MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Magnaporthe oryzae* CAUSING RICE BLAST AND ITS REACTION TO RESISTANT ADVANCED BREEDING LINES

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JUNE, 2020

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A Thesis
Submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
IN
PLANT PATHOLOGY
SEMESTER: JANUARY-JUNE, 2020

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CERTIFICATE

This is to certify that the thesis entitled **"MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Magnaporthe oryzae* CAUSING RICE BLAST AND ITS REACTION TO RESISTANT ADVANCED BREEDING LINES"** submitted to the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **PLANT PATHOLOGY**, embodies the result of a piece of bona fide research work carried out by **SHARMIN AKTER**, **Registration No. 13-05486** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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.

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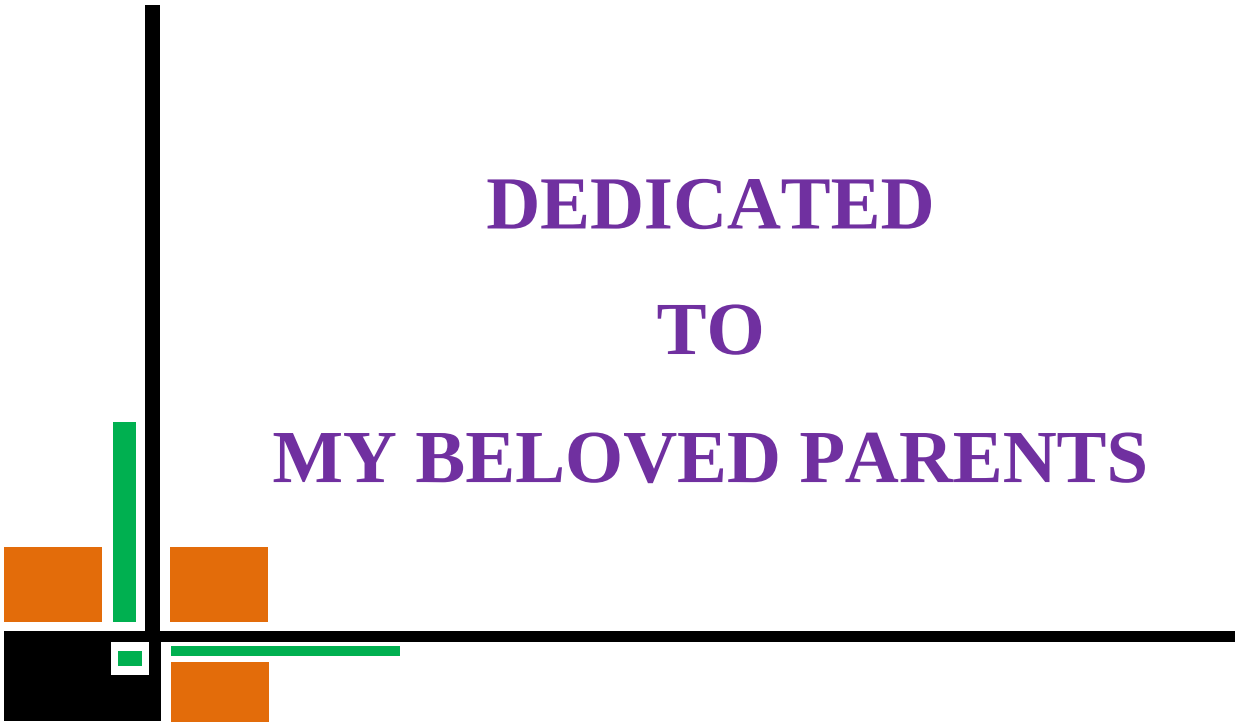

DEDICATED

TO

MY BELOVED PARENTS

LIST OF ABBREVIATIONS OF SYMBOLS AND TERMS

% : Percent

^{0}C : Degree Celsius

AEZ : Agro-Ecological Zones

bp : base pair

cm : centimeter

cm^2 : Square centimeter

CRD : Completely Randomized Design

DAI : Days After Inoculation

DAT : Days After Transplantation

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

et al. : and others

gm : gram

i.e.: That is

kb : kilobase

Kg : kilogram

L : Liter

LSD : Least Significant Difference

M : Molar

mL : Milliliter

mm : millimeter

mM : milli Molar

MPa : Mega Pascal

MMT : Million Metric Ton

NBS-LRR : Nucleotide-Binding Site Leucine-Rich Repeat

No. : Number

& : And

PCR : Polymerase Chain Reaction

pH : Hydrogen ion concentration

psi : pound per square inch

R gene : Resistant gene

RCBD : Randomized Complete Block Design

RMG : Radial Mycelial Growth

rpm : revolutions per minute

SSR : Simple Sequence Repeat

STAR : Statistical Tool for Agricultural Research

TBE : Tris Borate EDTA

TE : Tris EDTA

UBN : Uniform Blast Nursery

UV : Ultra Violet

viz. : Namely

µL:Microliter

ACKNOWLEDGEMENTS

All the praises and gratitude are due to Almighty Allah, who has kindly enabled the author to complete this research work and complete this thesis successfully for increasing knowledge and wisdom.

The author sincerely expresses her cordial gratitude, deep sense of respect and enormous indebtedness to her research Supervisor, ***Dr. M. Salahuddin M. Chowdhury,*** *Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for his scholastic supervision, incessant encouragement, positive suggestion, unvarying inspiration, co-operation and constructive criticisms throughout the entire period of research work and the preparation of the thesis.*

The author expresses heartfelt gratitude and indebtedness to her Co-supervisor, ***Dr. Tahmid Hossain Ansari****, Principal Scientific Officer, Plant Pathology Division, Bangladesh Rice Research Institute, Gazipur for his constant guidance, helpful suggestions, timely directions and inspirations on research for the successful completion of the research work and manuscript preparation.*

The author also wishes to pay her deep respect to ***Dr. Fatema Begum,*** *Chairman, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for her continuous support and valuable advices throughout the study period.*

The author also wishes to express her sincere gratitude to all other ***Respected Teachers*** *of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for their valuable suggestions and helps during the study period.*

The author expresses her immense gratitude to ***Montasir Ahmed,*** *Scientific Officer, Plant Pathology Division, Bangladesh Rice Research Institute, Gazipur for his continuous help and valuable suggestions throughout the whole experiment and preparation of the thesis.*

The author also likes to thank ***Ministry of Science and Technology*** *for facilitating* ***NST Fellowship*** *that helped to complete her research.*

The Author is also grateful to her friends, specially ***Yasin Afroja*** *and* ***Jannatus Sakib*** *for their support and valuable suggestions throughout the experiment.*

The author would like to expresses cordial thanks to all of the ***staffs*** *and* ***labors*** *of Plant Pathology Division, BRRI, Gazipur, for their valuable suggestions and helps throughout the whole research work.*

The author expresses his massive thankfulness to all of them who supported and encouraged her to pursue higher education and regret for her inability for not to mention every one by name who also contributed in pursuing the research works

The Author

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ABSTRACT

Experiments were conducted for identification and characterization to study pathogenic variation among *Magnaporthe oryzae* isolates. Eight blast resistant advanced lines of rice developed by BRRI were tested phenotypically and at molecular level for the presence of *Pi9* gene and their yield and yield attributes were studied. The experiments were carried out at Bangladesh Rice Research Institute (BRRI). The three isolates of Gazipur, Rangpur and Khulna were morphologically confirmed on the basis of three celled (2 septate) and pyriform hyaline conidia. Cultural characteristics on PDA, PSA and OMA media and mycelial growth as well as sporulation of the isolates were considered for determination of morphological variation. Cultural characteristics of three isolates showed different color (olivaceous, brownish or greyish to black), margin (filamentous, entire or undulate), surface texture (velvety or wrinkled) and colony form (regular or irregular) but same in case of elevation (flat) in different media. Sporulation of Khulna isolate was higher compared to others. Molecular detection of *M. oryzae* was confirmed using four specific primers namely; Bt1a/Bt1b, CAL-228/CAL-737, ACT-512/ACT-783, ITS1/ITS4 by amplifying fungal targeted region; beta tubulin (515 bp), calmodulin (510 bp), actin (260 bp), Internal Transcribed Spacer (520 bp), respectively in expected position. Eight *Pi9* gene introgressed blast resistant lines were evaluated along with donor parent (IRBL9-W) as resistant check and recurrent parent (BRRI dhan28) as susceptible check variety. Phenotypic reaction for blast resistance indicated that six lines (PL-6, PL-17, PL-21, PL-22, PL-26, PL-35) showed resistant reaction (score 0-2) and only one line (PL-34) showed susceptible reaction (score 4). BRRI dhan28 gave average score 3.75 (highest score 5) which was susceptible and IRBL9-W gave average score 0.5 (highest score 1) which was resistant to blast. These resistant lines were amplified with two linked markers RM8225 (172 bp) and NMSMPi9-1(168 bp) and all the phenotypically resistant lines gave the band at similar position with the linked markers. PL-21 gave maximum yield (4.47 t/ha) among the tested lines which was similar to BRRI dhan28 (4.94 t/ha). Therefore, it could be concluded that except PL-34, all the developed lines have *Pi9* gene and resistant to rice blast disease. The line PL-21 could be advanced for releasing as variety.

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa* L.) is the world's most important grain crop that belongs to the family Poaceae (USDA-NRCS, 2019). It is the staple food for more than half of the world's population (Yadav *et al*., 2015). In 2002, rice provided more than 500 calories person^{-1} d^{-1} for over three billion people and a substantial amount of protein for about 520 million people globally (FAOSTAT, 2019; Muthayya *et al*., 2014). It is also becoming a model plant for monocot species in molecular biology and genomics studies (Jo *et al*., 2007). Worldwide rice production is about 496.1 MMT of which 36 MMT is produced in Bangladesh holding 3rd position (USDA, 2020). Asia dominates as the top producing continent with India and China producing over 52% of the total rice in the world together (USDA, 2017). About 77% of small farmers of Bangladesh depend on the production and sale of rice for their food security and livelihood (Sayeed and Yunus, 2018). It has an important role in the economy of Bangladesh, accounting for nearly 70% of agricultural Gross Domestic Product (GDP) and providing about one-sixth of the national income of Bangladesh (Sayeed and Yunus, 2018). It is predicted that to meet global food demand, annual shortage of rice is estimated to increase the production of grains from 400,000 tons in 2016 to 800,000 tons by 2030 (Thirze, 2016).

The growth and productivity of rice is influenced by numerous biotic and abiotic factors. Every year around 37% of rice yield is reduced due to various biotic stresses (IRRI, 2019; Mondal *et al.*, 2017). Among different biotic stresses, diseases are of prime importance. Major diseases like blast, brown spot, sheath blight, bacterial blight and emerging diseases like bakanae, sheath rot and false smut are causing significant damage to the rice crop all over the world (Raghu *et al*., 2018). Among the fungal diseases, rice blast is the most important and destructive disease of rice, which is caused by an ascomycetous fungus *Magnaporthe oryzae* (Anamorph *Pyricularia oryzae* Couch) formerly

known as *Pyricularia grisea* (Cooke) Sacc. (Couch and Kohn, 2002). Several species of *Magnaporthe* cause blast disease on a wide range of graminaceous plant hosts. Recently, based on the phylogenetic analyses and mating tests, isolates from grasses including rice were named as *M. oryzae* and isolates from crabgrass were separated from the species complex and named *M. grisea* (Choi *et al*., 2013). *Magnaporthe oryzae* causes disease on more than 50 graminaceous species including important food crops such as rice (Tosa *et al*., 2004).

Rice blast was one of the ancient diseases of rice probably known as rice fever disease in China as early as 1637 and then reported in Japan in 1704, Italy 1828, USA 1876 and India in 1913 (Ou *et al*., 1971). Rice blast is a major concern in temperate areas as well as in tropical uplands in West Africa, Iran (Mousanejad *et al*., 2010), Malaysia (Rahim, 2010) and the savannas of South America (Bonman *et al*., 1989). Blast affects 12% of the total rice cultivation area in Indonesia (BPS, 2008). Rice blast, a severe fungal disease causing significant yield loss in rice (Aravindan *et al*., 2016). This infectious fungal disease is distributed worldwide and prevails in more than 85 countries of the world (Jamal *et al*., 2012), responsible for yield losses of about 10-30% annually (Sakulkoo *et al*., 2018; Ashkani *et al*., 2015; Wilson and Talbot, 2009). In favorable conditions, this disease can devastate entire rice plants within 15-20 days and cause yield loss of up to 100% (Musiime *et al*., 2005). It is estimated that more than 60 million people could be fed by the rice destroyed by blast disease annually (Pennisi, 2010).

Incidence and severity of blast disease in Bangladesh is increasing day by day, especially in the Boro season. Outbreak of the disease is a recurrent problem and is very difficult to control in all rice-growing regions of the world, including Bangladesh (Khan *et al*., 2014; Dean *et al*., 2005; Talbot, 2003; Valent and Chumley, 1991). The country has experienced several epidemic outbreaks of rice blast since 1980 (Ahmed *et al*., 1985; Shahjahan, 1994). Neck blast disease was severe in aromatic rice in Aman and BRRI dhan28 in Boro

season and recent year neck blast disease was found so devastating in BRRI dhan28 (BRRI, 2019). Severe blast attack occurred in Boro fields during the flowering stage in Northern part of the country viz. Bogra, Gaibandha, Naogaon, Rajshahi, Dinajpur, Rangpur, Nilphamari, Kurigram and Lalmonirhat, the country's largest rice farming hub (Mohibub-Uz-Zaman, 2017). BRRI dhan29 and BRRI dhan28 are the most popular and mega varieties of rice but they are highly susceptible to blast disease (BRRI, 2011). Disease incidence and severity is higher in irrigated ecosystems (Boro season) (21.19%) than in rain fed ecosystems (Transplanted Aman season) (11.98%) regardless of different AEZs (Hossain *et al.*, 2017). Its occurrence is as high as 68.7% in hybrid rice variety Jhalak followed by high yielding rice cultivar BRRI dhan47 (58.2%), BRRI dhan29 (39.8%), BRRI dhan28 (20.3%) during Boro and BRRI dhan34 (59.8%) during T. Aman season (Hossain *et al.*, 2017).

To increase rice yield by disease management strategies, chemical control of the disease is possible, but it is costly for resource poor farmers and environmentally dangerous to health. In such a situation, resistant cultivar is one of the potential ways of blast disease management. The resistant variety can be used as preventive measure. It is environment-friendly and at the same time a cost effective way to reduce the adverse effect of the blast disease (Yadav *et al*., 2017). Varietal resistances generally declined due to the appearance of new virulent races of the blast pathogen, cause difficulties to control the disease (Laha *et al*., 2017). Virulence of the *M. oryzae* isolates differ from place to place. In Bangladesh, wide variaton of blast isolates are reported by different researchers (Hossain *et al*., 2017; Khan *et al.*, 2016). Among 331 isolates of rice blast pathogen, 267 blast races are identified in Bangladesh (Khan *et al.*, 2016). In this regard, the structure and dynamics of the population of *Magnaporthe oryzae* need to be understood clearly if resistant genes are to be utilized to implement the strategies for management of blast (Vanaraj *et al*., 2013). In this study, it is therefore, given importance on the identification of blast isolates using ITS marker and virulence of those identified isolates which severely affected rice crop in different regions of

Bangladesh. Further, a number of advanced fixed lines possessing blast resistant *Pi9* gene have been developed in the background of BRRI dhan28 in Plant Pathology Division, BRRI (Ansari, 2019a). These lines have challenged by these virulence blast isolates before its utilization as variety for blast management or pre-breeding materials in the development of new blast resistant variety. The information obtained from this study will be helpful to develop management strategies for rice blast disease in Bangladesh and to devise strategies for developing durable resistant rice cultivars.

Objectives:

The present research work was undertaken to achieve the following objectives:

1. To identify the rice blast pathogen, *Magnaporthe oryzae* using molecular markers as well as cultural and morphological characterization.
2. To evaluate the blast resistant advanced breeding lines on the basis of disease reaction and field performance.

CHAPTER 2

REVIEW OF LITERATURE

The available literature of work was done on molecular and morphological identification of blast pathogen and its resistance in rice. The review of literature pertaining to this study is presented in foregoing pages.

