PREVALENCE OF SEED BORNE PATHOGENS AND THEIR EFFECT ON SEEDLINGS OF SELECTED IMPORTED HYBRID RICE VARIETIES

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CERTIFICATE

This is to certify that thesis entitled, ***"PREVALENCE OF SEED BORNE PATHOGENS AND THEIR EFFECT ON SEEDLINGS OF SELECTED IMPORTED HYBRID RICE VARIETIES"*** submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of ***MASTER OF SCIENCE*** *in* ***PLANT PATHOLOGY,*** embodies the result of a piece of bona fide research work carried out by **NARGIS ISLAM RONI** bearing Registration No. **06-02097** 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 been duly been acknowledged by her.

……..
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Dated: 31st December, 2013
Place: Dhaka, Bangladesh

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ABSTRACT

Experiments were conducted to evaluate the effect of seed borne pathogens of selected imported hybrid rice varieties on seed germination and seedling vigor during the period of January to December, 2012 at Department of Plant Pathology, Sher-e-Bangla Agricultural

University, Dhaka, Bangladesh. Five fungal species and two bacterial strains of a bacterium were isolated and identified from selected imported hybrid rice varieties namely Tia, Moyna and Richer. Seed borne fungal genera *Aspergillus*, *Fusarium*, *Bipolaris* and *Alternaria* were isolated and identified from blotter method. Most frequently isolated fungi were *Aspergillus flavus* (12.90% from Richer, 11.95% from Moyna and 10.40% from Tia), *Aspergillus niger* (9.55% from Richer, 8.15% from Tia, 6.52% from Moyna), *Bipolaris oryzae* (13.45% from Moyna, 10.15% from Tia, 9.52% from Richer), *Fusarium moniliforme* (2.2% from Richer, 1.57% from Tia) and *Alternaria* spp. (2.17% only found in Moyna). Seed borne bacteria *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola* were highest in Moyna (15.25%) and Richer (5.37%), respectively and minimum number in rice variety Tia 12.25% and in Moyna 3.62%, respectively. *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola* were identified by pathogenicity test and growth on differential media. In seedling symptoms test by water agar test tube method and seedling vigor index test by rolled paper towel method, rice variety Tia showed good performance in terms of minimum number of diseased seedlings (6.36%) and dead seed (5.57%) and maximum germination (96%) and vigor index (2260).

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LIST OF SYMBOLS AND ABBREVIATIONS

%	=	Percentage
et al.	=	And others
spp.	=	Species
J.	=	Journal
viz.	=	Namely
&	=	And
etc	=	Etcetera
^{o}C	=	Degree Celsius

cm	=	Centimeter
cfu	=	Colony forming unit
NaCl	=	Sodium chloride
Kg	=	Kilogram
g	=	Gram
ml	=	Mililiter
hrs	=	Hours
pv.	=	Pathovars
i.e.	=	That is
SAU	=	Sher-e-Bangla Agricultural University
BAU	=	Bangladesh Agricultural University
BBS	=	Bangladesh Bureau of Statistics
NA	=	Nutrient Agar
PDA	=	Potato Dextrose Agar
YDCA	=	Yeast Dextrose Calcium Carbonate Agar
LSD	=	Least Significant Difference
CV%	=	Percentage of Co-eficient of Variance
IRRI	=	International Rice Research Institute
BLB	=	Bacterial leaf blight
BLS	=	Bacterial leaf streak
cv.	=	Cultivar(s)
DAS	=	Day after sowing
Min	=	Minute(s)
PSI	=	Per square inch
WATT	=	Water agar test tube (method)
RPT	=	Rolled paper towel (method)
SDW	=	Sterilized distilled water

CHAPTER 1
INTRODUCTION

Rice (*Oryza sativa* L) is a self pollinated cereal crops under the family of Gramineae. It is one of the world's primary cereal crops used as staple food by 60 % of the world population and grows in more than 100 countries. It is also the most important cereal crops in Asia producing about 96 % of the world rice production (IRRI, 2006). It also used as the staple food crop of people of Bangladesh which covers 92 % of food grain production. About 84.50 % of cropped area of Bangladesh is used for rice production (BBS, 2012). In Bangladesh most of the hybrid rice seeds are imported from china. High quality seed is not only important for increased crop production but also for proper establishment of sound seed industry in the country. Among the important characteristics of seed quality, purity, germination, high yielding potentiality and seed health quality are of major importance. Of these major characteristics of seed quality health is immensely important. Seed health refers to whether a seed or a seed lot is infected by pathogens or not. Infected seeds fail to germinate and the pathogens from infected seeds may be transmitted to seedlings and growing plants in field causes disease. Therefore it is important to know whether a seed lot is free from seed-borne infection of pathogen(s) or the lot contains pathogen(s) with its maximum acceptable limit. Pathogen free seed is the vital input in agriculture. The average yield of rice in this country is low compared to other countries due to seed borne diseases. In Bangladesh, approximately 2.5 million tons of rice worth more than TK. 12 thousand million is lost due to seed borne pathogens (Fakir *et al*., 2003). Without improving the quality of seed, the improved technology can hardly improve the production potentially. Normally farmers do not test the quality and health status of rice seed, but so many devastating diseases can be carried by seed and there is a great possibility to remain pathogen within the seed. There are many causes of low yield of rice in Bangladesh of which disease and pest plays a major role (Fakir, 1982). Among them seed borne diseases are more destructive. Rice seeds are known to harbor a wide range of both fungi and bacteria (Neergaard, 1977).

Seed borne diseases create a great threat to the production of crops in Bangladesh. Rice suffers from more than 60 different diseases. In Bangladesh, 43 diseases are known to occur on the rice crop. Among these diseases, 27 are seed borne of which 14 are of major importance. The infected seeds may fail to germinate, transmit disease from seed to seedling and from seedling to growing plants (Fakir *et al.*, 2002). Infected seeds germinate poorly and could be a major source of inoculums for new crops raised from

them. For example, most pathogens causing abnormal seedling of rice are seed borne (Guerrero *et al.,* 1972). Seed borne pathogens affect seed quality (Khare, 1999). Islam *et al*. (2000) observed that highest lethal seed infection caused by *Fusarium moniliforme, Trichoconis padwickii* and *Curvularia* spp. About 20 species of fungal pathogens were detected from rice seed at any one time (Mew and Gonzales, 2002). The crop is affected by as many as 36 seed-borne diseases of which 31 were caused by fungi (Ou, 1985). Totally 8 genera of fungi viz., *Alternaria, Aspergillus, Bipolaris, Chaetomium, Curvularia, Fusarium, Sarocladium* and *Trichoderma* comprising twelve species were found to be associated with the seed samples. Among them, the most predominant one was *Bipolaris oryzae* (Gopalakrishnan and Valluvaparidasan, 2010).

Among seed borne diseases of rice 6 are bacterial. BLB under mild infection causes yield reduction ranging from 10-12% (Mew *et al*., 1993) whereas under severe condition, it can be as high as 50% (Ou, 1985). The extremely seed borne bacteria are *Xanthomonas oryzae* pv. *oryzae, Xanthomonas oryzae* pv. *oryzicola, Pseudomonas, Acedovorax* etc (Agarwal *et al*, 1990). Bacterial leaf blight of rice (BLB) was first reported in Fukuoka Prefecture, Japan, during 1884 in rice affected by *X. oryzae* pv. *oryzae*. Bacterial leaf streak of rice (BLS) is caused by *X. oryzae* pv. *oryzicola*. *Xanthomonas oryzae* pv. *oryzicola* has reached epidemic proportions in recent years in China. Rice seed play an important role to carry pathogen in quarantine aspect. Farmers generally use different hybrid rice varieties and face the difficulties of many diseases. In the last few years the cultivation of imported hybrid rice in Bangladesh increased rapidly. Recently bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*), bacterial leaf streak (*Xanthomonas oryzae* pv. *oryzicola*) disease appeared seriously in the boro (cultivation period: December-February) rice. As the pathogens of BLB and BLS are seed borne, there is a chance to transmit new race of the pathogen in the country by imported hybrid rice seed. So, assessment of the seed health standard of imported hybrid rice is very important for farmer and food security. Seed is common carrier of plant pathogens. It carries several destructive pathogens that often take heavy toll causing diseases of crops raised from them. Seed borne diseases are very important from the following points of view; i. introduction of new pathogens (ii) quantitative and qualitative crop losses and (iii) permanent contamination of soil (Anselme, 1981).

Considering the above facts the present experiment were undertaken with hybrid rice varieties collected from the seed importer and local market of the country with the following objectives:

i) To identify different seed borne pathogens and their incidence on selected imported hybrid rice varieties in Bangladesh and

ii) To determine the effect of seed borne pathogens on germination and seedling of selected imported hybrid rice varieties in Bangladesh

CHAPTER 2
REVIEW OF LITERATUR

Fungi associated with rice seed

Archana and Prakash (2013) performed a survey and a total of 69 rice seed samples obtained from different states of India. Totally sixteen genera of fungi *viz*. *Acremonium*, *Alternaria*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Exserohilum*, *Fusarium*, *Microdochium*, *Nigrospora*, *Phoma*, *Pyricularia*, *Rhizoctonia*, *Rhizopus* and *Verticillium* comprising 27 species were found to be associated with the rice seed samples. Among them the most predominant was *Bipolaris oryzae* which is associated

with 82.08% seed samples, followed by *Alternaria padwickii* (63.36%). A least incidence of 4.32% was observed with *Bipolaris halodes* and *Acremonium* spp.

Mansur *et al*. (2013) conducted experiment to detect the fungi associate with the seed samples and to record the germination of seed samples of Parshuram upazila of Feni district. Three rice varieties are collected for the studies were BR6, Pajam and Joya (Local) from Parshuram upazila of Feni district to determine the seed health and quality. The germination of rice seeds of the variety BR6 was 54.67%, while the varieties Joya and Pajam showed 58.00% germination respectively. Nine seed-borne fungi were detected from these seed samples. The identified fungi were *Fusarium oxysporum, F. moniliforme, Bipolaris oryzae, Alternaria padwickii, Curvularia lunata, Aspergillus flavus, Aspergillus niger, Penicillium* sp. and *Nigrospora oryzae*.

Islam and Borthakur (2012) conducted experiment to evaluate the effect of some dominant seed borne fungi of *Aijung* rice variety on seed germination and seedling vigour. Twenty dominant fungi were found associated with *Aijung* rice seeds. Analysis of seed borne fungi by blotter method and agar plate method showed that species of *Aspergillus, Fusarium, Alternaria* and *Curvularia* are the dominant genera. Seed germination and seedling vigour tests were conducted using seed inoculation, soil inoculation and seed submergence method. Maximum reduction in seed germination and seedling vigour was caused by species of *Fusarium* in seed inoculation method, by species of *Rhizopus* and *Fusarium* in soil inoculation method and by species of *Aspergillus* in seed submergence method. In another experiment healthy rice seeds were soaked in 25, 50, 75 and 100% concentration of 7, 14 and 21 days old culture filtrates of the isolated seed borne fungi. Maximum reduction in seed germination was recorded from 21 days old culture filtrates. The inhibitory effect on seed germination was found to decrease with increase in dilution of the filtrates.

Islam *et al*. (2012) examined ten rice cultivars grown in non saline tidal zones of Patuakhali district were examined to identify seed-borne fungi and their effect on germination. The observed fungi were *Trichoconis padwickii, Curvularia lunata, Fusarium moniliforme, Bipolaris oryzae, Aspergillus flavus, Rhizopus* sp., *Aspergillus clavatus, Aspergillus niger* and *Chaetomium* sp. Among the fungi detected, *Trichoconis padwickii* and *Aspergillus flavus* were most predominant. Seed germination is decreased with increased the seed infection regardless of the rice cultivars tested. High negative significant correlation was obtained between all isolated fungi and seed germination in

the laboratory for all seed samples tested, Aus rice cultivars (r = -0. 97) and Aman rice cultivars (r = -0.90).

Butt *et al*. (2011) studied seed borne mycoflora of different stored grain of rice varieties by using blotter method and its chemical control they reporeted varieties of rice (*Oryzae sativa* L.) viz. KS-282, Basmati-385, Basmati-370, Basmati Kernal and Basmati-198 were investigated the occurrence of seed-borne mycoflora using blotter paper method and 27%, 19%, 17%, 16% and 14% mycoflora was found associated with the seeds of Basmati kernel, Basmati-385, Basmati- 370, Basmati-198 and KS-282, respectively. Four fungal species namely *Fusarium moniliforme*, *Alternaria* sp., *Helminthosporium* sp. and *Curvularia* sp. were isolated from different test rice varieties.

Utobo *et al*. (2011) studied seed borne fungi associated with eight hybrid (H) and three local check (LC) rice varieties and their effects on grain germination and seedling vigour during the 2007 and 2008 harvesting seasons. A total of 9 fungal genera were isolated and identified from the seed samples. Most frequently isolated fungi were *Trichoconis padwickii, Helminthosporium oryzae* and *Fusarium moniliforme* for hybrid and local check rice varieties respectively. Percentage of germination and seedling vigour were found significant ($p<0.05$) from hybrid to local check rice varieties. Maximum numbers of germinated seed at 5, 9 and 14 DAS were recorded from seed samples of hybrid rice varieties and minimum numbers of germinated seeds at 5, 9 and 14 DAS were observed from that of local check rice varieties. Hybrid rice showed higher vigour in terms of germination, root length, root weight, shoot length, root weight and vigor index when compared to local check rice varieties.

Gopalakrishnan *et al*. (2010) conducted an experiment to identify the seed borne pathogen associated with rice seed and recorded 8 genera of fungi viz. *Alternaria, Aspergillus, Bipolaris, Chaetomium, Curvularia, Fusarium, Sarocladium* and *Trichoderma* comprising twelve species. Among them, the most predominant one was *Bipolaris oryzae* which was associated with 58.89 percent seed samples followed by *Alternaria padwickii* (52.96%).

Ibiam *et al*. (2008) examined seeds of 3 varieties of rice both in storage and in the field reported that *Fusarium moniliforme, Bipolaris oryzae, Fusarium oxysporum, Chaetomium globosum, Curvularia lunata, Aspergillus niger, Aspergillus flavus,*

Aspergillus terreus, *Alternaria tenuis* and *Penicillium* sp. were isolated from seeds of three varieties of rice in storage. *Fusarium moniliforme, Bipolaris oryzae, Fusarium oxysporum, Chaetomium globosum, Curvularia lunata* and *Trichoderma harzianum* were isolated from the seeds of the three varieties from the field. *Fusarium moniliforme* was more prevalent than the other fungi.

Tripathi and Dubey (2004) reported that the most destructive seed-borne fungi of rice are *Bipolaris oryzae*, *Pyricularia oryzae*, *Sarocladium oryzae*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Fusarium* spp., *Curvularia oryzae* and *Nigrospora oryzae*.

Mew and Gonzales (2002) detected more than 100 fungal species on rice seeds. However, the detection frequency varied considerably. About 20 species of fungal pathogens were detected from rice seed at a time.

Javaid *et al. (*2002), Wahid *et al.* (1993 and 2001) and Khan *et al.* (2000) isolated *Alternaria alternata, A. padwickii, A. longissima, Aspergillus niger, Curvularia oryzae, C. lunata, Drchslera oryzae, Fusarium miniliforme, F. semitectum, F. oxysporum, F. soalni*, *Pyricularia oryzae*, and species of *Phoma, Cercospora, Chaetomium, Sclerotium, Pecicillium, Myrothecium* and *Colletotrichum* from seeds of different varieties of rice collected from different regions of the Pakistan.

Fakir *et al.* (2002) determine the quality of farmer's saved rice seeds of Rajshahi, Rangpur aand Bogra in Bangladesh before sowing. Total number of rice 354 seed samples was collected from farmer's storage of three different locations (Bogra, Rajshai and Rangpur) and determind the quality of farmer's saved seeds. Also five important pathogenic fungi viz. *Alternaria padwickii, Fusarium moniliforme, Bipolaris oryzae, Pyricularia oryzae* and *Sarocladium oryzae* were detected in rice seed samples varied in prevalence with respect to season and sites of seed collection.

Naeem Khalid *et al.* (2001) determind the incidence of micro flora, their frequency and impact on germination of four different rice cultivars. They reported five strong fungi viz. *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* spp. *Chaetomium globosum* and *Rhizopus stolonifer* were associated with rice seeds. The associated microflora reduced the seed germination of all the cultivars.

