GROWTH AND SPORULATION OF *Magnaporthe oryzae* CAUSING BLAST OF RICE IN SELECTED CULTURE MEDIA AND ITS *In vitro* MANAGEMENT

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Growth and Sporulation of *Magnaporthe oryzae* Causing Blast of Rice in Selected Culture Media and Its *In vitro* Management

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CERTIFICATE

This is to certify that the thesis entitled **GROWTH AND SPORULATION OF *Magnaporthe oryzae* CAUSING BLAST OF RICE IN SELECTED CULTURE MEDIA AND ITS *In vitro* MANAGEMENT** '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 results of a piece of bona fide research work carried out by **Afsana Akhter, Registration no. 18-09014** 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.

Dated-

Dhaka, Bangladesh.

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DEDICATED
TO
Maj. Muktadir
Aaiman
And
THE FARMERS WHO FEED THE NATION

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The Author

Growth and Sporulation of *Magnaporthe oryzae* Causing Blast of Rice in Selected Culture Media and Its *In vitro* Management

Abstract

An experiment was carried out at Molecular Biology and Plant Virology Laboratory, under the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207. The study was designed to aid in further experiments and find out the suitable measures to control rice blast pathogen. The study consisted of the isolation of rice blast pathogen from the field sample for morphological study, the evaluation of 12 different selected solid media to have a comparative analysis of their performance on morphological study of *Magnaporthe oryzae*, the pathogenicity test through Koch's postulates and evaluation of efficacy of four latest chemicals against *Magnaporthe oryzae*. Diseased samples were collected randomly from the severely rice blast infected field from Mymensingh and Thakurgaon districts. The disease affected plant parts were collected and conveyed to the laboratory for *in-vitro* experiments. The morphological study was done by observing two isolates from the collected samples. It is evident that the disease was caused by *Magnaporthe oryzae* as the isolates produced pyriform, 3 celled hyaline conidia which had two septa. Among the 12 selected media PDA, CDA and PSA gave better performance in terms of mycelial growth and PSA, PR$_S$DA for sporulation. On the other hand, PA (Prune agar) and SA (Starch agar) also gave good performance and these media can be a good alternative for morphological study and isolation purpose. Among the four selected fungicides, Amister top 325 SC and Blastin 75 WDG gave 100% mycelial growth inhibition with 500 ppm, 1000 ppm and 1500 ppm doses against *Magnaporthe oryzae in-vitro*. Besides, Acibean 28 SC and Deconil 500 SC had lower effect on rice blast pathogen over control.

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LIST OF SYMBOLS AND ABBREVIATIONS

% : per cent

C : Celsius

µm : micrometer

cm : centimeter

ml : milliliter

mm : millimeter

g : gram

kg :kilogram

hr : hours

ha : hectare

i.e., : that is

L : liter

µl : microliter

°C : degree celsius

mg : milligram

ppm : Parts per million

MT : Million Tons

CRD : Completely Randomized Design

DS : Disease severity

DI :Disease incidence

et al. : and others

No. : Number

RH : Relative Humidity

LSD :Least significant difference

CV : Co-efficient of varience

Viz., : Namely

PCR : Polymerase chain reaction

DAI : Days After Inoculation

AUGPC: Area under growth progress curve

SC :Soluble concentrate

WDG :Water dissolved granule

kg ha^{-1} : Kilogram per hectare

BRRI- Bangladesh Rice Research Institute

Introduction

Rice (*Oryza sativa* L.) is the world's most recognized cereal and main source of carbohydrate for more than half of the world's population. Rice belongs to the family Poaceae (Gramineae), the tribe is Oryzeae and genus is *Oryza. Oryza* has two cultivated and 22 wild species and from those two cultivated species, *Oryza sativa* is known as Asian rice. It is not just a grain, it is seemed lifeline and the second most cultivated crop next to wheat (Tony, 2005) and by production it is also second after Maize (Boumas, 1985). It is taken as a staple food by 60% of the world's population and approximately 90% of the total rice is consumed by the people of Asian countries (Kole, 2006). Globally rice cultivation occupies in an area of 166 M ha with a production of 758.8 MT per annum which produce 21% of world's food calorie supply (FAO, 2017). The major rice producing countries are China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Nepal, Brazil and Japan. China is the leading rice producer followed by India, Indonesia and Bangladesh in 2016 (FAO, 2017). Rice production needs more attention as it has been predicted that 800 million tons of rice will be required by the year 2025 (Kubo and Purevdorj, 2004).

Bangladesh is a developing country and still striving hard for rapid development of its economy. Approximately, 12 % of GDP has been derived from cultivated crops where rice contributes 9.5 % to the agricultural GDP (BBS, 2017). Nearly 20 % of nation's gross domestic product (GDP) and about one-sixth of the national income of Bangladesh comes from rice. More or less 13 million farm families of our country cultivate rice. About 75% of the total cropped area and over 80% of the total irrigated area is planted to rice. The production per annum of rice in Bangladesh is 33.804 million tons from 11.01 million ha. of land (BBS, 2017). The average yield in Bangladesh around 3.07 t/ha. With the time come, rice production is needed to be increased. This increased rice production has been possible largely due to the adoption of modern rice varieties on around 66% of the rice land which contributes to about 73% of the country's total rice production (DAE, 2010).

With a view to focus attention on the importance of rice in global food security and the necessity to increase rice production, United Nations General Assembly was declared to celebrate the year 2004 as "International year of rice "IYR- 2004" with the title of "Rice is

life. Rice provides 20% of the world's dietary energy supply, on the other hand wheat contributes 19% and maize 5%. Rice grain contains 7% protein, 62-65 % starch, 1.3% fiber and 0.7% fat on an average and it is also a source of vitamin B1 (thiamin), B2 (riboflavin), B3 (niacin) and B5 (pantothenic acid). The biological value of rice is 63% whereas Biological value of wheat and maize is 49% & 36%, respectively. In Bangladesh more than 95% of population consumes rice and it alone provides 76% of calorie and 66% of total protein requirement of daily food intake (Bhuiyan *et al.,* 2002).

The production of rice faces numerous biotic and abiotic stresses. Among the biotic stresses, diseases are of main threat in rice production. Rice suffers from many diseases which are caused by fungi, bacteria, viruses, phytoplasma, nematodes and other non-parasitic disorders. As a field crop rice is prone to attack by more or less 50 diseases include 6 bacterial, 21 fungal, 4 nematodes, 12 viral and 7 miscellaneous diseases and disorders (Jabeen *et al*., 2012). Fungal diseases are most devastating and cause epidemic condition in rice. Major fungal diseases like blast, brown spot, sheath blight, and now a days some 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 these fungal diseases, blast is considered as major threat to rice production for its wide spread distribution and destructiveness under favorable environmental conditions for last 3 to 4 years here in Bangladesh.

Rice blast caused by a filamentous, ascomycete fungus named *Magnaporthe oryzae* and it is reported in 85 countries wherever rice is grown (Gilbert *et al*., 2004). This disease can occur in all rice growing regions of the world and the most serious in temperate and tropical area of non-irrigated conditions. Blast problem is stabilized by the high pathogenic variability of the fungus in temperate regions. Rice blast was reported to reduce yield by 30-50% in Southeast Asia and South America (Shimamoto, 2001). It is estimated that each year enough rice is destroyed by rice blast alone which can feed 60 million people worldwide (Zeigler *et al*., 1994). Among the major rice diseases that often cause great losses, rice blast is a vicious threat to the country's economy and Boro faces it worst. Most popularly affected Boro rice is BRRI dhan28 (29.6% disease severity) followed by BRRI dhan29 (25.9% disease severity) and T. Aman rice was BRRI dhan34 (22.9% disease severity) (Hossain *et al.,* 2017).

The fungus can infect rice plants at any growth stage. The mycelium may survive within the tissues of embryo, endosperm and glumes. There are certain cultural practices that encourage blast such as use of excessive use of nitrogen (by chemical fertilizers) increases susceptibility of rice to the fungus, and inadequate spacing. Rice blast results four typical disease symptoms such as leaf blast, node blast, neck blast and panicle blast. The major symptoms of this disease are found on leaves with brown spot turning to grey center, on neck of the panicle sunken lesion on nodal area with brown or black lesion. Panicle blast shows brown spot on grain with destroying whole inner materials. It is most devastating and sometimes causes 100 % yield loss. On the other hand, neck blast causes the highest yield loss since it affects the panicle directly. It is occurring seriously but new hybrid line development is time needed job. In order to reduce huge crop loss, present situation turned to its chemical and biological management until resistant variety developed.

By realizing the importance of the crop and economic threat and unsatisfactory management of the disease, urgent considerable attention need to be given on the detailed studies of development of resistant cultivars and evaluation of new molecules for the management of rice blast.

Objectives

The present research work was planned to achieve with the following objectives:

- ✓ To isolate and study the morphological and cultural characteristics of *Magnaporthe oryzae*
- ✓ To find out effective, cheap, easily prepared culture media for the growth and sporulation of the pathogen
- ✓ To evaluate the efficiency of selected phytochemicals against *Magnaporthe oryzae in vitro.*

Review of Literature

The available literature of work was done on blast disease of rice. Pathogen isolation, effective and cheap media evaluation and its management strategies have been reviewed in this chapter. The review of literature of this dissertation is discussed in the following heading and sub-heading titles.

2.1. Rice

According to FAO (1995) Rice is the 1st economically important crop in India, China, East-Asia, South East Asia, Africa and Latin America catering to nutritional needs of 70% of the population in these countries.

Molina *et al.,* (2012) reported, Rice (Oryza sativa L.) is consumed as a staple food for half of the world's population.

A report by Yaduraju, (2013) In Asia and the Pacific region, it is the main staple food, providing almost 39 % of calories.

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

Luo *et al*., (1998) said, Rice (*Oryza sativa L*.) is one of the most important cereals of the world and is consumed by 50% of the world population.

Silue and Notteghem, (1991) reported that, there are two species cultivated *Oryza sativa L* (Asian rice) and *Oryza glaberrima* S (African rice).

Seebold *et al*., (2004) said that, *Oryza glaberrima* is traditionally found in diverse West African agroecosystems but it is largely abandoned in favor of high yielding *Oryza sativa* cultivar that has higher agronomic performance.

Rice provides 20% of the world's dietary energy supply than wheat and maize which provide 19% and 5%. During 2012-13 and 2013-14, the worlds production has increased to 1% from 472 M. Tons to 476 M. Tons, and trade by 8% from 38 M. Tons to 41M.Tons

and consumption by 3% from 469 M. Tons to 481 M. Tons (FAO Commodity profile for rice - January 2015).

Khandakar *et al.,* (2013), In Bangladesh, rice is the most staple cereal crop and central to Bangladesh's economy, accounting for nearly 20 percent of gross domestic product (GDP) and providing about one-sixth of the national income of Bangladesh.

Swaminathan (1995) provide information that, Rice is a main source of carbohydrates. Rather this proteins and vitamins providing 84 to 310 kilo calories of energy. From nutritionally point of view, hundred grams of rice is a source of 6.9 g of protein, 1.3 g of dietary fiber, 0.2 g of sugar, 3.7 g of carbohydrates, 0.008 g calcium, 0.23 g of iron, 0.049mg of riboflavin, 0.10 mg of thiamine, 0.018 mg of pantothenic Acid, 1.3 g of nicotinic acid, 0.158 mg of vitamin B6 and also 1.05 mg of manganese, 115 g of phosphorus, 25 mg of magnesium, 115 mg of potassium , 1.09 mg of zinc and 0.01 mg of vitamin C.

2.2 Blast disease of Rice

Hasinur and Jashim (2017) collected information and reported that, the rice blast disease is caused by the fungi *Pyricularia oryzae* (renamed as *Magnaporthe oryzae)* and first documented in 1637 in China, then in Japan in 1704. In Italy, the USA and India the disease was also identified in 1828, 1876 and 1913, respectively.

Verma and Sengupta (1985) said, rice is one of the principal cereal crops. It is affected by more or less 17 diseases caused by fungi, bacteria, viruses and nematodes. The major diseases were blast, brown spot and bacterial leaf blight, wilt etc.

Biswas (2017) said, at present 267 races of rice blast have already been identified in Bangladesh's environment.

According to BBS. (2017); Chiba *et al.,* (1996); Kato, (2001); Rice blast disease occurs in rice cultivation areas with the pathogen *Magnaporthe grisea* (Anamorph *Pyricularia grisea* Sacc. synonym *Pyricularia oryzae* Cav.)

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

The perfect stage of *Pyricularia grisea* was earlier named as *Ceratosphaeria grisea* (Hebert, 1971). Later Nishihara and Yaegashi (1976) suggested the genus *Magnaporthe*.

SYSTEMATIC POSITION:

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Order: Magnaporthales

Family: Magnaporthaceae

Genus: *Pyricularia* **(**Anamorph**)**

Magnaporthe **(**Teleomorph**)**

Species: *P. oryzae*

M. oryzae

Blast disease causes yield losses from between 1- 100% in Japan (Kato, 2001), 70% in China, 21-37% in Bali Indonesia (Suprapta and Khalimi, 2012), 30-100% in Bangladesh (Singh G. and Prasad C.S. 2007) and 30-50% in South America and Southeast Asia (Baker *et al.,* 1997; Scardaci, 2003).

According to Chiba *et al*., (1996) and Liu *et al*., (2004), Rice blast is distributed about 85 countries where the rice plant is cultivated, 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 system. Environments with frequent and prolonged dew periods and with cool temperature in daytime are more favorable to blast.

Ravindramalviya, (2014) reported that, Outbreaks of rice blast is a serious and recurrent problem in all rice growing regions of the world and it's a widespread and damaging disease of cultivated rice caused by *Pyricularia oryzae.*

Zeigler, Leong, &Teng, (1994) said, it is estimated that each year enough of rice is destroyed by rice blast alone to feed 60 million people.

Supriya Devi and G. D. Sharma, (2010) studied that, outbreaks of rice blast are a serious and recurrent problem in all rice growing regions. Rice blast is a very widespread disease

of cultivated rice caused by *Pyricularia oryzae*. Around 50% of production may be lost in a field moderately affected by infection.

Hossain *et al.,* (2017) reported the incidence and severity of blast disease of rice in ten Agro-ecological zones (AEZs) of Bangladesh. They conduct the survey during Boro and Transplanted Aman seasons. Disease incidence and severity was higher in Boro season (21.19%) than Aman season (11.98%) on those AEZs.

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

Tilva *et al*., (2013) reported that, for blast disease three conditions must be happening. A susceptible host plant, in a stage that can easily infected by the disease agent. Presence of an active pathogen. Without pathogen there can be no disease. Third, a favorable environment for the pathogen to occur the disease.

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

Patel and Tripathi (1998) reported that blast caused by *M. oryzae* was epidemic on finger millet (Eleusine coracana) during a short period of time within favourable weather conditions in Madhya Pradesh, India. The average temperatures were 22 - 29^0 C with 88-99°/o RH. Disease intensity showed positive correlation with temperature, rainfall and RH.

