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Biological Characterization and Management of Rhizoctonia oryzae-sativae Causal Agent of Aggregate Sheath Spot Disease of Rice

Jahan, Suriya Bilkis

University of Rajshahi

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A Thesis Submitted to the University of Rajshahi for the Degree of Doctor of Philosophy in Botany

By

Suriya Bilkis Jahan

Mycology and Plant Pathology Laboratory
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Roll no: 10504
Registration no: 0022
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University of Rajshahi
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DEDICATION

This piece of work is dedicated to my dear husband and beloved sons Md. Ishmum Tasnim Dibbo and Md. Faiyad Tasnim Kabbo

DECLARATION

I hereby declare that this thesis is my own effort and work and it has not been submitted to anywhere for any other degree. This work was done under the guidance of Dr. Mohammad Shahidul Alam, Professor, Department of Botany, University of Rajshahi, Rajshahi, Bangladesh and Dr. Md. Ansar Ali, Chief Scientific Officer and Head, Plant Pathology Division, Bangladesh Rice Research Institute, Gazipur, Bangladesh.

October, 2014
Rajshahi University

(Suriya Bilkis Jahan)
Candidate

CERTIFICATE

This is to certify that the thesis entitled **"Biological Characterization and Management of *Rhizoctonia oryzae-sativae* Causal Agent of Aggregate Sheath Spot Disease of Rice"** submitted by Suriya Bilkis Jahan, Roll No. 10504, Registration No. 0022 (Session: 2010-2011), Department of Botany, University of Rajshahi, Bangladesh, embodies original work done by her under our guidance and supervision for the degree of Doctor of Philosophy.

Supervisors

(Dr. Md. Ansar Ali)
Chief Scientific Officer and Head
Plant Pathology Division
Bangladesh Rice Research Institute
Gazipur-1701
Bangladesh.

(Dr. Mohammad Shahidul Alam)
Professor
Department of Botany
University of Rajshahi
Rajshahi-6205
Bangladesh.

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ABSTRACT

Plant pathogenic fungus *Rhizoctonia oryzae-sativae* (teleomorph *Ceratbasidium oryzae-sativae*) caused aggregate sheath spot disease of rice. The disease is considered as minor on rice. However, in rice–wheat/potato cropping system the disease has emerged in higher incidence in North-West region of Bangladesh. Research studies of *R. oryzae-sativae* in particular are still limited hence very little information concerning the fungus is available in Bangladesh. Therefore, the present study was undertaken with aims to generate preliminary information on the population biology and structure of *R. oryzae-sativae.* Perhaps this is the first comprehensive report in which thirty isolates of *R. oryzae-sativae* collected from rice fields of ten different districts of Bangladesh. Multidisciplinary characterization approach or technique viz. culture morphology, virulence test, somatic compatibility groups (SCGs) and DNA fingerprinting were adopted. Isolates were confirmed using *R. oryzae-sativae* specific diagnostic markers in which a single band of 1200 bp was amplified. Data analysis of the mycelial growth rate separated the isolates into two distinct groups relatively slow and faster. Pathogenicity test on cultivar BR11 revealed that all the tested isolates were pathogenic and found significant correlation between mycelial growth rate and relative lesion height (RLH). There were 27 SCGs from 30 isolates indicated 90% diversity among the isolates. Variable number tandem repeat (VNTR) and Repetitive-element Polymerase Chain Reaction (Rep-PCR) were conducted using MR, RY, GF and BOXA1R primers respectively. DNA bands of four primers ranged from 0.25 to 2.21 kb. A combined dendrogram was constructed by NTSYS-pc software (2.20 e) which separated the isolates into three groups at 69.6% similarity level. Among them, all isolates placed in two major clusters except isolate RA-1, which placed in cluster group III. Fast growing isolates have been placed in Group II, while slow growing isolates in cluster group I. The

similarity coefficient values of the dendrogram profile ranged from 0.36 to 0.98 with an average of 0.67. The effects of eight fungicides with six concentrations were tested *in vitro* on mycelial growth of *R. oryzae-sativae*. *R. oryzae-sativae* found sensitive to all the fungicides tested. Carbendazim showed strong activity against the pathogen with the lowest LD (lethal dose) 90 values (0.8 ppm) and LD50 (0.1 ppm) *in vitro*. All the fungicides significantly reduced disease development in field trials but only Carbendazim, Tebuconazole and Trifloxystroin-tebuconazole increased rice yield. However, aggregate sheath spot reduced yields by up to 14.74% under Bangladeshi conditions. The maximum growth of the pathogen on PDA measured at 6.0-9.0 pH level. The highest mycelial growth was recorded in dextrose (0.47 mm/hr) followed by sucrose (0.44 mm/hr). The best mycelial growth rate of *R. oryzae-sativae* found in peptone (0.43 mm/hr) followed by yeast (0.37 mm/hr). Among the tested phytoextracts, garlic and henna found most effective against *R. oryzae-sativae.* None of the tested germplasm or cultivars found immune to aggregate sheath spot disease. T. Aus germplasm Akuee, Kalosoti and T. Aman varieties Kurchi jira, Kurchi binni, Shabichi dhan, BRRI dhan39, BRRI dhan40, BRRI dhan46 found moderately susceptible while no susceptible variety found in Boro season. Multidisciplinary characterization of *R. oryzae-sativae* revealed that isolates of Bangladesh origin is not genetically identical.

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ABBREVIATIONS

AEZ	:	Agro- ecological zone
AFLP	:	Amplified fragment length polymorphism
ASS	:	Aggregate Sheath spot
BRRI	:	Bangladesh Rice research Institute
DI	:	Disease incidence
DNA	:	Deoxyribo nucleic acid
DS	:	Disease severity
EDTA	:	Ethylene di amine tetra acetic acid
GRS	:	Genetic Resources and Seed
ha	:	Hectare
ITS	:	Internal transcribed spacer
Kg	:	Kilogram
LD	:	Lethal dose
LSD	:	Least significant difference
MLMs	:	Multilocus microsatellites
MOP	:	Muriate of potash
MVSP	:	MultiVariate Statistical Package
PCR	:	Polymerase Chain Reaction
PCoA	:	Principle co-ordinate analysis
PDA	:	Potato dextrose agar
PD	:	Potato dextrose
PI	:	Panicle initiation
ppm	:	Parts per million
RAPD	:	Randomly amplified polymorphic DNA

RCBD	:	Completely randomized block design
Rep-PCR	:	Repetitive-element Polymerase Chain Reaction
RFLP	:	Restriction fragment length polymorphism
r DNA	:	Ribosomal DNA
RLH	:	Relative Lesion Height
SAHN	:	Sequential agglomerative hierarchical and nested clustering
SCG	:	Somatic compatibility group
SDS	:	Sodium dodecyl sulphate
SES	:	Standard Evaluation system
SLMs	:	Single locus microsatellites
SSR	:	Simple sequence repeats
STMS	:	Sequence tagged microsatellite sites
SWD	:	Sterile distilled water
TAE	:	Tris- acetate-EDAT
TSP	:	Triple super phosphate
TGW	:	Thousand grain weight
UN	:	United Nations
UPGMA	:	Unweighted pair group method with arithmetic mean
USDA	:	United States department of agriculture
UV	:	Ultra violate
VNTR	:	Variable Number Tandem Repeat
WA	:	Water agar

General Introduction

Rice (*Oryza sativa* L.) is one of the most important food crop and a primary source of food for more than half of the world's population (Khush, 2005). According to the United Nations (UN) estimates, the current world population is 6.1 billion and expected to reach 8.0 billion by 2025. Most of this increase (93%) will take place in the developing world. Rice is the predominant dietary energy source for 17 countries in Asia and the pacific, 9 countries in North and South America and 8 countries in Africa. Rice provides 27% of the world's dietary energy supply and 20% dietary protein intake in the developing world (www. fao.org/newsroom /EN/focus/2004/36887/index.html). The nutritional value of rice per 100 g is 365 kcal, which is the source of 80 g carbohydrate, 7.13 g protein and so on (www.nal.usda.gov/finc/foodcomp/search). Now-a-days economic growth, food security and poverty reduction in Asia largely depend on adequate and stable supply of Rice. Almost 92% of total rice produced in Asia (McDonald, 1996), where 3.1 billion people consumed rice as staple food. The current population of Bangladesh is 163.65 million (www.inde xmundi.com/bangladesh/demographics _profile.html), where rice is the staple food for 135 million people (www.knowled gebank-brri.org/riceinban.php). The demand of rice in Bangladesh is increasing with the increasing of population over time. In Bangladesh Average clean rice production is 2.27 ton/hector, 1.92 ton/hector and 3.90 ton/hector in Aman, Aus and Boro respectively in Bangladesh (Anon, 2012), which is relatively lower than the other rice growing countries of the world. Low production of rice in unit area is attributed to various biotic and abiotic factors. Rice disease is one of the most important limitations for increasing rice yield. Different groups of micro-organisms are involved to cause diseases on rice such as virus, bacteria, fungi and nematodes. So far, 31 rice diseases have been identified; ten of them are considered as major (Miah and Shahjahan, 1987; Anon, 1994). Among them five

diseases, like, tungro, bacterial blight, Sheath blight, blast and ufra have been considered as most important because of their wide spread occurrence and significant damage potential. Sheath diseases of rice are widely observed in many rice growing regions of the world. Aggregate Sheath Spot (ASS) caused by *Rhizoctonia oryzae-sativae* (Sawada) Mordue. It is usually considered as a minor disease but it can be a very aggressive disease of rice (Lanoiselet *et al*., 2005b). Aggregate sheath spot regarded as minor until the disease become increasingly important in temperate rice growing regions such as- California and south eastern Australia. The potential yield loss caused by this disease was unknown until recent research clearly demonstrated the potential threat aggregate sheath spot represent of temperate rice industries. Worldwide, aggregate sheath spot has received little attention compared with the other diseases caused by *Rhizoctonia*. In the tropical and sub-tropical regions including Bangladesh, aggregate sheath spot could be a serious disease of rice. In Bangladesh, until 1988 there was no report of *Rhizoctonia oryzae* and *Rhizoctonia oryzae-sativae* except *Rhizoctonia solani*, which is the most common pathogen of rice sheath blight disease. A survey of rice crops conducted by Shahjahan *et al*. (1988) revealed that aggregate sheath spot caused by *R. oryzae-sativae* was prevalent in the Bangladesh Rice Research Institute (BRRI) farm during all the three rice growing seasons. It was observed on both local and modern cultivars and advanced breeding lines.

R. oryzae-sativae was first described by Sawada in Taiwan. He named the fungus *Sclerotium oryzae-sativae.* Mordue (1974) transferred it to *Rhizoctonia* as *Rhizoctonia oryzae-sativae* (Sawada) Mordue. Recently, stem rot of rice caused by *Sclerotium hydrophillum* was reported in Myanmar (Aye *et al*., 2009) which was then initially identified as *R. oryzae-sativae.* First comprehensive review of sheath spot and aggregate sheath spot of rice was reported in Australia by Lanoiselet *et al.,* 2007. By the first quantifying potential yield loss caused by aggregate sheath spot of rice under Australian condition is as high as 20.3% (Lanoiselet *et al*., 2005b).

The importance of aggregate sheath spot was enhanced with the introduction of the semi-dwarf cultivars and has increased with the practice of incorporating residue. The most effective means of reducing aggregate sheath spot is to limit the carry over inocula as is the case with stem rot residue management.

R. oryzae and *R. oryzae-sativae* have been recognized as causal agents of rice sheath blight complex in some geographic locations. Knowledge of the epidemiology of the pathogens and also the significance of each of pathogens is essential to implementing appropriate disease control measures. Accurate diagnosis of pathogen is also essential to ensure success in developing ASS resistant varieties. Bangladesh is still deficit in production of food grains. Globally the demand of rice is rising at an alarming rate primarily to population growth. The demand is being minimized by green revolution. The advances made in food production in developing countries due to the green revolution can be lost if proper attention is not given to plant diseases.

Symptoms of Aggregate sheath spot disease of Rice

Symptoms of aggregate sheath spot can be easily confused with the symptoms of sheath spot (caused by *Rhizoctonia oryzae*) and sheath blight (caused by *R. solani)*. *R. oryzae-sativae* causes lesions similar to those of sheath blight but they are smaller with distinct margins (Ou, 1985). In aggregate sheath spot first lesions appear on the rice plant near the water lines as water-soaked spots. Lesions are oval 0.5-4 cm. long and present grey-green to straw coloured centers surrounded by a brown margin and often have a thin, vertical, necrotic line spanning the centre from top to bottom. The spots often coalesce, giving the impression of an aggregation of concentric lesion bands. Under favourable conditions, the disease can progress upward and reach the upper leaf sheaths and even the panicle. Leaves of infected sheaths usually turn yellow and die. The fungus can sometimes infect the panicle rachis, resulting in grain sterility. *R. oryzae-sativae* can also infect the culms which may cause it to rot. This symptom rarely occurs in California

(Gunnell and Webster, 1984) but has been reported in India (Mukherjee *et al*., 1980). The pathogen is also thought to infect rice roots (Mordue, 1974).

Host range

R. oryzae-sativae has been recorded on *Oryza cubensis* Eckman ex Gotoh & Okura (perennial wild red rice), *Juncellus serotinus* (Rottoell) C. B. Clarke (tidalmarsh flatsedge) and *Zizania latifolia* (Grise.) Turez. ex Stapf (Manchurian wild rice) (Mordue, 1974).

Biology of the pathogen *Rhizoctonia oryzae-sativae*

Mycelium

On potato dextrose agar medium *R. oryzae-sativae* mycelium is colourless when young, later turning pale brown. The cells of hyphal tips measure 5-6.5 µm wide and up to 300 µm long. On older mycelium, the diameter of cells can very from 3.5-7 µm. Some isolates can produce moniloid cells (21 to 37 x 6 to 11 µm). Hyphal branches are constricted near there point of emergence, usually the middle of the cell. Hyphal branches are also septate, just above their point of origin (Mordue, 1974). Mycelium is superficial in culture. Maximum growth was observed in *R. oryzae-sativae* at 6-8 pH level.

Sclerotia

Sclerotial cells of *R. oryzae-sativae* are globose, white when young, then become brown when mature and their diameter can ranged from 0.5 to 2 mm due to their aggregation (Mordue, 1974). Sclerotia produced within rice tissue ranged from 360-1250 µm x 270-620 µm (Miller and Webster, 2001).

Basidiospore

The teleomorph of *R. oryzae-sativae* (*Ceratobasidium oryzae-sativae*) is known to occur on rice plants in the field in California as whitish hymenia present on the outside of infected leaf sheaths (Gunnell and Webster, 1987). Even though the teleomorph exists in the field, the disease thought to be mainly monocyclic (Miller

and Webster, 2001). Gunnell and Webster (1987) described the basidiospores of *C. oryzae- sativae* as globose to sub globose to broadly ellipsoid, occasionally ellipsoid, (8-)9-17(-21) x (8-)9-16 (-19) µm in size and smooth. They also described a method to produce fructification in the glasshouse. Rice plants were inoculated with a mixture of unhulled rice+rice hulls, colonised with *R. oryzae-sativae*, by adding the inocula on the surface of the water in which the plants were growing. The rice plants were misted every 2 hrs.

Nucleus

The rapid stain, Safranin O, reported by Bandoni (1979) can be used to rapidly determine the number of nuclei per cell. *R. oryzae-sativae* is binucleate.

Disease cycle, Epidemiology, and Pathogenicity

R. oryzae-sativae survives the over wintering season as sclerotia or mycelium present in the soil or in rice crop debris (Endo, 1931; Mordue, 1974; Gunnell, 1992; Rush, 1992; Miller and Webster, 2001). At the start of rice growing season, when rice fields are flooded, the hydrophobic sclerotia float on the water surface, germinate and infect the rice sheaths at, or just above, the water line. Epidemiological studies suggest that *R. oryzae-sativae* infect the rice plant early in the season without showing any symptoms (Kadowaki and Isota, 1993b) and the pathogen is present on the rice plant during the whole rice-growing season (Kadowaki and Isota, 1993a), even though the fungus can sporulate on rice plants, the aggregate sheath spot disease is believed to be predominantly monocyclic (Gunnell, 1986; Miller and Webster, 2001).

Ray and Pan (1989) studied the effect of temperature, soil moisture and soil pH on sclerotial germination of *R. oryzae-sativae*. The optimal conditions for sclerotial germination were at 30ºC and 75% soil moisture. Sclerotia germinated well when incubated at 20-30ºC, but germination was better between 30 and 40ºC. Up to 12 germ tubes were produced per sclerotium. When incubated in natural soils, a fungistatic effect was observed, resulting in delayed sclerotial germination, then

sudden sclerotial germination, reduced number of germ tubes and poor to nil colonisation. Several authors reported that isolates of *R. oryzae-sativae* were less aggressive than *Rhizoctonia oryzae* on rice plants (Shangzhi and Mew, 1987; Kim and Yu, 1990). Aggregate sheath spot is not favored by high nitrogen applications (Gunnel, 1986). Recent field trials suggest that aggregate sheath might be favored by potassium deficient soils (Williams and Smith, 2001).

Yield losses

In Australia, field trials showed that aggregate sheath spot caused yield losses as high as 20% (Lanoiselet *et al*., 2005b. In Uruguay, potential yield losses on cultivar Tacuari, ranging from 4% to 9%, have been attributed to aggregate sheath spot (Lanoiselet *et al*., 2007).

Disease management

Chemical control

A recent study (Lanoiselet *et al*., 2005b) found that pyraclostrobin and propiconazole were strong inhibitors of pathogen *in vitro* and that both fungicides reduced disease development in the field, but failed to increase rice yield. The fungicide Quadris (azoxystrobin) has been registered for the control of aggregate sheath spot in California. California rice-growers are currently advised to regularly monitor aggregate sheath spot progression up the leaf sheaths and to use Quadris if the disease threatens the upper sheath leaves (Webster and Greer, 2004). In Missouri, azoxystrobin and propiconazole are registered for the control of aggregate sheath spot.

Cultural practices

In California, chemical control and straw management practices aimed at reducing the carryover of *R. oryzae-sativae* inoculam are currently recommended practices (Lanoiselet *et al*., 2005a). Miller and Webster, (2001) demonstrated that straw management practices such as burning and bailing stubbles could reduce the number of *R. oryzae-sativae* sclerotia present in the soils but they also showed that disease

incidence was not always correlated with the number of sclerotia present in the seedbed. They reported a decrease in disease incidence when rice straw was incorporated into soil in autumn compared with late spring incorporation. Lanoiselet *et al.*, (2005a) studied the effect of burning stubble on the survival of laboratory-produced sclerotia of *R. oryzae-sativae*. Experiment revealed that even if a large proportion of sclerotia present on the soil were killed, many of them were survived the stubble burning regardless of whether it was a cold burn or a hot burn, and burning certainly did not eradicate the entire inoculam. They underlined the importance of managing rice straw to reduce the inocula and concluded that burning rice straw greatly contributed to keeping the disease at a relatively low level in Australian rice fields. *R. oryzae-sativae* has only been reported on rice, perennial wild red rice, tidalmarsh flatsedge and Manchurian wild rice (Mordue, 1974). So crop following a rice crop is likely to break the disease cycle of aggregate sheath spot.

Resistance

Gunnel and Webster (1984) attributed the increase in both incidence and severity of aggregate sheath spot in California to the introduction of semi-dwarf rice cultivars. In 1986, Gunnel reported the effect of cultural practices on aggregate sheath spot in California and stated that with the exception of cultivar S-201. Californian semi-dwarf cultivars were more susceptible than tall cultivars. A field trial in Australia in 2003-04 showed that Australian semi-dwarf cultivars were also more susceptible than a tall Australian cultivar (Lanoiselet *et al*., 2005b). Research from Uruguay suggests that varietal susceptibility to aggregate sheath spot also exists among Uruguayan rice cultivars and breeding lines (Lanoiselet *et al*., 2005b).

Gunnell (1986) reported that, under natural inoculation, time of maturity did not affect disease severity. It is not known if rice is more susceptible to *R. orizae-sativae* at a particular growth stage but symptoms do not appear before the late tillering (Gunnell, 1986) to heading stage (Kadowaki and Isota, 1995). Good levels

of resistance to aggregate sheath spot were found in *Oryza ruftpogon* and successfully transferred into long grain germplasm during the 1990s. (McKenzie *et al*., 1994).

Characterization by means of molecular genetics

Polymerase Chain Reaction (PCR) is defined as a molecular technique which allows the production of large quantities of a specific DNA from a DNA template using a simple enzymatic reaction without a living organism. It is a recently developed molecular biological procedure for *in vitro* amplification of DNA. Starting from a vary low amount of template DNA (mostly in the nanogram range), millions of copies of one or more particular target DNA fragments are produced which can be electrophoresed and visualized by staining or autoradiography. PCR is characterized by its high speed, sensitivity and selectivity. Its application in diverse variants and for many different purposes has opened up a multitude of new possibilities in molecular biology. One main reason for the versatility of the PCR technique is that any kind of primers can be chosen, depending on the purpose of the study. Based on variation in the base sequence of nucleic acids, different methods have been developed and different approaches have been pursued to characterize remotely related fungi, as well as distinguishing between closely related strains. A unique pattern of DNA fragment or polymerase chain reaction (PCR) products that can be used to distinguish the DNA of a specific individuals or strain. AFLP, RAPD, RFLP, SSR and VNTR are the examples of fingerprinting techniques. DNA Fingerprinting refers as a reproducible banding pattern created by a number of biochemical techniques of DNA isolation and/or amplification that clearly differentiate a genetic component in an individual, group or population. DNA of an organism contains the blueprint of its characteristics. Every human, lower animal, sexually reproduced plant has a characteristics phenotype physical appearance because each possesses unique hereditary composition. Since each living creature is unique, each has a unique DNA recipe. Using the techniques of DNA fingerprinting and PCR, scientists can

easily distinguish between closely related individual, map the location of specific genes along the vast length of DNA molecules in the cells or viral or fungal infection. DNA fingerprinting can be used to identify a fungus, bacterium or virus that is present on a rice seed or causing a disease. DNA fingerprinting also identifies the variety of rice. Discrimination between similar DNA samples at a species or subspecies level is provided by RFLP analysis. Often, ribosomal DNA (r DNA) is target regions for RFLP analysis. Only a few works has been done by the researchers in molecular level to study the characteristics of *R. oryzae-sativae*. Chaijuckam and Davis (2010a) studied the population structure of *R. oryzae-sativae* by using single locus microsatellites (SLMs) and multilocus microsatellites (MLMs). Taheri *et al*. (2007) characterized *R. oryzae-sativae* by using amplified fragment length polymorphism (AFLP) and found two groups from eleven Indian isolates of *R. oryzae-sativae*. Aye and Matsumoto (2010) were differentiated three types of *R. oryzae-sativae* by Repetitive-element Polymerase Chain Reaction (Rep-PCR).

This work continues to focus on developing a greater understanding of the biology of rice disease like Aggregate sheath spot that occurs in Bangladesh and to develop methods for control. Keeping this in mind the present project is undertaken the following objectives.

Research objectives

1. To understand the biology and genetic diversity of the pathogen.
2. To estimate yield loss by the aggregate sheath spot disease.
3. Screening rice cultivar, fungicide and integration to design better management strategies.
4. To examine the efficacy of different plant extracts on *R. oryzae-sativae*.

Materials and Methods

2.1 Collection, isolation, purification and preservation of *R. oryzae-sativae* isolates

Forty-five isolates of *R. oryzae-sativae* were collected randomly from different rice fields with aggregate sheath spot symptoms in different districts of Bangladesh. Out of these, three isolates each from ten districts were selected based on their geographical region (Table 1). Samples were cut into small pieces (approximately 5 x 5 mm per section) from the margin of the expanding lesion and soaked in sterile distilled water (SDW) for 2-3 minutes. Then surface sterilized by 0.6% sodium hypochlorite solution for 3 minutes and rinsed 3 times in SDW. Samples were then transferred on water agar (WA) plates and incubated 27°C. After three days, single hyphal tip were cut from the margin of the colonies showing mycelial characteristics of *Rhizoctonia* spp. and transferred Potato dextrose agar plates. A piece of each culture of *R. oryzae-sativae* isolates on PDA was placed on slant media in test tubes. The cultured tubes were kept at (25 ± 2°C) and refreshed after every 4 months.

2.2 Identification of the isolates

All isolates were identified as *R. oryzae-sativae* based on colony characteristics. The production of globose brown sclerotia on white to pale-brown mycelia on PDA after 2 weeks was diagnostic (Gunnell, 1992). The identity of 30 isolates were confirmed by the examination of morphological characteristics (the appearance of T-cells and constricted hyphae) (Mordue, 1974), bi-nucleate condition of cells. Bi-nucleate cells were observed under light microscope and characteristics of the isolates were matched with the key characteristics described by Sneh *et al*. (1991).

Table 1 : List of fungal isolates with their origin during 2010-2011 at different crop stage used in this study

District	Agro-ecological zone (AEZ)	Isolate code	Season	Crop stage	Cultivar	Lesion size(cm)
		DI-1	Aman	Flower	Malshira	1.4
Dinajpur	0 1	DI-2	Aman	Mature	BR 10	0.9
		DI-3	Aman	Flower	Bashphul	1.2
		TH-1	Aman	Mature	BR 4	0.8
Thakurgaon	0 1	TH-2	Aman	Mature	BR 7	1.6
		TH-3	Aman	Mature	BRRI Dhan 39	1.1
		RA-1	Aman	Boot	BRRI Dhan32	2.1
Rajshahi	11	RA-2	Boro	Max-T	BRRI Dhan 29	1.9
		RA-3	Boro	Flower	BR 18	2.3
		GA-1	Aman	Flower	BR 4	1.8
Gazipur	28	GA-2	Boro	Max-T	BR 2	1.3
		GA-3	Aus	Boot	BR 26	2.0
		NE-1	Boro	Mature	Balam	0.9
Netrakona	0 9	NE-2	Boro	Mature	BRRI Dhan 47	1.7
		NE-3	Boro	Mature	BRRI Dhan 28	2.0
		SH-1	Aus	Max-T	Shathi	2.7
Shrepur	0 9	SH-2	Aus	Max-T	IR-108	1.9
		SH-3	Boro	Flower	Chandan	2.3
		MY-1	Boro	Mature	BRRI Dhan 28	2.9
Mymensingh	0 9	MY-2	Aman	Mature	BRRI Dhan 40	1.8
		MY-3	Boro	Mature	BRRI Dhan 29	2.6
		NA-1	Aman	Flower	BRRI Dhan32	1.7
Narshingdi	0 9	NA-2	Aman	Flower	Bashpata	0.9
		NA-3	Aman	Flower	BRRI Dhan30	2.4
		TA-1	Boro	Mature	BRRI Dhan 28	2.8
Tangail	0 9	TA-2	Aus	Mature	Chapila	1.5
		TA-3	Aus	Mature	Muktahor	2.6
		JA-1	Aman	Mature	BRRI Dhan 40	2.4
Jamalpur	0 9	JA-2	Aman	Mature	BR 5	1.3
		JA-3	Aman	Mature	BRRI Dhan 49	2.0

2.3 Molecular characterization

2.3.1 Raising culture of *R. oryzae-sativae* for DNA extraction

i. Preparation of culture

Cultures of 30 isolates of *R. oryzae-sativae* were grown on PDA. Hyphal tip of 72 hours old purified cultures were transferred into 250 ml conical flask containing 50 ml potato dextrose (PD) broth and placed on electric shaker for four days at 120 rpm at 25°C following the procedure of Anon (1994). Four days old mycelia of *R. oryzae-sativae* from potato dextrose broth were filtered and squeezed to remove water using cheese cloth. The squeezed culture was blotted dry to remove moisture as much as possible. The squeezed and blotted, flattened mycelium of *R. oryzae-sativae* was folded into an aluminum foil paper and frozen at -20°C.

ii. Extraction of DNA from *R. oryzae-sativae*

DNA was extracted from 30 isolates of *R. oryzae-sativae* following the methods of Raeder and Broda (1985) and Anon (2001). Frozen mycelia were grounded to powder using pestle and mortar. An amount of 25 µg powdered mycelia were poured into 1.5 µl centrifuge tube and homogenized with 800 µl of extraction buffer (250mM NaCl, 100mM Tris-HCl, 100mM EDTA, all Sigma) by stirring on vortex mixer for lyses of the cells. An amount of 80 ml of 10% sodium dodecyl sulphate (SDS) was added to centrifuge tube containing powdered mycelia and extraction buffer and then mixed by inverting a few times and incubated for 30 minutes at 65°C in water bath. After incubation, 352 µl of 3 M sodium acetate (pH.5.2) was added into the centrifuge tube, mixed gently and kept in ice for 15 minutes to burst the cells and denature the protein. The mixture was then centrifuge in a refrigerated centrifuge (Hawkd15/05) at 12500 g at 4°C for 10 minutes and the water soluble supernatant were colleted and transferred into a new centrifuge tube. The previous tube containing much water insoluble impurities in the mycelial pellet were discarded. After that, Chloroform (500 µl) was added to the collected supernatant in new centrifuge tube and mixed thoroughly on vortex mixer. The mixture was centrifuged at 12500 g at 4°C for 30 minutes to precipitate

the protein and long chain polysaccharides from the DNA. The upper aqueous phase (the DNA solution) was collected into clean tubes very carefully with a pipette without disturbing the amphibathic natured protein interface. The organic phase layer beneath the protein interface was discarded.

iii. Precipitation and re-suspension of DNA

Precipitation of DNA was done following the procedure of Anon (2001). Two volumes of pure ethanol were added into the DNA suspension, kept on ice for 10 minutes to allow the DNA molecules to aggregate. The DNA was precipitated down to the bottom of the centrifuge tube as pellet by centrifuging at 12500 g at 4°C for five minutes. Then the supernatant was discarded using a wide bore pipette and preserved the centrifuge tube with the DNA pellet. An amount of 200 µl of 70% ethanol was added into the centrifuge tube, stirred on vortex mixer for washing the DNA pellet and centrifuge at 12500 g at 4°C for two minutes, ethanol was discarded using a micropipette. The centrifuged tube containing DNA pellet was dried into a vacuum desiccators for two minutes. The DNA pellet was re-suspended in required amount of tris-EDTA (TE) buffer (10mM Tris, pH-8.0, 1M EDTA, all Sigma) depending on the size of DNA pellet. The DNA solution was preserved at 20°C for further studies.

iv. Assessment of genomic DNA quality:

Quality and quantity of extracted DNA were measured by NanoDrop 1000 spectrophotometer (Thermo Scientific). Data was shown in Appendix (3). Those were the stock solution. Final working DNA concentration for setting up PCR were made with the mixing of 5 µl sample DNA and 45 µl of sterile distilled water.