Islam *et al*. (2000) conducted an experiment with the nine seed samples of rice cultivar BR11 collected from farmer's storage and analyzed for *B. oryzae* incidence using blotter method. Incidence of *B. oryzae, Trichoconis padwickii, Curvularia lunata, Aspergillus* spp. and *Penicillium* spp. ranged from 0.0-64%, 16-48%, 1.2 21%, 0.9-19.5% and 0.0-4% respectively. The presence of spotted seeds produced low number of seedlings.

Rahman *et al.* (2000) tested the efficacy of seed cleaning method (manual seed sorting and floatation in water) to improve the seed quality in rice cv. BB11. The seed borne fungi were associated with the treated and untreated seeds were *Bipolaris oryzae, Trichoconis padwickii, Curvularia lunata, Nigrospora oryzae, Alternaria tenuis, Aspergillus* spp. and *Penicillium* spp.

Fakir (2000) reported that rice suffers from more than 60 different diseases. In Bangladesh, 43 diseases are known to occur on the rice. Among these diseases, 27 are seed borne of which 14 are of major importance. He mentioned that major seed-borne diseases were brown spot (*Bipolaris oryzae*), Blast (*Pyricularia oryzae*), sheath rot (*Sarocladium oryzae*), sheath blight (*Rhizoctonia solani*) leaf scaled (*Microdochium oryzae*), seed rot and seedling blight (*Bipolaris oryzae, Sclerotium rolfsii and Fusarium* spp.) and grain spot (*Curvularia lunata, Nigrospora oryzae, Phoma glumarum, Cladosporium* sp.).

Khan *et al*. (1999) isolated varius fungi viz. *Fusarium moniliforme (Gibberella fujikuroi,) F. semitectum (F. pallidoroseum), F. oxysporium, Alternaria alternata, A. padwickii, Curvularia oryzae, C. lunata (Cochlibolus lunata), Dreschslera oryzae (Cochlibolusmiyabeanus) Pyricularia oryzae (Magniporthe grisea), Nigrospora* spp., *Phoma* spp., *Aspergillus* spp. and *Penicillium* spp. from 38 rice samples of 16 different varieties/lines.

Radha jeyalakshmi (1998) reported that totally 18 fungal species belonging to twelve genera were found to be associated with the rice field seed samples in Tamil Nadu.

Sharma and Viad (1997) studied rice samples were collected from Himachal states of India and showed that the extent of grain discoloration varied between 4.35 to 79.82%. 10 fungi fungi viz. *Alternaria alternata, Aspergillus niger, Curvularia oryzae, Curvularia lunata (Cochlibolus smiyabeanus), Tilletia barclayana (Khuskia oryzae), Pestolatia oryzae, Phylostictia glumarum (Phoma sorghina), Penicillium* spp., *Sarocladium oryzae*

(Magnapothe salvinii), were detected. *A. alternata* occurred most commonly followed by *Curvularia lunata.*

Ali and Deka (1996) reported that 10 fungal species from seven fungi *(Curvularia, Dreschslera, Fusarium, Nigrospora, Aspergillus, Penicillium,* and *Chicothcium)* were associated in discoloration grain of 16 rice cultivars. The frequency of occurrence of these fungi varied considerably on different cultivars. The frequency of *Fusarium monilifome* and *Penicillium* were most frequent among the storage fungi after 8-10 months storage.

Ilyas and Javaid (1995) found that out of 46 samples 30 yielded *Fusarium moniliforme (Gibberella fujikuroi),* 45 *Alternaria padwickii,* 7 *Alternaria longissima,* 41 *Dreschslera oryzae,* 2 *Phoma* sp. and 1 each *Curvularia oryzae* and *Cercospora* sp.

Riaz *et al.* (1995) examined 255 accessions of rice seeds and found most of the accessions were contaminated with species of 16 fungal genera. *Alternaria* and *Helminthosporium* spp. occurred most frequently and followed by *Curvularia, Fusarium,* and *Aspergillus* spp.

Mirsa *et al.* (1994) screend 144 seed samples collected from 7 different regions of Philipines during dry and wet season of 1988-89 using standard blotter method. A total of 39 fungal species belonging to 30 genera were isolated. The common species excepting *Pyricularia oryzae* and *Nakatia sigmoideum* were evenly distributed during dry season. During wet season distribution of *Dreschslera* sp. and *Mcrodochium oryzae* was even. Infection of both apparently healthy and discolored seeds was highest with *Alternaria padwickii* followed by *Curvularia* sp.

Sisterna *et al.* (1994) isolated *Fusarium semitectum, F. equiseti, F. graminerum, F. oxysporum, Alternaria* sp., *Bipolaris oryzae, Epicoccum* spp., *Curvularia lunata, C. protuberate* and an unidentified species from 9 rice seed samples with black dots, discoloration, chalky spots and other symptoms from two province in Argentina during 1988-1989.

Bhuiyan *et al*. (1994) detected the incidence of *Pyricularia oryzae* in 28 seed samples and of rice out of 173 samples tested. The highest incidence in individual sample recorded was found in unfilled grains copared to filled grains.

Roy (1993) conducted experiment to determine the rice seed discoloration in Assam, and reported that *Curvularia lunata* was the most common (37%) associated with discolored grains, followed by *Fusarium* sp. (13%) and *Chaetomium* (6%).

Bokhary (1991) reported that the most frequent genera isolated *Curvularia* (5 spp.), *Ulocladium* (5 spp.), *Aspergillus* (4 spp.) *Alternaria* (4 spp.), *Fusarium, Mucor* and *Penicillium* (2 spp.) each. Discolored grain has lower persentage germination than normal grains and had a higher percentage of fungal infection.

Vallejos and Mattos (1990) isolated fungal species from milled rice; most frequently occurred were *Aspergillus candidus, A. versicolor, A. fumigates, A. niger*, *Trichoconis* spp., *Alternaria padwickii, Nigospora oryzae,* and *Penicillium* spp.

Fakir *et al.* (1990) detected seed borne fungal pathogens of rice seed in Bangladesh; this were *Fusarium* spp. *Trichoconis padwickii, Dreschslera oryzae, Phoma* sp. and *Curvularia lunata.* Among these *F. moniliforme* found to be the most common occurring in 58 and 59 seed sample of Pajam and Mala respectively out of 60 samples of each two varieties. As high 55% seed borne infection of the pathogen was detected in Mala. Seed borne infection by *D. oryzae* causing brown spot in rice higher than the normal seed health standard fixed for those pathogens. Average germination of most of the seed samples was below 80.5 which were lower than the national germination standard.

Mian and Fakir (1989) studied on fungi, moisture content and germinability of rough rice grain during storage and observed that the most predominant fungi were *Helminthosporium oryzae, Curvularia lunata, Cladosporium cladosporioides, Aspergillus* spp. and *Trichoconis padwickii.*

Ahmad *et al*. (1989) detected *Fusarium moniliforme, Trichoconis padwickii, Dreschslera oryzae and Curvularia lunata* from rice seed.

Odebunmi and Osikanlu (1989) isolated *Fusarium moniliforme, C. lunata, H. oryzae, Rhynchosporium oryzae* from the six rice seed varieties: IRAT.110, COL.38, C22, TOX494-SLR, DJII-509, and F.H. 109.

Gajapathy and Kalyansundram (1988) studied on distribution of rice seed micro flora within grain with special reference to storage fungi. Storage fungi found to be invading rice and remain mainly husk and outer layer of karnel. The fungi invading the potential layer were mainly *Aspergillus flavus, A. nidulus, A niger*, to some extent. The more common ones being *A. candidus, A. glacucus* and sometimes *A. versicolor, Penicillium* spp. were less common there.

Jayaweera *et al*. (1998) reported that 17 fungi namely *Bipolaris oryzae, Curvularia pallescens, C. verruculosa, C. eragrostidis, C. afflnis, Pyrenochaeta terrestris, Trichoconis padwickii, Sodaria fimicola, Fusarium* spp. and *Penicillium citreoviride* significantly reduced the germination of rice seed.

Basak and Mridha (1988) studied the seeds of different varieties of Amon rice collected from Chittagong and Chittagong Hill tracts district of Bangladesh. Prevalence of fungi in 44 seed samples tested by the blotter methods varied with cultivar and location. Among those isolates *Rhizopus* spp. had the maximum prevalence in seeds.

Shahjahan *et al.* (1988) conducted an experiment during Aman season with rice varieties and lines and found that 75% entries with more than 10% grain spotting. Modern varieties had more spotted grain than the local ones. A total of 23 fungal species (17 genera) 1 actimomycetes and 2 bacteria were found associated with spotted rice grains. In Aus, Aman and Boro season, 23, 16 and 22 organisms were recorded froin seed, respectively. Thirteen of these organisms were both externally and internally seed borne namely *Drechslera oryzae, Fusarium* sp., *Chaetominuim* sp., *Sarocladium oryzae* and *Trichoconis padwicku* were predominant. The kinds of organism and their frequency of association with the spotted grains were found to vary depending on the variety/line and season.

Imolehin (1987) studied rice seed multiplication centres in relation to seed- borne pathogens of rice: A case study of on do State Rice Multiplication Centers and stated that *Fusarium moniliforme* and *Drechslera oryzae* were the major pathogens that caused devastating seedling disease of rice in the field (*D. oryzae* 12%, *F. moniliforme* 40%). This work is a survey of the incidence of seed-borne fungi of rice associated with three varieties of rice: Faros 12, 15, and 29 popularly cultivated in Afikpo North local government area of

Ebony State and isolated *B. oryzae* (*Drechslera oryzae*), *Curvularia lunata*, *Chaetomium* spp., *Trichoderma* spp., *Aspergillus* spp. and *Penicillium* spp. from twenty-two different rice cultivars from South West Nigeria.

Singh and Kang (1987) observed that the major seed borne pathogens of rice were *Helminthosporium oryzae, Fusarium moniliforme, Curvularia lunata, Aspergillus flavus, Alternaria* and *Penicillium* spp.

Sharma *et al*. (1987) detected 10 fungal species of fungi from the rice seeds where *Fusarium moniliforme (Gibberella fujikuroi), Curvularia lunata (Cochliobolus lunata)* and *Aspergillus flavus* were the most common.

Aruna and Chaudhary (1986) listed 34 fungi in 23 rice seed samples from different locations. More were detected by blotter method than by deep freeze and agar plate methods.

Ou (1985) reported that the rice is affected by as many as 36 seed-borne diseases of which 31 were caused by fungi.

Ramadoss (1985) observed the discoloration of grains caused by *Drechslera oryzae*, *Curvularia lunata*, *Fusarium moniliforme*, *Alternaria padwickii* and *Sarocladium oryzae* decreased seed germination by 10% in CO44 and 3% in IR50.

Kim *et al.* (1984) found that the fungus *Monographella albescens* occurred at a frequency of 1-4% in 2 seed samples, among the 21 fungi detected in 26 samples from Chungnan province. Results obtained indicated that *Gerlachina oryzae* was present only in the chaff, endosperm and seed coat but also in the embryo. Seed borne infection caused seed rot seedling blight and brown discoloration of the coleoptiles, primary and secondary leaf when infected seeds were sown in agar or in soil.

Sovae *et al.* (1983) reported the association of *Alternaria tenuis, Cladosporium herbarum, Curvularia lunata, Epicoccum purpurascens, Helminthosporium oryzae, Rhizoctonia solanii, Pyricularia oryzae, Phoma* spp. in rice seed. The average incidence of these fungi were 12%, 13%, 35, 28%, 2%, 6%, 6%, 33%, and 1% respectively.

Imolehin (1983) studied on rice seed-borne fungi and their effect on seed germination and reported that seed-borne fungi affected rice seed germination. Fungal pathogens recorded on twenty-two seed samples of rice cultivars from south-western Nigeria included *Drechslera oryzae, Curvularia lunata, Fusarium moniliforme*, *Penicillium* spp., *Rhizopus* spp., *Chaetomium* spp., *Trichoderma* spp. and *Cladosporum* spp.

Mia and Mathur (1983) investigated seed microflora of rice in Bangladesh. They tested seed health of 75 seed samples from different parts of country in the Aus, T- Aman, and Boro seasons and observed that more than 90% samples were infected with *Drechslera oryzae*, and *Trichonis padwickii* and the highest infection in individual samples were 88.5% and 63.0% respectively.

Caratelli and Saponaro (1983) in Brazil isolated *Drechslera oryzae*, *Pyricularia oryzae* and *Alternaria padwickii* from rice seed, among others *Curvularia* spp. were also found in some cases.

Riberio (1980) examined 79 samples of rice in Brazil, incidence of *Helminthosporium oryzae* was higher in seed sample tested by the filter paper method, indicating it's presence inside the seeds and its high transmissibility through them, washing and centrifuging showed the incidence of *Pyricularia oryzae* (26.3), *Cochliobolus miyabeanus* (13.9%), *Curvularia lunata* (44.3%), *Nigospora oryzae* (22.7%), *Fusarium* sp. (12.6%) and *Alternaria* sp. (44.4%).

Ranganathaiah *et al.* (1979) reported that *Pyricularia oryzae* is one of the most serious pathogens of rice in Kamataka. Out of 50 samples tested 12 were found to be infected with this fungus.

Asokhan *et al*. (1979) studied on the influence of seed borne fungi on germination and post imergence mortality of rice (ADT31) and Ragi (Co7) seedling on treatment of seed with spore suspensions of 12 fungi *Helminthosporium* sp., *Curvularia* sp. and *Fusarium* sp. were most inhibitory on rice seed germination.

Reddy and Khare (1978) in India, noted 4 fungi in 42 rice seed samples collected from 41 districts, of which *Drechslera oryzae* and *Trichonis padwickii* were associated with 18 samples. Individual sample the highest incidence of these fungi was 32% and 40% respectively and both were internally seed-borne.

Zainun and Nik (1977) collected 23 rice varieties from 11 different locations of Malaysia and isolated 33 seed-borne fungi from such seed, commonly encountered pathogens were *Drechslera oryzae, Trichonis padwickii, Fusarium moniliforme, Pyricularia oryzae* and *Nigospora oryzae.*

Shrestha *et al*. (1977) isolated *Drechslera, Trichonis, Fusarium, Pyricularia, Nigospora, Curvularia, Alternaria, Phoma* and *Cercospora* from rice seed.

Esuroso *et al*. (1975) conducted an experiment over three years on the seed borne fungi of rice in Nigeria following blotter method. It was revealed that *Drechslera oryzae, Trichonis padwickii* and *Pyricularia oryzae* were seed borne inculding someother fungi.

Hossain and Fakir (1974) studied on the seed borne microflora of freshly harvested rough rice varieties, which revealed the association of 10 fungal genera. In order of prevalence these were *Fusarium, Nigospora, Curvularia, Alternaria, Aspergillus Helminthosporium Penicillium, Rhizopus, Chaetomium* and *Sordida*. *Curvularia,* the most predominant genus constituted 28.9% of total fungal isolation and 59.5% of the grains yieldedthis fungus.

Fakir and Ahmed (1974) investigated the association of seed borne micro flora with the freshly harvested rough rice of Tepi-boro, collected from Bangladesh Agricultural University farm during 1970. About 400 bacterial and more than 11000 fngal colonies were isolated form a total of 7000 grains.Different genera of fungi were identified viz. *Fusarium* spp. (3.5%), *Alternaria tenuis* (7.5%), *Aspergillus* spp. (26.7%), *Helminthosporium oryzae* (4.3%), *Penicillium* spp. (0.5%), *Rhizopus nigricans* (0.6%), *Curvularia lunata (*21.7%) *Tilletia barclayana* (19.1%), *Chaetomium* spp. (0.7%), *Zygorhynchus* sp. (0.7%) and *Sordida* spp. (0.1%).

Agarwal and Singh (1974) observed 7 fungal species with *Trichonis padwickii* as the most common one. They alos observed varietal differences on seed borne fungi. Seeds of IR8 had the least infection and highest infection was recorded on Krisna. Grain discoloration was associated with heavy infection of *Trichonis padwickii, Fusarium moniliforme, Fusarium semitectum* and *Trichotheccium* sp.

Marthur and Neergaard (1970) and Neergaard *et al.* (1970) reported that a myriad of seed borne fungi that caused serious diseases of rice in nurseries, fields and storage were seed-borne.

Bacteria associated with rice seed

Rafi *et al.* (2013) conducted an experiment on hundred and twenty-five *Xanthomonas oryzae* pv. *oryzae* isolates were collected from twelve districts of different agro-ecological zones of Khyber Pakhtunkhwa province of Pakistan for characterization through a number of biochemical tests. HR- response was found negative for 34 isolates while 45 isolates showed negative results in terms for Pathogenicity. However, egg yolk reaction, tetrazolium tolerance test, anaerobic growth test and oxidase test was negative for all 125 isolates. Therefore, identity of the all candidate of *Xanthomonas oryzae* pv. *oryzae* isolates were verified through polymerase chain reaction (PCR).