Childs (2020) provided the information that global rice production in 2020/21 is forecast at 499.6 million tons (milled basis), down 0.5 million tons from the previous forecast but up almost 1 percent from a year earlier and the highest on record. The small monthly reduction was based on downward revisions in crop forecasts, for Bangladesh, Burma, Iraq, and Thailand that more than offset higher production forecasts for Brazil, Chile, EU, India, the United States, and Uruguay. India's production increase of 1.0 million tons was the largest increase.

USDA (2020) informed that worldwide rice production is about 496.1 MMT of which 36 MMT is produced in Bangladesh holding third position.

Ansari (2019b) experimented on pyramiding blast resistant *Pita2* and *Pi9* genes into Boro rice. He introgressed the monogenic resistant gene, *Pi9* and *Pita2* in the background of BRRI dhan28, BRRI dhan29 and BRRI dhan63 during T. Aman season, 2018. The seeds produced in different crossings and confirmation of the crosses was done through marker assisted selection. *Pi9* and *Pita2* were detected using the primer NMSMPi9-1 and YL155/YL87 respectively.

Asibi *et al*. (2019) found that the pathogen produces lesions on leaves (leaf blast), leaf collars (collar blast), culms, culm nodes, panicle neck nodes (neck rot), and panicles (panicle blast), which vary in color, shape and size depending on varietal resistance, environmental conditions and plant age.

Gowrisri *et al*. (2019) found that PCR amplification of ITS1 and ITS4 region and Pot2 transposon region of *M. oryzae* were done accordingly with pfh2a and

pfh2b primers, respectively. Amplicon size ranged between ~550 bp and ~680 bp band for ITS and Pot 2 transposon, respectively were observed in all the six isolates taken for analysis and the result showed the expected amplicon size in all the isolates.

Khan *et al*. (2019) said that a short duration popular variety named BRRI dhan33 is a blast resistant variety and was used as a blast resistant check in Plant Pathology Division, BRRI, from a long time.

Tian *et al*. (2019) experimented with the assistance of blast resistant gene, *Pi9/2*, *Pi9* and *Pi2*, from donor lines 75-1-127 and C101A51, respectively, were separately introgressed into the strong rice restorer line, Hui 316, by backcrossing. Two improved representative Hui 316 lines, Hui 316-*Pi9* and Hui 316-*Pi2*, displayed similar agronomic traits to Hui 316. Inoculation tests for blast resistance revealed that Hui 316-*Pi9* and Hui 316-*Pi2* showed markedly enhanced resistance to blast fungi compared with Hui 316. Investigation of agronomic traits for yield and rice quality indicated that the hybrid combinations from Hui 316-*Pi9* or Hui 316-*Pi2* crossed with male sterility lines were similar to those derived from Hui 316. Therefore, introgression of *Pi9* or *Pi2* into with Hui 316 could improve its resistance to blast with little influence on its restoring ability, which will facilitate the application of Hui 316 in rice production.

UNFPA (2019) narrated that about 0.16 billion people of Bangladesh consume rice as principal food.

BRRI (2018) informed that BR3, BR14, BR15, BR16, BR24, BRRI dhan32, BRRI dhan33, BRRI dhan44, BRRI dhan45, BRRI dhan74 are BRRI invented blast resistant varieties.

Sayeed and Yunus (2018) reported that seventy-seven percent of small farmers depend on the production and sale of rice for their food security and livelihood in Bangladesh. It has an important role in the economy, about 70 percent of

agricultural Gross Domestic Product(GDP) and providing about one-sixth of the national income of Bangladesh.

Zhao *et al.* (2018) stated that all of the cloned R genes except for *Pi-d2*, *pi21* and *Ptr* contain nucleotide-binding protein leucine-rich repeat (NLR) that actually responsible for the resistance.

FAO (2017) also reported that rice cultivation occupies 166 M ha area with a production of 758.8 MT per annum which produce 21% of world's food calorie supply.

Hossain *et al.* (2017) showed that the incidence and severity of blast disease of rice in ten agro-ecological zones (AEZs) of Bangladesh during Boro (irrigated ecosystem) and Transplanted Aman (Julrain fed ecosystem) seasons. Disease incidence and severity was higher in irrigated ecosystem (Boro season) (21.19%) than rain fed ecosystem (Transplanted Aman season) (11.98%) regardless of locations (AEZs).

Kulmitra *et al.* (2017) noticed that growth of *M. oryzae* in different solid, semi solid and liquid media. The colony color differed from greyish black to dark black color, smooth to irregular margin, medium to good growth of the pathogen.

Abed-ashtiani *et al*. (2016) used four primers in their experiment and found that the ITS, actin, β-tubulin and calmodulin gene regions of all *Magnaporthe* spp. isolates were successfully amplified with 505, 336, 536, and 512 bp long, respectively. BLASTn queries based on the four regions indicated that all isolates used in this study were 100% identical to those of *P. oryzae* in the GenBank, except for the three isolates (PO-FA01, PO-FA02, and PO-FA04) which were collected from the Selangor state which had 99% identity by substitution in one locus at position 397 (C/T) of the calmodulin gene.

Fang *et al.* (2016) informed that more than a hundred blast resistance loci or genes have been mapped to rice chromosomes.

Kariaga *et al*. (2016) studied on cultural character of different isolates of *P. oryza* on PDA media and found variation of colors was from white (isolate 4), light gray (isolate 2 and 3), dark brown (isolate 5) and black (isolate 1, 6, 7 and 8). Major differences in the colony textures were found in isolate 4 and 5 with cartilage like texture. Isolate 5 formed rings and Isolate 4 formed pits. The outstanding differences between the black colonies was in the edges; Isolate 1, 6 and 7 had smooth edges with clear emission of a yellow metabolite while 8 had a ragged edge and no metabolite.

Khan *et al*. (2016) mentioned that at present 267 races of rice blast have already been identified in Bangladesh's environment.

Scheuermann and Jia (2016) stated that the broad-spectrum, nucleotide-binding site leucine-rich repeat (NBS-LRR)-type disease resistance protein encoded *Pi9* gene in rice, was originated from Wild rice (*Oryzae minuta*) is an effective resistance gene for controlling rice blast disease.

Ramesh *et al.* (2015) verified introgressed different lines for blast resistance and revealed that the introgressed lines (ILM-16 and ILM-29) with gene pyramiding of three genes (*Pi1*, *Pi2* and *Pi54*) showed complete resistant reaction to all locations. The introgressed lines (ILM-10, ILM-11, ILM-15 and ILM-30-4) with two resistance genes (*Pi1* and *Pi2*) showed moderate resistant reaction. The introgressed line (ILM-30) with two resistance genes (*Pi2* and *Pi54*) showed moderate resistant reaction at three different locations.

Yadav *et al*. (2015) stated that Rice (*Oryza sativa* L.) is consumed as a staple food for more than half of the world's population.

Chuwa *et al*. (2014) used four primers Bt1a and Bt1b, CAL-228F and CAL-737R, ACT-512F and ACT-783R, ITS1 and ITS4 for molecular analyses of seven *P. oryzae* strains. The results showed no promising differences in banding patterns between individual strains, indicating that all strains analyzed were genetically homogeneous in nature but pathogenically heterogeneous.

Divya *et al*. (2014) experimented on Near Isogenic Lines (NILs) harboring different blast resistant *Pi* genes along with resistant and susceptible varieties were surveyed for blast resistance. These genotypes were randomly crossed to transfer disease resistance gene to agronomically superior varieties ADT 43, Improved White Ponni and BPT5204. Disease reaction was recorded in both artificial and natural epiphytotic conditions. The minimum blast incidence was observed in F1 generation of ADT 43/CT13432-3R, ADT 43/C101A51 and ADT 43/C101LAC across the environments. Advanced back cross inbred lines developed from the cross combination of ADT 43/CT13432-3R were also tested against blast disease. Gene pyramided back cross lines exhibited higher resistance than susceptible ones. Among the genotypes tested under epiphytotic conditions at different environments, lines with gene combinations *Pi1+Pi2+Pi33+Pi54* and *Pi1+Pi2+Pi33* showed highly resistance nature to blast disease than those with single genes indicating that these non-allelic genes have a complementary effect.

Gashaw *et al.* (2014) observed that blast fungal isolates produced ring like, circular, irregular colonies with rough and smooth borders on oat meal agar media having buff color, greyish black to black color. The colony diameter of different groups ranges from 67.40 to 82.50 mm and the conidial shape of the different groups is pyriform (pear-shaped) with rounded base and narrow towards the tip. On oat meal agar, colony color of all the isolates is usually grey with good growth. All the isolates showed raised mycelial growth with smooth colony margin.

Kapoor and Katoch (2014) said that rice blast is of immense importance in temperate, tropical, subtropical Asia, Latin America and Africa and found in approximately 85 countries throughout the world**.**

Ravindramalviya (2014) showed mycelial growth of *P. grisea* under *in vitro* in four culture media. Among them PDA media supported maximum mycelial growth followed by Richard's Agar medium after 168 hours of incubation. Sporulation of *P. grisea* was then observed in traces of Potato dextrose agar

medium and Richard's Agar medium after 168 hrs of incubation. However, Czapek-Dox medium was not found effective in case of both vegetative growth and sporulation of the test pathogen.

Wang *et al.* (2014) reported that 99 blast R genes have been identified so far till this day. Among them 45% were found in japonica cultivars, 51% in indica cultivars, and the rest 4% in wild rice species.

Lau (2013) investigated on the identification and characterization of rice blast fungus (*M. oryzae*) based on the morphology and with molecular examinations. He concluded that misidentification of various fungal isolates occurred due to the characterization on the basis of morphology, but ITS sequencing achieved more accurate recognition of all isolates at species level. Based on his findings, the ITS sequencing technology is a comparatively reliable and rapid molecular tool for the characterization of pathogenic fungal isolates.

Mahdieh *et al.* (2013) concluded that PDA culture medium could provide the best medium for *P. oryzae* vegetative growth regardless of light condition. However, *P. oryzae* caused sporulation when light was provided either continuously or at intervals. A combination of 16/8 hr light/darkness intervals and adding rice materials to culture media induced better sporulation of *P. oryzae.*

Vanaraj *et al*. (2013) recorded a fine experiment result on rice blast disease. The blast lesions were first surface sterilized with 0.1% mercuric chloride for a minute, then placed over clean glass slide and kept it in a sterile petri dish padded with wet cotton. The dishes were incubated at 28±2° Celsius for 48 hours. Single conidia were aseptically transferred to potato dextrose agar (PDA) media for maintenance. He also studied on different *Pyricularia oryzae* isolates and observed that colonies of *P. oryzae* appeared white on oat meal, rice polish and malt extract agar, grey on potato dextrose agar and whitish grey on rice agar media.

Yaduraju and Rao (2013) reported that rice is the staple food in Asia and the Pacific region and providing almost 39 % of calorie intake.

Khadka *et al.* (2012) found isolates of the fungus from different hosts differed in their response in media for mycelial growth and sporulation. Radial mycelial growth and days of sporulation of *P. grisea* were studied by culturing three fungal isolates from rice, finger millet and *Panicum* sp. on six different media: prune agar (PA), oat meal agar (OMA), potato dextrose agar (PDA), finger millet leaf decoction agar, finger millet polish agar (FPA) and finger millet meal agar. The highest RMG was found in the isolates from finger millet and the lowest in the isolates from rice. The shortest days of sporulation (1 week) was found in the isolate from rice and the longest (>2 weeks) in the isolate from finger millet. Among the different media used, PA and OMA were found to be the best for mycelial growth and sporulation of the isolates both from rice and finger millet. The shape, color and compactness of the fungal colonies varied with the media and isolates used. Cross inoculation studies showed that the fungus isolates from rice were able to infect all the plant species while isolates from finger millet were only able to infect three plant species (*E. coracana*, *Setaria* sp. and *E. indica*).

You *et al*. (2012) reported that the mycelium consists of septate, uninucleate and branched type hyphae. However, as the fungus gets older, the hypha become brownish. Generally, growth of the pathogen is relatively more on upper surface than lower surface making the spot darker on upper side. Conidiophores are simple, septate and basal portion is relatively darker than the rest. Conidia are pyriform in shape and hyaline in color, produced acrogenously, one after another. Conidia is three celled, the middle cell being much wider and darker, and end cell germinates giving out germ tube. Conidium is rarely two celled or four celled. Formation of intercalary or terminal chlamydospores is common, which are globose, olive brown and thick walled.

Shafaullah *et al.* (2011) said that blast is known to attack nearly all above ground parts as well as during all growth stages of plant. Recent reports shows that the fungus has the capacity to infect plant roots as well.

Javadzadeh and Motlagh (2010) isolated from small leaf pieces having conspicuous lesions were surface sterilized with 0.5% sodium hypochlorite solution. Then they were rinsed with sterile distilled water and placed on Potato Dextrose Agar (PDA) media in Petri dishes at 25ºC for 48 hours. Later, Petri dishes were incubated at dark or artificial fluorescent light for 12 hours by alternating the light and dark photoperiod for up to 25 days. Monoconidial isolates of the recovered fungi were maintained on half-strength potato dextrose agar media in test tubes and stored as stock culture.

Supriya Devi and Sharma (2010) reported that it is the most destructive pathogen of rice worldwide and around 50% of production may be lost in a field moderately affected by blast.

Castilla *et al.* (2009) said that the environment with frequent and prolonged dew periods and with cool temperature in day time is most favorable for the spread of the blast disease.

Cruz *et al.* (2009) observed the higher sporulation on wheat meal culture medium in alternate light and dark regime.

Silva *et al.* (2009) isolated the pathogen from eight samples of rice leaves in Goias, Brazil. They took 2-3 lesions from each leaf and one conidium from each lesion and transferred to 5% water agar media. Thus monoconidial isolate was obtained. The collected isolates were then conserved on sterilized filter paper disc and stored in refrigerator at -20ºC.

Skamnioti and Gurr. (2009) stated that two types of resistant genes are responsible for rice blast resistance viz. major resistance (R) genes that induce race-specific resistance and quantitative trait loci (QTLs) that control partial, nonrace-specific resistance.

Arshad *et al*. (2008) reported that rice blast was mostly found in Pakistan during the last two decades in districts of Faisalabad, Toba Tek Singh, Vehari and places like Gaggoo Mandi.

Nutsugah *et al*. (2008) stated that *M. oryzae* is able to infect plants at all stages of growth and development in both upland and lowland rice production systems. Lowland rice produced in both temperate and subtropical climates of Asia are highly susceptible to the pathogen, while tropical upland areas are susceptible only under irrigation.

Ram *et al.* (2007) reported that leaf blast fungus can attack the rice plant at any growth stage and can cause severe leaf necrosis resulting in decreased grain yield and weight. When the fungus attacks node, it causes sterility. Rice blast pathogen infects all the above ground parts of rice plants at different growth stages. It causes a typical blast lesion on rice leaf as grey at the center with a dark margin and gradually becomes spindle shaped.

TeBeest *et al*. (2007) stated that *Magnaporthe oryzae* produces sexual spores (ascospores) in structures called asci, that's why it's an ascomycete and is classified in the newly established family Magnaporthaceae. The asexual stage of *M. oryzae* is named as *Pyricularia oryza.*

Sharma (2006) showed that 12 elite germplasm *viz*; HPR- 917, HPR- 933, HPR- 977, HPR- 1001, HPR- 1009, HPR- 1020, HPR- 1062, HPR- 1064, HPR-1153, HPR- 1155, HPR-1161 and HPR- 1174 and six released varieties *viz;* Himalaya 741, Himalaya 799, Himalaya 2216, RP-2421, IR 64 and Palam Dhan 957 are resistant against rice blast.

Agrios (2005) stated in his book that rice blast is one of the most devastating diseases all over the world.

Chadha and Gopalakrishna (2005) showed that primers were designed based on the nucleotide sequence of the mif 23, an infection-specific gene of *M. grisea*. The primers amplified target DNA from genetically and geographically diverse

isolates of the pathogen. The lowest concentration of template DNA was 20gg that led to amplification. When DNA from other fungi was used, no PCR product was detected. That indicates the specificity of the primers. With this PCR based seed assay, *M. grisea* was detected in different rice seed lots with infestation rates as low as 0.2%.