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 and weight. When the node is attacked, it causes sterility. Rice blast pathogen infect all the above ground parts of rice plants at different growth stages. It starts a typical blast lesion on rice leaf as grey at the center with a dark border and gradually become spindle shaped.

According to Padmanabhan (1974), The spots first appear as minute brown dot. Then become spindle shaped. It occurs in several cm long and about 0.4-1.2 cm wide. The center is greenish showing a brown margin. The size, color and shape of the lesions may vary with different climatic conditions and varietal response. Under favorable conditions on a

susceptible cultivar several greyish spots may appear and coalesced which leads to browning of the whole leaf.

2.3 Isolation of *Magnaporthe oryzae*

Xia *et al.*, (1993) had collected the panicles of neck blast. They washed with distilled water and placed on moist filter paper in Petri dishes at room temperature. Conidia from the surface were spread on 3% water agar. Then single germinating conidium was transferred to potato dextrose agar media.

According to Gashaw *et al*., (2014), blast fungal isolates produced ring shaped, irregular colonies with rough or smooth margins on oat meal agar media. It becomes greyish black to black color. The colony diameters of different groups ranged from 67.50 to 83 mm and the conidial shape was pyriform and narrowed towards the tiM. All the isolates showed raised mycelial growth with smooth colony margin.

Vanaraj *et al*., (2013) obtained a fine experiment result on rice blast disease. The blast lesions were surface sterilized with 0.1% mercuric chloride for 1 minute. Then placed over clean glass slide and kept it in a sterile Petri dish which was padded with moist cotton. The dishes were incubated for 48 hours at 28±2°C. Single conidia were aseptically transferred to potato dextrose agar (PDA) for maintenance.

Rahnema (1979) observed that, longer duration of susceptive condition such as relative humidity and darkness increased conidia germination and appressoria formation.

Mijan Hossain (2000) showed that mycelium was first hyaline then to olive in color. It usually 0.5 – 5µm, septation occurs and branched hypha. The spores were 10 – 25µm x 2 – 8µm.

Hawksworth, (1990) contributed on Commonwealth mycological institute (CMI) description about blast. The Cultures are greyish in color, conidiophores single, either simple or rarely branched and show sympodial growth. Conidia formed singly at the tip of the conidiophore, pyriform and narrowed toward tip which is hyaline to pale olive in color.

You *et al*., (2012) reported that, the mycelium consists of septate, uninucleate, branched hyphae. However, as the fungus gets older, the hypha become brown. Generally, growth

of the pathogen is relatively more on upper surface making the spot darker on upper side. Conidiophores are simple, septate, basal portion being relatively darker. 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. Conidia is rarely two celled or four celled. Formation of intercalary or terminal chlamydospores is common, which are globose, thick walled and olive brown.

Javadzadeh and Motlagh (2010) studied on *Magnaporthe oryzae* isolation. Small leaf pieces with lesions were surface sterilized with 0.5% sodium hypochlorite solution. Then they were washed with sterile distilled water and placed on potato dextrose agar in Petri dishes at 25ºC for 48 hours. Later, Petri dishes were incubated at dark or artificial fluorescent light on 12hours alternating light and dark photoperiod for up to 25 days. As stock cultures monoconidial isolates of the recovered fungi were maintained on half-strength potato dextrose agar slants in test tubes and stored.

Silva *et al.,* (2009) worked on eight samples of rice leaves in the state of Goias, Brazil. Directly transferred one conidium per lesion on 5% water agar media from two to three lesions per leaf. This become Monoconidial isolates. The collected isolates were conserved on sterilized filter paper discs and stored in refrigerator at -20ºC.

2.4. Morphological characters of the pathogen

Aoki, (1955) measured 16 isolates in potato dextrose agar culture and showed that, the average length of mycelia the isolate ranged from 21.2 to 28.4μm, and the average width from 7.3 to 14 9.0μm.

Ono and Nakazato (1958) observed that, the size of conidia of *M. grisea* varied with the culture media also.

Mijan Hossain (2000) observed mycelium in cultures was first hyaline in colour, then changed to olivaceous, 1 – 5.2 μm in width, septate and branched. The spore measurements were 15 – 22 μm × 4 – 7 μm (Average, 17.4 μm × 5.2μm). Linear growth of the colonies of the Pyricularia 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. He 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.

Nishikado (1917) described the morphology of *M. oryzae* spores. Which 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 (1.6) μm in width, basal cell 4.8 – 11.5 (7.8μm), middle cell 1.8 – 11.5 (6.6 μm), apical cell 6 – 14 (7 μm) in length.

Reddy and Bonman (1987) said, the isolates produced good growth on the decoctions of their host material.

Padmanabhan (1965) observed the dimensions 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.

2.5 Evaluation of Different Solid Media

According to Ramakrishnan (1948), The linear growth of *Magnaporthe* isolated on standard medium agar, Oat meal agar, French bean agar and decoction agar was made. He also determined the weight of mycelial mat produced by the isolates. The isolates produced good growth on the dextrose agar of their host material.

Sun *et al.,* (1989) conducted studies the effects of 17 media on 41 isolates of *Magnaporthe oryzae*. Corn meal and rice straw agar media were best for spore production.

Awoderu *et al.,* (1991) showed that linear growth of *M. oryzae* in Potato dextrose agar (PDA) medium was best and conidial production was at 1% soluble starch yeast extract agar.

Arunkumar and Singh (1995) used different solid culture media for isolating *Magnaporthe oryzae* from rice. They found maximum colony growth occurred on malt extract agar and Leonin agar.

Mijan Hossain (2000) observed that among the all non-synthetic media used in his study the potato dextrose agar gives maximum radial growth (85.00 mm) than host extract + 2 per cent sucrose agar medium (80.33 mm) followed by oat meal agar (75.00mm).

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

Vanaraj *et al.,* (2013) studied culturing of different isolates of *M. oryzae* and reported that colonies appeared as white on oat meal agar, rice polish and malt extract agar. Color turns grey on potato dextrose agar and whitish grey on rice agar.

Padmanabhan *et al.,* (1970) isolated *Magnaporthe oryzae* from infected rice plant on oat meal agar (OMA) with biotin and thiamine (B and T). Cultures were purified by dilution method to use the single spore isolates. It was grown and multiplied on OMA + B & T at 25^0C.

Correa *et al.,* (1993) collected infected rice plants leaves and panicles from rice cultivars of germplasm bank at CIAT and IRRI. They derived cultures from either mass or single conidial isolates. Cultures were maintained on V8 juice agar and multiplied for inoculations on rice-polish agar at 28^0C under light. They stated that *Magnaporthe oryzae* expressed its virulence spectrum irrespective of geographical location.

Srivastava *et al.,* (2014) said, blast fungal isolates produce ring like, circular, irregular colonies with rough and smooth margins on OMA media with buff color, greyish black to black color.

Gashaw *et al., (*2014) showed, the colony diameters of different groups ranged from 67.50 to 82.50 mm and the conidial shape of the different groups was pear-shaped (Pyriform) with rounded base and narrowed towards the tiM. On oat meal agar, colony color of all the isolates was usually grey. All the isolates showed raised mycelial growth and smooth colony margin.

Ram *et al.,* (2007) reported that, leaf blast fungus can attack the rice plant at any growth stage. When the node is attacked by *Magnaporthe oryzae* it can cause partial to complete sterility. Radial mycelial growth and days of sporulation were studied by culturing three fungal isolates from rice, finger millet and Panicum sM. on six different media named 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 mycelial growth was found in the isolates from finger millet and the lowest in the isolates

from rice. Among the different media PA and OMA were found to be the best for mycelial growth and sporulation. The shape, color and compactness of the fungal colonies varied with the media and isolates.

Meena (2005) found that, colony color of all the rice blast isolates was usually buff with good growth on Oat meal agar. Greyish black with medium growth on host seed extract with 2% sucrose agar. Raised mycelial growth with smooth colony margin on potato dextrose agar (PDA). And raised mycelium with concentric ring pattern on Richard's agar medium. On host seed extract with 2% sucrose agar all the isolates give black to greyish black color and good growth.

Ravindramalviya (2014) used four culture media for the study of mycelial growth of *M. oryzae*. Among them PDA media supported maximum mycelial growth than Richard's Agar medium after 168 hours of incubation. Then sporulation was observed in traces in Potato dextrose agar medium and Richard's Agar medium. However, Czapek-Dox medium was not effective for both vegetative growth and sporulation.

Mahdieh S. (2013) reported that PDA culture medium could provide the best medium for vegetative growth. However, *M. oryzae* could sporulate 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 could induce *M. oryzae* a better sporulation.

Awoderu *et al.,* (1991) reported that linear growth of *M. oryzae* was best on PDA media while conidial production was greatest on soluble starch yeast extract agar (1%).

Jamal-U-ddin Hajano *et al.,* (2013) said, Abiotic factors like culture media, photo-periods and temperature greatly influenced mycelial growth and sporulation of rice blast fungus *Magnaporthe oryzae*. Under laboratory conditions maximum colony growth was recorded on potato dextrose agar (PDA) media followed by potato carrot agar (PCA). whereas oat meal agar (OMA) produced minimum mycelial growth. For sporulation, PDA served as favorable medium. Alternate 12 hours light and 12 hours dark appeared best solution for mycelial growth of *M. oryzae*, whereas maximum sporulation occurred when culture was exposed to 8 light and 16 hours darkness cycles. Studies indicated that *M. oryzae* grew between 10-35°C and 30°C was the optimum temperature for mycelia growth.

Kandhari (1996) cultured *Magnaporthe oryzae* from pearl millet on 10 different liquid media. Among them Ramakrishnan's medium was the best for growth but sporulation was best on Glucose Asparagine Vitamin (GAV) medium. The maximum growth and excellent sporulation on GAV were recorded at 25°C and pH 5.5. Again 11 inorganic and 8 organic N sources were tested. Maximum growth and sporulation occurred on nitrate N, while serine and glycine were excellent sources of organic N. There was no correlation between growth and sporulation.

Du Xinfa *et al.,* (1995) stated that *Magnaporthe* isolates from hosts including rice and common weeds in rice fields sporulated huge on sterilized barley or sorghum grains.

2.6 Evaluation of different chemical treatment

Kishan Lal *et al.,* (2015) conducted experiments on the effect of mycelial growth of *Magnaporthe oryzae in-vitro* by two fungicides i.e. Carbendazim 50% WP (100% effective at 0.1%, 0.2% and 0.4% concentration) and Tricyclazole 75% WP (100% effective 0.06 % and 0.12 % concentration). Bioagent *Pseudomonas fluorescens* (2×108 cfu/ml) were evaluated by dual culture technique which is 59.72 % effective.

According to Jamadar and Naik (2014), Among the non-systemic fungicides mancozeb 75WP renders highest inhibition which is 93.30% of the mycelial growth. The captan 70 + hexaconazole 5 gives 93.17% and copper oxychloride gives 89.41%. It was found to be significantly superior over the combined chemicals, where tricyclazole18 + mancozeb 62 inhibit 87.38% and chlorothalonil 75WP inhibit 83.81%. Further, it was observed that, there was no interaction effect among the fungicides and concentrations. Among systemic fungicides tricyclazole 75WP gave maximum inhibition of the mycelial growth (87.78%) of *Pyricularia oryzae* followed by difenoconazole 25EC (86.91%), hexaconazole 5E (85.33%) and propiconazole 25EC (75.92%) and carbendazim 50WP (54.23%) which was found to be the least efficient in inhibiting mycelial growth of *M. oryzae*. There was no significant difference among the different concentrations tested as well as there was no interaction effect between the fungicides and concentrations.

Pal (2014) provide an information by conducting the study on six fungicides. They are Kresoxim methyl, Azoxystrobin, Propiconiazole, Trifloxystrobin + Tebuconazole,

Difeconazole, and Tricyclazole. He evaluated the mycelial growth control of *Magnaporthe oryzae* in vitro and found that Azoxystrobin & Tricyclazole were the most effective.

Prasanna *et al.,* (2014) used various fungicides against neck and leaf blast and sheath blight of rice. Among them, Conika 50% WP (Kasugamycin 5% + Copper Oxychloride 45% WP), Dhanucop Team (Tricyclazole 75% WP) and RIL-068/F1 48 WG (Kresoxim methyl 40% + Hexaconazole 8% WG) were found effective.

Singh *et al*., (2014) reported that tebuconazole completely inhibited the colony growth of *Magnaporthe oryzae* at 10 µg/ml. Rather this azoxystrobin + difenoconazole, propiconazole and difenoconazole inhibited the colony growth 100% of the pathogen at 25 µg/ml. The remaining fungicides named zineb, tricyclazole, kasugamycin and azoxystrobin proved least effective as high concentration as 200 µg/ml.

Chander Mohan *et al*., (2013) evaluated the fungicides named Folicur (tebuconazole), Tilt (propiconazole), Score (difenconazole), Dithane-78 (zineb), Kasu-B (kasugamycin), Amistar top (azoxystrobin + difenoconazole), Baan (tricyclazole) and Merger (tricyclazole + mancozeb) under in vitro conditions. The concentration was 0.1, 1, 10, 25, 50 and 100 ppm. Among them Tilt, Amistartop, Score and Folicur were significantly effective. Tilt showed 100% growth inhibition at 10 ppm. Folicur, Amistar top and Score exhibited 100% growth inhibition at 25 ppm. Merger and Baan exhibited 50% growth inhibition at 10 and 25 ppm respectively. However, Kasu-B registered the least 60% growth inhibition at 100 ppm.

Kunova *et al.,* (2013) screened the fungicides against the mycelium growth of *Magnaporthe oryzae*. Mycelial growth inhibited at low concentrations of Azoxystrobin and relatively high concentrations of Tricyclazole. Again, infection efficiency of conidia was affected to a higher extent to Tricyclazole than for Azoxystrobin-amended media. Even though germination of such conidia was reduced after Azoxystrobin treatment.

Debashis *et al.,* (2012) screened five fungicides i.e., Nativo 75WG, Gain75 WP, Score 250 EC, Hexacon Super 5% SC, and Tilt 25 EC against rice blast with the dose rates of 0.4 g/l, 0.6 g/l, 1.25ml/l, 1.5ml/l, 1 ml/l water respectively. Among them Nativo, Gain and Score proved effective in reducing the disease more. In 3rd week with 10.15%, 12.85% and

11.46% disease. In case of neck blast, it shown by Score, Tilt and Nativo with 11.63%, 14.29% and 18.98% disease respectively. Tilt was the least effective in controlling leaf blast where Hexacone is greatest in controlling neck blast.

Hajano *et al.,* (2012) conducted a study to determine the efficacy of four fungicides named thiophanate methyl, carbendazim, fosetyl-aluminum, mancozeb and copper oxychloride. Only mancozeb appeared as the highly effective fungicide that can 100% inhibited the mycelial growth of the fungus. The rest fungicides showed little effect at low to higher concentrations.