2.4 Establishment of field experiment

2.4.1 Land preparation

A levee was made surrounding plots to maintain standing water up to 5.0 cm in side. Land was prepared 15 days before transplanting. Ploughing and cross ploughing following by laddering was done by power tiller to attain puddle condition. Weeds were cleaned manually.

2.4.2 Seedling rising

Seeds of tested variety (BR11) for pathogenicity test were soaked overnight in water then excess water removed and seeds were incubated for 48 hours at ambient temperature. Sprouted seeds were separated and soaked further for overnight until radicals attain length 2-3 cm. Seeds were sown in wet seedbed. Standing water was removed from seedbed until seedling had grown up to 5 cm long. No fertilizer or pesticides were applied in seedbed.

Seeds of tested 60 varieties for germplasm/cultivar screening were treated with hot water (52°C for 20 min). Treated seeds were then soaked for 24 hours. Sprouted seeds were then sown in pot (tray). Seedlings were transplanted into the field after 21 days, 30 days, and 35 days for T. Aus, T. Aman and Boro respectively.

2.4.3 Transplanting

For pathogenicity test thirty days old seedling were transplanted having 2-3 seedlings per hill. Spacing was maintained at 15 cm x 20 cm.

2.4.4 Fertilizer application

Fertilizers were applied as per recommendation of BRRI (Adhunik Dhaner Chash, Publication 2007). All fertilizers were applied in basal except urea. Urea was applied in three steps at 15, 35 and 55 days after transplanting.

Table 2: Dose of fertilizers were applied in all the experimental fields

Fertilizer	Dose(kg/ha)
Urea	220
TSP	78
MOP	120
Gypsum	60
Zinc sulphate	10

2.5 Virulence of *R. oryzae-sativae* isolates

2.5.1 Cultivar

BR11 was used for pathogenicity test. Growth duration of BR11 is 145 days. Thirty days old rice seedlings were transplanted in single line. Each isolates were inoculated in every row intervals. For each isolates, nine rice plants were artificially inoculated in a randomized completely block design (RCBD) with three replications.

2.5.2 Preparation of inoculum and inoculation

Pure culture of each isolate were grown on 9 cm PDA plate and incubated at ambient temperature for 7 days to allow approximately full plate growth. PDA plates with 7days old inoculum were divided into 8 equal splits. Followed by the process of Lanoiselet *et al.* (2001), each portion of the PDA with mycelial plugs was inserted at the base of each hill.

2.5.3 Data recording for virulence test

The inoculated hills were cut from the ground level after 14 days of inoculation. Data ie. plant height, lesion height, incidence were taken at the plant pathology lab in BRRI. The relative lesion height were calculated using the following formula, RLH (%) = (Lesion height/Plant height) X 100. The scale was developed by Ahn *et al*. (1986). This is a qualitative scale which differentiates varietals reactions. Incidence was calculated by the following formula, Incidence (%) = No. of infected plant / No. of total plant X 100.

2.6 Disease Management

2.6.1 Fungicide screening *in vitro*

Eight fungicides were used in this study. Out of them, two were new generation with two active molecules and others were single with only one active molecule fungicide. Details of fungicide are shown in table 3.

2.6.2 Fungicide screening *in vivo*

BRRI dhan 48 was used *in vivo* trial. Seeds of tested variety for field trial were treated with hot water (52°C for 20 min). Treated seeds were then soaked for 24 hours. Sprouted seeds were then sown in pot (tray). Seedlings were transplanted into the field after 30 days. Carbendazim, Differ, Folicur, Nativo and Propiconazole were tested in this experiment.

Table 3 : List of fungicides used *in vitro* trial

Fungicide (Trade name)	Group (Chemical name)
Amistar Top	Azoxystrobin (325 Sc.)
Roly poly	Carbendazim (50% WC.)
Differ	Difeconazole + Propiconazole (300 EC)
Folicur	Tebuconazole (250 EW)
Mancodazim plus	Mancozeb (63%) + Carbendazim (12%)
Monceren	Pencycuron (250 SC)
Nativo	Trifloxystroin + Tebuconazole (75% WG)
Propi	Propiconazole (25% EC)

2.7 Germplasm/cultivar screening against aggregate sheath spot disease of rice

2.7.1 Germplasm/cultivar

A total of 60 germplasm/cultivar (T-Aus, T-Aman, and Boro) which was collected from GRS **(**Genetic Resources and Seed**)** Division of BRRI were tested in this experiment.

2.7.2 Crop management

Fertilizers such Urea, TSP, MOP, Gypsum, and Zinc sulphate were applied as described in 2.4.4. Weeding and water management were done as and when necessary.

Table 4 : List and accession no. of different germplasm/cultivar used in the screening test

Aus			T- Aman			Boro	
Sl No.	**BRRI ACC No.**	**Local Name**	**Sl No.**	**BRRI ACC No.**	**Local Name**	**Sl No.**	**Cultivar Name**
1	4927	Musur	21	7076	Kurchi jira	41	BR-1
2	4928	Koisramuri	22	7077	Kurchi binni	42	BR-2
3	4929	Ogoan	23	7078	Shabichi dhan	43	BR-3
4	4932	Akuee	24	7079	Chabichi dhan	44	BR-6
5	4933	CM25 (indian variety)	25	7080	Mosolla jira	45	BR-7
6	4934	Aus Balam	26	7081	Kocheri jira	46	BR-8
7	4935	Aus Khusni	27	7082	Katari bhog	47	BR-9
8	4936	Kataktara	28	7083	Khirsapat	48	BR-12
9	4937	Kobila Rus	29	7085	Deshi malshira	49	BR-14
10	4938	Kaloburi	30	7086	Panisail	50	BR-15
11	4939	Ratol	31	7088	BR-4	51	BR-16
12	4940	Kalosoti	32	7089	BR-10	52	BR-17
13	4941	Aus Boilam	33	7090	BR-22	53	BR-18
14	4942	Iet (Indian variety)	34	7091	BR-23	54	BR-19
15	4943	Kataktara	35	7095	BRRI Dhan38	55	BRRI Dhan26
16	4944	CN (Indian variety)	36	7096	BRRI Dhan 39	56	BRRI Dhan27
17	4945	Balam	37	7097	BRRI Dhan40	57	BRRI Dhan28
18	4946	Lotabhog	38	7098	BRRI Dhan41	58	BRRI Dhan29
19	4947	Ausboro	39	7099	BRRI Dhan44	59	BRRI Dhan35
20	4948	Nuncha	40	7100	BRRI Dhan46	60	BRRI Dhan36

2.7.3 Preparation of inoculum and inoculation

Isolates My-1 was used for germplasm screening. Preparation of inoculum and inoculation process was the same of 2.5.2

2.7.4 Data recording for germplasm/cultivar screening against aggregate sheath spot of rice

The inoculated hills were cut from the ground level after 21 days of inoculation. Data were recorded as 2.5.3. Aggregate sheath spot of rice considered as a minor

disease and there is no standard Evaluation system (SES) therefore, a system is constructed here based on RLH (%) and the table is given below-

Table 5 : Evaluation System used for aggregate sheath spot disease

Scale/ (RLH %)	Categories of resistance
0	Immune
1 to 20	Moderately resistant
>20	Moderately susceptible

2.8 Effects of different plant extracts on *R. oryzae-sativae*

2.8.1 Plants

The common and locally available medicinal plants were selected for screening antifungal activity against the pathogen. Details of plants used in this experiment are shown in table 6.

Table 6: List of the plants common name, botanical name with family and used part applied to assess antifungal activity

Common name	Botanical name	Family	Used part
Garlic	*Allium sativam*	Liliaceae	Bulb
Ginger	*Zingiber officinales*	Zingiberaceae	Rhizome
Henna	*Lawsonia inermis*	Lythraceae	Leaf
Water pepper	*Poligonum hydropiper*	Polygonaceae	Stem
Ivy gourd	*Coccinia cordifolia*	Cucurbitaceae	Stem
Neem	*Azadirachta indica*	Meliaceae	Leaf

2.8.2 Antifungal activity assay

About 75 ml of each plant extract diluted to 5%, 10%, 15%, 20% and 25% in potato dextrose agar (PDA) were poured into sterile 90 mm diameter Petri plates. PDA without any extract served as the control. Isolate no MY-1 was grown for 5 days on PDA. A mycelial plug (6 mm diameter) cut from 5- day-old culture of *R.*

oryzae-sativae isolate on PDA was placed at the center of each Petri plate. The plates were kept at 25 ± 2°C. The redial mycelial growth of the tested fungus in the treated plates was measured in millimeters in all treatments when the pathogen growth touched the edge of the controlled Petri plate. Percent growth inhibition was calculated using the following equation: I=(C-T)/C X 100, Where I=Percent inhibition, C=Growth of fungus in control, T=Growth of fungus in Treatment. Four replicated plates were prepared for each concentration.

Inhibition of sclerotia germination was determined by a method of Chaizuckam and Davis (2010b). Ten sclerotia, harvested from MY-1 isolate grown on PDA for 14 days, were transferred to PDA after sclerotia were soaked for 10 min in each 5%, 10%, 15%, 20% and 25% plant extract in sterile water. After incubation at room temperature for 3 days, the numbers of germinated sclerotia were counted. Sclerotia soaked in sterile water served as controls. The experiment was repeated once with four replicated plates in each trial.

Biological Characterization

3.1 Introduction

Sheath diseases of rice are the major constraints of rice production in Bangladesh. Among them *Rhizoctonia* sheath diseases of rice are predominant, comprising sheath blight, sheath spot and aggregate sheath spot. Significant yield losses in many rice growing regions of the world have been reported by Kobayashi *et al.* (1997) and Johanson *et al.* (1998). The emergence of *Rhizoctonia* sheath diseases as economically important rice diseases that attributed to the intensifications of rice-cropping; development of high-yielding rice cultivar with dwarf plant type, high-tillering along with high plant densities. The plant architecture, favorable environment, along with high nitrogenous fertilizer use in cultivation has aggravated the Rhizoctonia disease spread. Moreover, due to a dense leaf canopy with an increased leaf-to-leaf and leaf-to-sheath contact (Banniza *et al.*, 1999, Jones and Blemar, 1989).

Various methods have been used by different researchers to study the population structures of different *Rhizoctonia* species including morphological characteristics (Vijayan and Nair, 1985), entra and extracellular enzymes and proteins (Liu and Sinclair, 1993; Zuber and Manibhushanrao, 1982), total cellular fatty acid analysis (Matsumoto and Matsuyama, 1999) and molecular techniques (Matsumoto *et al*., 1997; Matsumoto, 2002). Rice sheath blight caused by *Rhizoctonia solani* is the most widely studied species in the genus *Rhizoctoni* (Sneh *et al*., 1991). The genetics, anastomosis grouping and pathogenicity of *Rhizoctonia solani* are well known due to economically significance soilborne pathogen with wider host range (Ogoshi, 1996). In contrast, only a few studies have been concerned with *Rhizoctonia oryzae-sativae*. Aggregate sheath spot of rice caused by *Rhizoctonia oryzae-sativae*, occurs in Bangladesh and in other countries including United states (Gunnel and Webster, 1984), Japan, Vietnam, India, Thailand, Iran, Venezuela, Uruguay and Australia (Cedeno *et al*., 1998, Rahimian, 1989, Gunnel, 1992,

Lanoiselet *et al*., 2001). This pathogen belongs to the AG-Bb anastomosis group of binucleate *Rhizoctonia* spp. (Gunnel, 1992). The sexual stage of the fungus was first reported in California rice fields in 1987 (Gunnel and Webster, 1987) on the outside of leaf sheaths as a distinct whitish layer (Gunnel, 1992). Despite the occurrence of a sexual stage, aggregate sheath spot is considered a mono-cyclic disease with predominantly asexual reproduction (Miller and Webster, 2001). Over wintering sclerotia or mycelia in soil or crop residue serve as reservoirs of inocula (Mordue, 1974). A subsequent rice crop is infected by floating infected crop residue, sclerotia in the rice paddy. Teleomorph induction can be difficult in *Rhizoctonia* spp. therefore; classification of new isolates of *Rhizoctonia* spp. has mostly been based on nuclear condition, colony morphology, anastomosis and pathogenicity (Sneh *et al*., 1991, Sen *et al*., 1999). *Rhizoctonia oryzae-sativae* has been distinguished from other *Rhizoctonia* spp. by amplified fragment length polymorphism (Taheri *et al*., 2007) specific primers (Johanson *et al.*, 1998) and fatty acid analysis (Lanoiselet *et al*., 2005c). However, intraspecific genetic diversity of *Rhizoctonia oryzae-sativae* had examined by only a few workers in the world (Chaizuckam and Davis, 2010a).

Somatic incompatibility has been used as a marker to identify clones of *Rhizoctonia oryzae-sativae* in order to determine the survival of ovewintering inoculam and dissemination of the fungus in rice field in Japan (Guo *et al*., 2006). Isolates that have somatic incompatibility reactions produced growth inhibition zones or barrage zones due to plasmolysis of fused cells; this reaction occurs between genetically distinct isolates, whereas a compatible reaction produces confluent growth and living fusion of cytoplasm between isolates as heterokaryon (Sneh *et al*., 1991, Adams, 1996). Compatible reactions usually observed in self pairing and considered as isolates are genetically similar. However, some isolates of *Suillus granulatus* were genetically different after examination of DNA markers (Jacobson *et al*., 1993). Therefore, in this study multidisciplinary approaches viz. cultural charactistics, pathogenicity test and molecular studies were adopted to characterize *Rhizoctonia oryzae-sativae* along with somatic compatibility.

Study of genetic diversity is the process by which variation among individuals or groups of individuals or populations is analyzed by a specific method or a combination of methods. Molecular techniques such as restriction fragment length polymorphism (RFLP), sequence analysis of rDNA, random amplified polymorphism DNAs (RAPD), and random amplified microsatellites have been used to study genetic and taxonomic relationships within specific anastomosis groups of *Rhizoctonia* isolates (Elbakali *et al*., 2003, Sen *et al*., 1999, Sharma *et al*., 2005). In addition, microsatellites or simple sequence repeats, which are widely dispersed in most eukaryotic and prokaryotic genomes (Tenzer *et al*., 1999, Douhan and Rizzo, 2003), have also been used to characterize diversity of *Rhizoctonia* spp. (Meinhardt *et al*., 2002). The number of repeat units in a particular microsatellite region generates DNA polymorphism (Razavi and Hughes, 2004). These powerful tools have been used in genetic analysis of numerous organisms, including animals, plants, gene flow, intraspecific phylogeny, mating systems and gene mapping (Barroso *et al*., 2000, Douhan and Rizzo, 2003).

Since breeding for disease resistance has become an important program taken up by many workers, an attempt was made in this study to differentiate the isolates of the pathogen and establish the type of resistance in term of vertical or horizontal, as defined, and offered by the rice cultivars against the pathogen studies at the DNA level have also advanced our understanding of the structure of *Rhizoctonia* populations (Vandermark, 1999). Molecular tools are being increasingly used to characterize fungal plant pathogen and to evaluate level of genetic diversity within and between species and to identify particular race and pathotypes. Variable Number Tandem Repeat (VNTR) includes micro and mini-satellites and hyper variable region. Microsatellites are arrays of randomly repeated DNA sequences which are dispersed through out the genomes (Jeffeys *et al*., 1985a) and are also referred to as “Sequence tagged microsatellite sites (STMS).” Microsatellite comprises a class of VNTR loci in which the repeated sequences are short (< 65 bp) and are frequently GC rich (Jeffeys *et al*., 1985b; Nakamura *et al*., 1987). Simplified techniques such as VNTR-PCR of rDNA and ITS region analysis have been utilized for rapid detection of variation in different fungi (Matsumoto *et al*.,

1996; Pascual *et al.*, 2000). Ribosomal RNA in fungi are conserved and contain sequence components reflection different evolutionary rates and that are phylogenetically and taxonomocally informative in studying relationship and patterns of genetic variation in fungi (Bruns *et al*., 1991).

Repetitive-element Polymerase Chain Reaction (Rep-PCR) primers (such as THE BOX, ERIC and PER primers) which were originally designed for repeated elements in prokaryotic genomes, have been used to characterize several fungal genera including *Verticillium*, *Fusarium*, *Stagonospora* and *Septoria* (Arora *et al*., 1996; Czembor and Arseniuk, 1999; Edel *et al*., 1995). Recently, ERIC2 and BOXA1R primers have been used to characterize clonal population of *Rhizoctonia* fungi because of their potential of distinguishing fungal isolates, and because PCR-based fingerprinting has the advantage of being relatively simple and widely applicable in any laboratory with PCR capability.

In Bangladesh, until 1988 there was no report on *Rhizoctonia oryzae-sativae* except *Rhizoctonia solani*. Scientific information on aggregate sheath spot of rice is scanty in Bangladesh and the population diversity of the causal agent has not yet been studied. However, knowledge of the population of pathogenic *Rhizoctonia oryzae-sativae* is essential for understanding population structure, integrated control strategies including pathogenicity, host range and adaptability to environmental conditions.

In this study, three types of VNTR primers (MR, RY and GF) and one Rep-PCR primer (BOXA1R) were employed to investigate diversity of *Rhizoctonia oryzae-sativae* for the first time. This chapter describes multi-disciplinary approach of diagnostics techniques to characterized Bangladeshi isolates of *Rhizoctonia oryzae-sativae* collected different locations with the following objectives

1. Characterization of morphological diversity of the isolates.
2. To study somatic compatibility of the pathogen.
3. To investigate genetic variability and relationship among the isolates.
4. To identify the virulence diversity of *Rhizoctonia oryzae-sativae* isolates.

3.2 Materials & Methods

3.2.1 Identification of *R. oryzae-sativae* by specific primer

Identification of *R. oryzae-sativae* isolates collected from rice field done using diagnostic primers, forward Primer GMROS-6 (5'-GAA AGA GAG AGA GGT CGC CTC-3') and reverse primer R635 (5'-GGT CCG TGT TTC AAG ACG G-3'). PCR was performed using DNA of each of 30 isolates. DNA of *R. oryzae-sativae* was extracted with the procedure mentioned as chapter 2(2.3). PCR amplification (50 µl) was performed using 2µl of genomic DNA, 1.5 mM $MgCl_2$, 2 unit Taq polymerase, 0.2 mM each of dNTPs, 10 Mm Tris-HCL (pH 8.3), 0.2 µM of each primer and rest with distilled H_2O. PCR amplifications was carried out in an Eppendrof Mastercycler personal machine with the cycling program 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. A 5 min extension at 72°C was used to complete the reaction. Aliquots 10 µl of the amplified products were taken to electrophoresis using 2% agarose gel, 2 and ½ hrs at 80 V. Gel was stained in ethidium bromide for 20 min and visualized under BioDoc Analyze.

3.2.2 Cultural characterization

3.2.2.1 Mycelial growth

Collected thirty isolates were used for this experiment. Mycelial disks of 6 mm diameter were transferred aseptically to PDA plates and incubated at 25 ± 2°C. Radial mycelial growth were taken from the center of inoculated plug after 24, 48, 72 and 96 hours of incubation. A CRD design was followed with 3 replications. Data were analyzed by CropStat (version 7.2) computer software.

3.2.2.2 Morphological characterization

Thirty isolates were used for this experiment. Mycelial block of six mm diameter from the margin of 3 days old colonies were placed aseptically to PDA plates and incubated at room temperature for 14 days. Three replications were used for each isolate. All the isolates were grouped into different category based on the appearance of the colony and the formation of pattern, size, and shape of the

sclerotia. Modified morphological characters were recorded as Banniza (1997) and Ali (2002). The cultural characteristics and character states are shown in Table 7. Isolates were grouped with the maximum similarities by MVSP (MultiVariate Statistical Package) version 3.2.

Table 7 : Morphological characteristics used for the description of *R. oryzae-sativae* isolates and there attributes (after Banniza, 1997 and Ali, 2002)

Sl no.	Characters	Character state
1	Mycelial color on PDA	0=hyaline, 1=cream 2= faint brown, 3=medium brown.
2	Arial mycelial quantity	0=absent, 1=few, 2=moderate, 3=abundant.
3	Color of sclerotia	1=light brown, 2= brown, 3 =dark brown.
4	Quantity of sclerotia	0=absent, 1=few, 2=moderate, 3=abundant
5	Topography of sclerotia	0=Immersed, 1=superficial
6	Shape of sclerotia	1= round , 2=irregular, 3=round and irregular
7	Superficial sclerotia(ss) discrete(<1mm)	0=absent, 1=few, 2=moderate, 3=abundant
8	SS discrete(>1mm)	0=absent, 1=few, 2=moderate, 3=abundant
9	SS aggregated	0=absent, 1=few, 2=moderate, 3=abundant
10	SS scattered	1=few, 2=moderate, 3=abundant
11	SS near inoculam	0=absent, 1=few, 2=moderate, 3=abundant
12	SS near margin	0=absent, 1=few, 2=moderate, 3=abundant
13	SS dispersed on whole colony	0=absent, 1=present
14	Exudate droplets on sclerotium surface	0=absent, 1=present
15	Fluffy mycelium	0=absent, 1=present
16	Quantity of fluffy mycelium	0=not present, 1=few, 2=moderate, 3=abundant.
17	Colony reverse pigment	0=not present,1=cream or faint brown,2=light brown,3=medium brown,4=dark brown
18	Pseudo sclerotia	0 = absent, 1=few, 2=moderate, 3=abundant
19	Sclerotia on lid	0=absent, 1=present
20	Zonation	0=absent, 1=present
21	Zonation type	0=no zonation,1=cr (centrel ring) weakly developed 2= cr sharply developed
22	Mycelial growth on lid	0=absent, 1=few, 2=moderate, 3=abundant

3.2.3 Somatic compatibility test

Mycelial plugs having 6 mm diameter were taken from growing culture of 4 days old and placed on 9 cm diameter Petri plates were approximately 2 cm apart from each other. All plates were kept at 25 ± 2°C in the dark for 10 days. Thirty isolates were tested in all possible combinations. The experiment included three replications and was repeated once. Isolates that grew together and failed to show a barrage reaction at the colony junction were classified as same somatic compatibility group (SCGs), while isolates exhibiting a barrage reaction were classified into different SCGs.

3.2.4 Characterization of *R. oryzae-sativae* through variable number of tendem repeats (VNTR) analysis

For standardization of $MgCl_2$ concentration, primer and Taq polymerase enzyme, a master-mix of 20 µl reactions was prepared with the components as shown in Appendix (2). Two concentrations of each of $MgCl_2$ concentration, primer and Taq polymerase enzyme was used for a series of experiments and selected concentrations were used for the rest of the study.

3.2.4.1 Primer selection for VNTR analysis

Three primers, MR, RY and GF were used for VNTR analysis and the sequences of the primers are listed in the Table 8.

Table 8 : List of primers and their sequence used for VNTR analysis

Name of primers	Sequence of primers
MR	GAG GGT GGC GGT TCT
RY	CAG CAG CAG CAG CAG
GF	TCC TCC TCC TCC TCC

The DNA was amplified on the basis of the polymerase chain reaction (PCR). A master-mix of 20 µl reactions was prepared with the components as shown in Appendix 2. Then 19 µl of this master-mix was aliquot into PCR tube and 1µl of genomic DNA of selected isolates added into it. One drop of mineral oil was also

added to each PCR tube to prevent evaporation. The PCR tubes were fitted into the blocks of PCR machine (Eppendrof Mastercycler personal). The PCR machine was then run with 103°C lid temperature following VNTR-PCR protocol as shown below-

a) 94°C for 2 minutes
b) 94°C for 20 seconds
c) 45°C for 45 seconds
d) 72°C for 2 minutes
e) 72°C for 5 minutes and held at 4°C.

The tubes with the PCR products were taken out from the PCR machine after completed thirty five cycles and then added 2 µl of 6x loading dye into it and mixed thoroughly. Amplified PCR products were separated on agarose gel.

3.2.4.2 Agarose gel electrophoresis for VNTR analysis with Primer MR

i. Preparation of 2% agarose gel

Agarose (3.6 g) was weighed in a conical flask for preparation of 180 ml of 2% agarose gel. An amount of 180 ml of 1xTAE was poured into the conical flask having 3.6 g agarose and melted by heating in a microwave oven until dissolved fully. The melted agarose gel was cooled down under flowing tap water. The plate of the gel tank was placed flat on the table. The gel was poured smoothly and continuously starting from one corner until reached to the other corner. Then comb was inserted halfway into the gel immediately for making well, allowing an hour for polymerization before removing the comb gently. The casted plate was assembled in the tank. It was made sure that there was no bubble at the bottom to avoid short circuit. The gel was submerged into the 1xTAE running buffers in gel tank.

ii. Gel loading and running

PCR product (10 µl) stained with 2 µl of 6x loading dye was loaded into the well of the agarose gel and electrophoresed for three hours at 80 volts to move the negatively charged DNA towards the anode.

iii. Visualization of the gel

The gel was taken out from the gel tank and stained with ethidium bromide solution (0.5µg/ml) for an hour. The stained gel was rinsed with water for distaining, illuminated on UV transilluminator and photographed by gel documentation (BioDoc Analyze) for measuring the bands of amplified DNA fragments.

The selected primer MR was used for the rest of the studies with 30 selected isolates for amplification of DNA. The gel used to resolve the amplified DNA was stained, illuminated under UV light and the fingerprints photographed by gel documentation.

iv. Data analysis

Bands of DNA fingerprints were counted and recorded according to the position of bands for each of the 30 isolates. Genotypic data were obtained by considering different product sizes as different alleles at each locus, which were measured by AlphaEaseFC 4.0 computer software and then scored for the presence (1) or absence (0) of the bands at the certain position for each isolate. These data were used to calculate the genetic diversity. A genetic similarity matrix was calculated with the Simqual subprogram using Dice's coefficient, followed by cluster analysis with the SAHN (sequential agglomerative hierarchical and nested clustering) subprogram using UPGMA (unweighted pair group method with arithmetic mean) clustering method as implemented in numerical taxonomy and multivariate analysis system (NTSYS-pc. version 2.20e.) software. The similarity matrix was also used for principal coordinate analysis with the Eigen and MXPlot subprogram in NTSYS-pc. Principle coordinate analysis (PCoA) was performed in order to highlight the resolving power of the ordination. Similarly primer GF and RY were used for all these isolates.

3.2.5 Characterization of *R. oryzae-sativae* by REP-PCR analysis

Repetitive-element Polymerase Chain Reaction (REP-PCR) primer such as BOX was originally designed for repeated elements in prokaryotic genomes, have been used to characterize several fungal genera including *Verticillium*, *Fusarium*, *Stagonospora* and *Septoria* (Arora *et al*., 1996; Czembor and Arseniuk,1999; Edel *et al*., 1995). Recently, BOXA1R primer has been used to characterize clonal populations of *Rhizoctonia* fungi because of its potential of distinguishing fungal isolates. In this study BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') is used for REP-PCR analysis. Amplifications was done in a 15 µl reaction volume containing 2 µl of template DNA, 1.5 µl of 10 x reaction buffer, 0.3 µl of dNTPs (0.2mM), 4.5 µl of the primer (6pM), 0.1 µl of *Taq* DNA polymerase (5units/sample) and 6.6 µl of sterile distilled water. Amplifications were performed in a Thermal cycler with slight modifications to the temperature profiles reported by Toda *et al*., (1999), i.e., one cycle of initial denaturation (95°C, 7 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (52 °C for 1 min) and extension (72°C for 8 min) with a final extension at 72°C for 16 min. PCR products were electrophoresed in 2.0% agarose gels stained with ethidium bromide and the gels were visualized under UV light (BioDoc Analyze). Genotypic data was analyzed by following as described as 3.1.4.2 **(**iv**).**

3.2.6 Virulence of the isolates of *R. oryzae-sativae* on cultivar BR11

Virulence tests of the isolates against rice plant were done in T. Aman, 2011 in a field of Bangladesh Rice Research Institute (BRRI), Gazipur. A total of 30 isolates were evaluated through artificial inoculation (5 days old culture) for virulence test using a rice variety BR11 and following standard cultivation and management practices recommended by BRRI. Detail descriptions of experimental technique, transplanting and inoculation method are mentioned under General Materials and Methods in chapter two (2.4 and 2.5). 12.4 m meter long (each replication) and 1.05 m breath plot was transplanted in 15 cm x 20 cm spacing. Artificial inoculation was done at the PI stage. A RCB design was followed with three replications. Three hills of each replication were inoculated and data were collected from all the tillers of these inoculated hills. RLH (Relative Lesion Height) and incidence were taken after 14 days of inoculation.

