 1999-2006 from state of Punjab (North western India) bearing *Xa* genes on NRLs found any of these strains were not compatible with the *Xa13 R*-genes (Lore *et al*., 2011). Overall, *Xa21* resistance gene were incompatible with 93% of the isolates as well as *Xa5* resistance genes were showed 95% compatibilityof the isolates (Singh *et al*., 2001). Pyramid lines have displayed higher levels and/or wider spectra of resistance to BB than the parental NILs with single *R*-genes (Adhikari *et al*., 1999a; Huang *et al*., 1997). Specifically, *R*-gene pyramiding is a fruitful approach to apply more *R*-genes which ultimately confer wide resistance as well as sustainable against various races or pathogens (Kim *et al*., 2018).

However, none of the breeding approaches found effective due to dynamic changes of pathogen population. The widespread repeated use of a few resistance genes might accelerate the selection of new pathogenic races of the isolates followed by selection of resistant host plant after a single crop cycle. This leads to a change in pathogen population structure through either mutation or recombination to adapt itself to the new resistant host plant or environmental changes. Thus, identification of the effective *R*-genes against BB of rice will be a good choice for the development of BB resistant rice cultivars in the country. All of these aforementioned studies were conducted within a limited condition or in a specific geographical area. Anyway, previously no such research was conducted to evaluate the genetic mitigation of resistance as well as pathogenic diversity analyses of *Xoo* isolates particularly for Bangladesh. Analysis of the virulance of the dominant *Xoo* isolates on a series of standard inequitable hosts and relevant information is inevitable to implement resistance transfer in a sustainable and systematic way. Considering all of these, the present work was experimented by using 239 strains of *X. oryzae* pv. *Oryzae* obtained from 30 districts were carried out i) to get a more comprehensive idea of pathotype diversity analysis in Bangladesh including the existence of the dominant pathotype (s) using 10 near isogenic lines (NILs) and ii) to evaluate the performance of some selected *R*-genes against these pathotypes using some pyramided rice lines.

MATERIALS AND METHODS

Collection of bacterial blight infected rice leaf samples
Plant samples those were infected with bacterial blight exhibiting typical symptom were coalesced randomly from 30 districts of Bangladesh. Each district was comprised five to ten different locations and from each location, 10 plants were gathered and each of them had 3 leaves combinely build composite sample and a representative sample was gathered for the isolation of

X. oryzae pv. *oryzae,* those were brought into laboratory and preserved those in the refrigerator for isolation.

Isolation and identification of *X. oryzae* pv. *oryzae* isolates

During 2015-2016, 239 strains of *X. oryzae* pv. *oryzae* were isolated from infected rice leaf samples as described previously by Alam *et al*. (2016). At each location, all *X. oryzae* pv. *oryzae* strains were independent isolates confirming by the pathogenicity test using susceptible check rice cultivar IR24 in the net house.

Identification and purification of *X. oryzae* pv. *oryzae* cultures

Plates containing bacterial growth was measured and from those which exhibited similar to the chriterion of *X. oryzae* pv. *Oryzae* were pull out by sterilized loop and purified cultures were prepared by streaking on both YDC and NBY agar media.

Confirmation of *X. oryzae* pv. *oryzae* isolates by pathogenicity test

Rice cultiver IR24 which was susceptible used in the pathogenicity test to confirmed the isolates of *X. oryzae* pv. *oryzae* carried out in Professor Golam Ali Fakir Seed Pathology Centre, Bangladesh Agricultural University, Mymensingh, Bangladesh. Seedlings were emerged when seeds were sown in the plastic pot. Then seedlings were transplanted for inoculation in the net house. NBY agar medium were utilized to culture the bacterial isolates for 48 hours at 28°C and then using Spectrophotometer cell density was detected at 10^8 cells/ml by resuspending in sterile distilled water. leaf clipping method of Kauffman *et al*. (1973) was practised in plants containing youngest leaves and subsequently plants were monitored for development of symptoms at net house.

Confirmation of *X. oryzae* pv. *oryzae* isolates by hypersensitivity test

Hypersensitivity response of the isolates was assessed on tobacco (cv. *virginia*) plant grown in earthen pots containing loamy soil. Approximately 10^8-10^9 CFU ml^{-1} of freshly cultured bacteria were injected onto the abaxial surface of tobacco leaf with a hypodermic syringe at 5-6-leaf stage. Inoculated plants were then kept in a moist chamber for a few hours to promote symptom development which was later shifted to the screen house. Controls were similarly inoculated with Sterile distilled water. Complete collapse of tissue after 24 hrs, followed by necrosis was interpreted as positive reaction (Klement and Goodman, 1967).

Confirmation of *X. oryzae* pv. *oryzae* isolates by PCR

The isolates with similar morphology and positive in pathogenicity were used as representative isolates of *X. oryzae* pv. *oryzae* from each growing area for PCR confirmation. The PCR based confirmatory test of *X. oryzae* pv. *oryzae* was performed by using primers XOR-F and XOR-R2 as reported by Adachi *et al*. (2000).