Gohel *et al.,* (2008) screened that tricyclazole, mancozeb, carbendazim, iprobenfos, propiconazole and edifenphos were highly fungitoxic. They can inhibit 100% of mycelial growth in vitro of *Pyricularia oryzae*.

Boza *et al.,* (2006) evaluate the race pattern of nine isolates of *Magnaporthe oryzae*. Thirtythree rice varieties were inoculated with conidia @ 2.0×10^5 of pathogen at three leaf stage. Two per cent Tween 20 was added as a sticking agent. The plants were placed in a chamber at nearly 100% relative humidity at 20-22^{o}C for 24 h. Plants were then transferred to green house for 7 days. Then disease reaction was observed.

Zhu *et al.,* (2005) said neck blast as the most destructive phase of the blast disease of pathogen *PyrIicularia oryzae*. Node infection includes infected nodes appearing black-brown and dry with a band pattern. This kind of infection often causes the culm to break.

Haq *et al.,* (2002) conducted an experiment to evaluate seven fungicides like Captan, Acrobat, Bayeltan, Sunlet, Dithane M-45, Trimiltox and Derosal in inhibiting the growth in in vitro conditions. He found that Captan and Acrobat were the most effective fungicides that time.

Arun kumar and Singh (1995) evaluated the efficiency of several fungicides on the mycelial growth of blast pathogen isolated from rice, finger millet, pearl millet. He observed that, isolates from rice were most sensitive to Bavistin(carbendazim) then Topsin-M (thiophanate methyl), Hinosan(edifenphos), Iproben(fosmancozeb), Dithane M-45, Blitox 50(copper oxychoride)) and Fongonrene(pyroquilon).

El-Kazzaz *et al.,* (1990) found that two fungicide, Hinosan (edifenphos) and Kitazin (iprobenfos) inhibited mycelial growth of *Magnaporthe oryzae* in culture at 75 and 100 ppm respectively. The minimum concentration of tricyclazole (Beam) was 500 ppm and pyroquilon was 700 ppm.

Kamalakannan *et al.,* (2001) inoculated rice seedlings of 10 days old with *Magnaporthe oryzae* spore suspension. He evaluated the efficiency of botanicals as leaf extract. Among them *Prosopis juliflora* (93°/o), then by *Ziziphus jujuba* (82°/o) *Abutilon indicum, Cynodon dactylon* (78°/o), *Cyperus rotunduns* (74%) and *Clerodendrum inerme* recorded reduction in mycelial growth than the control. Rather this *Eclipta alba* showed minimum reduction (22°/o).

Jagannathan and Narasimhan (1988) screened 66 plant extracts against *Magnaporthe oryzae* under *in-vitro* condition. The garlic oil, neem seed oil, neem leaf, parthenium leaf, turmeric rhizomes and garlic bulb extracts were effective in inhibiting the spore germination and mycelial growth of *Magnaporthe oryzae* isolates.

Amadioha (2000) reported that leaf extract contains water and ethanol and oil extract of neem seed can significantly reduce the redial growth of *Magnaporthe oryzae* in greenhouse. Neem is the potential inhibitor for managing rice blast in the field.

Rajni *et al*., (2015), studied the mycelial growth of *Magnaporthe oryzae* with 8 systemic, 2 contact and 6 combi products in vitro. Among systemics tricyclazole, carbendazim, hexaconazole, kitazin-p and azoxystrobin gives 100% mycelial inhibition.

Dey *et al.*, (2013) At present Chemical control is more effective for management of blast of rice caused by *Magnaporthe oryzae*.

Pal (2014) evaluated six fungicides which are named Kresoxim methyl, Azoxystrobin, Propiconazole, Trifloxystrobin +Tebuconazole (Nativo), Difeconazole, and Tricyclazole. To control the leaf blast of rice among them Trifloxystrobin + Tebuconazole (Nativo) was found to be a most effective.

Ravindramalviya (2014) tested newly evolved fungicides such as Trifloxystrobin 25% + Tebuconazole 50% (Nativo 75 WG), Kresoxim methyl (Ergon 44.3 SC), Thifluzamide 24

SC, Metaminostrobin 20 SC, Azoxystrobin 25 SC (Amistar), Tricyclazole 75 WP (Beam), Carbendazim 50WP (Bavistin), Propiconiazole 25EC (Tilt) against leaf blast of rice under natural conditions. Among them Azoxystrobin and Tricyclazole shows better result than others.

Singh and Prasad (2007) reported that BEAM(Tricyclazole) as most effective fungicide for the control of rice blast and increasing the yield.

Jamal-u-Ddin *et al.*, (2012) recorded that Mancozeb is the most effective fungicide that completely inhibited the mycelial growth of the *Magnaporthe oryzae.*

Nasruddin and Amin (2013) studied that Difenoconazole and Difenoconazole + Propiconazole were evaluated against the rice blast disease and found effective in suppressing blast and protecting yield as compared to the other tested fungicides.

Materials and Methods

The present study was conducted in Molecular Biology and Plant Virology Laboratory under the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207 during 2018 to 2019. The samples were collected from the field of Mymensingh and Thakurgaon districts. The selected areas were reported to have blast infestation by the regional agricultural extension centers. The details of the materials required and the methodology adopted during the study are described in this chapter.

The experiment is conducted under four sections, namely-

a. Isolation of rice blast pathogen and its cultural and morphological study
b. Evaluation of different solid media for its mycelial growth and sporulation
c. Conduction of Pathogenicity test
d. Determination of fungicidal efficiency against identified rice blast isolate.

3.1. Sample collection from diseased fields

3.1.1. Location of the field

The samples were collected from selected two districts reported to have rice blast infestation in 2018. The Mymensingh district samples were collected from Bharadoba village under Bhaluka upazila and Thakurgaon district samples were collected from Horihorpur village under Thakugaon sadar. The collected sample fields were in different AEZ's (AEZ 9 and AEZ 1).

AEZ 9 (Old Brahmaputra floodplain)- It's around 7230 sq. km. This region contains larger area of Brahmaputra sediments before the river turned to its present Jamuna channel about 200 years ago. Most of Large area of Sherpur, Jamalpur, Tangail, Mymensingh, Netrokona, Kishoreganj, Narsingdi, Narayanganj districts. Small areas of East Dhaka and Gazipur (FAO/UNDP, 1988).

AEZ 1 (Old Himalayan Piedmont Plain) - Its around 4,008 sq. km. This region is developed by extension of old Tista alluvial fan from the foot of the Himalayas. Most of Panchagarh and Thakurgaon and the north-western part of Dinajpur district are included in this AEZ (FAO/UNDP, 1988).

3.1.2. Climate of the Areas

Mymensingh is situated in north eastern part of Bangladesh at 24º 45’ 14” north latitude, 90º 24’11” east longitude. The climate of Mymensingh is moderate and much cooler as it is close to Himalayas. Monsoon starts in May-June and stays till late August. During the monsoon, the temperature varies between 16 and 22 degrees. The temperature falls below 15 °C (59 °F) in December to January. The highest temperature occurs during April to May period, then the temperature may find to 40 °C (104 °F). The annual rainfall of this region is 2000 to 2250 mm (BBS, 2017).

Thakurgaon is a district in the north-western corner of Bangladesh at 25º 95’ north latitude and 88º25’ east longitude. It consists of a part of the Himalayan plain land. Highest average temperature of this district is 33.5° C during April to May and lowest average is 08.05° to 15.5° C in January. It is one of the coolest areas in Bangladesh. The average temperature in Thakurgaon is 24°C to 28°C. Monsoon starts at May to till October. More or less 2147 mm of precipitation falls annually (BBS, 2017).

3.1.3. Type of soil

Soils of Mymensingh area are silt loams to silty clay loams on uplands and clay in the basins. Mymensingh soil texture is 52-66 % sand, 30-33% silt and 14-21% clay. General soil type is Dark Grey Floodplain soil. Organic matter content is low on the ridges 0.33-0.89% and moderate in the basins 1.21 to 1.49%. The topsoil is acidic but subsoil is neutral in nature. pH value ranges 5.33 to 6.46.

Thakurgaon has deep rapidly permeable sandy loams and sandy clay loams soil. Soil types in the region are Non-calcareous Brown Floodplain soils, Black Terrain soils and Non-calcareous Dark Grey Floodplain soils. This area contains 47-58% sand 20-30% silt 21-27% clay. They are strongly acidic in topsoil and moderately acidic in subsoil as well as rich in sand minerals. pH ranges 5.4 to 6.67% and organic matter content 1.32 to 1.98%.

3.1.4. Season and crop stage in time of sampling

The sampling was carried out in the period of January 2018 to May 2018. Samples were collected during milking stage of the rice crop (BRRI dhan28) to observe neck blast and

leaf blast symptoms. The climate of the field areas was of sub-tropical characterized by high temperature and heavy rainfall during March to June and moderately to very low temperature during November to February.

3.1.5. Cropping Sequence

Crop sequences were divided by 2 major groups on the basis rice cultivation intensity, they are (a) Cropping Sequence-1 (annual cycle consisting one-rice followed by fallow/other crop) and (b) Cropping Sequence-2 (annual cycle with two times rice cultivation followed by fallow or other crop or rice) (Hossain *et al.*, 2017).

3.1.6 Sampling pattern

Three diseased fields were selected from each location. From each field three spots were selected by simple random sampling method which was based on visual inspection.

Figure 1. Rice blast affected plants

3.2. Isolation of *Magnaporthe oryzae*

3.2.1. List of equipment's

The following equipment's were used in present investigation:

- Incubator for incubation
- Autoclave for media sterilization

- Oven for glassware sterilization and for media melting
- Thermometer
- Inoculation needle, forceps, knife, 9 cm Petri dish, conical flask, scissor, scotch tape, pipette, p^H meter and blotter paper etc.
- Laminar air flow cabinet as working chamber
- Compound Microscope
- Weighing balance
- Refrigerator for sample and pure culture storage (4^0C and -20^0 C)
- Spirit lamp

3.2.2. Cleaning and sterilization of glassware

The glass-wares were first cleaned with detergent, then thorough cleaning with tap water. Then they were air dried and sterilized in hot air oven at 160^0C for one hour. Working media and water were sterilized at 121^0C and 15 psi pressure for 40 minutes in an autoclave machine. Whole Laminar air flow cabinet and work benches were sterilized with 70% ethyl alcohol. Needle, scalpel and inoculation loop were sterilized by spirit lamps flame (Darmady *et al*., 1961).

3.2.3. Collection of blast diseased specimens from selected area

Sampling was done where Blast affect at severe form. Infected rice leaf and neck samples were collected from fields. Collected leaves and necks were separated from the mother plant and kept in a zip lock plastic bag with needed information in a small paper (Name of the sample, district, village, cultivars and date of collection). Then the samples were carried to Molecular Biology and Plant Virology Laboratory under the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka 1207 for pathogen isolation and characterization.

3.2.4. The isolation of pathogen from collected sample

3.2.4.1. Sample placement

The infected parts of diseased leaves were cut into small pieces (0.5-1 cm) with diseased and healthy parts. Samples were surface sterilized by dipping in 1000 times diluted (0.1%)

mercuric chloride solution for 30 seconds and rinsed by sterilized water for 1 min and repeated the washing 3 times (Sahu *et al.*,2018). Then they were placed and soaked in sterilized blotter paper to remove excess water. Then immediately sample pieces were inoculated in another wet sterilized blotter paper and water agar medium (Sileshi *et al*,2016).

For blotter paper, small leaves were also inoculated in sterilized Petri dish containing blotter paper which were wetted with distilled water. Wet paper supplied moisture for rapid sporulation on plant parts (Sileshi *et al*,2016).

For wet blotter paper preparation, at first 9 cm radial blotter papers were sterilized in hot air oven for 10 minutes at 160^0C temperature. After that, the blotter papers were placed in sterilized plastic petri dishes. Two papers per dish were placed. Then sterilized distilled water was added with sterilized dropper aseptically. When the whole paper become wet but no excess water was floated than it was ready for next step. Infected sample pieces were then placed on over the dish containing wet blotter papers. It was covered with lid and transparent scotch tape was used to seal it as it could be easily visible with transparent scotch tape. The whole process was done in Laminar air flow cabinet.

Preparation of Water Agar (WA) Media

Amount of ingredients for WA media preparation (Lingappa, Y. and J. L. Lockwood, 1960) were-

Distilled water - 1L

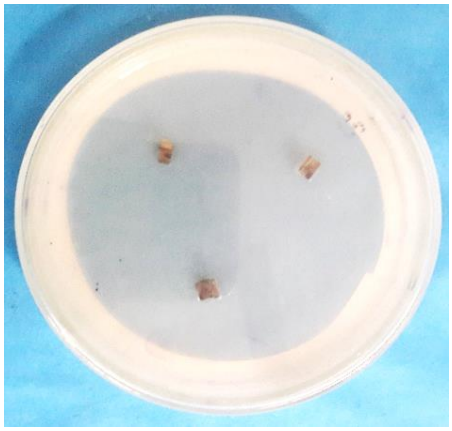
Agar - 15g

An amount of 15 grams of agar powder was weighed in electric balance and it was added in 1L distilled water. A sterilized glass rod was used to mix up the ingredients. Then the mixture was heated until dissolve. It was then sterilized at 121^0C under 15 psi pressure for 40 minutes in autoclave. All the instruments needed to be present in laminar air flow cabinet before the inoculation work started. After that the media needs to be cooled at a temperature in which the conical flask could be touched with bare hand. Then the media should be poured in sterilized petri dish and kept for solidification. When the media totally

cooled and solidified, small diseased samples were placed on over the water agar media and lid was covered.

After that both the water agar and blotter paper petri dishes were incubated at 28±1^{0}C for mycelial growth. After 48-72 hours of inoculation, a tiny portion of culture was placed on slide and observed under microscope and identified the pathogenic fungi with the help of literature cited characters (Barnett and Hunter, 1972; Ellis, 2001; Mew and Gonzales, 2002).

Small pieces placed on wet blotter paper



Small pieces placed on water Agar

Figure 2. Sample placement on Blotter paper and Water Agar media

Two types of media were used for isolation of blast pathogen. PDA media was used for pathogen isolation and PR$_S$DA media was used for sporulation purpose and spore study. Both media were prepared in conical flasks and autoclaved at 121^{0}C under 15 psi pressure for 40 minutes.

Preparation of Potato Dextrose Agar (PDA) media

Composition of Potato Dextrose Agar (PDA) media (Ricker and Ricker, 1936).

Components	**amount**
Potato	200 g
Dextrose	20 g

Agar	20 g
Distilled water	1000 ml

At first 200 gm of diseased free potatoes were peeled and cut into small cubes. Then they were boiled in 500 ml water for 15 to 20 minutes. After that the potato extract was collected by filtration with muslin cloth filter. Then 20.0 g dextrose was added and dissolved by stirring with glass rod. Agar 20 g was also added and shaked until a uniform solution was prepared. The final volume was made up to 1000 ml with distilled water. Then the open mouth of conical flask was temporarily covered by wrapping with aluminum foil paper. It was sterilized by autoclaving at 121^{o}C under 15 psi pressure for 40 minutes.