Meena (2005) found the grayish black and raised mycelial growth of the blast isolates.

Leong (2004) reported that rice blast is the most serious disease found in the extensive rice growing areas of Latin America, Africa, and Southeast Asia and a worldwide problem in rice production. Rice blast disease is a significant constraint to global food security and agricultural trade.

Liu *et al*. (2004) and Chiba *et al*. (1996) informed that rice blast disease is distributed in about 85 countries in all continents where the rice is grown, in both low land and upland conditions. Rice blast is present wherever rice is cultivated, but the disease occurs with highly variable intensities depending on climate and cropping pattern. Environment with frequent and prolonged dew periods and with cool temperature in daytime are more favorable to blast occurrence.

Oerke and Dehne (2004) informed that rice blast caused an estimated annual loss of 35% of the worldwide rice production during 90's.

Seebold *et al.* (2004) found that the fungus *Pyricularia oryzae* attacks at all stages of the crop and symptoms appear on leaves and nodes.

Sesma and Osbourn (2004) said that blast is very devastating for its ability to attack nearly all above ground parts as well as during all growth stages of a plant. Recent reports have shown that the fungus has the capacity to infect plant roots as well.

Harmon *et al*. (2003) used a method to confirm the isolates of *M. oryzae* on rice. They have developed and evaluated a PCR based method to detect *M.*

oryzae in infected perennial rice tissue. They have designed the primers in such a way that will amplify a 687 bp fragment of the Pot 2 transposon found in multiple copies in the genome of the pathogen.

Talbot (2003) stated that fungus produce sexual fruiting bodies called perithecia within 3 weeks. Perithecia are flask-shaped and carry asci containing ascospores, the products of meiosis. Ascospores are arranged as unordered octads or as larger populations of randomly selected ascospores.

Couch and Kohn (2002) reported that rice blast is one of the most important diseases of rice and caused by the fungus, *Magnaporthe oryzae*.

Liu *et al*., (2002) stated that *Pi9* is the first blast resistant gene identified from wild rice *Oryzae minuta* and they used a total of 43 blast isolates collected from 13 countries to inoculate the *Pi2*(t) and *Pi9*(t) plants. *Pi9*(t)-bearing lines were highly resistant to all isolates tested than *Pi2*(t) bearing lines.

Hajimo (2001) stated that the fungus *P. grisea* was able to infect and produce lesions on all organs of the rice plant and when the fungus attacks young leaves, purple spots were observed changing into spindle shape which has a grey centre and purple to brown margin. Brown spots appeared only on older leaves or leaves of resistant cultivars. In young or susceptible leaves, lesions coalesce together and cause withering of the leaves, especially at seedling and tillering stage. Infection to the neck results in the formation of triangular purplish lesions followed by elongation on both sides of neck. When young necks are infected, the panicles become white in color and later infection caused incomplete grain filling and poor grain quality.

Kato *et al.* (2000) said that the disease generally spreads by infected seeds and airborne spores, and the fungus can survive in infected crop residues and seeds.

Mijan Hossain (2000) observed that mycelium in cultures was hyaline initially and gradually changed to olivaceous, 1-5.2 µm in width, septate and branched. The spores were 15-22 µm × 4-7 µm (Average, 17.4 µm × 5.2µm) in size.

Among different non-synthetic media, Potato Dextrose Agar (PDA) supported maximum radial growth (85.00 mm) followed by host extract + 2 per cent sucrose agar medium (80.33 mm) and Oat Meal Agar(OMA) (75.00 mm) gave minimum radial growth.

Manandhar *et al.* (1998) reported that *P. grisea* is one of the most important fungal pathogens of rice because of its widespread occurrence and destructive nature. The fungus can attack any aerial part of the rice plant, including seeds making it very destructive. They also suggested systemic transmission of the fungus from seeds to seedlings.

Dean (1997) and Hamer *et al.* (1988) reported that the germinating spore of *M. oryzae* develops an appressorium, a specialized infection cell which generates enormous turgor pressure (up to 8MPa) that ruptures the leaf cuticle, allowing invasion of the underlying leaf tissue.

Faure and Mazaud (1996) found that in several developed countries such as North America and European Union (EU) also, rice consumption is increasing due to food diversification and immigration.

Manibhushanrao (1994); Bhatt and Singh (1992) and Padmanabhan (1974) observed that *Magnaporthe oryzae* infects and produces lesions on different parts of the rice plant viz. leaf (leaf blast), leaf collar (collar blast) and panicle (panicle blast). In leaf blast, initial lesions/spots are white to gray-green with darker borders around. Older lesions are white-grey, surrounded with a red-brown margin and are of diamond shape (wide centre and pointed toward either end). Lesion size is commonly 1-1.5 cm in length and 0.3-0.5 cm in width. Under favorable conditions, lesions can coalesce and covers the entire leaf which eventually kills the leaf. In collar rot, lesions are located at the junction of the leaf blade and leaf sheath and it can kill the entire leaf.

Xia *et al*. (1993) also isolated *Magnaporthe oryzae.* The infected panicles were collected with the symptoms of neck blast, washed well with sterile distilled water, and placed on wet filter paper in Petri dishes at room temperature to

initiate sporulation. Conidia from the lesion surface were spread over 3% water agar with a sterile loop and incubated overnight. Single germinating conidium was isolated and transferred to Potato Dextrose Agar (PDA) medium.

Bonman (1992) said that infection to the neck node produces triangle shaped purplish lesions, followed by lesion elongation to both sides of the neck node symptoms which are very crucial for grain development. When young neck nodes invasion occurs, the panicles become white in color, so called 'white head' that is sometimes misinterpreted as insect damage. Infected panicles appear white and are partially or completely unfilled. The white head symptoms are easily confused with a stem borer attack which also results in a white and dead panicle. Panicle branches are also infected. Spikelets attacked by the fungus change to white in color from top and produce many conidia, which become the inoculum source after heading phase. Panicle blast symptom includes the panicle appearing brown or black. Node infection includes infected nodes appearing black-brown and dry. This kind of infection often causes the culm breakage, resulting in the death of the rice plant. The pathogen is most common on leaves, causing leaf blast during the vegetative stage of growth, or on neck nodes and panicle branches during the reproductive stage, causing neck blast.

Awoderu *et al.* (1991) recorded that linear growth of *P. oryzae* was greatest on Potato Dextrose Agar (PDA), while conidial production was greatest on 1 percent soluble starch yeast extract agar. He also observed that the minimum, optimum and maximum temperature for mycelial growth and conidial production of *P. grisea* were 10^{o}C, 25^{o}C and 37^{o}C, respectively.

Bastiaans (1991) observed that leaf blast lesions reduce the net photosynthetic area of individual leaves to an extent far beyond the visible diseased leaf fraction.

Bourett and Howard (1990) informed that spores of *M. grisea* germinates rapidly and within two hours of landing on the leaf, a polarized germ tube is formed and then appressorium develops.

Hawksworth (1990) narrated the description of the culture from Commonwealth Mycological Institute (CMI) that cultures greyish in color, conidiophores single or in fascicles, simple or rarely branched, shows sympodial growth. Conidia formed singly at the tip of the conidiophore at different points arising sympodially and in succession, pyriform to obclavate in shape, narrowed toward tip, rounded at the base, normally three celled, rarely one or two celled, hyaline to pale olive, 19-23 × 7-9 μm diameter, with a distinct protruding basal hilum. Chlamydospores often produced in culture, thick-walled, 5-12 μm diameter.

Sun *et al.* (1989) studied the effects of 17 media on 41 isolates of *P. oryzae.* They found that rice straw and corn meal agar media were most effective for sporulation.

Reddy and Bonman (1987) linear growth of *Pyricularia* colonies isolated from rice was measured on standard medium agar, oat meal agar, french bean agar and decoction agar made out of the leaf material of rice. They also determined the weight of mycelial mat produced by the isolates in the standard medium, Richards's medium, Browns medium and decoctions of leaf material of rice. The isolates produced good growth on the decoctions by their host material.

Rahnema (1979) recorded that longer duration of susceptive condition such as relative humidity and darkness increases conidia germination percentage and appressoria formation.

Yaegashi and Udagawa (1978) suggested *M. grisea* as a perfect stage of *Pyricularia grisea* (cooke.) Sacc. instead of *Ceratosphaeria grisea*.

Yaegashi and Nishihara (1976) expressed the genus *Magnaporthe* for the first time.

Ou *et al.* (1971) reported that rice blast probably the disease known as rice fever disease in China as early as 1637 and then reported in Japan in 1704, Italy 1828, USA 1876 and in India in 1913.

Chakrabatri and Wilcoxson (1970) showed the effect of light on sporulation. They reported that light stimulates sporulation of *P. oryzae*. They found that there was a distinct interaction between temperature and light. The experiment showed that ultra-violate light was most favorable for conidia production of *P. oryzae*.

Padmanabhan (1965) showed that the dimension of conidia produced by *M. oryzae* ranged from 17.6 to 24.0 µm in length and 8.0 to 9.6 µm in width.

Ono and Nakazato (1958) observed that the size of conidia of *P. grisea* varied with the nature of culture media as well.

Aoki (1955) measured 16 isolates in Potato Dextrose Agar (PDA) culture and result showed that the average length of the isolate ranged from 21.2 to 28.4µm and the average width from 7.3 to 9.0µm.

Nishikado (1917) found that the dimension of *P. oryzae* spores was measured 16-33 × 5-9 µm. Usually 22-27 × 7-8 µm with a small basal appendage. Other dimensions were, basal appendage 1.2-1.8 µm (1.6µm) in width, basal cell 4.8-11.5 µm (7.8µm), middle cell 1.8-11.5 µm (6.6 µm), apical cell 6-14 µm (7 µm) in length.

CHAPTER 3

MATERIALS AND METHODS

The details of the materials used and methodologies adopted during present study are described in this chapter. The research activities were conducted under 3 experiments-

- **Experiment-1**: Isolation, identification and characterization of rice blast pathogen, *Magnaporthe oryzae.*
- **Experiment-2**: Phenotypic evaluation and molecular confirmation of *Pi9* gene in blast resistant advanced breeding lines.
- **Experiment-3**: Field performance evaluation of the resistant advanced breeding lines.

3.1. Experimental Site and Period

The present research was carried out at Bangladesh Rice Research Institute (BRRI), Gazipur-1701 during June 2019 to February 2020. This experiment was a part of the core research of BRRI. The laboratory work was done at Fungi Culture Lab, phenotypic screening at Uniform Blast Nursery (UBN), molecular work at Molecular Plant Pathology Laboratory, Plant Pathology Division, BRRI and the field experiment was conducted at research field, BRRI. The disease infected samples and seeds of advanced breeding lines were provided by the Plant Pathology Division, BRRI, Gazipur.

3.2. Preparation of culture media and culture plates

3.2.1. Glassware and equipments used

The following equipments and materials were used in present investigation:

1. Incubator (HettCube 400 R, Hettich, Germany)

2. Autoclave (Astell, Astell Scientific, UK)

3. Dry oven (BINDER, Yang Ming, China)

5. Inoculation glass needle, forceps, needles, blades, scalpel, scale, aluminum foil, cotton, parafilm, petridish, conical flask, blotter paper etc.

6. Laminar air flow cabinet (LABCAIRE, Labcaire Systems Ltd, UK)

7. Electronic weighing balance (FSH, A&D Company Ltd., Korea)

8. Refrigerator

9. Spirit lamp

10. Compound microscope (CX41RF, Olympus, Japan)

11. Stereo microscope (Leica ES2, Leica Microsystems, Germany)

3.2.2. Cleaning and sterilization of glassware

All the *in vitro* experiments were conducted under aseptic conditions. The equipment used for the experiments e.g. glassware, inoculation needles, scissors etc. were sterilized by keeping them in a dry oven at 180^0C for at least six hours before starting the experiment.

The glassware was cleaned first with a detergent followed by thoroughly rinsing with tap water. The cleaned glassware were then placed in potassium dichromate solution for 24 hours and finally washed with distilled water for 3-4 times. Then they were air dried and sterilized in oven at 180^0C for 1 hour (Darmady *et al*., 1961).

Different media and water used in the study were sterilized in an autoclave under 15 psi at 121.6^0C for 20 minutes. Work benches were sterilized by 70% ethanol. Scissors, forceps, needles were also sterilized in the autoclave and by flame before working.

3.2.3. Preparation of culture media

For isolation and *in vitro* growing of *M. oryzae*, the causal fungus of blast disease of rice, five culture media namely Water Agar, Potato Dextrose Agar, Potato Dextrose Broth, Potato Sucrose Agar and Oat Meal Agar were used. Each medium was prepared following standard procedures.

3.2.3.1. Water Agar (WA)

Water Agar was used for separation of fungus mycelium from diseased samples (Lingappa and Lockwood, 1960).

To prepare 1L of WA medium, 15 gm agar powder was added to 1000 mL distilled water in a beaker and thoroughly mixed with a glass rod. The mixture was heated in an oven until the agar powder completely dissolved. Additional distilled water was added to the beaker to make the volume 1000 mL to compensate evaporation loss during heating and dispensed into 250 mL conical flasks at 200 mL per flask. The mouth of the flask was closed with non-absorbent cotton, covered with aluminium foil and autoclaved at 121^0C under 15 psi for 20 minutes. Autoclaved medium was stored in a refrigerator at 4^0C.

3.2.3.2. Potato Dextrose Agar (PDA)

To prepare 1L of PDA medium, 200 gm sliced potato was cooked in 500mL distilled water until the potato slices boiled. Water extract of potato was filtered into 1000 mL measuring beaker through a cheese cloth, 20 gm dextrose and 20 gm agar powder and sufficient quantity of distilled water was added to make the volume 1000 mL. The mixture was heated in a oven until the agar dissolved and poured into 250 mL conical flask at 200 mL/flask. The mouth of the flask was closed with non-absorbent cotton, covered with aluminium foil and autoclaved at 121^0C under 15 psi for 20 minutes. Autoclaved medium was stored in a refrigerator at 4^0C (Ricker and Ricker, 1936).

3.2.3.3. Potato Dextrose Broth (PDB)

To prepare 1L of PDB medium, 200 gm sliced potato was cooked in 500 mL distilled water until the potato slices boiled. Water extract of potato was filtered into 1000 mL measuring beaker through a cheese cloth, 20 gm dextrose and sufficient quantity of distilled water was added to make the volume 1000 mL. The mixture was heated until the agar dissolved and poured into 250 mL conical flask at 200 mL/flask. The mouth of the flask was closed with non-

absorbent cotton, covered with aluminium foil and autoclaved at 121^0C under 15 psi for 20 minutes. Autoclaved medium was stored in a refrigerator at 4^0C.

3.2.3.4. Potato Sucrose Agar (PSA)

To prepare 1L of PSA medium, 200 gm sliced potato was cooked in 500 mL distilled water until the potato slices boiled. Water extract of potato was filtered into 1000 mL measuring beaker through a cheese cloth, 20 gm sucrose and 20 gm agar powder and sufficient quantity of distilled water was added to make the volume 1000 mL. The mixture was heated until the agar dissolved and poured into 250 mL conical flask at 200 mL/flask. The mouth of the flask was closed with non-absorbent cotton, covered with aluminium foil and autoclaved at 121^0C under 15 psi for 20 minutes. Autoclaved medium was stored in a refrigerator at 4^0C.

3.2.3.5. Oat Meal Agar (OMA)

To prepare 1L of OMA, 70 gm of oat flakes was boiled in 500 mL distilled water for 15 minutes and passed through cheese cloth. Exactly, 20 gm agar powder was heated in a oven with another 500 mL distilled water until the agar completely dissolved. Both mixtures were mixed in a 1000 mL beaker. Required quantity of distilled water was added to make the final volume 1000 mL. The final mixture poured into 250 mL conical flask at 200 mL/flask. The mouth of the flask was closed with non-absorbent cotton, covered with aluminium foil and autoclaved at 121^0C under 15 psi for 20 minutes. Autoclaved medium was stored in a refrigerator at 4^0C (Johnson and Curl, 1972).