Extraction of genomic DNA from *X. oryzae* pv. *oryzae*

Genomic DNA of *X. oryzae* pv. *oryzae* was extracted by using wizard® genomic DNA purification kit (Promega, Madison, WI, USA) from 1 ml of liquid culture of each isolate and was quantified using an UV spectrophotometer absorbance at 260 nm with a model T-80 UV/VIS and stored at -20^{0}C. DNA concentration was adjusted to 100ng/μl and verified by comparing with a 100bp/1kb plus DNA ladder (Invitrogen, USA) on 1.5% agarose gel. After electrophoresis, the gel was placed under UV trans illuminator using the Gel View Master, Dynamica, UK for visualization of DNA bands. The UV light of the apparatus switched on, the image of the desired bands on the gel was viewed on the monitor and saved on the computer disc (CD-R) for taking photograph.

Determination of pathotypes of *X. oryzae* pv. *oryzae* isolates

Pathotypic analyses of *X. oryzae* pv. *oryzae* were experimented with a number of NILs (Near-Isogenic lines) of rice viz. IRBB1, IRBB2, IRBB4, IRBB5, IRBB7, IRBB8, IRBB10, IRBB11, IRBB13, IRBB14 and IRBB21 conveying single known bacterial blight resistance gene (*R*-gene) in the background of IR24 (susceptible check cultivar). Each pot comprising three hills where plants were raised. Three pots were designated as replications for individual isolate.

Plant materials

Three sets of bacterial blight differential rice lines were used in the study for inoculation with a total of 239*X. oryzae pv. oryzae* isolates (180 isolates from rainfed and 59 isolates obtained from irrigated condition) *to* monitor the pathotypic changes of *X. oryzae* pv. *oryzae* isolates as compared to the pathotypes reported in Bangladesh previously by Alam *et al*. (2016). The first set consists of ten NILs with a single *R* gene in an IR24 background viz. NILs (IRBB-1, 2, 4, 5, 8, 10, 11, 13, 14 and 21). The second set included 11 different gene pyramid lines (IRBB- 52, 53, 54, 57, 58, 59, 60, 61, 63, 64 and 65), carrying *R*-genes (*Xa4, Xa5, Xa7, Xa13*, and *Xa21*) in all possible combinations in an IR24 background. Seeds of the near-isogenic and pyramid lines were obtained from the Bangladesh Rice Research Institute (BRRI), and sown in plastic pots. Thirty days after sowing, three seedlings of each line were transplanted into 100-cm diameter plastic buckets in the net house. Each pot was filled with 8kg of soil and farm yard manure mixed at a ratio of (1:1) wt/wt (N/P/K was applied approximately at the proportion of 1:1/1:2/3) 10g, mixer of N/P/K per pot (as the basal dose in the form of ammonium sulphate, triple super phosphate, and muriate of potash). Plants were watered daily and were top dressed with urea 46% N (at the rate of 5g per pot at 30 days after transplanting. Isolates of *X. oryzae* pv. *oryzae* that were maintained in 20% glycerol at -80°C were revived on NBY plates and incubated for 72h at 28°C

Method of inoculation

Inoculums of each strain was prepared by suspending the bacterial cells in 50 ml of sterile distilled water and adjusting (optical density at 600nm=0.3) to approximately 1.2 X 10^9 CFU/ ml. Leaf tips of rice plants at the late tillering stage (45-50 days

Table 1. Response of near-isogenic lines to the eight pathotypes of *X. oryzae* pv. *oryzae* isolates in irrigated and rainfed season during 2016.

Pathotype	Total no. of Isolates	% of Isolates	Near-isogenic lines (NILs) and known resistance genes[a]										
			IRBB1 (Xa1)	IRBB2 (Xa2)	IRBB4 (Xa4)	IRBB5 (xa5)	IRBB8 (xa8)	IRBB10 (Xa10)	IRBB11 (Xa11)	IRBB13 (xa13)	IRBB14 (Xa14)	IRBB21 (Xa21)	IR24 (susceptible)
I	17	7.11	S	S	S	S	S	S	S	S	S	S	S
II	28	11.72	R	S	S	S	R	S	S	S	S	R	S
III	36	15.06	R	R	S	S	S	R	R	S	S	S	S
IV	72	30.13	R	S	S	R	S	S	R	R	S	S	S
V	55	23.01	R	S	R	S	S	R	R	S	S	R	S
VI	13	5.44	R	S	S	S	R	R	S	S	R	R	S
VII	12	5.02	R	R	S	R	R	S	R	S	S	R	S
VIII	6	2.51	S	R	S	S	R	R	R	R	R	R	S

S=Susceptible and R=Resistant.

after transplanting) were clip-inoculation method with scissors that had been dipped in the inoculum (Mew and Vera Cruz, 1978). Individual isolate was used for inoculation on all the leaves of five plants which was around a total of 10 leaves. The plants were cultivated in a net house under natural photoperiodic conditions. Twenty one days after inoculation, the lesion length formed due to inoculation was measured with a ruler.

Classification of pathotypes or races

The different *X. oryzae* pv. *oryzae* isolates were classified into pathotypes/races based on their virulence on the differential NILs, pyramid lines and cultivated varieties. Reaction showing lesion length <3 cm was considered as resistant and >3 cm was considered as susceptible (Adhikari *et al.*, 1995). The collected *X. oryzae* pv. *oryzae* pathotypes in 2015-2016 was compared based on their reactions against each NILs and the pyramid lines.

Data analyses

Data sets of measured lesion lengths for each set of rice lines or varieties against the selected pathotypes of *X. oryzae* pv. *oryzae* were used to know the effect of single *R*-genes and their combined effects on BB resistance. A dendrogram was constructed based on the reactions (lesion lengths) of each line against the tested isolates to evaluate and compare the discriminating powers of cultivated rice varieties and single-*R*-gene NILs on the *X. oryzae* pv. *oryzae* isolates. Disease severity patterns of cultivated rice varieties and IR24 against the selected *X. oryzae* pv. *oryzae* isolates were compared by using correlation analysis.