Preparation of Potato-Rice straw Dextrose agar (PRsDA)

Components	**Amount**
Potato	200 g
Dextrose	20 g
Agar	20 g
Rice straw	80 gm
Distilled water	1000 ml

At first 80 gm disease free chopped BRRI dhan28 rice straw was boiled in 400 ml water for 15 minutes. Then the infusion was filtrated by muslin cloth filter. 200 gm of diseased free potatoes were peeled and cut into small cubes. Then they were boiled in 500 ml water for 15 to 20 minutes. After that the potato extract was collected by filtration with muslin cloth filter. The straw extract and potato extract were mixed. Then 20.0 g dextrose was added and dissolved by stirring with glass rod. Agar 20 g was also added and shaked until a uniform solution prepared. The final volume was made up to 1000 ml with distilled water. Then the open mouth of conical flask was temporarily covered by wrapping with aluminum foil paper. It was sterilized by autoclaving at 121^{o}C under 15 psi pressure for 40 minutes.

3.2.4.2. Preparation of the culture plates and inoculation

All the sterilized equipment's required for this study were opened only in the laminar air flow chamber to avoid contaminations. The sterilized media were cooled to near 45^0C so that it can be handled and poured into sterilized glass petridish inside laminar airflow. Then 10 ml lactic acid was added on 1 L media broth just before pouring in a plate in laminar airflow cabinet to maintain the p^H near 5 to 6. The plates were labelled with distinct marks. Then, 18 to 20 ml liquid media were poured in each petri plate. It was kept until the liquid media became solidified. To sterilize the needle, it was hold by the handle and placed in a flame until it turns bright red. After burning it was allowed to cool for 3-5 seconds before touching the media. The needle was re-sterilized after each inoculation. Petri plate lid was uncovered just small enough to inoculate the medium and just enough to allow the needle inside to prevent contamination from air-borne particles. After the media become solidified and totally cooled the plates are prepared. It will take one to two hours for proper solidification and cooling of media. In case of later use, the plates were sealed with scotch tape and kept in refrigerator at 4^0C for highest 7 days until inoculation. By this process all the culture plates were prepared throughout the experiments.

The growing mycelia on water agar and blotter plates were incubated at 27 to 28°C for 24 to 36 hours to induce sporulation. A piece of sporulated fungus was picked by using needle tiM. The growing mycelia was placed on a slide with a drop of water and covered by a cover sliM. Then it was observed under the microscope for general characterization. The fungus was identified as *Magnaporthe oryzae* by following mycological characteristics given by Ou and Nuque (1985). After confirmation, small mycelial parts were carefully picked and inoculated on PDA media with the help of needle for morphological study as mycelial growth was best in PDA media by literatures. Then it was incubated at 26^0 to 28°C for at least 7-10 days and examined. A large number of replicates were made for the culture and it was stored in the room temperature at 28°C. For sporulation purpose Potato-rice straw Dextrose Agar (PR_SDA) media was used. A small mycelial part from the pure culture in PDA media was inoculated in PR_SDA media and incubated for 10 days at 26^0 to 28^0C. After 10 days the inoculated PR_SDA plates were kept under continuous light condition to induce sporulation for another 7 to 10 days. In this continuous light duration,

a very small mycelial part was picked and observed under microscope to confirm sporulation at 24 hours interval until spore observed.

3.2.5. Characterizations of *Magnaporthe oryzae* isolates

Two isolates were identified based on their morphological growth pattern and spore. On PDA media the morphological variation among the isolates were examined and characterized based on mycelial size, shape, color through necked eye. Colony characteristics were observed at 48 hours interval from inoculation for 21 days. For conidia, number of septation and color were also recorded (Mebratu *et al.,* 2015). Photographs were also taken to show the typical spore morphology. Then they were identified as isolates of *Magnaporthe oryzae*. Two purified isolates were stored on PDA media at 4^0C and -20^0C. The diameters of the fungal mycelial growth were measured with a measuring scale in millimeter (Sreenivasprasad *et al*., 2005). Two cross lines with semi-permanent pen were drawn on the under surface of the lower plate by taking a center. Mycelial growth was measured according to the scale provided by Narendra (2006). According to this scale very good mycelial growth gives 76-90 mm, moderate 56-75 mm, and poor gives <56 mm mycelial diameter. It is done at 5 days interval for 25 days after inoculation. Each treatment was replicated three times. Sporulation of *Magnaporthe oryzae* was detected by electric microscope from 14 days after inoculation in PR_SDA media at the interval of 24 hours up to 30 days. Conidial size was measured by ocular microscope. Slide preparation was done with lactophenol cotton blue by "teased mount techniques". For observing number of septations per conidia the prepared slide was placed under microscope at 100X. Other cultural characters like mycelial growth rate, type of margin, colony color was also recorded (Padmanabhan, 1974).

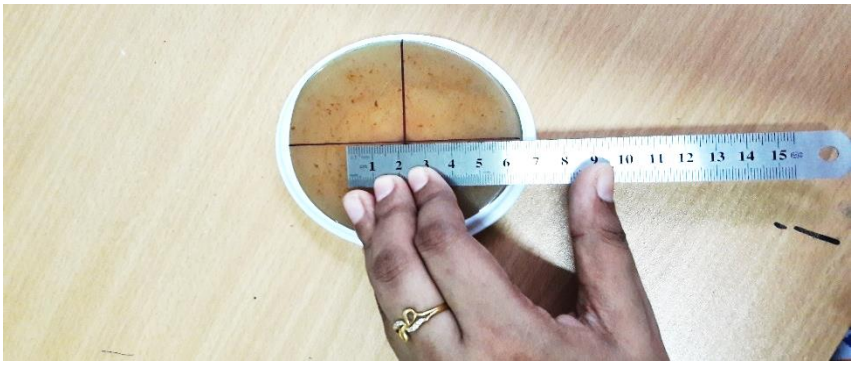


Figure 3. Radial growth measurement of *Magnaporthe oryzae*

3.2.6. Purification of the pathogen

In case of contamination purification of culture media was done. The marginal mycelial growth of fungus was picked-up aseptically with needle and inoculated in sterilized PDA media for sub-culturing in Laminar air flow cabinet. It was done at an interval of 3-5 days.

3.2.7. Storage of fungal isolates

The fungus was grown on PDA medium for 7 days at 28±1^{0}C at incubator. Then the fungus with media was poured in plastic zip lock bags and stored for short term preservation at 4^{0}C and for long term in -20^{0}C.

3.3. Evaluation of different solid media for mycelial growth and sporulation

Mycelial growth and sporulation of *Magnaporthe oryzae* was studied in twelve selected solid culture media.

3.3.1. Composition of different media

The selected culture media were prepared according to standard composition and protocol.

3.3.1.1. Preparation of Potato Dextrose Agar (PDA) media

The potato dextrose agar media was prepared according to standard procedure that was mentioned earlier in section 3.2.4.1.

3.3.1.2. Preparation of Carrot Dextrose Agar (CDA)

Components	**amount**
Carrot	280 g
Dextrose	20 g
Agar	20 g
Distilled water	1000 ml

At first 280 gm of diseased free carrots were peeled and cut into small cubes. Then they were boiled in 500 ml water for 20 to 30 minutes. After that the carrot extract was collected

by filtration with muslin cloth filter. Then 20.0 g dextrose was added and dissolved by stirring with glass rod. Agar 20 g was also added and shake until a uniform solution prepared. The final volume was made up to 1000 ml with distilled water and sterilized by autoclaving at 121^{o}C under 15 psi pressure for 40 minutes.

3.3.1.3. Preparation of Potato Sucrose Agar (PSA)

Components	**Amount**
Potato	200 g
Sucrose	20 g
Agar	20 g
Distilled water	1000 ml

200 gm of potatoes were cut into small cubes and boiled in water. Then potato extract was collected by filtration. Agar 20.0 g was added and dissolved than Sucrose 20 g was also added. The final volume was made up to 1000 ml with distilled water and sterilized in autoclave.

3.3.1.4. Preparation of Oatmeal agar (OMA)

Components	**Amount**
Oat flakes	70 g
Agar	20 g
Distilled water	1000 ml

70 gm oat flakes were boiled in 500 ml distilled water for 15 minutes. Then it was filtered by muslin cloth filter. Twenty-gram Agar was melted in another 500 ml of distilled water separately. After that both the solutions were mixed. The final volume was made 1000 ml and then sterilized in autoclave (Johnson and Curl, 1972).

3.3.1.5. Preparation of Corn meal Agar (CMA)

Components	**Amount**

Corn meal agar powder	17 g
Distilled water	1000 ml

17 gm ready-made corn meal agar powder was measured in electric balance. Then it was added at 500 ml distilled water and mixed. The final volume was made up to 1000 ml and sterilized.

3.3.1.6. Prune Agar (PA)

Components	**Amount**
Prune	25 g
Sucrose	2.5 g
Agar	20 g
Distilled water	1000 ml

25 g Prunes were boiled in 200 ml water for 15 minutes. Then filtration was done and included as much pulp as possible by pressing with finger. After that 2.5 g sucrose was mixed and 20 g agar was added and stirred until dissolved. The final volume was made up to 1000 ml. Finally, autoclaving was done.

3.3.1.7. Starch Media (SA)

Components	**Amount**
Boiled rice infusion	500 ml
Agar	10 g
Distilled water	1000 ml

250 gm rice was boiled in 750 ml water. Then 500 ml boiled rice infusion was mixed with 10 g Agar and mixed thoroughly. Water was mixed to make the volume up to 1000 ml mark.

3.3.1.8. Potato-Rice straw Dextrose agar (PR_SDA)

The potato rice straw dextrose agar media was prepared by following the standard protocol which was mentioned in section 3.2.4.1.

3.3.1.9. Carrot-Straw Dextrose Agar (CR_SDA)

Components	**Amount**
Carrot	280 g
Dextrose	20 g
Agar	20 g
Rice straw	80 gm
Distilled water	1000 ml

At first 80gm disease free chopped rice straw was boiled in 500 ml water. Then 280 gm of carrots were cut into small cubes and boiled in water. Then carrot extract was collected by filtration. Dextrose 20.0 g was added and dissolved then agar 20 g was also added. At last rice straw extract was added and the final volume was made up to 1000 ml with distilled water and sterilized.

3.3.1.10. Potato Rice leaf Dextrose Agar (PR_LDA)

Components	**Amount**
Potato	200 g
Dextrose	20 g
Agar	20 g
Rice leaf	80 gm
Distilled water	1000 ml

At first 80gm disease free freshly green rice leaves was chopped and boiled in 500 ml water. 200 gm of potatoes were cut into small cubes and boiled in water. Then potato extract was collected by filtration. Dextrose 20.0 g was added and dissolved than 20 g agar

was also added. At last the rice leaf extract was added and the final volume was made up to 1000 ml with distilled water and sterilized.

3.3.1.11. Potato Rice grain Dextrose Agar (PR$_G$DA)

Components	Amount
Potato	200 g
Dextrose	20 g
Agar	20 g
Rice grain	80 gm
Distilled water	1000 ml

At first 100gm diseased free fresh milking stage rice grain was boiled in 500 ml water. Then 200 gm of potatoes were cut into small cubes and boiled in 500 ml water. Then potato extract was collected by filtration. Dextrose 20.0 g was added and dissolved than agar 20 g was also added. At last rice grain extract was added and the final volume was made up to 1000 ml with distilled water and sterilized.

3.3.1.12. Potato Carrot Dextrose Agar (PCDA)

Components	Amount
Potato	100 g
Carrot	140 g
Dextrose	20 g
Agar	20 g
Distilled water	1000 ml

At first 100 gm potato was boiled in 500 ml distilled water and 140 gm Carrot in another 500 ml distilled water. After boiling both extracts were collected by filtration in Muslin cloth. 1000 ml jar was taken. Both the potato and carrot extract were added on it. Then 20 gm dextrose was added and mixed through a glass pipe. A 20 gm of agar was also added and mixed. Finally, the solution was taken for sterilization.

3.3.2. Preparation of plates and inoculation

All the sterilized equipment's required for this study were opened only in the laminar air flow chamber to avoid contaminations. The sterilized media were cooled to near 45^0C so that it can be handled and poured into sterilized glass petridish with bare hand inside laminar airflow. Then 10 ml lactic acid was added on 1 L media broth just before pouring in a plate in laminar airflow cabinet to maintain the p^H near 5 to 6. The plates were labelled with distinct marks. Then, 18 to 20 ml liquid media were poured in each petri plate. It was kept until the liquid media gets solidified. To sterilize the needle, it was hold by the handle and placed in a flame until it turns bright red. After burning it was allowed to cool for 3-5 seconds before touching the media. The needle was re-sterilized after each inoculation. Petri plate lid was uncovered just small enough to inoculate the medium and just enough to allow the needle inside to prevent contamination from air-borne particles. After the media become solidified and totally cooled the plates are prepared. It will take one to two hours for proper solidification and cooling of media. After solidification 3-5 mm mycelial disc was inoculated on each petri plate and incubated at 28 ± 1^0C. Each treatment was replicated for three times.

In case of later use, the plates were sealed with scotch tape and kept in refrigerator at 4^0C for highest 7 days until inoculation. By this process all the culture plates were prepared throughout the experiments.

3.3.3. Measurement of Mycelial Growth

After that observation was done after 48 hours of inoculation at 5 days interval. The radial growth of mycelia was measured in mm. Two cross lines with semi-permanent black marker pen were drawn on the under surface of the lower plate by taking a center. Then radial growth was measured by taking the intersect point as the center. It was done until the petri plates were covered with mycelia. Colony characteristics (Growth type, growth pattern, colony color etc.) were observed by visual observation. Area Under Growth Progress Curve (AUGPC) was calculated by the following formula:

$$\mathbf{AUGPC} = \boldsymbol{\Sigma}\left\{\frac{\boldsymbol{X1}+\boldsymbol{X2}}{\mathbf{2}} * (\boldsymbol{T2}-\boldsymbol{T1})\right\}$$

Where, X1 is the growth of the pathogen on 1st date, X2 is the growth of second day. T1 is the time when X1 measured and T2 is the time when X2 measured.

3.4. Pathogenicity test

Pathogenicity test was done in the net house of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207. For this test, pathogen free viable seeds of BRRI dhan28 was collected. Seed treatment was done by Tricyclazole 75% WP @1.5 gm/1kg seeds. Then water soaked sprouted rice seeds were broadcasted on the seed bed. After 7 days, seeds were germinated. Properly mature seedlings were grown at 3 weeks. The 21 days old seedlings were transplanted in a "cup tray". A "cup tray" is a tray containing 50 small cup sized pot in a plastic tray. Its measurement was 30 inches×18 inches. Each cups diameter was 2 inches.