3.2.4. Preparation of the culture plates

Whenever necessary, preserved medium in conical flask was melted in a laboratory micro oven. Melted medium was cooled down to 40^0C and poured into sterilized glass petri dishes inside a laminar airflow cabinet. Each plate was labelled with the type of media to be poured into it. After solidification of medium, the plates were sealed with parafilm tape.

3.3. Isolation, identification and preservation of *Magnaporthe oryzae* isolates

In vitro experiments were carried out following Completely Randomized Design (CRD) with four replications.

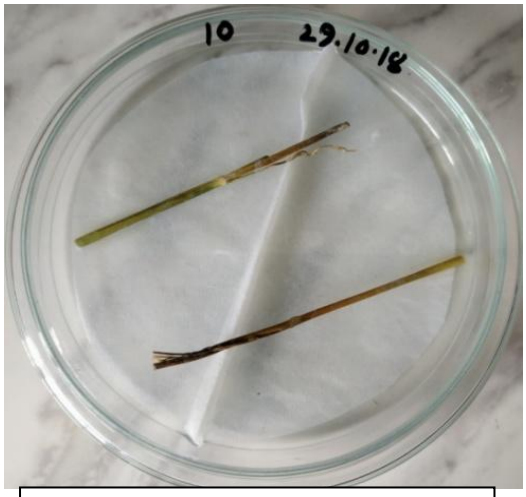
3.3.1. Collection of Blast disease infected sample

Blast disease infected leaf and panicle samples were collected from farmers' fields of Dhormodas Lokkhonpara village under Rangpur Sadar Upazila of Rangpur district, Sajiara village under Dumuria Upazila of Khulna district and Gazipur Sadar Upazila of Gazipur district. Collected samples were put into envelop separately and brought to the laboratory.

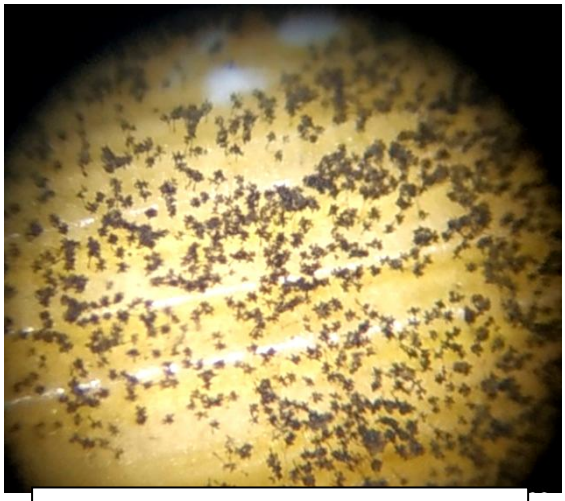
3.3.2. Isolation and identification of test isolates

Blast causing fungus was isolated from infected panicle node. Approximately 5 cm long plant pieces with diseased and healthy parts were cut with a sharp knife, washed with sterile water and plated on sterilized folded moist filter paper in glass petri dishes (Plate 1). The petri dishes were placed in an incubation chamber under a fluorescent light. Before putting in the incubation chamber, lid of each petri dish was removed and all the petri dishes were placed inside a transparent plastic box to prevent loss of moisture and contamination. After 24 hours of incubation, plant pieces were observed under stereo microscope. Fungal spores observed on infected tissues were collected with a needle, placed on a glass slide, covered with cover slip and observed under compound microscope (Plate 1).

Single spore of test pathogen was transferred to petri dishes containing WA media with the help of glass needle. The petri dishes were sealed with parafilm tape and incubated at 25^{o}C for 2-3 days. Culture growth occurs within 2-3 days and mycelial blocks were cut from the culture and placed in PDA plate. All these transfer works were done under aseptic condition in a laminar airflow cabinet. The inoculated petri dishes were labelled and incubated at 25^{o}C in an incubator until the mycelium growth reached the rim of the plate. The culture plates were used for further experimentation.



Infected panicle on moist filter paper



Spores on panicle under stereo microscope (30X)

Plate 1. Isolation of *M. oryzae* from infected panicle

3.3.3. Purification of the pathogen culture

To avoid any kind of contamination, purification of culture was done. It was done by sub-culturing through hyphal tip method. Sub-culture of isolates was done for rejuvenation of growth. Hyphal tips of isolated fungus were collected aseptically in Laminar Air Flow Cabinet from edge of an actively growing fungal colony along with a bit of culture medium for sub-culturing fortnightly and preserved at low temperature (4^{o}C) in refrigerator (Ellis, 2001).

3.3.4. Cultural and morphological characterization of test isolates

Cultural and morphological characters of all monoconidial isolates of test pathogen were observed by growing them on different medium for 2 weeks at 25^{o}C. Cultural characters *viz*., colony color, margin, elevation, surface texture and colony form were observed on PDA, PSA and OMA media and radial growth (cm) of the fungal mycelium were recorded on PDA medium. Morphological characteristics *viz.* size and shape of conidia, septa formation and sporulation were observed on OMA medium. Spores of test pathogen of different isolates were collected from the culture plate and mounted in glycerin on a clean slide. Spores were measured digitally under high power objective (40X) using compound microscope. The average size of spore was then

determined and shape of the spores were recorded. Microphotographs were also taken to observe the typical spore morphology of the pathogen (Srivastava *et al*., 2014).

3.3.4.1. Measurement of mycelial growth

First observation and radial growth was done on PDA media after 72 hours of inoculation and the subsequent growth was measured at 3 days interval. Four cross lines with semi-permanent black marker pen were drawn on the under surface of the plate i.e., the lower portion of the plate by taking a center (Plate 2).

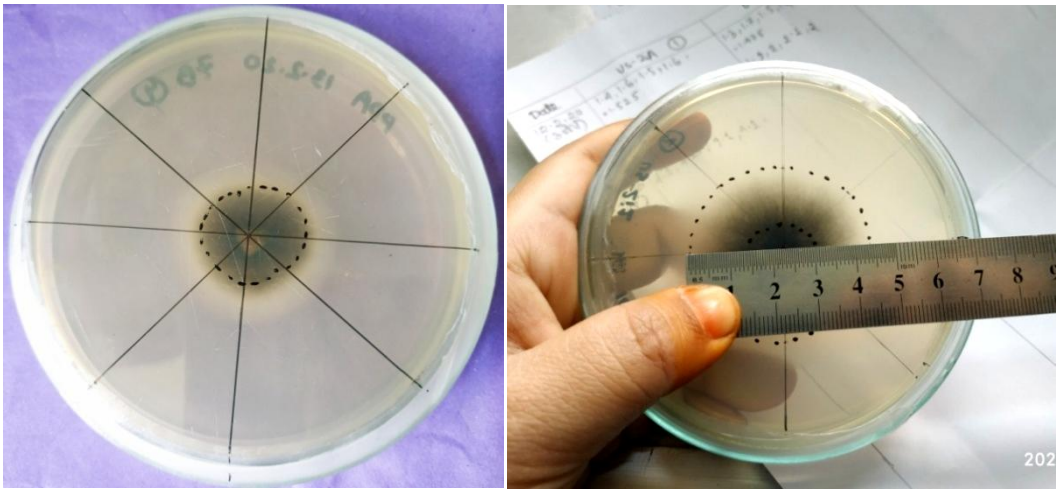


Plate 2. Measurement of mycelial growth on culture

The radial growth area was measured in cm^2 by taking the intersect point as the center. The diameter of the petri plate was 9 cm and the measurement was continued until the colony margin reached rim of petri plate. Colony characteristics (Growth type, growth pattern, colony color etc.) were recorded by visual observation (Plate 2).

3.3.4.2. Morphology of conidia

Each test isolate was grown on OMA media and incubated at 25^{o}C for 2 weeks. The morphological characters such as shape, color and size (length and breadth) of the conidia were measured using 5 spores for each isolate and the number of septation per conidia was determined under microscope at 40X

(Plate 3). Based on these features all the three isolates were identified using key manual developed by Barnett and Hunter (1960). Sporulation was measured by microscopic observation from five days after incubation at the interval of two days up to 25 days after inoculation transferring on glass slides, mixing well with uniform distribution of glycerin mounting and covered with cover slip. Prepared slides were then observed under compound microscope and number of spores per microscopic field was counted with four replications (Plate 3). Microphotographs were taken to observe the typical spore morphology of the *Magnaporthe oryzae* isolates. Sporulation rate was recorded by following index (Table 1) used by Meena (2005).

Table 1. Sporulation index of *Magnaporthe oryzae* isolates

Sporulation	No. of spores/ microscopic field	Index
Excellent	>30	4
Good	20-30	3
Fair	10-20	2
Poor	<10	1
Nil	0	0

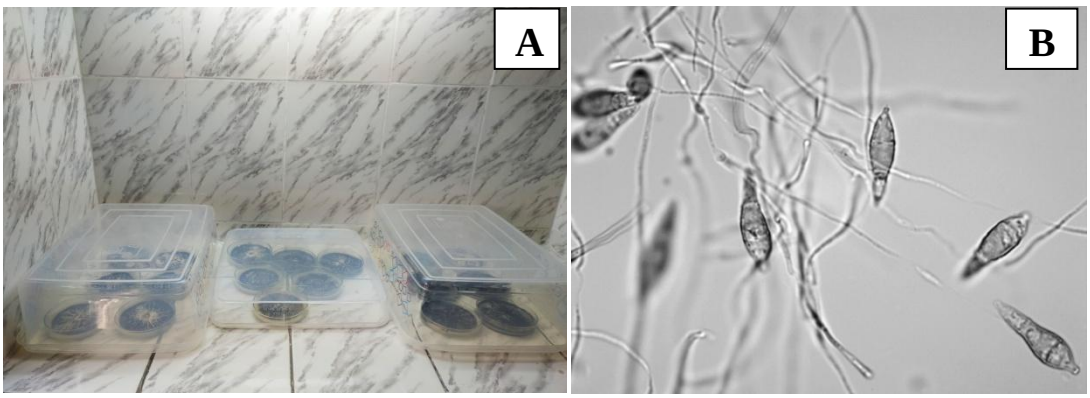


Plate 3. For sporulation, A. culture under fluorescent light, B. conidia under compound microscope (40X)

3.3.5. Preservation of fungal isolates

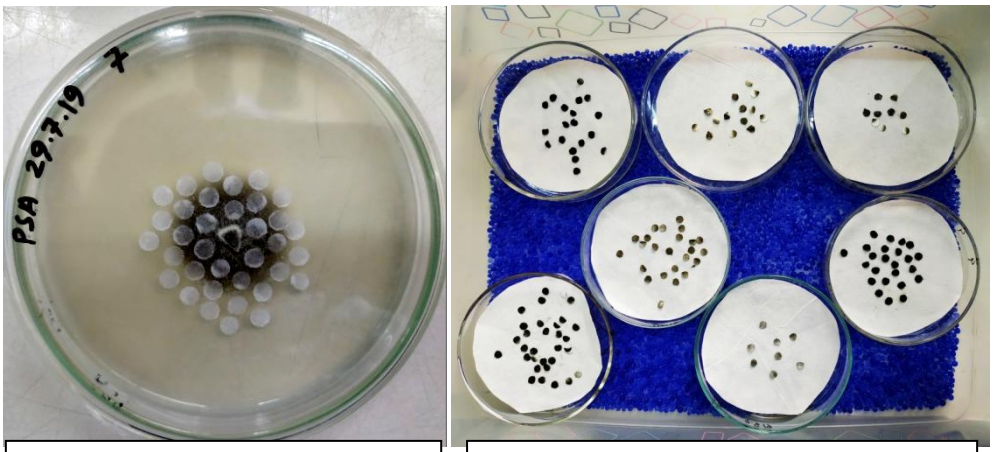
3.3.5.1. Preservation on PDA slants

The fungus was allowed to grow on PDA slants in test tube for 14 days at 25^0C in an incubator. These test tubes were sealed in plastic zippy bags and stored at -50^0C as medium term preservation for further studies (Figure 1).

Figure 1. Culture on PDA slants for medium term preservation

3.3.5.2. Preservation on filter paper discs

Established cultures were also subsequently maintained according to the method of Hayashi *et al*. (2009). At first, Whatman No.3 filter papers were cut into 0.5 cm^2 discs and autoclaved. The discs were placed on PSA medium and



Mycelium grown on filter paper block	Filter paper block with mycelium in desiccator

Plate 4. Culture on paper disc for long term preservation

inoculate by fungal mycelium block from pure culture. These inoculated plates were incubated at 25ºC until the filter paper discs were covered by fungal mycelium (Plate 4). Then filter paper disc, covered with fungal mycelia, were carefully removed with the help of needle and placed in an empty petri dish laid a sterilized filter paper. The paper discs were dried in silica gel containing airtight desiccator at room temperature for 7 days (Plate 4). After dried, the paper discs were placed in sterilized coin envelopes with the help of a sterilized forcep. The envelopes were placed in ziplock plastic bag containing silica gel. Then the bag were sealed and stored in -20ºC refrigerator. The silica gel should be changed whenever the color changes from blue to pink. Stocked isolates preserved for long term storage.

3.4. Reagent preparation for molecular study

3.4.1. Preparation of 1M Tris-HCL, pH 8.0

For 1L solution preparation, 121.1 g Tris, UltraPure was added to 700 mL distilled water and dissolved. Then pH of this solution was adjusted to 8.0 using HCL, fuming 37%. Finally the volume was adjusted to 1L using distilled water.

3.4.2. Preparation of 0.5M EDTA, pH 8.0

For 1L solution preparation, 186.12 g EDTA was added to 700 mL distilled water. pH of the solution was adjusted to 8.0 by adding NaOH. Finally, the volume was adjusted to 1L adding distilled water.

3.4.3. Preparation of 5M NaCl

For 1L solution preparation, 292.2 g NaCl was added to 700 mL distilled water. The solution was completely dissolved using magnetic stirrer. Then the final volume was adjusted to 1L adding distilled water.

3.4.4. Preparation of 2X Cetyl Trimethyl Ammonium Bromide (CTAB)

For 1L solution preparation, 100 ML, 40 mL, 280 mL of previously made Tris-HCL (1M, pH 8.0), EDTA (0.5M, pH 8.0), NaCl (5M), respectively, were added to 500 mL distilled water. 10.0 g Polyvinyl pyrrolidone (PVP-40) and

20.0 g CTAB were also added to the solution. The solution was completely dissolved using magnetic stirrer. Then the final volume was adjusted to 1L adding distilled water.

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

For 1L solution preparation of CIP, 912.0 mL Chloroform, 38.0 mL Isoamyl Alcohol and 50.0 mL Phenol were mixed properly. Each combination of chemicals should be freshly prepared every time.

3.4.6. Preparation of TE buffer

For 1L buffer preparation, 10 mL 1M Tris-HCL (pH 8.0, final conc. was adjusted to 10 mM) and 2 mL 0.5M EDTA (pH 8.0, final conc. was adjusted to 1 mM) were added to 700 mL distilled water and dissolved. Finally the volume was adjusted to 1L adding distilled water.

3.4.7. Preparation of 10X and 1X TBE buffer

For preparation of 1L 10X TBE buffer preparation, 108 g Tris, 55 g Boric Acid and 9.3 g EDTA were added to 700 mL distilled water and dissolved. Finally the volume was adjusted to 1L adding 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. Molecular detection of *Magnaporthe oryzae*

3.5.1. Fungal DNA extraction

Genomic DNA were extracted from fungal mycelia of *Magnaporthe oryzae* pure culture following the CTAB method (Ahmed, 2019).

3.5.1.1. Fungal isolates

Three *Magnaporthe oryzae* isolates were used for DNA harvest.