Najeeya *et al*. (2007) conducted experiment to isolate and identification of *Xanthomonas oryzae* pv. *oryzae,* the causal agent of bacterial leaf blight (BLB) of rice was characterized through pathogenicity and biochemical assays. The isolates obtained were subjected to pathogenicity test on six rice cultivars that varied significantly in terms of disease severity among each other. However, non-significant results were recorded when clip and pinprick methods of leaf inoculation were compared for pathogenicity test. The isolates were also subjected to different biochemical tests and were found to be negative for oxidase, lecithinase and gram reactions. Results of biochemical tests like Tween 80 and starch hydrolysis, anaerobic nature and acid production from carbohydrates varied among the isolates. Only 20% isolates were similar in terms of their reactions to these tests. Based on biochemical responses it was established that despite the small sample size (n =15) genetic variability was detected in *Xathamonas oryzae* pv. *oryzae* isolates.

Jalaluddin et al. (1999) screened and evaluated four somaclonal progenies of rice variety BR3 along with four check varieties for their resistance to bacterial leaf blight (BLB)

caused by Xanthomonas oryzae pv. oryzae and sheath blight caused by Rhizoctina solani during the Aman and Boro seasons of 1990-1993. All the somaclonal progenies were moderately susceptible to BLB in both Aman rice and Boro season.

Xie *et al.* (1999) conducted a field survey in the Zhejiang province of China (subtropical) and Lunzon island of Philippines (tropical) during 1993-98. They screened about two hundred and eighty pathogenic bacterial isolates from over 3500 isolates associated with rice seeds from 116 seed samples collected in the subtropics and 129 seed samples from the tropics. The frequency of pathogenic bacteria was about 9% and 6% in the tropics and subtropics respectively. Eleven bacterial species *(Acidovorax avenae, Burkholderia glomae, Erwina chrysanthelne* pv. *oryzae, Pantoea agglomerans, Pseudomonas fuscovaginae, X. oryzae* pv. *oryzae, X. oryzae* pv. *oryzicola, Pseudomonas aeruginosa, P. fluorscens, P. fulva* and *P. putida)* were differentiated by bacteriological and numeric taxonomy (Biology) methods.

Ise *et al*. (1998) screened 16 lines of rice (pedigree of Asominori) against bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*). They reported that only Saikai 85 showed resistance reaction on bacterial leaf blight isolates. In second study, bacterial leaf blight resistance of these lines, and their F_1 and F_2 was assessed using clip inoculation at heading. All F_1 plants were resistant to bacterial leaf blight isolates, which were collected in Japan.

Jalaluddin *et al*. (1998) screened and evaluated fourteen advanced mutants along with five check varieties of rice for their resistance to bacterial leaf blight *(Xanthomonas oryzae* pv. *oryzae)* and sheath blight *(Rhzoctina solani)* during four consecutive T. aman seasons from 1994-1997. For bacterial blight, flag leaves were inoculated with the causal bacterium ($l0^3$ cell/ml) by clipping method. All the induced mutants and the check varieties TKM6, Binasail, BR9 and BR14 were moderately susceptible to bacterial leaf blight.

Sharma and Viad (1997) studied rice samples were collected from Himachal states of India and showed that the extent of grain discoloration varied between 4.35 to 79.82%. 10 fungi fungi viz. *Alternaria alternate, Aspergillus niger, Curvularia*

oryzae, Curvularia lunata (Cochlibolus smiyabeanus), Tilletia barclayana (Khuskia oryzae), Pestolatia oryzae, Phylostictia glumarum (Phoma sorghina), Penicillium spp., *Sarocladium oryzae (Magnapothe salvinii),* were detected. *A. alternat* occurred most commonly followed by *Curvularia lunata.*

Mukhopadhyay *et al.* (1996) isolated bacteria from rice seedling were grow from surface sterilized seeds. Three isolates were associated with, the rice seed husk and while 4 others were growing endophytically within the seed. Microscopic study revealed that the endophytes were concentration in the root stele region. Some of the bacteria exhibited strong anitifungal activity.

Cottyn *et al.* (1996) conducted an experiment and isolated 5600 from rice plants with sheath rot complex and grain discoloration syndrome and 2 batches of 1 kg of rice seed (cvs. IR54 and IR8866), 204 pathogen were initially characterized by phenotypic tests, serology and growth selective media. The non fluorescent pathogens were represented by clusters D_l *(Pseudomonas glumae)* and *E (P. avenae).* Seven clusters were distinguished among the fluorescent strains associated with sheath rot complex and grain discoloration. Cluster A_5 was identified as *P. aeruginosa* and cluster B_1 as *P. fuscovaginae an*d *P.marginals* clusters B_l and B_2 were only slightly different. The strains identified as P. *fuscovaginae* were different from the type strains in 2-ketogluconate production.

Kumar *et al.* (1995) reported that a high yielding variety, Kranti, susceptible to bactiral blight was used to study the induction of resistance against *Xanthomonas campestris* pv. *oryzicola* by differentially killed cells of the same bacterium. Two criteria were used (1) development of disease by re-inoculation with live bacteria 7 days after pretreatment (2) conc. of orthodilydri phenols. Pretreatment of plants with killed bacteria did not induce resistance to the disease and orthodihydric phenol levels did not increase. It is concluded that either elicitors are not present on the cell surface of the bacteria or that they were destroyed the heat treatment or antibiotics used to kill them.

Singh and Dodan (1995) conducted an experiment where a large number of rice genotype was screened for resistant to bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*). Among the 73 promising genotypes, only 3 genotypes consistently resistant

reaction to Bacterial leaf blight during Kharif 1992 and 1993. Twelve entires showed moderate resistance to this disease.

Sahu and Jena (1995) found that seed micro flora of 15 semi deep water rice varieties cultivated in India was studied by standard blotter method and direct seed inoculation in agar. In total, 16 fungi belonging to 9 genera and a single bacterium (*Xanthomonas campetris*) were isolated. Among the seed varieties contained the highest % of incidence of fungi and the lowest % is bacteria. Higher levels of micro flora infection were associated with decrease rate of seed germination.

Bhutta and Ahmed (1994) collected 153 rice seed samples from paddy crop growing area in Pakistan and tested for the presence of seed borne bacterial pathogen using seedling symptoms test maximum seed infection due to *Xanthomonas oryzae* pv. *oryzae* was 11 and 12% in variety IR-6 at Lahore and Hyderabad respectively. *Acidovorax avenae* pv. *avenae* infection was 13% in variety B-385 from Sahiwal. Percentage seed infection due to bacterial pathogen varied from cultivar to cultivar in different locations.

Lin *et al.* (1994) studied with 3 media (SX, NBY and Wakimoto) for detecting the bacteria *Xanthoraionus campestries* pv. *oryzicola* from rice seed, they found that Wakimoto is the basic media for *Xanthomonas oryzicola.*

Ziao et al. (1998) evaluated 185 accessions of rice from IRRI, Philippines to 7 pathotypes of bacterial leaf blight (Xanthomonas oryzae) during 1991-93 in Zhejlang, China. They reported that among 185 accessions, 18 were highly or moderately resistant to the pathotypes, of which 14 showed potential as good sources of resistance in breeding programs. Evaluation of agronomic traits and field resistance indicated that Suweon 290, Milying 46 and Si-Pi 692033 had the potential for commercial production.

Shen (1993) evaluated the resistance of some rice genotypes to *Xanthomonas oryzae* pv. *oryzae* at 15 institutes during 1986-90. IR 26 and Jin Gang 30 were respectively the resistant and susceptible controls. They observed 7 accession with immunity and 31 with a high degree of resistance to *Xanthomonas oryzae* pv. *oryzae.*

Mew *et al.* (1993) described that reduction in rice yield might be as high as 50% in fields where the crop was severely infected, and infection at the tillering stage could lead to total-crop losses. More commonly, however, plants were affected at the maximum tillering stage, and yields were reduced by 10-20%.

Ashrafuzzaman (1992) reported that the severity of BLB of rice was high in tropical Asia. He also reported that the BLB of rice was one of the most damaging diseases in South East Asia and losses due to this disease vary from 6 to 60%.

Li *et al.* (1992) detected *Xanthomonas campestris* pv. *oryzae* from rice seed by using DAS-ELISA.

Swings *et al.* (1990) recently considered the bacterium to be a distinct species from *Xanthomonas campestris* on the basis of phenotypic, genotypic, and chemotaxonomic data, and proposed the name *Xanthomonas oryzae* pv. *oryzae* (Ishiyama, 1922). This name is now used widely by researchers of bacterial blight.

Sharada *et al.* (1990) isolated bacteria externally and internally from seed of 5 cultivars. Responses of sprouted rice seed to the bacterial cultures differed between the bacteria in terms of germination, root and shoot growth and vigor, but the stimulatory or inhibitory effects followed no particular pattern. The vigour of cv. Crowrisanna was increased, suggesting that colonization of its seeds by bacteria may be beneficial. Most of the cultures that reduced, vigor were capable of producing symptoms when inoculated to rice seedlings.

Lu (1990) investigated the effect of bacterial leaf blight *(Xanthomonas oryzae* pv. *oryzae)* on 8 rice hybrids in Hangzhow, China. They reported that inoculation of susceptible hybrids at the booting stage decreased the number of filled grains plant^{-1}, 1000-grain weight, and grain yield and increased the number of empty husks plant.

Bai and Mew (1990) studied the infection types of rice bacterial blight (BB) on eight isogenic lines (with Xa-1, 3, 7, 10, and 11 and Xa-5, 8) using six inoculation method. Two infection types were commonly observed on the same combination inoculated at 65 DAS, which were similar to that of 85 DAS inoculated leaves. In inoculum density and temperature studies, no symptom Philippine races of *Xanthomonas campestris* pv. *oryzae* by prickling inoculation method. Two infection types were commonly observed on the same combination inoculated at 65 DAS, Which were similar to that of 85 DAS inoculated leaves. In inoculums density and temperature studies, no

symptom was evident when an inoculum density of 10^5 was used. Increasing the inoculum density 10^7 to 10^9 is increased in lesion size and reduction in latent period. Generally no changes in the infection types were observed, although no symptom occurred some line-race combinations. Increasing the temperatures (20/17° C, 29/21° C, and 35/27° C in day/night) resulted in shortening the latent period and increasing the lesion size. Infection type was not affected by temperatures.

Ikeda and Busto (1990) evaluated 198 rice accession including 10 wild species and 22 natural hybrids against six races of BLB caused by *Xanthomonas campestris* pv. *oryzae* in February-May, 1989 in the screen house at IRRI Reaction to the BLB races were tested using he clipping method. Five seedlings/pot per accession were inoculated at booting to heading stage. Lesion length were measured 18 days after inoculation resistance was measured using lesion length, which was less than 10 cm and the lesion length longer than 20 cm was susceptible.

Xie *et al.* (1990) isolated *Xanthomonas oryzae* pv. *oryzicola* from rice seed by using immunoradiometric assay which is one kind of antiserum.

Agarwal *et al.* (1989) worked on seed borne diseases and seed health testing of rice and found 20 seed borne diseases of rice (13 fungal, 6 bacterial and 1 caused by nematode). The bacterial diseases were grain discoluration, bacterial leaf blight *(Xanthonzonas ccrtripestris* pv. *oryzae),* leaf, stiak *(Xanthomvnas oryzae* pv. *oryzicola),* strip *(Pseudomonas avenee),* sheath brown rot *(P.fuscovaginae),* grain rot *(P. glumae)*and sheath rot *(P. syringae).*

Raj and Pal (1988) failed to obtain overwintering of *Xanthomonas oryzae* pv. *oryzae* in soil or seed, and found survival only in infected leaves. Reddy (1972) states that *X. oryzae* pv. *oryzae* survives for 7-8 months in seed, but for only 3-4 months in straw and stubble; Kauffman & Reddy (1975) reported that, although glumes were readily infected, viable bacteria could not be detected on seed stored for 2 months. It is thought that bacteriophages play a role in reducing bacteria in germinating seed.

Natrajan *et al.* (1988) evaluated 235 rice cultivars for their resistance to bacterial leaf blight *(Xanthomonas oryzae* pv. *oryzae)* using the clip inoculation method. Four lines were free from infection and 9 recorded grade 1 infection. Four showed multiple resistances by recording grade l infection to bacterial leaf blight.

Chandrasekaran et al. (1988) studied the reaction of some rice strains against a pathotype of Xanthomonas campestris pv.oryzae in International Rice Bacterial Blight Nursery (IRBBN) cultures to an Aduthurai, The Aduthural pathotype showed different reactions from those of other Indian isolates. They reported that the rice strains RP-2151-:173-1-8, RP-2151-40-1, Kachmota and DV85 showed resistance to the aduthural pathotype.

Malik and Hasi (1987) recorded of nineteen cultivars under X. campestris pv. oryzae inoculation and in disease free conditions. The yield was correlated with flowering date, panicle length and grains/panicle in both environments with height in disease free environment and with panicles/m^2 under disease stress. Path co-efficient analysis showed that panicles/m^2 and grain /panicle had the greatest direct effect on yield in both environment. Plant height had a greater effect on yield under disease stress, while seed weight had a positive effect under normal conditions but a negative effect under disease control.

Lu and Shen (1987) conducted a field experiment on interaction between 8 hybrid rice combinations and *Xanthomonas campestris* pv. *oryzae* isolates in Hangzhou, 1981. A clipping method was used for inoculating both adult and seedlings. They reported that Japonica rice was more resistant than indica rice. Among the rice combinations, Shanyou No. 6, whose restorer line was IR 26, had higher resistance and Nanyou No. 2, whose restorer line was IR 24, had lower resistance. The degree of resistance of the restorer line was highest among 3 lines and their F, while resistance of sterile line was lowest in most cases. The resistance of the maintainer line was similar to that of the sterile line. The three isolates were different in aggressiveness.

Potential inoculum sources of *Xanthomonas oryzae* include infected planting material, volunteer rice plants (Durgapal, 1985), infected straw or chaff (Devadath & Dath, 1985), and weed hosts, although the exact role of these sources in nature is poorly understood.

The bacterium enters by way of hydathodes and wounds on the roots or leaves. Penetration may also occur via stomata, where there will be a resultant build-up of bacteria which subsequently exude onto the leaf surface and re-enter the plant through the

hydathodes. Once inside the vascular system, the bacterium multiplies and moves in both directions. Spread takes place in wind and rain, but primarily in flood and irrigation water (Dath & Devadath, 1983).

Choi *et al.* (1985) observed varietal difference in field resistance to *X. campestries* pv. *oryzae* recognized more clearly by the test plant inoculation method (measuring disease leaf area on new leaves expanded after inoculation) than by the neighbor plant inoculation method. A highly significantly correlation was observed between results obtained by two methods. Some cultivars showed remarkable resistance to the disease.

Qi and Mew (1984) conducted an experiment with nine Chinese rice varieties which have adult plant resistance to bacterial blight of rice four races of' bacterial blight pathogen *(X. campestries* pv. *oryzae*) in the Philippines while others have resistance to one or two races only. The disease score of some of these varieties was gradually decreased from seedling to flag leaf while other showed a clear cut susceptible and resistant reaction the leaf position of the varieties showing resistance was different.

Sharma and Kaul (1984) tested the pathogenicity of *X. campestries* pv. *oryzae* on 2 susceptible and 2 resistance rice cultivars. They reported that all yield components were adversely affected to varying degrees at different growth stages; significant reduction in grain filled leaf to a high degree of seed sterility yield loss due to reduction in productive tiller number was high for susceptible varieties. In breeding for resistance, increased fertile grain number rather than heavy grain weight should be considered.

Jain *et al.* (1984) conducted an experiment and revealed that <2% seed infection in 8 test cultivars with disease scores of 9 at the flag leaf stage. Though the cultivars had similar disease scores they varied in percentage seed infection. A fluorescence technique was unable to detect the bacterium in the seed.

Miah and Hossain (1981) evaluated the symptoms of pale yellow leaf were reproduced artificially by clip inoculation or by root dip inoculation.

Skerman *et al.* (1980) described on the basis of the International Committee on Systematic Bacteriology adopted in based on 1975 revision, the Approved Lists of Bacterial Names in which neither *Xanthomonas oryzae.* In this list, the name *Xanthomonas oryzae* was revisesd to *Xanthomonas campestris* pv. *oryzae* (lshiyama, 1922).