Figure 4. Cup tray

Fertilizer dose

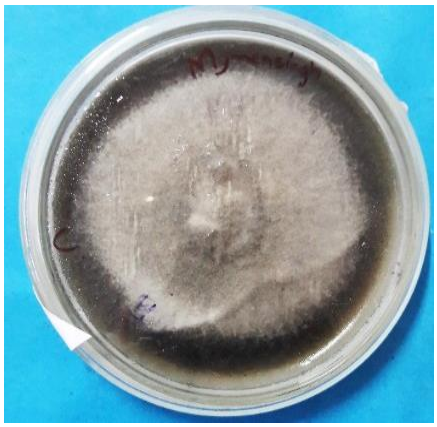
The doses of fertilizers were used is mentioned below.

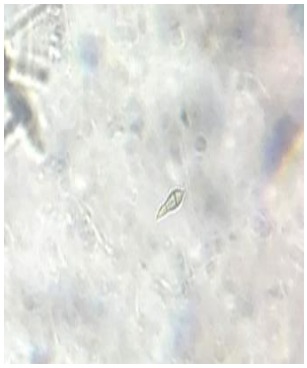
Name	**Amount**
Cow dung	1 kg /5 kg soil
Urea	200 g /5 kg soil

TSP	200 g /5 kg soil
Gypsum	200 g /5 kg soil
Boron	12 g /5 kg soil
Zinc Sulphate	12 g / 5 kg soil

All fertilizers were mixed properly with 5 kg sun dry soil and water was added for muddy consistency, and then the mud was placed in cup tray. One seedling per cup was transplanted in each cup and the tray contained total 50 seedlings. Everyday water was provided on the tray for two times to keep soil always moist. After 7 days of transplanting when the seedlings were at three to four leaf stage than inoculation of fungus in the plant was done. Inoculation was done by spraying spore suspension. Each plant was inoculated with the test pathogen isolate. Spore suspensions of $1x10^5$ spore/ml sprayed on leaves by using hand sprayer with 2.5ml of 20% Tween 20 to 1000ml spore suspension (Han *et al.*, 2003). For maintaining environment that is needed for successful infection a large plastic box was used. It was internally wrapped with wet paper to seal moisture. Then the cup tray containing inoculated plants was placed in the box. Nearly 95% humidity for 24 hours give better inoculation (Sreenivasaprasad *et al*., 2005). The box provided nearly 98% humidity. The tray was there for 24 hours darkness for quick penetration. This box method is one of the successful and cheap method for quick penetration process. After that the tray was placed in natural condition. Then waited for development of blast symptoms on the leaves. It was replicated three times for each of two isolates. The temperature in the net house during the study period were 24 ± 5^0C in late January. After that the plants were taken care for 45 days after transplantation for necessary study.

a. Collected sample





b. Pure culture and conidia of *Magnaporthe*

c. Transplanting seeding in cup tray

d. Inoculation with *Magnaporthe oryzae* suspension

e. Placing in customized moisture restored box for suitable environment

f. Replacement in natural condition for disease

Figure 5. Steps involved in pathogenicity test for *Magnaporthe oryzae*

3.5. Efficacy of different fungicides against blast isolates *in-vitro*

Five fungicides were evaluated against *M. oryzae* isolates by poured plate technique (Grover and Moore, 1962) at selected three concentrations to assess fungicidal effectiveness.

Table 1. List of fungicides and dosages used for evaluation of the fungicidal efficiency

Treatment no.	**Trade name**	**Company name**	**Active ingredient**	**Dose (ppm)**
T1	Amister Top 325 SC	Syngenta Bangladesh Ltd.	Azoxystrobin 20% Difeconazole 12.5%	500, 1000, 1500
T2	Blastin 75 WDG	ACI crop care Ltd.	Trifloxystrobin 25% Tebuconazole 50%	500, 1000, 1500
T3	Acibean 28 SC	ACI crop care Ltd.	Azoxystrobin 20% Cyproconazole 8%	500, 1000, 1500
T4	Deconil 500 SC	Haychem Bangladesh Ltd	Chlorothalonil 50%	500, 1000, 1500

The required quantities of fungicides were weighed to get the desired concentration of active ingredient of each fungicide (Table 1). The requisite quantities of fungicides were incorporated aseptically to PDA medium by poured plate technique. The fungicides were added to the autoclaved potato dextrose agar (PDA) media broth after cooled to 45°C to prevent denaturation. Then,18 to 20 ml of fungicide treated media broth was poured into sterile Petri dish. The plates were kept 2 hours for proper solidification. The plates were inoculated by cutting 3 to 5 mm of mycelial discs of *Magnaporthe oryzae*, and incubated

at 28±1°C. Three replications were made for each treatment. The fungus growth on the PDA without any fungicide termed as control. The growth of isolates was determined by measuring radial colony diameters in two perpendicular directions on each culture plate using a center. The radial mycelial growth (mm) of the mycelia was recorded when maximum growth (21 days) in control plates was noticed. The percent inhibition of mycelial growth was calculated using the formula (Riungu *et al.*, 2008)

$$I = \frac{C-T}{C} * 100$$

Where,

I = Per cent inhibition of mycelial growth

C = Colony diameter in control (mm)

T = Colony diameter in treatment (mm)

3.6. Statistical Analysis

The laboratory trials were conducted following Completely Randomized Design (CRD). The replicated data generated from different experiments were tabulated and analyzed by using STATISTIX 10 software. Treatment means were compared by LSD range test at 5% level of significance.

a. Results

This chapter provided necessary explanation including graphs and photographs obtained from the *in-vitro* experiment on rice blast pathogen *Magnaporthe oryzae* for isolation, morphology study, media evaluation and chemical efficacy determination.

4.1. Symptomology of different types of rice blast

The samples were collected from Mymensingh and Thakurgaon district. The collected samples had typical blast symptoms on leaves, nodes and panicles. The leaves were showed brown colored spindle shaped spots with greyish center. In severely affected leaves spots were gradually coalesce together and blighted the whole leaf area. Infection to the node produced purplish lesions called node blast, followed by lesion elongation to both sides of the neck node. Dark brown to black colored lesion was found on infected panicle represented the characteristic panicle blast symptom (Figure 6).

A. leaf blast B. Panicle blast

Figure 6. Rice blast symptoms.

4.2 Isolation and morphological study of the pathogen

The pathogen was isolated on Potato Dextrose Agar (PDA) media (Section 3.2) and purified (Section 3.2.6.). Sporulation on PDA is very rare and needs special treatment and for this reason for better sporulation the pathogen was grown in PR_SDA. The isolates were identified as *Magnaporthe oryzae* based on the spore and colony morphology (section 3.2.5). It produced whiteish mycelia at first but it turned into grey to black in color by time. Sporulation was observed in PR_SDA after 30 to 35 days old culture. The spore counting was done by using hemocytometer. Based on the location of collection the isolates were labelled as $M_{rs}28$ (Mymensingh) and $T_{rs}28$ (Thakurgaon).

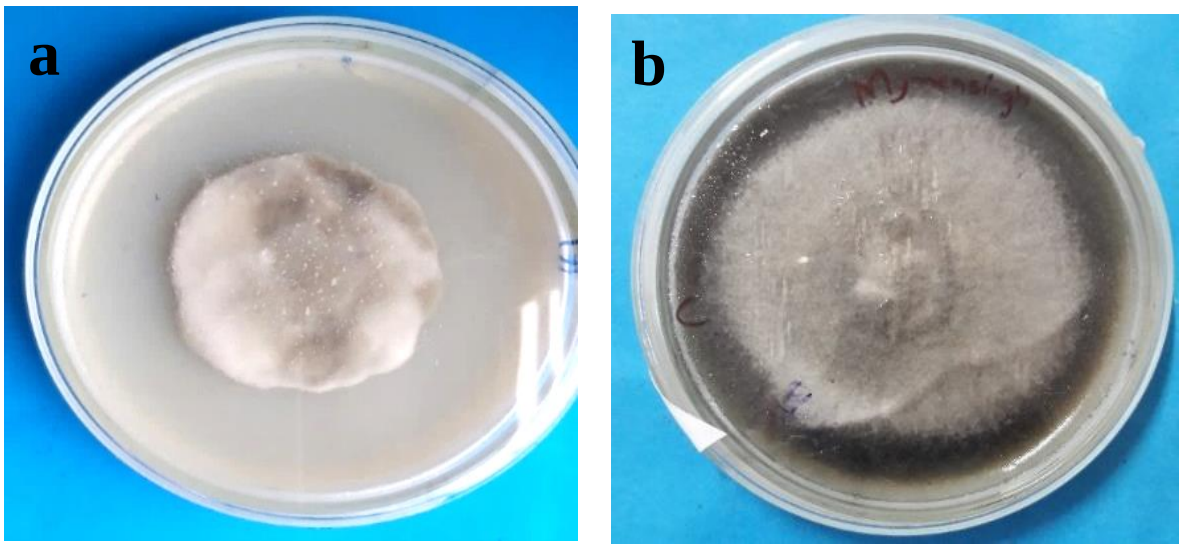


Figure 7. $M_{rs}28$ (Mymensingh) isolates (a) 5 DAI. (b) 21 DAI

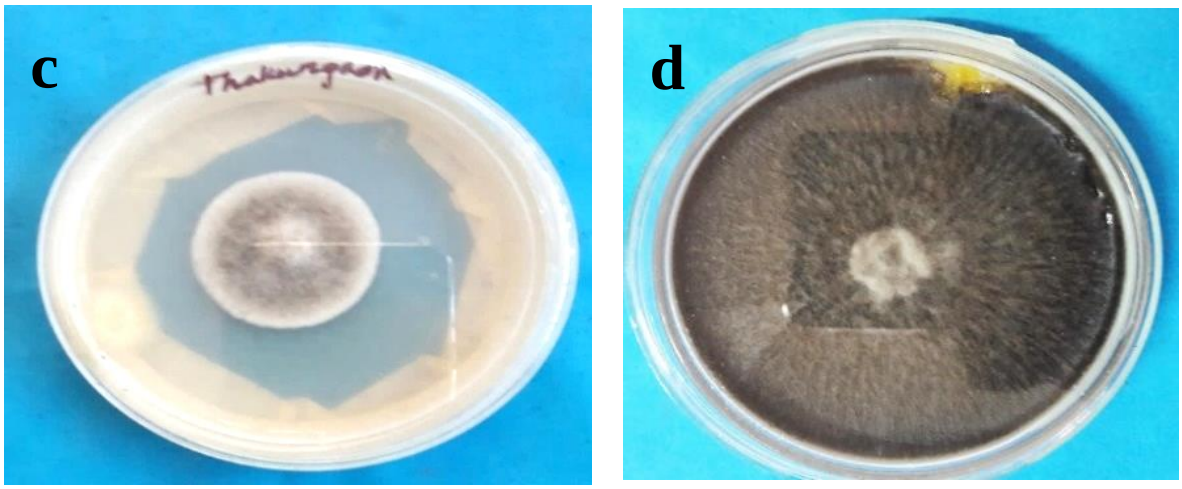


Figure 8. T_{rs}28 (Thakurgaon) isolates (c) 5 DAI. (d) 21 DAI

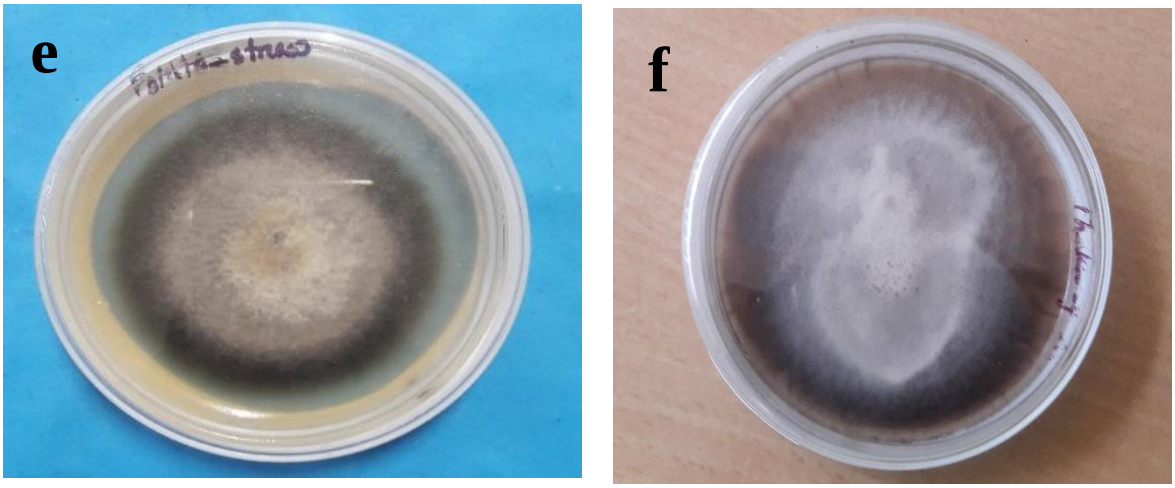


Figure 9. ***Magnaporthe oryzae*** **culture in PRSDA (e) Mrs28 (f) Trs28**

Table 2. Mycelial characteristics of both isolates on PDA Culture media after 21 days at 28 ±1ºC

Collection area	**Isolates**	**Visible fungal characters**					
		colour	**texture**	**Colony form**	**margin**	**elevation**	**Mycelial growth***
Mymensingh	$M_{rs}28$	Greyish black	Cottony velvet	Initially irregular to circular	Undulated	Flat with slightly raised middle	Very good
Thakurgaon	$T_{rs}28$	Deep black	Mass Velvety	Circular	Entire	Flat	Very good

*mycelial growth (mentioned in section 3.2.5)

The cultures of the isolates were ash to black in color. In case of $T_{rs}28$, characteristic was deep black in color. The culture was first in whitish color and under microscope only mycelia were observed at that time. In this study, for sporulation of the pathogen, PR_SDA media was used (Section 3.2.5). In PR_SDA media, up to 20 days it remained the same and at 25-40 days the conidia of *Magnaporthe oryzae* was observed under the electric microscope. The mycelium in cultures was hyaline at first, then turned in to olivaceous, septate and branched. The conidia were usually pyriform in shape and had 2 septa. They are almost hyaline, pyriform to obclavate, narrowed toward tip, rounded at the base, 2-septate. The size of conidia was measured about 22.13 - 28.47 µm × 9.13 - 11.72 µm (average 25.30 × 10.43 µm). Conidia had a characteristic basal appendage hilum. Conidiophores were either single or in fascicles, simple, rarely branched. The shape of conidia for both isolates were same. The $T_{rs}28$ isolate's conidial basal part is slightly round than $M_{rs}28$ isolate's conidia. Both were hyaline. The results of morphological study are presented in Figure 10 and 11.