RESULTS AND DISCUSSION

Determination of pathotypes of *X. oryzae* pv. *oryzae* field isolates

Pathotypic analyses of 239 *X. oryzae* pv. *oryzae* isolates were conducted based on their reactions against 10 Near Isogenic Lines (NILs) and a total of 8 pathotypes (I-VIII) were recorded. Among 8 pathotypes, pathotypes IV and V considered as major, containing maximum number of isolates, (30.13% and 23.01% respectively) whereas pathotype VIII considered as minor, containing only 2.51% isolates recorded from Khulna and Barishal districts. Pathotype I showed highest virulence or aggressiveness compatible to all NILs, whereas pathotype VIII exposed lowest virulence incompatible to NILs. One study conducted by Suparyono *et al.* (2003) that at panicle initiation and tillering growth stages the most influential bacterial pathotype was VIII and at maturity, the most eminent was pathotypes III and IV. Pathotype VIII also exhibited a virulence reactions to almost all *R*-genes except *Xa1, Xa4* &*Xa5*. Pathotype II performed better virulence by showing maximum compatible reaction to NILs. Pathotype II showed a virulence reaction only with *Xa1, Xa8* and *Xa21*. Pathotypes III performed a virulence interactions to 4 *R*-genes and showed virulence reactions to *Xa4, Xa5, Xa8, Xa13, Xa14* & *Xa21*. The major pathotype IV exposed virulence reactions to *Xa2, Xa4, Xa8, Xa10, Xa14* and *Xa21*, whereas

Table 2. Reactions of *X. oryzae* pv. *oryzae* on *Xa* gene pyramid lines.

| Pathotype[b]	Pyramid lines[a]										
	IRBB52 (Xa4+Xa21)	IRBB53 (Xa4+Xa21)	IRBB54 (Xa5+Xa21)	IRBB57 (Xa4+Xa5+Xa21)	IRBB58 (Xa4+Xa13+Xa21)	IRBB59 (Xa5+Xa13+Xa21)	IRBB60 (Xa4+Xa5+Xa13+Xa21)	IRBB61 (Xa4+Xa5+Xa7)	IRBB63 (Xa5+Xa7+Xa13)	IRBB64 (Xa4+Xa5+Xa7+Xa21)	IRBB65 (Xa4+Xa7+Xa13+Xa21)
I	S[d]	R[c]	S	R	S	S	S	R	R	S	S
II	S	S	S	R	S	R	S	S	S	S	R
III	S	S	S	S	R	S	S	S	R	R	S
IV	R	S	S	S	R	S	S	S	R	R	R
V	R	S	S	S	R	R	S	S	R	S	R
VI	R	S	S	R	S	S	S	S	R	R	R
VII	R	R	R	R	R	R	S	S	R	R	S
VIII	S	R	R	R	R	R	R	S	S	R	S

[a]The *Xa* genes that are present in the gene pyramid line is indicated in parentheses; [b]Based on mean lesion length, the nature of responses was classified into susceptible (S) (above 3 cm), and resistant (R) (up to 3 cm); [c]The gene pyramid line exhibits less resistance than the lines having the single 'R' genes.

pathotypes V showed virulence reactions to *Xa2*, *Xa5*, *Xa8*, *Xa13*, *Xa13* and *Xa14*. Pathotypes VI exhibited virulence reactions to *Xa2*, *Xa4*, *Xa5*, *Xa11* and *Xa13*, whereas VII pathotypes showed virulence reactions with *Xa4*, *Xa10*, *Xa13* and *Xa14*. Similarly, all other pathotypes showed distinct virulence and a virulence reactions to each other with NILs tested (Table 1). From 1980s the highest exploited *R*-genes are *Xa4*, *Xa7*, *Xa3* and *Xa21* for rice breeding purposes to enhance BB resistance. The most utilized R genes are *Xa3*, *Xa4*, *Xa7*, and *Xa21* for rice breeding to improve BB resistance from the 1980s (Wang *et al.*, 2020). Moreover, a crucial factor was analysed by a number of researchers named gene for gene theory exhibiting pathogen and host interaction (Alam *et al.*, 2016) and a similar approach was conducted by Wolfe *et al.* (1976); Browder and Eversmeyer, (1977); Lebeda *et al.* (1982), based on gene for gene theory. Dynamic changes of *X. oryzae* pv. *oryzae* field isolates were also observed in Bangladesh by Khan *et al.* (2009); Alam *et al.* (2016); Rashid *et al.* (2021). This occurred due to expansion of overused a number of resistance genes which also triggered completely unknown pathogenic races with an increasing rate of 1.64 times compared to the level of virulence in the particular isolate following resistance host-plant selection pressure (Nayak, 1986). Another study conducted by focused that the diversity in Vietnam exhibited due to comparatively wide rage of diverse rice cultivars which was traditional and also been suggested that cultivar difference plays a crucial role to make the changes in the *Xoo* pathogen structure (Furuya *et al.*, 2012) and breakdown of *R*-genes was detected when these genes have been applied frequently for a number of years in a gigantic population (Chen *et al.*, 2020). A hypothesis was adopted by Bai and Shi, (1993) and Leung *et al.* (1993) that applying cultivars throughout many decades share the similar genetic background with resistance genes, however, in China they proposed that planting susceptible hybrid line as well as signification infection by leaf blight pathogen, besides, cultival diversity and mass propagation of the bacteria, all are the potential causes of frequently diverse virulence mutation (Li *et al.*, 2009). Thus, these incidents will enhance the possibility of population of pathogens structure through mutation or modification to adapt itself within unfavorable environmental changes or in the new host plant which is resistance.