Buddenhagen *et al.* (1979) reported that the first bacterial blight was reported in West Africa from Mali. Consequently it was found to occur in Upper Volta (Aqoderu and John, 1984); Mauritania, Gambia, Burkina Faso, Guinea Bissau, Guinea, Ghana, Benin, Nigeria (Awoderu *et al.*, 1991) and Cameroon (Jones *et al.*, 1991).

Mew (1979) and Mew (1987) considered the symptoms a secondary effect of kresek or leaf blight, apparently due to the effect of toxins produced by the pathogen than to interruption of nutrient transfer from roots to shoots.

Mohiuddin et al. (1978) estimated yield losses from infection by 2 isolates of Xanthomonas oryzae by 2 critical methods, inducing the disease at different stages. Inoculation of flag leaf resulted in 38-40% loss in yield respective of virulence of the isolates. Losses were also significant when disease pressure approach 50%.

Lozano (1977) identified American isolates from affected rice as *Xanthomonas oryzae,* and reported the disease to be found in most rice growing areas of tropical America including the Caribbean region (Mexico, Costa Rica, Honduras, Salvador and Panama) and South America (Colombia, Venezuela and Bolivia).

Kauffman *et al.* (1973) reported a faster method for artificial inoculation, the clipping method, was later developed at IRRI. The leaves of each plant are grasped and the tops of all leaves tire clipped by a pair of scissors wetted with bacterial suspension, so that the cut ends of the leaves are inoculated with the bacteria. The disease severity is evaluated by determining the disease based on the ratio of lesion length to whole leaf length remaining. The clipping method was reported to be useful not only for evaluating qualitative resistance but also quantitative resistance by Kaku *et al*. (1977and 1980).

The bacterium can persist from season to season on infected leaves and leaf debris, but is unable to survive in non-sterile soil (Devadath & Dath, 1970). No distinct race or varietal specificity has been reported.

According to Srivastava and Rao (1963) in North India, a severe outbreak of a disease of unknown origin occurred in the early 1960s. They examined the pathogen and proved to be, *Xanthomonas oryzae,* the incident of bacterial blight, which was first reported from India in 1959. The disease was also reported from Sri Lanka (Seneviratne, 1962); the Philippines (Goto, 1964); Bangladesh (Alim, 1967); Vietnam, Malaysia (Hashioka, 1969); Camnodia (OTCA, 1970) and Pakistan (Mew and Majiid, 1977). Ou (1977) observed rice and several weeds showing the symptoms of bacterial blight in several Latin American counrrics.

Yamanuki *et al.* (1962) studied that bacterial blight of rice has been first seen by farmers in Fukuoka prefecture, Kyushu Island, Japan as early as in 1884-1885. They stated that surveys were conducted in 1908 and 1910, the disease was found to be distributed widely from central to southwestern parts of Japan. From 1926 it spread to northeast of Honshu Island, and was reported from the northern island of Hokkaido in 1962. The disease increased from the 1960s throughout Japan, being especially prevalent in Kyushu Island. Occurrence of bacterial blight was then reported from Korea (Takeuchi, 1930); Taiwan (Hashioka, 1951); Mainland China (Fang *et al.*, 1957) and Thailand (Jalavicharana, 1958).

According to Tabei and Mukoo (1960), the causal organism of BLB invades the rice plant through water pores and wounds. As the water pores are located on the margin of the upper part of the leaf, the lesions usually start from the leaf margin near the top. The border of the lesion adjoining the healthy part shows a wavy margin. Lesions may start at one or both margins of the leaf. Bacterial ooze, small, yellowish, spherical masses, may sometimes be seen on the margins or on veins of the fresh infected leaf under moist conditions. As the disease advances, the lesions may cover the entire blade, turn white and later become grayish from the growth of various saprophytic fungi. When a leaf is injured naturally or artificially, the pathogen invades through the wound to develop a lesion from that portion (OT CA, 1970 and Ou, 1985).

Ishiyama (1922) estimated the yield losses in individual affected fields which were at 20% or 20-30% with the extreme of over 50% in severely affected fields. In the tropics, the damage is usually more severe than in temperature zones since the kresek type of attack kills young plants completely or nearly so, and the lesions on leaves are often large and progress rapidly (Ou, 1985).

According to Ishiyama (1922) the causal bacterium is short and rod-shaped, with round ends, 0.5-0.8x l.0-2.0µm (0.8-LOX 1.7µm in host), with monotrichous flagellum of 6-8 pm, Gram negative and non-spore- forming. Iatcr, Yoshimura (1963) reported by electron microscopic observation that the size of the bacterial cells was 0.55-0.75 X 1.35-2.17µnn in culture, and 0.45 - 0.60 x 0.65-1.40µm in host tissue. The flagellum was 8.75µm in the longest one, and about 30 nm thick. The latest

description by Swings *et al.* (1990) is as follows: Cells are straight rods, 0.4-0.8 X1.5-2.9µm, Gram negative, motile by means of a single polar flagellum. Cells occur singly, in pairs, or sometimes in chains, filaments may occur as morphological characters. He also described the physiological characters of *Xanthornonas oryzae* is as follows: obligately aerobic, catalase present, indole formation, 2-ketogluconate formation, urease, egg yolk reaction, nitrate reduction, and oxidase are all-negative.

Takaishi (1909) first asserted a bacterium to be the major factor causing the disease BLB. He observed dew drops on diseased leaves to be turbid with bacteria, which formed yellow bacterial masses when dried. Inoculation with these bacterial masses produced disease in healthy leaves.

CHAPTER 3
MATERIALS AND METHODS

3.1 Experimental Site:

The experiment was carried out in the Disease Diagnostic Laboratory and Net house of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

3.2 Time of Experiment:

This study was conducted during the period of January to December 2012.

3.3 Collection of Seeds:

Seeds of rice selected imported hybrid varieties were collected from Lal Teer Seed Company Limited, Bangladesh.

3.4 Rice varieties used in this experiment

Altogether 3 seed samples of hybrid rice varieties namely Moyna, Tia and Richer were used in these experiments which were imported from China.

3.5 Determination of occurrence of seed borne fungi on selected imported rice seed

Standard blotter method was used to determine the occurrence of seed borne fungi on rice seeds.

3.5.1 Blotter method

The collected seed samples of rice were analyzed for the presence of major seed borne pathogens by blotter method following the International rules for Seed Testing Agency (ISTA, 2001). Four hundred seeds were tested for each variety. Seeds were surface sterilized by 3% chlorax (seeds were dipped into 3% chlorox for 30 seconds then washed

3 times with distilled water).Three piece of blotter paper were soaked in sterilized water and were placed at the bottom of 9 cm well labeled plastic petridishes. 25 seeds of each rice variety selected at random from each sample and were placed in each plastic petrdish using a pair of forceps, making sure that seeds are placed epuidistantly with 15 seeds on the outer ring, 9 seeds at inner ring and and 1 seed at center (Figure 2). The lid of each petridish was held in place with gummy cello tape.

Figure 1. Collected hybrid rice seeds

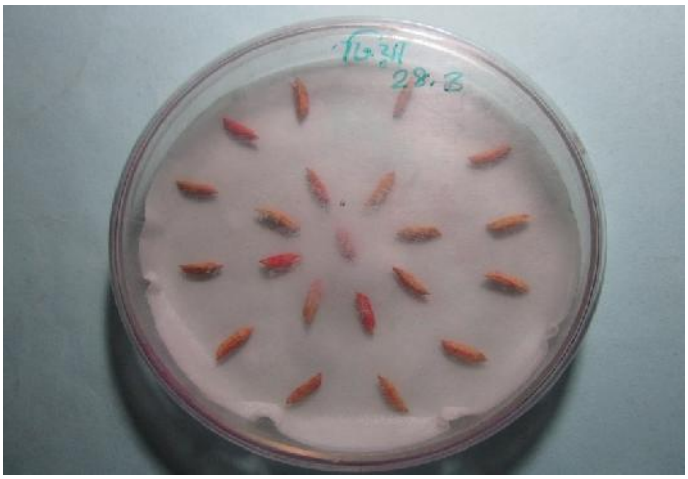

Figure 2. Seed health test by blotter method

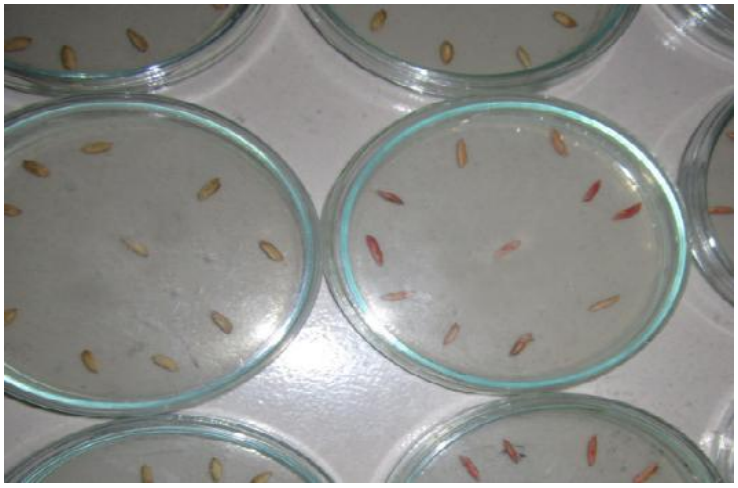

Figure 3. Seed health test by agar plate method

The petridishes containing seeds were incubated at 20±2°C for 7 days under 12 hours alternate cycle of Near Ultra Violet (NUV) light and darkness. After 7-10 days of incubation period, individual rice seeds were examined under stereomicroscope in order to record the incidence of different seed borne fungi. With flamed sterilized needles fungal growths on the grains were aseptically mounted in glycerine or laetophanol placed slides and examined under the bionocular compound microscope for fungal diagnostics characteristics. A list of morphological characters of taxonomic important such as spore size, shape, septation, color and their arrangement of the mycelium, density of the colony were compiled for each fungus. Identification of fungus were performed using all the characteristics observed and identification reference manuals of Booth (1971), Barnett and Hunter (1992) and Watanabe (2000). Number of germinated seeds was recorded along with the seed-borne fungi after 7-10 days of incubation. The results were expressed in percentage.

3.5.2. Preparation of potato dextrose agar (PDA)

PDA (appendix-**I**) was prepared as described by Islam (2009). 200 g peeled and sliced potato was boiled in 500ml water in a bowl for about half an hour. Then the extract of the potato was filtered through was cheese cloth. The other two ingredients viz. 20g dextrose and 20g agar were added in the extract and the volume was made up to 1L mark. Then the prepared standard PDA was poured in 1000ml conical flask and sterilized (121˚C, 15 psi for 15 min.) in an Autoclave.

3.5.3 Isolation, purification and preservation of seed borne fungal pathogens of rice

Isolation of the seed borne fungi was carried out on PDA medium. PDA plates were inoculated by taking a bit of mycelia from the incubated seed surface and transferred on PDA plates. The fungi were isolated, purified using the hyphal tip culture techniques. Purification was done by reculture. Identification was done following the keys of Barnett and Hunter (1992). The pure cultures were also maintained on PDA slants kept at 5 ^{0}C for further studies.

3.6 Determination of occurrence of seed borne bacteria on selected imported rice seed

Nutrient agar plate method was used to determine the occurrence of seed borne bacteria on rice seeds.

3.6.1 Preparation of nutrient agar medium:

Nutrient agar media (appendix-I) was prepared according to the method followed by Hossain (2006). Thirty five gram Nutrient agar mixed well in 1000 ml distilled water. It was then autoclaved at 121^0 C under 15 PSI pressure for 15 minutes.

3.6.2 Agar plate method

In the NA plate method, 400 hundred seeds were tested. Each plate was containing 10 seeds. Seeds were dipped into 10% chlorax for 1 minute, then wash 3 times with distilled water. 10 seeds were placed on the NA medium 1 at center and 9 at outer ring (Figure 3) and the plated seeds were usually incubated for 5-7 days at 20±1ºC under 12hrs alternating cycles of light and darkness. After incubation, bacterial ooz were coming out from the seeds on seed surface. Incubated seeds examined and germination (%) and of seed borne bacteria were determined in percentage.

3.6.3 Isolation, purification and preservation of seed borne bacteria of rice

Bacterial ooz were streaked on NA plate with help of sterilized loop. The streaked plates were kept in incubation chamber at 30^o C. The plates were observed after 24 hrs and 48 hrs. The single colony grown over agar plate was restreaked on another agar plate with the help of a loop get pure colony. After purification the bacteria streaked on NA slants and incubated for 48 hours. Then these slants were kept at refrigerator at 4ºC for stock culture.

3.7 Characterization of bacteria

3.7.1 Cultural characters

3.7.1.1 Growth on NA medium

Nutrient agar mediu was poured into a sterile petri dish and after cooling, colony of bacteria were streakinoculated on the plate with the help of a sterile transfer loop. Then it was incubated at 30^{o} C for at least 24 hrs in incubation chamber and observed the colony characters.

3.7.1.2 Growth on differential YDCA (Yeast extract-dextrose-$CaCO_3$) medium

Yeast extract-dextrose-$CaCO_3$ agar medium (Appendix-I) according to the method followed by Najeeya *et al*. (2007). For preparation of 1 liter YDCA medium yeast extract (10 g), dextrose (20 g), and agar (15 g) were needed. All the ingredients were mixed well and heated them. 20 g $CaCO_3$ light powder then adde to it and finally it was autoclaved at 121 ^{o}C under 15 PSI pressure for 15 minutes. The autoclaved medium was cooled and poured into a sterile petri dish. Then isolates were streaked the on YDCA plate with help of a sterile transfer loop. Then it was incubated at 30^{o} C for at least 24 hrs in incubation chamber and observed the colony characters.

3.7.1.3 Growth on SX agar medium

SX agar medium (Appendix-I) was prepared according to the method followed by Schaad *et al*. (2001). Starch (10 g), beef extract (1 g), ammonium chloride (5g), K_2HPO_4 (2 g), methyl violet 2B (1 ml), methyl green (2ml) and agar (15 g) were needed to prepare 1 liter SX agar medium. After autotoclaving at 121 ^{o}C 5 under 15 PSI pressure for 15 minutes, 2 ml cycloheximide (100 g/100 ml in ethanol) was added. Plates containing SX medium were inoculated with bacterial isolates and incubated at 30^{o} C for 48 hrs in an incubator chamber and observed colony characters.

3.7.2. Morphology of the bacteria

For morphological characters, colony color, shape and surface textures were carefully studied and recorded as all bacterial isolate developed after 24hrs of incubation in NA medium.

3.7.2.1Gram's Reaction

3.7.2. 1.1. Gram's staining

24 hrs old bacterial culture was used for tests. Gram staining (Gerhardt, 1981) consisted of subjecting a thin bacterial film on a glass slide to aqueous Crystal Violet, Iodine,

Ethanol and Safranin solutions for various periods of time and washing with tap water. Gram stain was done on a clean slide, dried a thinly spreads bacterial film in air without heat. Then lightly flamed underside of the slide twice to fix the bacteria to the slide. Then the smear was flooded with Crystal violet solution for 60 seconds. It was washed with running tap water for a few seconds and excess water removed by air. Then the smear was flooded with Iodine solution (Lugol's Iodine) for 60 seconds and then washed with running tap water for few seconds and excess water removed by air. After that the smear was decolorized with 95% ethanol for 30 seconds and again washed with tap water and dried by air. Then the smear was counterstained with 0.5% safranine for 10 seconds and washed briefly in tap water and excess water was removed by air. Finally it was examined under microscope at 100X was oil immersion objective.

3.7.2.1.2. KOH solubility test

Gram staining results were confirmed with reaction to Potassium hydroxide (3% KOH) test (Ryu, 1940). During this lest, a loopful of bacteria (24 hrs old) was stirred in 3% KOH and change in the viscosity was recorded. Gram negative bacteria forms thread like slime when picked with a tooth pick while gram positive bacteria disperses and forms no slime.

3.7.3 Biochemical characters

Different chemical tests were done for each bacterial isolate such as starch hydrolysis, citrate utilization test, catalase test, oxidase test, pecteolytic test and gelatin liquefaction test.