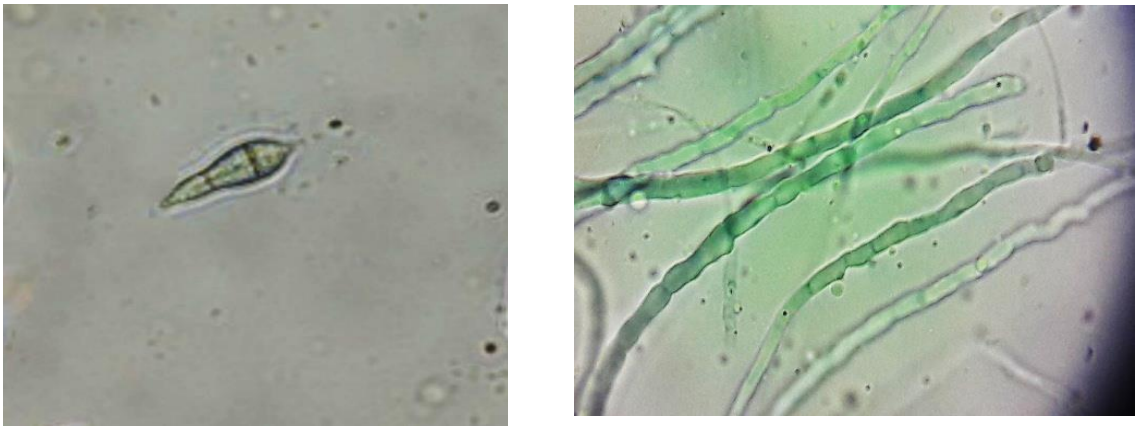


Figure 10. Microscopic view of conidia and Mycelia of *Magnaporthe oryzae for* Mrs28

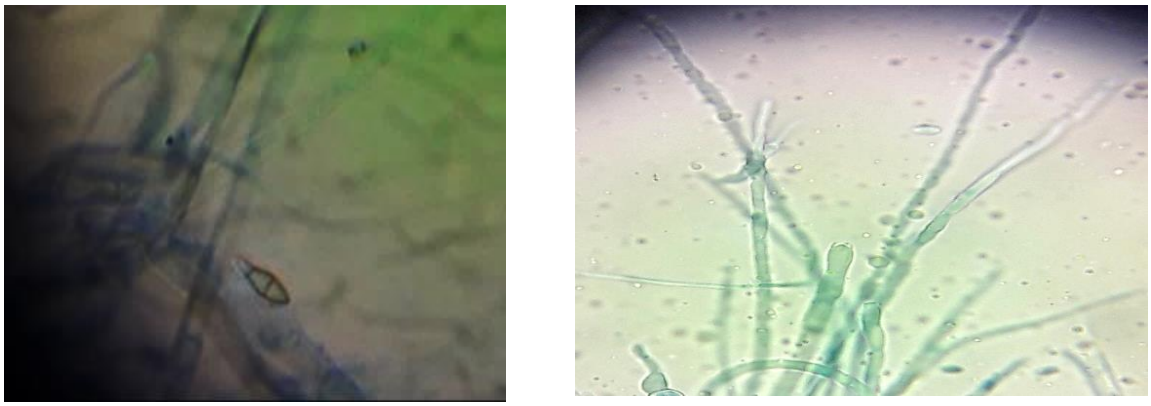


Figure 11. Microscopic view of conidia and Mycelia of *Magnaporthe oryzae for* Trs28

4.3. Evaluation of different selected solid media against *M. oryzae*

Table 3: Mycelial growth of *Magnaporthe oryzae* in different solid media

Media	Mycelial growth (mm)				AUGPC value
	5th DAI	10th DAI	15th DAI	25th DAI	
PDA	49.2 a	90.0 a	90.0 a	90.0 a	**1698.00**
CDA	45.5 b	72.0 c	90.0 a	90.0 a	1598.75
PSA	37.7 c	78.0 b	90.0 a	90.0 a	1609.25
OMA	0.0 i	6.1 i	15.9 h	35.3 g	326.25
CMA	10.5 g	20.7 h	32.2 f	49.2 f	617.25
PA	9.1 h	31.3 e	48.4 c	67.1 c	877.75
SA	14.5 e	27.2 g	34.2 e	84.7 b	852.25
PR_SDA	11.9 f	29.3 f	38.8 d	65.4 d	794.25
CR_SDA	0.0 i	0.0 j	8.0 i	21.9 i	**169.50**
PR_LDA	10.5 g	20.6 h	26.5 g	56.7 e	611.50
PR_GDA	0.0 i	0.0 j	9.4 i	26.7 h	204.00
PCAD	29.2 d	51.2 d	62.9 b	90.0 a	1250.75
CV	6.84	4.56	1.57	1.12	
LSD (0.05)	2.18	2.99	1.20	1.21	

Note: DAI-Days After Inoculation.
AUGPC-Area under growth progress curve.

(In a column those having similar letter(s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of significance)

The *Magnaporthe oryzae* pathogen was grown in 12 different selected solid media and the mycelial growth was noted at 5,10,15 and 25 DAI. AUGPC was also calculated (Section 3.3.2). From the table (Table 3.), it sees that the highest mycelial growth of 49.2 mm and 90.0 mm was recorded in Potato dextrose agar (PDA) medium at 5 and 10 DAI respectively. It was the highest growth for *Magnaporthe oryzae* in any media where pathogen attained 90.0 mm mycelial growth within 10 DAI. In PSA, mycelial growth was lower in 5 DAI than PDA and CDA but at 10 DAI it was 78.0 mm which was higher than CDA but lower than PDA. Where OMA, CR_SDA and PR_GDA did not give any mycelial growth at 5 DAI. The OMA gave visible mycelia within 10 DAI, where CR_SDA and PR_GDA initiated mycelial growth after 10 DAI. Whereas at 20 DAI the lowest growth was achieved in CR_SDA medium (21.9mm). From the table and figure (Table 3 & figure 14) the highest AUGPC was calculated 1698.00 for PDA and the lowest AUGPC from CR_SDA which was 169.50.

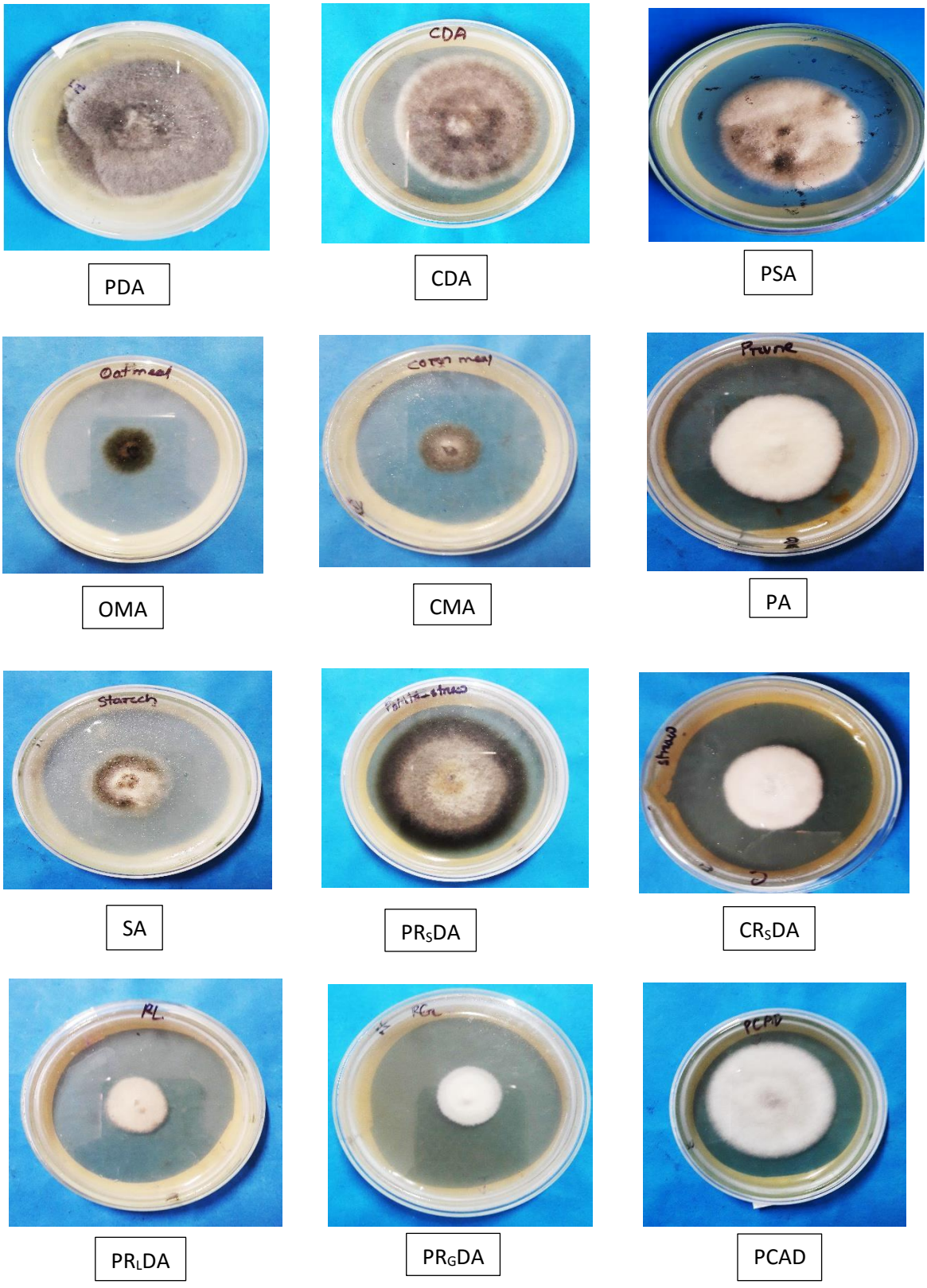
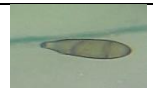


Figure 12. Mycelial growth of *Magnaporthe oryzae* on different selected solid media at 5-25 DAI.

Table 4. Comparative analysis of different selected solid media for *M. oryzae*

Media	Colour	Texture	Colony form	Margin	Elevation	Mycelial growth*	Spore (within 40 DAI)
PDA	Greyish black	Cottony	Irregular	Undulated	Raised middle	Very good	Not found
CDA	Greyish black	Cottony	Circular	Entire	Raised middle	Very good	Not found
PSA	Greyish black	Cottony	Circular	Undulated	Raised middle	Very good	
OMA	Black	Velvety	Circular	Entire	Flat	Poor	Not found
CMA	Black	Velvety	Circular	Entire	Flat	Poor	Not found
PA	White	Cottony	Circular	Entire	Raised middle	Moderate	Not found
SA	Black	Velvety	Circular	Entire	Flat	Very good	Not found
PR_SDA	Greyish black	Velvety	Circular	Entire	Flat	Moderate	
CR_SDA	White	Cottony	Circular	Entire	Raised middle	Poor	Not found
PR_LDA	White	Velvety	Irregular	Entire	Flat	Moderate	Not found
PR_GDA	White	Velvety	Circular	Entire	Flat	Poor	Not found
PCAD	White	Cottony	Circular	Entire	Raised middle	Very good	Not found

* mycelial growth (mentioned in section 3.2.5)

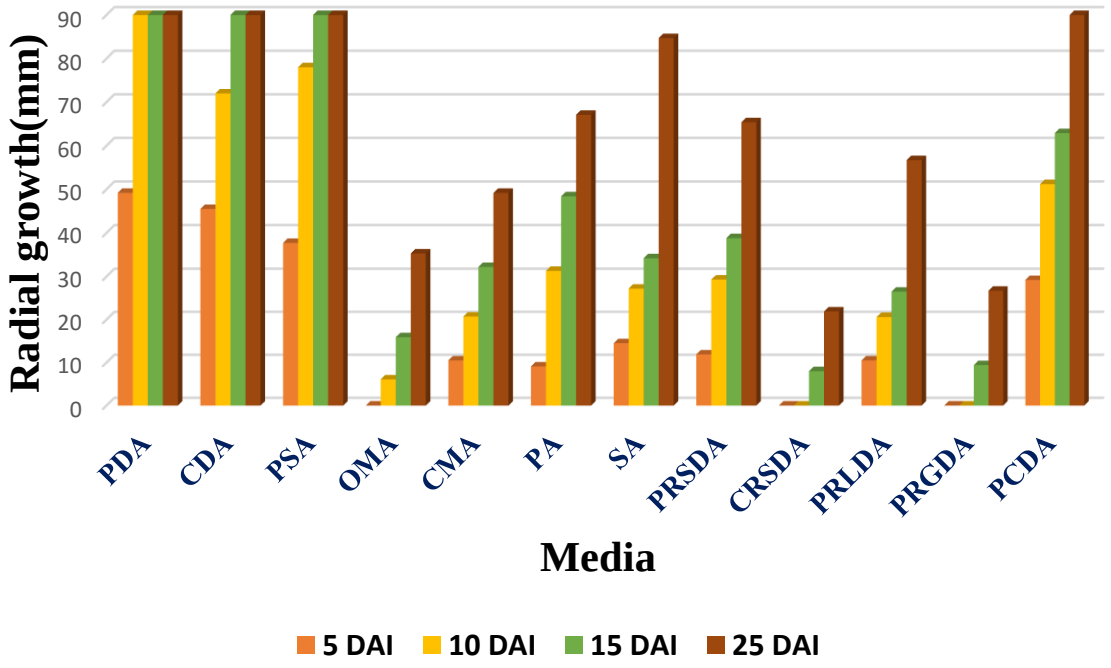


Figure 13. Comparative range of mycelial growth within 12 selected solid media from 5 to 25 DAI

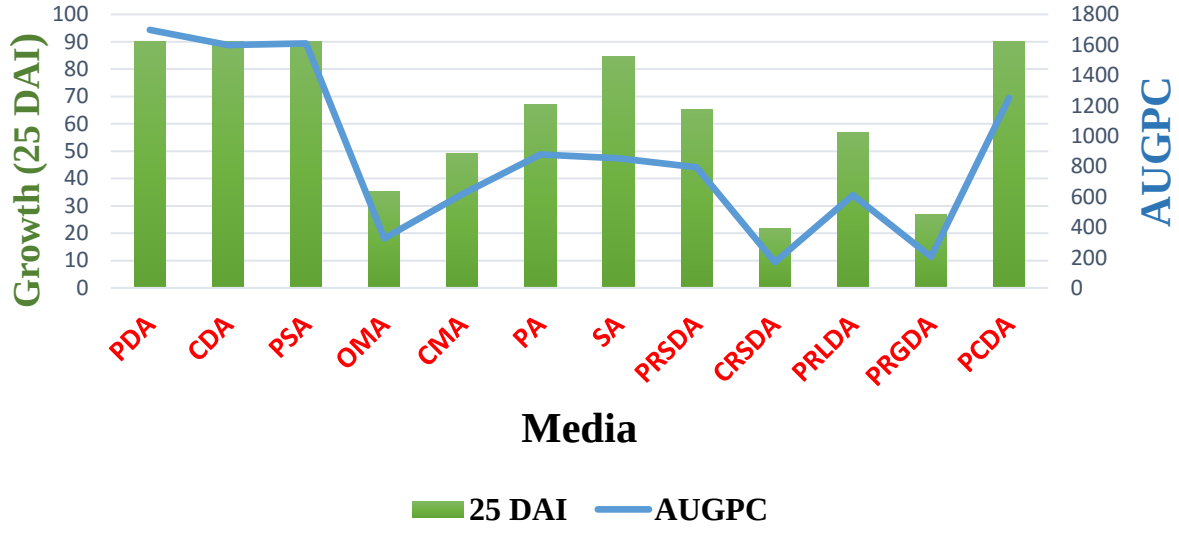


Figure 14. Mycelial growth and AUGPC analysis for different solid media in *M. oryzae* at 25 DAI

4.4. Pathogenicity test for blast pathogen

In order to prove the pathogenic nature of *M. oryzae* producing blast disease, Koch's postulates test was conducted on leaves of the rice plants by detached leaf technique (section 3.4). After 3/4 days of inoculation, small spot started to form. Initially the spots were very small brown color like pinpoint along the leaf margins then turned into yellow, round to oval. later stage the spots became spindle shaped enlarged having ash center. The spot was similar in appearance with the rice blast spots found in the field. The leaf sample with developed spots were placed in PDA media and kept in aseptic condition. It developed greyish to black mycelial growth and 3 celled *Magnaporthe* conidia was observed under the microscope. So, the re-isolated pathogen from the developed spots in the net house was producing similar type of growth and spore characteristics which was found in first isolation of the pathogen *in vitro*. In this way Koch's postulate was established.