District-wise distribution of *X. oryzae* pv. *oryzae* strains collected from thirty districts of Bangladesh

The frequent distributions of major pathotypes within each district along with total number of isolates are shown. Maximum Four (4) pathotypes were recorded from Dinajpur, Gaibandha, Natore, Bhola and Habigonj districts, whereas only single pathotype was recorded from Jamalpur, Moulovibazar, Gazipur and Manikganj districts. Three (3) pathotypes were recorded from Thakurgaon, Nilphamari, Bogura, Mymensingh, Netrokona, Jhalokhati, Perojpur, Bagerhat, Satkhira, Feni, and Loxmipur. Two (2) pathotypes were recorded from Panchagar, Rangpur, Rajshahi, Sirajganj, Sherpur, Tangail, Pabna, Jessore, Jhenaidah, Gopalganj, Barishal, Khulna, Sylhet, Sunamganj, Cumilla,
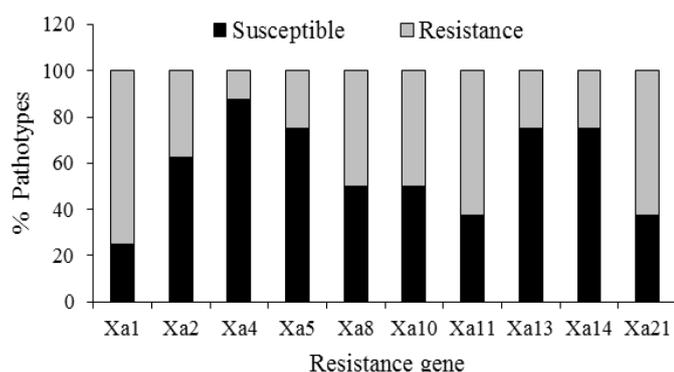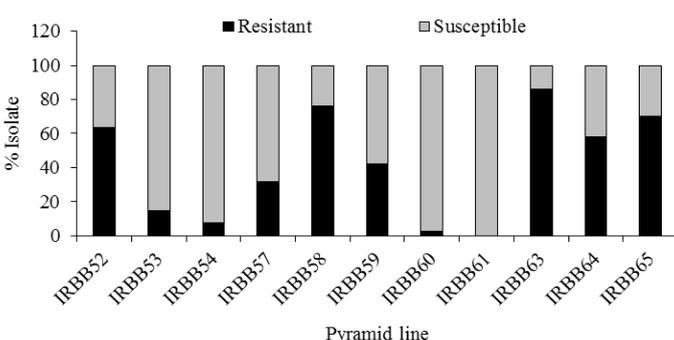
Figure 2. *Performance of Xa genes against X. oryzae pv. oryzae pathotypes. The X-axis indicates Pathotypes and the Y-axis indicates the frequency of pathotypes against which these genes confer resistance. Reactions showing lesion length <3 cm were considered as resistant (R) and >3 cm were considered as susceptible (S).*
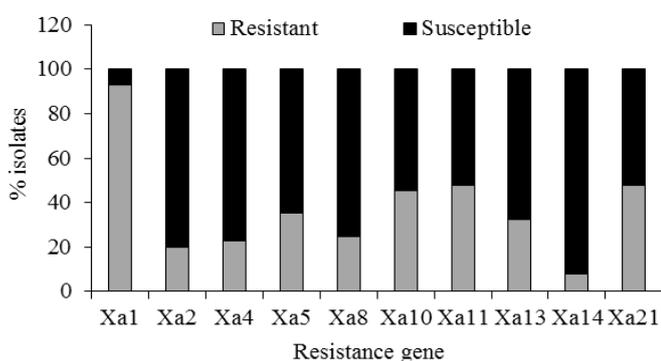
Figure 3. *Performance of Xa genes against X. oryzae pv. oryzae isolates. The X-axis indicates Pathotypes and the Y-axis indicates the frequency of pathotypes against which these genes confer resistance. Reactions showing lesion length <3 cm were considered as resistant (R) and >3 cm were considered as susceptible (S).*
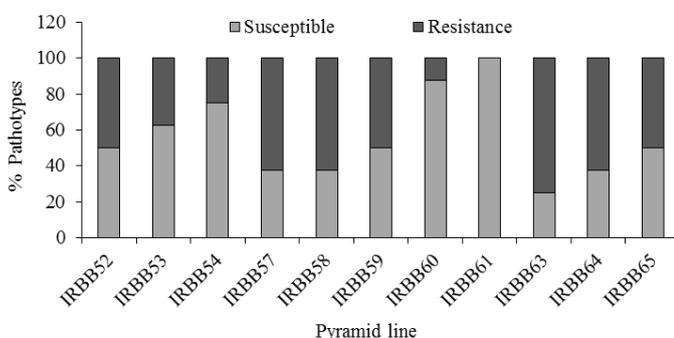
Figure 4. *Performance of Xa gene combinations against X. oryzae pv. oryzae pathotypes. The X-axis indicates Pathotypes and the Y-axis indicates the frequency of pathotypes against which these genes confer resistance. Reaction showing lesion length <3 cm was considered as resistant (R) and >3 cm was considered as susceptible (S).*