3.3 Results

3.3.1 Identification of *R. oryzae-sativae*

The key characteristics of *R. oryzae-sativae* are described below. Colonies were white to pale brown on PDA; hyphae were 5-7 µm in diameter, composed of cells 13-30 µm across; monilioid cells were 21-37 x 6-11µm (Mordue, 1974).

Hyphae of all isolates were bi nucleated along with typical constriction of *Rhizoctonia* sp. at the point of branching (Fig. 1) while *R. solani and R. oryzae* are multinucleated cell (Taheri *et al.*, 2007). This result was further confirmed by diagnostic molecular marker of *R. oryzae-sativae*. A single band of 1200 bp size was visualized in agarose gel among all the isolate tested with forward primer GMROS-6 combination of reverse primer R635 (Fig. 6). There by confirmed that isolates tested in the subsequent investigation were all *R. oryzae-sativae*.

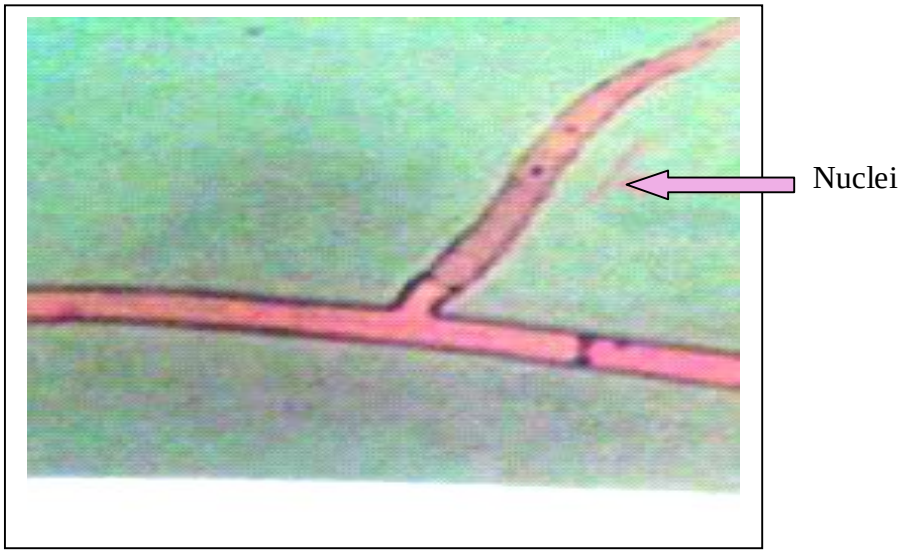


Figure 1 : Binunleate cell of *R. oryzae-sativae*

3.3.2 Cultural characterization

3.3.2.1 Mycelial growth

Considerable variation in mycelial growth of *R. oryzae-sativae* was recognized among the isolates. Accordingly, the studied isolates by Least Significant Difference Test indicated that mycelial growth rate significantly varied at $\alpha = 0.050$ among the isolates. Isolates were divided into two distinct groups according to growth rate such as slow and fast. Slow growing isolates ranged from 0.26-0.32 mm per hour with an average of 0.29 mm per hour while mycelial growth rate showed by the comparatively fast growing isolates ranged from 0.39-0.45 mm per hour with an average of 0.42 mm per hour (Fig. 2). According to growth rate, DI-1, DI-2, DI-3, TH-1, TH-2, TH-3, RA-1, RA-2, RA-3, NE-1, NE-2, Ne-3, GA-1, GA-2 and GA-3 belongs to slow growing group while MY-1, MY-2, MY-3, TA-1, TA-2, TA-3, JA-1, JA-2, JA-3, SH-1, SH-2, SH-3,NA-1, NA-2, and NA-3 were fast growing isolates. Growth rate of isolates ranged from 0.26 to 0.45 mm per hour with an average of 0.35 mm. Among the isolates, maximum growth rate (0.45 mm/hr) was associated with MY-1 collected from Mymensingh. In contrast, minimum growth rate (0.26 mm/hr) found in NE-2 from Netrokona (Plate 1). Variations in mean mycelial growth among the isolates were found within the location as well as across the locations (Table 9). Mycelial growth rate increased up to 72 hours but slightly decreased until PDA plates completely filled up (after 96 hours of incubation). Intra district isolates variations in mycelial growth were also observed almost in all isolates accept collected from Thakurgaon and Sherpur districts. However, inter isolates variability in mycelial growth was perhaps associated with genetic variation as well as distant geographical reason (ecology).

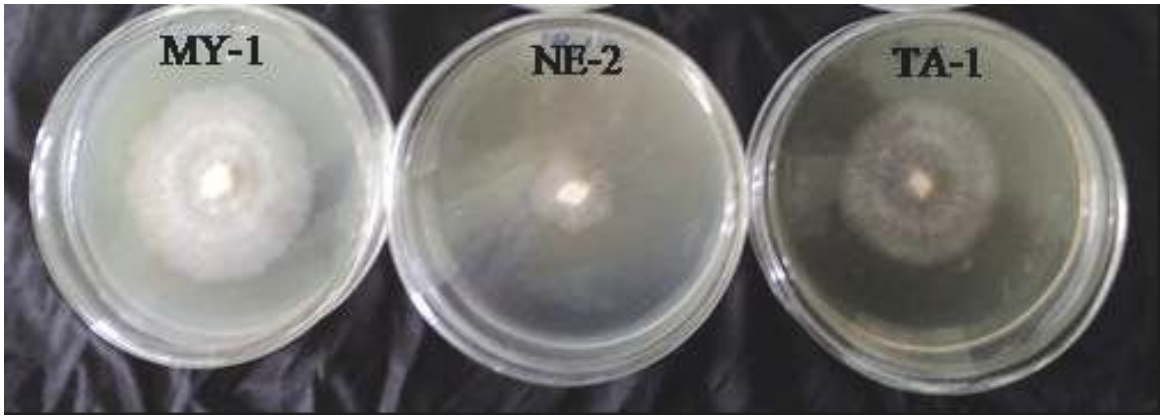


Plate 1 : Variation in mycelial growth of different isolates after 48 hours of incubation

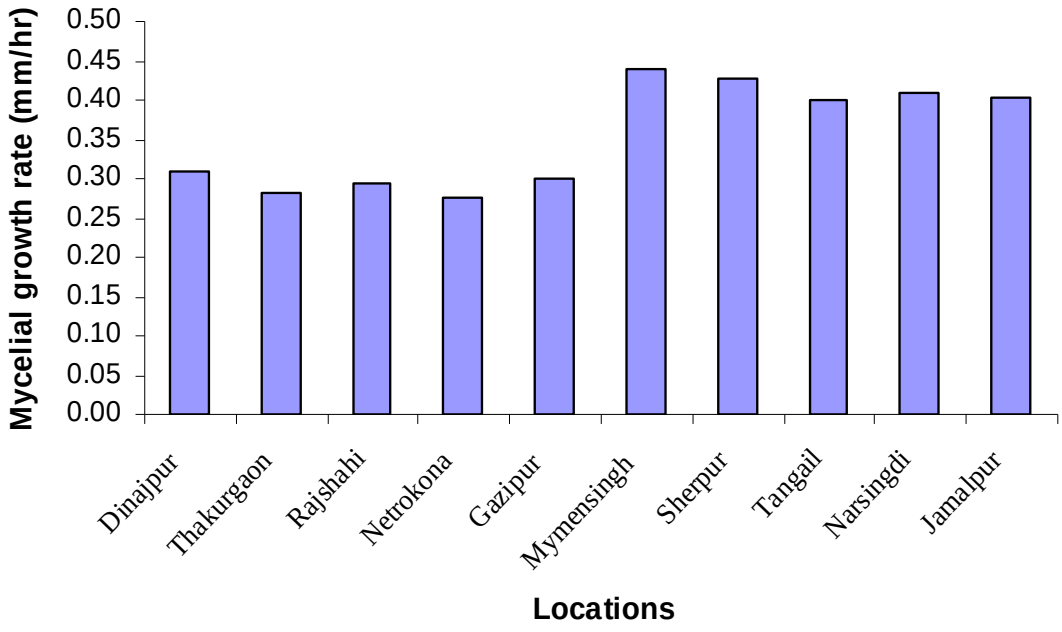


Figure 2 : Mycelial growth rate of isolates from each district showing variation (slow and fast) in growth after 96 hr. of inoculation

Table 9 : Mean mycelial growth of *R. oryzae-sativae* isolates in different incubation period

Location	Isolate	Mycelial growth rate(mm[a])* over incubation period (hr[b])				Growth rate (mm/hr)
		24	48	72	96	
Dinajpur	DI-1	5.50	12.16	21.66	30.33	0.32
	DI-2	5.83	13.66	22.00	29.83	0.31
	DI-3	5.16	12.83	21.50	29.00	0.30
Thakurgaon	TH-1	6.16	12.83	20.33	27.33	0.28
	TH-2	5.00	11.5	19.33	27.00	0.28
	TH-3	5.33	12.16	19.66	26.66	0.28
Rajshahi	RA-1	6.50	13.33	20.66	27.66	0.29
	RA-2	6.66	13.66	21.00	28.00	0.29
	RA-3	6.16	13.5	21.33	28.83	0.30
Tangail	TA-1	7.33	17.16	27.66	37.50	0.39
	TA-2	8.16	17.83	28.33	38.50	0.40
	TA-3	8.83	18.33	29.00	39.33	0.41
Mymensingh	MY-1	9.00	20.66	32.66	43.00	0.45
	MY-2	8.33	19.33	30.66	41.00	0.43
	MY-3	8.16	19.00	31.00	42.00	0.44
Jamalpur	JA-1	8.66	18.66	28.83	38.00	0.40
	JA-2	7.83	17.66	29.33	40.00	0.42
	JA-3	9.00	18.33	28.66	38.00	0.40
Netrokona	NE-1	5.50	12.33	19.66	26.66	0.28
	NE-2	4.66	11.16	18.33	25.16	0.26
	NE-3	5.00	12.16	20.00	27.66	0.29
Gazipur	GA-1	5.50	13.33	21.66	29.33	0.31
	GA-2	5.66	12.33	21.16	29.66	0.31
	GA-3	5.16	12.5	20.33	27.66	0.29
Sherpur	SH-1	7.16	17.33	29.66	41.00	0.43
	SH-2	8.50	18.83	30.00	40.33	0.42
	SH-3	8.83	19.33	31.00	41.66	0.43
Narshindi	NA-1	8.16	17.66	28.50	39.66	0.41
	NA-2	7.00	16.16	27.33	38.33	0.40
	NA-3	7.33	17.33	29.16	40.00	0.42
SE(N=3)		0.27	0.32	0.39	0.42	
LSD(0.05)		0.77	0.92	1.11	1.20	

* = Each value is an average of three replications, [a] mean mycelial growth in mean

[b] mean mycelial growth in hour

Investigation on mycelial growth indicated that some isolates produced a moderate quantity of aerial mycelium on the colony surface as well as on the lid and produced few sclerotia on the lid. Combined morphological data of twenty two characters were analyzed with UPGMA (MVSP) showing four groups at 66% similarity level (Fig. 3). Thirteen isolates were clustered in group I, which further constituted three subgroups at 75% similarity level. Group II consisted of 13 isolates and also further constituted three subgroups at 75% similarity level. Group III and IV contained 2 isolates each. Mycelial growth of Group-I and Group-III was slower than the Group-II and Group-IV. However, Group-IV consisted of two isolates eg., JA-1 and JA-3 containing light brown sclerotia and abundant fluffy type mycelia which was characteristically different from other cluster groups (Appendix 1).

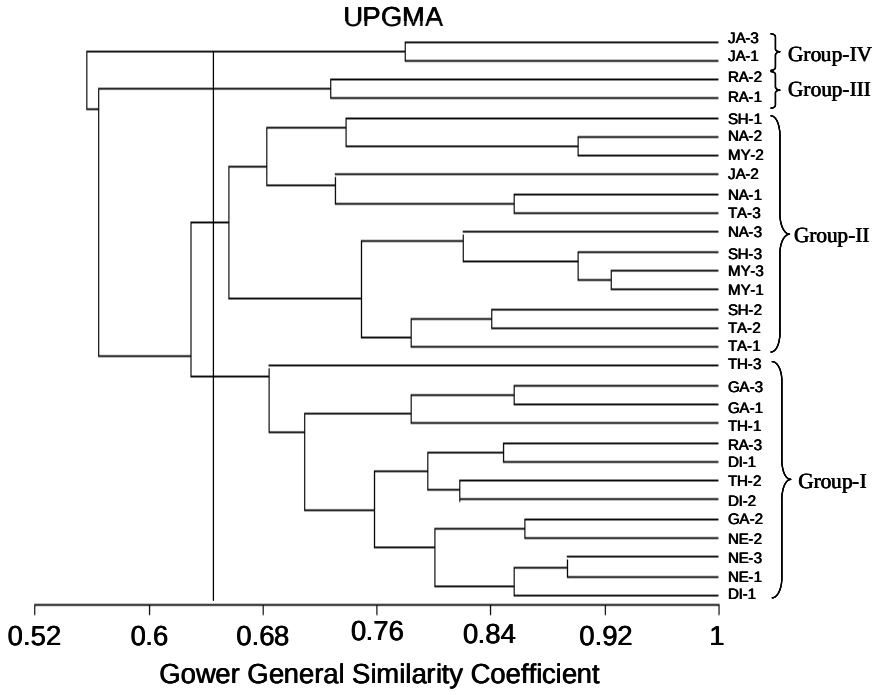


Figure 3 : Relatedness of *R. oryzae-sativae* isolates based on morphological characteristics by MVSP

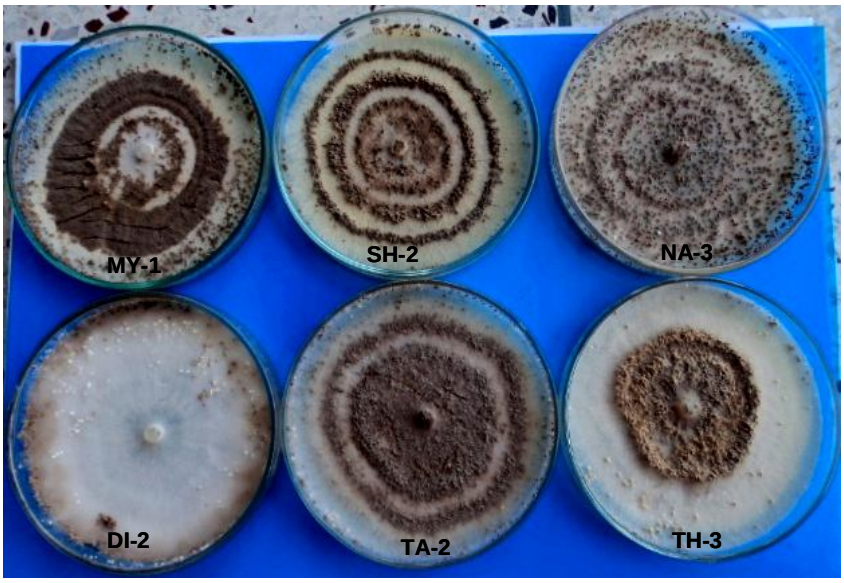


Plate 2: Morphological variation of some *R. oryzae-sativae* isolates showing sclerotial distribution

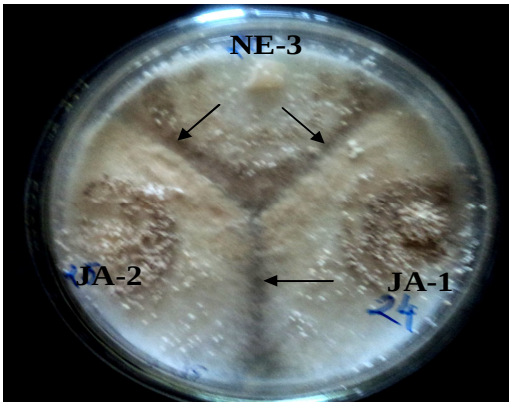


Plate 3 : Somatic compatibility reactions among *R. oryzae-sativae* isolates (after 10 days of incubation). Barrage zones developed between NE-3, JA-1 and JA-2

3.3.3 Somatic compatibility test

Barrage zone was observed at the colony interface of two different isolates. Only three pairs of isolates displayed somatic compatible reactions; one pairs from Tangail district (TA-1+ TA-3), one from Gazipur (GA-2+GA-3) districts and one pair between Mymensingh and Sherpur (MY-1+SH-3) districts (Appendix 6). Barrage zones were shown in plate 3. There are 27 SCGs from 30 isolates indicated 90% diversity among the isolates by Somatic compatibility test.

3.3.4 Characterization of *R. oryzae-sativae* through variable number of tendem repeats (VNTR) and REP-PCR combined analysis

3.3.4.1 DNA fingerprint analysis

Genetic variation was determined among thirty *R. oryzae-sativae* isolates using VNTR primers eg., GF, MR, RY and BOXA1R. PCR with 3 VNTR primers and 1 rep-PCR primer was carried out 2 times. Simple banding patterns were produced by VNTR primers and complex banding patterns by BOXA1R primer (Fig 7-10). All the primers amplified reproducible bands and band size ranged from 0.26 to 2.21 kb for VNTR primers and 0.25 to 2.02 kb for BOXA1R primer. The number of DNA fragments ranged from 8 bands in primer RY to 13 bands in primer GF irrespective of VNTR and BOXA1R primers. The primer RY, GF, MR and BOXA1R gave 8, 13, 9 and 11 bands respectively. Totally 41 distinct and clear bands were recorded of which only 4 bands were shared by all isolates. The rest 37 bands were polymorphic resulting 90% polymorphism. VNTR primers amplified 30 bands from which GF showed the highest polymorphism (100%) and the lowest polymorphism showed by RY (75%). Out of 11 bands, one band was monomorphic resulting 90% polymorphism by BOXA1R primer.

3.3.4.2 Cluster analysis

"Cluster analysis" refers to "a group of multivariate techniques whose primary purpose is to group individuals or objects based on the characteristics they possess, so that individuals with similar descriptions are mathematically gathered into the same cluster" (Hair *et al.,* 1995). The resulting clusters of

individuals should then exhibit high internal (within cluster) homogeneity and high internal (between clusters) heterogeneity. Combined data sets from the selected primers (MR, GF, RY and BOXA1R) were subjected to cluster analysis using NTSYS-pc software (version 2.20e) program (Fig.4). Under SIMQUAL program, similarity matrix was constructed using Dice coefficient method. This similarity matrix was used to generate a dendrogram using unweighted pair group method with arithmetic mean (UPGMA) method of SHAN. Application of the UPGMA clustering method gave the cophenetic correlation coefficient, where r = 0.82 indicates a good fit correlation value. The constructed dendrogram showed the similarities of the isolates clustered in together. The similarity coefficient values varied between 0.36 and 0.98 with an average of 0.67 which indicating variation among the isolates. Twenty nine isolates grouped into 2 clusters at 69.6% similarity level. Group I composed of 14 isolates. This cluster consisted of all comparatively slow growing isolates. Group II composed of 15 comparatively fast growing isolates. One separate solitary group resulted from the DNA finger printing in RA-1which comprised Group III.

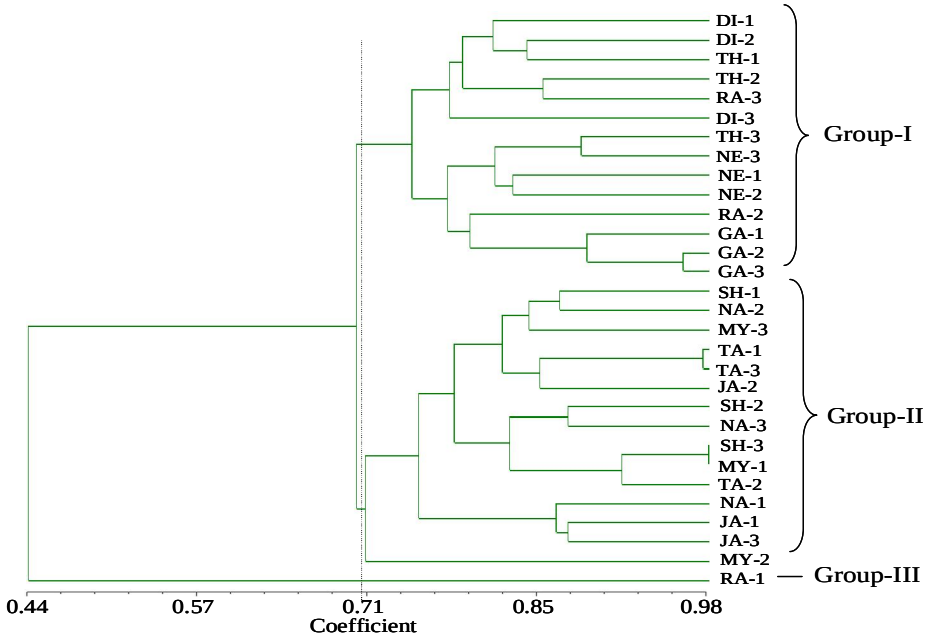


Figure 4 : Dendrogram showing relatedness among thirty *R. oryzae-sativae* isolates based on UPGMA method

3.3.4.3 Principle Coordinate Analysis

Principle Coordinate Analysis (PCoA) is a scaling or ordination method that starts with a matrix of similarities or dissimilarities between a set of individuals and aims to produce a low-dimensional graphical plot of the data in such a way that distances between points in the plot are close to original dissimilarities. The first principal component (PCo) summarizes most of the variability present in the original data relative to all remaining PCos. The second PCo explains most of the variability not summarized by the first PCo and uncorrelated with the first, and so on (Jolliffe, 1986). In our experiment, the result of PCoA was similar to that of clustering in the dendrogram. The first three PCos totally accounted 80.50% of the variation. First, second and third PCos explained 72.09%, 4.75%, and 3.66% variation respectively. The 3-dimensional view of plot showed that all isolates grouped as like UPGMA cluster groups (Fig. 5). Eigenvalues from Principle Coordinate Analysis was shown in Appendix 5.

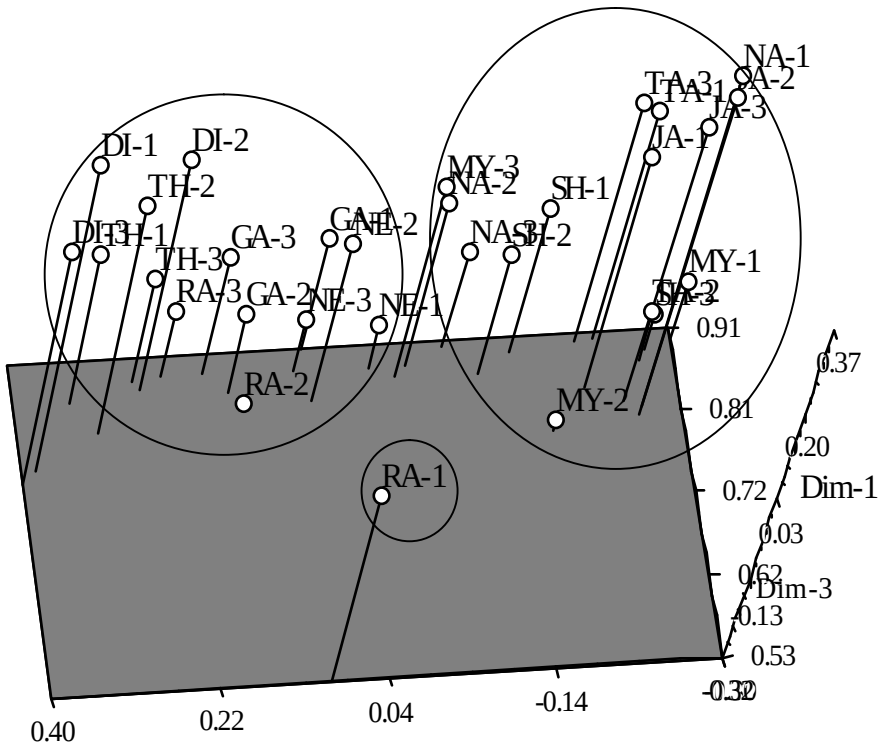
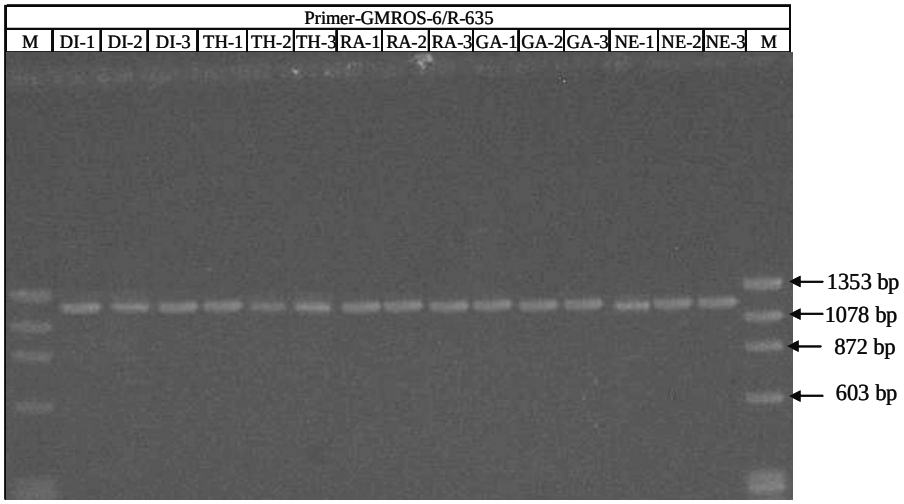


Figure 5 : Three dimensional plotting views of 30 *R. oryzae-sativae* isolates obtained from principle coordinate analysis

(a)



(b)

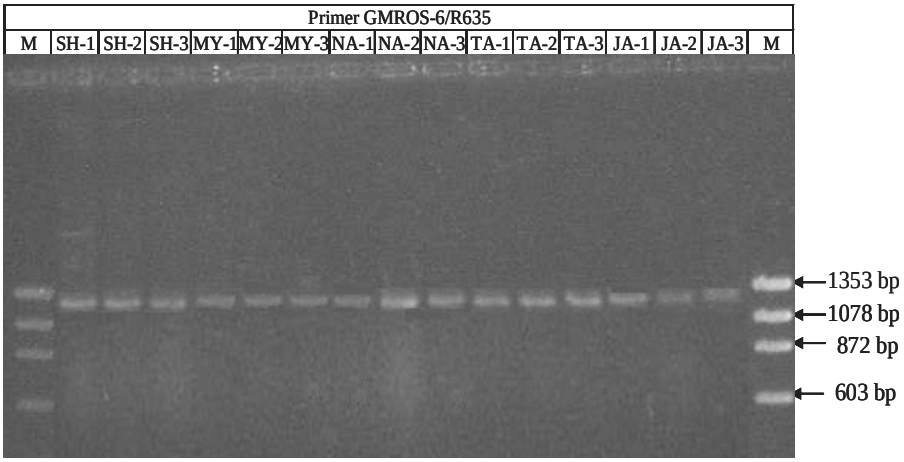


Figure 6 : PCR amplification of total genomic DNA from 30 isolates of *R. oryzae-sativae* using the primer pairs of GMROS-6 with R635; (a) Isolates DE-1-NE-3 (b) Isolates SH-1-JA-3; Lanes: M, PhiX174/HaeIII markers (Promega)