3.7.3.1. Starch hydrolysis

Starch hydrolysis test consisted of treating the inoculated basal medium of powdered agar and dissolved starch with Lugol’s iodine and recording the absence or presence of clear zones in stained media (Cowan, 1974). A nutrient agar plate containing 2% soluble starch was inoculated with the bacterium isolate to be tested. Then incubated at optimum temperature for at least 48 hours. After incubation, the plate was flooded with Lugol’s iodine and observed.

3.7.3.2. Catalase test

One colony of the organism from the agar plate was taken on a slide onto which one drop of 3% H_2O_2 (Hydrogen Peroxide) was added and observed.

3.7.3.3. Oxidase test

Kovacs Oxidase test (Kovacs, 1956) was carried out using a filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride solution and rubbing a loopful of inoculum on it. The appearance or absence of color on these filter papers was recorded.

3.7.3.4. P**ecteolytic test**

Potato tubers were disinfected with 99% ethanol, cut up into slices of about 7-8 mm thick, and then placed on moistened sterile filter paper in sterile Petri dishes. Bacterial cell suspension was pipetted into a depression cut in the potato slices. One potato slice pipetted with sterile water was treated as control. Development of rot on the slices was examined 24–48 h after incubation at 25°C. Examination was done for 5 days after inoculation. Two slices were inoculated for each isolate.

3.7.3.5. Citrate utilization test:

A portion of the test organism was picked up from the agar plate with a sterile inoculating loop and streaked into Simmon's citrate agar slants. Following incubation at 30^oC for 24 hours changing of the green bromothymol blue indicator positive results.

3.7.3.6 Gelatin liquefaction test:

One loop-full bacterial culture was inoculated with a sterile straight wire stabbed into the media and incubated at 30^oC for 24 hours. Gelatin liquefied microorganisms is detected by the formation liquid culture in the presence of 4^oC at refrigerator.

3.7.4. Pathogenicity test

3.7.4.1. Preparation of soil

The soil was prepared by sterilizing with 4% formalin at the rate of 2000 ml per 30 kg soil. At first, soil was mixed with formaldehyde and kept covered with polythene sheet for three days. After then, the sheet was kept out and the soil was pulverized. Then it was kept for three days. Thus, the soil was prepared.

3.7.4.2. Raising of seedlings

Seeds were sown at a constant rate on sterilized soil. Seeds are soaked in sterilized water then kept polythene sheet. After 24 hrs seeds are sown. Time to time observation and watering was done daily and whenever necessary.

3.7 .4.3. Inoculation of bacteria

For pathogenicity test, inoculum was prepared by streaking a loopful of each isolate in the middle of nutrient agar plates and incubating at 25-27 ^{o}C. The bacteria were washed from the plate surface after 24 h with 5 ml of SDW. The inoculum thus prepared was serially diluted and adjusted to a concentration of 10^{7}- 10^{8} cfu/ml. Based on its susceptible nature to BB, Moyna and Tia were used for pathogenicity test. Two weeks old seedling were transplanted to small plastic pots (13cm dia) and placed on a nethouse bench. At pre-tillering stage, plants were inoculated with clip inoculation method at panicle initiation stage (60-70 days old plants) as outlined below. Plants were inoculated using clip method of inoculation by dipping a pair of scissors in the inoculum and clipping off three leaves approximately 2-3 cm from their tip (Kauffman *et al*., 1973). Three leaves per isolate were thus testing inoculated (Di Ming *et al*., 1991) whereas control was similarly inoculated with SDW. The plants were wrapped in moist plastic bags to conserve moisture and placed in a net house at 25-27^{o}C immediately after inoculation (Schaad, 1980) until optimum development. After inoculation, the pots were kept in a net house and regular observation was done. To confirm Koch's postulates, bacteria were then re-isolated from leaves which developed symptoms.

3.8 Effect of seed borne pathogens on seedlings (Water agar test tube method)

This test was done by following methods of Khare *et al.* (1999). In this technique, test tube were prepared by pouring 10 ml of 1% water agar in each test tube (2 cm in diameter and 15 cm in length) and then sterilized in autoclave for 15 minute under 15 lbs pressure at 121ºC. The water agar in the test tube was solidified at an angle of 60ºC. 200 seeds for each variety, Tia and Moyna and Richer were tested. Seeds were dipped into 10% chlorax for 1 minute, then wash 3 times with sterilized water and then dried and one seed per test tube were placed on solidified water agar. The tubes were then incubated at erect condition in an air cooled room (temperature 22ºC) under fluorescent day light tube (Fig.5). The cotton plugs were removed when the seedlings

Figure 4. Raising of rice seedling for pathogenisity test

Figure 5. Seedling symptom test (Water agar test tube method)

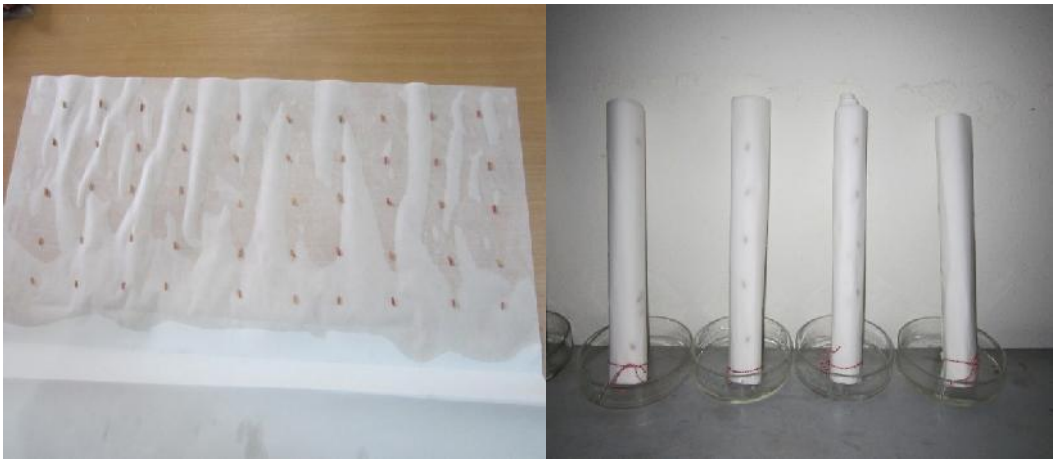

Figure 6. Seedling vigor test by rolled paper towel method

reached the rim of the test tube. Data on germination, number of abnormal seedlings, number of diseased seedlings and number dead seeds were recorded.

3.9 Effect of seed borne pathogens on seedling vigor (Rolled paper towel method)

Seedling infection and seedling vigor test was done in Rolled Paper Towel method (Warham, 1990). In this method, 400 seeds were randomly taken from each variety and 50 seeds were placed between a pair of moist paper towels in 10x5 direction The towels were rolled and the two ends were closed with rubber band as the moist could not remove easily (Fig. 6). Then the rolled papers containing seeds were placed in water containing large sized Petridis in an upright position in order to supply required moisture for 7 days at room temperature under normal 12/12 light and darkness cycle. After 10 days of incubation observation pertaining to (a) % germination (b) % diseased seedling (c) % dead seed (d) seedling weight (e) root length (f) shoot length and (g) vigor index. For determination of seedling vigor 10 seedlings (normal/ abnormal) were randomly selected from each paper and their individual root and shoot was measured. Length of shoot was measured from the base of the stem up to the growing point of the youngest leaf. Similarly, length of root was measured from starting point of the root to the largest available lateral root apex. Fresh weight of seedlings was taken before the materials could get desiccated. Vigor of the seedling was determined by the following formula (Baki and Anderson, 1972).

Vigor Index= (Mean of root length + Mean of shoot length) × % of seed Germination

3.10 Design of Experiment

The experiment was conducted following Completely Randomized Design (CRD) with four replication. Data collected during experimental period were tabulated and analyzed following Statistical package MSTAT-C. Treatment means were compared with Least Significant Difference Test (LSD) (Gomez and Gomez, 1984).

CHAPTER 4

RESULTS

4.1. Determination of occurrence of seed borne fungi of selected imported hybrid rice varieties

Significant variations among the varieties in respect of percent seed germination and incidence of seed borne fungi were observed (Table-1). Incase of germination, significant variation observed among the varieties. The highest seed germination was recorded in Tia (97 %) followed by Moyna (94 %). And the lowest germination was recorded in Richer (93%). Five fungal species viz. *Bipolaris oryzae, Aspergillus flavus, Aspergillus niger, Alternaria* spp. and *Fusarium moniliforme* (Figure 7-21) were detected from rice seed. The incidence of *Alternaria* spp. ranged from 0 to 2.17 % where the highest incidence was recorded in Moyna (2.17%) and no incidence was observed both varieties viz. Richer and Tia. The highest incidence of *Aspergillus flavus* was recorded in Richer (12.90%) where the lowest incidence was recorded in Tia (10.40%). The incidence of *Aspergillus niger* ranged from 6.52% to 9.55 %. The highest incidence was recorded in Richer (9.55%) where the lowest was in Moyna (6.52%). The incidence of *Bipolaris oryzae* ranged from 9.52% to 10.15 % where the highest incidence was in Moyna (10.15%) and lowest incidence was in Richer (9.25%). The incidence of *Fusarium moniliforme* was varied from 0 to 2.20 % and the highest incidence was recorded in Richer (2.20%) followed by Tia (1.57%).

Table 1. Occurrence of seed borne fungi on selected imported hybrid rice varieties

Hybrid rice varieties	% Pathogen incidence					
	% Seed germination	***Alternaria* spp.**	***Aspergillus flavus***	***Aspergillus niger***	***Bipolaris oryzae***	***Fusarium moniliforme***
Tia	97.00 a	0.00 b	10.40 c	8.15 b	10.15 b	1.57 b
Moyna	94.00 b	2.17 a	11.95 b	6.52 c	13.45 a	0.00 c
Richer	93.00 c	0.00 b	12.90 a	9.55 a	9.52 c	2.20 a
$LSD_{(0.05)}$	0.957	0.09	0.34	0.35	0.23	0.09
CV%	0.58	7.62	1.68	2.50	1.20	4.39

Figure 7. *Aspergillus flavus* on rice seed (naked eye view)

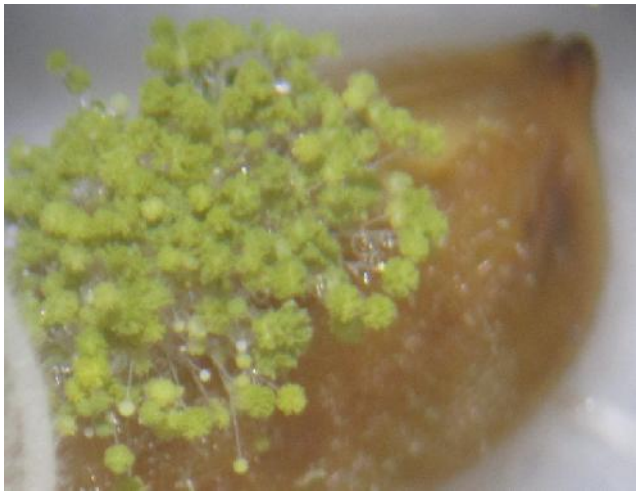

Figure 8. *Aspergillus flavus* on rice seed (under sterio microscope)

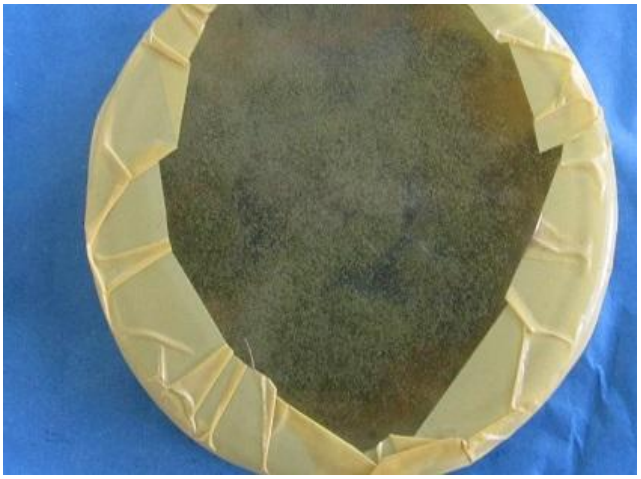

Figure 9. Pure culture of *Aspergillus flavus* on PDA medium

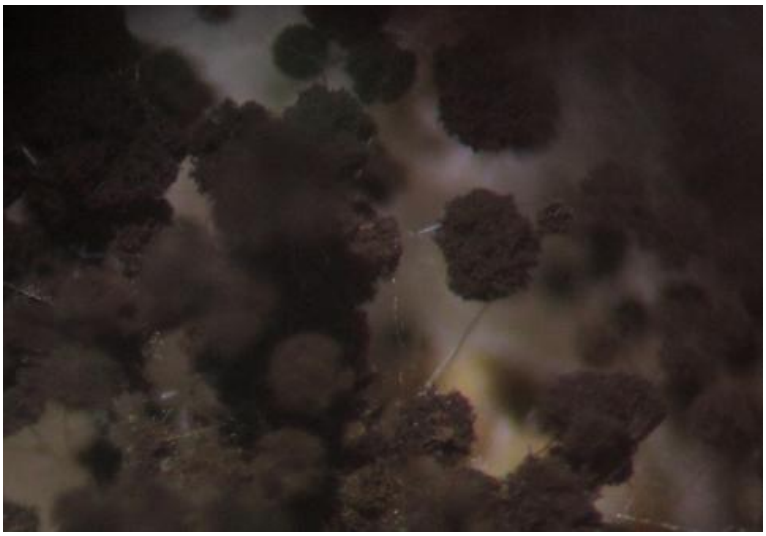

Figure10. *Aspergillus niger* on rice seed (under stereo microscope)

Figure11. Pure culture of *Aspergillus niger* on PDA medium

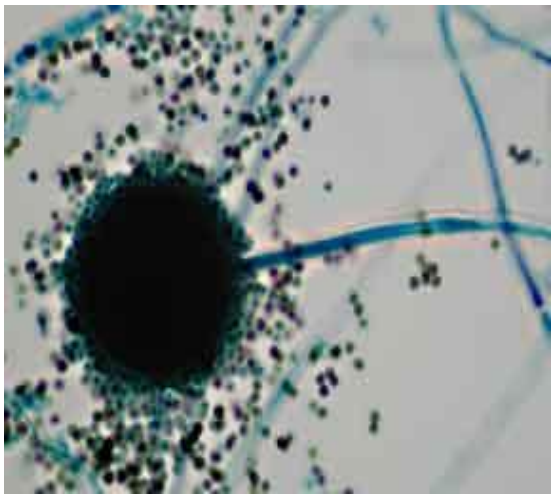

Figure12. Conidia of *Aspergillus niger* (under compound microscope at 40 X)

Figure13. *Bipolaris oryzae* on rice seed (under stereomicroscope)

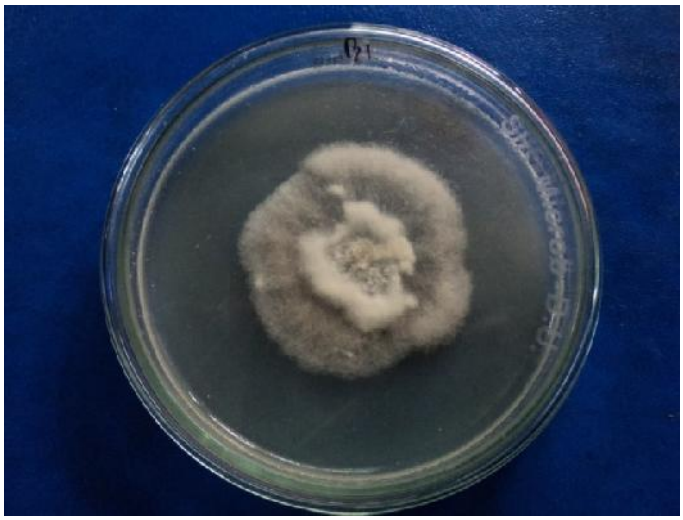

Figure 14. Pure culture of *Bipolaris oryzae* on PDA medium

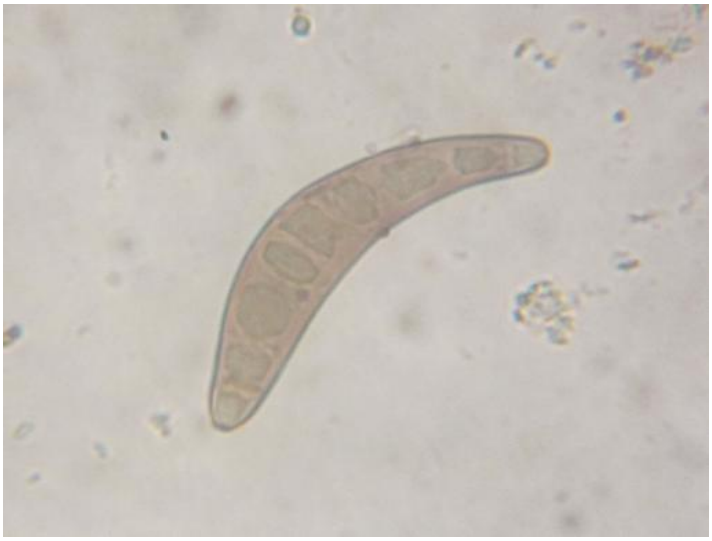

Figure 15. Conidium of *Bipolaris oryzae* (under compound microscope at 40 X)