Figure 5. *Performance of Xa gene combinations against X. oryzae pv. oryzae isolates. The X-axis indicates Pathotypes and the Y-axis indicates the frequency of pathotypes against which these genes confer resistance. Reaction showing lesion length <3 cm was considered as resistant (R) and >3 cm was considered as susceptible (S).*

Chandpur, Brahmanbaria, Chattagram, Khagrachari and Narsingdi. Others pathotypes also frequently distributed in all surveyed 40 districts recorded from both irrigated and rainfed seasons 2016–2017 (Figure 8). This relationship formed due to a high level of DNA polymorphism within all isolates which were collected district wise. Islam *et al.* (2016) also conducted similar approaches reporting 17 molecular lineages were detected from 96 *Xoo* isolates under 10 clusters covering 19 several geographic districts.

Hypersensitive Reaction (HR) test

The representative isolates of *X. oryzae* pv. *oryzae* collected major growing areas in Bangladesh were tested for hypersensitivity reaction (HR) test on tobacco plant (cv. MT 95). Tobacco leaves infiltrated with bacterial suspension induced a distinct yellow zone at the spreading edge of the lesion. Tobacco leaves infiltrated with sterile water were maintained as control and found not infected. Slight localized chlorosis followed by necrosis and collapse of whole tissue was evident in HR positive isolates. The result showed that all the 8 tested pathotypes were able to induce HR (death of local cells of tissues) between veins of tobacco leaves within 24 hrs (Figure 6). One study exposed by Li *et al.* (2009) that tested *X. oryzae* pv. *oryzae* strains had lessened their capability to engender a non-host HR (hypersensitivity reaction) when experimented with a syringe inoculation without needle in *Nicotiana tabacum* L as well as in Philippines same kind of experiment was also excecuted with other strains (Gonzalez *et al.*, 2007).

### Confirmation of *X. oryzae* pv. *oryzae* by PCR

To test the specificity of the pathogen, amplification was carried out with genomic DNA of all the 8 representative isolates and negative control was maintained with nuclease free water instead of DNA template. Results showed that the primers XOR-F and XOR-R2 amplified a specific DNA fragment in the size of 470-bp with DNA of all *X. oryzae* pv. *oryzae* isolates (Figure 7). These results are in accordance with the PCR confirmatory test of *X. oryzae* pv. *oryzae* by using primers XOR-F and XOR-R2 as reported by Adachi *et al.* (2000).

### Performance of *R* genes against *X. oryzae* pv. *oryzae* pathotypes

Considering the performance of individual *R*-genes against the *X. oryzae* pv. *oryzae* pathotypes, *R*-genes *Xa1* (75.00%), *Xa11*, *Xa21* (62.50% each) and *Xa8*, *Xa10* (50.00% each) performed better resistance followed by *Xa2* (37.50%), *Xa5*, *Xa13* and *Xa14* (25.00% each), whereas *Xa4* (12.50%) containing lowest resistance value. *Xa1* gene showed resistance to around 75.00% of total pathotypes followed by *Xa11*, *Xa21*, *Xa8* and *Xa10* while *Xa4* showed resistance to only around 12.50% of pathotypes (Figure 2). Over time *Xa4* gene might become ineffectual in rice producing countries of South Asia, thus it's quite substantial to pyramid this gene with other striking genes like *Xa5*, *Xa13*, *Xa21* and *Xa7* (Khush *et al.*, 1989). Numerous studies revealed that resistance denotes by *Xa8* varies within the areas of China (Li *et al.*, 2009; Liu *et al.*, 2007) and almost 85% virulent on *Xa8*