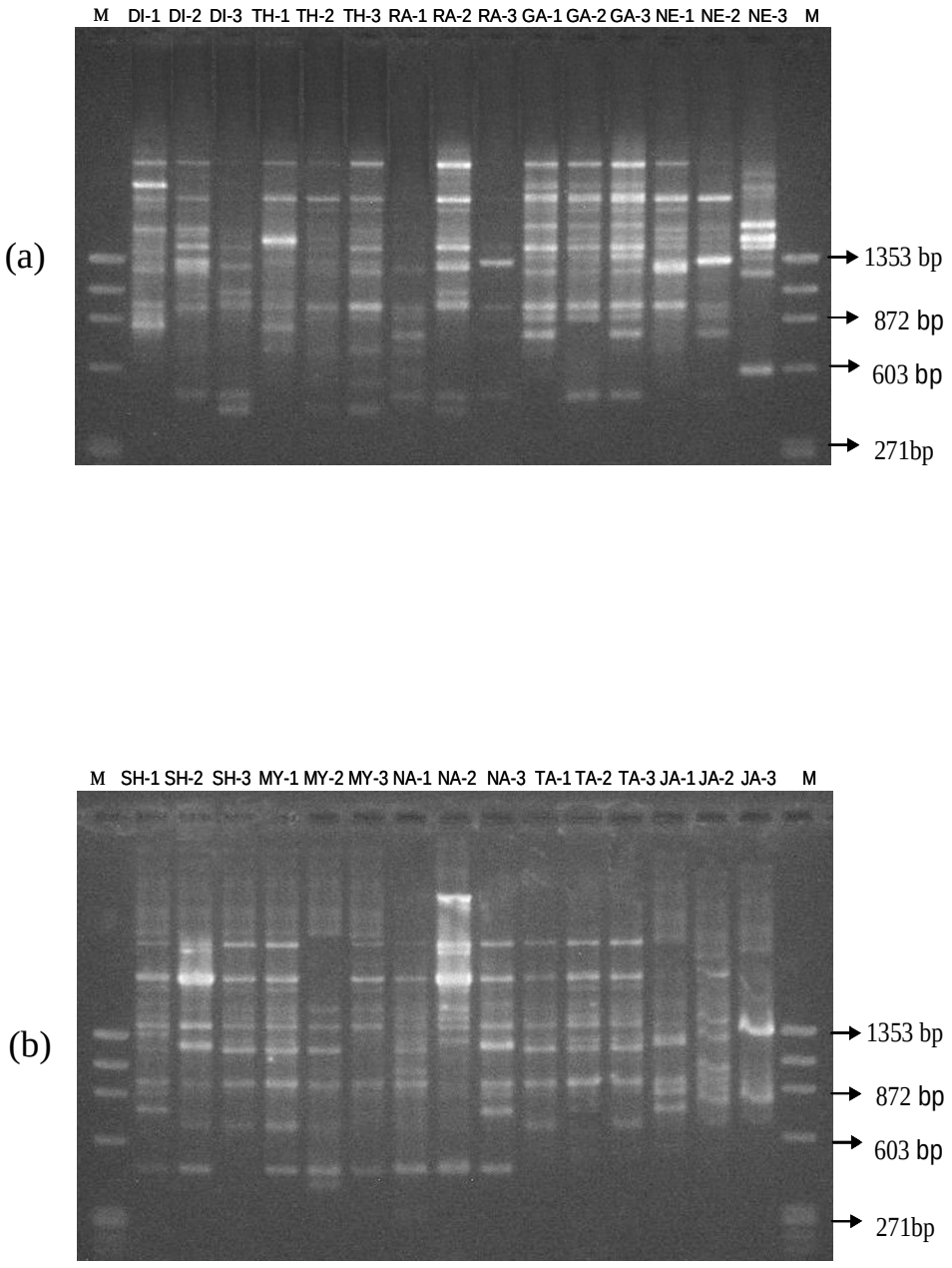


Figure 7 : DNA fingerprint profiles of 30 isolates of *R. oryzae-sativae* obtained with GF primer; (a) Isolates DE-1-NE-3 (b) Isolates SH-1-JA-3; Lanes: M, PhiX174/HaeIII markers (Promega)

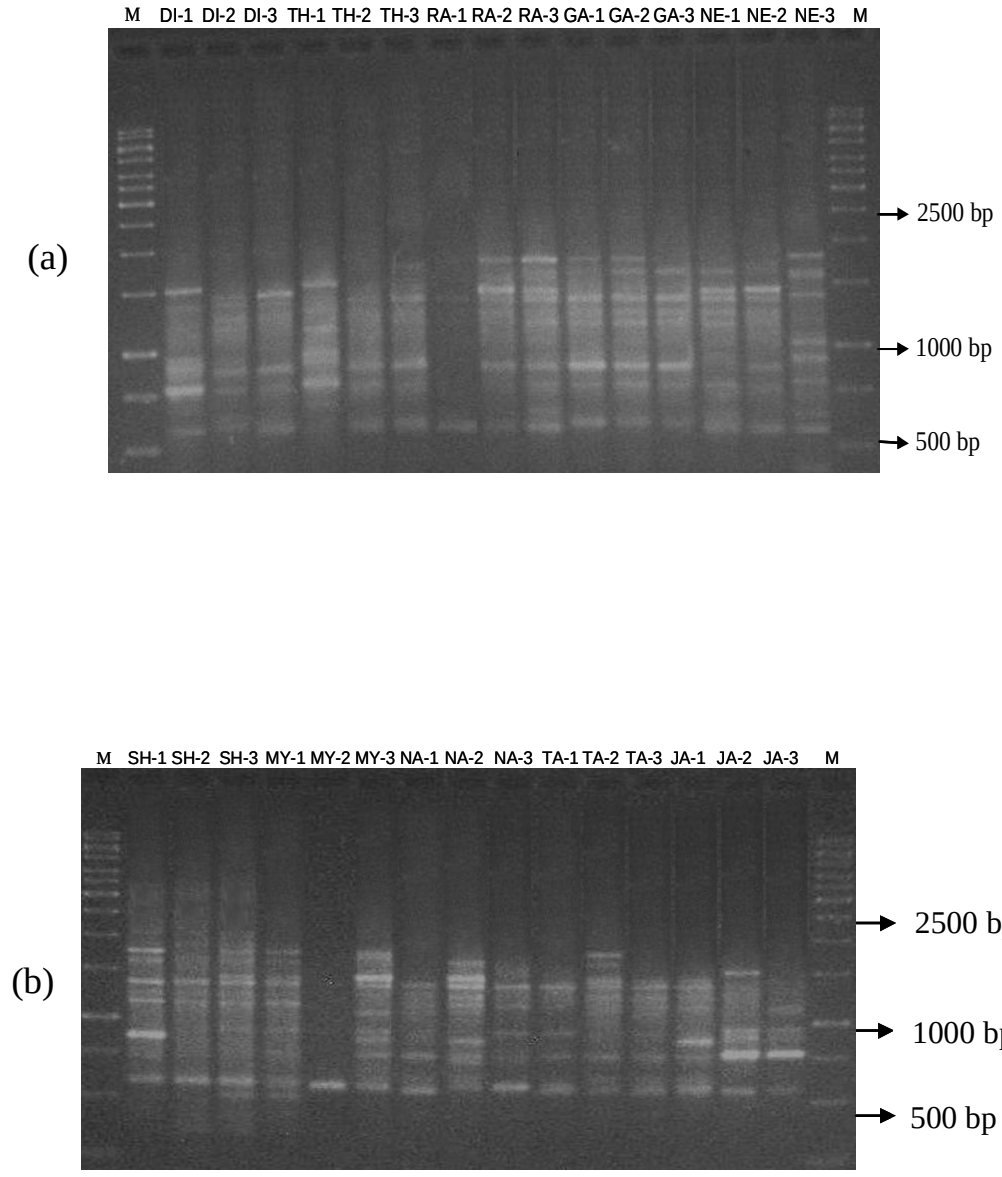


Figure 8 : DNA fingerprint profiles of 30 isolates of *R. oryzae-sativae* obtained with MR primer; (a) Isolates DE-1-NE-3 (b) Isolates SH-1-JA-3; Lanes M: size marker (1Kb ladder)

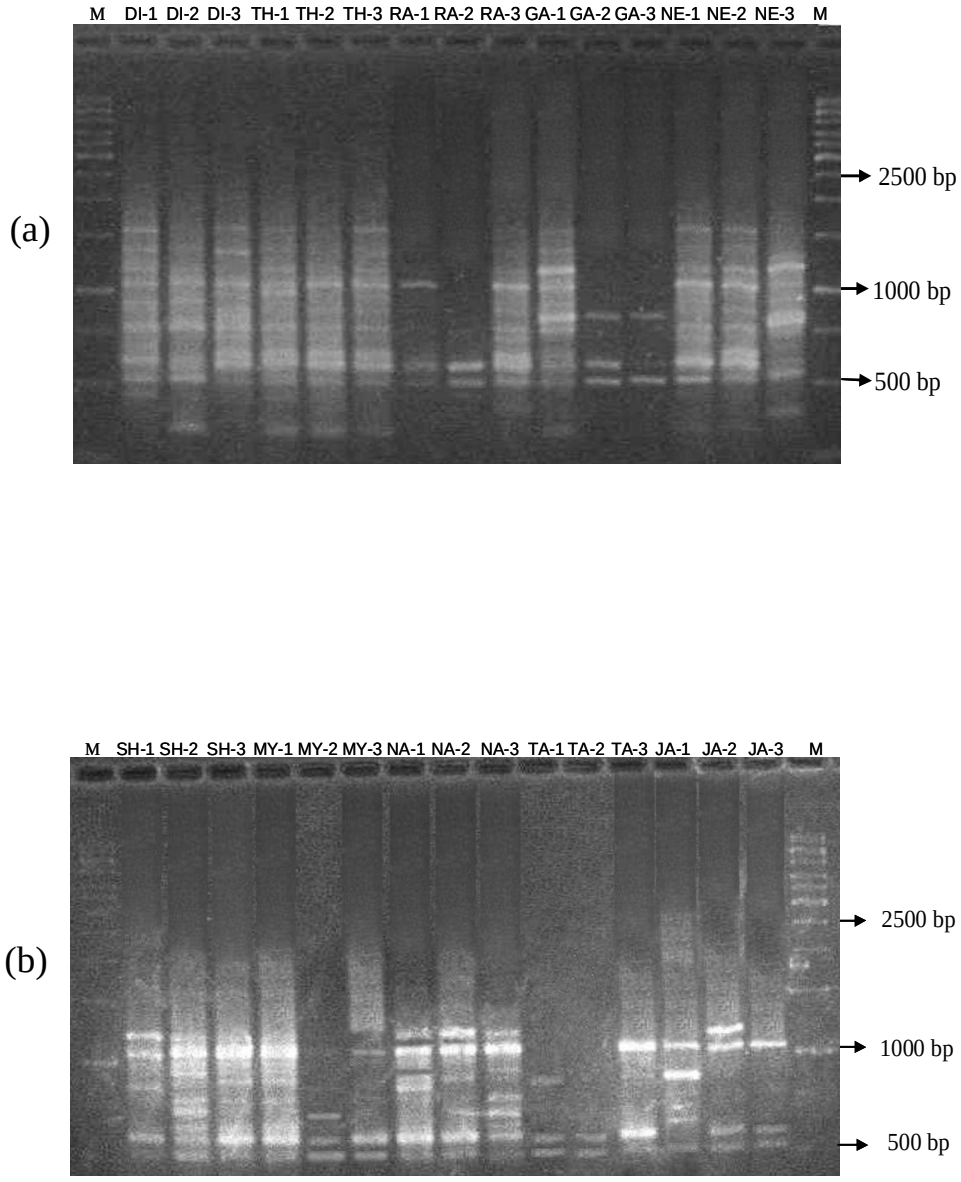
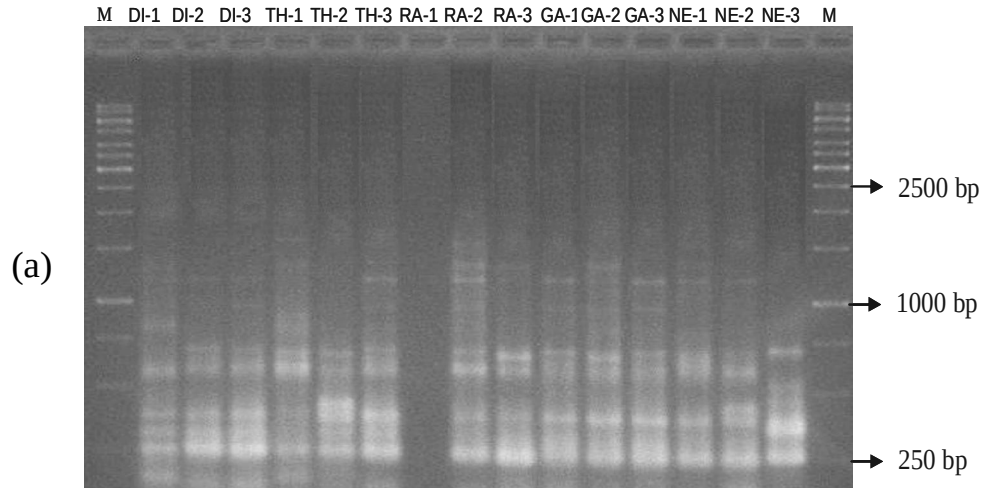
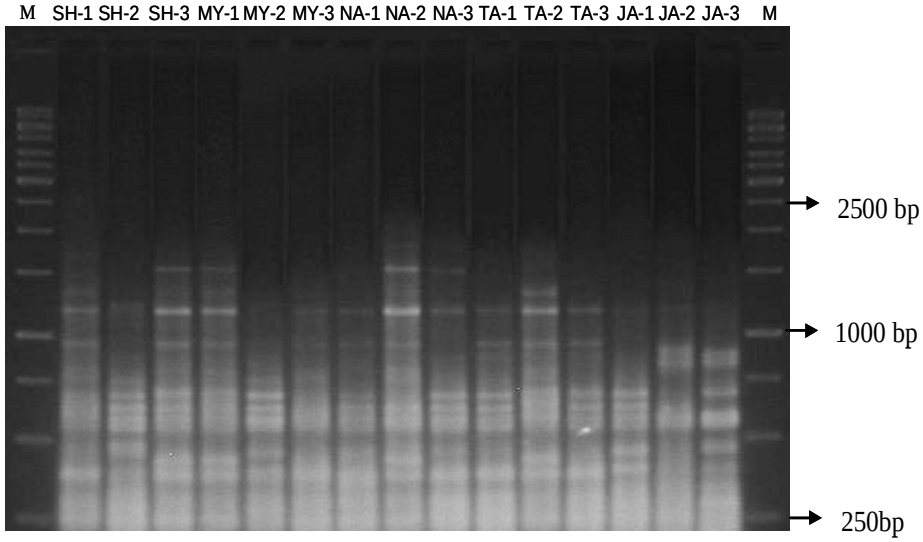


Figure 9 : DNA fingerprint profiles of 30 isolates of *R. oryzae-sativae* obtained with RY primer; (a) Isolates DE-1-NE-3 (b) Isolates SH-1-JA-3; Lanes M: size marker (1Kb ladder)



(a)



(b)

Figure 10: DNA fingerprint profiles of 30 isolates of *R. oryzae-sativae* obtained with BOXA1R primer; (a) Isolates DE-1-NE-3 (b)Isolates SH-1-JA-3; Lanes M: size marker (1Kb ladder)

3.3.5 Virulence of the isolates of *R. oryzae-sativae*

After artificial inoculation, all isolates induced typical rice aggregate sheath spot symptoms. The lesions were round to ellipsoid with grey-green to straw color in the center surrounded by distinct brown margin (Fig. 13 and 14). The RLH ranged from 6.58% to 18.62%. Significant variation in virulence was found among the isolates but the level of RLH was low. However the highest RLH was found in MY-3 (18.62%) followed by NA-2 (15.07%) and MY-1 (14.90%). Least RLH was found in TH-2 (6.58%) followed by TH-3(7.02%). On the other hand, the highest incidence was found in TA-1 (69.90%) followed by GA-1 (65.58%) and NA-2 (65.22%) while lowest incidence found in DI-1 (44.19%) followed by DI-3 (45.83%) as well as TH-1 (45.65%).

Mean RLH of Mymensingh isolates (15.97%) was found the highest followed by Narsingdi isolates (13.73%) while the lowest mean RLH observed in Thakurgaon isolates (7.23%). Furthermore, the mean incidence of Gazipur isolates was found the highest (62.54%) and similar incidence was observed in the isolates of Tangail (62.13%). The lowest incidence was observed in Dinajpur (46.26%) followed by Thakurgaon (48.44%). Disease reactions of ten districts presented in Table 10 and Fig. 11. Significant correlation (R^2=0.69) was found between mycelial growth rate and RLH (Fig. 12). However, no significant correlation was found neither between mycelial growth rate and incidence (R^2=0.28) nor with RLH and incidence (R^2=0.27).

Table 10 : Disease reaction of thirty *R. oryzae-sativae* isolates on BR11 (T. Aman)

Location	Isolate	Disease reaction			
		RLH (cm)		Incidence(%)	
Dinajpur	DI-1	6.97	lm	44.19	n
	DI-2	9.44	ghi	48.78	klm
	DI-3	7.85	ijkl	45.83	mn
Thakurgaon	TH-1	8.07	jkl	45.65	mn
	TH-2	6.58	m	47.83	lmn
	TH-3	7.03	lm	51.85	hijk
Rajshahi	RA-1	8.41	ijk	52.94	ghij
	RA-2	7.83	jklm	57.45	f
	RA-3	8.34	ijk	51.16	ijkl
Mymensingh	MY-1	14.90	b	56.52	fg
	MY-2	14.38	bc	62.26	bcde
	MY-3	18.62	a	58.54	def
Gazipur	GA-1	7.89	jkl	65.58	b
	GA-2	8.95	hij	62.96	bc
	GA-3	8.28	ijkl	59.09	cdef
Tangail	TA-1	11.77	ef	69.90	a
	TA-2	8.75	ijk	57.78	f
	TA-3	12.21	e	58.70	def
Netrokona	NE-1	8.21	ijkl	52.83	ghij
	NE-2	7.79	jklm	49.06	jklm
	NE-3	7.47	klm	48.21	klm
Jamalpur	JA-1	10.57	fg	57.50	f
	JA-2	12.75	de	58.33	ef
	JA-3	14.26	bc	62.50	bcd
Sherpur	SH-1	9.41	ghi	58.93	def
	SH-2	14.30	bc	62.50	bcd
	SH-3	10.26	gh	53.33	ghi
Narsingdi	NA-1	13.52	cd	50.98	ijkl
	NA-2	15.07	b	65.22	b
	NA-3	12.61	de	55.56	fgh

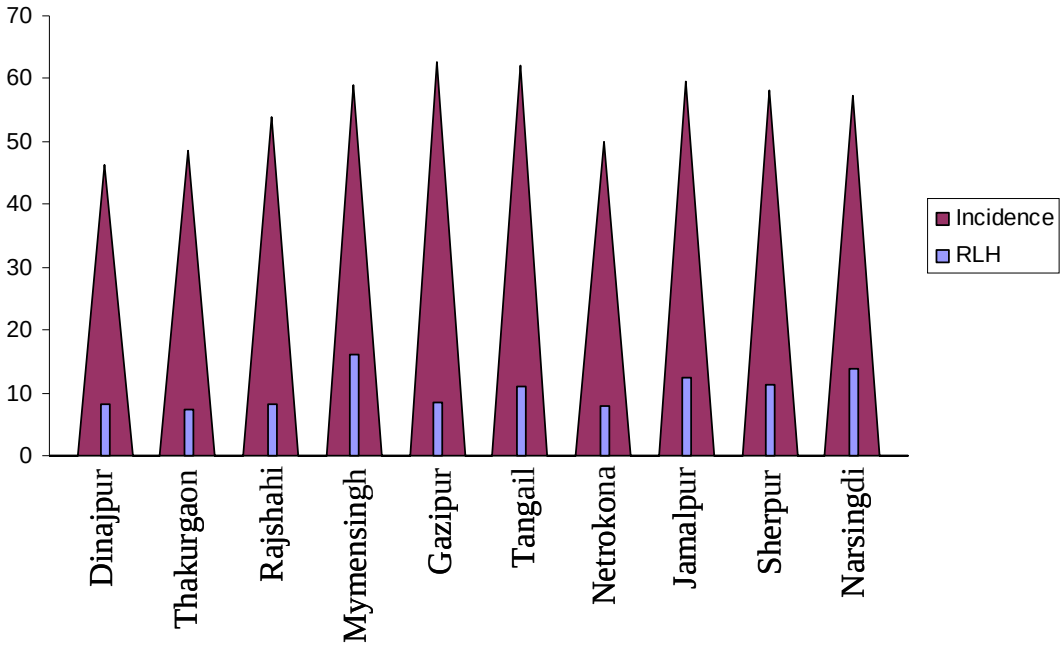


Figure 11: Disease reaction of *R. oryzae-sativae* isolates of ten districts on BR 11 (T. Aman)

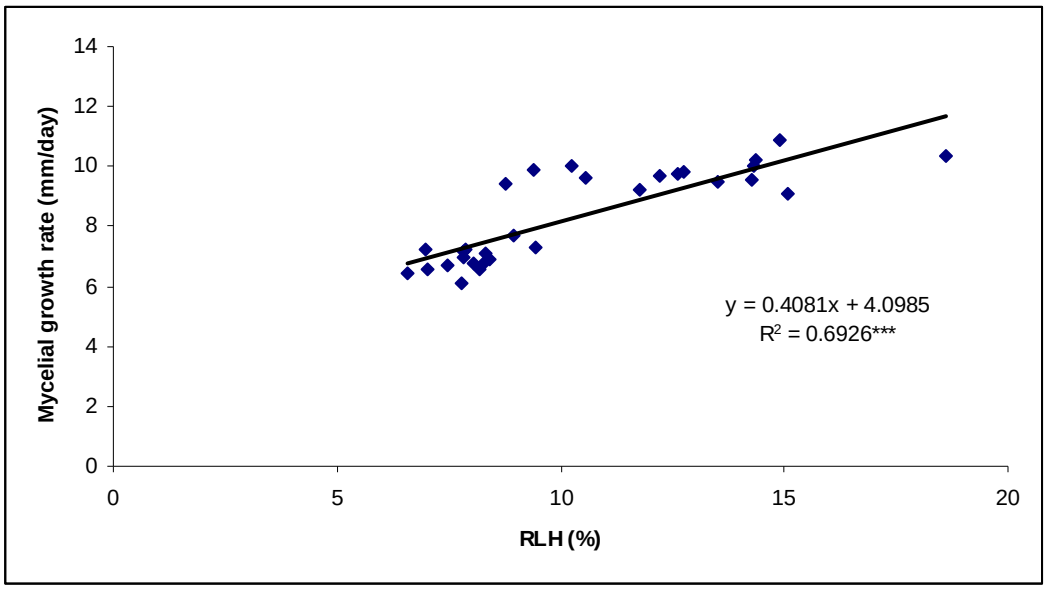


Figure 12 : Correlation between RLH and mycelial growth rate.
***** indicated significant at 0.1% level**

Figure 13 : PDA split with mycelial plug inserted between the tillers in artificial inoculaion

Figure 14 : Aggregate sheath spot disease symptoms produced by *R. oryzae-sativae*

3.4 Discussion

R. oryzae-sativae first reported during 1988 in Bangladesh. Shahjahan *et al*., (1988) revealed that aggregate sheath spot was prevalent in the Bangladesh Rice Research Institute experimental farm during all the three rice growing seasons. It was found on local and modern cultivars as well as advanced breeding lines. Aggregate sheath spot has been occurring throughout the country, but the characteristics and diversity of *R. oryzae-sativae* population is poorly understood in Bangladesh. Therefore, investigation on morphological characteristics of *R. oryzae-sativae* isolates as well as their genetic variation was analyzed using VNTR and Rep-PCR analysis.

In this investigation, a simple PCR based technology using ITS1 specific primer GMROS-6 was used for rapid and accurate identification of thirty *R. oryzae-sativae* isolates. GMROS-6 with the combination of R635 gave a single product of 1200bp resulting a convenient molecular tool for an easy and rapid species detection without hyphal anastomosis test with the tester isolates (Johanson *et al*; 1998).

Morphological qualitative characters like aerial mycelium, colour of sclerotia, and position of sclerotia (scattered, aggregated, on mycelia and on mycelia+embeded in agar) were found and consonant with Chaijuckam *et al*. (2010a). *R. oryzae-sativae* isolates which produced abundant sclerotia were found more virulent and similar observations were also reported by many workers in *R. solani* (Chien *et al*., 1969; Dath, 1984; Basu and Gupta, 1992; Banniza *et al*., 1999; Ali, 2002). Some characters were noted identical where all the isolates produced globose and irregular shape of sclerotia. This type of sclerotia was also found by Lanoiselet *et al*. (2001) in Australia. Most of the isolates with fast mycelial growth rate produced no pesudo sclerotia while the isolates with slow mycelial growth rate produced few to abundant pseudo sclerotia (Appendix 1).

Mycelial growth rate of isolates varied from 0.26-0.45 mm per hour. Variation in growth rate was also found by Lanoiselet *et al*. (2005c) and Chaijuckam *et al.* (2010a).

Virulence test was conducted on cultivar BR11 under field condition at BRRI farm, Gazipur. All isolates of *R. oryzae-sativae* produced typical symptoms of aggregate sheath spot within 7-10 days. The highest RLH showed by MY-3 (18.62%). Variability in virulence among the isolates could be explained in several ways. Firstly, variation in cultural and genetic characteristics of the isolates might be contributed to variability in virulence. Similar observation was reported by Taheri *et al.* (2007) in Indian *R. oryzae-sativae* isolates. Banniza, (1997) also found similar results in *R. solani* isolates. Secondly, Vidhyasekaran *et al*. (1997) reported that variation was found in toxin production among the isolates of *R. solani.* They mentioned that there is a positive correlation between virulence and toxin production. Furthermore, Singh *et al.* (2002) reported that pathogenic variation is related to the distribution of the isolates in different environment across the climatic regions. These might be influenced the pathogenic variability so far. However, the isolates failed to show high level of RLH. The experimental plot was drenched by continuous rainfall from seven days after inoculation. Low temperature at that period might be another cause for lower disease development. Mycelial growth rate showed significant correlation with the RLH (R^2= 0.69) which indicated that isolates with faster mycelial growth was virulent among the isolates. The results were in agreement with Akai *et al.* (1960) and similar to *R. solani* by Basu and Gupta (1992). However, no significant correlation was found neither between growth rate and incidence (R^2= 0.28) nor with RLH and incidence (R^2= 0.27).

SCGs displayed high resolution showing 90% diversity. In this experiment isolates with same multilocus genotypes in a same SCG were found both from specific (TA-1+TA-3, GA1+GA2) or different locations (MY-1+SH-3). Chaijuckam and Davis (2010a) mentioned that some isolates in the same SCG had different

multilocus genotypes but the isolates with the same multilocus genotypes were alaways in the same SCG. The isolates collected from different location like MY-1+SH-3 showed same multilocus genotypes meant that same *R. oryzae-sativae* clones were exist across the locations were separated by up to 70 km. This is evidence of dissemination of asexual propagules (sclerotia or mycelia) over relatively long distance. In Texas, Rosewich *et al.* (1999) identified clones in populations were separated by up to 280 km using an RFLP fingerprint probe with large populations. Dissemination of asexual propagules is made possible by the transport of sclerotia or other asexual fungal structures in soil, flash flood or by contaminated seeds.

The tested isolates of *R. oryzae-sativae* were classified genetically into three groups using VNTR and Rep-PCR primers at 69.6% similarity level. There were two main groups and another one isolate made a separate group. However, some relationship was observed between genetic variation and morphological characteristics. Group I produced by DNA fingerprint dendrogram exhibited 92% similarity with Group I produced by MVSP dendrogram. On the other hand Group II produced by DNA fingerprint dendrogram showed 86% similarity with Group II produced by MVSP dendrogram. The dissimilarities between two types of dendrogram might be occurred because of morphological characters which generally influenced by the environment and cultural conditions. Therefore, problems associated with studying different levels of genetic diversity in pathogen have been suggested to be best addressed by the use of molecular techniques (Toda *et al*; 1999). The similarities of cultural and molecular characteristics clustered the isolates from Dinajpur, Thakurgaon, Rashahi, Gazipur and Netrokona in group I with slow mycelial growth rate, small size sclerotia with less intensity. These results are in accordance with Singh *et al.* (2002), Sharma *et al.* (2005), and Khodayari *et al.* (2009) who have also reported that most of the microsclerotia forming isolates of *R. solani* were grouped together using RAPD and ISSR and ERIC markers analysis. The isolates which clustered in group II showed fast mycelial growth rate with intensified larger size of sclerotia. This isolates were

collected from Mymensingh, Tangail, Jamalpur, Sherpur and Narshingdi areas. It indicated that all the fast growing isolates existed in the middle part of the country (AEZ 9). A third group solitary isolate RA-1 collected from Rajshahi were characterised with slow mycelial growth rate and less sclerotial intensity. The difference of this RA-1 isolate from group-1 because of its null alleles in several primers, possibly caused by a mutation at the 3' end of the primer binding sites (Butler, 2005; (Chaijuckam *et al*., 2010c). However, at 90% similarity level, the isolates were categorized into 26 groups, which indicated a high genetic diversity of rice aggregate sheath spot fungi as revealed by VNTR and rep-PCR primers. Such kind of variation was observed in other pathogen like *Pyricularia grisea* (Mian *et al*., 2002 and 2003) and *Bipolaris oryz*ae (Kamal, 2006). The highest genetic similarity was found between MY-1 and SH-3 (98%) followed by TA-1 and TA-3 (97%) while the lowest genetic similarity shared by RA-1 and SH-1(36%) followed by RA-1 and JA-1(38%) (Appendix 4). Genetic variability indicated sexual recombination in this fungus. The genetic variation among the isolates could be explained by sexual reproduction, which is common in California rice fields (Chaijuckam and Davis 2010a). Airborne basidiospores may have the potential to disperse long distances. More focus on the genetic structure of *R. oryzae-sativae* is necessary in order to compare population structure and biological evolution of different taxonomic groups of this soil borne pathogen. Variation in the isolates of *R. oryzae-sativae* indicated that various genotypes of *R. oryzae-sativae* present in Bangladesh and should be included in screens of resistance genes in rice and fungicides

Physiological Effect on *R. oryzae-sativae*

4.1 Introduction

Soilborne phytopathogen affects rice production by inhabiting inoculum permanently in the soil. Sheath diseases of rice caused by *Rhizoctonia solani, R. oryzae* and *R. oryzae-sativae* are important soilborne phytopathogens distributed worldwide and cause yield losses in rice growing countries. The growth rates and sclerotia formation of different *Rhizoctonia* spp. are not uniform at the same level of two growth factors. Even it differs among the isolates of specific pathogen. Many researchers reported that mycelial growths of fungi were significantly influenced by physiological condition (Stamet, 1993, Kadiri and Kehindi, 1999, Ibekwe *et al*., 2008). Fungi secure food and energy from the substrates upon which they live in nature. In order to culture fungi in the laboratory, it is necessary to furnish in the medium with those essential elements and compounds they require for the synthesis of their cell constituents and for the operation of their life processes. All the fungi require much the same essential elements for functional as well as structural purposes but differ widely in their ability to utilize compounds in which these elements occur. Aggregate sheath spot caused by *R. oryzae-sativae* is one of the major rice disease in California (Linquist *et al*., 2008). In Bangladesh, the disease was observed on both local and modern cultivars and advanced breeding lines (Shahjahan *et al*., 1988). Variation in mycelial growth of *R. oryzae-sativae* was found by Sharma (2002) at different pH level. Ray and Pan (1989) studied the effect of temperature, soil moisture and soil pH on sclerotial germination of *R. oryzae-sativae*. They observed the optimal condition for sclerotial germination was at 30ºC and 75% soil moisture. Sclerotia germinated

well when incubated at 20-30ºC, but germination was better between 30ºC and 40ºC. Gunnell and Webster (1987) found *R. oryzae-sativae* was not favoured by high nitrogen applications in field. Another field trial suggested that aggregate sheath spot might be favoured by potassium deficient soils (Willams and Smith, 2001). A lot of information exists on the *R. solani* but, very little is known about *R. oryzae-sativae* for physiological condition affect the growth. Although aggregate sheath spot has frequent occurrence in the rice field in Bangladesh but unfortunately, no works had been done to evaluate the nutritional factors that affect on the growth of *R. oryzae-sativae in vitro*. The carbon and nitrogen sources are the important constituents of every medium. Therefore, this study is aimed to find out useful information about the effect of different carbon sources, nitrogen sources and different pH level on mecelial growth and sclerotia formation of *R. oryzae-sativae in vitro*.