Figure 15. Visible blast symptom on infected rice plant

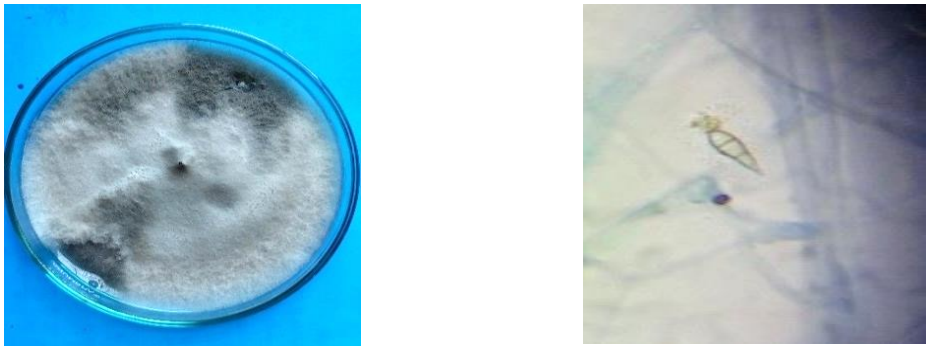


Figure 16. Re-isolated *Magnaporthe oryzae* fungal mycelia and spore

4.5. Evaluation of four different fungicides against *Magnaporthe oryzae in vitro*

The mycelial growth of the pathogen against fungicide treated media and control media were being observed and their mycelial growth was recorded. The percent inhibition of mycelial growth was calculated using the formula mentioned in section 3.4. No growth was observed for Amister top 325 SC and Blastin 75 WDG even after 15 days of incubation. It was found that Amister top 325 SC and Blastin 75 WDG gave 100% mycelial inhibition capacity over control. Acibean 28 SC and Deconil 500 SC inhibited 87.67% and 84.74% over the control respectively (Table 5 & Figure 16).

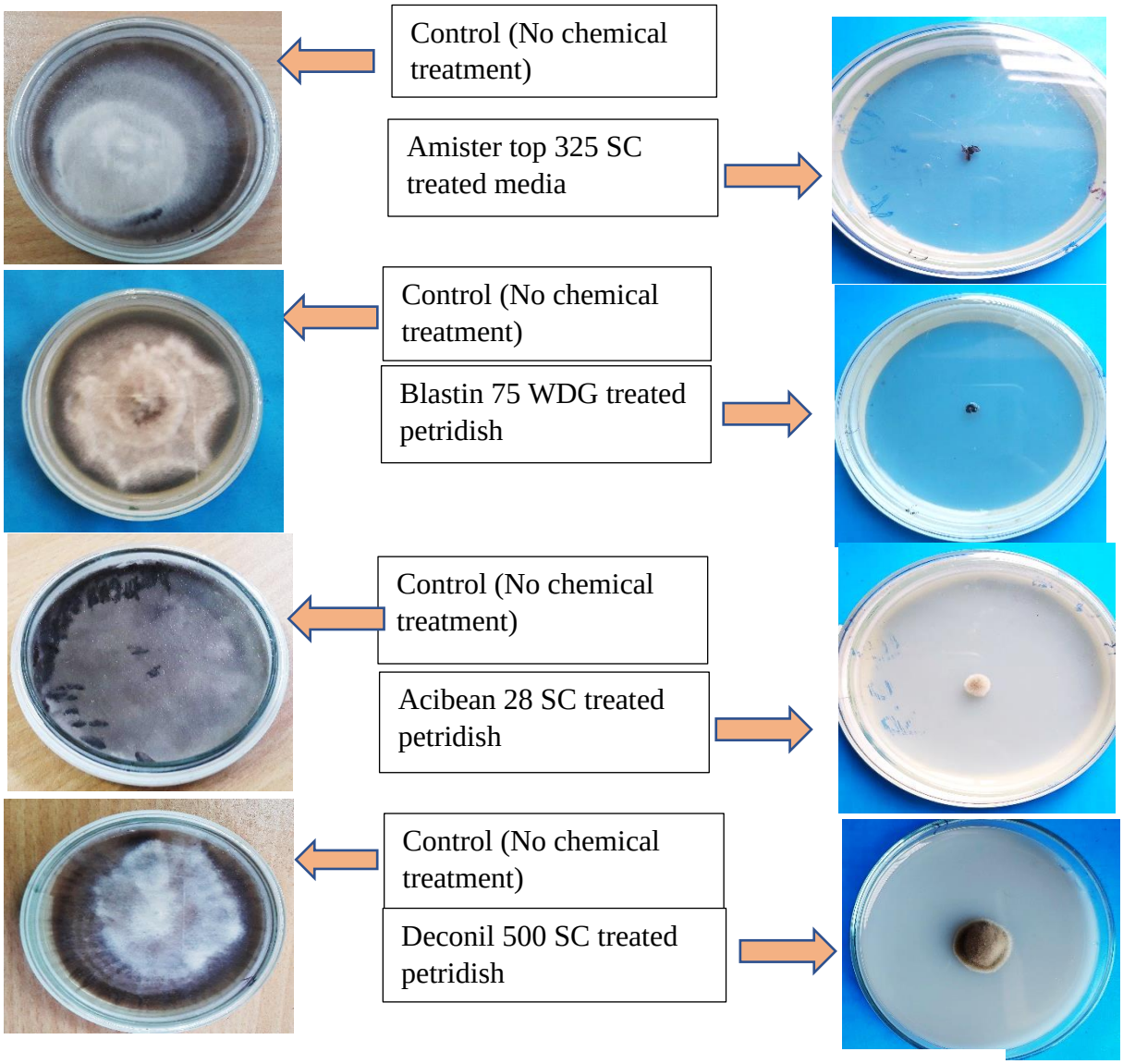


Figure 17. Comparison between control and fungicide treated plates

Table 5. Effect of Fungicide on mycelial growth of *Magnaporthe oryzae in-vitro* (% inhibition over control)

Name of the chemicals	5 DAI			10 DAI			15 DAI			Average inhibiti-on (at 15 DAI)
	500 ppm	1000 ppm	1500 ppm	500 ppm	1000 ppm	1500 ppm	500 ppm	1000 ppm	1500 ppm	
T1 (Amister top 325 SC)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	T1=100 %
T2 (Blastin 75 WDG)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	0.0 (100)	T2=100 %
T3(Acibean 28 SC)	7.5 (83.58)	7.1 (84.46)	5.7 (87.52)	11.2 (87.55)	9.4 (89.56)	7.2 (92)	13 (85.56)	11.5 (87.22)	8.8 (90.22)	T3=87.67%
T4(Deconil 500 SC)	11.9 (73.96)	10.3 (77.46)	9.9 (78.33)	15.6 (82.67)	11.1 (87.67)	11.0 (87.78)	20.0 (77.78)	12.0 (86.66)	11.9 (86.78)	T4=84.74%
Control (No chemical)	45.7	45.7	45.7	90.0	90.0	90.0	90.0	90.0	90.0	
CV (%)	11.13	2.60	5.67	3.60	3.27	5.28	5.08	1.68	2.56	
LSD (0.05)	2.39	1.11	2.62	0.86	1.36	2.27	1.17	0.67	1.05	

N.B. ( ) indicates % inhibition over control

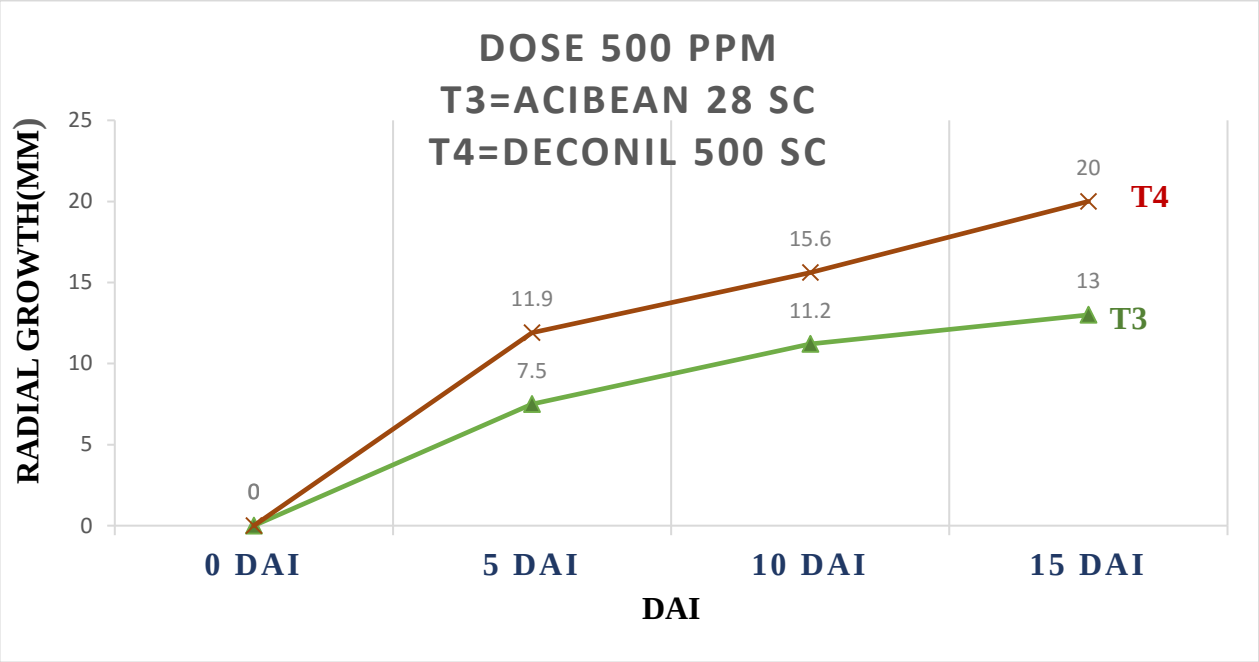


Figure 18. Mycelial growth for 500 ppm at 5,10 and 15 DAI

This figure shows that T3 and T4 gave mycelial growth at 500 ppm concentration. With the increase of time mycelial growth was also increasing.

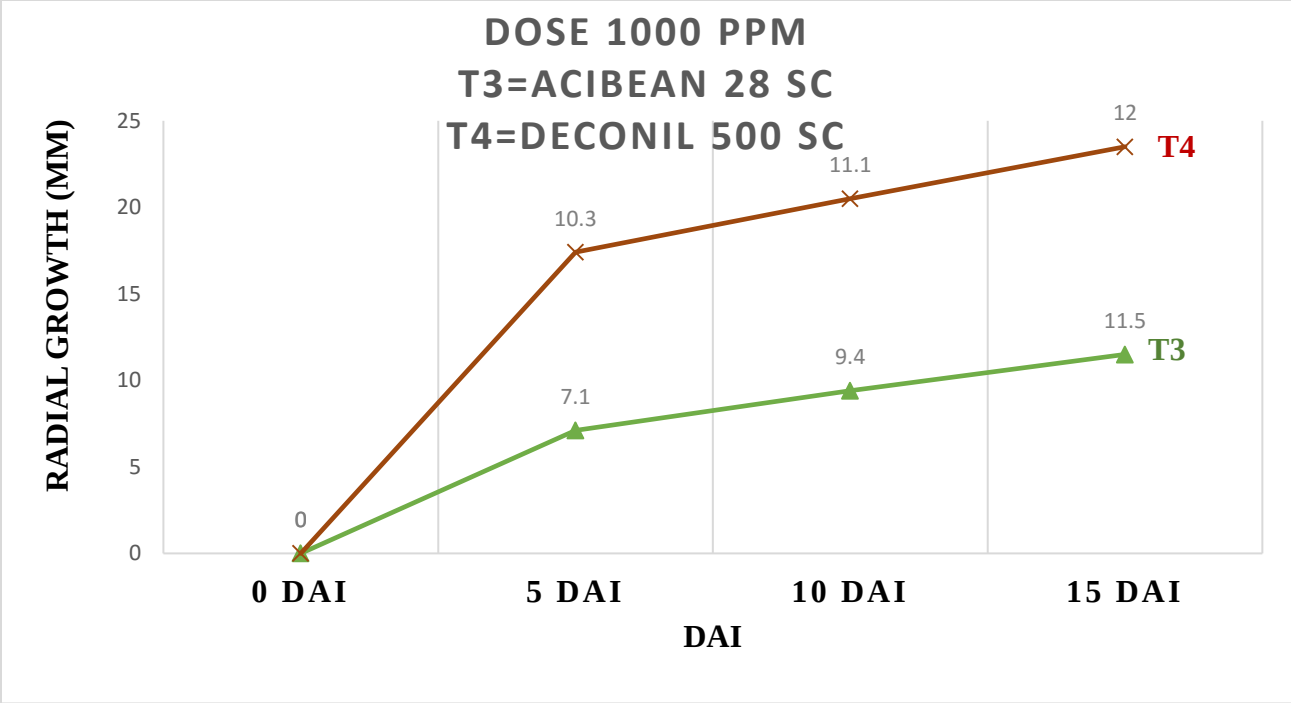


Figure 19. Mycelial growth for 1000 ppm at 5,10 and 15 DAI

This figure also shows that T3 and T4 gave mycelial growth at 1000 ppm concentration. With the increase of time mycelial growth was also increasing.