Figure 6. *Representative photographs showing hypersensitive response (HR) test on tobacco leaves (cv.) of different X. oryzae pv. oryzae pathotypes. Infiltrated tobacco leaves with sterile water (A) and with bacterial cell suspension of X. oryzae pv. oryzae (B).*
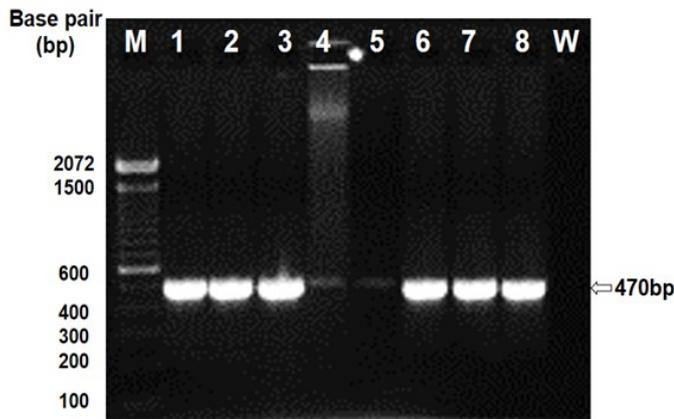
Figure 7. *Agarose gel electrophoresis of PCR products amplified from X. oryzae pv. oryzae using primer XOR-F and XOR-R2. Lanes 1-8 are the PCR products of isolates BDXO91, BDXO68, BDXO34, BDXOS6, BDXO210, BDXO251, BDXO309 and DXO0319 control W (Lane 9) respectively. The molecular size marker is a 100-bp DNA ladder (Invitrogen).*
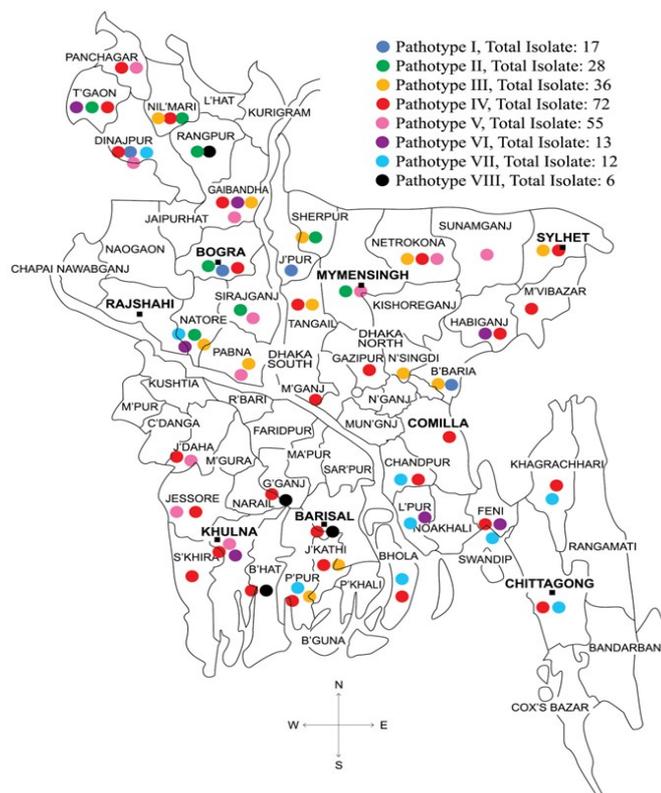
Figure 8. *Distribution of X. oryzae pv. oryzae pathotypes in the 40 districts covering 30 Agro-ecological Zones (AEZs) of Bangladesh.*

found from 33 strains, suggesting that *Xa8* cannot provide effectual against BB disease of rice in Fangchenggang, China. In case of individual *R*-genes, *Xa21* depicted wider resistance against 88% of *X. oryzae* pv. *oryzae* isolates in India (Mishra *et al.*, 2013). When screening genotypes of 57 basmati rice, *Xa5*, *Xa7*, Xa4 and *Xa13* detected for resistance activity and chronicity of *Xa7* in the experimental phase (Ullah *et al.*, 2012), and same outcomes found has also been confirmed by Jeung *et al.* (2006) in Korea, Khan *et al.* (2012) in Pakistan and Shanti *et al.* (2001) in eastern India and these findings were quite matched in our studies.

Performance of *Xa* genes against *X. oryzae* pv. *oryzae* isolates
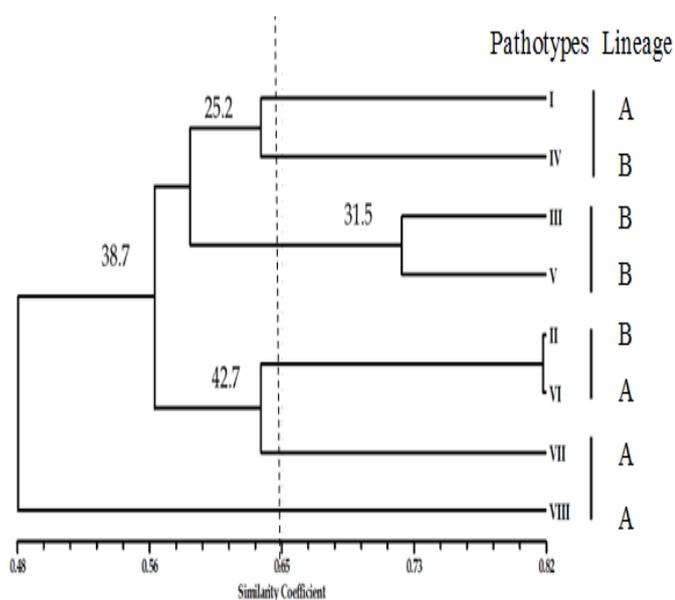
Considering the performance of individual *R*-genes against the *X. oryzae* pv. *oryzae* isolates, *R*-genes *Xa1* (92.89%), *Xa11*, *Xa21* (47.70% each) and *Xa10* (45.61%), *Xa5* (35.15%) performed better resistance followed by *Xa13* (32.64%), *Xa8* (24.69%), *Xa4* (23.01%) and *Xa2* (20.08%), whereas *Xa14* (7.95%) containing lowest resistance value. Xa1 gene showed resistance to around 92.89% of total isolates and *Xa14* showed resistance to only around 7.95% of total isolates (Figures 3). The IRBB-MR (pyramid lines) + IR24 containing *R* genes (*Xa4*, *Xa5*, *Xa7*, *Xa13*, and *Xa21*) in different combinations exhibited higher resistance levels than IRBB-NILs. In a number of countries *Xa21* gene was also manifested extensive resistance significantly (Song *et al.*, 1995; Wang *et al.*, 1996) and in Fangchenggang, China *Xa5* gene depicted efficacious activity in case of resistance (Yang *et al.*, 2012) and *Xa21* unveils much utter resistance than rest of the *R*-genes (Li *et al.*, 2009). Considering isolates, IRBB63 showed resistant to around 85.77% of total isolates followed by IRBB58, IRBB64, IRBB65 and IRBB52 while IRBB60 and IRBB61 showed susceptibility to more than 95% of total isolates. These results are agreement with the findings of Li *et al.* (2001). They reported that there is a strong interaction leading to increased resistance between *Xa13* and *Xa5* and between either of them *Xa4* or *Xa21*.