4.2 Materials and Methods

4.2.1 Effect of different pH level on mycelial growth and sclerotial intensity of *R. oryzae-sativae*

Seven pH levels such as 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were investigated for the growth of MY-1 isolates on PDA medium. The pH was adjusted with 50% lactic acid in the range of 3.0-6.0 and 10N NaOH in the range of 7.0-9.0 before sterilization. Mycelial disks of 6 mm diameter were transferred aseptically from the margin of 3-day-old colonies at the centre of PDA plates (90 mm diameter) and incubated at 27ºC. The mycelial colony diameter was recorded every day until the complete coverage with fungal growth in any plate (Verma and Prasad, 1975; Sonai and Muthukrishnan, 2010). Four replicate plates were used for each treatment. Sclerotial intensity was also observed. Data were analyzed by CropStat (version 7.2) computer software.

4.2.2 Effect of different carbon sources on mycelial growth and sclerotia formation of *R. oryzae-sativae*

Carbon sources i.e. Dextrose (pH 6.7), Sucrose (6.8), Galactose (6.8), Xylose (6.6), Fructose (6.7) and Lactose (6.8) were examined in this experiment. 2% Carbon source and 2% agar were mixed with 100 ml potato extract and autoclaved. Mycelial disks of 6 mm diameter were transferred aseptically from the margin of 3-day-old colony at the centre of PDA plates (90 mm diameter). Four replicate plates were used for each treatment. Radial mycelial growth and sclerotial data (sclerotial intensity) were measured as 4.1.1. Data were analyzed by CropStat (version 7.2) computer software.

4.2.3 Effect of different nitrogen sources on mycelial growth and sclerotia formation of *R. oryzae-sativae*

Nitrogen sources i.e. Peptone (pH 6.3), Yeast (6.0), KNO_3 (7.1), $NaNO_3$ (7.2), NH_4NO_3 (6.1) and Urea (7.3) were examined in this experiment. 2% Nitrogen source and 2% agar were mixed with 100 ml potato extract and autoclaved. Mycelial disks of 6 mm diameter were transferred from the margin of 3-day-old colony in the centre of PDA plates and incubated at 27ºC. Four replicated plates were used for each treatment. Control plate with only PDA (6.5) was also observed and compared with treated plates. Radial mycelial growth and sclerotial data (sclerotial intensity) were measured as 4.1.1. Data were analyzed by CropStat (version 7.2) computer software.

4.3 Results

4.3.1 Effect of different pH level on mycelial growth and sclerotial intensity of *R. oryzae-sativae*

To determine the suitable pH on PDA media with the pH range of 3.0 to 9.0 were used on mycelial growth of *R. oryzae-sativae*. Significant differences in mycelial growth were observed within the pH levels. The pathogen grew on PDA with maximum growth at 6.0-9.0. Minimum growths were recorded at pH level 3.0-5.0 (Table 11 and Plate 4). The highest growth rate (0.46 mm/hr) was recorded at pH 7.0 followed by 6.0 (0.45 mm/hr) and 8.0 (0.43 mm/hr). The lowest growth was found at pH 3.0 (0.33 mm/hr) followed by pH 4 (0.37 mm/hr) and 5.0 (0.40 mm/hr). Abundant sclerotial intensity was recorded also at pH 7.0, while moderate sclerotial intensity recorded at pH 8.0 and 9.0 after 96 hours of incubation. Poor sclerotia formed at pH level 5.0 and 6.0, but no sclerotia formation was observed at pH level 3.0 and 4.0 after 96 hours of incubation.

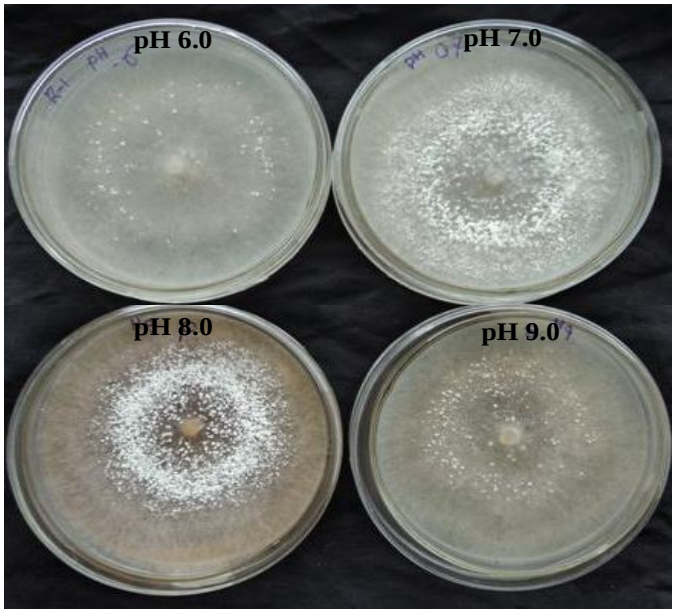


Plate 4 : Sclerotial intensity of *R. oryzae-sativae* on different pH level after 96 hours of incubation

Table 11 : Effect of different pH level on mycelial growth and sclerotial intensity of *R. oryzae-sativae*

pH	Mycelial growth rate (mm[a]) * over incubation period (hr[b])			Growth rate (mm/hr)	Sclerotial Intensity[c]
	48	72	96		
3.0	11.50	20.00	31.25	0.33	-
4.0	14.00	23.50	35.75	0.37	-
5.0	16.25	27.00	38.00	0.40	+
6.0	22.50	33.50	43.00	0.45	+
7.0	23.50	34.25	44.50	0.46	+ + +
8.0	19.75	31.25	41.50	0.43	+ +
9.0	19.00	30.50	40.25	0.42	+ +
SE(N=4)	0.35	0.32	0.38		
5% LSD 21DF	1.03	0.93	1.12		

* = Each value is an average of four replications, [a] mean mycelial growth in mean, [b] mean mycelial growth in hour. [c] Intensity: - = No sclerotia, + = poor; ++ = moderate; +++ = Abundant.

4.3.2 Effect of different carbon sources on mycelial growth and sclerotia formation

Six different Carbon sources were tested in this experiment to know their effect on the growth and sclerotia formation of *R. oryzae-sativae*. All the Carbon sources showed statistically significant different mycelial growth at 48, 72, and 96 hours after incubation (Table 12 and Plate 5). The highest mycelial growth was recorded in dextrose (0.47 mm/hr) followed by sucrose (0.44 mm/hr) and galactose (0.41 mm/hr), while the lowest mycelial growth was noted in fructose (0.24 mm/hr), xylose (0.32 mm/hr) and lactose (0.39 mm/hr) after 96 hours of incubation. Moderate sclerotial intensity was found in dextrose while sucrose, galactose and lactose showed slight sclerotial intensity. On the other hand no, sclerotium was formed in xylose and fructose after 96 hours of incubation.

Table 12 : Effect of different carbon sources on mycelial growth and sclerotial intensity of *R. oryzae-sativae*

Carbon sources	pH	Mycelial growth rate (mm[a]) * over incubation period (hr[b])			Growth rate (mm/hr)	Sclerotial Intensity[c]
		48	72	96		
Dextrose	6.7	24.00	34.50	45.00	0.47	+ +
Sucrose	6.8	21.50	31.00	42.00	0.44	+
Galactose	6.8	17.50	29.50	39.00	0.41	+
Xylose	6.6	12.75	21.25	31.00	0.32	-
Fructose	6.7	10.75	16.75	23.50	0.24	-
Lactose	6.8	16.50	27.00	37.75	0.39	+
SE (N=1)		0.56	0.59	0.96		
5% LSD		1.73	1.82	2.97		

* = Each value is an average of four replications, [a] mean mycelial growth in mean, [b] mean mycelial growth in hour. [c] Intensity: - = No sclerotia, + = poor; ++ = moderate

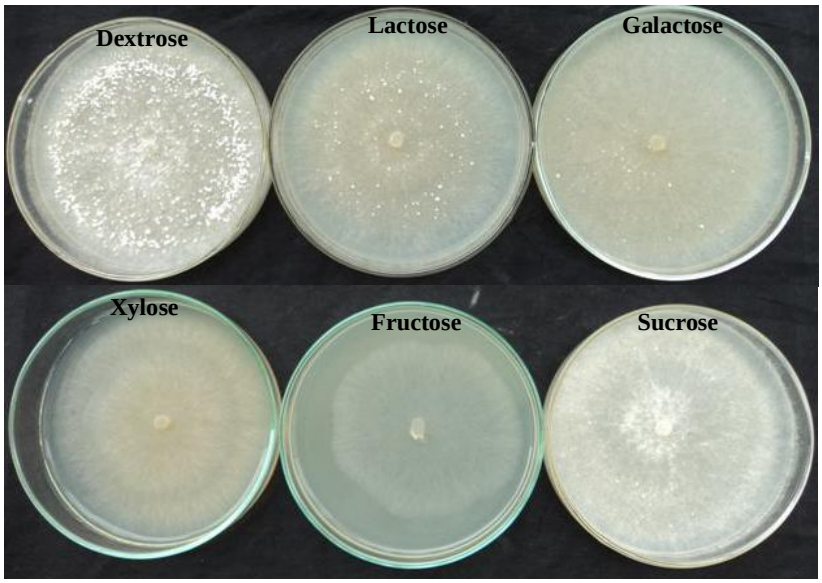


Plate 5 : Effect of different carbon sources on mycelial growth and sclerotial intensity of *R. oryzae-sativae* after 96 hours of incubation

4.3.3 Effect of different nitrogen sources on mycelial growth and sclerotia formation

Among the six different nitrogen sources used to find out the optimal culture condition in this experiment, the best mycelial growth rate of *R. oryzae-sativae* measured in peptone (0.43 mm/hr) followed by yeast (0.37 mm/hr), and $NaNO_3$ (0.34 mm/hr), while the least growth was recorded in NH_4NO_3 (0.17 mm/hr) after four days of incubation (Table 13 and Plate 6). Though peptone was found to be the best for mycelial growth, but there was no sclerotia formed after 4 days of incubation. However, the highest mycelial growth was found in the control plate (without nitrogen source) and moderate sclerotial intensity was observed after 4 days of incubation. The most unsuitable source was urea. There was no mycelial growth and sclerotial formation observed in urea (47% Nitrogen) treated plates. In general, mycelial growth of *R. oryzae-sativae* increased with the lower nitrogen percentage.

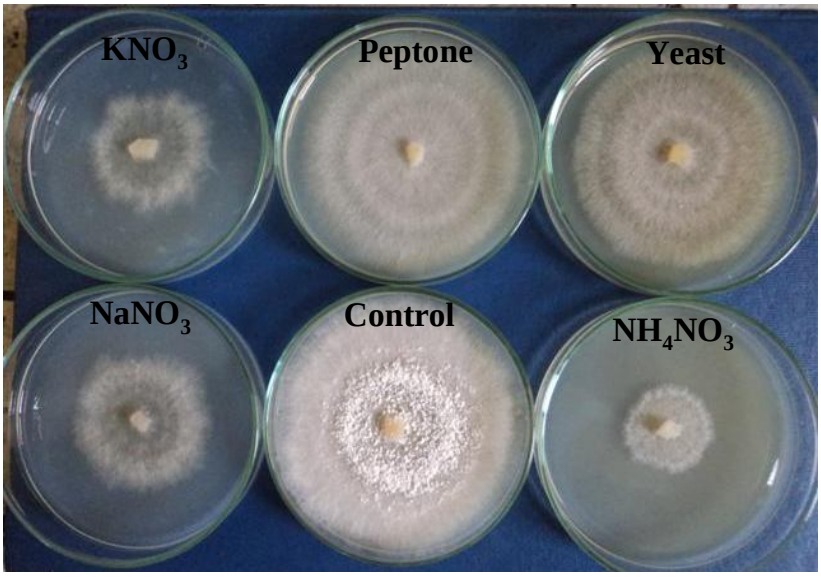


Plate 6 : Effect of different nitrogen sources on mycelial growth and sclerotial intensity of *R. oryzae-sativae* after 96 hours of incubation

Table 13 : Effect of different nitrogen sources on mycelial growth and sclerotial intensity of *R. oryzae-sativae*

Nitrogen sources	pH	Nitrogen (%)	Mycelial growth rate (mm[a]) * over incubation period (hr[b])			Growth rate (mm/hr)	Sclerotial Intensity
			48	72	96		
Peptone	6.3	10.0	20.25	31.25	41.75	0.43	-
Yeast	6.0	*	16.75	26.50	35.75	0.37	-
KNO_3	7.1	13.86	10.50	17.00	26.75	0.28	-
$NaNO_3$	7.2	16.47	15.00	23.00	32.50	0.34	-
NH_4NO_3	6.1	35.0	7.50	12.25	16.50	0.17	-
Urea	7.3	47.0	0.00	0.00	0.00	0.00	-
Control (PDA)	6.7	*	22.50	32.50	43.50	0.45	+
SE (N=1)			0.82	0.72	0.62		
5% LSD 18DF			2.43	2.14	1.83		

* = Each value is an average of four replications, [a] mean mycelial growth in mean, [b] mean mycelial growth in hour. - = N0 sclerotia, + Moderate sclerotia

4.4 Discussion

Seven different pH levels, six different carbon sources and six different nitrogen sources were examined to determine the optimal cultural condition of *R. oryzae-sativae* after 96 hours of incubation. The pathogen *R. oryzae-sativae* grew on PDA with the maximum growth at 6.0-9.0. The minimum growth was recorded at pH ranged from 3.0-5.0. The growth of this fungus reduced at very strong acidic and alkaline pH. This result agrees with the findings of Sharma (2002) in *R. oryzae-sativae* and in basidiomycetous fungus *Volvariella volvacea* (Moonmoon *et al.*, 2008).

Among the carbon sources maximum mycelial growth rate was recorded in dextrose (0.47 mm/hr). Whether a sugar is utilized or not, depends upon both the configuration (arrangement of the hydrogen and hydroxyl groups) of the sugar and the particular abilities of the specific fungus. Dextrose (D-Glucose) is the most widely utilizable carbon source and PDA is the most commonly used laboratory medium for fungi due to its good and balanced nutrient content. Dextrose helps to formation of an adaptive enzyme which helps the fungi to grow well in medium. However, when optimized or modified by nutrients viz. sucrose, and peptone, it gives better result of fungal growth rate (Gupta *et al.*, 2012). Several workers stated PDA to be the best media for mycelial growth of fungi (Xu *et al.*, 1984, Maheshwari *et al.*, 1999, Saha *et al.*, 2008). Next to dextrose, sucrose showed a better performance on mycelial growth (0.44 mm/hr) of *R. oryzae-sativae.* This result is similar to that of Shim *et al.*, 2005, Moonmoon *et al.*, 2008, and Imtiaj *et al.*, 2008 who found sucrose was the best for mycelial propagation of *Volvariella volvacea*, *Agrocybe cylindracea*, and *Macrolepiota procera* which belongs to basidiomycetes class. The next best in order to merit were galactose, lactose, xylose, and fructose (Table 12 and Plate 5). The fungus produced moderate sclerotial intensity in dextrose while slight sclerotial intensity was observed in sucrose, galactose and lactose after 96 hours of incubation. There was no sclerotia formed recorded within the xylose and furctose treated media at the same time.

Among the six different nitrogen sources used in this experiment, the best mycelial growth of *R. oryzae-sativae* found in peptone (0.43 mm/hr) followed by yeast (0.37 mm/hr), while the least growth was found in NH_4NO_3 (0.17mm/hr) after four days of incubation (Table 13 and Plate 6). This result is similar to that of Moonmoon *et al.* (2008) in *Volvariella volvacea*. Kadiri and Fasidi (1994) found peptone was the best nitrogen source for mycelial propagation of *Lentinus subnudus*. Luo (1993) reported that peptone is the preferred nitrogen source for mycelium growth of *Auricularia auricular*. Though peptone is found to be the best for mycelial growth, but there were no sclerotia formed after 96 hours of incubation. However, the highest mycelial growth was measured in the control plate (without nitrogen source) and moderate sclerotial intensity was produced after 96 hours of incubation. Most unsuitable source was urea and no mycelial growth and sclerotia observed in urea (47% Nitrogen) treated plates. This result is also agreed with the findings of Moonmoon *et al*. (2008) in *Volvariella volvacea*, who have found urea as the most unsuitable nitrogen source and no result found in urea. Gunnell and Webster (1987) found *R. oryzae-sativae* was not favoured by high nitrogen applications in field. It was proved in this experiment that, high nitrogen percentage disfavoured the growth and sclerotia formation of *R. oryzae-sativae* not only in field condition but also *in vitro* test. In general, mycelial growth of *R. oryzae-sativae* increased with the lower nitrogen percentage. Though KNO_3 have lower nitrogen percentage compared to $NaNO_3$, mycelial growth of *R. oryzae-sativae* found lower than $NaNO_3$ treated plates. In this experiment, mycelial growth might be decreased for simultaneous presence of potassium and nitrogen which is agreed with the findings of Willams and Smith (2001). They suggest that aggregate sheath spot might be favoured by potassium deficient soils.

Management of Aggregate Sheath Spot Disease of Rice

5.1 Introduction

Rice is grown as an important cereal crop all over the world, but mostly in Southeast Asian countries. Among the different diseases that attack rice crops, rice sheath diseases are considered as one of the dominant diseases causing significant reduction of rice yield. In Bangladesh the disease is spreading in wheat and potato growing areas on Northwest Bangladesh and yield loss was about 1.0% (Ali *et al.* 2004). However, in Australia yield loss reported up to 20% and 4 to 9% in Uruguay (Lanoiselet, 2005b). Many methods of plant disease control are presently being used to control sheath diseases of rice such as chemical, physical, cultural methods etc. Selection of control measure options is largely based on rice production system that allows one to live with disease, reducing the inevitable loss as much as possible. Crop rotations may be ineffective even if long because of wide host range or may be uneconomical to farmers (Lee and Rush, 1983). *R. oryzae-sativae* has only been reported on rice, perennial wild red rice, tidal marsh flat sedge and Manchurian wild rice (Mordue, 1974), so any of those crops in the cropping pattern is likely to continue disease spread in following rice crop. In California, currently recommended practices (Lanoiselet *et al*., 2005a and Miller and Webster, 2001) demonstrated that straw management practices such as burning or bailing stubble could reduce the number of *R. oryzae-sativae* sclerotia present in the soils but they also showed that disease incidence was not always correlated with the number of sclerotia present in the seedbed. These authors also reported that a decrease in disease incidence when rice straw was incorporated into soil in autumn compared with late spring incorporation. Lanoiselet *et al*. (2005a) studied the effect of burning stubble on the survival of laboratory-produced sclerotia of *R. oryzae-sativae* revealed that even if a large proportion of sclerotia present on the soil were killed, many of them were survived the stubble burning

regardless of cold burn or hot burn, and burning certainly did not eradicate the entire inoculums from the field. The authors underlined the importance of managing rice straw to reduce the inoculums and concluded that burning rice straw greatly contributed to keep the disease at a relatively low level in Australian rice fields. In modern agriculture, the diseases are controlled by the use of fungicides. Chemical fungicides can effectively control several plant diseases. However, indiscriminate use of the fungicides is not only hazardous to living beings but also adversely affects the microbial population present in the ecosystem. There is an increasing awareness about the risks involved in chemical fungicides, and therefore much attention is being focused on alternative methods of plant pathogen control. On the other hand, the world market continues to be extremely competitive and the growers compete to supply quality and safe products. Many researchers emphasised for biocontrol. *Trichderma viride, T. harzianum, Aspergillus niger, A. terreus, Neurospora crassa* etc. have been reported mycelium inhibition, suppress formation and germination of sclerotia of *Rhizoctonia* spp. (Mew and Resales, 1984; Roy, 1996). Biocontrol agents (*T. harzianum* and *T. viride*) were effective in mycelial inhibition and sclerotia formation but limited or ineffective under field condition (Ali *et al*., 2004) perhaps the favorable condition for pathogen could have disfavored the growth and development of *Trichoderma* sp. Similar observation was also reported by many authors. However, some authors found the effectiveness of biocontrol agents in experimental field but not in commercial cultivation.

Alternative disease control options that may benefit organic rice growers include application of natural plant products. Many plant species possess antifungal and antibacterial properties (Hasan *et al*., 2005; Ogbo and Oyibo, 2008; Dubey *et al*., 2009). Several higher plants and their constituents have been used successfully in plant disease control, and also have been proven to be harmless and non-phytotoxic unlike chemical fungicides (Singh *et al*., 1993). Some plant extracts possesses cidal properties as contact fungicides; some disrupt cell membrane integrity at different stages of fungal development, while the others inactivate important enzymes and

interfere with metabolic processes. Antifungal activities were found in *Eucalyptus, Syzygium aromticum, Azadirachta indica, Rosmarinus officinalis* on *Rhizoctonia solani, R. oryzae, R. oryzae-sativae* and *Sclrotium hydrophilum* (Aye and Matsumoto, 2011). Chaijuckam and Davis (2010b) found cinnamon oil as efficient plant product that inhibit vegetative growth of *R. oryzae-sativae in vitro* as well as suppression of aggregate sheath spot disease of rice. Adityachaudhury (1991) mentioned that the use of plant extracts and phytoproducts in gaining attention due to their biodegradability, low toxicity and minimum residual toxicity in the ecosystem. Though plant extracts have the potential to inhibit mycelial growth *in vitro* and to reduce disease in a small experimental pot or plot. Globally chemical control represents the most significant practice for *Rhizoctonia* disease management due unavailability of resistant sources. Present cultivation practices are not likely to be changed shortly. In California, Quadris (azoxystrobin) has been registered for the control of rice aggregate sheath spot disease. In Australia, pyraclostrobin tolclofos-methyl and propiconazole found effective fungicides, reducing mycelial growth of *R. oryzae-sativae in vitro,* while disease severity was significantly reduced by pyraclostrobin and propiconazole. The strobilurin fungicides were introduced recently against a board range of pathogens (Ali and Archer, 2003; Bertelsen *et al*., 2001; Chen *et al.*, 2004). These fungicides reduced disease development and subsequent inoculum production and increased yield. In Bangladesh, Sharma (2002) found the growth of *R. oryzae-sativae* was completely inhibited at 1 ppm Carbendazim *in vitro.*

Though completely resistant sources against *Rhizoctonia* spp. is still unavailable but partial resistance in many rice varieties or germplasm have been reported by many authors (McKenzie *et al*., 1994). In Bangladesh, about 8000 germplasm are available in BRRI gene bank of which about 4000 from wet season T. Aman, 2500 Boro and 1500 Aus. These landraces adapted in different parts of this country. Many of them possesses unique in terms of quality, adaptation, aroma, taste, drought, submergence, salinity, disease-insect pest resistance, high amylose, Zn, protein contents etc. However, native germplasm of Bangladesh has not been

screened against aggregate sheath spot disease earlier. Many countries in the world characterize their indigenous crop landraces at both molecular and phenotypic level. They have been done it for their crop identity and search for new sources of disease resistant gene for further crop improvement. Considerable numbers of resistant materials have been reported in *Oryza ruftpogon* to aggregate sheath spot (McKenzie *et al*., 1994). Since, native germplasms harboring many traits including disease resistance; inadequate availability of management information particularly on botanicals and chemicals against *R. oryzae-sativae* therefore, this study was under taken with the following objectives:

1. To identify most effective fungicide/(s) against *R. oryzae-sativae* following *in vitro* and field evaluation.
2. To find out native landraces and varieties those are tolerant / resistant against the disease.
3. To evaluate the effect of various plant extracts on the growth of *R. oryzae-sativae in vitro.*

5.2 Materials and Methods

5.2.1 Chemical control against aggregate sheath spot disease

5.2.2 Fungicides

Eight fungicides were used in this experiment, of which two were mixed fungicides. Details of fungicides used in this study are shown in Table 3 (chapter 2).

5.2.3 *In vitro* evaluation of fungicides

Fungicides were screened against *R. oryzae-sativae* following Poison food technique (Dhingra and Sinclair, 1985; Ali and Archer, 2003) using six different concentrations (100, 10, 1, 0.5, 0.25, and 0.1ppm). An appropriate amount of stock solution (1000 ppm concentration) was added to each flask of Potato Dextrose Agar (PDA) medium (100ml) following the equation C1V1=C2V2 before autoclave (15 psi for 15 min). Three replicated plates were prepared for each concentration. One treatment with no fungicides was maintained as control. Autoclaved PDA was poured into Petri dishes at the rate of 20 ml/dish. After solidification of the medium, a mycelial plug (6 mm diameter) cut from 3-days-old growing culture of *R. oryzae-sativae* on PDA was placed at the alongside of each Petri plate. The plates were incubated at 27°C in incubator. Mycelial growth was measured when mycelial growth reached the opposite site of control plate. Percent growth inhibition was calculated using the following equation: I=(C-T)/C X 100, Where, I=Percent inhibition, C= Growth of fungus in control plate, T = Growth of fungus in Treated plates.

5.2.4 *In vivo* trial

Two field trials were conducted in the BRRI (Bangladesh Rice Research Institute) farm of Gazipur in T.Aus on 2012 and 2013. The experimental site was under the sub-tropical climatic zone. BRRI dhan48 was used. The experiments were laid out in completely randomized block design (RCBD) with three replications in both trials and the unit plot size was 1 m x 1 m. Seven days old culture of MY-1 isolate was used for artificial inoculation at maximum tillering stage. Nine hills in the middle of each plot were inoculated. Two consecutive sprays applied at 7 days and 15 days after

inoculation. Data on infected and healthy tillers, plant height and topmost lesion height were recorded from which incidence, relative lesion height (RLH) and severity was calculated following Ahn *et al.* (1986); Yoshimura (1954) stated below:

i. e. RLH (%) = (Lesion height/Plant height) X 100.

Incidence (%) = No. of infected plant/No. of total plant sampled X 100.

Degree of severity (%) = (3N1+ 2N2 + N3 + 0N4)/3N) X 100, where N1 is number of tillers with all four uppermost sheaths infected; N2, number of tillers with the three uppermost sheaths infected; N3, number of tillers with the two uppermost sheaths infected and N4, number of tillers with the four uppermost sheaths disease free.

The crops were hand harvested whole plot at 80% maturity. After threshing, the grain samples were weighed and moisture content of paddy was determined using Grain moisture meter GMK-303, G-WON MACHINERY CO. LTD, Korea and adjusted at 14% moisture for yield calculation. Sterility and thousand grain weight were also recorded from nine selected inoculated hills. There were two control plots for each replication, one was disease another was healthy control.

5.2.5 Data analysis

Standard errors of means of three replications (mycelial inhibition) were calculated by using computer software Stata (version 12). The LD_{50} and LD_{90} values were also calculated by using software Origin 7.0. Field data were analyzed using MSTATC (version 2.10), Mean separation was done using least significant difference (LSD) test at α=0.05.