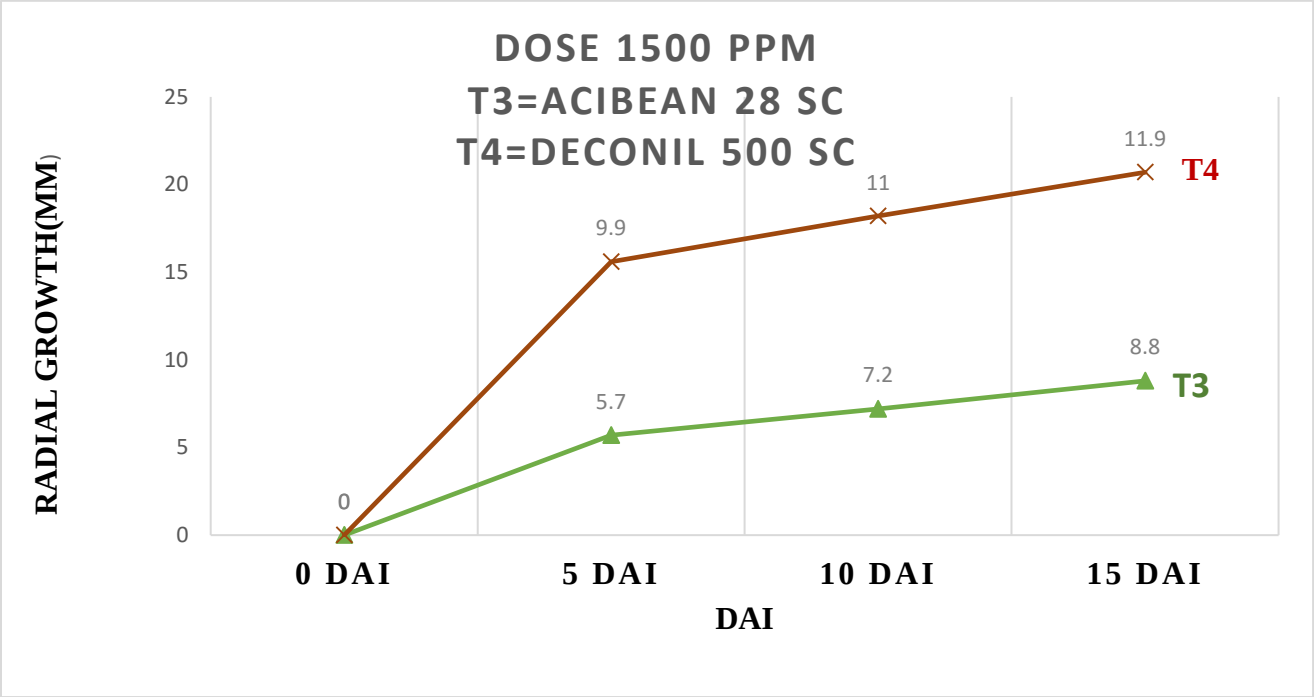


Figure 20. Mycelial growth for 1500 ppm at 5,10 and 15 DAI

This figure also shows that T3 and T4 gave mycelial growth at 1500 ppm concentration. With the increase of time mycelial growth was also increasing. But in this figure growth rate of mycelia is much lower than initial 500 ppm and 1000 ppm concentration. That means 1500 ppm concentration is better for these chemicals.

b. Discussion

Rice (*Oryza sativa*) plays a significant role in achieving global food security. However, it suffers from several biotic and abiotic stresses that seriously affect its production. Rice blast caused by hemi-biotrophic fungal pathogen *Magnaporthe oryzae* is one of the most widespread and devastating diseases of rice. The crop rice is vulnerable to this pathogen from growing to adult plant stages affecting leaves, nodes, panicles and roots. Rather this, *Magnaporthe oryzae* is a non-predictable pathogen. It can change its genetic makeup easily to adapt in any adverse condition (Chadha,2014). So, to understand the overall character of that pathogen it's more and more studies are needed. For this purpose, an effective and comparatively cheap media can be helpful for the researchers. At field level chemical control is mainly practiced for blast disease management because other management options are mostly tricky to practice. So far, no resistant variety of rice identified against blast and no specific treatment to control this disease. To analyze and control blast disease suitable media for its mycelial growth and sporulation must be identified and a suitable chemical and cultural measures need to integrated. In this study emphasizes are given to isolation and morphology of *Magnaporthe oryzae* cause blast disease of rice in terms of mycelial growth and sporulation, evaluation of effective and cheap media for *Magnaporthe oryzae* and it's *in vitro* management by using latest phytochemicals.

5.1 Isolation and morphological study of *Magnaporthe oryzae*

For sampling, rice blast infected plant parts were collected from Mymensingh and Thakurgaon districts randomly. Blast infected leaves and panicles were collected for isolation of the pathogen. The pathogen was first isolated carefully at PDA media. The mycelial growth of the isolated pathogen *Magnaporthe oryzae* was greyish to black in color. The Isolate that was from Mymensingh ($M_{rs}28$) had characteristic cottony greyish black colony with raised middle mycelial growth. On the other hand, Thakurgaon's Isolate ($T_{rs}28$) had black, velvety and flat mycelial growth. The pathogen was grown in PR_SDA media for sporulation purpose. Conidiophores were either single or in fascicles, simple, rarely branched, showing sympodial growth. Conidia were pyriform (pear-shaped with pointed tip), narrowed toward tip, rounded at the base, 2 septate, hyaline in color with a

distinct basal hilum. The size of conidia was measured about 22.13 - 28.47 µm × 9.13 - 11.72 µm (average 25.30 × 10.43 µm).

Bonman *et al.,* (1992) conducted a research on *Magnaporthe oryzae*. For isolation of *Magnaporthe oryzae,* rice blast infected leaves were collected and each lesion was placed in a moist petri dish and incubated at 25ºC for isolation purpose. PDA was used for isolation of *Magnaporthe grisea* by Motlagh and Javadzadeh (2010) and Priya Vanaraj *et al*., (2013). Leong (2004), Mukund Variar *et al*., (2006) and Mebratu *et al.,* (2015) observed *Magnaporthe* culture black to olive gray color. Kulmitra *et al.,* (2017) observed growth of *M. oryzae* in different solid, semi solid and liquid media. The colony color varied from grayish black to dark black color, smooth to irregular margin, medium to good growth of the pathogen was observed. Meena (2005) also demonstrated the grayish black and raised mycelial growth of the blast isolates. Sun *et al*., (1989) reported that rice straw agar media is most supporting medium for sporulation of *M. oryzae* and observe that the spore measurements were 15 – 22 µm× 4 – 7 µm (Average, 17.4 µm × 5.2 µm). Mijan Hossain (2000) also observed *Magnaporthe* cultures and reported that, mycelia were first hyaline in color, then changed to olivaceous. It was observed that mostly 2 celled conidia were found from rice grain media and 3 celled conidia were found in infected leaf sample.

5.2 Evaluation of effective solid media for *Magnaporthe oryzae* culture

The *Magnaporthe oryzae* pathogen was grown in 12 different selected solid media and the growth was noted at 5,10,15 and 25 days after inoculation (DAI). AUGPC was also calculated. It was found that the highest growth of 49.2 mm and 90 mm was recorded in Potato dextrose agar (PDA) medium at 5 and 10 DAI respectively. In PDA media *Magnaporthe* gave greyish black mycelia with cottony structure. The highest AUGPC was calculated 1698.0 in PDA media. Conidia was not observed within 60 DAI in PDA media. The CDA media gave 45.5 mm and 72.0 mm mycelial growth at 5 and 10 DAI respectively, which is second best for mycelial growth but no sporulation was observed. In PSA, mycelial growth was lower in 5 and 10 DAI than PDA media. PSA gave 37.7 mm radial mycelial growth at 5 DAI which is lower than CDA, but at 10 DAI it was 78 mm which was higher than CDA. In PSA media, mycelial growth was good and few conidia were observed after 45 DAI. On the other hand, PR$_S$DA was used for both mycelial growth and

sporulation. At 25 DAI its radial mycelial growth was 65.4 mm. This media gave profound spore after 35 DAI. SA and PA media had average result as cheap media. SA needs very small amount of agar (50% of PDA) as starch has natural capability to solidify and no dextrose was added (mentioned in 3.3.1.7). OMA, CR_SDA and PR_GDA did not give satisfactory result. OMA gave visible mycelia at 10 DAI, where CR_SDA and PR_GDA initiated mycelia at 15 DAI. Whereas at 20 days after inoculation the lowest growth was achieved in CR_SDA medium (21.9mm). And the lowest AUGPC from CR_SDA which was 169.5. So, PDA, CDA and PSA are good for mycelia but for sporulation PR_SDA and PSA is preferable.

This result is in agreement with Ravindramalviya (2014) who studied that PDA media supported maximum mycelial growth of *M. grisea* after 168 hr of incubation. Sun *et al*., (1989) reported that rice straw agar media is most supporting medium for sporulation of *M. oryzae*. This result is in accordance with the findings of Mahdieh S. (2013) who reported that PDA culture medium could provide the best medium for *Magnaporthe* mycelia than Potato carrot agar and rice potato extract agar. Vanaraj *et al*., (2013) found almost similar result by using 5 different media. The growth of *M. oryzae* was rapid on PDA which significantly differed from others but not found any sporulation even after 60 days of culture maintenance in proper conditions.

5.3 Pathogenicity test

The pathogen was isolated from the infected leaf sample from the field in PDA and PR_SDA. The isolated pathogen was inoculated in the healthy plants. Same type of symptom was noticed. It was re-isolated again in the same media and the mycelial growth and spore characters were similar to the earlier growth in the media. In this way Koch's postulates was established.

Pathogenicity test was conducted for *Magnaporthe oryzae* by Perello *et al.*, (2015). Artificially inoculated Plants were incubated in a moist chamber at 22°C and 100% RH for 48 hours. Inoculated plants gave symptoms which were similar to those observed in the field. Dutta (2017) was conducted koch’s postulates for *Magnaporthe* for the conformity of the Pathogen, and found the exact fungus and symptoms from artificially inoculated plants that was found in natural condition.

5.4 Evaluation of different chemicals against *Magnaporthe oryzae*

The isolates were treated with four latest common fungicides by pour plate technique *in vitro* and the result showed that Amister top 325 SC (Azoxystrobin+ Difeconazole) and Blastin 75 WDG (Trifloxystrobin+Tebuconazole) hinder 100% mycelial growth of *Magnaporthe oryzae* isolate while Acibean 28 SC (Azoxystrobin+ Cyproconazole) and Deconil 500 SC (Chlorothalonil) had less inhibition on mycelial growth of *Magnaporthe oryzae* isolates than Amister top 325 SC and Blastin 75 WDG. The percent inhibition of Acibean 28 SC and Deconil 500 SC were 87.67% and 84.74% respectively. From this study is was concluded that Amister top 325 SC and Blatin 75 WDG has best result against *Magnaporthe oryzae*. Among the three doses 1500 ppm gave satisfactory result for the four fungicides than other two 500 ppm and 1000 ppm.

Hossain and Kulkarni (2001) evaluated the efficacy of fungicides; Azoxystrobin, carbendazim, tebuconazole, mancozeb and chlorothalonil tested against *Magnaporthe oryzae*, all other fungicides showed significant effect to inhibit mycelial growth at even lower concentrations. Hajano *et al*., (2012) stated in their study that lantadenes and chlorothalonil are inactive on *Magnaporthe oryzae* which is apparently the active ingredient of Deconil 50 SC. The present finding was in agreement with Kulmitra *et al*. (2015), who found that under *in vitro* conditions, Tricyclazole, Azoxystrobin+ Difenoconazole and Azoxystrobin were the most effective fungicides against *M. oryzae*. This result is also agreement with Chander Mohan *et al.*, (2013) who found that under *in vitro* conditions Amistar top (azoxystrobin+ difenoconazole), Score (difenconazole) and Folicur (tebuconazole) were significantly effective in growth inhibition of *Magnaporthe oryzae*. Tebuconazole is active ingredient of Blastin. Singh *et al*., (2014) also give similar type of results that tebuconazole was most effective as it completely inhibited the colony growth of *M. oryzae* at very low concentration whereas azoxystrobin + difenoconazole, propiconazole and difeconazole completely inhibited the colony growth of the pathogen. This results also agreed with Kunova *et al.,* (2013) who observed that *Magnaporthe grisea* mycelium growth was inhibited at low concentrations of Azoxystrobin and sporulation was sensitive to the fungicides and was affected at low dose.

Summary and Conclusion

The present investigation was carried out in the laboratory in aseptic condition. An *in vitro* study was done for isolation, identification, characterization and pathogenicity test for *Magnaporthe oryzae* derived from the diseased samples. From the study it can be said that, the Pathogen *Magnaporthe oryzae* could be effectively grown in different media and blast pathogen of rice could be controlled by chemical management. The findings are summarized as follows:

The *Magnaporthe oryzae* infected leaf and panicle sample which were showing typical blast symptom were collected from highly infected rice field from two selected districts (Mymensingh and Thakurgaon). The pathogen was isolated successfully from the infected sample in PDA media and PR_SDA media. Characteristic mycelia and conidia were observed in isolated pathogen. The isolated pathogen was inoculated in the healthy seedlings with preferable condition for disease development and exactly same type of symptom was noticed which was seen earlier. It was placed for re-isolation purpose again in the PDA and PR_SDA media and the growth was similar to the earlier pathogen's colony growth in the same media with conidia.

The cultures of the isolated pathogen *Magnaporthe oryzae* were greyish to black in color. Conidiophores were either single or in fascicles, rarely branched. The conidia were pyriform (pear-shaped) and had 2 septa. The spores almost hyaline and had a characteristic basal appendage named hilum.

The pathogen was grown in 12 different selected solid media for observing their growth characters. The pathogen was good at PDA (90mm) within 10^{th} days, CDA (90mm) within 15 days and PSA (90mm) media within 15 days to cover the entire Petri dish. For mycelial growth PDA is very good for its very fast mycelial development. But for sporulation purpose PR_SDA is better than PDA as PDA has very rare sporulation capacity in spite of having suitable condition. Good results were also found in PA, SA media. PA and SA media can be a good alternative media for *Magnaporthe* as its preparation is very easy and cheap than PDA media because SA needs very small amount of agar (50% of PDA) as

starch has natural capability to solidify and no dextrose was added. All the media patronize the growth of *Magnaporthe oryzae.*

Under *in-vitro* condition, all the four available latest fungicides tested showed growth inhibition over control. Isolate was treated against four fungicides for blast treatment by pour plate technique. The result showed 100% mycelial inhibition of *Magnaporthe oryzae* for Amister top 325 SC and Blastin 75 WDG while Acibean 28 SC and Deconil 500 SC had 86.67% and 84.74% inhibition on mycelial growth of *Magnaporthe oryzae* isolates. So, this experiment resulted that two most available chemicals Amister top 325 SC and Blastin 75 WDG can be the suitable option for controlling rice blast pathogen. The best dose could be 1500 ppm. However, the selected fungicides need to be explored in field condition against blast disease of rice for final recommendation.

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