Isolates in Bangladesh, Nepal, Pakistan, Korea and Sri Lanka exhibited on *Xa21* due to having mutation in pathogen of avr*Xa21* gene (Lee *et al.*, 1999) and developing wider and persistent impact (Jeung *et al.*, 2006). In this study we observed the interaction effect between combined *R*-gene and pathotypes on lesion length was statistically significant. Numerically, the highest (12.50cm) lesion length was found in (IRBB60 × pathotypes I) and the lowest (0.66 cm) lesion length was found in (IRBB57 × pathotype I) and (IRBB60 × pathotype VIII). Khan *et al.* (2010) reported that the IRBB65 (*Xa4*, *Xa7*, *Xa13* and *Xa*21) was the best among the tested entries for gene pyramiding against the BB pathogen in Bangladesh. Jeung *et al.* (2006) found that the pyramid line containing gene *Xa4*, *Xa5* and *Xa21* would be the most promising and valuable genotype for improving Korean cultivars for BB resistance. These increased resistance levels might be due to the synergistic effects of the *R*-genes, and each *R*-gene combination expressed a unique genetic effect against the corresponding *X. oryzae* pv. *oryzae* isolates. The pathotypic reactions of eight pathotypes against

11 pyramided lines have been shown in Table 2. Pathotypes I, II and III showed that highest virulence or aggressiveness compatible with all pyramided lines, whereas pathotype VII and VIII exposed lowest virulence to pyramided lines. Pathotype VIII also exhibited a virulence reactions with almost all *R*-genes except IRBB52, IRBB61, IRBB63, IRBB65. Pathotype IV, and V showed avirulence reaction only with IRBB52, IRBB58, IRBB59, IRBB63, IRBB64, and IRBB65. Pathotypes VI showed virulence reactions to IRBB53, IRBB54, IRBB59, IRBB60, and IRBB61). Significant reductions in lesion length were not frequently observed between the gene pairs *Xa4* and *Xa5* or *Xa7*, probably because of the effective resistance spectrum of *Xa5*, *Xa7* and *Xa13* against all tested *X. oryzae* pv. *oryzae* isolates (Table 2). Therefore, in the gene pairs *Xa5+Xa7+Xa13* (IRBB63), *Xa4+Xa5+Xa21* (IRBB57), and *Xa4+Xa13+Xa21* (IRBB58), the *R* genes interacted with each other independently and additively. Expression of the three (*Xa5 + Xa13 + Xa21*) gene pyramid as the impact of genetic background expressing resistance was also discussed previously (Yoshimura, 1989; Gautam *et al.*, 2005). Inoculation experiments on IR24 pyramid lines with a newly evolved field isolates showed high susceptibility to bacterial blight in IRBB61 (*Xa4+Xa5+Xa7*), IRBB60 (*Xa4+Xa5+Xa13+Xa21*), IRBB54 (*Xa5+Xa21*), and IRBB53 (*Xa4+Xa21*), and highly resistance to IRBBB63 (*Xa5+Xa7+Xa13*), IRBB57 (*Xa5+Xa5+Xa21*), IRBB58 (*Xa4+Xa13+Xa21)*, and IRBB64 (*Xa4+Xa5+Xa7+Xa21*). Around 2 -5 resistance genes from 16 gene pyramids derived in 10 NILs (near isogenic lines) and IRBB lines were detected against 61 isolates that *Xa21* and *Xa13* genes accorded resistance to more than average *Xoo* isolates in Pakistan (Khan *et al.*, 2012). A combination of three *R*-genes *Xa1*, *Xa3*, and *Xa4* in promising lines could lead to a significant outcome controlling BLB in suitable areas of Mali reported by Tekete *et al.* (2020)

Figure 9. *Phenogram of X. oryzae pv. oryzae strains based on virulence to 11 near isogenic lines containing a single gene for resistance. A data matrix was generated for the virulence data by scoring avirulence as 0 and virulence as 1. From these data, a similarity matrix was derived with the SIMQUAL program (NTSYSpc, version 2.02i, Exeter Biological Software, Setauket, NY) using Jaccard's coefficient of similarity. A phenogram was reproduced by the unweighted pair group method for arithmetic average in the SAHN Program.*

Pyramiding line bearing *Xa2*, *Xa4*, *Xa5* would be the highest fruitful genotype for developing Korean cultivars against BB resistance (Jeung *et al.* 2006) as similar to our findings. IRBB63 showed resistance to around 75% of total pathotypes followed by IRBB57, IRBB58, IRBB64 (62.50% each) and IRBB65, IRBB52 (50.00% each), while IRBB61 showed susceptibility to all of the pathotypes followed by IRBB60, IRBB53, IRBB54. Other lines showed moderate performance. Considering the performance of pyramid lines against the *X. oryzae* pv. *oryzae* isolates, pyramid lines IRBB63 (85.77%), IRBB58 (76.15%), IRBB65 (70.29%), IRBB52 (63.60%) and IRBB64 (58.16%) performed better resistant followed by IRBB59 (42.26%), IRBB57 (31.80%), IRBB53 (14.64%) whereas IRBB61 (1%), IRBB60 (2.51%), containing lowest resistance value. IRBB63 showed resistant to around 85.77% of total isolates followed by IRBB58, IRBB65, IRBB52 and IRBB64 while IRBB61 and IRBB60 showed susceptibility to more than 98% of total isolates (Figures 4 and 5).

