PHENOTYPIC AND MOLECULAR ASSESSMENT OF BLB RESISTANT ADVANCED RICE LINES AND THEIR YIELD PERFORMANCE

MD. JANNATUS SAKIB

DEPARTMENT OF PLANT PATHOLOGY
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA-1207

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BY

MD. JANNATUS SAKIB
REGISTRATION NO. 13-05697

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Approved by:

Dr. M. Salahuddin M. Chowdhury **Professor** **Department of Plant Pathology** **Sher-e-Bangla Agricultural University** **Supervisor**	**Dr. Tahmid Hossain Ansari** **Principle Scientific Officer** **Plant Pathology Division** **Bangladesh Rice Research Institute** **Co-Supervisor**

Dr. Fatema Begum
Chairman
Examination Committee
Department of Plant Pathology
Sher-e-Bangla Agricultural University

Dr. M. Salahuddin M. Chowdhury
Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207
Contact: 01713 382588

CERTIFICATE

This is to certify that the thesis entitled, **"PHENOTYPIC AND MOLECULAR ASSESSMENT OF BLB RESISTANT ADVANCED RICE LINES AND THEIR YIELD PERFORMANCE"** *submitted to the Department of Plant pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural university, Dhaka, in partial fulfillment of the requirement for the degree of* **MASTER OF SCIENCE IN PLANT PATHOLGY** *embodies the results of a piece of bona fide research work carried out by* **MD. JANNATUS SAKIB** *bearing Registration No.* **13-05697** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma elsewhere in the country or abroad.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: 20 December, 2020

Place: Dhaka, Bangladesh

(Dr. M. Salahuddin M. Chowdhury)

Professor
Department of Plant Pathology
Supervisor

Dedicated to my
beloved parents and
respected teachers

ABBREVIATIONS

Full Word	Abbreviation
and others	*et al.*
base pair	bp
Centimeter	cm
Degree Celsius	°C
Deoxyribonucleic acid	DNA
Gram	g
Hydrogen ion concentration	pH
Insertion Deletion	InDel
Kilobase	Kb
Liter	L
Location severity index	LSI
Microliter	µL
Milliliter	mL
Millimeter	mm
Millimolar	mM
Molar	M
Namely	*viz.*
Nanogram	Ng
Percent	%
Polymerase chain reaction	PCR
Polymorphic information content	PIC
Revolutions per minute	Rpm
That is	i.e.
Volt	V
Revolutions per minute	Rpm
ANOVA	analysis of variance
BLB	bacterial leaf blight
CF:	colony forming units
DFF	days to 50% flowering
FG/P	filled grains per panicle
G	genotype
GW	grain weight
LSD	least significant difference
MAS	marker assisted selection
SES	Standard Evaluation System for Rice

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The Author

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PHENOTYPIC AND MOLECULAR ASSESSMENT OF BLB RESISTANT ADVANCED RICE LINES AND THEIR YIELD PERFORMANCE

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ABSTRACT

Four of *Xanthomonas oryzae pv. oryzae* isolates viz. Iso1 to Iso4 were isolated from different diseased samples collected from BRRI during T. Aman 2019 season. Isolates phenotypic characters e.g, colony color, shape, growth patterns were examined and found similar. Typical slimy and cream colored bacterial growth was observed in peptone sucrose agar medium for all four isolates. Molecular markers were used to confirm the *Xanthomonas oryzae pv. oryzae* isolates and two isolates namely Iso1 and Iso4 were confirmed as *Xoo.* Five BLB resistant advanced breeding lines, P-3-58, P-5-1, P-7-70, P-7-82 and P-8-78 were tested against *Xoo* with susceptible check variety Purbachi and all five advanced lines showed resistance reaction. These five advanced lines were molecularly screened for four BLB resistance genes *Xa4, xa5, xa13* and *Xa21*using four molecular markers *MP*, *xa5* Multiplex, *xa13*-prom and *pTA248*. Among the advanced lines, P-5-1, P-7-70, P-7-82 and P-8-78 possessed *Xa4* gene and P-3-58, P-5-1, P-7-70 and P-8-78 possessed *xa5* gene. A field experiment was set with five advanced lines along with one susceptible check (Purbachi) and two standard checks (BRRI dhan39 and BRRI dhan49) to find out the BLB resistant advanced line. All the selected lines showed good performance in terms of BLB resistance and yield. The highest yield (3.32 t ha^{-1}) was recorded in P-7-70. Considering all other agronomic traits and resistance reaction, P-7-70 line can be used in further BLB resistance variety development programs.

CHAPTER-1

INTRODUCTION

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops in the world under the family Poaceae (USDA-NRCS, 2019). Around a third of the world population depends on rice as an important source of energy (Muthayya *et al*., 2014). Bangladesh is one of the major producers of rice and currently it is the fourth-largest producer among the world (FAO, 2018).

Rice is the staple food of the peoples of Bangladesh. This country is used its 75% arable land for rice production and yearly produced 36.3 million tons of rice (BBS, 2018). But still a considerable amount of yield gap is existed in Bangladesh. There are several reasons for these yield gap and biotic stress is one of the main causes. Because of these biotic stresses, yearly around 37% of rice yield is reduced (Mondal *et al*., 2017).

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae pv. oryzae* (*Xoo*) is one of the most serious disease of rice. It occurs at all the growth stages of rice and is manifested by leaf blight symptoms. It is one of the oldest recorded rice diseases and first reported from Kyushu, Japan in 1884 (Tagami and Mizukami, 1962).

Bacterial leaf Blight lead to 16% of global crop yield losses. (Oerke, 2006). BLB limits rice production and can cause up to 81% yield loss in countries like India (Kumar *et al*., 2012). Report from various countries reveals that BLB can cause yield losses of 2.0 to 81.3% (Soga, 1918; Srivastava *et al*., 1966; Mew *et al*., 1993). In Bangladesh, the disease appears apparently every year with different degree of severity (Jalaluddin *et al*., 2005). The introduction of the high yielding varieties (HYV) during mid 1960s is the major cause of BLB spreading in Bangladesh (Adhikari *et al*., 1994a). However, the disease may cause up to 32% yield loss in Bangladesh (Anon, 1988; Adhikari *et al*., 1999a; Farooq and Ahmad, 2007).

Identification of *Xoo* is most important job for culturing and performing other experiments. Identification only based on colony color and other characteristics may mislead the proper detection of the bacteria. In recent years, molecular detection of an organism becomes more popular due to its accuracy and authentication (Khan *et al*., 2004).

Molecular *Xoo* markers is used to identify the unique regions of *Xanthomonas oryzae*. Several molecular marker has been developed for the detection of *Xoo* bacteria. For accurate identification of pathogen precise diagnostic tools is actually important for regulatory reason. The *Xanthomonas oryzae pv. oryzae* specific primer is *Xoo80, Xoo3350, Xoo4009. Xoo80* amplified all reported *X. oryzae* strains including weakly pathogenic strains of texas and lousiana, USA. But it does not amplify any resistant band in USA strains. It amplifies resistant band only in Asian strains. (Jones *et al*., 1959). *Xoo3350* and *Xoo4009* are not normally used for multiplex analysis, because they normally amplified Asian strain specially Philippines strain. (Lang *et al.,* 2010).

Some efficient functional markers are developed for *xa13* and *xa5* (Hajira *et al*., 2016). IRBB60 (*Xa4* + *xa5* + *xa13* + *Xa21*) conveyed effective resistance against tested isolates. (Gautam *et al*., 2014). *Xa21* gene is most effective against BLB in south and south east Asia. It is first discovered in the wild species *Oryza longistaminata*. The marker *pTA248* specific for the resistant allele of *Xa21* developed by Ronald *et al* (1992) was used as the functional marker for the gene (Perumalsamy *et al.*, 2010; Salgotra *et al*., 2012). The *Xa4* and *xa5* genes were identified by (Petpisit *et al*., 1977). *Xa4* gene is most effective against BLB in Philippines. The marker *MP* specific for the resistant allele of *Xa4* developed by Sun *et al*., 2003). It is first discovered in primer RFLP marker G181 which is developed by (Yoshimura *et al*., 1995) to detect *Xa4* gene. *xa5* gene is most effective against BLB in Philippines. The marker *xa5* Multiplex specific for the resistant allele of *xa5* developed by Sundaram *et al*., (2011) and Chu *et al.,* (2006). The *xa13* gene was first discovered in the rice variety BJ1 and this gene

specifically confers resistance to the Philippine *Xoo* race (Bhatia and Vikal, 2017).

Plant pathology division of Bangladesh Rice Research Institute (BRRI) developed some bacterial blight resistance advance lines through head to row selection. Some of these lines are excellent BLB resistant but unknown about the resistance genes it possessed. Detection of resistance genes using molecular marker from these advanced lines can answer the host pathogen interaction process among these lines. Besides the lines can be a potential source of resistance genes which can utilize for durable BLB resistant variety. Present research was therefore carried out with the following objectives:

Objectives:

1. To isolate and identify the *Xanthomonas oryzae pv. oryzae* through phenotypic characteristics and molecular markers.
2. To screen out presence of resistance genes in selected BLB resistant advanced lines at BRRI using linked molecular markers.
3. To evaluate the yield performance of the selected resistant advanced lines.

CHAPTER-2

REVIEW OF LITERATURE

CHAPTER II
REVIEW OF LITERATURE

Alejendra *et al.* (2020) reported that effective and durable disease resistance for bacterial blight (BB) of rice is a continuous challenge due to the evolution and adaptation of the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), on cultivated rice varieties. Fundamental to this pathogens virulence is transcription activator-like (TAL) effectors that activate transcription of host genes and contribute differently to pathogen virulence, fitness or both. Host plant resistance is predicted to be more durable if directed at strategic virulence factors that impact both pathogen virulence and fitness. Of 18 QTL revealed by genome-wide association studies and interval mapping analysis, six were specific to Tal7b (qBB-*tal7b*). Overall, 150 predicted Tal7b gene targets overlapped with qBB-*tal7b* QTL. Of these, 21 showed polymorphisms in the predicted effector binding element (EBE) site and 23 lost the EBE sequence altogether. Inoculation and bioinformatics studies suggest that the Tal7b target in one of the Tal7b-specific QTL, qBB-*tal7b*-8, is a disease susceptibility gene and that the resistance mechanism for this locus may be through loss of susceptibility.

Nino *et al*. (2020) concluded that bacterial blight is a common disease found in the rice-growing regions in the Korean peninsula. Identification of the gene network involved against *Xanthomonas oryzae pv. oryzae* Korean race K2 in popular japonica cultivars is essential in molecular mechanism of resistance. Microarray of two popular Korean *japonica* rice cultivars, bacterial blight susceptible Dongjin and resistant Jinbaek, was performed to investigate the transcripts of inducible genes at 48 h post-inoculation. A total of 771 differentially expressed genes were identified in Jinbaek, whereas 298 were found in Dongjin. To discover genes essential to bacterial blight resistance in Jinbaek, 13 highly expressed genes encoding different protein classes were cloned and overexpressed in rice. These findings revealed the complexity of key immune signaling conduits critical to mounting a full defense against *Xanthomonas. oryzae* pv. *oryzae* race K2 in japonica rice.

CGIAR (2019) and FAO (2018) reported that rice production and consumption are among the highest in Asian populations. It is the main staple food in the Asia and the Pacific region, providing almost 39% of calories. Bangladesh is the fourth-largest rice producer in the world. Besides, about 160 million people of Bangladesh consume rice as principal food (UNFPA, 2019). Almost all of the 13 million farm families of the country grow rice and about 75% of the total cropped area and over 80% of the total irrigated area is planted to rice (BRKB, 2019).

Shaheen *et al.* (2019) reported that Bacterial blight caused by *Xanthomonas oryzae pv. oryzae (Xoo)* is an important bacterial disease in rice leading to heavy yield and economic losses. They investigated the rice leaf samples from infested regions were screened for *Xoo* strains linked to incidence of this disease. Subsequently, 17 different isolates were identified based on PCR analysis. Moreover, the percentage diseases incidence and weight loss of 1000-grains of each sample from four zones of rice production were recorded. In addition, the molecular recognition by means of a 16S rRNA universal primer revealed DNA amplification in 15 out of 17 isolates which confirmed the pathogen as "*Xoo*". The data assessed for disease incidence in all investigated districts ranged between 70.12%- 49.23%. While, the maximum and minimum weight losses of 17.84% and 11.17% from Sialkot and Narowal were recorded respectively. The application of such tools for *Xoo* detection and its impact on crop yield are contested in this investigation.

Banerjee *et al*. (2018) reported that Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*), is one of the most serious diseases of rice causing a significant yield loss mostly in Asia and parts of Africa and poses a threat to the breakdown of varietal resistance. Development of resistant varieties carrying major resistance (*R*) gene(s) has been the effective way for controlling BB. The type of *R* gene(s) to be deployed depends on the predominant *Xoo* pathotypes. In their experiment, phenotyping for BB resistance was carried out in 210 rice germplasm comprised of released varieties and landraces from eastern and northeastern India. Based on disease scoring, 95

released varieties being categorized into 29 resistant, 42 moderately resistant and 24 susceptible, while, 115 rice landraces were grouped into eight resistant, 38 moderately resistant and 69 susceptible accessions. Molecular screening for the presence and frequency of 10 BB resistance genes was made from a sub set of 70 genotypes, comprising 35 resistant, 21 moderately resistant and 14 susceptible entries. The frequency of *R* genes varied from 0 to 5 per genotype. The most frequent gene was *Xa1* followed by *Xa7* > *Xa4* > *Xa10* > *Xa11*. A few entries such as Nua Kalajeera, Kalinga III, Naveen, CR Dhan 701, Swarna Sub1, Kalajeera, and ARC5791 possessed 3–5 genes. The findings indicated that *Xa1*, *Xa7*, and *Xa11* had been frequently selected in breeding programmes, and the frequency of *xa5*, *Xa8*, *xa13* and *Xa21* should be increased in the released varieties in different combinations to achieve durable resistance.

