# BIOLOGICAL CONTROL OF SEED BORNE FUNGAL PATHOGENS OF RICE BY USING ANTAGONISTIC BACTERIA

**BY**

**EINUN NAHAR MAJUMDER**


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

**JUNE, 2011**

# BIOLOGICAL CONTROL OF SEED BORNE FUNGAL PATHOGENS OF RICE BY USING ANTAGONISTIC BACTERIA

**Submitted by:**
**EINUN NAHAR MAJUMDER**
**Registration No. 05-1636**

**A thesis**
**Submitted to the Faculty of Agriculture,**
**Sher-e-Bangla Agricultural University, Dhaka**
**In partial fulfillment of the requirements**
**For the degree of**

**MASTER OF SCIENCE (MS)**
**IN**
**PLANT PATHOLOGY**
**SEMESTER: January-June, 2011**

**Approved by:**

| | |
|---|---|
| **Nazneen Sultana** | **Dr. Md. Rafiqul Islam** |
| **Associate Professor** | **Professor** |
| **Supervisor** | **Co- Supervisor** |

**Nazneen Sultana**
**Associate Professor**
**And**
**Chairman**
**Examination Committee**

**Nazneen Sultana**
**Associate Professor**
**Department of Plant Pathology**
**Sher-e Bangla Agricultural University**
Dhaka-1207, Bangladesh
Mob: 01733955171

# CERTIFICATE

This is to certify that thesis entitled, **"BIOLOGICAL CONTROL OF SEED BORNE FUNGAL PATHOGENS OF RICE BY USING ANTAGONISTIC BACTERIA"** submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN PLANT PATHOLOGY,** embodies the result of a piece of bona fide research work carried out by **Einun Nahar Majumder, Registration No. 05-1636** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. Further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

**Dated: JUNE, 2011**
**Dhaka, Bangladesh**

**Nazneen Sultana**
**Supervisor**
Associate Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207


Dedicated to
My
Beloved Parents

## *ACKNOWLEDGEMENTS*

*Having unflinching faith on to "omnipotent creator" for his never ending blessing, the author deems it a great pleasure to express her profound gratitude to her respected parents, who digested much hardship inspiring for prosecuting her studies, thereby receiving proper education.*

*The author feels proud to convey her earnest respect, sincere appreciation and enormous indebtedness to her supervisor* ***Nazneen Sultana, Associate Professor and Chairman,*** *Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for her continuous direction, constructive criticism and valuable suggestions in carrying out the research work and preparation of this thesis.*

*The author expresses her gratitude and best regards to her respected Co-Supervisor,* ***Dr. Md. Rafiqul Islam, Professor,*** *Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for his scholastic supervision, helpful commentary and unvarying inspiration throughout the research work and preparation of the thesis.*

*The author highly grateful to my honorable teachers,* ***Professor Dr. Salahuddin M. Chowdhury; Associate Professor Dr. F. M. Aminuzzaman; Assistant Professors Khadija Akhter and Abu Noman Faruq Ahmmed,*** *Department of Plant Pathology, Sher-e-Bangla Agricultural University, for their valuable teaching, direct and indirect advice, and encouragement and co-operation during the whole study period.*

*The author pleased to thank all stuffs of the Department of Plant Pathology of Sher-e-Bangla Agricultural University, Dhaka for their valuable and sincere help in carring out the research work.*

*The author also likes to give thanks to all of her friends for their support and inspiration throughout his study period in Sher-e-Bangla Agricultural University, Dhaka.*

*The author found no words to thank her parents, her sister Kamrun Nahar, Nazmun Nahar and her brother Jahedul Huq Majumder, Asaduzzaman Milon for their unquantifiable love and continuous support, their sacrifice never ending affection, immense strength and untiring efforts for bringing her dream to proper shape. They were constant source of inspiration and enthusiasm in the critical moment of her studies.*

***The Author***

# BIOLOGICAL CONTROL OF SEED BORNE FUNGAL PATHOGENS OF RICE BY USING ANTAGONISTIC BACTERIA

## ABASTRACT

The experiment was conducted during the period of June, 2011 to May, 2012 to determine the prevalence of seed borne fungal pathogens of BRRI Dhan 46 and to search suitable antagonistic bacteria against seed borne fungi of rice and also to evaluate the effectiveness of antagonistic bacteria in controlling seed borne disease of rice. Five seed borne fungi viz. *Fusarium* spp., *Bipolaris oryzae*, *Curvularia* spp., *Gliocladium* sp and *Aspergillus flavus* were isolated from the seeds of rice. Fifteen probable antagonistic bacterial isolates were isolated from rhizosphere soil and screening was done to detect the most suitable antagonistic bacterial isolates against seed borne fungi of rice. Among fifteen isolates six isolates (Iso-1, Iso-2, Iso-3, Iso-5, Iso-7 and Iso-10) were found effective in dual culture method. Seeds of rice variety, BRRI Dhan 46 yielded 21.5 % *Fusarium* spp., 6.25% *Bipolaris oryzae*, 4.75% *Curvularia* sp, 0.25% *Gliocliodium* sp. and 3.5% *Aspergillus flavus* infection in blotter method. In dual culture method, the bacterial Iso-2 produced higher inhibition of mycelia. Bacterial antagonist Iso-2 performed better than other isolates on seed borne fungal prevalence in blotter method. Seed treated with Iso-2 completely controlled seed borne *Bipolaris oryze*, *Aspergillus flavus*, and *Gliocladium*. The highest seed germination (98%) was recorded in seeds treated with Iso-2 and Iso-1. In water agar test tube method the highest germination (96.67%) was found in Iso-2 treated seed. In rolled paper towel method seed treated with iso-2 gave the highest Vigor Index (1424) and the lowest vigor index was noted (753) in untreated control.