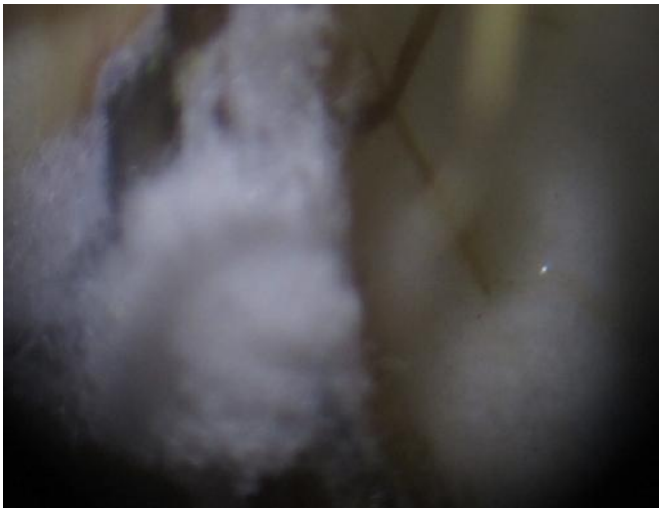

Figure 16. *Fusarium moniliforme* on rice seed (under stereomicroscope)

Figure 17. Pure culture of *Fusarium moniliforme* on PDA medium

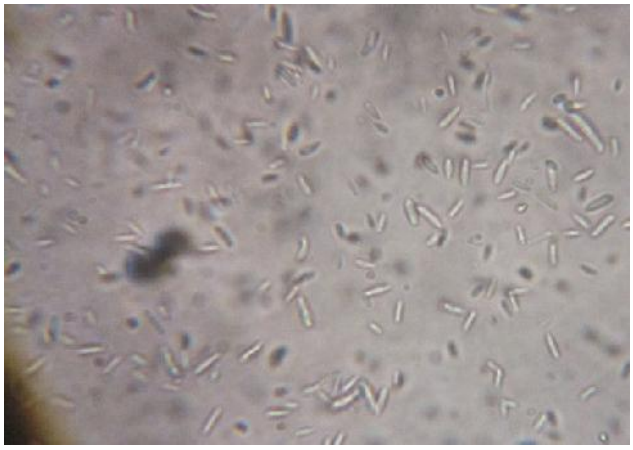

Figure 18. Conidia of *Fusarium moniliforme* (under compound microscope at 40 X)

Figure 19. *Alternaria* sp. on rice seed (under stereo microscope)

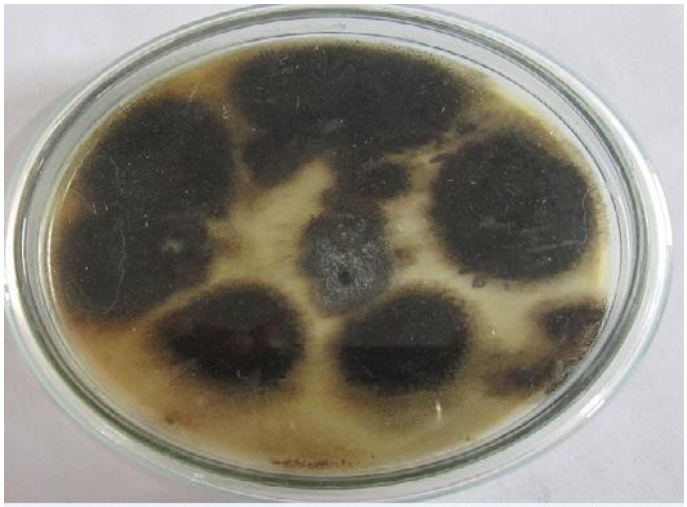

Figure 20. Pure culture of *Alternaria* sp. on PDA medium

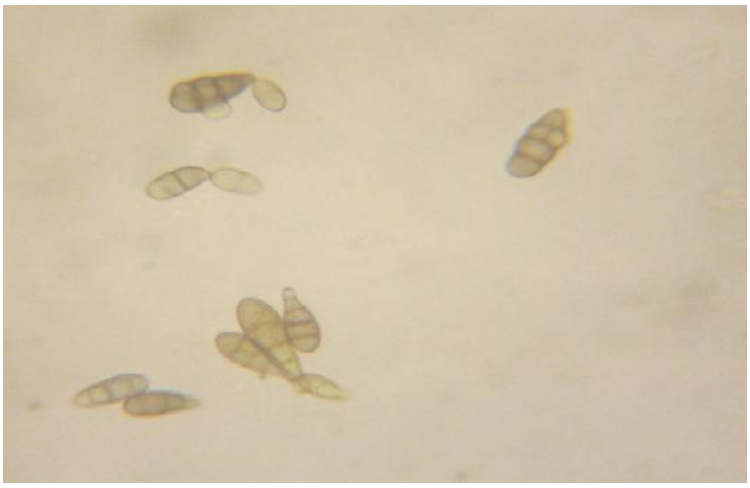

Figure 21. Conidia of *Alternaria* sp. under compound microscope (40 X)

4.2. Determination of occurrence of seed borne bacteria on selected imported hybrid rice varieties

Significant variations among the varieties were observed regarding germination and the incidence of seed borne bacteria on agar plate method (Table-2). Seed germination differs significantly among the varieties on agar plate methods. The highest seed germination (94%) was recorded in the variety Tia followed by Moyna (89%) and the lowest seed germination was observed in the variety Richer (87%). Two strains of a bacterium viz. *Xanthomonas oryzae* pv. *oryzae, Xanthomonas oryzae* pv. *oryzicola* were detected and identified from rice seed. The incidence of *Xanthomonas oryzae* pv. *oryzae* varied from 12.25% to 15.25 %. The highest incidence of *Xanthomonas oryzae* pv. *oryzae* was observed in Moyna (15.25%) followed by Richer (13.75%) and the lowest incidence was

recorded in Tia (12.25%). The highest incidence of *Xanthomonas oryzae* pv. *oryzicola* was observed in Richer (5.37%) and the lowest incidence was observed in Moyna (3.62%) which was statistically identical with Moyna (3.62%).

Table 2: Occurrence of seed borne bacteria on selected imported hybrid rice varieties

Hybrid rice varieties	% Seed germination	% Pathogen incidence	
		Xanthomonas oryzae* pv. *oryzae	***Xanthomonas oryzae* pv. *oryzicola***
Tia	94.00 a	12.25 c	4.25 b
Moyna	89.00 b	15.25 a	3.62 b
Richer	87.00 c	13.75 b	5.37 a
$LSD_{(0.05)}$	0.8152	0.576	0.968
CV%	0.52	2.42	12.66

4.3 Identification of bacteria

Identification of bacteria was done by conducting studies on cultural, morphological and biochemical features of the bacteria as per standard microbiological procedures.

4.3.1 Colony morphology of bacteria on different media

Morphological test was carried out on the basis of colony color on NA medium. Colonies of *Xanthomonas oryzae* are fairly slow-growing, usually pale-yellow, round, smooth, entire, domed and mucoid. (Table 3 and Figure. 22).

Circular, flattended, or slightly raised, yellow to bright yellow colour, mucoid colonies were found on YADC medium (Table 3 and Figure. 23).

Bacteria showed very poor growth with light yellow to slight blue, mostly circular, small, flatended, mucoid colonies were found on SX medium (Table 3 and Figure. 24).

Table 3. Cultural characteristics of *Xanthomonas oryzae* on different growth media

Media	Colony characters

	Colour	**Shape**	**Appearance**
NA	Yellow to orange	Circular	Mucoid, convex
YDCA	Yellow to bright yellow	Circular	Mucoid, flattended
SX	Light yellow to slightly blue	Irregularly circular	Mucoid, raised

Figure 22. Cultural characteristics of *Xanthomonas oryzae* on NA medium

Figure 23. Cultural characteristics of *Xanthomonas oryzae* on YDCA medium

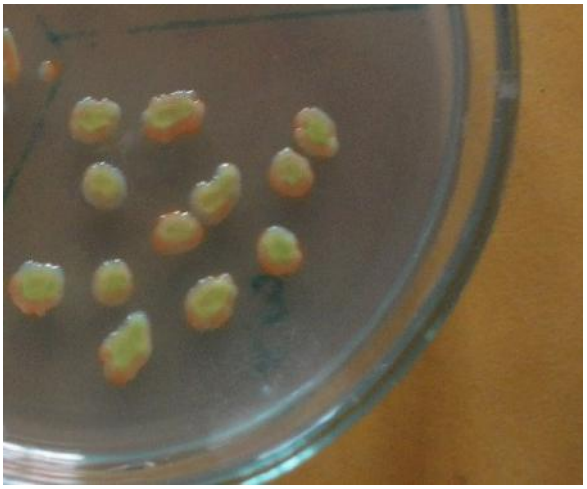

Figure 24. Cultural characteristics of *Xanthomonas oryzae* on SX medium

4.3.2. Morphological characters

Under compound microscope at 10 X magnification with oil immersion, bacteria were rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red colour) and capsulated. (Figure. 26)

In reaction with 3% KOH, mucoid thread was lifted from the glass slide with the loop (Figure. 27), thus the test was positive ie. the bacteria were gram negative that supports the results of grams staining test.

4.3.3 Identification of isolated bacteria by different biochemical tests

The biochemical tests performed for the identification of the isolated bacteria and different test results are presented in Table 4.

Both isolates were tested in starch hydrolysis and the speice was showed positive result (Figure 28. A) *i.e.* a clear zone appeared within 10 seconds after adding Indols Iodine.

The Isolates of Bacteria were tested with H_2O_2 (Hydrogen Peroxide) as described in materials methods. It was observed that both isolates of bacteria were formed bubbles within a few seconds indicted a positive (Figure 28. B) result for catalase.

Both the isolates were tested in Oxidase where the filter paper soaked with oxidase reagent it gave positive reaction. After 25-30 seconds blue color (Figure 28. D) was developed in the filter paper.

The Isolates of bacteria were tested with pectiolytic test described in materials methods. It was observed that 5 days after incubation of bacteria with potato slices, the potato slices became rotten and the result indicated positive (Figure 28. C).

The Isolates of bacteria were tested Simmon's citrate agar slants as described in materials methods. After incubation at 30^{o}C for 24 hours both isolates showed (Figure 28. F) positive results by changing of the green bromothymol blue.

After incubation with gelatin liquefaction, tubes were kept in the refrigerator for 20 minutes and liquefaction was observed (Figure 28. E).

Table 4. Morphological and biochemical characteristics of isolated bacteria

Biochemical test	***Xanthomonas oryzae* pv. *oryzae***	***Xanthomonas oryzae* pv. *oryzicola***
Starch hydrolysis test	+	+
Catalase test	+	+
Oxidase test	+	+
Gilatine liquifection test	+	+
Citrate utilization test	+	+
Pectiolytic test	+	+

Figure 25. Bacterial ooze on rice seed surface in NA method

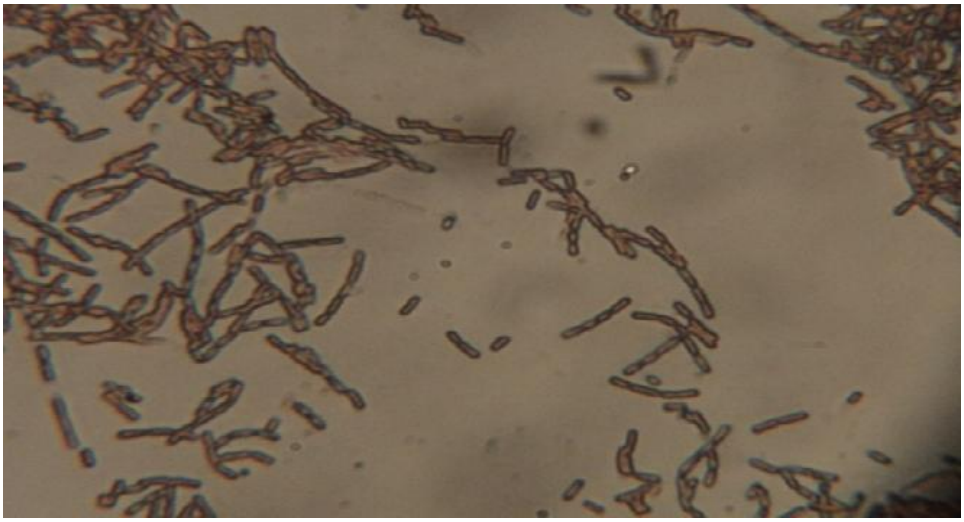

Figure 26. Gram staining of bacteria showing gram negative, pink color, chained rod shaped characteristics

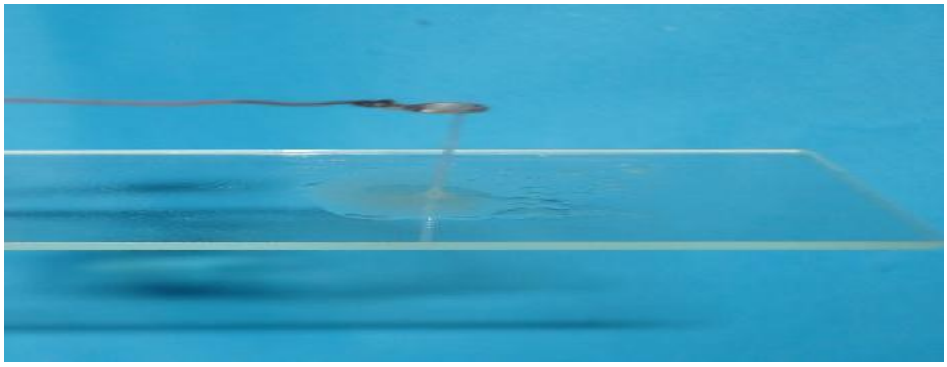

Figure 27. Gram reaction with 3% KOH test showing positive reaction.