Christine Jade Dilla-ermita *et al.* (2017) investigated a range of resistance loci against different races of *Xanthomonas oryzae pv. oryzae* (*Xoo*), the pathogen causing bacterial blight (BB) disease of rice, have been discovered and characterized. Several have been deployed in modern varieties, however, due to rapid evolution of *Xoo*, a number have already become ineffective. The continuous "arms race" between *Xoo* and rice makes it imperative to discover new resistance loci to enable durable deployment of multiple resistance genes in modern breeding lines. Rice diversity panels can be exploited as reservoirs of useful genetic variation for bacterial blight (BB) resistance.

Chen *et al*. (2016) concluded that bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most widely distributed and devastating diseases of rice (*Oryza sativa*) in Asia. BLB can cause yield losses ranging from 20 to 30% and as high as 50% (Mew, 1987). Typical BLB symptoms, including tannish gray or white lesions along the veins, were observed in the rice fields in Guangxi, China, in October 2013. Fifteen rice leaves with such symptoms were collected from the infected rice fields. These leaves were cut into approximately 1-cm pieces and homogenized in 9ml of sterile water by grinding after 1% sodium hypochlorite solution treatment.

Diluted homogenates were plated on peptone sucrose agar (PSA) and incubated at 28°C for 3 to 4 days. The non-flat, mucous colonies with yellow, round and smooth margins that developed on the plates were selected for further analysis.

Suryadi *et al.* (2016) stated that the virulence of 15 *Xanthomonas oryzae pv. oryzae (Xoo)* isolates collected in three provinces in Indonesia (North Sumatra, South Sumatra, and South Sulawesi). Eight different pathotypes were present, as demonstrated by a particular virulence pattern of each isolate on the genotypes. Determination of *Xoo* pathotype revealed that *Xoo* pathotypes responded differently based on their reaction to NILs and Indonesian differential genotypes. The field assessment demonstrated the incidence and severity of BLB disease on rice genotypes ranging from 25% to 100% and 5.5% to 72.91%, respectively, while the mean disease index (*Xa*4+*xa*5+*Xa*7+*xa*13+*Xa*21), IRBB7(*Xa*7), Angke (*Xa*4+*xa*5) and Code (*Xa*4+*Xa*7) were revealed to be highly resistant to the BLB pathogen. These genotypes have potential as genetic material for the pyramiding of several resistance genes for the development of rice resistance to BLB disease in Indonesia.

Gautam *et al*. (2014) reported that bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major disease of rice in the tropics for which This study aimed at identification of resistance gene combinations effective against *Xoo* isolates and fingerprinting of the *Xoo* isolates of Andaman Islands (India). of 21 rice BB differentials possessing *Xa1* to *Xa21* genes individually and in different combinations to various isolates of pathogen collected from Andaman Islands. Pathological screening results of 14 isolates revealed that among individual genes tested across 2 years, *Xa4*, *Xa7* and *Xa21* conferred resistance reaction across all isolates, whereas among combinations, IRBB 50 (*Xa4 + xa5*), IRBB 52 (*Xa4 + Xa21*) and IRBB 60 (*Xa4 + xa5 + xa13 + Xa21*) conveyed effective resistance against tested isolates. The nature of genetic diversity among four isolates selected on the basis of geographical isolation in the islands was studied through DNA finger printing. The RAPD primers S111, S119, S1117, S1109, S1103, S109 and S105 were found to be better indicators

of molecular diversity among isolates than JEL primers. The diversity analysis grouped 14 isolates into three major clusters based on disease reaction wherein isolate no. 8 was found the most divergent as well as highly virulent. The remaining isolates were classified into two distinct groups. The importance of the study in the context of transfer of resistance gene(s) in the local cultivars specifically for tropical island conditions is presented and discussed.

Akhtar *et al.* (2011) reported that the disease reduces grain yield to varying levels depending on the stage of the crop, degree of cultivar susceptibility and a great extent to the conduciveness of the environment in which it occurs.

Basso *et al.* (2011) reported that recent studies in West African countries such as Burkina Faso, Niger and Mali revealed the occurrence of BLB causing significant crop damages.

Rani *et al*. (2011) reported that over 1035 rice varieties with varying maturity duration (early, mid-early, medium, and late) were also developed in these released varieties, maturity duration, grain size, appearance, scent and quality, resistance to biotic and abiotic stresses could be tailored to suit consumer preferences and needs.

Kadai (2010) reported that occurrence of bacterial leaf blight in most of rice growing ecozones of Togo with high incidence and severity, and the virulence of the pathogen was determined.

Ali *et al*. (2009) screened out 15 Pakistani rice genotypes and revealed Kashmir Basmati as a highly resistant genotype which showed ≥75% resistance to all the tested strains/isolates of *Xanthomonas oryzae pv. oryzae*, only YR6W14D3 infect the genotype but the severity was not divesting. IR-6, Basmati-370, JP-5 and KSK-370 were ≥50% resistant to all the tested strains, while the remaining genotypes were susceptible to all the strains/isolates of *Xanthomonas oryzae pv. oryzae.* Among the four strains/isolates LKA4 (i) showed 38% severity. As Kashmir Basmati showed resistance to LKA4 (i), so plant breeder can easily

transfer the gene into high yielding and susceptible genotype to enhance the food quality and quantity.

Khan *et al.* (2009) worked on pathogenic diversity of *Xanthomonas oryzae pv. oryzae* in Bangladesh. The collected 41 isolates of *X. oryzae pv. oryzae* from 7 rice growing districts of Bangladesh. The pathotypes of *X. oryzae py. oryzae* were classified by inoculating 9 near-isogenic lines (NILs), each NIL carrying a single specific bacterial leaf blight (BLB) resistance gene (*Xa*-gene) and showed different types of virulence. Six pathotypes were identified based on 9 NILs. Among those, pathotype 1 was virulent to almost all tested NILS except IRBB21 carrying BLB resistance gene *Xa21*. The resistance gene *Xa21* has broader resistance than others. The isolates BXO80 followed by BXO9 showed the most virulent activities on NILS but it was also avirulent to IRBB21. Pathotype 1 constituted 80.5% of the total population of 41 isolates and was predominant across the seven locations of the country. Results indicated that the gene *Xa21* would be there most promising gene for developing BLB resistant varieties in Bangladesh.

Waheed *et al.* (2009) reported that Bacterial leaf blight has the potential to become a destructive bacterial disease of rice in Pakistan and can cause huge losses mainly because of the lack of information regarding the pathogen and its effective measure of control.

Dinh *et al.* (2008) used 41 isolates of *Xanthomonas oryzae pv. oryzae* for inoculation on 10 differential rice varieties containing different single resistant genes. The results showed that the infection responses were clearly compatible and incompatible reactions on differential rice varieties. Gene *xa*5 was highest effective against to disease, the next were *Xa7, Xa21* and *xa13*. Six pathogenic races were identified and their distribution was varied across the provinces in Mekong Delta. Race A, E and F were predominant, they occurred and infected on rice in 6 to 8 provinces, while race B, C and D in 3 to 5 provinces in Mekong Delta.

Dai *et al.* (2007) reported that Bacterial Leaf Blight Disease (BLB), one of the important diseases of rice, known to occur in epidemic proportion in many part of the world, can reduce production by more than 50%.

Kabir *et al*. (2007) exploited bacterial leaf blight (BLB) resistant sources, a total of 14,000 entries were screened from 1980 to 2006 in Bangladesh Rice Research Institute (BRRI) of which 2,241 entries were found resistance to moderately resistance to BLB. Seven entries were found consistently resistant and twenty entries was reconfirmed that could serve as a donor source of developing BB resistance variety. Among the screening IRBB21 showed moderately resistant and stable reactions in IRBB lines. In phenotypic analyses of *Xanthomonas oryzae pv. oryzae* based on 66 morphological, biochemical and physiological traits of 56 strains, no variation was found among the isolates and showed existence of homogeneous populations.

Liu *et al.* (2007) collected 285 isolates of *Xanthomonas oryzae pv. oryzae* randomly from 22 rice-growing provinces in China. They chose 91 representative isolates to assess the differential characteristics of 24 near-isogenic rice lines containing a single resistance gene or two to four genes. Most isolates were avirulent on pyramided lines, except IRBB51, and hence, the pyramided lines cannot be used as differentials for the virulence analysis of *X. oryzae pv. oryzae* in China. The 13 rice lines with a single gene were used further to establish a system of races classification of *X. oryzae pv. oryzae* in China. IR24 and IBBB10 were susceptible to the isolates with several exceptions, whereas IRBB5, IRBB7 and IRBB21 were resistant. Based on the interactions between the isolates of *X. oryzae pv. oryzae* and the 13 near-isogenic rice lines, six single-gene rice cultivars (IRBB5, IRBB13, IRBB3, IRBB14, IRBB2 and IR24) were chosen as differentials and the 285 tested isolates were classified into nine races.

Yu *et al*. (2006) demonstrated the pathogenicity of 36 isolates of *Xanthomonas oryzae pv. oryzae* (*Xoo*), which were collected from japonica rice cultivars in

the Yunnan Plateau, China. Pathogenicity was evaluated on 29 rice cultivars including a set of seven cultivars (i.e., Haonuoyang, TNT, Kogyoku, Zhenzhuai, IR26, Nanjing 33 and Kinmaze) to identify pathogenicity, which may be considered as a set of differential cultivars for *Xoo* races from Yunnan japonica rice. The efficiency of the seven cultivars was further confirmed. The results showed reversible and specific interactions between isolates and cultivars. The isolates were classified into nine pathotypes (pathotypes I to IX) according to their pathogenic reactions on the seven rice cultivars. Pathogen VII was the most pathogenic. Most japonica cultivars grown in the Yunnan Plateau were susceptible to *Xoo*. The rice lines IRBB21 (*Xa*-21), Zhachanglong (*Xa*-22t, *Xa*-24t), and IR1545-339 (*xa*-5), which were resistant to all the isolates tested, can be used as donors of resistant genes for bacterial blight in japonica rice breeding in the Yunnan Plateau.

Yang *et al.* (2006) reported that the diversity of *Xanthomonas oryzae pv. oryzae* virulence was found in China. Some 108 and 177 strains of *Xoo* were collected from China during 1970-92 and 2003-04, respectively. The virulence variation of 285 strains was tested by the clipping method using 13 rice near isogenic lines with single resistance gene. The variation in virulence and races of *Xoo* population were compared systemically, and based on the results, the causes of virulence variation were analysed.

Bharathkumar-Srinivasan and Gnanamanickam (2005), determined the new sources of resistance to *Xanthomonas oryzae pv. oryzae (Xoo),* wild rice plants, *Oryza malampuzhaensis* and *O. rufipogon* were collected from Tamil Nadu and Kerala, India, respectively. To determine their resistance to *Xoo*, fully grown leaves of the wild rices were clip-inoculated with diagnostic *Xoo* strains PX061 (race I strain that detects the presence of *Xa*4), PX099 (race 6 strain that detects the presence of *Xa*21) and 50 strains of *Xoo* from southern India. Control plants of IR24 and IRBB21 (*Xa*21) and IRBB4 (*Xa*4) rices were also inoculated. *O. malampuzhaensis* and *O. rufipogon* were resistant to *Xoo* strain PX061 and

susceptible to PX099, indicating the absence of *Xa*21 in both wild rice. *O. malampuzhaensis* was susceptible to all 50 pathogen strains from southern India. On the other hand, *O. rufipogon* showed uniform resistance to all 50 strains from southern India, indicating that it has a major R-gene different from *Xa*21. Marker assisted selection on leaf DNA, using a sequence tagged site marker (U 1/11) specific to the *Xa*21 locus, was used to detect polymorphisms between IRBB21 and *O. rufipogon*. Banding profiles of IRBB21 (band at 1.4 kb) differed from those observed in *O. rufipogon* (band at 1.3 kb). In fulther analysis of PCR-based polymorphisms, the R-gene that contributes to BB resistance in *O. rufipogon* appears to be distinct from *Xa*21, suggesting that it is perhaps *Xa*23(t), based on the observed DNA polymorphisms and its pattern of resistance to *Xoo* strains.

Jalaluddin *et al.* (2005) studied the level of resistance of twelve Bangladeshi modern rice cultivars along with M95 (radiation induced mutant of the Japonica rice cultivar "Harebare"), TKM6 and IR24 to five major Japanese and 13 Bangladeshi races of *Xanthomonas oryzae py oryzae.* Among the tested materials, the induced mutant M95 and the Boro (January to May growing season) rice cultivar BR14 showed resistant reactions to all the five Japanese races (races I to V) of *X. oryzae pv. oryzae.* The Aman (July to December growing season) rice cultivar BRRI dhan33 showed resistant reactions to the Japanese races I, II, III and V. The induced mutant M95 also showed high level of resistant reactions to all the 13 Bangladeshi races of *X. oryzae pv. oryzae* indicating the non-specific, horizontal or quantitative nature of resistance to both Japanese and Bangladeshi races. The resistance genes from M95, BR14 and Brridhan33 will be useful for the development of durable BLB resistant rice cultivars in Bangladesh, Japan and other Asian countries.