3.5.1.2. DNA harvest procedure

A 5 mm mycelia mats from the edge of an actively growing 10 day old culture was used. Mycelial mats were gently scrubbed with the spatula and placed in 50 ml of PDB media containing in a conical flask. Cultures were grown for 2 days at 25^0C in a rotary shaker (Plate 5). The culture filtrate was removed and the mycelia were blot dried completely. The dried mycelia (around 20 gm) were placed by a forcep into a 2 mL microcentrifuge tube for each isolate. A zirconium oxide ball (3mm) was put into each tube followed by 200 µL of 2X CTAB into the tube and the tubes were placed into Mixer Mill (MM 400, RETSCH, Germany) and ruptured the mycelia at 30 rpm for 4 minutes. 200 µL CIP (Chloroform:Iso-amylalcohol:Phenol) was further added into the tubes after rupturing the mycelia and placed on vortex mixer. After mixing well by vortex mixer (Fisherbrand, fisher scientific, UK), tubes were centrifuged at 13500 rpm for 10 minutes. 150 µL supernatant was taken and taken into a new 1.5 mL micro centrifuge tube. Then 150µL of cold isopropanol was mixed with the supernatant. The tubes were inverted for few minutes and rested at room temperature for 20 minutes. After 20 minutes, the tubes were centrifuged at 13500 rpm for 12 minutes. At last, supernatants were removed and DNA pellet was at the bottom of the tube. 700 µL 70% ethanol was added on the pellet and centrifuged at 10000 rpm for 5 minutes. Then the ethanol was removed and 300 µL 100% ethanol was added. Then the tubes were centrifuged at 10000 rpm for 3 min. Finally the ethanol was removed from the tubes.

DNA pellets were air dried on laminar air flow cabinet for 3 hours and then re-suspended with 100 µL ultra-pure water and this solution were used as stock DNA solution. The DNA samples were later diluted with ultra-pure water to the concentration of 20 ng/µL for Polymerase Chain Reaction (PCR).

3.5.2. Molecular markers for *M. oryzae* detection

Targeting four DNA regions includes the ITS (Internal Transcribed Spacer, ITS1 and ITS4), actin, β-tubulin and calmodulin genes were chosen to identify *Magnaporthe oryzae* isolates using universal primers (Table 2). All molecular markers were synthesized by Integrated DNA Technologies, Inc., USA.

3.5.2.1. PCR amplification

Individual DNA samples were run into PCR Thermal Cycler (PeQSTAR, PeQlab, UK) to amplify the targeted sequence for a specific region using specific marker (Table 2).

3.5.2.2. PCR reaction volume preparation

A 20 µL PCR reaction volume was made for each PCR sample. Each PCR sample containing 20 ng template DNA, 1.0 µL of each primer (0.4 µ mol/L), 10 µL of Taq PCR Master Mix and nuclease-free water to make final volume of 20 µL.

3.5.2.3. PCR protocol

PCR program was arranged as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds. Primers annealed at 55°C for 45 seconds, and extension at 72°C for 2 minutes, followed by a final extension at 72°C for 10 minutes.

3.5.3. Gel electrophoresis and gel documentation

The amplified PCR products along with 100 bp DNA ladder were separated using gel electrophoresis method in 2.5% Ultra Pure agarose gel in 1X TBE buffer at 90V for 1.5 to 2 hours. After electrophoresis was completed, the gel was stained with Ultra Pure ethidium bromide (0.5 µg/mL) and visualized under ultra-violet (UV) illumination. Banding patterns were photographed using a gel documentation system (BioDocAnalyze, Biometra, Germany).

All PCR reactions were repeated twice for each sample to confirm the results.

Table 2. List of molecular markers used for molecular detection of *Magnaporthe oryzae*

Target region, gene	Primers	Primer sequence (5'-3')	References
Actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)
	ACT-783R	TACGAGTCCTTCTGGCCCAT	
β-tubulin	Bt1a	TTCCCCCGTCTCCACTTCTTCATG	Glass and Donaldson (1995)
	Bt1b	GACGAGATCGTTCATGTTGAACTC	
Calmodulin	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	Carbone and Kohn (1999)
	CAL-737R	CATCTTTCTGGCCATCATGG	
Internal transcribed spacer	ITS1	TCCGTAGGTGAACCTGCGG	White *et al*. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	

M. oryzae isolates mycelia collection

Isolates culture in PDB media on rotary shaker

Plate 5. Initial steps of molecular detection process of *Magnaporthe oryzae*

3.6. Phenotypic reaction of advanced blast resistant lines

Eight blast resistant advanced lines viz. PL-5, PL-6, PL-17, PL-21, PL-22, PL-26, PL-34, PL-35 and two parents viz., BRRI dhan28 (recurrent parent) and

IRBL9-W (donor parent) were collected from BRRI and used to find out the phenotypic reaction pattern of these blast resistant advanced lines. These developed advanced lines possessing a major blast resistance gene, *Pi9*.

3.6.1. The method of growing seedling following UBN method

All the tested advanced lines were developed from a Boro variety (BRRI dhan28), with introgression of *Pi9* gene. Tested advanced lines with susceptible check (BRRI dhan28) were screened to find their reaction against leaf blast in Uniform Blast Nursery (UBN), BRRI, Gazipur. The screening was done in Boro 2019-20 season with four replications for each entry. Nursery bed was prepared to sow each entry in different rows. Individual row was maintained 50 cm long keeping 10 cm row to row distance. Susceptible check, US2, were sown after every 10 entries and two rows in border around the rows (Appendix-II). The blast isolate 7B, collected from Rangpur, was inoculated at 3 weeks after seeding (Figure 2). Disease reaction was recorded visually at 7 days after inoculation following the scoring system developed by Japan International Research Center for Agricultural Sciences (JIRCAS) (Hayashi *et al.*, 2009) (Table 3). Infection type 0 to 2 was considered as resistance where 3 to 5 was considered as susceptible. Disease scoring was completed considering the highest disease score among different replications (Appendix-III).

Figure 2. Inoculation by spraying spore suspension at UBN

Table 3. Leaf blast scoring system of JIRCAS

Disease score	Leaf blast symptom
0	No lesions.
1	Uniform or scattered brown specks.
2	Small lesions with distinct tan centers surrounded by a darker brown margin approximately 1 mm in diameter.
3	Small eye spot lesions less than one and a half times of the interval between thin veins or less than 1.5mm in diameter surrounded by dark brown.
4	Intermediate size eye spot lesions less than twice the interval between thin veins or less than 2 mm in diameter.
5	Large eye spot lesions more than twice the interval between thin veins or more than 2 mm in diameter.

3.7. Molecular confirmation of *Pi9* gene in advanced lines

3.7.1. Genomic DNA extraction

Genomic DNA were extracted from leaves of each advanced lines along with two parents (PL-5, PL-6, PL-17, PL-21, PL-22, PL-26, PL-34, PL-35, BRRI dhan28 and IRBL9-W) following the CTAB method (Ahmed, 2019).

3.7.1.1. DNA harvest procedure

Required quantity of young leaves was collected from three-week-old seedling of each rice plants. Leaves were cut into small pieces of around 1-1.5 cm size and placed into a separate 2 mL microcentrifuge tube. A zirconium oxide ball (3 mm) was put into each tube followed by 300 µL of 2X CTAB into the tube and the tubes were placed into Mixer Mill (MM 400, RETSCH, Germany). The leaves were then crashed at 30 rpm for 2 minutes. 300 µL CIP was further added into the tubes after crashing the leaves and placed on vortex mixer. After mixing well by vortex mixer (Fisherbrand, fisher scientific, UK), tubes were centrifuged at 14000 rpm for 12 minutes. 150 µL supernatant was taken and taken into a new 1.5 mL microcentrifuge tube. Then 150 µL of cold isopropanol was mixed with the supernatant. The tubes were inverted for few

minutes and rested at room temperature for 10 minutes. The samples were again centrifuged at 14000 rpm for 12 minutes. At last, supernatants were removed and the pellets were washed with 70% ethanol at 12000 rpm for 5 minutes.

DNA pellets were air dried on laminar air flow cabinet and re-suspended with 100 µL ultra-pure water and that solution were used as stock DNA solution. The samples were later diluted with ultra-pure water to the concentration of 20 ng/µL for Polymerase Chain Reaction (PCR) (Plate 6).

3.7.2. Molecular markers for gene confirmation

Tested blast resistance advanced lines were developed through backcrossing method and introgressed one blast resistance gene, *Pi9*. Two molecular markers tightly linked with *Pi9* gene were used for gene confirmation (Table 4). All molecular markers were synthesized by Integrated DNA Technologies, Inc., USA.

3.7.2.1. PCR amplification

Individual DNA samples were run into PCR Thermal Cycler (PeQSTAR, PeQlab, UK) to amplify the targeted sequence for *Pi9* gene using specific marker (Table 4).

3.7.2.2. PCR reaction volume preparation

A 20 µL PCR reaction volume was made for each PCR sample. Each PCR sample containing 20 ng template DNA, 1.0 µL of each primer (0.4 µmol/L), 10 µL of Taq PCR Master Mix and nuclease-free water to make final volume of 20 µL.

3.7.2.3. PCR protocol

PCR program was arranged as follows: Initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds. Primers annealed at 55^0 C for 45 seconds, and extension at 72°C for 2 minutes, followed by a final extension at 72°C for 10 minutes.

3.7.3. Gel electrophoresis and gel documentation

The amplified PCR products with 100 bp DNA ladder were separated using gel electrophoresis method in 2% Ultra Pure agarose gel in 1X TBE buffer at 90V for 2 to 2.5 hours. After electrophoresis was completed, the gel was stained with Ultra Pure ethidium bromide (0.5 μg/mL) and visualized under ultra violet (UV) illumination (Plate 6). Banding pattern photographs were taken using a gel documentation system (BioDocAnalyze, Biometra, Germany).

All PCR reactions were repeated twice for each sample to confirm the results.

Table 4. Molecular markers used for confirmation of *Pi9* gene in advanced breeding lines

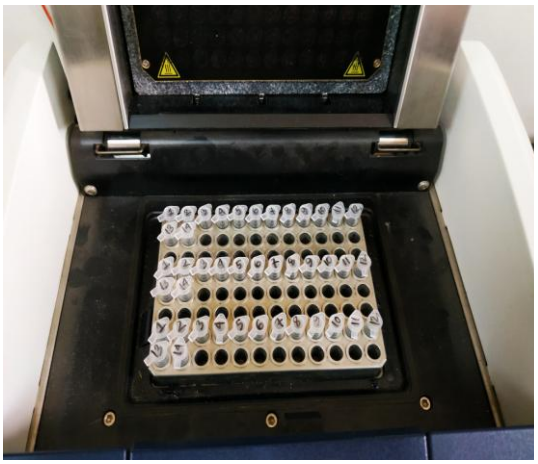
Primers	Primer sequence (5'-3')	Types of marker	Annealing temp. (^{0}C)	Expd. Size (bp)
RM8225	F: ATGCGTGTTCAGAAATTAGG R: TTGTTGTATACCTCATCGACAG	SSR	55	172
NMSMPi9-1	F: CGAGAAGGACATCTGGTACG R: GAGATGCTTGGATTTAGAAGAC	Allele specific	55	168

*SSR-Simple Sequence Repeat

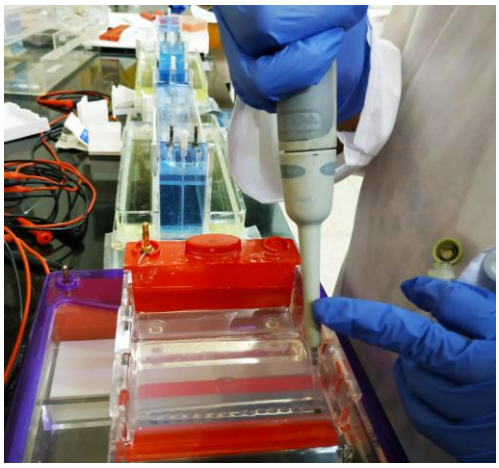
Genomic DNA extraction

Harvested DNA

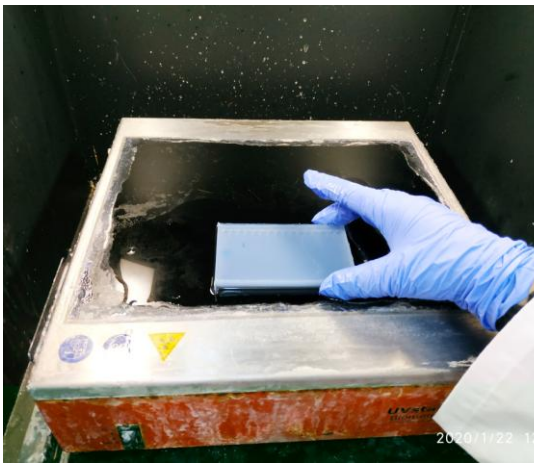
PCR reaction volume preparation



Polymerase Chain Reaction (PCR)



Gel electrophoresis



Gel documentation

Plate 6. Some laboratory activities for molecular confirmation of *Pi9* gene

3.8. Field performance of the advanced blast resistant lines

3.8.1. Field experiment

The experiment was conducted in the Research Field of Plant Pathology Division, BRRI, Gazipur, Bangladesh during the T. aman season, 2019. The experiment was laid out in a Randomized Complete Block Design (RCBD) with two replications. The unit plot size was $6m^2$ (3 m x 2 m). Eight blast resistant previously mentioned advanced lines and two parents were the treatments (Appendix-IV).

3.8.2. Fertilizer dose

A fertilizer dose of 165-60-105-68-0 kg NPKSZn ha^{-1} was applied in the form of urea, triple super phosphate, muriate of potash, gypsum, and zinc sulphate.

3.8.3. Seedling transplantation

Twenty five days old seedlings were transplanted on 4th August 2019 in the main plots. Plant spacing was maintained at 20 cm x 20 cm. Seedlings were transplanted in the field at a rate of one seedlings per hill.

3.8.4. Intercultural operations

Weeding and other intercultural operations were done properly according to agricultural practices after transplantation to mature stage. These are given below:

3.8.4.1. Weeding

Hand weeding was done for three times. First one was done at 15 DAT (Days after Transplantation) and second and third weeding were done at 25 and 40 DAT respectively.

3.8.4.2. Manure and fertilizer management

All the fertilizers were applied in basal dose except urea. Urea was applied in three splits at 15 days interval starting from 10 days after transplanting (DAT).

3.8.4.3. Irrigation water

Irrigation was done according to necessity of the crop.

3.8.5. Yield attributes and their procedure

The yield contributing characters were recorded for comparing the yield and yield components among the advanced lines of rice. For each line, four randomly selected plant hills were tagged from each plot and used for further observations. The mean values of the recorded data were taken as the actual values of the respective characters. The crop was harvested at full ripening stage. The data on the following yield contributing parameters were recorded: i) Number of panicle per hill ii) Panicle length (cm) iii) Number of grains per panicle iv) Grain sterility percentage (%) v) Grain yield (t/ha), were taken and significantly influenced by the combined effect of cultural conditions and rice cultivars (Appendix-V).

Number of panicle/hill: Total number of panicle of a hill was counted. Then average from two replications of each treatment was calculated.

Panicle length (cm): Panicle length of all panicles of a hill was calculated by a scale in centimeter (cm). Then average value from two replications of each treatment was calculated.

Number of grains / panicle: Total number of grains per panicle was counted manually from the panicles which were selected randomly from 4 hills of each plot. The average of four randomly selected panicles from plot were used to determine the number of grains per panicle. Then average from two replication of each treatment was calculated.

Grain sterility percentage (%): The total number of spikelets and the number of filled grains per panicle was recorded in each replication in each treatment. Total number of grains and unfilled grains per panicle was used to calculate sterility percentage as per given formula-

$$\text{Grain Sterility \%} = \frac{No.of\ unfilled\ \ grains}{Total\ \ no.of\ grains} \times 100$$

Grain yield (t/ha): The yield of grains obtained from each of the plots per treatment was recorded in kilograms (kg) after sun drying of grains and the

moisture percentage was recorded at the same time in each cases separately. The grain yield per plot was converted into tonnes per hectare (t/ha).

3.9. Statistical analysis

All the data were analyzed by using a computer program Statistical Tool for Agricultural Research (STAR) Version: 2.0.1 (IRRI, 2013). The treatment means were compared using Least Significant Difference (LSD) at 5% level of significance.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter provides the presentation and explanation of the results attained from the *in-vitro* experiment, nursery experiment, molecular lab experiment and the field experiment on rice blast pathogen *Magnaporthe oryzae* with relevant discussion.