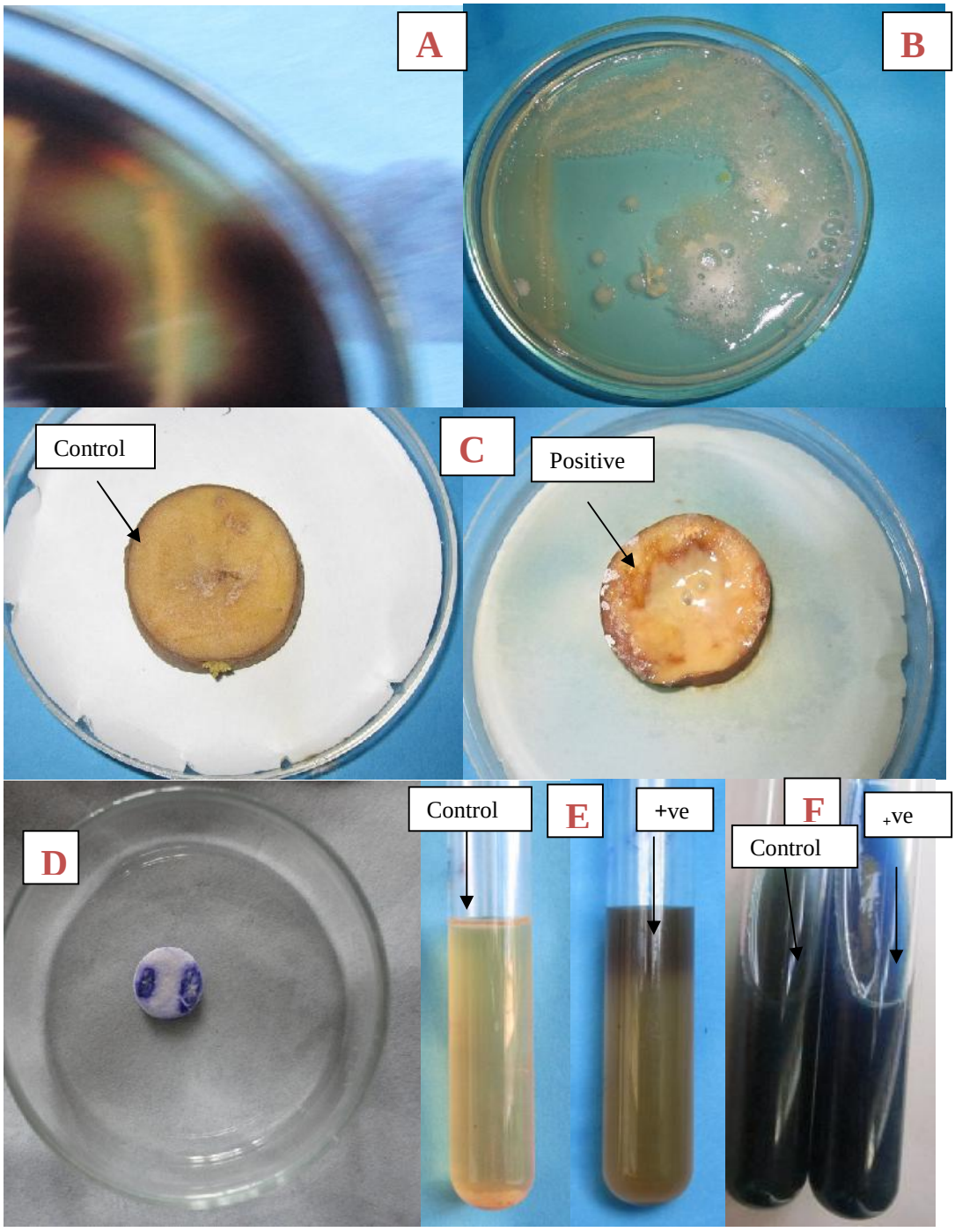

Figure 28. Different biochemical test

A.Starch hydrolysis test (positive)

B.Catalase test (positive)

C. Pectolytic test

D. Oxidase test (positive)

E.Gelatin lequefication test

F. Simmon's citrate agar test

4.3.4 Pathogenicity test

Characteristics symptoms were observed on rice leaves after 20 days of inoculation. Bacterial leaf blight (BLB) is characterized by wavy elongated lesions, which developed along the leaf margins. They start as small water-soaked stripes from the tips where water pores are found and rapidly enlarge in length and width, forming a yellow lesion with a wavy margin along the lead edges (Figure 30. A). Later on, diseased areas turned white to grey. These lesions developed on both sides of the leaf (Figure 30. B).

Initial symptoms of (BLS) were small water-soaked, transparent interveinal to elongate streaks (Figure 31.A). The transparent streaks differentiated leaf streak lesions from those of *Xanthomonas oryzae* pv. *oryzae* that are opaque against the light. Finally the narrow, long, translucent lesions (Figure 31.B) were found on the leaf of rice seedlings. The narrow, long, translucent lesions may coalesce, forming large patches, and severely affected fields appear burnt.

Bacteria were reisolated from infected rice (BLB and BLS) leaves and identified with the help of growth on YDCA and SX medium.

4.4 Comparative study of germination in different methods

Seed germination (%) varied from 94% - 97%, 89%-94%, 93% - 96%, and 94% - 96% in blotter method, agar plate method, water agar test tube method, and rolled paper towel method, respectively (Fig. 29). In all cases the maximum germination percent was recorded from the variety Tia followed by Moyna except in rolled paper towel method. And the minimum germination percent was recorded from Richer in all method except rolled paper towel method.

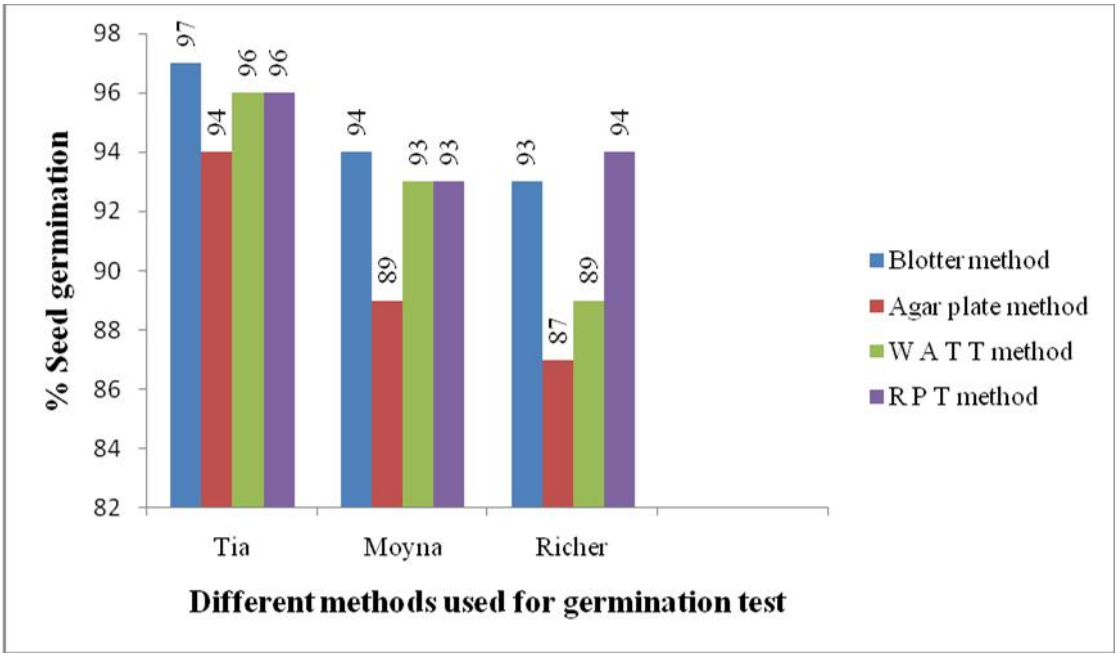

Figure 29. Comparative study of germination (%) in different methods

Figure 30. Symptom of bacterial leaf blight in pathogenicity test

A. BLB at early stage B. BLB at severe stage

Figure 31. Symptom of Bacterial leaf streak observed in pathogenicity test

A. BLS at early stage B. BLS at severe stage

4.5. Effect of seed borne pathogens on seedlings (water agar method)

Effect of seed borne pathogens on seedling of selected imported hybrid rice varieties were determined (Table 5). Significant variation was observed among the varieties regarding germination, abnormal seedling, diseased seedling and dead seed. Incase of germination, the highest germination was recorded in Tia (96%) followed by Moyna (93%) and the lowest was in Richer (89%). Incase of abnormal seedling, the number of abnormal seedlings was highest in Richer (7.90%) and the lowest was recorded in Tia (4.13%).

Diseased seedling varied from 9.11% to 13.45% and the highest percent of diseased seedlings was recorded in Richer (13.80%) and the lowest percent of diseased seedlings was observed in Tia 9.11%. Dead seed ranged from 3.25% to 10.25%. The highest percent of dead seed was recorded in Richer (10.25%) and the lowest percent of dead seed was observed in Tia (3.25%).

Table 5. Effect of seed borne pathogens on seedlings of imported hybrid rice varieties

Treatments	% Germination	%Abnormal seedling	%Diseased seedling	%Dead Seed
Tia	96.00 a	4.13 c	9.11 c	3.25 c
Moyna	93.00 b	7.48 b	11.72 b	6.50 b
Richer	89.00 c	7.90 a	13.45 a	10.25 a
$LSD_{(0.05)}$	0.9571	0.1094	0.2119	0.9571
CV%	0.59	0.97	1.06	8.29

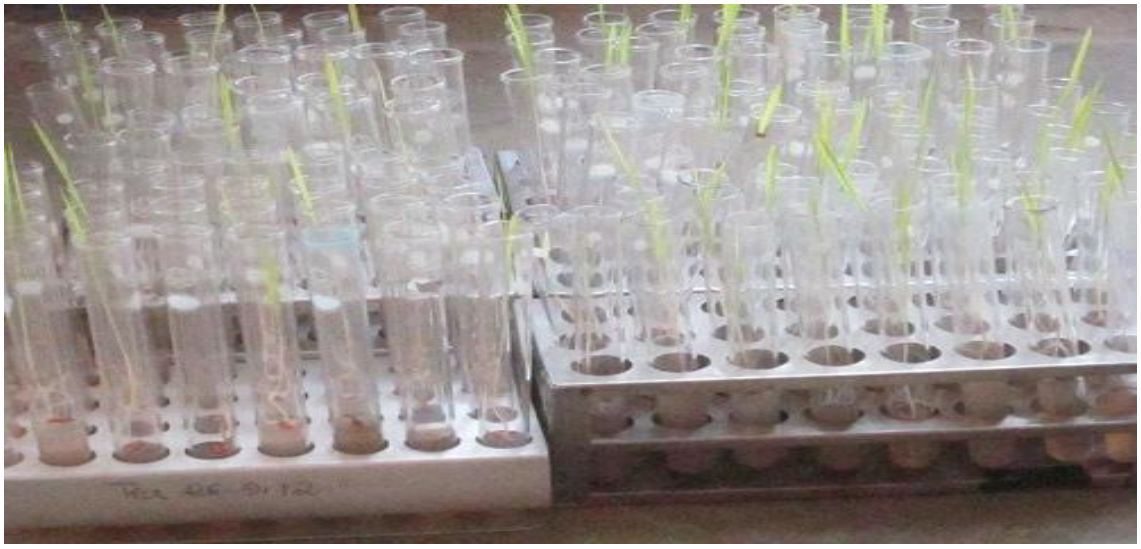

Figure 32. Seedling symptom test on water ager test tube method.

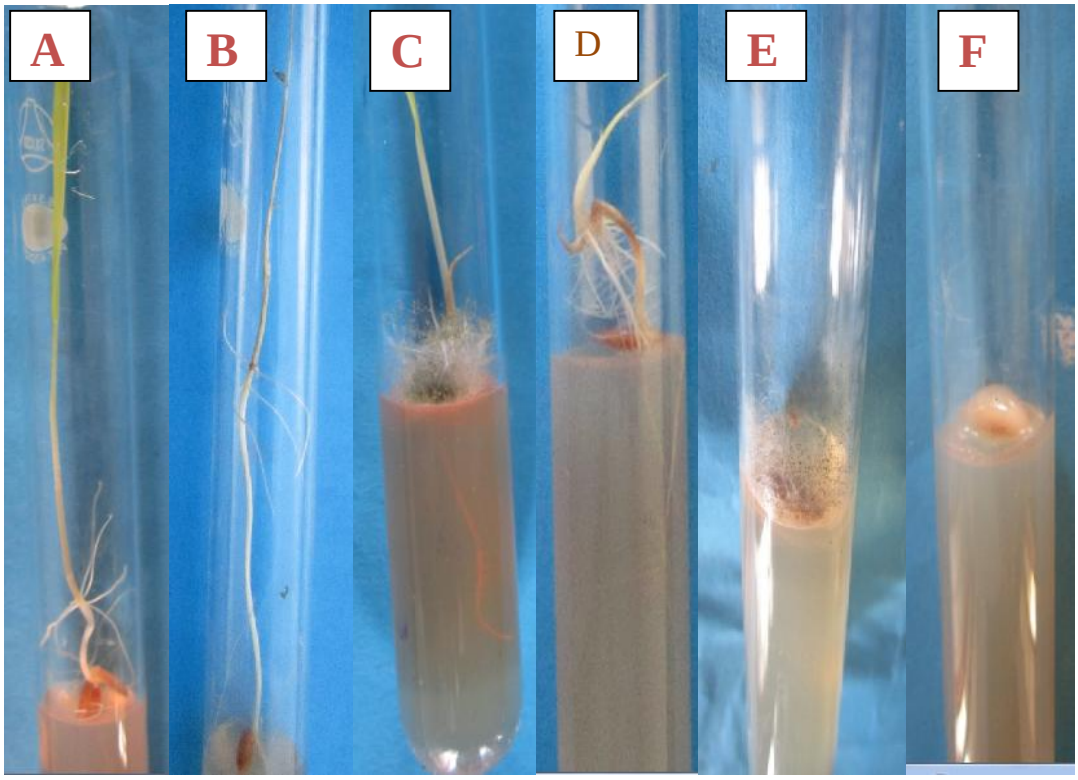

Figure 33**.** Seedling symptoms on water ager test tube method.

A. Normal seedling

B. Diseased seedling

C. Seedling with fungus

D. Abnormal seedling

E. Dead seed with seed borne fungus

F. Dead seed with bacterial ooze

4.6 Effect of seed borne pathogens on seedling vigor (Rolled paper towel method)

Effect of seed borne pathogens on seedling vigor of selected imported hybrid rice varieties were determined and significant results found regarding germination, diseased seedling, dead sead, seedlings weight, root length, shoot length and vigor index (Table 6). The highest seed germination percentage was recorded in Tia (96%) and the lowest germination percent was recorded in Moyna (93%). Percent of diseased seedlings varied from 6.36% to 13.80% where the highest value was recorded in Richer (13.80%) and the lowest value was recorded in Tia (6.36%). Incase of dead seed highest value was counted in Richer (12.75%) followed by Moyna (10.75%) and the lowest value was counted from Tia (5.75%). Seedling weight ranged from 0.049 g to 0.051 g where the highest value was counted from Tia (0.051 g) and the lowest from Richer (0.049 g). But there is no significant difference of seedling weight among three selected imported hybrid rice varieties. The highest root length was recorded in Tia (14.98 cm) which was statistically similar with Moyna (14.88 cm) and the lowest root length was recorded in Richer (13.13 cm). Shoot length varied from 7.47 cm to 9.05 cm. The highest shoot length was recorded in Moyna (9.05 cm) which was statistically similar with Tia (9 cm) and the lowest shoot length was recorded in Richer (7.475 cm). Incase of vigor index the maximum vigor index was recorded in Tia (2260) and the minimum vigor index was observed in Richer (1797).

Table 6. Effect of seed borne pathogens on seedling vigor of selected imported hybrid rice varieties

Treatments	Germination (%)	Diseased seedlings (%)	Dead seed (%)	Seedling weight (g)	Root length (cm)	Shoot length (cm)	Vigor index
Tia	96.00 a	6.36 c	5.750 c	0.051 a	14.98 a	9.00 a	2260 a
Moyna	93.00 c	10.20 b	10.75 b	0.050 a	14.88 a	9.05 a	2135 b
Richer	94.00 b	13.80 a	12.75 a	0.049 a	13.13 b	7.47 b	1797 c
$LSD_{(0.05)}$	0.998	0.1895	0.8152	0.0573	0.2257	0.3046	22.58
CV%	0.61	1.07	4.83	1.10	0.90	2.08	0.63

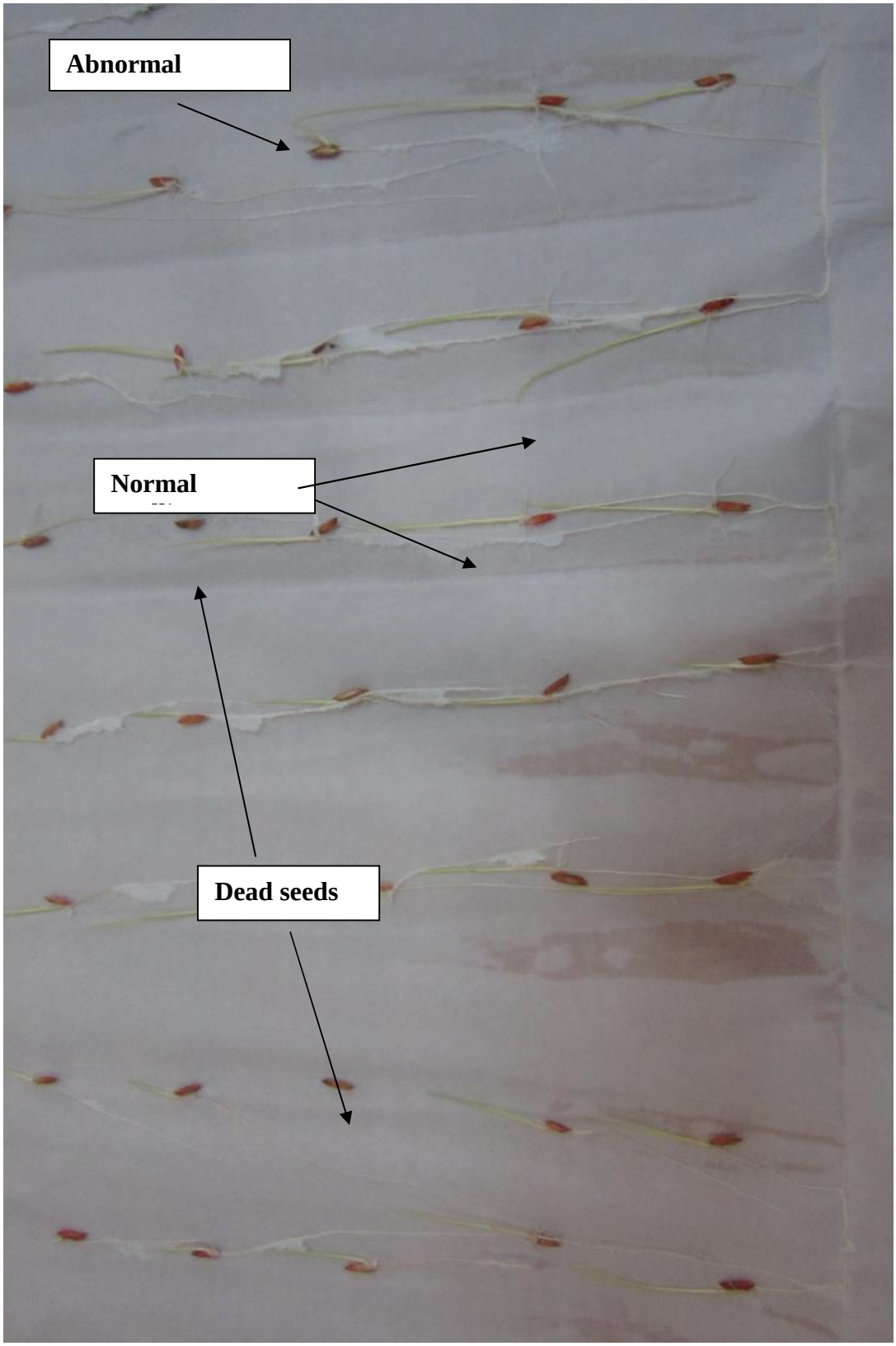

Figure 34. Seedling vigor test by rolled paper towel method.