Sere *et al*. (2005) carried out a disease survey and samplings in Niger, Burkina Faso and Mali which indicated a wide spread of Bacterial Leaf Blight (BLB) in farmer's fields. Sixty pure BLB isolates cultures were obtained. Pathogenicity of 4 Malian isolates against four important rice varieties revealed differences in

pathogenicity among isolates and in resistance of the varieties tested. The results obtained in these initial studies revealed the future research directions to increasing rice production in West Africa.

Satya *et al*. (2005) stated that pathogenic variability and identification of resistance genes are key factors in breeding against bacterial leaf blight disease of rice. Five isolates of *X. oryzae pv. oryzae* collected from different parts of north western India were screened against eight aromatic genotypes to identify variability in virulence. Most virulent isolate was used to screen forty-eight rice genotypes collected from different regions of Bihar, Jharkhand and UP to identify resistant sources. All the aromatic genotypes were found to be either moderately or highly susceptible against all the isolates with significant differences in disease progress. Isolate 5 was found to be most virulent against the aromatic lines while isolate 4 was least virulent. Isolate-5 was used to screen forty-eight genotypes. Thirteen lines showed high resistance against the highly virulent isolate. Inheritance of resistance in these rice lines should, therefore, be investigated further.

Liu *et al*. (2004) worked on Ninety-one isolates of *X. oryzae pv. oryzae* and collected during 1975-2003 from 20 rice-growing regions in China and evaluated for their virulence on 24 rice near-isogenic lines containing single resistance gene and 2-4 genes: IRBB1 (*Xa1*), IRBB2 (*Xa2*), IRBB3 (*Xa3*), IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB8 (*xa8*), IRBB10 (*Xa10*), IRBB11 (*Xa11*), IRBB13 (*xa13*), IRBB14 (*Xa14*), IRBB21 (*Xa21*), IR24 (*Xa18*), IRBB50 (*Xa4+xa5*), IRBB51(*Xa4+xa13*), IRBB52 (*Xa4+Xa21*), IRBB53 (*xa5+xa13*), IRBB54 (*xa5+Xa21*), IRBB55 (*xa13+Xa21*), IRBB56 (*Xa4+xa5+xa13*), IRBB57 (*Xa4+xa5+Xa21*), IRBB58 (*Xa4+xa13+Xa21*), IRBB59 (*xa5+xa13+Xa21*) and IRBB60 (*Xa*4+*xa*5+*xa*13+*Xa*21).The results showed that most isolates were less virulent on lines with more than one gene pyramided than those with single resistance gene.

Xu *et al*. (2004) used six rice cultivars (Jingan 30, IRBB3, IRBB4, IRBB5, IRBB14 and Java14) for monitoring *X. oryzae pv. oryzae* races in China. The

pathogen population in the country can be divided into 8 races, named C1-C8. Races Cl and C2 were predominant and stable. Results indicated that rice plants with the resistance gene *Xa3* can be widely used. 'The population of races C4 and C5 increased in the last 10 years. The resistance gene *Xa4* will be arrested by races C4 and C5. Genes, *xa5* and *Xa7* provided qualitative resistance to all races.

Yu *et al*. (2003) collected isolates of *X. oryzae pv. oryzae* from japonica rice grown in the Yunnan, China, plateau region was preliminary classified into 7 pathotypes according to the pathogenic reactions on 30 rice cultivars tested. The 7 pathotypes were named pathotype I to VII. Pathotype V is an epidemic one in the Yunnan plateau rice region now. Seven rice cultivars are considered as a of differential cultivars for bacterial blight race in the Yunnan plateau japonica rice, i.e. Kogyoku, Haonuoyang, TNI, Zhenzhu'ai, IR26, Nanjing 33, and Kinmaze. The disease reactions of the 7 pathotypes were determined on the set of differential varieties. The cultivars Wase Aikoku 3 (*Xa3*), IRI 545-339 (*xa5*), IRBB21 (*Xa21*), and Zhachanglong (*Xa22t*, *Xa24*t) were resistant to all strains tested, and useful for resistance breeding in Yunnan.

Nakatsu *et al*. (2003) evaluated Rice cultivars from the Hiroshima Prefecture Plant Gene Bank in Japan for resistance to bacterial blight (*Xanthomonas oryzae pv. oryzae*) races I and II. "The level of resistance was evaluated based on a 1-7 scale (0.0-2.0 for resistance and 4.1-7.0 for susceptibility). Among the 504 cultivars evaluated for resistance to race I, 27 cultivars (8 were developed in Japan, 8 were local cultivars, and 11 were introduced from other countries) were resistant. Among the 470 cultivars evaluated for resistance to race Il, 13 cultivars (5 were developed in Japan and 8 were local cultivars) were resistant. Seven cultivars from other countries were resistant to both races.

Qian *et al*. (2003) evaluated 300 accessions of rice, including cultivars, near isogenic lines (NILS) and pyramid lines, for their resistance to bacterial blight (*Xanthomonas oryzae pv. oryzae* (*Xoo*) in 2000-2001. Fifty-one germplasn-t accessions (17% of total) were identified as resistant to the isolates of the

disease. Cultivars widely cultivated in the province, such as japonica rice cultivar Hexi and indica hybrid rice cultivars, appeared highly susceptible to the disease, indicating that most of the cultivars widely grown in Yunnan are susceptible. The 16 NILS tested varied in their response to *Xoo* infection. The pyramid lines showed a wide resistance spectrum and a high level of resistance to all the isolates.

Li *et al*. (2001) investigated the resistance of rice to its bacterial blight pathogen *X. oryzae pv. oryzae (Xoo)* has both qualitative and quantitative components that using sets for four resistance (R) genes (*Xa4*, *xa5*, *xa13* and *Xa21*) and 12 *Xoo* races. Two R genes, *Xa4* and *Xa21*, showed complete dominance against the avirulent *Xoo* races and had large residual effects against virulent ones. The third R gene, *xa*5, showed partial dominance or additivity to the avirulent *Xoo* races and had relatively small but significant residual effects against the virulent races. In contrast, *xa*13 was completely recessive, had no residual effects against the virulent races, and showed more pronounced race specificity. There was a strong interaction leading to increased resistance between *xa13* and *xa5* and between either of them and *Xa4* or *Xa21*, suggesting their regulatory roles in the rice defensive pathway(s). Our results indicated that high level and durable resistance to *Xoo* should be more efficiently achieved by pyramiding different types of R genes.

Noda *et al*. (2001) collected 138 strains of *Xanthomonas oryzae pv. oryzae* from Yunnan province, China, during the period from 1994 to 1996. The strains were polymorphic for virulence to the 12 advanced lines harboring the resistance genes *Xa1, Xa2, Xa3* and *Xa4, xa5, Xa7, xa8, Xa10, Xa11, xa13 and Xa14.* The three check varieties IR24, Toyonishiki and Sigadagabo. The strains were classified into 10 pathogenic groups (tentatively designated as pathotypes A to J) based on their pathogenicity. In this study, they showed that 2 differentials, IRBB13 (*xa13*) and IRBB21 (*Xa21*), were resistant to all the strains tested, while IRBB5 (*xa5*) was susceptible only to 6 strains (4.3%). These resistance

genes could be used for a breeding program for resistance to BLB in Yunnan province.

Veena *et al.* (2000) reported that in India, the yield loss due to this disease is up to 81.3%.

Adhikari *et al*. (1999b) evaluated 171 strains of *Xanthomonas oryzae pv. oryzae* (the bacterial blight pathogen of rice) which was collected from eight rice producing zones in Nepal. Multiple correspondence analyses divided the collection into five putative genetic lineages. Twenty-six pathotypes (virulence phenotypes) of *X. oryzae pv. oryzae* were identified using 11 advanced rice lines, each containing a single gene for resistance. The 26 pathotypes were grouped into five clusters, and cluster1 contained wide virulence spectrum strains from all geographic populations.

Ou (1985) and Sere *et al.* (2005) reported that Yield losses due to BLB ranging from 50 to 90%.

Mew *et al.* (1987,1989). Bacterial leaf blight of rice (BLB), caused by *Xanthomonas oryzae pv. oryzae* (Swings *et al.* 1990), is one of the most widespread and destructive diseases of rice in several countries in tropical rice-growing areas of Asia, Australia, United States, Latin America and Africa.

Zhao *et al*. (1994) cited that response of 285 accessions of rice from IRRI in the Philippines to 7 pathotypes of bacterial leaf blight (*Xa*nthomonas oryzae) was evaluated during 1991-93 in Zhejiang, China. Local rice varieties Tesanai 2 and Jingang 30 were used as controls. Among the accessions, 18 were highly or moderately resistant to the pathotypes, of which 14 showed potential as good sources of resistance in breeding programmes. Evaluation of agronomic traits and field resistance indicated that Suweon 290, Milyang 46 and Si-Pi 692033 have potential for commercial production.

Ashrafuzzaman *et al. (*1992) reported that the severity of BLB (the most damaging disease of rice) in tropical Asia (South East Asia) was high and losses varied from 6-60%.

Ou *et al.* (1985) reported that disease incidence increases with stages of plant growth and peaking at the flowering stage. An infection could reduce rice yields by more than 50% and even lead to a complete crop damaged when it occurs during the tillering stage.

(Goto, 1992; Watanabe, 1975) concluded that the "Kresek" phase of bacterial leaf blight disease was characterized mainly by the systemic infection due to this phase the symptoms of disease usually appear1-2 weeks after transplantation of the nursery into the field. Under severe conditions leaves of the rice plant become grayish green to whitish and suddenly withered and sometimes "Kresek" phase of the disease happens on mature plants. In this phase usually, symptoms appear on foliar parts, related those on younger plants yet the rotting of the stem also reaches the upper part of the leaf.

Dath (1983) *et al.* described that the pathogen might be transmitted to basal parts of the leaf sheath through the contaminated roots or by the lower leaves which encounter contaminated water and becomes the source of infection for the rest of crop period.

Reddy and Shukla (1978) estimated 72.7% loss in Koruna and 43% in Sona when both were infected with BLB panicle initiation stage. Inoculation of flag leaf with two isolates of *Xanthomonas oryzae pv.oryzae* resulted 38%-40% loss in yield. Inoculation of susceptible hybrids at the booting stage decreased the number of filled grains per plant,1000-grain weight, grain yield and increased the number of empty husk plant-^{1}.

Olufowote *et al*. (1977) also reported that some varieties carried genes *Xa*-4 or *xa*-5 and that breeding lines IR944-102 and IR1698-241 carried another gene closely linked to *Xa*-4.

Petpisit *et al*. (1976) concluded that resistance in IR20, IR22, and IR1529-680-3 showed incomplete dominance, and these cultivars carried the same gene for resistance, which they designated *Xa*-4. They also reported that IR1545-284 and

RP291-7 carried the same recessive genes for resistance, designating it *xa*-5. Genes *Xa*-4 and *xa*-5 segregated independently.

Mizukami and Wakimoto (1969) concluded that the bacterium remains in roots of the weed "*Leersia haxandra"* form where in the rice growing season it reaches the rice nursery beds and further spreads in the channels by the irrigation water applied to the young plants. Besides this, infected straw present in the field or the infected seeds may also introduce the pathogen into the rice nursery. They also severe attack of the disease yellow to white stripes appeared just inside the margins of the leaf blades which later on turned to pale yellow and become necrotic.

Wakimoto *et al.* (1969) reported that the word "Kresek" is derived by the Vernacular term in Java, meaning "the sound of dead leaves" stroked with one another.

CHAPTER-3

MATERIALS AND METHODS

CHAPTER III
MATERIALS AND METHODS

In this chapter the details of different materials used and methodology followed during the experimental period are described. This research is a part of core research program of Plant Pathology Division, Bangladesh Rice Research Institute (BRRI).

3.1. Experimental site

The pathological research was performed at general Plant Pathology Laboratory, Plant Pathology Division, Bangladesh Rice Research Institute (BRRI), Gazipur. Molecular works were done at molecular Plant Pathology Laboratory, Plant Pathology Division, BRRI, Gazipur. The field experiment was conducted at the research field of Plant Pathology Division, BRRI, Gazipur.

3.2. Experimental time

Pathological and molecular works were conducted during August, 2019 to February, 2020. Field research was done during *T. aman* season, 2019.

3.3. Culture media preparation

3.3.1. Peptone Sucrose Agar (PSA) Medium

PSA media was prepared according to Fahy, P. C., and G. J. Persley. 1983. For 1L Peptone Sucrose Medium, 20g of Agar (Bio Basic Inc., Canada), 20g of sucrose (Duchefa Biochemie, Netherlands) and 5g peptone were poured in 800 mL of distilled water and then boiled until dissolved. Then the solution was adjusted to 1L with distilled water. Finally, it was autoclaved for 15 min at 121°C (Plate 1).

Plate 1. PSA (Peptone Sucrose Agar) Medium

3.3.2. Peptone Sucrose Broth (PSB)

For the preparation of 1L peptone sucrose broth 20g of sucrose and 5g of peptone were poured in 800mL of distilled water and boiled until dissolved. Then the volume was adjusted up to 1L with distilled water. Finally, it was autoclaved for 15 min at 121°C (Plate 2).