5.3 Germplasm/cultivar screening against aggregate sheath spot disease of rice

5.3.1 Germplasm/cultivar

A total of 60 germplasm/cultivars were investigated in this experiment. Among them 20 T-Aus germplasm, 20 (10 T. Aus germplasm and 10 BRRI released cultivars) T-

Aman and 20 BRRI released as Boro cultivars. The list of the materials including their BRRI accession number is given in Table 4 in Chapter 2.

5.3.2 Data analysis

Data were subjected to analysis of variance followed by mean separation through least significant difference (LSD) test at α=0.05 using CropStat (version 7.2). The correlation analyses were done by using computer software Stata (version 12).

5.4 Effects of different plant extracts on *R. oryzae-sativae*

5.4.1 Preparation of plant extract

Aqueous extracts from garlic, ginger, henna, water pepper, ivy gourd and neem leaves were evaluated for their antifungal effects *in vitro*. Details of plants/plant parts used in this experiment are shown in chapter 2 (2.8.1).

Plant extracts were prepared from each of 100 gm material in sterile distilled water (SDW). Each extract was prepared with sterile water with ratio (1:1 w/v) of Plant materials: Water using mortar and pestle. Then it was passed through a double layered cheese cotton cloth. Again each plant extracts passed through a double layered filter paper (Whatman no.1). Finally the extracts were considered as standard plant extracts and used for antifungal activity. Poison food technique (Dhingra and Sinclair, 1985; Ali and Archer, 2003) was used to test different concentrations (5%, 10%, 15%, 20%, and 25%) of plant extracts against *R. oryzae-sativae.*

5.4.2 Data analysis

Standard errors of means of four replications were computed using Stata (version 12). ANOVA and LSD (0.05) test was carried out for mycelial growth for each plant extract using CropStat (version 7.2). Data of sclerotial germination between the treatments, plant extracts and the controls were tested by paired *t* test. The LD_{50} and LD_{90} values were calculated by using software Origin (version 7.0).

5.5 Result

5.5.1 Chemical control against aggregate sheath spot disease

5.5.1.1 *In vitro* evaluation of fungicides

All the tested fungicides inhibited the mycelial growth of *R. oryzae-sativae* on PDA. A positive relationship was observed between growth inhibition and dose of the fungicides. Irrespective of fungicide mycelial growth inhibition increased with the increasing concentration. The highest inhibition (93.55%) recorded with the highest concentration (100 ppm) used in this experiment. In contrast the lowest inhibition (24.36%) recorded with the lowest concentration (0.1ppm). Irrespective of concentration fungicide, Carbendazim found to be the best in mycelial inhibition (80.16%). This was followed by fungicide Folicur (69.38%). Fungicide Nativo and Propiconazole behaved almost the same (65.51% and 63.10%). Relation between concentration and inhibition of different fungicides are shown in appendix 7.

Growth in different fungicides at 10 ppm, 1 ppm, and 0.5 ppm are shown in Plate no. 7, 8, and 9 respectively. Complete inhibition was recorded in all fungicides at 100 ppm except Amistar top and Monceren compared to control plate. While Carbendazim showed 100% inhibition at 100ppm, 10 ppm and 1ppm concentration. Among the fungicide tested, the least LD 50 value 0.1 ppm was noted for Carbendazim. This was followed by Nativo and Propiconazole having same concentration (0.4 ppm). Folicur was in the next order (0.5 ppm) (Table 15 and Fig 16). Carbendazim further showed strong activity against the pathogen with the lowest LD 90 value (0.8 ppm) followed by Folicur (0.9 ppm). LD 90 values could not be calculated for Monceren within the concentration range tested. LD 90 value for Nativo, Propiconazole, Differ and Amistar top were 5.0 8.0, 13.0 and 15.0 ppm respectively.

Table 14 : Effect of different concentrations of eight fungicides on the mycelial growth (mm) of *R. oryzae-sativae in vitro*

Fungicide	Mycelial growth[a] (mm) at various Concentration[b] (ppm)						
	0	100	10	1	0.5	0.25	0.1
Control	84						
Amistar Top		5.00	21.67	25.67	68.67	69.33	70.00
Carbendazim		0.00	0.00	0.00	26.67	31.67	41.67
Differ		0.00	21.00	43.33	46.33	60.33	73.33
Folicur		0.00	0.00	1.33	44.67	52.00	56.33
Mancodazim		0.00	9.33	59.33	68.67	72.00	72.67
Monceren		38.33	41.67	57.00	72.33	72.67	73.67
Nativo		0.00	1.50	25.67	32.67	54.00	60.00
Propiconazole		0.00	6.67	26.00	33.67	59.00	60.67
LSD[A] (0.05)				4.07			
LSD[B] (0.05)				3.73			

[A]LSD to be used to compare all means except control mean

[B]LSD figure to be used to compare control mean *v.* all means

[a] Means of mycelial growth

[b] Concentration of fungicides

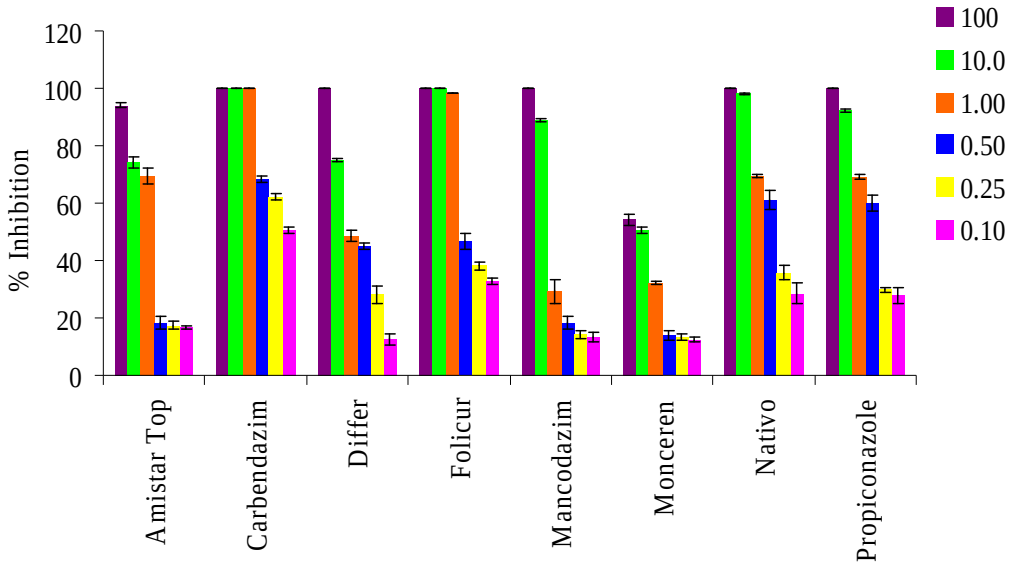


Figure 15 : Effect of different concentrations of eight fungicides on the mycelial growth inhibition (%) of *R. oryzae-sativae in vitro*

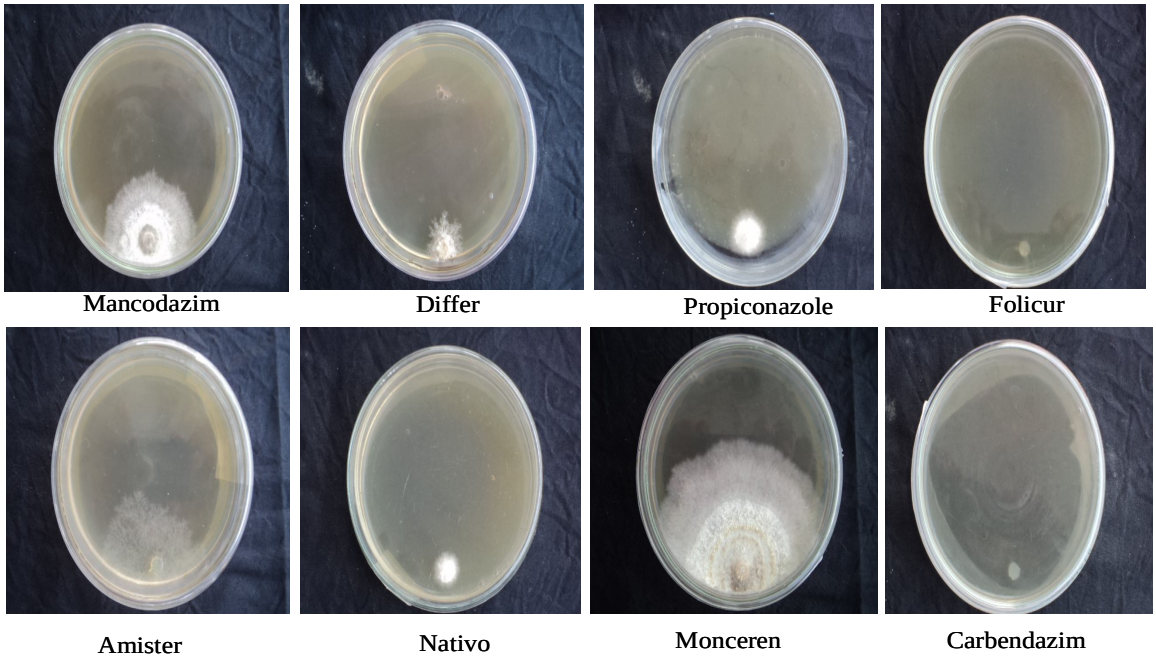


Plate 7 : Growth of *R. oryzae-sativae* in different fungicides at 10 ppm

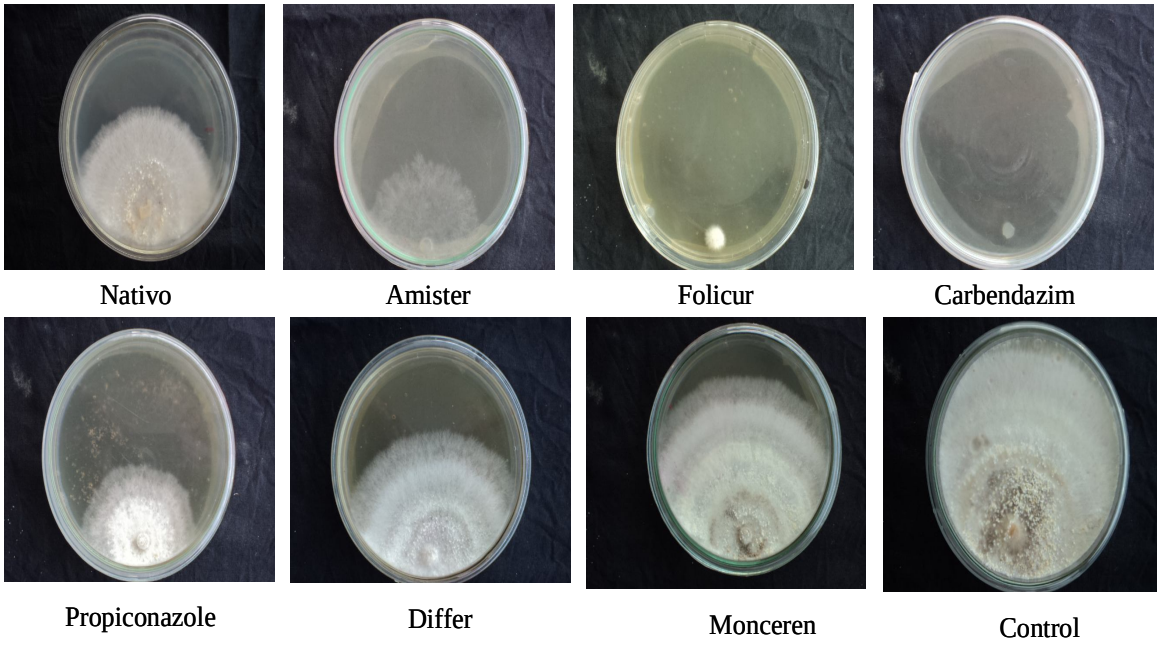


Plate 8 : Growth of *R. oryzae-sativae* in different fungicides at 1 ppm

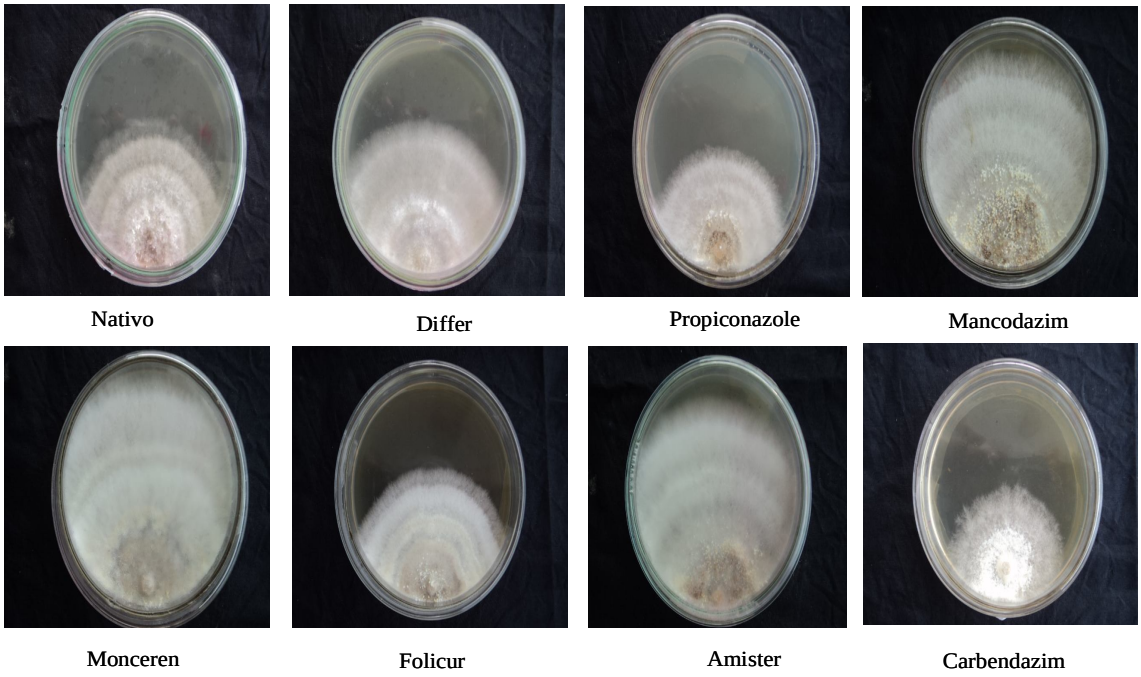


Plate 9 : Growth of *R. oryzae-sativae* in different fungicides at 0.5 ppm

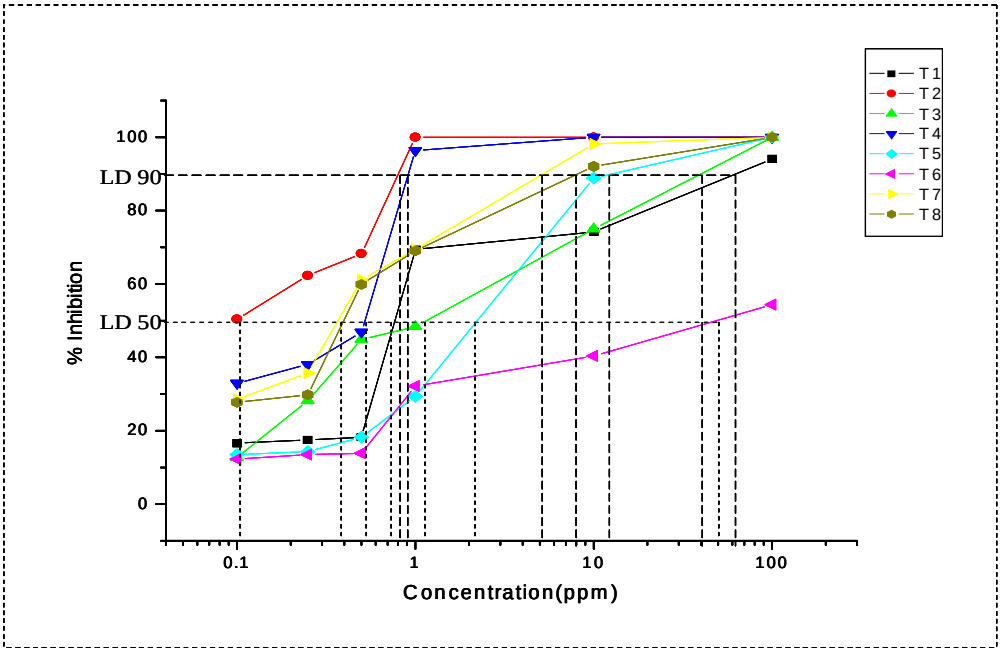


Figure 16 : LD $_{50}$ and LD $_{90}$ values of different fungicides on growth inhibition of *R. oryzae-sativae*

Table 15 : Values of LD 50 and LD 90 of different fungicides used against mycelial growth of *R. oryzae-sativae*

Trade / Common name	LD 50 values (ppm)	LD 90 values (ppm)
1. Amistar Top	0.75	15.0
2. Carbendazim	0.10	0.80
3. Differ	1.10	13.0
4. Folicur	0.50	0.90
5. Mancodazim plus	2.10	10.2
6. Monceren	14	-
7. Nativo	0.4	5.0
8. Propi	0.4	8.0

5.5.1.2 *In vivo* trial

Five fungicides (Carbendazim, Differ, Folocur, Nativo and Propiconazole) were tested in field under artificial inoculated condition. Results of two years 2012 and 2013 field trial data are presented in Table 16 and Table 17 seperately. Fungicide application had significantly reduced the disease parameters (RLH, DI and DS) and sterility that in consequent increased TGW and yield at $\alpha = 0.05$ level compared to the control. Among the fungicides, Carbendazim found to be the best and highly effective against aggregate sheath spot disease. In general, Folicur and Nativo performed almost similar and were in the next order of effectiveness. Differ and Propiconazole were least effective and similar with disease control in case of yield however some disease parameters differed. In T. Aus 2012, the lowest RLH (10.92%) were noted in Carbendazim applied treatment followed by Folicur (15.01%) and Nativo (17.15%). These three fungicides reduced the RLH significantly compared to other fungicides (Table 16). Efficacy of Differ and Propiconazole was almost similar and did not significantly different each other for RLH. Analysis of variance of disease incidence varied among the treatments at

(α=0.05). The lowest disease incidence was recorded after treated with Carbendazim (38.89%) followed by Folicur (52.27%) and Nativo (55.56%). Statistically similar results were found in Differ (62.22%) and Propiconazole (64.44%). In case of DS Carbendazim, Folicur and Nativo performed significantly better over other fungicides tested. Sterility decreased significantly with carbendazim and folicur that were at par with healthy control. Other fungicides had narrowly decreased sterility compared to disease control. TGW did not significantly differ over diseased control except carbendazim that was related with least RLH, DI and DS. (Table 16). The disease incidence, RLH and disease sterility were lower in all treated plots in 2013 compared to 2012 (Table 17). However, a similar trend of effectiveness was recorded for all the fungicides tested that confirmed the superior performance of Carbendazim, Folicur and Nativo against aggregate sheath spot.

Yield was influenced by the disease variables investigated in both 2012 and 2013 and significantly varied among the treatments. In 2012, the highest yield (5.43 t/ha) was recorded in healthy control followed by Carbendazim (5.2 t/ha), Folicur (5.1 t/ha) and Nativo (5.0 t/ha). Yield in Differ and Propiconazole were statistically similar with diseased control. In this investigation, *R. oryzae-sativae* reduced yield by 0.73 t/ha that counts to 13.44% in disease control compared to healthy control. Application of Carbendazim, Folicur and Nativo increased rice yield by 10.63%, 8.51% and 6.38% respectively compared with diseased control. In 2013, yield was also significantly affected by aggregate sheath spot disease (α = 0.05). *R. oryzae sativae* reduced yield of BRRI dhan48 by 0.83 t/ha (14.74% yield reduction) over healthy control. Yields recovery in Differ and Propiconazole treatments over disease control were not significantly different (α = 0.05). In contrast, Carbendazim, Folicur and Nativo increased yield by 14.58%, 11.88% and 9.79% respectively compared with diseased control. These comprehensive studies revealed that Carbendazim, Folicur and Nativo were very effective fungicides for aggregated sheath spot disease management.

Table 16 : Evaluation of fungicides against aggregate sheath spot disease of rice during T. Aus 2012

Treatment	*RLH (%)	*DI (%)	*DS (%)	*TGW (g)	Sterility (%)	Yield (t/h)
Carbendazim	10.92 D	38.89 E	45.40D	22.26 AB	21.02 D	5.2 B
Differ	27.39 B	62.22 B	59.57B	21.52 CD	27.31 AB	4.8 D
Folicur	15.01 C	52.27 D	51.21C	21.92 BC	22.61 CD	5.1 BC
Nativo	17.15 C	55.56 C	53.61C	21.85 BCD	25.58 BC	5.0 C
Propiconazole	28.14 B	64.44B	62.24B	21.24 DE	27.77 AB	4.7 D
Diseased Control	31.08 A	67.78A	66.91A	20.80 E	29.76 A	4.7 D
Healthy Control	7.82 E	6.67F	18.68E	22.58 A	20.53 D	5.43 A

*****RLH- Relative Lesion Height, DI-Disease Incidence, DS- Disease Severity, TGW- Thousand Grain Weight**.** In a column, means followed by the same letter are not significantly different at $\alpha = 0.05$ after ANOVA-LSD test

Table 17 : Evaluation of fungicides against aggregate sheath spot disease of rice during T. Aus 2013

Treatment	*RLH (%)	*DI (%)	*DS (%)	*TGW (g)	Sterility (%)	Yield (t/h)
Carbendazim	6.93 E	12.22 D	40.79 E	22.44 B	20.87 E	5.50 AB
Differ	18.63 C	53.33 B	55.53 B	21.80 D	26.01 BC	4.90 D
Folicur	12.69 D	34.44 C	51.11 C	22.17 C	22.40 DE	5.37 BC
Nativo	14.12 D	37.78 C	47.29D	22.09 C	23.56 CD	5.27 C
Propiconazole	27.74 B	58.89 A	58.17 B	21.37 E	26.50 AB	4.83 D
Diseased Control	30.98 A	61.11 A	65.94 A	21.02 F	28.95 A	4.80 D
Healthy Control	5.48 F	4.44 E	14.50 F	22.80 A	18.12 F	5.63 A

*****RLH- Relative Lesion Height, DI-Disease Incidence, DS- Disease Severity, TGW- Thousand Grain Weight**.** In a column, means followed by the same letter are not significantly different at $\alpha = 0.05$ after ANOVA-LSD test

5.5.2 Germplasm/cultivar screening against aggregate sheath spot disease of rice

Sixty germplasm/cultivar (T. Aus, T. Aman, and Boro) were tested in this experiment. There was no immune germplasm or cultivar found in this screening experiment. Aggregated sheath spot disease found in all the germplasm and cultivar tested and produced typical aggregated sheath spot disease symptoms on the rice sheath. Resistance levels was calculated based on RLH (%) (Table 21).

In T. Aus season the highest RLH found in Akuee (24.18%) and Kalosoti (22.21%) followed by Lotabhog (19.26%), Ausboro (18.58%) and Indian variety CM25 (18.14%). The lowest RLH found in Balam (10.41%) followed by Agoan (10.65%) and in Ratol (11.13%) which were statistically similar. Details result shown in the Table 18. The highest incidence in Aus season showed by the Akuee (75.78%) followed by Lotabhog (75.43%) and Aus boro (73.26%). The lowest incidence found in Balam (59.73%) followed by Ratol (60.19%), Kaloburi (61.28%).

In T. Aman, among the germplasm and cultivars tested the highest RLH found in Kurchi binni (26.24%) followed by Kurchi jira (24.04%), Shabichi dhan (22.62%) and BRRI dhan46 (22.39%). In contrast, the lowest RLH showed by the germplasm Panisail (9.80%) followed by BRRI dhan44 (11.57%) and Katari bhog (12.74%) (Table19). The highest incidence was recorded in Kurchi jira (71.37%) and Shabichi dhan (69.69%) followed by Kurchi binni (67.53%) while the lowest incidence showed by Panisail (49.46%), Chabichi dhan (51.04%), and BRRI dhan44 (51.48%). They showed statistically similar incidence.

In Boro season, the highest RLH found in BR-1(18.89%), followed by BRRI dhan28 (18.43%), BR-16 (17.75%) and BR-15 (17.52%) while the lowest RLH showed by BRRI dhan27 (7.94%) followed by BR-7 (9.73%). BR-9 (10.39%) and BR-8 (11.38%) were in the middle of disease reaction (Table20). The highest

incidence noted in the same variety BR-1(40.16%) followed by BR-15 (35.89%), BR-16 (35.16%) and BRRI dhan29 (33.80%). In contrast, the lowest incidence recorded in BR-7(17.74%) followed by BR-17 (18.64%), BR-9 (19.02%).

Irrespective of season, among 60 germplasm and cultivars tested in this experiment, the highest RLH found in Kurchi binni (26.24%) (T. Aman) followed by Akuee (24.18%) (T. Aus). In contrast, the RLH was lower in Boro season in almost all varieties compared to either T. Aman or T. Aus. In case of incidence, the highest number of tillers infected in Akuee (75.78%) followed by Lotabhog (75.43%) and Aus boro (73.26%) interestingly all of them are aus varieties. On the other hand, the lowest incidence found in Boro season, from BR-7(17.74%) followed by BR-17 (18.64%), BR-9 (19.02%) and BR-8 (20.02%). Though the highest RLH found in T. Aman season but the highest incidence occurred in T. Aus season this perhaps varietal differences and local microclimatic condition induced by plant architecture and inherent resistance capability. Lowest RLH and incidence were found in Boro season also perhaps due to low temperature. It was observed that RLH and plant height were negative correlated with each other. The correlation between RLH and plant height were significant at R^2 = 0.89, 0.88 and 0.91 for T. Aus, T. Aman and Boro respectively (Fig. 17, 19 and 21). Similar results were found for disease incidence at R^2 values 0.76, 0.72 and 0.68 in T. Aus, T. Aman and Boro respectively (Fig. 18, 20 and 22).