CHAPTER 5
DISCUSSION

Selected imported hybrid varieties of rice seeds were evaluated to determine the seed health status of imported hybrid rice seed in Bangladesh. A considerable amount of seed borne pathogenic fungi and bacteria were detected by using blotter method, rolled paper towel method, nutrient agar plate method and water agar test tube method. In total 5 pathogens were associated with the collected seed samples as detected by blotter method. These incidence of different pathogens were found to vary individually and independently the hybrid varieties of rice seeds.

In blotter method, five genera of fungi viz. *Bipolaris oryzae, Aspergillus flavus, Aspergillus niger, Alternaria* spp. and *Fusarium moniliforme* were identified. A considerable number of seed borne fungi belonging to the genera *Aspergillus, Bipolaris, Alternaria* and *Fusarium.* Esuroso *et al.* (1975) reported that a wide range of fungi occurred on rice seeds in Nigeria. Imolehin (1987) observed that *B. oryzae* (*Drechslera oryzae*), *Curvularia lunata*, *Chaetomium* spp. *Trichoderma* spp. *Aspergillus* spp. and *Penicillium* spp. were isolated from twenty-two different rice cultivars from South West Nigeria. Bora and Gogoi (1993) isolated *Fusarium moniliforme* and *Bipolaris oryzae* from discoloured seeds from deep water rice, in Sialkot district in Pakistan (Ilyas and Javaid, (1995). Riaz and Ahmed, (1995) isolated *Helminthosporum* spp. *Curvularia*, *Fusarium* and *Aspergillus* from various seeds from North Southern provinces of Pakistan. Odebunmi–Osikanlu (1989) isolated *Fusarium moniliforme, C. lunata*. *H. oryzae, Rhynchosporium oryzae* from the six seed varieties (IRAT.110, COL.38, C22, TOX494-SLR, DJII-509 and F.H. 109). The incidence of *Bipolaris oryzae* significantly differs from variety to variety. The incidence of seed borne fungi significantly varied among different varieties. The highest incidence of *Aspergillus* spp., *Alternaria* spp., *Bipolaris oryzae* and *Fusarium moniliforme* were detected from Richer, Moyna, Moyna and Richer, respectively. Similar results were obtained by some other researcher like Archana and Prakash (2013); Ora *et al*. (2011) and Gopalakrishnan *et al*. (2010). Gopalakrishnan *et al*. (2010) conducted experiment to identify the seed borne pathogen associated with rice seed and recorded 8 genera of fungi viz. *Alternaria, Aspergillus, Bipolaris, Chaetomium, Curvularia, Fusarium, Sarocladium* and *Trichoderma* comprising twelve species. Among them, the most predominant one was *Bipolaris oryzae* which was associated with 58.89% seed samples followed by *Alternaria padwickii* (52.96%). From the present study it was

observed that presence of seed borne fungi in seed reduced the germination percent which is supported by (Naeem Khalid *et al*. (2001); Islam and Borthakur (2012) and Islam *et al*. (2012). Fungi reduced seed germination. *Fusarium* is known to invade the seed coat, endosperm and embryo resulting in failure in germination. The role of *Fusarium* inhibiting germination has been reported by Utobo *et al*. (2011). The adverse effect of *Aspergillus* on the germination of cereals has been reported in the recent years (Kanujia and Singh, 1975). Species of *Aspergillus* although reduced as surface contaminant were also responsible for production of aflatoxins and also been known to deteriorate rice grain (Imolehin, 1987). Bora and Gogoi (1993) reported that *F moniliforme* and *B. oryzae* reduced the germinablility of seeds. Islam *et al*. (2012) examined ten rice cultivars grown in non saline tidal zones of Patuakhali district were examined to identify seed-borne fungi and their effect on germination. They found that seed germination is decreased with increased the seed infection regardless of the rice cultivars tested. High negative significant correlation was obtained between all isolated fungi and seed germination in the laboratory for all seed samples tested.

Significance variation among the varieties was observed regarding the incidence of seed borne bacteria. Two strains of a bacterium viz. *Xanthomonas oryzae* pv. *oryzae, Xanthomonas oryzae* pv. *oryzicola* were isolated and identified from rice seed. Bhutta and Ahmed (1994), Xie *et al.* (1999), Jalaluddin *et al*.(1998) Agarwal *et al.* (1989) also stated the presence of *Xanthomonas oryzae* pv. *oryzae, Xanthomonas oryzae* pv. *oryzicola* in rice seed. Xie *et al.* (1999) conducted a field survey in the Zhejiang province of China (subtropical) and Lunzon island of Philippines (tropical) during 1993-98. They were screened about two hundred and eighty pathogenic bacterial isolates from over 3500 isolates associated with rice seeds from 116 seed samples collected in the subtropics and 129 seed samples from the tropics. Eleven bacterial species (*Acidovorax avenae, Burkholderia glome, Erwina chrysanthelne* pv. *oryzae, Pantoea agglomerans, Pseudomonas fuscovaginae, Xanthomonas oryzae* pv. *oryzae, Xanthomonas oryzae* pv. *oryzicola, Pseudomonas aeruginosa, P. fluorscens, P. fulva* and *P. putida*) were differentiated by bacteriological and numeric taxonomy (Biology) methods.

At the present study, it was recorded that the most prevalent bacteria is *Xanthomonas oryzae* pv. *oryzae* over *Xanthomonas oryzae* pv. *oryzicola* which supports the findings of Bhutta and Ahmed (1994) who reported that maximum seed infection due to

Xanthomonas oryzae pv. *oryzae* was 11% and 12% in variety IRRI-6 at Lahore and Hydrabad, respectively.

In present study *Xanthomonas oryzae* produced Circular, flattended, or slightly raised, yellow to bright yellow colour, mucoid colonies were found on YADC medium Jabeen *et a.l* (2012) observed that *Xanthomonas oryzae* pv. *oryzae* produced yellow circular, smooth, convex and viscous bacterial colonies on Yeast extract-dextrose-$CaCO_3$ medium (YDCA) after 48-72 hrs of incubation at 28^o C. In SX medium *Xanthomonas oryzae* pv. *oryzae Xanthomonas oryzae* pv. *oryzicola* grow poorly with light yellow to slight blue, mostly circular, small, flatended, mucoid colonies were found on SX medium. Both isolates showed positive in starch hydrolysis test, catalase, oxidase, gelatin liguifection test, citrate utilization test and pectiolytic test. Similar results have been reported by Schaad (1980) and Gerhardt (1981). A similar finding was reported by Lin *et al.* (1994). In case of both diseases viz. BLB and BLS developed characteristics symptoms having varied lesion length and size. Kauffman and Rao (1972) have also reported differential interactions between the isolates and the cultivars even without major gene resistance.

The maximum germination was counted from blotter method and water agar test tube metod. Islam and Borthakur (2012) showed that percent incidence of seed mycota was higher in agar plates as compared to blotter method. This may be attributed to the ability of agar plates to support growth of various fungal species, and due to the higher osmotic potential of PDA medium that showed its substrate efficiency for promoting the colonising rates of both external and internal seed fungal types. Further, species of *Fusarium* are known to produce phytotoxins which probably interfere with germination (Ellis, 1971; Neergard, 1977; Suryanarayana, 1978; Kanapathipillai and Hashim, 1982).

In water agar test tube method highest germination (96%), minimum number of abnormal seedling (4.13%), minimum number of diseased seedling (9.11%) and minimum number of dead seed (3.25%) were recorded from rice variety Tia. In all aspects Richer showed competively poor performance. The present findings support the findings of Guerrero *et al.* (1972) and Islam *et al*. (2000). Guerrero *et al.* (1972) showed that most of the seed borne pathogens cause abnormal seedlings and Islam *et al*. (2000) observed that highest lethal seed infection caused by seed borne pathogens.

Germination and seedling vigor of selected imported hybrid rice varieties showed significant variation. Seeds of Tia gave maximum germination and also yielded maximum number of seedling vigor. From this study it it had been found that seeds of rice variety Richer resulted comperetively poor seedling vigor. This may be attributed to improved genetic characteristics. The present findings support the findings of Islam and Borthakur (2012) and Utobo *et al.* (2011). Islam and Borthakur (2012) reported that *Fusarium moniliforme*, *Rhizopus nigricans* and *Penicillium oxalicum* caused marked reduction in shoot length, whereas *Chaetomium herbasum* and *Fusarium moniliforme* caused marked reduction in root length. *Fusarium moniliforme, F. chlamydosporum* and *Aspergillus niger* caused reduction in vigour index.

The present study showed that hybrid rice seeds were infected by harmful pathogens and the pathogens were capable to cause diseases of rice and that may cause yield loss.

CHAPTER 6
SUMMARY AND CONCLUSION

The present study was conducted to isolate and identify the seed borne pathogens of selected imported hybrid rice varieties in Bangladesh and to determine their effect on germination and seedling vigor. The research work was carried out in the Seed Pathology Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207 during the period of January to December 2012. The experiment was carried

out according to the rules of International Seed Testing Agency (ISTA) with selected imported hybrid rice varieties namely Tia, Moyna and Richer.

In blotter method, five fungal species were identified. The identified fungi were *Bipolaris oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Alternaria* spp. and *Fusarium moniliforme*. The incidence of different seed borne pathogens was found to vary individually and independently among the hybrid varieties. In blotter method the highest incidence of *Bipolaris oryzae* (13.45%), *Aspergillus flavus* (12.90%), *Aspergillus niger* (9.55%), *Alternaria* spp. (2.17%) and *Fusarium moniliforme* (2.20%) were noticed in Moyna, Richer, Richer, Moyna, and Richer, respectively. *Alternaria* spp. was found only on Moyna and *Fusarium moniliforme* was found on Tia and Richer.

In agar plate method, the highest incidence of *Xanthomonas oryzae* pv. *oryzae* (15.25%) was observed in Moyna where as *Xanthomonas oryzae* pv. *oryzicola* (5.37%) in Richer. *Xathomonas oryzae* are fairly slow-growing, usually pale-yellow, round, smooth, entire, domed and mucoid on NA medium. Circular, flattended, or slightly raised, yellow to bright yellow colour, mucoid colonies were found on YADC medium. Both pathovars showed poor growth with light yellow to slight blue, irregularly circular, small, raised, mucoid colonies on SX medium. In Gram staining and KOH test both the strains of *Xanthomonas oryzae* gave gram negative reaction. Under compound microscope produced pink color, straight and curved rod with no particular arrangement. Both isolates were positive in catalase, oxidase, pectiolysis, utilization of citrate, starch hydrolysis and Gelatin liquefaction test.

The maximum germination was counted from blotter method and water agar test tube metod. In water agar test tube method the highest seed germination percentage (96%) was found in Tia. The maximum number of abnormal seedling (7.90%) was found in Richer. The highest diseased seedlings (13.45%) were recorded from Richer. The highest percentage of dead seed was recorded from Richer (10.25%).

In rolled paper towel method, the maximum number of seed germination (96%), highest vigor index (2260) and the minimum number of diseased seedlings (6.36%) were recorded from Tia. The maximum number of diseased seedlings (13.80%), dead seed (12.75%) and lowest vigor index (1797) were recorded from Richer.

High quality seed is not only important for increasing crop production but also proper establishment of sound seed industry in the country. Seed is a common carrier of plant pathogens. Pathogen free seed is the important input material in agriculture. The present experiment showed that a lot of seed borne pathogens were associated with imported hybrid rice seeds though the hybrid rice seeds were treated by fungicides. Seed borne fungi appeared may be due to improper management of rice seeds in storage. Hybrid rice seeds also yielded *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola* because seed treatment with fungicides are not able to eradicate bacterial inocula from seed. Considering the over-all findings it was revealed that the seed health status of imported hybrid rice seeds is not a satisfactory level. Farmers are therefore advised to collect the seeds from reliable source and check their rice seed health status before sowing in the main field.

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APPENDIX I

Composition of Media:

The compositions of the media used in this thesis work are given below: Unless otherwise mentioned all media were autoclaved t 121^0c for 15 minutes at 15 lb pressure.

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g

Agar	20.0 g
Distilled water	1000 ml

Potato Dextrose Agar (PDA)

Peeled Potato	200g
Dextrose	20g
Agar	17g
Water	1000ml

SX Agar

Potato starch (soluble)	10.0 gm
Beef extract (Dico)	1.0 gm
NH_4Cl	5.0 gm
K_2HPO_4	2.0 gm
Methyl violet 2B(1% in 20%ethanol)	0.4 ml
Methyl green (1% in water)	2.0 ml
Bacto agar	15.0 gm
Cyclohexamide	2.0 gm
Distilled water	1000ml

Yeast extract-dextrose-$CaCO_3$ Agar (YDCA) medium

Yeast extract	10.0 gm
D-glucose	20.0 gm
Bacto agar	15.0 gm
$CaCO_3$	20.0 gm
Distilled water	1000ml

Gelatin liquifection media

Peotone	5.0g
Beef extract	3.0g
Gelatin	120.0g
Distilled water	1000 ml

Simmon's citrate agar

Magnesium Sulfate	0.2 gm

Sodium citrate	2.0 gm
NaCl	5.0 gm
Dipotassium Phosphate	1.0 gm
Monopotassium Phosphate	1.0 gm
Bromothymol Blue	0.08 gm
Distilled Water	1000ml
Agar	20.0 gm

Starch hydrolysis media and reagent:
Culture medium

Nutrient broth (Difco)	8.0 g
Soluble potato starch	10.0 g
Bacto agar (Difco)	15.0 g
Distilled water	1000 ml

Reagent (Lugol's iodine)

Iodine	5.0 g
Potassium iodide	10.0 g
Distilled water	100 ml

Preparation of Reagents

Gram Stain Solution

a. Stock Crystal violet

Crystal violet	10gm
Ethyl alcohol (95%)	1000ml

b. Stock oxalate

Ammonium Oxalate	1gm
Distilled Water	1000ml

Crystal violet working solution: 20 ml of solution no. a mixed with 80 ml solution no. b. Additional dilution was made when desired.

c. Lugol's Iodine Solution

Iodine Crystal	1gm

Potassium Iodide	2gm
Distilled water	300.0 ml

Dissolved completely in 10 ml of distilled water, and then added to distilled water to make 300 ml. Stored in amber bottle.

KOH solubility test

3% aqueous solution of KOH was prepared from the KOH granules.

Catalase test

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution.

Oxidase test

1% aqueous solution of N,N,N,'N-tetramethyl-p-phenylene dihydrochloride.