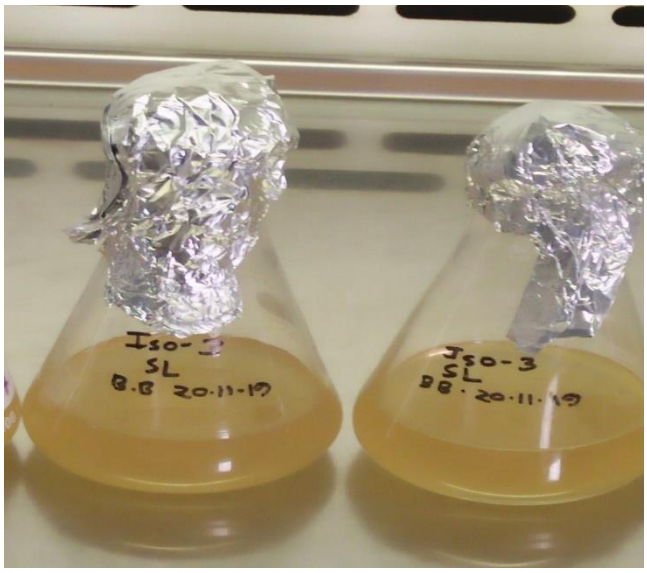


Plate 2. PSB (Peptone Sucrose Broth)

3.3.3. Slant preparation

Slant was prepared following the procedure described 3.1.1. Slants were prepared in a test tube containing PSA media. First the test tubes were autoclaved for sterilization. Peptone Sucrose Agar slants were created by bringing peptone and agar to the boiling point and it was poured into a test tube. Before the peptone and agar cooled and solidifies, the test tube was set on its side. Once the peptone and agar were cooled, the test tube can be stored upright, and the agar inside had a slanted appearance (Plate 3). Bacterial colony was transferred into slant from the sub-cultured bacteria.

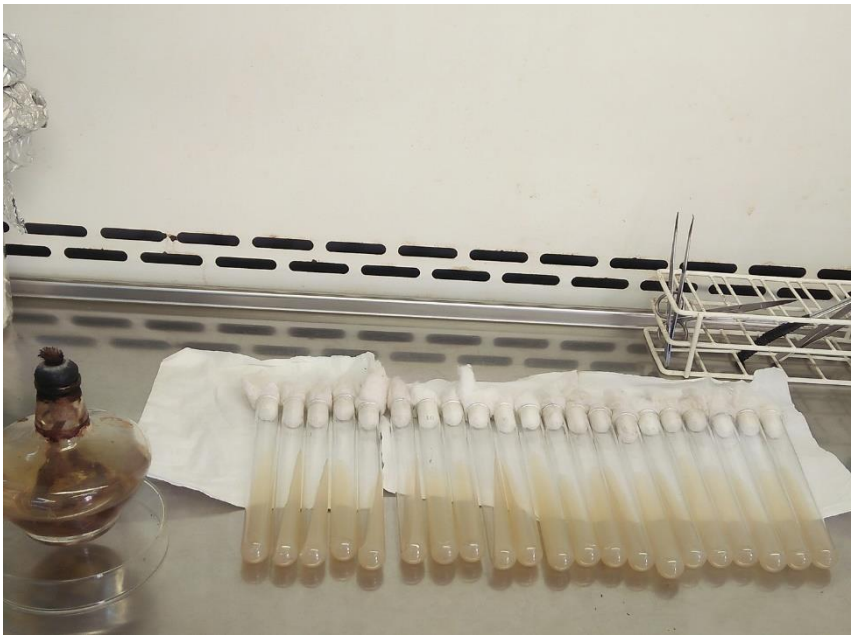


Plate 3. Preparation of slant

3.4. Reagent Preparation

3.4.1. 1M Tris-HCl, pH 8.0

121.14g Tris (Invitrogen, USA) was dissolved in 800ml distilled water. Fuming 37% HCl (Merck, Germany) was added with the solution until the pH was reached to 8.0. Then the final volume was made up to 1 liter with distilled water. Autoclaved and stored at room temperature.

3.4.2. 0.5M EDTA, pH 8.0

186.12g of EDTA (VWR International, USA) was added to 800mL of H_2O. Solution was stirred vigorously on a magnetic stirrer (IKA C-MAG HS 7, USA). During stirring, NaOH (Merck, Germany) pellets were added until the pH became 8.0. EDTA was not dissolved until the pH reached to 8.0. Finlay, the solution was adjusted to 1L volume with distilled water.

3.4.3. 5M NaCl

292g of NaCl (Duchefa Biochemie, Netherlands) was added in 800mL of H_2O. the solution was stirred until dissolved. Then the volume was adjusted to 1L with distilled water. Finally, the solution was autoclaved and stored at room temperature.

3.4.4. 2X Cetyl trimethylammonium bromide (CTAB)

100ml of 1M Tris HCl pH 8.0, 280ml of 5 M NaCl, 40ml of 0.5 M EDTA and 20 g of CTAB (cetyltrimethyl ammonium bromide) (Bio Basic Inc, Canada) were poured into 700mL of distilled water. Then the solution was stirred until the solution completely dissolved. Finally, the volume was adjusted to 1L with distilled water.

3.4.5. Chloroform: Iso-amyl alcohol: Phenol (CIP)

912mL Chloroform ((DAEJUNG Co. Ltd., Korea) was mixed with 38 mL Isoamyl Alcohol (AMRESCO, USA). Further 50mL Phenol (Wako Pure Chemical Industries, Ltd., Japan) was used with existing "Chloroform-Isoamyl Alcohol" solution. Finally, the solution was poured into a colored glass bottle and stored below 20˚C out from light.

3.4.6. TE buffer

To prepare 1L solution of TE Buffer, 10ml of 1M Tris base (pH 8.0) and 2ml EDTA (0.5 M) were added together and to the volume was adjusted to 1L with distilled water.

3.4.7. 10X and 1X TBE buffer

For 10X TBE buffer preparation, 108g of Tris (MP Biomedicals, USA), 55g of Boric Acid (Bio Basic Inc., Canada) and 9.3g of EDTA (VWR International, USA) were poured into 750mL distilled water and then stirred until dissolved. Finally, the solution volume was adjusted to 1L with distilled water.

1X TBE buffer was prepared from 10X TBE following the formula,

$V_1S_1=V_2S_2$

Where, S_1= Concentration of stock solution (10X)

S_2= Desired concentration of solution (1X)

V_1= Volume of stock solution (10X)

V_2= Final volume of buffer with desired concentration

3.5. Isolation and pure culture of *Xanthomonas oryzae pv. oryzae* (*Xoo*)

Xoo was isolated from different BLB infected rice leaves [collected from plant pathology division, BRRI]. Diseased leaves were cleaned with tap water, and air dried. These leaves were cut into small pieces about 5 to 7 cm and sterilized with 1% sodium hypochloride solution for 1 minute for surface sterilization. Then the samples were washed in sterilized distilled water. These pieces were cut into smaller pieces about 5 x 5 mm in size and put into the watch glass containing sterilized distilled water for about 10 to 15 minutes, to allow the bacteria to ooze out from the infected leaf tissue. By using the sterilized loop needle with bacterial suspension streak onto Petri dishes containing PSA medium (Ou, 1985). The plates were incubated in incubator (Hettich incubator, hettcube 200, USA) at 37ºC for 2 days. The single cream colored, round and smooth margin, non-flat, mucous colonies were selected and transferred into PSA medium slant as pure culture. This process is done for each collected sample and preserved at 4°C for further studies.

3.6. Preservation of pure culture in PSA slant

All isolates were preserved in PSA slant for medium term preservation (Plate 4). Slants were kept in -40^{0}C refrigerator when all the slants were fully covered.

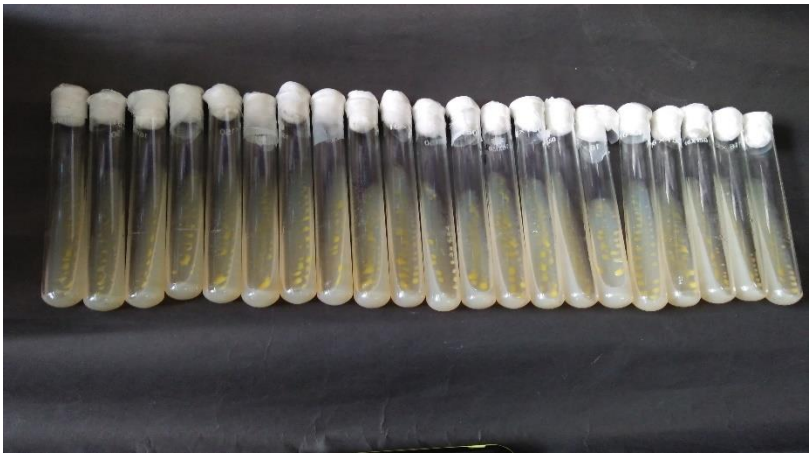


Plate 4. *Xoo* culture preserved in PSA slants

3.7. Pathogenicity test

Each isolates of *X. oryzae pv. oryzae* were cultured on PSA slants separately for 72 hours at 37 ^{0}C. Inoculum of each isolate was prepared by mixing the cultured bacteria with 10 ml sterile distilled water in each slant. Before inoculation the concentration of the bacterial suspension was adjusted to 1X10^{6}CFU/ml using sterile distilled water. The leaf clipping inoculation method was adopted in this experiment. The scissors were dipped into bacterial suspension, and then the tips of fully expanded leaves were clipped as described by Kauffman et al. (1973). At the maximum tillering stage of rice plants, 8-10 leaves/hill were clipped with a sterile scissor. Before clipping the scissor was dipped in the bacterial suspension.

3.8. Genomic DNA extraction from bacterial culture

3.8.1. Bacterial culture

Each isolate was cultured in PSB medium for DNA extraction (Plate 5). One loopful bacteria from each isolate was poured into a 100mL conical flask filled with 25mL of PSB medium respectively. Then each conical flask was sealed with cotton plug and aluminum foil. This whole process was done carefully in laminar airflow cabinet (LABCAIRE, USA). The conical flask then placed on a rotary shaker (Hz-300, China) and was shake overnight. The next day, the bacterial cells were harvested and proceed for further process.

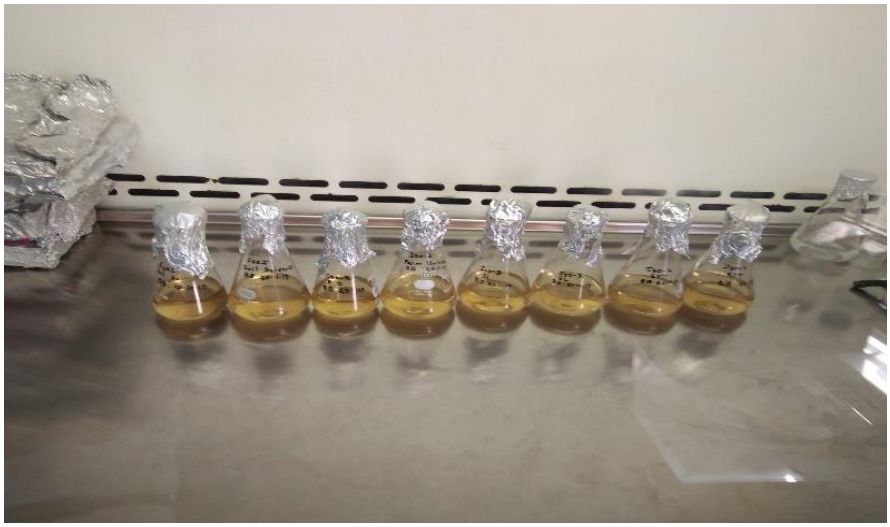


Plate 5. Liquid culture for four bacterial Isolates

3.8.2. DNA extraction from bacterial cells

The genomic DNA extracted from bacterial cells was done by heat shock/heat treatment method (Dashti *et al.,* 2009).

3.8.2.1. Protocol for Genomic DNA extraction

1000µl of bacterial cell culture was taken in a 1.5µl microcentrifuge tube (Axygen, USA). The tube was centrifuged (Microfuge 20R, Beckman Coulter, Inc., Germany) at 12000 rpm for 5 minutes. After centrifuge, the supernatant was removed; the bacterial cells were pelleted at the bottom of the tube. Saline water was added and centrifuged at 12000 rpm for 5 minutes to wash the DNA. The supernatant was removed and 200µL nuclease free water was added to the

microcentrifuge tube and mixed by pipetting. The mixture then vortexed (Whirl mixer, Fisher Scientific, UK) for 0.5-10 seconds for destroying the cell wall. The tube was placed on dry heat block (Techne, UK) and was heated at 60-65^{0}C for 40 min. After heating, the tube was thaw in ice for 15-20 minutes. Then it was vortexed for 5-10 seconds. Again, the tube was centrifuged at 12000 rpm for 5 minutes. 150µL aqueous supernatant was taken out in another 1.5mL microcentrifuge tube. The DNA was preserved at 4^{0}C for further use.

This process was followed for each isolate.

3.8.3. Molecular marker for *Xoo* detection

Bacterial isolates were confirmed using three highly specific diagnostic markers, *Xoo80, Xoo4009 and Xoo3350*. The details of markers are described in (Table 1) All molecular markers were synthesized by Integrated DNA Technologies, Inc., USA.

Table1. Molecular marker for *Xanthomonas oryzae pv.oryzae* detection

SL.	Primer	Primer Sequence Forward (5'-3') Reverse (5'-3')	Expected size(bp)	References
1	*Xoo80*	F=GCCGCTAGGAATGAGCAAT	162	Lang *et al.*, 2010
		R= GCGTCCTCGTCTAAGCGATA		
2	*Xoo4009*	F=CCTTCATTTCCGTCGTCAC	302	
		R=ATGCATGAAGAACCACCACA		
3	*Xoo3350*	F=GCAAGCTGATCGGTATCCC	300	
		R=GCGAGACCTTGAACTGGAAC		

3.9. PCR

Purified DNA collected from each *Xoo* isolate run through PCR Thermal Cycler (PEQLAB, VWR International, USA) to amplify the targeted sequence of specific marker.