4.1. Blast disease symptoms on rice

Characteristic symptoms of blast disease observed on leaves, nodes and panicles of infected rice plant samples collected from Rangpur, Khulna and Gazipur districts. On leaves, symptoms appeared first as minute brown spots which later became spindle shaped lesions with pointed ends at both sides. The centre of lesions was generally grey and showed distinct brown margins. Under favorable conditions, these lesions increased several centimeters long and about 0.5-1 cm wide. Later, the lesions coalesced together to form larger lesion and led to drying of whole leaf (Plate 7A). Symptoms on nodes appeared at the junction of the leaf and the stem sheath which showed necrotic area on union of two tissues. On panicles, symptoms appeared as dark brown area at the base of panicle, later on these panicles break off and the grains formed on the panicle were chaffy with brown in color (Plate 7B). Similar symptoms were also reported by Padmanabhan (1974), Bhatt and Singh (1992), Manibhushanrao (1994) and Tebeest *et al.* (2007).

Plate 7. Rice blast symptoms, A) Leaf blast, B) Panicle blast

4.2. *Magnaporthe oryzae* isolates

Fungal isolates from blast infected nodes of rice panicles were identified as *M. oryzae* based on cultural and morphological characteristics recorded from PDA cultures as suggested by Javadzadeh and Motlagh (2010) and Vanaraj *et al.* (2013). Initially the pathogen produced white mycelia, which gradually turned into grey, olive, brown and finally black in color (Plate 8). Three isolates viz. 7B was collected from Rangpur, 10C from Khulna and US-2i from Gazipur.

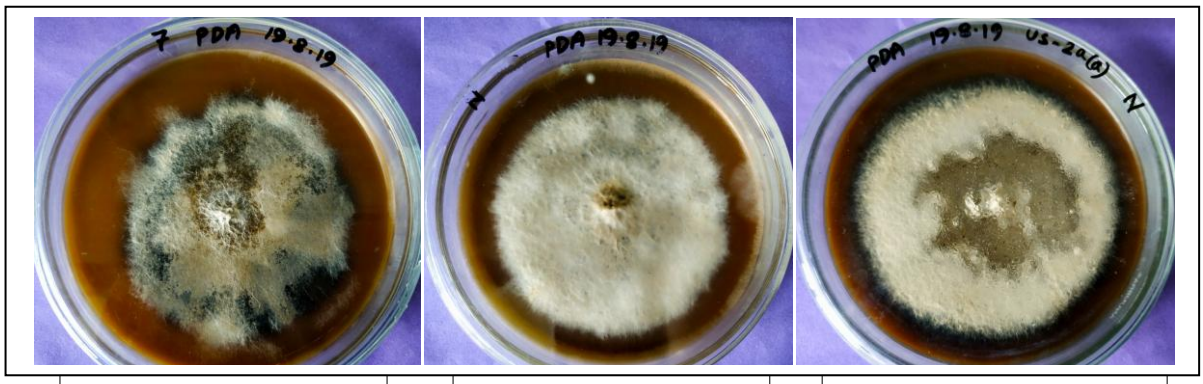


Plate 8. Pure culture of *Magnaporthe oryzae* on PDA media

4.3. Cultural and morphological characterization of *Magnaporthe oryzae* isolates

4.3.1. Colony characters on different media

Colony characters considered for determining variations among isolates of *M. oryzae* were diameter, color, margin, elevation, surface texture and form developed on three culture media are summarized in Table 5 and Figure 3.

4.3.1.1. Colony characters

On PDA medium, the color of the colony were olivaceous black, brownish black and greyish black coloured. Isolate 10C and US-2i showed filamentous margin and velvety surface texture except the isolate 7B which showed entire margin and velvety with white cottony texture. All the isolates showed flat elevation. Isolate 7B and US-2i showed regular colony form whereas isolate 10C showed irregular (Plate 9 & Table 5). Similar colony characters were observed and described by many researchers around the world. Asfaha *et al*. (2015) characterized the growth parameters of six isolates on four different media. On PDA media, they found grey to light grey colony color with black pigmentation, cottony surface texture, entire and irregular margin which was similar with present study. Kariaga *et al*. (2016) studied on cultural character of different isolates of *P. oryza* on PDA media and found variation of colors was from white, light gray, dark brown and black. They found cartilage like texture in the colony as major differences. The outstanding difference between the black colonies was in the edges; smooth edges with clear emission of a yellow metabolite and ragged edge without metabolite. Vanaraj *et al*. (2013) studied on different *Pyricularia oryzae* isolates and observed that colonies of *P. oryzae* appeared grey on potato dextrose agar media.

On PSA medium, colony color of isolate 7B and US-2i showed olivaceous black whereas isolate 10C was brownish black. All the isolates showed filamentous margin, flat elevation and velvety surface texture. Isolate 7B and US-2i showed regular colony form whereas isolate 10C showed irregular form of colony (Plate 9 & Table 5). However the present study was somewhat

different with the colony color findings of Bhaskar *et al*. (2018) and they found greyish colony on PSA media.

On OMA medium, colony color of isolate 7B and 10C were dark grey and velvety with white cottony surface whereas isolate US-2i showed light grey with wrinkled surface texture. All the isolates showed flat colony elevation and regular colony form. Isolate 7B and US-2i showed entire margin except isolate 10C which showed slightly undulate margin (Plate 9 & Table 5). In the present study colony characters are in conformity with Asfaha *et al*. (2015) who characterized the growth parameters of six isolates on four different media. On OMA media, they found dark grey, greyish black and off white colony color, velvety surface texture with entire margin. Srivastava *et al*. (2009) also observed that off white colony color with smooth margin on OMA media. Gashaw *et al.* (2014) observed similar colony color of all the isolates is usually grey on oat meal agar media with smooth colony margin. Vanaraj *et al*. (2013) also studied on different *Pyricularia oryzae* isolates and observed that colonies of *P. oryzae* appeared white on OMA media.

The present investigation found that three different isolates showed different color (olivaceous, brownish or greyish to black), margin (filamentous, entire or undulate), surface texture (velvety or wrinkled) and colony form (regular or irregular) in colony character but same in case of elevation (flat) in different media (Plate 9 & Table 5). Similar colony observation was found by other researchers. Kulmitra *et al.* (2017) studied growth of *M. oryzae* in different solid, semi solid and liquid media. The colony color differed from greyish black to dark black color, smooth to irregular margin, medium to good growth of the pathogen. Leong (2004), Variar *et al*. (2009) and Mebratu *et al.* (2015) found black to olive grey color for *Magnaporthe* colony. Meena (2005) also found the grayish black and raised mycelial growth of the blast isolates.

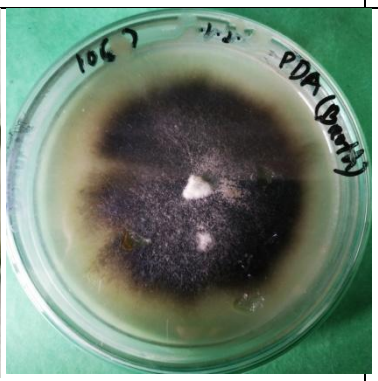
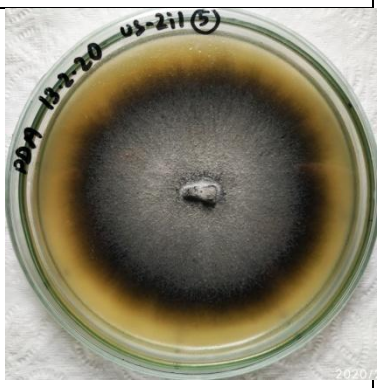
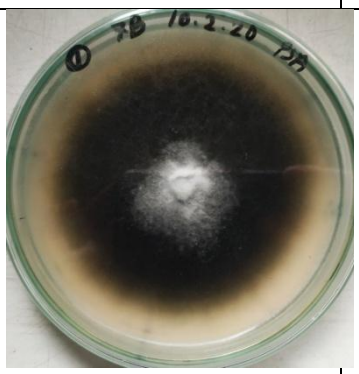
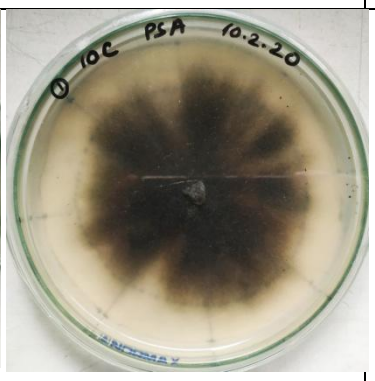
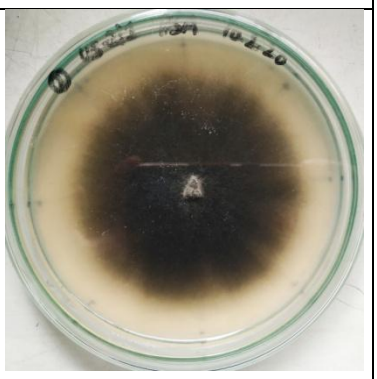
Media	Isolates Culture		
	7B (Rangpur)	10C (Khulna)	US-2i (Gazipur)
PDA			
PSA			
OMA			

Plate 9. Colony of *Magnaporthe oryzae* on different media after two weeks of incubation at 25^0C

Table 5. Characteristics of blast isolates cultured on PDA, PSA and OMA media after two weeks of incubation at 25°C

Media	Isolate name	Colony characters				
		Color	**Margin**	**Elevation**	**Surface texture**	**Colony form**
PDA	7B	Olivaceous black	Entire	Flat	Velvety with white cottony	Regular
	10C	Brownish black	Filamentous	Flat	Velvety	Irregular
	US-2i	Greyish black	Filamentous	Flat	Velvety	Regular
PSA	7B	Olivaceous black	Filamentous	Flat	Velvety	Regular
	10C	Brownish black	Filamentous	Flat	Velvety	Irregular
	US-2i	Olivaceous black	Filamentous	Flat	Velvety	Regular
OMA	7B	Dark grey	Entire	Flat	Velvety with white cottony	Regular
	10C	Dark grey	Slightly undulate	Flat	Velvety with white cottony	Regular
	US-2i	Light grey	Entire	Flat	Wrinkled	Regular

4.3.1.2. Mycelial growth of *M. oryzae* on PDA medium

Three isolates of *M. oryzae* namely 7B, 10C and US-2i were grown on PDA and their mycelial growth area was recorded at 3, 6, 9 and 12 Days After Incubation (DAI). Colony growth area was 1.55, 9.35, 26.38 and 58.27 cm^2 in

isolate 10C, 1.34, 9.89, 29.03 and 59.22 cm^2 in 7B and 3.19, 14.69, 36.41 and 61.23 cm^2 in isolate US-2i at 3, 6, 9 and 12 DAI, respectively. The colony area increased gradually with the increase of incubation period. In general, growth was the maximum in isolate US-2i followed by 7B and 10C (Figure 3).

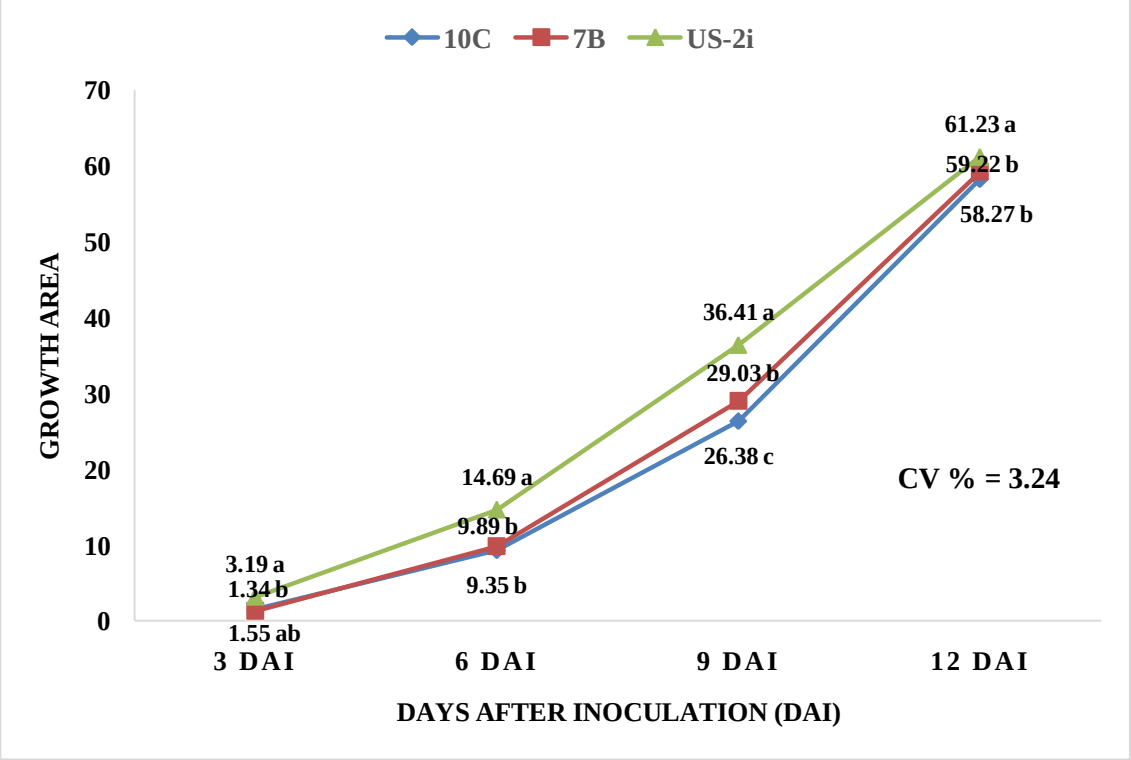


Figure 3. Mycelial growth area (cm^2) of three isolates of *M. oryzae* on PDA medium at 3, 6, 9 and 12 DAI

The findings of present study corroborates with Meena (2005) who used four different types of solid media *viz*. Host extract+ 2% sucrose agar, OMA, PDA and Richard's agar and found linear growth 5.03 cm to 8.99 cm growth of *M. oryzae* on PDA media that meant maximum 63.48 cm^2 growth on PDA which was close to 61.23 cm^2, maximum growth of isolate US-2i. Ibrahim (1975) found that PDA, OMA, CMA and SPDA were the best media for maximum growth of *M. oryzae.* This result is in conformity with Ravindramalviya (2014) who found that PDA media supported maximum mycelial growth of *M. grisea* after 168 hr of incubation. Awoderu *et al.* (1991) recorded that linear growth of *P. oryzae* was greatest on Potato Dextrose Agar (PDA).

4.3.2. Conidial characteristics of three isolates

Slide culture and microscopic study was done using pure culture of three isolates on OMA medium. Conidial characteristics were observed under compound microscope (Table 7).

4.3.2.1. Conidial shape and color

In all isolates, shape of the conidia was pyriform with round base and distinct hilum, apex narrowed, hyaline, 2-septate, 3 celled and middle cell was broader compared to others (Table 7).

4.3.2.2. The size of conidia

The conidial size was measured under 40X objective of a compound microscope. The length and breadth of conidia was 114.0 – 143.4 µm × 27.29 – 34.90 µm (average, 127.74 × 32.36 µm) in isolate 7B, 85.59 -126.6 µm × 31.79-38.33 µm (average 114.8 × 34.48 µm) in isolate 10C and 93.03-125.7 µm × 29.60-37.44 µm (average 110.44-32.85 µm) in isolate US-2i. The length and breadth ratio was 3.96, 3.29 and 3.53 in isolate 7B, 10C and US-2i, respectively (Plate 10 & Table 7).

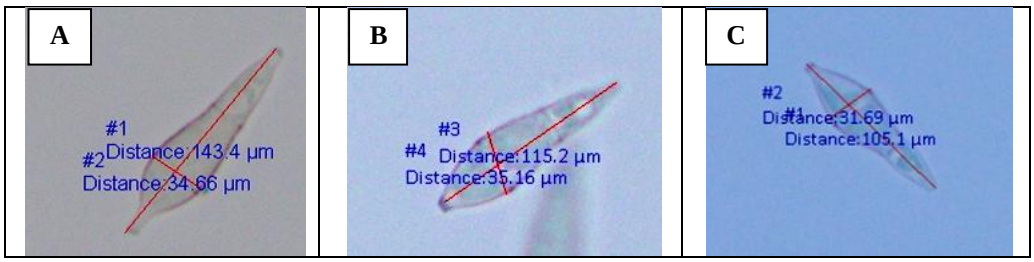


Plate 10. Measurement of size of conidia under compound microscope (40X), A) Isolate 7B, B) Isolate 10C, C) Isolate US-2i

4.3.2.3. Spore producing ability

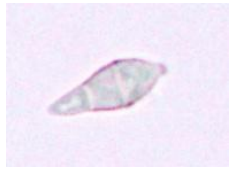
Sporulation ability of three isolates of *Magnaporthe oryzae* are shown in Table 6. In isolate 7B, average population of spores per microscopic field (40X) was 9, index 1 and spore producing ability was poor. In isolate 10C and US-2i, average population of spores per microscopic field was 18 and 15, respectively. Their index was 2 and spore producing ability was fair (Table 6).