Table 18 : Aggregate sheath spot disease reaction on T. Aus rice germplasm

Sl No.	Local Name	Plant height (cm)	Disease reaction	
			Incidence (%)	RLH (%)
1	Musur	98	64.06	13.00
2	Koisramuri	98	63.23	13.04
3	Iet (Indian variety)	90	70.38	15.99
4	Nuncha	98	64.69	12.90
5	CM25 (indian variety)	85	72.11	18.14
6	Balam	100	59.73	10.41
7	Aus Khusni	95	66.82	15.00
8	Kataktara	95	68.12	16.88
9	Kobila Rus	98	65.53	12.40
10	Kaloburi	100	61.28	15.01
11	Ratol	105	60.19	11.13
12	Agoan	100	62.30	10.65
13	Aus Boilam	95	67.17	15.41
14	Kalosoti	75	68.78	22.21
15	Kataktari	90	70.54	17.47
16	CN (Indian variety)	85	72.88	18.13
17	Aus Balam	95	68.33	15.01
18	Lotabhog	80	75.43	19.26
19	Ausboro	85	73.26	18.58
20	Akuee	75	75.78	24.18
SE (N=3)			2.99	1.02
5% LSD 38 DF			8.56	2.92

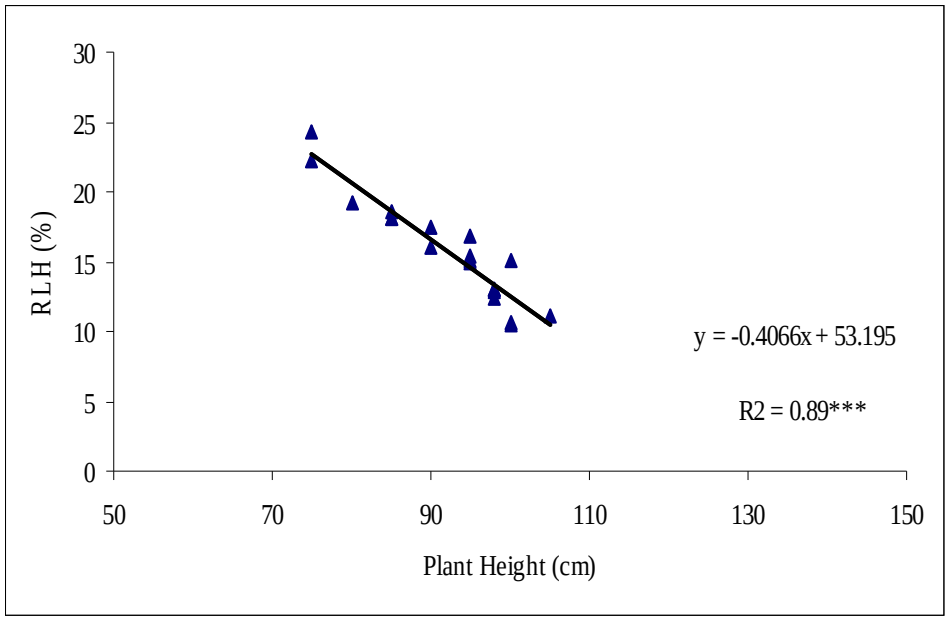


Figure 17: Correlation between RLH and plant height in disease reaction on T. Aus rice germplasm screening against ASS disease. * indicated significant correlation at 5%, 1% and 0.10% level**

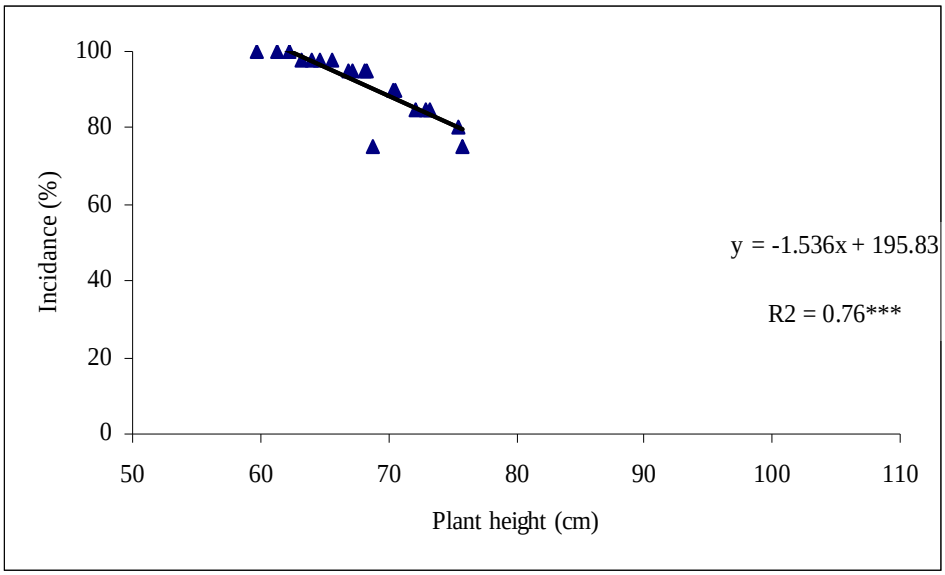


Figure 18 : Correlation between incidence and plant height in disease reaction on T. Aus rice germplasm screening against ASS disease. * indicated significant correlation at 5%, 1% and 0.10% level**

Table 19 : Aggregate sheath spot disease reaction on T. Aman rice germplasm/cultivar

Sl No.	Local Name	Plant height (cm)	Disease reaction	
			Incidence (%)	RLH (%)
1	Kurchi jira	97	71.37	24.04
2	Kurchi binni	90	67.53	26.24
3	Shabichi dhan	97	69.69	22.62
4	Chabichi dhan	138	51.04	15.01
5	Mosolla jira	113	62.12	18.86
6	Kocheri jira	120	66.94	14.49
7	Katari bhog	125	52.83	12.74
8	Khirsapat	102	56.89	18.75
9	Deshi malshira	115	61.13	17.88
10	Panisail	138	49.46	9.80
11	BR4	125	54.81	13.34
12	BR10	115	59.98	17.61
13	BR22	125	55.39	13.27
14	BR23	120	56.31	16.02
15	BRRI Dhan38	125	54.81	13.46
16	BRRI Dhan39	105	63.56	21.39
17	BRRI Dhan40	110	60.70	20.07
18	BRRI Dhan41	115	59.73	17.91
19	BRRI Dhan44	130	51.48	11.57
20	BRRI Dhan46	105	62.00	22.39
SE (N=3)			1.16	0.47
5% LSD 38 DF			3.33	1.34

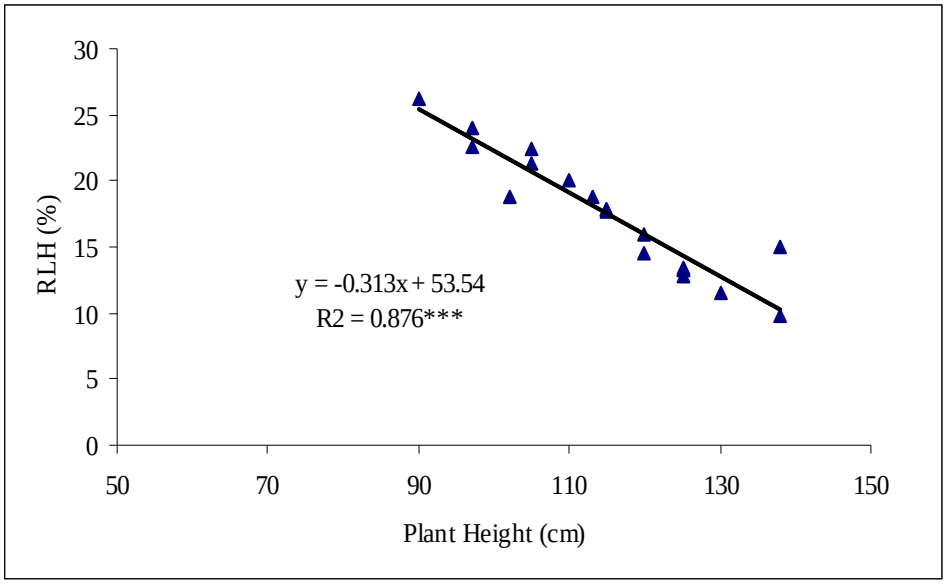


Figure 19 : Correlation between RLH and plant height in disease reaction on T. Aman rice germplasm/cultivar screening against ASS disease.*indicated significant correlation at 5%, 1% and 0.1% level.**

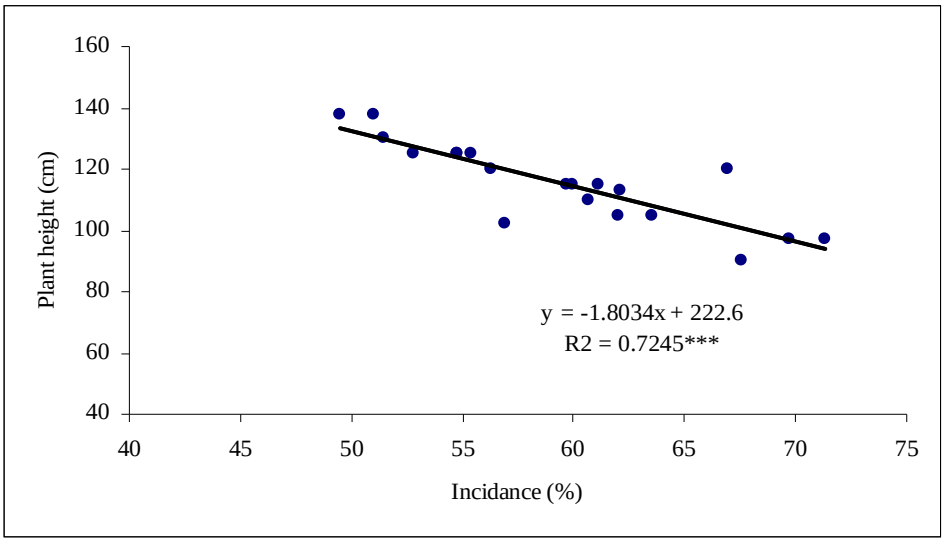


Figure 20 : Correlation between incidence and plant height in disease reaction on T. Aman rice germplasm/cultivar screening against ASS disease. * indicated significant correlation at 5%, 1% and 0.1% level.**

Table 20 : Aggregate sheath spot disease reaction on Boro rice cultivars

Sl No.	Cultivar Name	Plant height (cm)	Disease reaction	
			Incidence (%)	RLH (%)
1	BR1	90	40.16	18.89
2	BR2	120	28.30	14.24
3	BR3	95	30.59	16.01
4	BR6	100	28.84	15.21
5	BR7	125	17.74	9.73
6	BR8	125	20.02	11.38
7	BR9	125	19.02	10.39
8	BR12	105	27.43	14.31
9	BR14	120	22.05	12.75
10	BR15	90	35.89	17.52
11	BR16	90	35.16	17.75
12	BR17	125	18.64	13.02
13	BR18	115	23.02	12.92
14	BR19	110	25.84	14.11
15	BRRI Dhan26	115	23.72	13.08
16	BRRI Dhan27	140	24.77	7.94
17	BRRI Dhan28	90	27.81	18.43
18	BRRI Dhan29	95	33.80	17.28
19	BRRI Dhan35	105	26.19	14.26
20	BRRI Dhan36	90	30.29	17.11
SE (N=3)			1.87	0.57
5% LSD 38DF			5.36	1.64

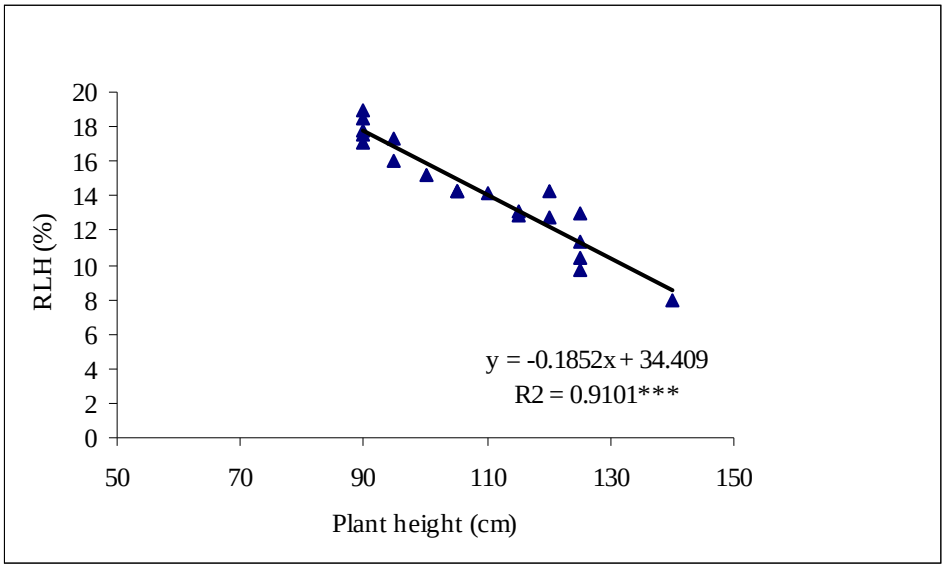


Figure 21 : Correlation between RLH and plant height in disease reaction on Boro rice cultivar screening against ASS disease. * indicated significant correlation at 5%, 1% and 0.10% level**

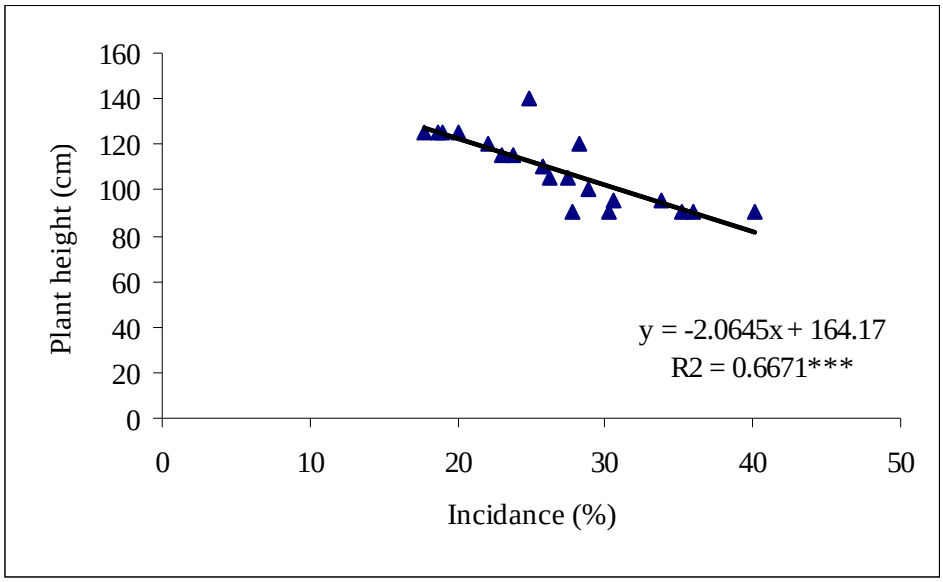


Figure 22 : Correlation between incidence and plant height in disease reaction on Boro rice cultivar screening against ASS disease. * indicated significant correlation at 5%, 1% and 0.10% level**

Table 21 : Resistant level of rice germplasm and cultivars based on RLH (%) of aggregate sheath spot disease reaction

Scale (RLH)	Categories of resistance	Germplasm/Cultivar		
		T. Aus	**A. Aman**	**Boro**
0	Immune	None	None	None
1 - 20	Moderately resistant	Balam, Agoan Musur, Koisramuri, Iet, Nuncha, CN, CM25, Aus Khusni, Kataktara, Kobila Rus, Kaloburi, Ratol, Aus Boilam, Aus Balam, Kataktari, Lotabhog, Ausboro	Panisail Chabichi dhan, Mosollajira, Kocheri jira, katari bhog, Khirsapat, Deshi malshira, BR4, BR10, BR22, BR23, BRRI dhan38, BRRI dhan41, BRRI dhan44	BR7, BR9, BRRI dhan27BR1, BR2, BR3, BR6, BR8, BR12, BR14, BR15, BR16, BR17, BR18, BR19, BRRI dhan26, BRRI dhan28, BRRI dhan29, BRRI dhan35, BRRI dhan36
>20	Moderately susceptible	Akuee, Kalosoti	Kurchi jira, Kurchi binni, Shabichi dhan, BRRI dhan39, BRRI dhan40, BRRI dhan46	None

5.5.3 Effect of different plant extracts on *R. oryzae-sativae*

Six common and locally available medicinal plants were tested to evaluate their antifungal activity on *R. oryzae-sativae*. The mycelial growth of *R. oryzae-sativae* in the control plates touched the wall of the Petri dishes at 4 days after incubation, and the data was recorded on that day. All plant extracts with different concentration showed antifungal activities with different degrees of intensity (Table 22). Radial growth of the isolate MY-1 was significantly affected by both plant extracts and plant extracts concentration. In general, garlic and henna extracts were the more effective to reduce growth of the isolate when used at the

lowest concentration (5%). Garlic extract effectively controlled the fungal growth 50% at 3.25% that indicated the lowest LD 50 value followed by henna extract at 3.75% (Table 23 and Fig 24). The lowest LD 90 value also showed by garlic extracts at 17.25% followed by henna extract at 19%. Mycelial growths of *R. oryzae sativae* on henna, ginger and ivy gourd with different concentrations are showed in Plate 10, 11 and 12 respectively.

A positive relationship was also observed between growth inhibition and concentration of the plant extracts. Irrespective of plant extracts inhibition percentage increased gradually with the increasing concentration. However, The growth of *R. oryzae-sativae* was highly inhibited by all the tested concentration (5-25%) of garlic extract compared with control, the corresponding inhibition ranging from 100% - 67.78%. 100% inhibition was comparable with chemical fungicide 1ppm Carbendazim 50% wp. (Table 22). The henna leaf extract caused 94.44% - 65.56% inhibition. While, neem leaf extract showed 78.33% - 33.89% inhibition. Ginger extract caused 70.56% - 15.56% inhibition. The lowest inhibition was recorded from *Polygonum* extract (32.78% - 12.22%) followed by telakucha extract (61.67% -27.22%). Garlic and henna extracts significantly decreased sclerotia germination at all the concentrations while, neem extract at 20% and 25% reduced sclerotia germination significantly compared to the control plate. Ginger and telakucha decreased sclerotia germination significantly only at 25% (Table 24).

Antifungal activity was confirmed by all of the selected plant species and the result revealed that garlic and henna are the most effective inhibitor for the mycelial growth of *R. oryzae-sativae in vitro*. The findings of the present investigation could be an important step towards the possibilities of using natural plant extracts as biopesticides in the control of aggregate sheath spot of rice caused by *R. oryzae-sativae*.

Table 22 : Effect of different concentration of six plant extracts on the radial mycelial growth (mm) of *R. oryzae-sativae in vitro*

Treatment	Radial mycelial growth[a] (mm) at various concentration[b] of plant extracts					
	[b]0%	5%	10%	15%	20%	25%
Control	45.00					
Allium sativam		[a] 14.50	12.25	8.25	0	0
Zingiber officinales		38.00	27.75	21.25	18.50	13.25
Lawsonia inermis		15.50	13.00	9.250	3.75	2.50
Poligonum hydropiper		39.50	36.50	34.75	33.00	30.25
Coccinia cordifolia		32.75	31.00	27.75	20.50	17.25
Azadirachta indica		29.75	23.00	17.75	13.25	9.75
LSD^A (5%)				1.47		
LSD^B (5%)				1.31		

[A]LSD to be used to compare all means except control mean
[B]LSD figure to be used to compare control mean *v.* all means
[a] Means of radial mycelial growth
[b] Concentration of plant extracts

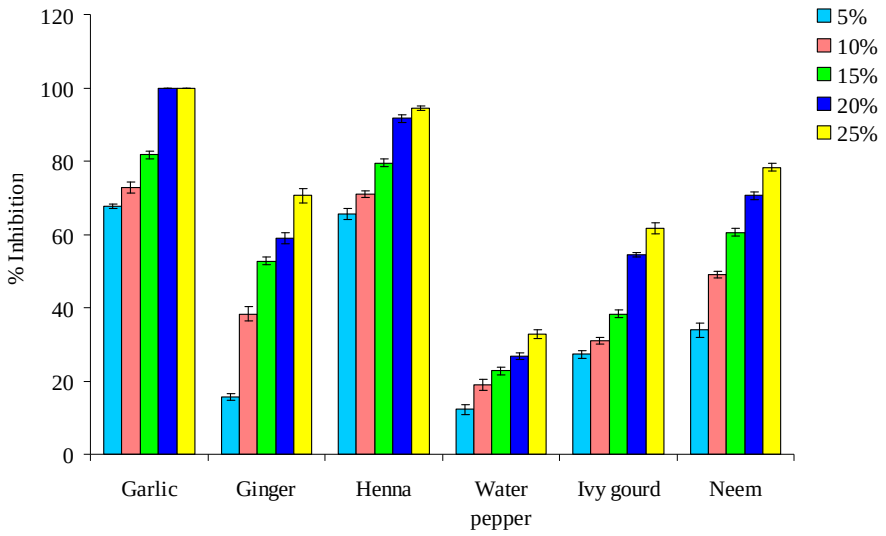


Figure 23 : Mycelial growth inhibition effect at various concentrations of different plant extracts against *R. oryzae-sativae*

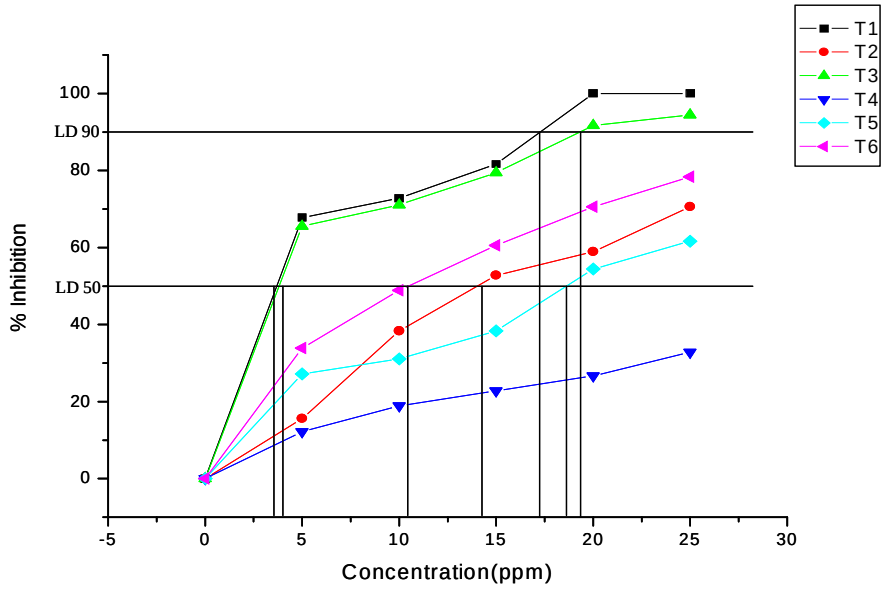


Figure 24 : LD 50 and LD 90 values of different plant extract on mycelial growth inhibition of *R. oryzae-sativae* Legend: T1 = Garlic, T2 = Ginger, T3 = Henna, T4 = Water pepper, T5 = Ivy gourd, T6 = Neem

Table 23 : Local name, Botanical name and LD 50 and LD 90 values of different plant extract used against mycelial growth of *R. oryzae-sativae*

Local name	Botanical name	LD 50 values (ppm)	LD 90 values (ppm)
Rashun	*Allium sativam*	3.25	17.25
Ada	*Zingiber officinales*	14.75	>25
Mehendi	*Lawsonia inermis*	3.75	19
Panimorich	*Poligonum hydropiper*	>25	>25
Telakucha	*Coccinia cordifolia*	18.75	>25
Neem	*Azadirachta indica*	10.5	>25

Table 24 : Antifungal activity of different plant extracts with different concentrations on sclerotia germination of *R. oryzae-sativae*

Plants	Concentration of plant extracts				
	5%	10%	15%	20%	25%
Allium sativam	8.0***	7.25***	7.0***	0.0	0.0
Zingiber officinales	9.5	9.0	9.0	8.75	8.25**
Lawsonia inermis	8.25**	8.25**	8.0***	7.5***	7.25***
Poligonum hydropiper	9.5	9.5	9.5	9.0	9.0
Coccinia cordifolia	9.5	9.5	9.25	8.75	8.5*
Azadirachta indica	9.25	9.0	8.75	8.5*	8.0***
Control (without plant extract)	9.5				

Significant difference between plant extracts with different concentrations and the controls at 0.1%, 1% and 5% level is indicated by ***, ** and * respectively

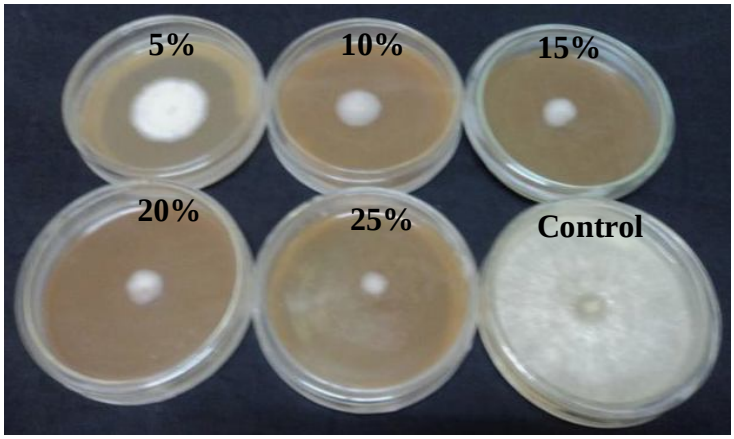


Plate 10 : Mycelial growth of *R. oryzae-sativae* on henna with different concentrations

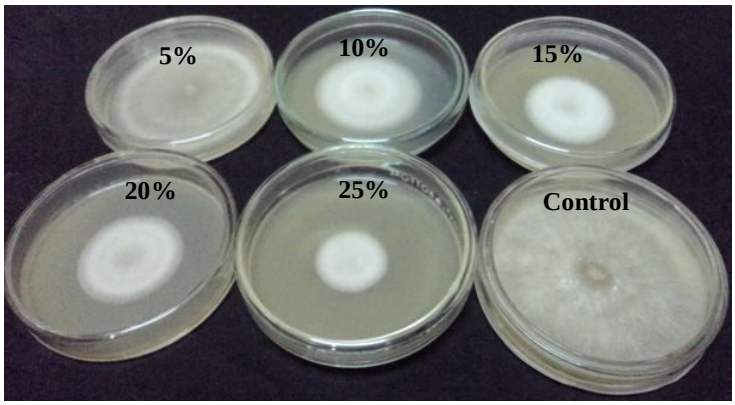


Plate 11 : Mycelial growth of *R. oryzae-sativae* on ginger with different concentrations

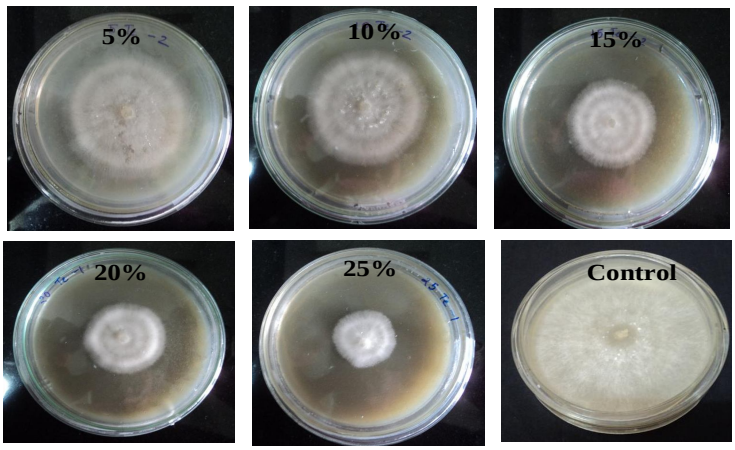


Plate 12: Mycelial growth of *R. oryzae-sativae* on ivy gourd with different concentrations

5.6 Discussion

Both *in vitro* and *in vivo* screening of fungicides against aggregate sheath spot disease caused by *R. oryzae-sativae* revealed that Carbendazim, Folicur and Nativo were the effective in controlling the disease with the highest control obtained in Carbendazim. Though, all the fungicides significantly inhibited the mycelial growth of *R. oryzae-sativae in vitro*. It was observed that mycelial growth decreased with increasing concentration from 0.1 to 100 ppm that agrees with the findings of Lanoiselet *et al.* (2005). They found significant reduction of *R. oryzae-sativae* growth when concentration increased from 0.1 to10 µg/ml. In this investigation, only Carbendazim inhibited 100% mycelial growth at the lowest dose at 1.0 ppm and onward concentration used. Similar result with Carbendazim was also reported by Sharma (2002) in *in vitro*. Amister top (azoxystrobin), Monceren (pencycuron) and Mancodazim were poorly effective in *in vitro* and excluded from field test. Poor efficacy of Azoxystrobin on mycelial growth of *R. oryzae-sativae* and *R. solani* has also been reported by Lanoiselet *et al.* (2005b) and Ali (2002). The present result of Axoxystrobin disagrees with the findings of Webster and Greer, 2004. In this experiment, Differ and Propiconazole though showed better inhibition in *in vitro* but did not perform well in *in vivo* under field test. These findings are in agreement of Carling *et al.*, 1990, Martin *et al.*, 1984a, Martin *et al.*, 1984b, Van der Hoeven and Bollen 1980.

Out of eight fungicides, five were selected *in vivo* trial under artificial inoculated condition. Among the fungicides Carbendazim was the best in controlling disease. No information is available of the supremacy of carbendazim and folicur against aggregated sheath spot disease however; this result will enrich scientific information and be useful for disease management where ASS is a problem in rice production. It is interest to note that Differ and Propiconazole though decreased disease severity significantly over disease control but yield was not increased accordingly. A similar result was also found by Lanoiselet *et al.* (2005b) with Propiconazole and Pyraclostrobin against *R. oryzae-sativae* in Australia.

The maximum yield loss caused by aggregate sheath spot was 14.74% (on BRRI dhan48 in 2013) that relates with the findings of Lanoiselet *et al.* (2005) whom found 20.3 % yield loss in Australia.

Screening of rice germplasm and cultivars is a continuous process to identify sources of resistance against disease. In this investigation, a total of 60 germplasm and cultivars were screened against aggregate sheath spot disease. However, none of them were immune although a large number of varieties showed moderately resistant and some of those could be useful for varietal improvement program. Year prevalence of the aggregate sheath spot disease has been reported by Shahjahan *et al.* (1988) that agrees with screening activities in all three rice growing seasons. They also observed the disease on both local and modern cultivars and advanced breeding lines. In this artificial screening experiment, growth rate and development of the disease in all the three rice growing season with varying degree of disease reaction among the germplasm and cultivars are tested. In Bangladesh, the RLH found maximum in Aman season, medium in Aus season and minimum in Boro season that may most instances were below economic threshold level. In cultivar screening experiment it was found significant negative correlation between plant height and relative lesion height (RLH) in all the three seasons with the highest RLH in dwarf and semi-dwarf plants (Akuee and Kurchi binni). This finding is in the line of Gunnel and Webster (1984) and; Gunnel and Webster (1987), they also found less disease in taller varieties than Californian dwarf and semi-dwarf cultivars. Field trials in Australia reported that Australian semi-dwarf cultivars were also more susceptible (Amaroo and Langi) than a tall (Kyeema) Australian cultivar (Lanoiselet *et al*., 2005b). Research from Uruguay suggests that varietals susceptibility to aggregate sheath spot also exists among Uruguayan rice cultivars and breeding lines (Lanoiselet *et al*., 2005b). Higher incidence associated with lower RLH by *R. oryzae-sativae* also supported by Lanoiselet *et al*. (2005b).