3.9.1 Preparation of PCR reaction

20µL PCR volume was prepared for every PCR sample. Each PCR sample was contained 6µL template DNA, 0.5µL of each primer, 10µL of Taq PCR Master Mix and nuclease-free water to final volume of 20µL.

3.9.2. PCR program

PCR program was set up as follows: Initial denaturing step at 94°C for 3 minutes; followed by 35 cycles of 94°C for 30 seconds, 55°C anneal for 30 seconds and 72°C for 1 minute 30 seconds; and a final extension at 72°C for 7 minutes.

3.9.3. Gel electrophoresis and gel documentation

Amplified PCR products were separated through Gel electrophoresis (multiSUB Mini, Cleaver Scientific Ltd., UK) system. Each PCR product along with 100 bp DNA ladder (Promega Corporation, USA) were run into 2% agarose gel with 1X TBE buffer with 90V for 1 hours. After electrophoresis, gel was stained into ethidium bromide (0.5µg/mL) (Invitrogen, USA) solution for 30 minutes. After staining, gel was washed in water and gel picture was taken in a gel documentation system (Bio Doc Analyze, Biometra, Germany). Every sample were repeated twice to confirm the results through PCR reaction.

3.10. Planting materials

Five BLB resistant advanced line with two standard check varieties and one susceptible check variety were used as planting materials. The details of the materials were in the (Table 2).

Table 2. Name and description of the selected lines and varieties.

Treatment	Designation*	Remarks
V_1=	P-7-70	Developed by Plant Pathology division, BRRI
V_2=	P-7-82	Developed by Plant Pathology division, BRRI
V_3=	P-3-58	Developed by Plant Pathology division, BRRI
V_4=	P-8-78	Developed by Plant Pathology division, BRRI
V_5=	P-5-1	Developed by Plant Pathology division, BRRI
V_6=	BRRI dhan39 (Standard check)	Released by BRRI
V7=	BRRI dhan49 (Standard check)	Developed by Plant Pathology division, BRRI
V8=	Purbachi (Susceptible check)	Original name: Chen-chu-ai from China
*All designation are code names of the original lines identified through pure line selection from IRRI bred line.		

3.11. Seed sprouting

Seeds were separately packed into different gunny bag and soaked into tap water for 24 hrs. After soaking, bags were placed into pile of straw for Zag. It took around 72 hours for sprouting.

3.12. Seed bed preparation and sowing of sprouted seeds

Seed bed was prepared by paddling the soil with the help of power tiller in Bangladesh Rice Research Institute (BRRI), Gazipur. As the land was rich in organic matters, so no manuring was done. Sprouted seeds were sown in wet seed bed on 15 July, 2019. Seedlings were properly taken care of. Irrigation was given in the seed bed as and when necessary.

3.13. Land preparation

The land was prepared by with the help of power tiller. The land was first opened on 30 July 2019 and ploughed. The final ploughing was performed with the help of power tiller followed by laddering in order to level the soil surface. Weeds and stubbles were removed from the land.

3.14. Fertilizer application

Fertilizers were applied as per recommendation of BRRI(BRRI,2019). The following doses of fertilizers were applied to the plots:

Fertilizers	**Kg/ha**
Urea	100.03
TSP	37.05
MoP	49.89
Gypsum	33.34
Zinc Sulphate	2.47

Total amount of Urea, TSP, Gypsum and Zinc and half of MoP was applied as basal fertilizer during final land preparation. Urea was applied as three equal splits at10, 25 and 35 days after transplanting. Rest half of the MoP was applied along with 2nd top dressing of Urea.

3.15. Design and field layout

The experiment was laid out in randomized complete block design (RCBD) with three replications. Blocks were representing the replication. Each block comprised 8unit plot and total number of plots were 24. Hill to hill distance was 20 cm X 20 cm. The plot size was 5m X 2.5 m. The distances between plot to plot was 0.50 m and block to block was 1m. (Appendix I)

3.16. Seedling transplantation

Seedlings were uprooted from the seed bed very carefully, and then transplanted on 20 august,2019 in the main field. 2-3seedlings were transplanted together in individual hill.

3.17. Intercultural operation

Weeding and irrigation was done in the field as and when necessary.

3.18. Genomic DNA extraction from leaf

Genomic DNA were extracted from the rice leaves by using the CTAB method (Ferdous et al., 2012) with some modifications.

3.18.1. Collection of leaf

The leaf was collected from each advanced line's plot in the field. Leaves were collected from twenty-five days young plant and kept in 4ºC for further process.

3.18.2. DNA extraction

Collected leaves of each samples were cut and turned it into small pieces (about1-1.5cm cut) and placed into 2mL microcentrifuge tube. 3mm zirconium oxide ball was putted into each tube and 300µL of 2X CTAB was added into the tubes. The tubes were put into the Mixer Mill MM400 (RETSCH, Germany) and start at 30 rpm for 2 minutes. Then 300µL CIP was added into the tubes and was vortexed with vortex mixer (Whirl mixer, Fisher Scientific, UK). After vortex was completed, tubes were centrifuged at 15000 rpm for 10 minutes. 200µL supernatant was taken and put into a new 1.5mL microcentrifuge tube 200µL cold isopropanol (Molecular Biology Grade, Fisher Bio Reagents, USA) was added and mixed. For uniform mixing the tubes were inverted for few minutes and kept at room temperature for 10 minutes. The samples were centrifuged again at 15000 rpm for 10 minutes. after centrifuge, all supernatants were removed and 700µL 70% ethanol (TEDIA, USA) was added for washing. Tubes were centrifuged again at 12000rpm for 5 minutes. Finally, ethanol was removed and the DNA pellets was air dried. After the drying, 100µL ultra-pure water (PURELAB Option-Q, ELGA Lab Water, UK) was added in each tube and vortex. The solution was conserved as stock DNA solution. Later the DNA samples were diluted with ultra-pure water to the concentration of 10µL for polymerase chain reaction (PCR).

3.19. Molecular screening for BLB resistance genes

In this experiment, four BLB resistance genes (*Xa21, xa13, Xa4* and *xa5*) were targeted for primarily detection from the advanced lines. Four tightly linked marker were used to confirm these genes. The details of markers were listed in (Table 3).

Table 3. List of molecular markers used for detection of BLB resistance genes in rice germplasm.

Marker/ Linked Gene	**Sequence**	**Expected band size**	**Target gene**	**Reference**
pTA248	F-3'AGACGCGGAAGGGTGGTTCCCGGA5' R-3'AGACGCGGTAATCGAAAGATGAAA5'	1000 bp	*Xa21*	Ronald *et al*., 1992; Huang *et al*., 1997; Sudarman *et al*., 2008
MP	*MP1*-3'ATCGATCGATCTTCACGAGG5' *MP2*-3'TGCTATAAAAGGCATTCGG5'	150 bp	*Xa4*	Sun *et al*., 2003
xa5 Multiplex	**SF=** 3'GTCTGGAATTTGCTCGCGTTCG5' &3'TGGTAAAGTAGATACCTTATCAAACTGGA5' **SR** **RF=** 3'AGCTCGCCATTCAAGTTCTTGAG 5' & 3'TGACTTGGTTCTCCAAGGCTT5' **RR**	424bp and 134bp	*xa5*	Sundaram *et al.*, 2011
xa13-prom	F-3'GGCCATGGCTCAGTGTTTAT5' R-3' GAGCTCCAGCTCTCCAAATG5'	450bp	xa13	Chu *et al*., 2006

3.19.1. PCR

3.19.2. PCR volume preparation

20µL PCR volume was prepared for every PCR sample. Each PCR sample was contained 6µL template DNA, 0.5µL of each primer, 10µL of Taq PCR Master Mix and nuclease-free water to final volume of 20µL (Plate 6).

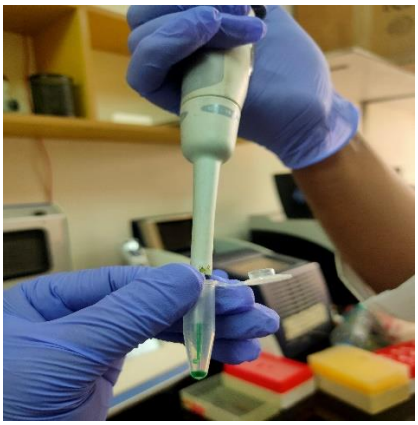


Plate 6. 20 µL PCR volume preparation

3.19.3. PCR programs

PCR program was set up as follows:

Initial denaturing step at 94°C for 3 minutes; followed by 35 cycles of 94°C for 30 seconds, 55°C anneal for 30 seconds and 72°C for 1 minute 30 seconds; and a final extension at 72°C for 7 minutes.

3.20. Gel electrophoresis and gel documentation

amplified PCR products were separated by Gel electrophoresis (multi SUB Maxi, Cleaver Scientific Ltd., UK) with DNA ladder (100 bp and 1kb) (Promega Corporation, USA). Gel electrophoresis was done in 2% agarose gel with 1X TBE buffer with 90V for 2 to 2.5 hours. Gel was stained by ethidium bromide (0.5µg/mL) after electrophoresis. Gel picture was taken in gel documentation system (Bio Doc Analyze, Biometra, Germany). Every sample were repeated twice to confirm the results through PCR reaction.

3.21. Bacterial inoculation on field

Bacterial inoculation was done with leaf clipping method in the field (Kauffman *et al.*,1973) at booting stage. Scissor tips was dipped into the

previously prepared *Xoo* suspension and the leaf tip was cut (approximately 4-5 cm) away from the leaf. Every 2nd line from every plot was clipped.

3.22. Assessment of the disease incidence and severity in the field

Each plot was investigated and recorded the incidence and severity of BLB disease. All data were recorded by observing visually through typical symptoms. All data were recorded through observing the typical symptoms of the leaf. Data was recorded at two times interval of 7 days (at 7 days after inoculation and at 14 days after inoculation). Each and every data was recorded on the basis of percent leaf area infected.

3.22.1. Disease Severity

Disease severity was recorded on the basis of leaf area infected.

3.22.2. Disease score

Disease score was recorded on the basis of severity (IRRI, 2013). The details of scale are described below:

Bacterial leaf blight (BLB) Scale

Field test, severity	% leaf area diseased
1	1-5%
3	6-12%
5	13-25%
7	26-50%
9	51-100%

3.23. Data collection

Maturity of crop was determined when 85-90% of the seed became golden yellow in color or filled properly. Data on yield components were recorded from 5 random hills of each plot excluding the border plant. After sampling central four rows of each plot were harvested to record grain and straw yields. The harvested crop of each plot was separately bundled, properly tagged and then

brought to the threshing floor to be threshed by peddle thresher. The following data on yield and yield contributing characters were recorded:

1.Panicle length

2. percent sterility

3. 1000 grain weight

4. Yield (ton/ha)

3.23.1. Panicle length

Panicle length was measured from the basal node of the rachis to the apex at each panicle.

3.23.2. percent sterility

For sample sterility 6 hills from each plot were selected. Number of sterile spikelets was counted from all the portions separately and then percentage of sterility (at the three portions) was calculated using the following formula:

$$\text{Grain sterility (\%)} = \frac{\text{Unfilled grains panicle}^{-1}}{\text{Total grains panicle}^{-1}} \times 100$$

3.23.3. 1000 grain weight

Thousand grains were randomly selected from sample plants of each plots and their weight were taken in an electric balance. The grain moisture content was adjusted to 14% moisture content and then it was converted to ton per hectare.

3.23.4. Yield (ton/ha)

An area of $5m^2$ harvested for yield measurement. A sample of 500 g of both rice grain and straw from each plot was taken separately and dried in the oven at 80^0c until a constant weight was obtained. This was done to find out the moisture percentage of grain and ± 41 straw samples. The grain and straw yields were adjusted at 14% level of moisture by using the formula suggested by Abedin and Chowdhury (1982).

3.24. Statistical analysis

The data were analyzed by using Statistical Tool for Agricultural Research (STAR) Version: 2.0.1 (IRRI, 2013) software. The treatment means were compared using Least Significant Difference (LSD).

CHAPTER-4

RESULT AND DISCUSSSION

CHAPTER IV
RESULT AND DISCUSSSION

4.0. Symptoms caused by *Xanthomonas oryzae* on infected leaves

Infected leaves were collected from experimental field of Plant Pathology division of BRRI Gazipur. Infected leaf exhibited water-soaked lesions (yellow in color) at the border of its leaf edge (Plate 7). Such lesions were parallel along the leaf, later merged together and then covered overall leaf. Sign of the causative agent as bacterial exudation was noticed on the boundaries and veins of newly infected leaf. The identical symptoms were also reported by several researchers around the world (Anon, 1970; Tagami & Mizukami,1962; Ou, 1985; Samanta *et al.*, 2014 and Afolabi *et al.*, 2016). In West Bangla, India and also in Benin BLB of rice showed sign of bacterial ooze formation on the veins or boundaries of the newly infected leaf under moist or humid conditions. Similar condition was also observed by Mukko *et al.* (1957) reported that the pathogenic bacterium invades the rice crop plants through the water pores using the fresh wounds of 24 hours. Reddy and Ou (1976); Ou (1985) also observed that the bacteria were percolated through the water pores and transferred to the leaf edges of the higher area of leave; so, lesions normally started at leaf margins on the upper part near the top area. Small water-soaked lesions appeared which later turned to yellowish white color expanding from the equal sides in a square form to produce elongated circular to quite uneven lesions. They also found the edges were adjoining the healthy areas on the leaves and showing the most characteristic symptom of the disease i.e. the wavy margins, which can be clearly seen on the leaf blade. The lesions normally started on one or both margins of the leaves or can be observed on the fresh infected leaf veins under humid conditions. Bacterial ooze was formed when the pathogen establishment on the leaf was completed. Ooze was formed on the veins and boundary of the leaf showed characteristics symptoms. In their study they reported environment played a key role for the development of the disease and the appearance of the symptoms in the field. They characterized the disease into two distinct phases; leaf blight

phase, and the “Kresek phase” which is the destructive one for the epidemic of disease.