Table 6. Sporulation of three isolates of *Magnaporthe oryzae*

Isolates	Average No. of spore/Microscopic field	Index	Spore producing ability
7B	9	1	Poor
10C	18	2	Fair
US-2i	15	2	Fair

The shape, size, septation and color characters of *M. oryzae* recorded in present study are in agreement with those described by Kariaga *et al*. (2016), Nishikado (1926), Mijan Hossain (2000) and You *et al*. (2012). Mijan Hossain (2000) observed that mycelium in cultures was first hyaline colored then changed to olivaceous, septate and branched. Mostly 2 celled conidia were found from rice grain media and 3 celled conidia were found in infected leaf sample. He found the conidia measurements under 10X microscopic view were 15 – 22µm x 4 – 7µm (Average, 17.4µm x 5.2 µm). Asfaha *et al*. (2015) also found the pyriform and hyline to pale olive conidia and size of the conidia on OMA was 14.5-26.5 µm x 5.1-8.3 µm (Average, 21.23 µm x 6.67 µm). Kulkarni (1973); Khadka *et al*. (2012) reported that among the solid media, OMA media was good for sporulation of the isolates.

Table 7. Conidial characteristics of *M. oryzae* isolates cultured on OMA media after 20 days of incubation at 25^0C (40X)

Isolate	Conidial size (in µm)				L-B Ratio of conidia	Conidial shape	Conidial color	Sporulation	Figure of conidia (microscopic view)
	Length (L)		Breadth (B)						
	Range	Average	Range	Average					
7B	114.0-143.4	127.74	27.29-34.90	32.362	3.96	Pyriform	Hyaline	Poor	
10C	85.59-126.6	114.8	31.79-38.33	34.48	3.29	Pyriform	Hyaline	Fair	
US-2i	93.03-125.7	110.44	29.60-37.44	32.85	3.53	Pyriform	Hyaline	Fair	
CV (%)					11.80				

4.4. Molecular detection of *Magnaporthe oryzae* isolates

Molecular analysis of three isolates of *M. oryzae* were studied using four specific primers, Bt1a/Bt1b, CAL-228/CAL-737, ACT-512/ACT-783, ITS1/ITS4 with amplified fungal targeted gene region; beta tubulin, calmodulin, actin, ITS (Internal Transcribed Spacer), respectively. The ITS region, β-tubulin and actin genes in chromosome of *M. oryzae* were successfully and strongly amplified with fragment of 520 bp, 515 bp and 260 bp, respectively (Figure 4A, 4C, 4D).

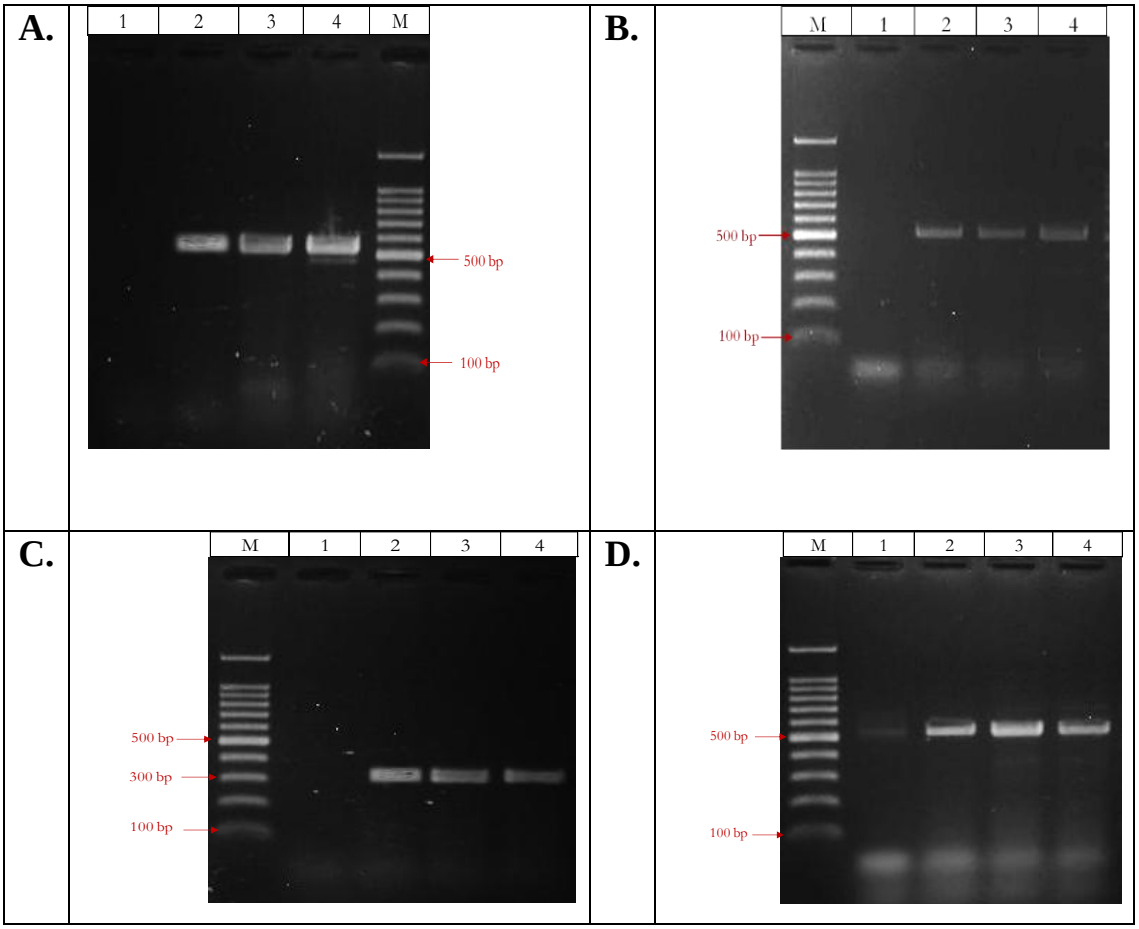


Figure 4. Agarose gel picture of PCR amplification products of *Magnaporthe oryzae* isolates genomic DNA with Primer A)Bt1a/Bt1b (515 bp), B) CAL-228/CAL-737 (510 bp), C) ACT- 512/ACT-783 (260 bp) and D) ITS1/ITS4 (520 bp). Here, 1= No DNA, 2=Isolate US-2i, 3=isolate 10C, 4=Isolate 7B, M=100bp DNA ladder.

The calmodulin gene in *M. oryzae* was also successfully but faintly amplified with fragment of 510 bp. These four primers confirmed all the isolates as *M. oryzae* by amplified their targeted region in expected band position (Figure 4B).

Similar findings were also observed and described by many other researchers around the world. The present study were closely related to the findings of Aslam *et al*. (2019) and Abed-Ashtiani *et al*. (2016). Aslam *et al*. (2019) used four primers for molecular analysis, ITS1-F and ITS4-R (ITS region), bt1a and bt1b (beta tubulin), ACT-512F and ACT-783R (actin), CAL-228F and CAL-737R (calmodulin) for amplification of 25 isolates of *M. oryzae*. The ITS region, beta tubulin, actin and calmodulin genes of *M. oryzae* were successfully amplified with fragment of 510 bp, 500 bp, 280 bp and 520 bp, respectively. BLASTn searches based on the four gene regions showed that the isolates used in this study were ≥99% similar to those of *M. oryzae* in GenBank (NCBI). Abed-Ashtiani *et al*. (2016) also worked on the characterization of *M. oryzae* collected from rice in Malaysia and they identified the rice blast pathogen as *M. oryzae* which was near about similar to present study. The ITS, actin, β-tubulin and calmodulin gene regions of all *Magnaporthe* spp. isolates were successfully amplified with 505, 336, 536, and 512 bp long, respectively.

4.5. Phenotypic reaction of blast resistance advanced lines of rice

Disease scores on PL-5, PL-6, PL-17, PL-21, PL-22, PL-26, PL-35 and the donor parent IRBL9-W were 0.5 – 1.5. The donor parent and the tested advanced lines were graded as Resistant (R). The recurrent parent BRRI dhan28 and the advanced line PL-34 showed the disease score of 3.75 and 4.00, respectively. These were graded as Susceptible (S) to rice blast pathogen (Table 8).

Table 8. Phenotypic disease reaction of advanced lines against blast disease

Designation	Disease score	Disease reaction
PL-5	-	-
PL-6	1.00 b	R
PL-17	1.00 b	R
PL-21	1.00 b	R
PL-22	0.75 b	R
PL-26	1.00 b	R
PL-34	4.00 a	S
PL-35	1.50 b	R
BRRI dhan28 (Recurrent parent)	3.75 a	S
IRBL9-W (donor parent)	0.50 b	R
CV (%)	42.76	

R= Resistant and S= Susceptible

Means with same letters are not significantly different at 5% level of significance.

4.6. Molecular confirmation of *Pi9* gene from advanced lines

Eight advanced blast resistant lines were tested at molecular level by using specific two molecular markers for the confirmation of *Pi9* gene. Tested advanced lines amplified in the expected band size confirmed resistant lines of the respective primers. It indicates that all the molecularly confirmed resistant lines carried *Pi9* gene. Gel picture denoted numbering of tested advanced lines of rice on gel electrophoresis are shown in Table 9. and gel electrophoresis results of 8 introgressed tested advanced lines for 2 linked markers are shown in Figure 5 where donor parent IRBL9-W was resistant check for having *Pi9* gene and recurrent parent BRRI dhan28 was as susceptible check.

Table 9. Detailed of gel picture denoted numbering of tested advanced lines of rice on gel electrophoresis

Denoted no.	Resistant Advanced line with check variety
1	IRBL9-W (Resistant check variety)
2	BRRI dhan28 (Susceptible check variety)
7	PL-35
8	PL-34
9	PL-26
10	PL-22
11	PL-21
12	PL-17
13	PL-6
14	PL-5

**7-14 denotes tested advanced lines

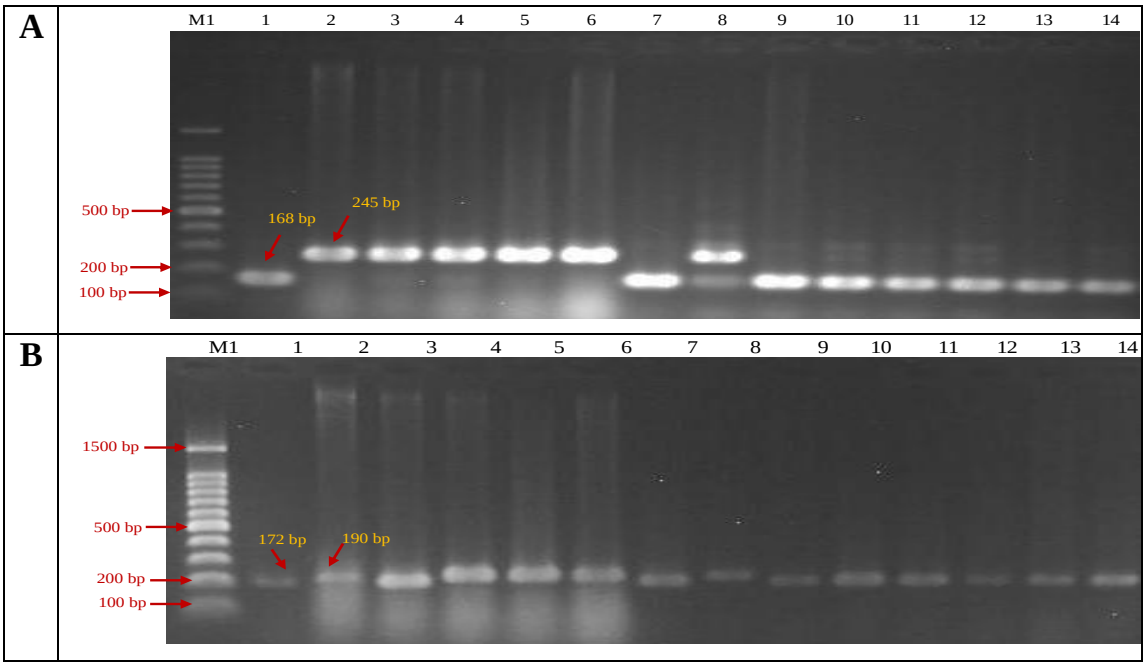


Figure 5. Agarose gel picture of PCR product of 8 tested advanced lines for resistance gene *Pi9* amplified with Primer A) NMSMPi9-1 (168 bp) and B) RM8225 (172 bp). Here, 1= IRBL9-W(*Pi9*), 2=BRRI dhan28, 7 to 14= tested advanced lines, M1= 100bp DNA ladder. (3-6 were not considered).

Among the tested advanced lines, PL-5, PL-6, PL-17, PL-21, PL-22, PL-26 and PL-35 gave 168 bp band for NMSMPi9-1 primer and 172 bp band for RM8225 primer which was resistant band for both primer. The results confirmed that *Pi9* gene was successfully introgressed in those lines and all of those lines were resistant to blast. P-34 line gave a susceptible band which indicate no *Pi9* gene was present in this line. This line is also susceptible to blast disease during phenotypic testing. Ansari *et al*. (2020) tested these advanced lines using the primer, RM8225 and NMSMPi9-1 and confirmed these advanced lines contain blast resistant gene, *Pi9*. Similar result was also found by Manoj Kumar *et al*. (2017), they conducted a research on molecular analysis for major blast resistance genes in rice and found the blast resistant *Pi9* gene in rice through NMSMPi9-1 marker amplified with 168 bp successfully.

4.7. Field performance of resistant advanced breeding lines of rice on yield attributing characters

Tested advanced lines were recorded under the following yield contributing parameters viz., Number of panicle per hill, Panicle length (cm), Number of grains per panicle, Grain sterility percentage (%), Grain yield (t/ha), were taken and significantly influenced by the combined effect of cultural conditions and rice cultivars. These tested advanced lines were developed by BRRI from Boro variety (BRRI dhan28), with introgression of *Pi9* gene by backcross breeding. The data were recorded and described under the mentioned parameters and tabulated (Table 10 & Figure 6).

4.7.1. Field performance on number of panicle/hill

Highest no. of panicle per hill was recorded 14 in BRRI dhan28 and lowest no. of panicle per hill was recorded 9 in IRBL9-W variety (Table 10). None of the lines were significantly different on the basis of number of panicle per hill.

4.7.2. Field performance on panicle length

The maximum panicle length was recorded 27.52 cm in PL-35 followed by 27.17 cm in PL-6 which were statistically similar to 26.28 cm in BRRI dhan28 whereas minimum panicle length was observed 20.22 cm in IRBL9-W (Table

10). Though these lines were developed from backcrossing population of BRRI dhan28 and IRBL9-W cross, the developed line will not gave the higher panicle length than BRRI dhan28.

4.7.3. Field performance on number of grains / panicle

In this present study, BRRI dhan28 produced the highest number (106) of grains per panicle followed by PL-21 (102), which was statistically similar with BRRI dhan28. The lowest number of grains per panicle was recorded 49 in PL-26 (Table 10).

Table 10. Mean performance of tested advanced lines and checks for yield attributing characters in T. Aman, 2019

Designation	No. of panicle/hill	Panicle length (cm)	No. of grain per panicle	Grain sterility (%)
BRRI dhan28(RP)	14	26.28 a	106 a	28.59 d
IRBL9-W(DP)	9	20.22 f	65 d	23.44 d
PL-17	11	22.1 de	76 c	38.25 bc
PL-21	11	23.61 bc	102 a	31.03 cd
PL-22	12	23 cd	77 c	31.02 cd
PL-26	12	24.75 d	49 e	51.03 a
PL-34	13	21.58 def	64 d	29.34 d
PL-35	10	27.52 a	62 d	40.16 b
PL-5	10	21.34 ef	86 b	26.65d
PL-6	11	27.17 a	84 bc	42.14 b
CV (%)	17.22	2.68	5.29	10.23

* RP= Recurrent Parent, DP= Donor Parent

Means with same letters are not significantly different at 5% level of significance.