Among the plant extract used, Garlic showed 100% inhibition against *R. oryzae-sativae* and comparable to 1ppm Carbendazim 50% WP *in vitro*. Sulfur rich

protein Ajoene derived from garlic had antifungal activities towards *Aspergillus niger*, *Candida albicans* (Yoshida *et al.*, 1987). Other workers also showed the presences of antifungal properties in *A. sativam* (Misra and Dixit, 1976; Agarwal, 1978). Garlic has already been reported to have antifungal activity against *R. oryzae-sativae* (Chaijuckam *et al.*, 2010b). The sensitivity of fungi and even isolates of the same species to plant extracts may vary. Such as, garlic extract at 5% completely inhibited vegetative growth of California isolates of *R. oryzae-sativae* (Chaijuckam *et al.*, 2010b) but in case of Bangladeshi isolate of *R. oryzae-sativae*, it required 20%. It may be due to different extraction methods. Moreover, Sensitivity to garlic extract may be genetically influenced because sclerotial germination at 5% garlic extract on California isolate of *R. oryzae-sativae* was not significantly different compared to the control, but significant reduced sclerotial germination were found at 5 concentrations of garlic extract used against *R. oryzae-sativae* in Bangladesh. Saha (1997) reported that, leaf extract of henna completely controlled the growth of *Drechslera oryzae*, *Sclerotium oryzae*, *S. rolfsii* and *Rhizoctonia solani* at 20% (w/v) concentration. In the present experiment, *R. oryzae-sativae* was added in the list of sensitivity to the henna extract. The presence of antifungal compound lawsone (2- hydroxyl- 1, 4 napthoquinine) in the leaf extract of henna had been identified by Tripathi *et al.* (1978) which might be responsible for the inhibition. Ginger showed moderate inhibition against *R. oryzae-sativae* in *in vitro.* Pakrashi and Pakrashi (2003) demonstrated that, Ginferenone A, a diarylheptenone constituent of ginger, showed strong antifungal action against *Pyricularia oryzae* and moderate anticoccidium effect *in vitro.*

Neem leaves extract inhibited the growth of *Rhizoctonia solani*, *R. oryzae sativae*, *R. oryzae*, and *Sclerotium hydrophilum* by 87.5%, 80.0%, 92.5% and 49.2% respectively (Aye and Matsumoto, 2011) in Japan. Similarly, present study expressed satisfactory inhibitory growth on *R. oryzae-sativae* by using neem leaf extract. Khatun *et al.* (2012) found antifungal activities of methanolic extract of *Coccinia cordifolia* on *Candida albicans*, *Aspergillus niger*, *Penicillium notatum*.

Hasan *et al.* (2009) observed *Polygonum hidropiper* root extract on chloroform had strong antifungal activities against *Aspergillus niger*, *A. fumigatus*, *A. flavus*, *C. albicans*, *Rhizopus oryzae* and *Tricophyton rubrum.* However, lower inhibition percents were noted in this experiment on *Polygonum hidropiper* (32.78% at 25% concentration) followed by *Coccinia cordifolia* (61.67% at 25% concentration). The differences in the inhibitory effect of various plant extracts may be due to qualitative and quantitative differences in the antifungal compounds presents in them. The extracts from garlic and henna proved to have strong antifungal activities against aggregate sheath spot *in-vitro* and these can be used for control of this disease under field condition as they are environmental safe alternatives.

General Discussions and Conclusion

Food is essential for survival and adequate intake of balanced food with required nutrients is vital to leading a healthy and productive life. Every food item has its own calorie, protein and other nutrient contents, which are essential for health. Virtually, rice is the synonym of food in Bangladesh. The per capita per day consumption of rice is 416.0 gram and 62% calories and 45.7% proteins come from rice (http://www.bbs.gov.bd/webtestapplication). The demand of rice in Bangladesh is increasing with the increasing population over time. Low production of rice in unit area is attributed to various biotic and abiotic factors. Rice disease is one of the most important limitations for increasing rice yield. Among various micro-organisms, fungi have a devastating position for decreasing rice yield. Sheath diseases of rice are most important fungal disease caused by *Rhizoctonia* spp. and cause significant yield losses in many rice-growing countries in the world. Among *Rhizoctonia* spp., *Rhizoctonia oryzae-sativae* causes aggregate sheath spot disease of rice. Historically aggregate sheath spot disease has been a minor disease across the world hence little attention was given in research priority. Until 1988, there was no report of *R. oryzae-sativae* on Bangladeshi rice cultivars under Bangladeshi climatic conditions. During the past few years however, incidence of the disease have increased markedly. This increase has coincided with an increase in the use of semi dwarf, high-yielding cultivars by growers. In Bangladesh, there are only a few repots about *R. oryzae-sativae.* The main thirsts of this study were to examine variation among the isolates which was collected from different locations using multidisciplinary characterization (cultural, morphological, molecular diagnostic) were utilized to detect diversity at the isolate level. A second objective was to find out the most virulent isolate among the collecting isolates. Thirdly attempts to identify the variety that are tolerant or resistant against the disease and finally fungicides were screened *in vitro* and *in vivo* and an attempt to select novel fungicide against the disease.

Thirty isolates were categorized for the study and details description was in chapter 2. Mycelial growth rate was measured after 24, 48, 72 and 96 hours of incubation. Isolates showed variation in mycelial growth rate observed after 24 hours of incubation and MY-1 and JA-3 found to be the highest mycelial growth (9.00 mm) which were collected from Mymensingh and Jamalpur districts respectively. MY-1 remains in the highest position in all the incubation period observed but JA-3 showed lower mycelial growth after 48, 72 and 96 hours of incubation period compared to MY-1. On the other hand, MY-3 showed statistically different mycelial growth after 24, 48 and 72 hours of incubation period but found statistically similar mycelial growth after 96 hours of incubation period compared to MY-1. Radial growth rates for all isolates were ranged from 0.26 mm/hr – 0.45 mm/hr with an average of 0.35 mm/hr. Least significant difference test indicated that there was a significant difference ($\alpha = 0.050$) within fungal isolates growth rates in which isolates were categorized into two distinct groups indicating slow and fast growth rate. These results are in accordance with Singh *et al.* (2002), Sharma *et al.*(2005), and Khodayari *et al.* (2009) in *R. solani*.

Young colonies of all isolates were identical; they were hyaline on PDA and produced white sclerotia at the outset. But after 14 days, they showed a high degree of variation in visual appearance. These results are in agreement with the observation of other workers (Taheri *et al.*, 2007; Chaijuckam *et al.*, 2010a). Studies on cultural characteristics of the colony of different *R. oryzae-sativae* isolates varied from white to pale brown on PDA, which produced light brown to dark brown, few to abundant sclerotia of different size either scattered near inoculum or scattered all over the plate, peripheral or aggregate only on zonal area, immersed or embedded in agar (Apendix 8.1). There are 27 SCGs from 30 isolates indicated 90% diversity among the isolates by Somatic compatibility test where as, the isolates were categorized into 26 groups at 90% similarity level as revealed by VNTR and rep-PCR primers. After tested thirty isolates on cultivar BR11, MY-3 found more virulent among the all tested isolates, which also showed faster mycelial growth rate and produced abundant sclerotia. The primary objective of

this study was to examine the genetic diversity of field population of *R. oryzae-sativae*. VNTR and Rep-PCR markers were used to find out genetic diversity of the isolates. The tested isolates of *R. oryzae-sativae* were classified genetically into three groups by using VNTR and rep-PCR primers at 69.6% similarity level. Among the groups there were two distinct groups. Group I isolates were obtained from different agroecological zones (Table 1). Slow growth rate, small sclerotia and short RLH of group I isolates indicated that, there is a possibility of environmental suppression on these isolates from these areas, and the influence of the environment on disease development will be an interesting subject for future epidemiological studies. Datta (1981) reported that the occurrence and distribution of rice sheath diseases is influenced by weather factors, Physiological responses and agricultural practices. Nevertheless, further studies are required to provide a more extensive analysis of the existing population structure of the species at different areas in Bangladesh. In addition, group I suggested also the possibility of gene flow of up to a distance of 378 km (maximum distance from Gazipur to Thakurgaon districts (www.discoverbangla.com/DistanceChart.pdf). This is an agreement with the observations of Aye and Matsumoto (2010), who have found gene flow up to a distance of 716 km in *Rhizoctonia solani* by Rep-PCR fingerprinting. However, rest of the isolates clustered in group II (same AEZ, maximum distance Mymensingh-Narsingdi 135 km. (www.discoverbangla. com/DistanceChart.pdf). The group II population was found dominant with relatively fast mycelial growth rate, relatively virulent and was obtained from the same AEZ in Bangladesh. Isolate RA-1 collected from Rajshahi made a solitary group though having slow growth rate and same sclerotial characteristics. RA-1 isolate exhibited null alleles in several primers, possibly caused by a mutation at the 3' end of the primer binding sites (Butler, 2005). Comparison of two molecular marker systems used in this study showed that the highest number of scored DNA bands and a higher percentage of polymorphism were produced by the GF primer indicated VNTR was more efficient compared to that of Rep-PCR. Because, fingerprints generated by BOXA1R seemed to overlap adjacent bands (Versalovic

et al., 1994). Though the primers and isolates used in this study were relatively few in number, they could effectively establish the molecular variability among the isolates of Bangladesh.

Out of six carbon, six nitrogen sources and various pH level tested, the growth and sclerotia formation of *R. oryzae-sativae* of carbon sources dextrose proved to be the best followed by sucrose and galactose. Out of nitrogen sources tested, peptone proved to be the best for the growth of the pathogen, but no sclerotia had formed after 96 hours of incubation compared to PDA (control, without nitrogen source) plates. It was observed in this *in vitro* experiment that, high nitrogen percentage disfavored by *R. oryzae-sativae* and no mycelial growth or sclerotia found in urea. Whereas studies on pH revealed that the highest mycelial growth and abundant sclerotial intensity found at pH 7.0 in PDA media. The growth of this fungus reduced at very strong in acidic and alkaline pH. So the aggregate sheath spot disease prevailed in the nitrogen deficient and impartial (pH) soils.

All the tested fungicides were effective against the aggregate sheath spot pathogen; however their relative efficacy and ancillary properties differed substantially. In *in vitro* fungicide test or mycelial growth inhibition test indicated that Carbendazim showed better performance than other fungicides. Folicur and Nativo behaved almost the same and showed better performance than Differ and Propiconazole in field trial. Foliar application of fungicides against aggregate sheath spot under inoculated condition suggested that Carbendazim exhibited the highest activity against aggregate sheath spot of rice and resulted in the highest crop yield (Table 16 and 17). Yields were increased by Carbendazim, Folicur and Nativo compared with disease control plot in both of the trial. Differ and Propiconazole showed better inhibition in *in vitro* test, but failed to show better performance at *in vivo* trial compared to others. Lanoiselet *et al.* (2005b) also found the same result with Propiconazole and Pyraclostrobin in Australia against *R. oryzae-sativae* isolates. Fungicide, dose and their interaction were all statistically significant suggesting that individual chemicals each gave a response with variable investigated parameter like incidence, yield, sterility etc.

Various management practices such as cultivar resistance, stabble burning, chemical control etc have been reported by different workers across the rice growing countries. Varietals screening experiment test showed no cultivars or germplasm are immune to aggregate sheath spot disease. The present study revealed that Balam, Agoan, Musur, Koisramuri, Iet, Nuncha, CN, CM25, Aus Khusni, Kataktara, Kobila Rus, Kaloburi, Ratol, Aus Boilam, Aus Balam, Kataktari, Lotabhog, Ausboro from T.Aus, Panisail Chabichi dhan, Mosollajira, Kocheri jira, katari bhog, Khirsapat, Deshi malshira, BR4, BR10, BR22, BR23, BRRI dhan38, BRRI dhan41, BRRI dhan44 from T. Aman and BR7, BR9, BRRI dhan27BR1, BR2, BR3, BR6, BR8, BR12, BR14, BR15, BR16, BR17, BR18, BR19, BRRI dhan26, BRRI dhan28, BRRI dhan29, BRRI dhan35, BRRI dhan36 from Boro found moderately resistant against the pathogen. Since resistance was dominated by plant height therefore modern varieties should be targeted to moderate plant height with high yield potential. Cultivars architecture showed greatly influenced by physiological, morphological and ecological factors (Lee and Rush, 1983). During winter when Boro season starts usually from November, (Boro season) temperature and humidity remain low up to the end of February, during this time the pathogen remain dormant at the base of the plant when temperature start to increase from March the pathogen become active until crop harvest in April and May. Therefore, low level of upward progress is not unusual as found in this experiment in boro season (Table 21) while T. Aus and T. Aman temperature and relative humidity remain optimum for the disease development. Therefore cultivars showed least disease reaction (incidence and RLH) may be useful for resistant breeding program. Field trial results also indicates that almost all Bangladeshi semi dwarf cultivars or germplasm were more vulnerable to the disease than tall cultivars that perhaps induces favorable microclimatic conditions for pathogen growth and development compared to the tall cultivars. Inherent genetic resistance might have minor role on disease susceptibility rather favorable climatic influences indicating that in order to utilize genetic resistance into dwarf and semi dwarf rice development other management practices would be necessary

to combat the disease. The dwarf and semi dwarf cultivars that have greater tillering capacity, pathogen could easily spread horizontally and vertically but in tall cultivars the microclimate particularly in the upper canopy level relative humidity become lower as breeze and higher sunlight penetration hampers mycelelial growth, colonization and disease establishment. Similar observation was found by Ali 2002 for *R. solani*. The finding also agrees with the observation of Gunnel and Webster (1984) who reported that denser rice stands may enhance disease incited by *R. oryzae-sativae*.

Several higher plants and their constituents have been used successfully in plant disease control. In our experiment out of six plant extracts, garlic and henna were found to be more effective plant extract which reduced the growth of *R. oryzae-sativae* isolate up to 50% with low concentration (3.25% and 3.75% respectively). Satisfactory inhibitory growth of *R. oryzae-sativae* by using neem leaf extract was also found in this experiment. In *in vitro* test, ginger showed moderate inhibition against *R. oryzae-sativae*. The lowest inhibition found from *Polygonum* extract followed by telakucha extract against the pathogen.

In conclusion, the study of *R. oryzae-sativae* isolates sampled from different districts and same districts, multidisciplinary approach demonstrated that diversity was present even at the level of same district population. There is a considerable polymorphism within *R. oryzae-sativae* isolates as detected by VNTR and Rep-PCR analysis.

The study will help finally the taxonomy of this species and help the breeder to develop resistant breeding against the pathogen and have increase knowledge about the spread of *R. oryzae-sativae* in Bangladesh. This experiment added new insights into the nature of variation generated in this pathogen and also relevant to the application of chemical control measure. This is the first study on morphological characterization and genetic diversity of *R. oryzae-sativae* in Bangladesh. It is evident from the study that incorporate larger sample sizes are needed to elucidate the genetic structure of *R. oryzae-sativae* which is less studied in Bangladesh.

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Appendices

Appendix 1: Morphological characteristics of *R. oryzae-sativae* isolates

Morphological characters	Isolates																														
	DI-1	DI-2	DI-3	TH-1	TH-2	TH-3	RA-1	RA-2	RA-3	TA-1	TA-2	TA-3	MY-1	MY-2	MY-3	JA-1	JA-2	JA-3	NE-1	NE-2	NE-3	GA-1	GA-2	GA-3	SH-1	SH-2	SH-3	NA-1	NA-2	NA-3
1. Mycelial color on PDA	2	1	1	1	1	2	3	1	1	2	2	2	2	2	2	3	2	3	1	2	1	1	1	1	1	2	2	2	2	2
2. Arial mycelial quantity	1	0	2	0	1	0	2	1	2	2	2	1	0	3	0	3	2	3	2	2	2	1	2	1	1	0	0	1	3	1
3. Color of sclerotia	2	2	3	2	2	2	1	2	2	3	3	3	3	3	3	1	3	1	2	2	2	2	3	2	3	3	3	3	2	3
4. Quantity of sclerotia	1	1	1	1	1	1	1	1	1	3	3	3	3	2	3	3	2	3	1	1	1	1	1	1	3	3	3	3	3	3
5. Topography of sclerotia	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1
6. Shape of sclerotia	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
7. Superficial sclerotia(ss) discrete(<1mm)	3	3	2	2	2	3	3	2	2	1	2	1	1	1	2	1	1	1	2	3	3	3	3	2	1	1	1	1	2	1
8. SS discretr(>1mm)	2	0	1	2	2	1	1	1	1	3	3	3	3	3	2	2	2	2	2	1	1	1	1	1	3	3	3	2	3	2
9. SS aggregated	2	3	3	3	3	3	1	1	3	3	3	3	3	3	3	1	3	3	3	3	3	3	3	2	2	3	3	3	3	2
10. SS scattered	2	2	3	3	2	1	2	2	2	3	3	2	2	3	2	1	2	2	2	2	2	2	2	2	3	2	2	2	3	3
11. SS near inoculam	2	2	3	2	3	3	1	0	3	1	1	1	2	2	1	1	1	3	2	3	3	3	3	3	1	1	2	1	2	2
12. SS near margin	2	2	3	3	2	1	3	3	2	3	3	1	3	3	3	0	1	3	2	3	2	2	3	2	3	2	3	2	3	3
13. SS dispersed on whole colony	1	1	1	1	1	0	0	0	1	1	0	1	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	0	1	1
14. Exudate droplets on sclerotium surface	1	0	0	1	0	1	0	0	0	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1
15. Fluffy mycelium	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0
16. Quantity of fluffy mycelium	1	2	1	1	2	2	2	2	1	0	0	1	0	2	0	3	1	3	2	2	2	1	1	2	2	0	0	1	2	0
17. Colony reverse pigment	2	2	4	4	2	2	2	1	2	3	3	3	3	3	1	2	3	3	2	1	3	1	1	1	1	3	2	3	3	1
18. Pseudo sclerotia	1	3	1	2	2	1	3	2	1	0	0	0	0	2	0	0	0	0	2	2	3	3	1	2	0	0	1	1	1	0
19. Sclerotia on lid	0	0	0	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	1	0	0	1	0	1	1	1	0	1	0
20. Zonation	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	0	1	0	0	1	1	1	0	1
21. Zonation type	1	1	2	0	2	1	1	2	2	2	2	0	1	0	1	0	0	0	2	2	1	0	2	0	0	2	1	1	0	2
22. Mycelial growth on lid	0	2	2	1	3	1	3	1	3	3	3	1	3	3	2	0	3	1	0	0	0	2	2	2	2	0	3	1	2	3

(Data were taken as follow as Table 7)

Appendix 2: Reagents of 20 µl reaction of a master-mix for VNTR-PCR

Name of the reagent	Stock Conc.	Required Conc.	Required Vol.
PCR Buffer	10X	1X	2 µl
$MgCl_2$	25mM	3mM	2.4µl
dNTPs	25mM	0.2mM	0.16µl
Primer MR/RY/GF	20 pmol/ µl	1pmol	1 µl
Taq Polymerase Enzyme	5 U	0.5 U	0.1 µl
Sterile Distilled Water	-	-	13.34 µl
Total	-	-	20 µl

Appendix 3: Quantification of DNA pellets by spectrophotometre

Isolates	260/280	260/230	ng/µl
DI-1	2.070	1.630	3239.300
DI-2	2.060	1.690	2989.900
DI-3	2.030	1.370	2217.400
TH-1	2.050	1.610	3000.700
TH-2	2.060	1.730	1745.200
TH-3	2.000	1.700	4019.700
RA-1	1.930	1.530	4445.000
RA-2	1.980	1.620	439.800
RA-3	2.060	1.620	2027.800
GA-1	2.110	1.510	2135.000
GA-2	2.110	1.650	1852.200
GA-3	2.060	1.390	3072.800
NE-1	2.020	1.540	3856.300
NE-2	2.060	1.350	3323.500
NE-3	2.010	1.760	3931.400
SH-1	2.110	1.790	2879.900
SH-2	2.120	1.420	2489.600
SH-3	2.090	1.720	2908.300
MY-1	2.050	1.600	1798.800
MY-2	2.040	1.660	3103.300
MY-3	2.050	1.740	2903.900
NA-1	2.050	1.650	2914.200
NA-2	1.940	1.380	4547.300
NA-3	2.070	1.420	2742.500
TA-1	2.040	1.670	2810.600
TA-2	2.030	1.600	469.500
TA-3	2.040	1.640	2761.400
JA-1	2.040	1.520	2749.700
JA-2	2.060	1.550	3125.000
JA-3	2.090	1.900	2910.300

Appendix 4: Genetic similarity index between pairs of isolate in BOXA1R and VNTR combined analysis

Isolates	DI-1	DI-2	DI-3	TH-1	TH-2	TH-3	RA-1	RA-2	RA-3	GA-1	GA-2	GA-3	NE-1	NE-2	NE-3	SH-1	SH-2	SH-3	MY-1	MY-2	MY-3	NA-1	NA-2	NA-3	TA-1	TA-2	TA-3	JA-1	JA-2	JA-3
DI-1	1.00																													
DI-2	0.82	1.00																												
DI-3	0.72	0.82	1.00																											
TH-1	0.80	0.84	0.80	1.00																										
TH-2	0.80	0.84	0.80	0.77	1.00																									
TH-3	0.76	0.80	0.76	0.83	0.78	1.00																								
RA-1	0.40	0.50	0.40	0.48	0.50	0.45	1.00																							
RA-2	0.65	0.74	0.65	0.73	0.72	0.83	0.48	1.00																						
RA-3	0.73	0.77	0.73	0.80	0.85	0.85	0.47	0.84	1.00																					
GA-1	0.73	0.81	0.68	0.83	0.74	0.80	0.42	0.79	0.82	1.00																				
GA-2	0.73	0.72	0.64	0.79	0.65	0.84	0.42	0.79	0.82	0.89	1.00																			
GA-3	0.77	0.77	0.68	0.83	0.70	0.88	0.42	0.79	0.82	0.89	0.96	1.00																		
NE-1	0.65	0.74	0.65	0.77	0.67	0.78	0.44	0.77	0.83	0.78	0.78	0.78	1.00																	
NE-2	0.67	0.71	0.72	0.79	0.74	0.80	0.43	0.65	0.77	0.72	0.68	0.72	0.83	1.00																
NE-3	0.64	0.77	0.73	0.79	0.70	0.88	0.42	0.79	0.82	0.81	0.77	0.77	0.86	0.81	1.00															
SH-1	0.68	0.72	0.64	0.75	0.65	0.68	0.36	0.75	0.78	0.81	0.77	0.77	0.78	0.72	0.77	1.00														
SH-2	0.60	0.74	0.70	0.68	0.72	0.70	0.41	0.77	0.76	0.79	0.71	0.71	0.77	0.74	0.75	0.79	1.00													
SH-3	0.49	0.67	0.58	0.69	0.64	0.75	0.41	0.78	0.76	0.79	0.72	0.72	0.81	0.75	0.83	0.83	0.82	1.00												
MY-1	0.52	0.69	0.57	0.68	0.62	0.73	0.40	0.76	0.75	0.82	0.74	0.74	0.83	0.74	0.82	0.85	0.84	0.98	1.00											
MY-2	0.44	0.62	0.50	0.65	0.63	0.67	0.56	0.75	0.68	0.68	0.64	0.64	0.74	0.67	0.68	0.68	0.70	0.80	0.78	1.00										
MY-3	0.72	0.76	0.67	0.74	0.74	0.71	0.43	0.74	0.77	0.77	0.72	0.72	0.74	0.71	0.72	0.85	0.74	0.71	0.74	0.77	1.00									
NA-1	0.63	0.63	0.58	0.62	0.60	0.64	0.44	0.52	0.61	0.70	0.65	0.70	0.67	0.73	0.65	0.78	0.71	0.72	0.75	0.53	0.68	1.00								
NA-2	0.70	0.78	0.65	0.81	0.67	0.74	0.44	0.77	0.75	0.82	0.75	0.78	0.76	0.70	0.78	0.86	0.68	0.77	0.73	0.70	0.83	0.71	1.00							
NA-3	0.67	0.76	0.62	0.74	0.73	0.79	0.45	0.78	0.81	0.84	0.80	0.84	0.78	0.71	0.76	0.80	0.87	0.82	0.85	0.76	0.76	0.73	0.74	1.00						
TA-1	0.63	0.82	0.63	0.71	0.70	0.72	0.47	0.71	0.70	0.82	0.69	0.74	0.75	0.68	0.78	0.82	0.80	0.84	0.86	0.73	0.82	0.84	0.83	0.81	1.00					
TA-2	0.57	0.67	0.52	0.65	0.63	0.71	0.39	0.74	0.77	0.80	0.72	0.72	0.82	0.71	0.76	0.84	0.78	0.90	0.92	0.81	0.76	0.68	0.78	0.83	0.81	1.00				
TA-3	0.65	0.84	0.65	0.73	0.72	0.70	0.48	0.68	0.71	0.79	0.67	0.71	0.77	0.70	0.75	0.83	0.77	0.82	0.84	0.75	0.84	0.81	0.85	0.78	0.98	0.83	1.00			
JA-1	0.67	0.67	0.57	0.65	0.63	0.75	0.39	0.61	0.68	0.72	0.72	0.76	0.74	0.76	0.72	0.72	0.74	0.78	0.81	0.62	0.62	0.86	0.74	0.79	0.81	0.79	0.78	1.00		
JA-2	0.63	0.68	0.58	0.62	0.65	0.59	0.44	0.57	0.61	0.74	0.65	0.65	0.62	0.63	0.61	0.78	0.71	0.72	0.75	0.68	0.83	0.80	0.76	0.73	0.84	0.73	0.86	0.73	1.00	
JA-3	0.65	0.65	0.55	0.55	0.67	0.74	0.41	0.64	0.67	0.67	0.67	0.71	0.68	0.74	0.67	0.71	0.73	0.78	0.80	0.60	0.70	0.86	0.68	0.74	0.80	0.74	0.77	0.87	0.81	1.00

Appendix 5: Eigenvalues from Principle coordinate analysis

Sl. No.	Eigenvalue	Percent	Cumulative
1	21.93921218	73.1307	73.1307
2	1.55027607	5.1676	78.2983
3	1.04535522	3.4845	81.7828
4	0.89696903	2.9899	84.7727
5	0.71820421	2.394	87.1667
6	0.62347163	2.0782	89.2450
7	0.47746527	1.5916	90.8366
8	0.39945181	1.3315	92.1681
9	0.34431727	1.1477	93.3158
10	0.28920152	0.964	94.2798
11	0.27789657	0.9263	95.2061
12	0.21393983	0.7131	95.9192
13	0.19427413	0.6476	96.5668
14	0.16352263	0.5451	97.1119
15	0.15225092	0.5075	97.6194
16	0.13357334	0.4452	98.0646
17	0.11777208	0.3926	98.4572
18	0.09186893	0.3062	98.7634
19	0.08929324	0.2976	99.0610
20	0.06899953	0.23	99.2910
21	0.0617577	0.2059	99.4969
22	0.05357911	0.1786	99.6755
23	0.03528304	0.1176	99.7931
24	0.01908393	0.0636	99.8567
25	0.01667644	0.0556	99.9123
26	0.00971455	0.0324	99.9447
27	0.0080643	0.0269	99.9716
28	0.00531036	0.0177	99.9893
29	0.00225274	0.0075	99.9968
30	0.00096242	0.0032	100.0000
Sum of eigenvalues =	30.000000		

Appendix 6 : Somatic compatibility reaction among the isolate of *R. oryzae-sativae* in all possible combination

***NB= no barrage zone and **B= barrage zone present**

Isolate	DI-1	DI-2	DI-3	TH-1	TH-2	TH-3	RA-1	RA-2	RA-3	GA-1	GA-2	GA-3	NE-1	NE-2	NE-3	SH-1	SH-2	SH-3	MY-1	MY-2	MY-3	NA-1	NA-2	NA-3	TA-1	TA-2	TA-3	JA-1	JA-2	JA-3
DI-1	NB*	B**	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	IB	B	B	B	B	B	B	B
DI-2		NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
DI-3			NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
TH-1				NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
TH-2					NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
TH-3						NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
RA-1							NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
RA-2								NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
RA-3									NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
GA-1										NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
GA-2											NB	NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
GA-3												NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
NE-1													NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
NE-2														NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
NE-3															NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
SH-1																NB	B	B	B	B	B	B	B	B	B	B	B	B	B	B
SH-2																	NB	B	B	B	B	B	B	B	B	B	B	B	B	B
SH-3																		NB	NB	B	B	B	B	B	B	B	B	B	B	B
MY-1																			NB	B	B	B	B	B	B	B	B	B	B	B
MY-2																				NB	B	B	B	B	B	B	B	B	B	B
MY-3																					NB	B	B	B	B	B	B	B	B	B
NA-1																						NB	B	B	B	B	B	B	B	B
NA-2																							NB	B	B	B	B	B	B	B
NA-3																								NB	B	B	B	B	B	B
TA-1																									NB	B	NB	B	B	B
TA-2																										NB	B	B	B	B
TA-3																											NB	B	B	B
JA-1																												NB	B	B
JA-2																													NB	B
JA-3																														NB

Appendix 7: Relation between Concentration and Inhibition of different Fungicides against *R. oryzae-sativae*

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% Inhibition							
Fungicide	Concentration (ppm)						Average
	100	10	1	0.5	0.25	0.1	
Amistar Top	94.05	74.21	69.44	18.25	17.46	16.67	48.35
Carbendazim	100	100	100	68.25	62.30	50.40	80.16
Differ	100	75.00	48.41	44.84	28.17	12.70	51.52
Folicur	100	100	98.41	46.83	38.1	32.94	69.38
Mancodazim	100	88.89	29.37	18.25	14.29	13.49	44.05
Monceren	54.37	40.40	32.14	13.89	13.49	12.30	27.77
Nativo	100	98.21	69.44	61.11	35.71	28.57	65.51
Propiconazole	100	92.06	69.05	59.92	29.76	27.78	63.10
Average	93.55	83.60	64.53	41.42	29.91	24.36	56.23