Plate 7. Different rice leaves showing BLB symptom

A. for old lesion in the midrib B. for young lesion to the margin C. for old lesion from the apical part and D. for undulated young leaf lesion from the midrib

4.1. Isolation and identification of *Xanthomonas oryzae pv. oryzae*

4.1.1. Isolation and pure culture of *Xanthomonas oryzae pv. oryzae*

Four Isolates viz. Iso1, Iso2, Iso3 and Iso4 were isolated from collected samples (Plate 8). All isolates were dome shaped, slimy, light yellow and mucoid colonies (1 to 2 mm in diameter) were formed after incubated at 25-30°C for 2 days. All the characteristics were much more similar with *Xathomonans oryzae*. So, we can say that this four isolates beared the characters of *Xanthomonas oryzae*. The stable temperature was needed for uniform growth of the colony. All isolates were sub-cultured from single colony to maintain pure culture.

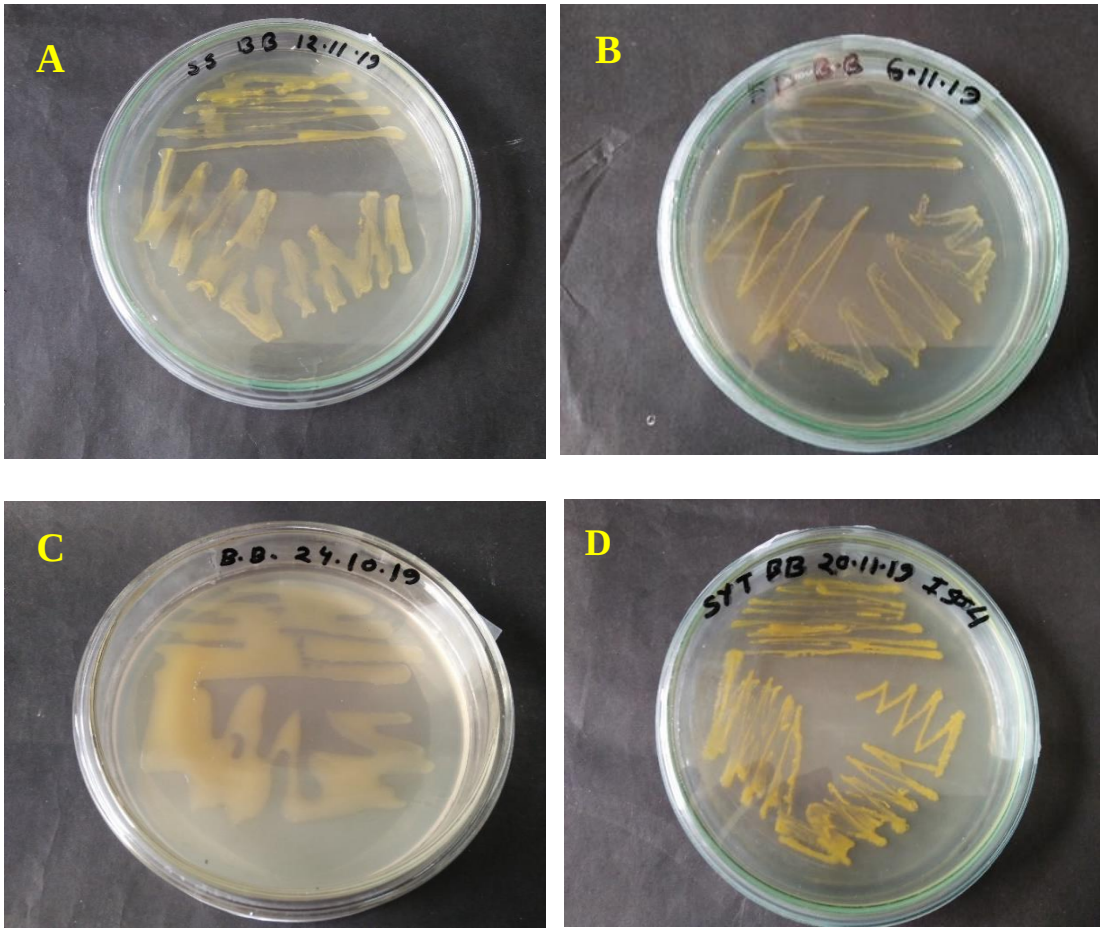


Plate 8. Pure culture of different Isolates of *Xanthomonas oryzae pv. oryzae*

A. for Isolate 1, B. for Isolate 2, C. for Isolate 3 and D. Isolate 4

Isaka *et al*. (1970) isolated the causal bacterium from green leaves with yellow BLB lesion and didn't from torned and rotten tissue that usually over grew by microorganism. Di *et al.* (1991) stated that the bacterial exudates from fresh lesion were better isolation material as compared to infected tissue because of less contamination and concluded that the recovery of *X. oryzae pv. oryzae* colonies from infected leaves sample was easy rather than infected seeds, due to presence of other bacteria and fungi in high population in seeds. In the present study PSA medium was used. In our study Dome shaped, slimy, light yellow and mucoid colonies were formed after incubated at 25-30°C for 2 days. Di *et al.* (1991) also used PSA medium. In his study he used Nutrient agar yeast extract medium (NYA), peptone sucrose agar (PSA), and nutrient agar (NA) for isolation and screening of the pathogens. After 48-72 hours of incubation at 28°C

they plated the infected leaf samples on peptone sucrose agar medium (PSA) and he found light yellow, circular dome shaped colonies. The yellow color and mucoid colonies were formed through *Xanthomonas oryzae pv. oryzae* due to the production of extracellular polysaccharides slime (EPS in media containing sugar). The samples also gave a light yellow, mucoid, round and smooth bacterial colony when streaked on nutrient agar yeast extract medium (NYA) and it was similar on peptone sucrose agar (PSA) which produces yellow water-soluble pigment, pale yellow colonies, mucoid and shiny. (Joint *et al*. 2016) described that all plates of isolates were fully grown after 3-5 days of incubation at uniform temperature of 25-30^{0}C.

This was also reported by Shankara *et al*. (2016), Joint *et al*. (2016) they also observe round and smooth margin non flat and mucous colonies in PSA (Peptone Sucrose Agar) medium, Jabeen *et al*. (2016) observed the colonies in wakimoto medium.

4.1.2. Pathogenicity test

In the pathogenicity test first symptom of the disease was initiated after 2 days of inoculation in the apical part of the leaf (Plate 8). After 21 days, the disease symptoms appeared clearly in the leaf (Plate 9). In our pathogenicity test Purbachi was used as susceptible variety. Purbachi was a highly susceptible variety against bacterial leaf blight disease (Latif *et al.,* 2011). Purbachi is not our native variety or neither it was developed by BRRI. Its original name was Chen-Chu-Ai (IRRI 1980), which was originated from china. According to BRRI annual research review report 2019 purbachi is recommended as susceptible variety for BLB of rice. In pathogenicity test in the field the cut leaves of Purbachi was totally damaged by bacterial leaf blight infestation. (Plate 10).

Plate 9. Pathogenicity test for BLB of rice leaves in *in-vitro* condition

A. for inoculation in the apical part of leaf

B. for after 21 days symptom appears on leaf

Plate 10. Purbachi in field

Ansari *et al.* (2019) conducted an experiment T. aman 2018. Seven BB resistant pure (P-7-70, P-7-72, P-7-82, P-3-58, P-8-78, P-5-1, P-9-17) lines along with a susceptible check (Purbachi) was used. It shows HS sign which means it was susceptible in their experiment. In our experiment we also used Purbachi as susceptible check in the field. In field it cannot withstand any disease condition.

In our present study our symptoms were developed in the field at 21 days. Shankara *et al*. (2016) observed pathogenicity by inoculating the plants with bacterial suspension for the development of symptoms. He also found that the infection of *Xanthomonas oryzae pv*. *oryzae* was developed in 21 days after the

pathogen inoculation. Joint *et al.* (2016) also observed the pathogen development of the leaves of *Xanthomonas oryzae pv. oryzae* in 21-23 days. The bacterium was re-isolate to prove the Koch's postulate and compared with the original culture.

4.1.3. Molecular detection of *Xoo*

In the present study for molecular detection specific primer *Xoo80, Xoo3350, Xoo4009* was used. In our experiment Iso1 and Iso4 gave expected band for all primers. For *Xoo80* primer Iso1 and Iso4 gave band in 162 bp. Iso1 and Iso4 gave 302 bp band and 500 bp band for *Xoo4009* and *Xoo3350* primers respectively (Figure 1). Iso2 and Iso3 gave no band for all primers. This result indicated that Iso1 and Iso4 were the *actual Xanthomonas oryzae pv. oryzae* bacteria. Iso2 and Iso4 were not *Xanthomonas oryzae pv. oryzae*. This result was similar with the experiment conducted by Lang *et al.* (2010)

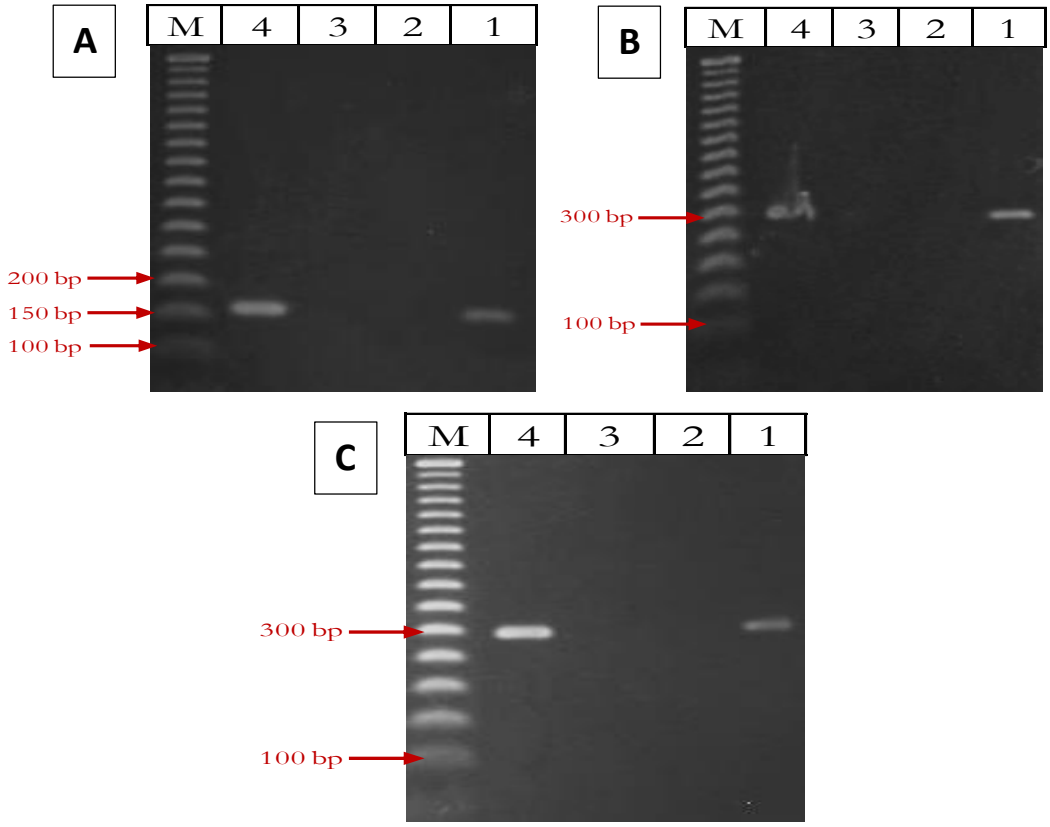


Figure 1. Molecular detection of *Xanthomonous oryzae* pv *oryzae* A. for *Xoo*80 primer, B. for *Xoo*4009 primer and C. for *Xoo*3350 primer. Here, M=100 bp ladder, 1 to 4 for Iso1 to Iso4 respectively.

Lang *et al.* (2010) also used these three primers for *Xanthomonas oryzae pv. oryzae (Xoo)* for gene detection. They studied 1697 primers to distinguish *X. oryzae pv. oryzae and x. oryzae pv. oryzicola*. They screened different primers *Xoo80, Xoo4009, Xo1321, Xo2207, Xo2967, Xoc3866,* and *Xoc3864* in PCR assays against *X. oryzae* strains for confirmation. Fourteen confirmed *X. oryzae pv. oryzae* strains and five confirmed *X. oryzae pv. oryzicola* strains from India were correctly identified using these primer pairs.