4.7.4. Field performance on grain sterility percentage (%)

Lowest percent grain sterility was recorded 29.34% in PL-34, 28.59% in BRRI dhan28, 26.65% in PL-5 and 23.44 % in IRBL9-W. Besides 31.02% in PL-22 and 31.03% in PL-21 were statistically similar with them. The highest sterility was observed 51.03% in PL-26. (Table 10).

4.7.5. Field performance on grain yield (t/ha)

The maximum yield among the tested lines was 4.47 t/ha in PL-21 which was statistically similar with recurrent parent BRRI dhan28 and gave 4.94 t/ha (Figure 6).

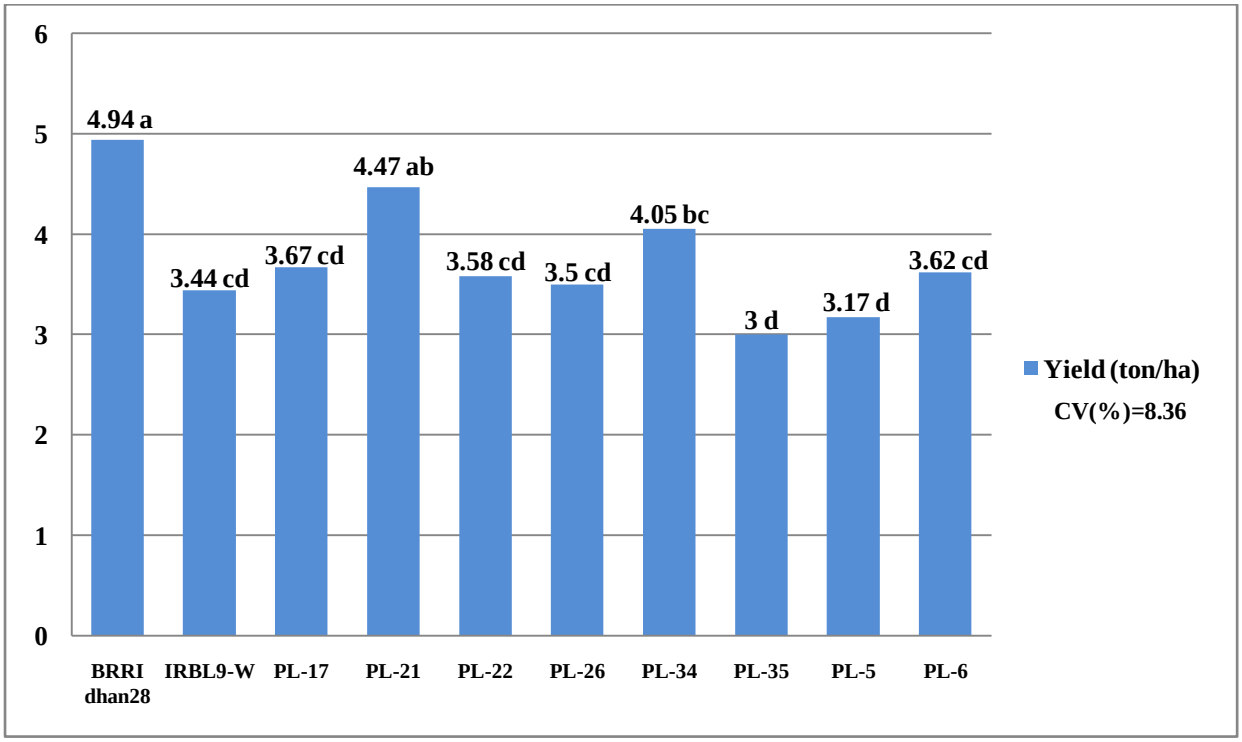


Figure 6. Mean performance of yield for tested advanced lines and checks

These parameters were analysed statistically which was significantly influenced by the advanced lines of rice. All the tested advanced lines were developed from Boro variety (BRRI dhan28) with introgression of *Pi9* gene. But for the continuous research and development of these advanced lines, it was grown, selected and tested over the seasons, irrespective of T. aman and Boro. Therefore all the introgressed lines (although Boro variety) were tested on T. aman season and produced lower yield including check material, BRRI dhan28. The results showed that PL-21 line was more recovered to recurrent parent and gave similar yield to BRRI dhan28. Since backcrossing population never gives higher yield than the recurrent parent, PL-21 gave maximum yield (4.47 t/ha) among the tested lines which was statistically similar with recurrent parent BRRI dhan28 (4.94 t/ha) (Figure 6). Besides this lines contain blast resistance gene (*Pi9*) and phenotypically blast resistant. The present study was closely related to the findings of Tian *et al*. (2019) and he found that introgression of two broad-spectrum blast resistance genes, *Pi9* and *Pi2* into with newly bred restorer line (Hui 316) could improve its resistance to blast under both greenhouse and field conditions with little influence on its yield and yield attributes.

CHAPTER 5

SUMMARY AND CONCLUSION

Morphological and molecular identification of *Magnaporthe oryzae*, and pathogenic variation among *M. oryzae* isolates was carried out to determine the morphological variation of blast isolates. Phenotypic disease reaction and molecular detection was done to understand the resistance level and field performance regarding yield and yield attributes of the tested advanced breeding lines of rice helped to find out the best line among them.

In this experiment, PDA media was used for isolation of three isolates from Gazipur (US-2i), Rangpur (7B) and Khulna (10C) from blast symptom containing panicle sample. Morphological identification was confirmed on the basis of three celled (2 septate) and pyriform shaped, hyaline conidia with cultural and morphological study and molecular detection of three blast isolates was confirmed using four primers by amplified targeted gene region. All the isolates gave 515 bp, 510 bp, 260 bp, 520 bp band with Bt1a/Bt1b (β-tubulin region), CAL-228/CAL-737 (calmodulin region), ACT- 512/ACT-783 (actin region), ITS1/ITS4 (Internal Transcribed Spacer region) primer, respectively.

Cultural characteristics of three isolates differed from different media (PDA, PSA and OMA) on the basis of colony form (regular or irregular), color (grey, brownish or olivacious to black), margin (entire or filamentous), elevation (all the cultures maintained flat elevation) and surface texture (velvety with or without white cottony except isolate US-2i, which has wrinkled surface texture). Mycelial growth area was observed on PDA media until the petri plates were fully covered with fungal mycelia. The highest mycelial growth area was recorded 3.19cm^2, 14.69cm^2, 36.41cm^2 and 61.23cm^2 in isolate US-2i at 3rd,6th, 9th and 12th days after inoculation(DAI), respectively. The average conidial size (length×breadth) of the conidia varied among the isolates (average 110.44-127.74×32.36-34.48) μm^2 and the highest sporulation was considered at isolate 10C (18 spore/microscopic field) on OMA media.

The eight introgressed lines (PL-5, PL-6, PL-17, PL-21, PL-22, PL-26, PL-34, PL-35) were evaluated along with donor parent as resistant check variety (IRBL9-W) and recurrent parent as susceptible check variety (BRRI dhan28). Phenotypic evaluation of introgressed lines for blast resistance *Pi9* gene, six lines (PL-6, PL-17, PL-21, PL-22, PL-26, PL-35) showed resistant reaction and only one line (PL-34) showed susceptible reaction. Phenotypic reaction of PL-5 was not done. These blast resistant lines including PL-5 amplified with two linked markers, RM8225 (172 bp) and NMSMPi9-1 (168 bp). All the lines amplified in the expected band size of the respective primers. It indicates that all the molecularly confirmed resistant lines including PL-5 which carried *Pi9* gene also showed phenotypically resistance to blast. BRRI dhan28 gave average score 3.75 (highest score 5) which was susceptible and IRBL9-W gave average score 0.5 (highest score 1) which was resistant to blast disease.

The field experiment of above mentioned eight introgressed lines were laid out in a Randomized Complete Block Design (RCBD) with two replications. Data were taken on number of panicle per hill, Panicle length (cm), Number of grains per panicle, Grain sterility percentage (%) and Grain yield (t/ha). These parameters were analysed statistically which was significantly influenced by the rice lines. Although all the tested lines were developed from Boro variety (BRRI dhan28), with introgression of *Pi9* gene. But for the continuous research and development of these advanced lines, it was grown, selected and tested over the seasons, irrespective of T. aman and Boro. Therefore all the introgressed lines (although Boro variety) were tested on T. aman season and produced lower yield including check material, BRRI dhan28. The results showed that, PL-21 gave maximum yield (4.47 t/ha) among the tested lines which was statistically similar to its recurrent parent BRRI dhan28 (4.94 t/ha).

In conclusion, The isolates collected from different region of Bangladesh was considered virulent on the basis of morphological characterization. Their growth and sporulation was as good as for blast disease development. On the basis of spore production, Khulna isolate is considered as virulent. All the

isolates were detected as blast as these were amplified with the Bt1a/Bt1b, CAL-228/CAL-737, ACT-512/ACT-783, ITS1/ITS4 primers. Phenotypic reactions indicated that all the developed lines were resistant to rice blast disease and possessing blast resistant *Pi9* gene. One blast resistant line PL-21 produced similar yield with the blast susceptible recurrent parent BRRI dhan28 and therefore, could be suggested for the advancement as a variety. Field evaluation of the selected resistant lines in the hot spots for phenotypic resistance and yield contributing attributes in the Boro season is suggested.

CHAPTER 6

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CHAPTER 7

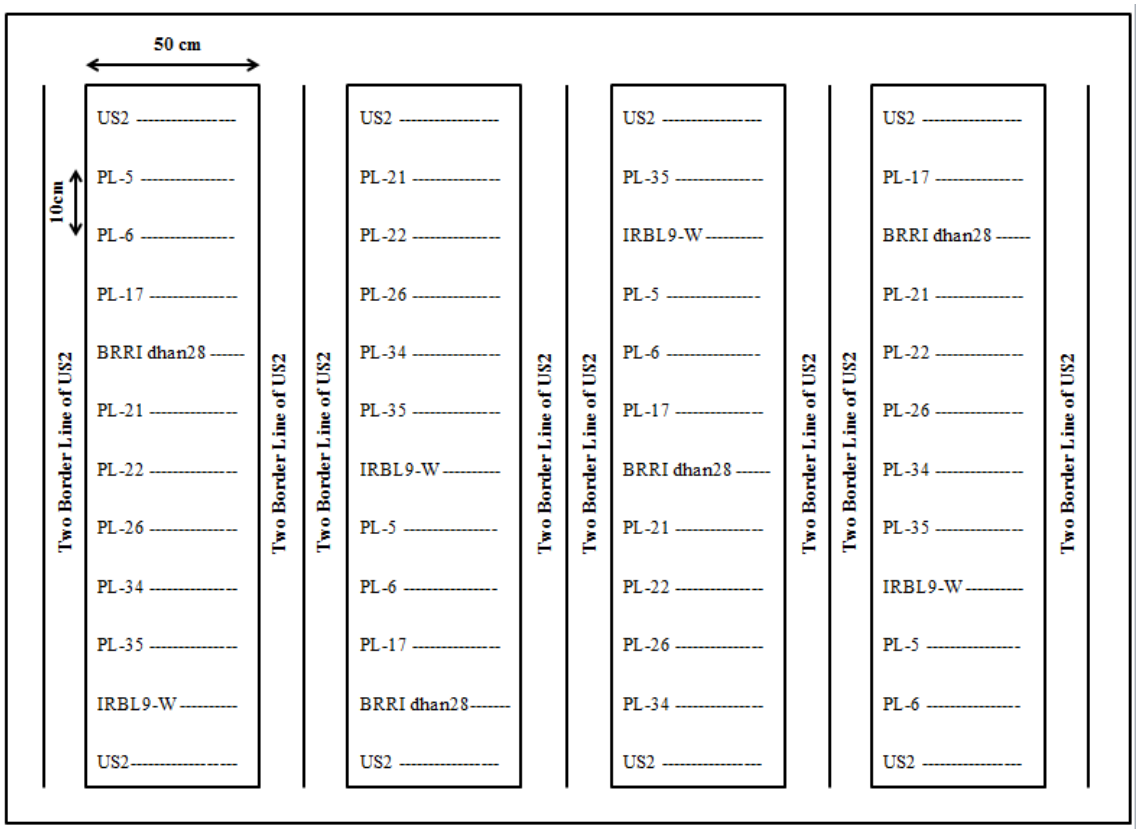
APPENDICES

Appendix-I: Analysis of variance for mycelial growth area of *Magnapothe oryzae* isolates in PDA media

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Isolate	2	167.7474	83.8737	45.89	0.0002
Error(a)	6	10.9654	1.8276		
Time	3	17449.77	5816.591	8286.61	0
Isolate:Time	6	66.0804	11.0134	15.69	0
Error(b)	18	12.6347	0.7019		
Total	35	17707.2			

***** Least Significant Difference (LSD) at 5% level of significance.

Appendix-II: Sample layout of nursery bed in uniform blast nursery (UBN)

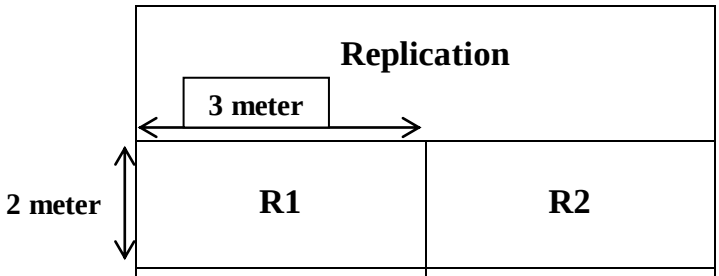


Appendix-III: Analysis of variance for disease scoring of resistant advanced breeding lines of rice against blast disease

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Rep	3	2.1111	0.7037	1.48	0.2443
Variety	8	55.0556	6.8819	14.5	0
Error	24	11.3889	0.4745		
Total	35	68.5556			

***** Least Significant Difference (LSD) at 5% level of significance.

Appendix-IV: Sample layout plan of the field experiment



R1	R2
PL-6	PL-21
PL-5	PL-22
PL-17	PL-26
BRRI dhan28	PL-34
PL-21	PL-35
PL-22	IRBL9-W
PL-26	PL-6
PL-34	PL-5
PL-35	PL-17
IRBL9-W	BRRI dhan28

Appendix-V: Analysis of variance for yield contributing parameters of resistant advanced breeding lines of rice

ANOVA: No. of panicle/hill

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	1	1.8	1.8	0.48	0.5043
Variety	9	46.9689	5.2188	1.4	0.3112
Error	9	33.4889	3.721		
Total	19	82.2578			

* Least Significant Difference (LSD) at 5% level of significance.

ANOVA: Panicle length (cm)

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	1	1.6704	1.6704	4.12	0.073
Variety	9	119.1742	13.2416	32.65	0
Error	9	3.6506	0.4056		
Total	19	124.4952			

* Least Significant Difference (LSD) at 5% level of significance.

ANOVA: No. of grain per panicle

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	1	335.2986	335.2986	20.08	0.0015
Variety	9	5772.302	641.3669	38.42	0
Error	9	150.2531	16.6948		
Total	19	6257.854			

* Least Significant Difference (LSD) at 5% level of significance.

ANOVA: Grain Sterility (%)

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	1	38.7254	38.7254	3.17	0.1086
Variety	9	1293.297	143.6997	11.77	0.0006
Error	9	109.8997	12.2111		
Total	19	1441.923			

* Least Significant Difference (LSD) at 5% level of significance.

ANOVA: Yield (ton/ha)

Source	DF	Sum of Square	Mean Square	F Value	Pr(> F)
Replication	1	0.0024	0.0024	0.02	0.8785
Variety	9	6.2896	0.6988	7.14	0.0037
Error	9	0.881	0.0979		
Total	19	7.173			

***** Least Significant Difference (LSD) at 5% level of significance.