Overall outcomes from one study suggested that several isolates on IRBB lines revealed the sequential order of resistance genes based on their effectiveness was *Xa4~Xa4 + Xa5~Xa4 + Xa21~Xa4 + Xa5 + Xa13 + Xa21 > Xa5 + Xa13 > Xa21 > Xa4 + Xa13 > Xa7 > Xa4 + Xa13 + Xa21* (Gautam *et al.*, 2015). However, in Punjab province of Pakistan, *Xa4 + xa13 + Xa21* followed by *Xa5 + Xa21, xa13 + Xa21, Xa4 + Xa21, Xa4 + Xa13* and *Xa4 + Xa5* were found effective in combating the *Xoo* pathogen (Khan *et al.*, 2012). With results on major aspects of our quantitative observations and statistical interpretation strong and broad resistance of the *Xa1*, *Xa11*, *Xa21* gene and combined *R*-gene (*Xa5+Xa7+Xa13*) and a strong quantitative complementation effect between the *Xa4*, *Xa5* and *Xa13* genes deduced that the *R* -gene pyramid of *Xa1*, *Xa5*, *Xa7*, *Xa11* and *Xa21* would be the most prospective genotype for improving Bangladeshi cultivars for BB resistance. A comparison of resistance spectra between the NILs with (*Xa5 + Xa7 + Xa13*) gene combinations and rice lines with known *R*-genes that have been used as genetic sources for improving BB resistance by Bangladeshi breeders also confirmed the effectiveness of the three *R*-gene pyramid against the BB population of Bangladeshi represented by the eight *X. oryzae* pv. *oryzae* pathotypes. Considering the above facts, *Xa1*, *Xa11* and *Xa21* genes (including *Xa5*, *Xa7*, and *Xa13*) may recommend for breeding rice cultivars resistant to BB disease in Bangladesh. The problems of understanding the biology of *X. oryzae* pv. *oryzae* and controlling bacterial blight have long been challenging. The known geographic distribution of the haplotypes provides a baseline for monitoring the future spread of *X. oryzae* pv. *oryzae* in other rice producing areas in Bangladesh.

Relationship between pathotypes and haplotypes of *X. oryzae* pv. *oryzae* in Bangladesh

A phenogram was constructed based on the virulence of *X. oryzae* pv. *oryzae* isolates to the 10 Near Isogenic Lines (NILs) of rice containing single gene for resistance to interpret the relationship between the pathotypes and the molecular

haplotypes (Figure 9). In the phenogram, constructed based on the findings of inoculation where, four haplotypes were observed at 63% similarity level. Among these haplotypes, haplotypes II considered as major which comprises number of pathotypes III, V) containing 91 isolates out of 239. Haplotype I contained number of isolates (89) belongs to pathotypes I and IV. Haplotype III comprises 2 pathotypes (II and VI) containing 41 isolates and haplotype IV represented by pathotypes VII, VIII, diverse than other haplotypes. From the phenogram, i) group of *X. oryzae* pv. *oryzae* isolates from different populations with same pathotypes showed multiple haplotypes, ii) group of *X. oryzae* pv. *oryzae* isolates from different populations had identical haplotypes in multiple pathotypes and iii) group of *X. oryzae* pv. *oryzae* isolates from different populations had identical pathotypes and haplotypes. These pathogenic strains showed an intricate relationship between molecular haplotypes and pathotypes as similar to the experimentation of Adhikari *et al*. (1999); Nelson *et al*. (1994); Ochiai *et al*. (2000) and Yashitola *et al*. (1997). Adhikari *et al*. (1999) delineated those 31 molecular haplotypes were found from 171 strains from 8 rice producing areas in Nepal by reacting under two PCR based assays, among them three were found highest intermittent withing a vast range of geographical population and some of them were dispersed geographically. Furthermore, Shanti *et al*. (2001) revealed that several haplotypes positioned in same pathotype belonging to several isolates and they also pointed out the genetic and pathotypic modification exits within the *X. oryzae* pv. *oryzae* isolates. Genetically similar haplotypes were identified from several districts of Bangladesh speculating due to national distribution or regional dispersion of contaminated germplasm (Islam *et al*., 2016) and same projection was speculated by George *et al*. (1997).

Conclusion

Potential *R*-genes viz. *Xa1, Xa4, Xa5, Xa7, Xa11, Xa13* and *Xa21* against the *X. oryzae* pv. *oryzae* Bangladeshi pathotypes can be deployed in a suitable combination in the promising rice varieties through pyramiding for example *Xa5+Xa7+Xa13* or *Xa5+Xa7+Xa2* or *Xa4+Xa5+Xa7* or *Xa4+Xa5+Xa13+Xa21* or *Xa5+Xa21* or *Xa4+Xa21* in developing BB resistant rice varieties in both rainfed and irrigated seasons of Bangladesh. However, frequent monitoring of the i) performance of these '*R*' genes against BB and ii) pathotypic changes of *X. oryzae* pv. *oryzae* population would be crucial to manage BB successfully through national rice resistant breeding programs under the global climatic change's scenario in the forthcoming years for ensuring food security in Bangladesh.

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