Lane *et al.* (1991) and Weisburg *et al.* (1991) reported about DNA-based approaches were used to distinguish *X. oryzae pv. oryzae* and *X. oryzae pv. oryzicola* involved amplification of the 16S rDNA followed by digestion with restriction enzymes. They found that 16S rDNA sequences exhibit 98.6% similarity within the genus *Xanthomonas* (Hauben *et al*.,1997), this approach did not reliably distinguish or differentiate these two *X. oryzae* pathovars. They also proposed that this approach was only useful if this sequence was supported by other sequence information such as the 16S-23S rRNA internal transcribed spacers. Primers based on the 16S-23S rDNA spacer region were designed for *X. oryzae pv. oryzae* but their design and testing were based on *X. oryzae* pv. *oryzae* isolates. In our experiment among the four-isolate contained 16S rDNA. But in Iso1 and Iso4 due to molecular characterization result both of them possessed band in expected area with specific primer so we can say that Iso1 and Iso4 were *Xanthomonas oryzae pv. oryzae* and other two isolates Iso2 and Iso3 might be *Xanthomonas oryzae pv. oryzicola.* Shaheen *et al*. (2019) also reported similar result proposed by (Lang *et al.* 2010) that the universal primers for 16S rRNA gene were used for the amplification of confirmed bacterial DNA in overall particular positive isolates to approve the pathogens. He achieved products of 1500 bp was achieved in 15 out of 17 isolates (The isolates at well 4 and 10 and those which were used as negative control (shown as zero "0") did not exhibit any amplification in polymerase chain reaction. Same the consequences of PCR detection were documented in Malaysia by Jonit *et al.* (2016). Furthermore, polymerase chain reaction analysis conducted by Shivalingaiah *et al.,* (2012) also detected this infection in India.

In testing the primers in different labs, calibration of PCR machines was so much important for accurate results. Jones *et al*. (1989) reported about the specific marker of *Xanthomonas oryzae pv. oryzae*. Bacterial leaf blight *Xanthomonas oryzae pv. oryzae* specific primer *Xoo80* were used in the multiplex set. This multiplex set were selected for the amplification of all reported *X. oryzae pv. oryzae* strains, including strains weakly pathogenic to rice from the United States (Texas and Louisiana. Although primer *Xoo80* amplified the U.S. strains, the product was consistently less intense in gels compared with other *X. oryzae pv. oryzae* strains. In our experiment we also use *Xanthomonas oryzae pv. oryzae Xoo80* specific primer along with two primer *Xoo3350, Xoo4009* for accurate identification.

4.2. Phenotypic screening of BLB resistance advanced line

All advanced lines were found resistant on screening against virulent bacterial blight isolate (Table 4). P-3-58, P-5-1, P-7-70, P-7-82, P-8-78, gave the lowest disease score 1 and the susceptible check (Purbachi) gave the highest score 9. These five lines indicate that it contained resitant gene that's why it gave disease score 1. This scoring was done by standard evaluation system for rice IRRI, 2013 scale.

In our experiment we have used five advanced line P-3-58, P-5-1, P-7-70, P-7-82, P-8-78 all of which found were resistant and their score was 1 so we could say that all the advanced line performed better against the bacterial leaf blight. 2 standard checks BRRI dhan39 and BRRI dhan49 were scored 5. From two standard checks of the scale we could say that they were moderately susceptible variety and they perform moderately against bacterial leaf blight. One highly susceptible check was used named Purbachi which scored 9 according to the scale. This purbachi variety cannot withstand against bacterial leaf blight disease in any condition. So, it scored high according to the scale.

Table 4. Phenotypic screening of BLB resistance advanced line

Advanced line	Disease Score
P-3-58	1*
P-5-1	1*
P-7-70	1*
P-7-82	1*
P-8-78	1*
BRRI dhan39(Standard Check)	5*
BRRI dhan49(Standard Check)	5*
Purbachi(Susceptible Check)	9*

IRRI scale: *1-(1-5) % leaf area disease; * 3-(6-12%) leaf area disease; *5-(13-25) % leaf area disease; *7-(26-50) % leaf area disease; *9-(51-100) % leaf area disease

Latif *et al.* 2019 investigated that a total of 35 materials (20 advanced breeding lines from Plant breeding division, 2 from biotechnology division ,2 resistant checks and 2 susceptible checks) were screened against bacterial leaf blight (*Xanthomonas oryzae pv. oryzae*) pathogen. The experiment was conducted BRRI farm using artificial inoculation. The disease severity was recorded 21 days of inoculation (10 leaves in each entry). On the basis of leaf damage area, disease severity of all the entries were classified as Resistant (R) (Disease score 1), Moderately resistant (MR) (Disease score 3), Moderately susceptible (MS) (disease score 5), Susceptible (HS) (disease score 9). The leaf damage area was measured by eye estimation and disease severity scale following SES, IRRI. Mean data of each entry was converted in to disease severity scale (0-9), SES 2013, IRRI.

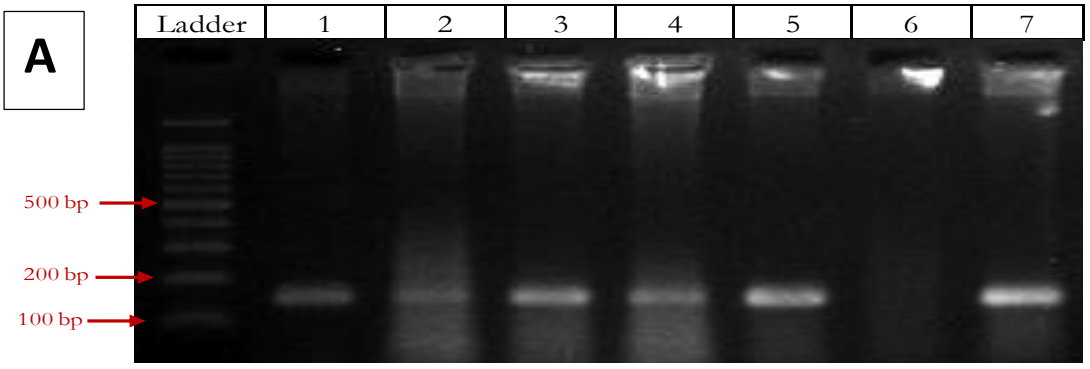
In their experiment they obtained among the tested genotypes none of the materials was found resistant to BLB. However only one material namely P-1 was found as moderately resistant among 22 breeding materials excluding checks against BLB. The resistant checks were also showed resistant to BLB.

4.2.1. Molecular screening of advanced lines for different BLB resistance gene

Molecular screening was practiced in the advanced line for establishing the bacterial leaf blight resitant gene (R) gene. We have screened five advanced line with one check IRBB60. Four resistant *Xa* genes was used for the screening of advanced line. All resistant gene was located by using those specific markers in gel electrophoresis. Among the five advanced lines, all advance lines except P-3-58 possessed *Xa4* gene (Table 5). Except P-7-82, all other lines having *Xa5* gene. No advanced line had *xa13* and *xa21* gene. All gel pictures are in (Figure 2).

Table 5. Detected resistance genes in the advanced lines

Advanced Lines	R genes			
	Xa4	***xa5***	***xa13***	***Xa21***
P-5-1	1	1	0	0
P-7-70	1	1	0	0
P-8-78	1	1	0	0
P-3-58	0	1	0	0
P-7-82	1	0	0	0



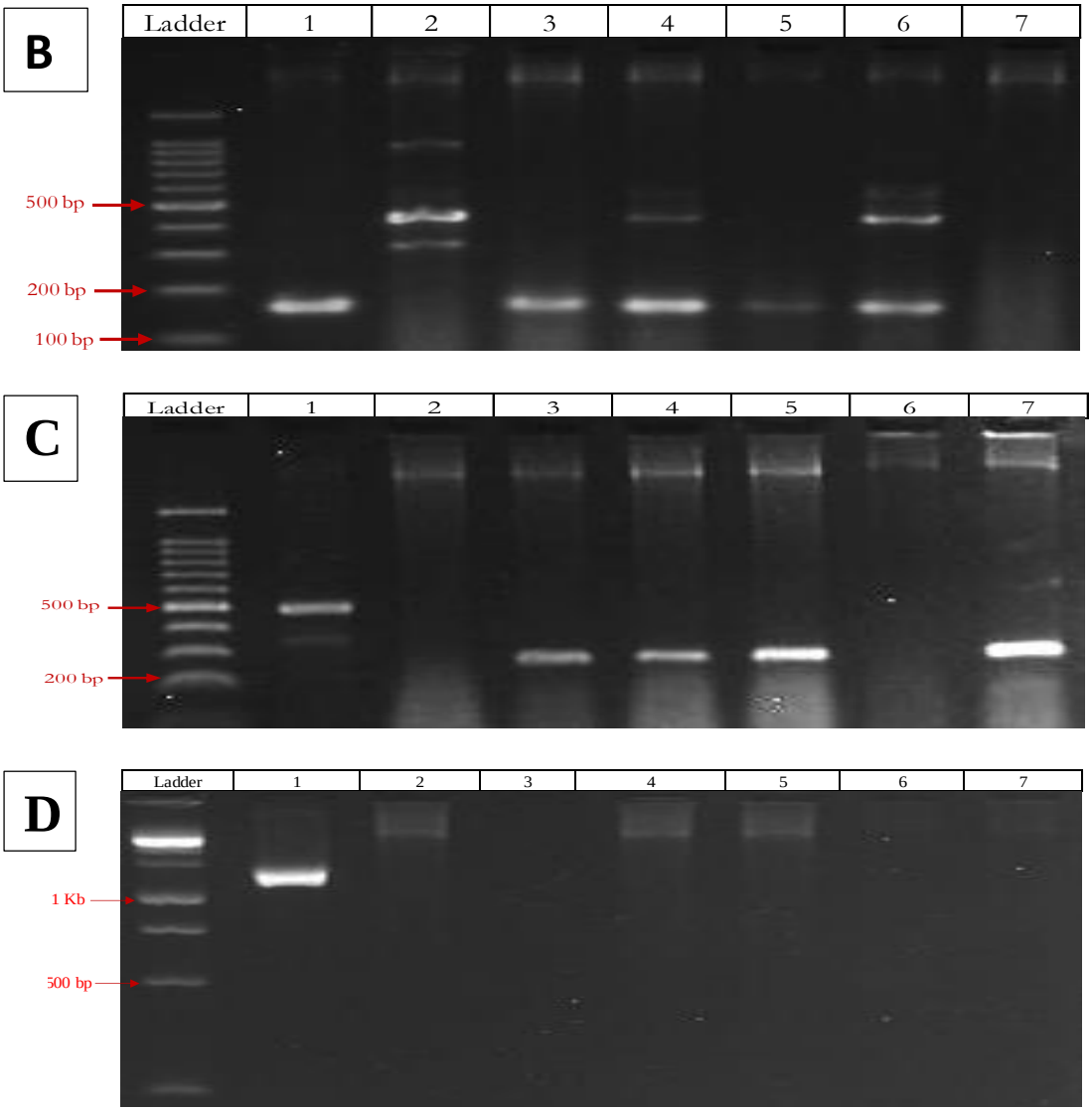


Figure 2. Gel picture of PCR product amplified for different resistance genes in advanced line. A. for *MP* primer (*Xa4* gene), **B.** for *xa*5 multiplex primer (*xa5* gene) **C.** for *xa13*-prom primer (*xa13* gene) **D.** for pTA248 primer (*Xa21* gene). Here 1=IRBB60 (check for all four genes), 2= Purbachi (contain no R gene), 3=P-5-1, 4=P-7-70, 5=P-8-78, 6=P-5-58, 7=P-7-72**.**

In our present study Four primer was used for the location detection of four *Xa* gene eg. *MP* primer was used for the detection of *Xa4* gene, *xa*5 multiplex primer was used for the detection of *xa5* gene, *xa13*-prom primer was used for the detection of *xa13* gene *pTA248* primer was used for the detection of *Xa21* gene. Pradhan *et al.* (2015) also used *pTA248*, RG 136 and *Xa*5S, R (multiplex) markers for the genes *Xa*21, *xa13* and *xa*5 gene detection. Wersinghe *et al.*

(2017) also used markers *pTA248* and *(MP)MP1+ MP2* markers for resistant gene detection.

Host plant resistance has been considered as the most economical and eco-friendly strategy for management of biotic stresses (Hulbert *et al*., 2001). For BLB there is no effective chemical control method practiced, hence, the only durable strategy is to grow resistant varieties. There are many BLB resistant germplasm available in the world, carrying multiple resistance alleles to the disease. So far 40 genes have been identified with alleles contributing to resistance against BLB (Khan, Naeem, & Iqbal, 2014). In our investigation, the resistant germplasm IRBB 60 carrying the resistance dominant alleles of *Xa4* and *Xa21* (Huang *et al*., 1997) and susceptible alleles *xa5 and xa13* (Hajira *et al*., 2016) were successfully pyramided to the backcross progeny of IRBB 60/ P-3-58, P-5-1, P-7-70, P-7-82, P-8-78.

Pradhan *et al*. (2015) carried a research about resistance gene in Jalmanga who found that *xa*5, x*a13* and *Xa*21 resistance genes was transferred in Swarna BB advanced line with (CRMAS 2232–85) check lines. He also detected the donor parent (CRMAS 2232–85) and recurrent parent (Jalmagna) with the markers *pTA248,* RG 136 and *Xa*5S, R (multiplex) for the genes *Xa*21, *xa13* and *xa*5 in parent polymorphism. Total of 122 lines were produced in his research of which, twenty-one, thirty-three and thirty-six lines showed presence of resistance genes, *Xa*21, *xa13* and *xa*5, respectively. He also described the parent polymorphism was detected for the donor (CRMAS 2232–85) and recurrent parent (Jalmagna) with the markers *pTA248*, RG 136 and *xa*5S, R (multiplex) for the genes *Xa21*, *xa133* and *xa5* respectively.

Wersinghe *et al.* (2017) reported *Xa*4 and *Xa*21 performed well with Ld-99-12-38 advanced line which is susceptible to BLB. Ld-99-12-38 backcrossed with 35 lines with check IRBB60 lines. BLB resistance gene (*Xa21* and *Xa4)* linked molecular markers *pTA248* and *(MP)MP1+ MP2* it *was confirmed* the resistance alleles from either *Xa21* or *Xa4*. Out of the tested 35 lines, eight were confirmed

with possessing both *Xa21 and Xa4* gene which was resistant to the disease, twenty-one lines carried only the resistant allele *Xa4.*

4.3. Field performance of BLB resistance advanced lines

4.3.1 Panicle length

Statistically significant differences were observed in terms of length of panicle in different lines and rice varieties (Table 6). The longest panicle was recorded from P-3-58 (26.02cm). Second longest was observed from P-5-1 (25.87cm), which was statistically similar with P-3-58 (26.02cm). However, the shortest panicle (24.34 cm) was observed in P-7-82(24.34cm) Among the check variety longest panicle length was observed with BRRI dhan39 (24.7cm) which was statistically similar with P-3-58 lines. Lowest panicle length from the check was obtained from Purbachi (22.7cm).

Rafi *et al. (*2015) reported that the lower and higher range for panicle length was 25.267cm and 33.583 cm after bacterial blight infestation in Kashmir Basmati and acc.0065025 respectively with overall average panicle length of 29.618cm. This experiment conducted in india was similar with our result.

4.3.2. percent grain sterility

Statistically significant differences were observed in terms of percent grain sterility in different lines and rice varieties (Table 6). The highest grain sterility was recorded from P-7-82 (34.47%) second highest grain sterility was observed from P-8-78(30.71%), which was statistically similar with P-7-82 (34.47%). the lowest sterility (26.45%) was observed from P-5-1. Among the check variety highest grain sterility was observed with BRRI dhan39 (23.41%) which was statistically similar with P-7-82 lines. Lowest grain sterility from the check was obtained from BRRI dhan49 (12.21%).

Singh and Deo *(*2019) also reported that the sterile grains per panicle (%) ranged from 26.37% to 35.10%. The highest number of sterile grains per panicle was recorded on T2 treated plots (35.10 %) followed by T1 (31.6 %). The lowest number of unfilled grains per panicle was observed in T5 (26.37 %) followed by T3 (27.76 %) and T4 (27.82 %).

4.3.3. 1000 grain weight

Statistically significant differences were observed in terms of weight of 1000 grains in different lines and rice varieties (Table 6). The highest weight of 1000 grains were recorded from P-5-1 (26.6 g). Second highest weight of 1000 grain was observed from P-7-70 (24.93g), which was statistically similar with P-5-1 (26.6 g). The lowest weight (23.74g) was observed from P-3-58. Among the check variety highest weight of 1000 grains was observed with BRRI dhan39 (24.65g) which was statistically similar with P-7-82, P-8-78, P-3-58 lines. Lowest grain sterility from the check was obtained from BRRI dhan49 (18.45g).

Rafi *et al. (*2015) who reported that average 1000 grain weight was recorded as 24.837 g. The minimum level (20.543g) was observed in acc. 006512 and higher level (44.463 g) was observed in acc. 006515. Acc. 006531 showed minimum value of biological yield per plot i.e. 1.433 kg while acc. 006509 showed maximum level of biological yield per plot i.e. 3.100 kg. Overall average was 2.090kg.

Shaheen *et al*. (2019) observed that the maximum weight loss discerned in 1000 rice grains was 17.84% at Sialkot while the minimum loss (11.17%) because of BLB infection was noted at Narowal. Other percentage losses noted at Hafizabad, Gujranwala and Nankana were 15.46%, 14.73% and 13.56% respectively. Maximum and minimum percentage losses in Grain Weight (GW) due to BLB infection were noticed in Sialkot and Narowal were 17.84% and 11.17% respectively. Such range of percentage weight losses (15.59%-11.94%) due to this pathogenic infection in Khyber Pakhtonkhwa province of Pakistan are also reported by Khan *et al.*, (2015). Intensification in disease severity is the main cause of 1000 grain weight reduction. Among all other pathogenic factors, BLB instigated due to *Xoo* interference is economically much important and cause considerable yield forfeiture each year in rice cultivating countries including Pakistan as well (Swing *et al.* 1990). It is surely a severe infection resulting annual grain losses of million tones. In case of Pakistan, the occurrence of BLB infection has increased in current years particularly in Kaller belt which

is eminent for the production of high-quality rice (Akhtar *et al.,* 2003; Ali *et al.,* 2009; Bashir *et al.,* 2010).

4.3.4. Yield (ton/ha)

Statistically significant differences were observed in terms of grain yield in different lines and rice varieties (Table 6). The highest grain yield (3.32 t ha^{-1}) was recorded from P-7-70. Second highest grain yield was observed from P-7-82 (3.15 t ha^{-1}). The lowest yield (2.46 t ha^{-1}) was observed in P-3-58. Among the check materials highest yield was observed from BRRI dhan49(3.45 t ha^{-1}) which was statistically similar with P-7-70. Lowest yield from the check was obtained from Purbachi (2.75 t ha^{-1}).

Similar findings were also recorded by Rafi *et al*. (2015) who reported that twenty-three indigenous rice germplasm and two commercial varieties i.e. Kashmir Basmati and IR-8 were studied. Out of all the tested genotypes thirteen lines were moderately resistant, five lines were moderately susceptible, six lines were susceptible and Kashmir Basmati showed highly susceptible response. None of the genotype was resistant to bacterial leaf blight. Among the tested genotypes Acc. 6505, 6508, 6509, 6515 6535 performed well for quantitative yield parameters along with moderately resistance response to BLB. Being highly susceptible variety Kashmir Basmati still showed good result for grain yield (0.78 kg/plot) relative to others.

Ansari *et al*. (2019) reported that the yield ranged from 2.4-3.4 t ha-1 among the treatments. The highest yield was recorded in the control (3.4 t ha-1) followed by heading (3.2 t ha-1) and flowering (2.8 t ha-1) stage inoculation with corresponding their lower disease severity. Similar yield at heading stage inoculation and control treatments were due to natural incidence of BB in control treatment occurred at heading and progress similarly until harvesting. BB inoculation in other three stages showed similar yield (2.4-2.7 t ha-1) which was significantly lower than the rest of the stages. Lower yield in MT, PI and booting stages was due to higher disease severity in these stages compared to the rest of the crop stages. Crop loss assessment is often reported as percent of the yield in

comparison with the control plot. The yield loss was observed 5.8-30.4% in the BB inoculated treatments.MT to Booting stages inoculation affected the yield much resulting 21-30.4% yield loss. Whereas later stage inoculation of BB at flowering or heading resulted considerably lower yield loss.

Similar findings were also recorded by Reddy *et al.* (1979) who reported that in tropical Asia, the yield losses of rice varied from 2 to 74% (depending on location, season, weather conditions and cultivars).

Mew *et al. (*1993) reported that the yield losses in individual affected fields were 20% or 20-30% while in severely affected field losses were over 50%. The severe infection at the tillering stage of rice could lead to yield loss of 50% or total crop losses.

Table 6. Field performance of BLB resistance advanced lines

Variety	**Panicle length**	**% sterility**	**1000 grain weight (g)**	**Yield (ton/ha)**
P-3-58	26.02 a	30.19 a	23.74 b	2.46 d
P-5-1	25.87 ab	26.45 ab	26.6 a	2.86 bcd
P-7-70	24.55 abc	29.97 a	24.93 ab	3.32 ab
P-7-82	24.34 bc	34.47 a	24.76 b	3.15 abc
P-8-78	24.52 abc	30.71 a	24.38 b	2.51 d
BRRI dhan39	24.7 abc	23.41 abc	24.65 b	2.84 bcd
BRRI dhan49	24.08 cd	12.21 c	18.45 c	3.45 a
Purbachi	22.7 d	16.3 bc	23.25 b	2.75 cd
CV(%)	3.79	30.24	4.2	10.15

CHAPTER-5

SUMMARY AND CONCLUSION

CHAPTER V
SUMMARY AND CONCLUSION

The present piece work was conducted at the Plant Pathology Laboratory, Bangladesh Rice Research Institute, Gazipur during the period from August, 2019 to February, 2020 to identify *Xanthomonous oryzae pv. oryzae* based on morphology and molecular marker and to identify the R genes using molecular marker and to evaluate the field performance of the five advanced lines.

Among the four isolated culture of *Xoo*, two isolates (Iso1 and Iso4) were detected as *Xoo* using three molecular markers (*Xoo80, Xoo4009 and Xoo3350*). Phenotypic screening of the bacterial blight resistance advanced lines along with susceptible check was conducted using virulent *Xoo* isolate and all lines were found resistant against BLB.

Molecular screening for bacterial blight resistance gene(s) from the selected advanced lines was conducted at the molecular laboratory of plant pathology BRRI, Gazipur. Four molecular markers *MP, xa5* Multiplex, *xa13*-prom and *pTA248* were used to identify *Xa4, xa5, xa13* and *Xa21* genes. From the study, all resistant line except P-3-58 possessed *Xa4* resistant gene. All resistant lines except P-7-82 possessed *xa5* gene resistant gene. No advanced line had possessed *xa13* and *Xa21* gene.

From present study it had been observed that, all the varieties developed bacterial leaf blight disease under field condition. Among all the varieties, the highest incidence and severity of BLB was found in Purbachi. The lowest incidence and severity of BLB were recorded in all five advanced resistance line (P-3-58, P-5-1, P-7-70, P-7-82, P-8-78).

From present study different yield data was recorded from the field. The data on growth parameters viz., Panicle length (cm), percent grain sterility (%), 1000 grain weight, yield (t ha^{-1}) were recorded. All the data were recorded after harvest.

The highest length of panicle was observed from P-3-58 (26.02cm) compared with other advanced lines and check variety. So, this line was resistant against bacterial leaf blight. Lowest panicle length was observed in P-7-82 line which was susceptible to bacterial leaf blight pathogen.

In terms of percent grain sterility, the highest sterility was observed from P-7-82 (34.47%) lines compared with other advanced line and check variety. So, this line was highly susceptible for disease against bacterial leaf blight. Lowest grain sterility was observed in P-5-1 (26.45) line.

In terms of thousand grain weight the highest weight was observed from P-5-1 (26.6g) compared with other four advanced lines whereas lowest weight was observed in P-3-58 which is disease susceptible against bacterial leaf blight pathogen.

In terms of yield, the highest yield was found in P-7-70 (3.32t ha^{-1}) compared with other four advanced line. This line is highly resistant compared to other four lines. The lowest yield (2.46 t ha^{-1}) was recorded in P-3-58 which was disease susceptible line against bacterial leaf blight pathogen.

Among the five lines, P-7-70 can be used for the development of durable bacterial blight resistant variety. Because this line provided the highest yield and both *Xa4* and *xa5* resistant genes were also detected in this advanced line. So in the present situation, it is preferable to cultivate P-7-70 lines for disease resistance and sustainable yield.

Further studies may be undertaken to find out more resistant lines for bacterial leaf Blight (BLB) disease under different agro ecological zones in the country for establishing those lines in the field and result validation.

CHAPTER-6

REFERENCES

CHAPTER VI
REFERENCES

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CHAPTER-7

APPENDICES

CHAPTER VII

APPENDICES

Appendix I. Layout of experimental field.

Replication 2	Replication 1	Replication3
BRRI dhan39	BRRI dhan49	5-1
Purbachi	8-78	7-70
8-78	BRRI dhan39	BRRI dhan39
7-82	Purbachi	8-78
7-70	7-70	3-58
5-1	3-58	7-82
BRRI dhan49	7-82	Purbachi
3-58	5-1	BRRI dhan49

5m

2.5m

Appendix II. Means square values for Panicle length of Aman rice at Field performance of BLB resistance advanced lines

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	2	3.2888	1.6444	1.9	0.1868
Variety	7	22.7626	3.2518	3.75	0.0169 *
Error	14	12.1448	0.8675		
Total	23	38.1962			

* Significant at 5% level, ** Significant at 1% level, *** Significant at 0% level

Appendix III. Means square values for Percent Sterility of Aman rice at Field performance of BLB resistance advanced lines

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	2	4.6295	2.3148	0.04	0.9618
Variety	7	1249.013	178.4304	3.01	0.0377*
Error	14	830.2443	59.3032		
Total	23	2083.887			

* Significant at 5% level, ** Significant at 1% level, *** Significant at 0% level

Appendix IV. Means square values for 1000 grain weight of Aman rice at Field performance of BLB resistance advanced lines

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	2	1.5444	0.7722	0.77	0.4818
Variety	7	120.0942	17.1563	17.1	0.0000***
Error	14	14.0469	1.0033		
Total	23	135.6856			

* Significant at 5% level, ** Significant at 1% level, *** Significant at 0% level

Appendix V. Means square values for Yield of T. aman rice at Field performance of BLB resistance advanced lines

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	2	0.2402	0.1201	1.37	0.2862
Variety	7	2.7331	0.3904	4.45	0.0085**
Error	14	1.2276	0.0877		
Total	23	4.2009			

* Significant at 5% level, ** Significant at 1% level, *** Significant at 0% level