# CONTENTS

# LIST OF TABLES

# LIST OF FIGURES



# LIST OF FLOWCHAT

# LIST OF PLATES

# CHAPTER 1
# INTRODUCTION

Rice (*Oryza sativa* L.) is one of the major cereal crops of the world used as the staple food by the 60% of the world population. It is also the most important cereal crop in Asia producing about 96% of the world rice production (IRRI, 2003). It also serves as staple food crop of the people of Bangladesh. About 79.77% of cropped area of this country is used for rice production (BBS, 2010). Rice is the second largest produced cereal in the world in 158.3 million hector area with annual production of about 685.24 million metric tons (Anonymous, 2011). Bangladesh is ranked as fourth in rice production with annual production of 47.72 million metric ton in the world (Anonymous, 2011). Rice provides 75% calories and 55% protein in the average daily diet of the people and shares 95% of cereal consumption (Bhuiyan, *et al.*, 2002).

There are many causes of low yield of rice in Bangladesh of which disease and pest plays a major role (Fakir, 1982). Among them seed borne diseases are more destructive. Rice seeds are known to harbor a wide range of both fungi and bacteria (Neergaard, 1977). A total of more than 100 fungi were detected on rice seeds. Rice seed very often infected by field fungi, *Bipolaris oryzae, Trichoconis paddwickii, Fusarium moniliforme, Fusarium oxysporum, Fusarium semitectum, Pyricularia grisea, Curvularia lunata, Alternata,* (Mia *et al.* 2002). The storage fungi such as *Aspergillus* spp., *Penicillium* spp., *Rhizopus* sp etc usually invade seed during preservation. There is no accurate estimate about the yield loss of rice due to diseases. However, roughly 10% yield loss of rice may be incurred annually due to seed borne diseases in the country. Among the seed borne pathogen of rice, fungi are most common.

Seed borne pathogens cause enormous losses to our crop. Seed borne disease causes seed rot, germination failure and seedling mortality and then reduce rice production. The infected seeds may fail to germinate, transmit disease from seed to seedling and from seedling to growing plants (Fakir *et al.*, 2002). Infected seeds germinate poorly

and could be a major source of inoculums for new crops raised from them. For example, most pathogens causing abnormal seedling of rice are seed borne (Guerrero *et al.,* 1972). Seed borne pathogens affect seed quality (Khare, 1999). Islam *et al*. (2000) observed that highest lethal seed infection caused by *Fusarium moniliforme, Trichoconis padwickii* and *Curvularia* spp. About 20 species of fungal pathogens were detected from rice seed at any one time (Mew and Gonzales, 2002). The crop is affected by as many as 36 seed-borne diseases of which 31 were caused by fungi (Ou, 1985). Totally 8 genera of fungi viz., *Alternaria, Aspergillus, Bipolaris, Chaetomium, Curvularia, Fusarium, Sarocladium* and *Trichoderma* comprising twelve species were found to be associated with the seed samples. Among them, the most predominant one was *Bipolaris oryzae* (Gopalakrishnan and Valluvaparidasan , 2007).

Seed borne pathogens can be effectively controlled by using seed treating fungicide and chemical. But the use of chemical also breaks the natural ecological balances by killing the beneficial antagonistic micro-organisms. The chemical fungicide use to control disease effectively but chemical application have long term residual effect; create environmental pollution and the development of resistant strain of pathogen. So an effective alternative is biological control such as antagonistic bacteria which is environmentally safe.

Biological control is an innovative, cost effective and eco-friendly approach. Use of natural enemies to control disease is termed biological control. Biological control is an alternative to the use of chemical pesticides. Biological agents may act to suppress the population of the pathogenic organisms through competition with pathogenic organisms, stimulated plant growth, which may allow plants to quickly outgrow any pathogen effects, or damage the pathogen by means of toxins produced (Cook, 2000 and Gilreath, 2002). On the other hand, it is safe and tends to maintain the ecological balance reducing the economic injury level of disease. Biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and the ecosystem, it neither eliminates the pathogen nor the disease, but brings them into natural balance (Ramanathan *et al*., 2002).

A diverse group of bio-control agents--such as bacteria, fungi, viruses--exist in nature. Among them bacterial antagonist are considered ideal candidates because of their rapid growth, ease of handling and aggressive colonizing character. Bio-control with beneficial bacteria is one promising alternative to fungicides (Li et al., 2008). Bacterial antagonists, *Pseudomonas*, and *Bacillus* in particular, are good candidates for biological control (Mew, *et al.,* 2003). Different strains of *Pseudomonas fluorescens sagainst* have antifungal activity against some plant pathogens such as *Alternaria cajani, curvularia lunata, Fusarium* sp, *Bipolaris* sp and *Helminthosporium* sp. in vitro with different concentrations (1000-2000, 3000, 4000 and 5000 ug/ml) of *Pseudomonas fluorescens* (Srivastava, 2009). Antagonistic and inhibitory activity of *Bacillus subtilis* isolate G-GANA7 (Gene Bank accession No. EF583053) obtained from Abo-Homos in Egypt against six fungal isolates belonging to four different genera, *Rhizoctonia solani, Helminthosporium* sp, *Alternaria* sp, and *Fusarium oxysporum* (Matar *et al,* 2009).

In Bangladesh, scientists are also now giving their efforts to control seed borne pathogens by using some bio-agents like *Trichoderma* spp. But no significant research was done regarding bacterial antagonists. Thus the study was undertaken with the following objectives:

1. To determine the prevalence of seed borne pathogens of rice.
2. To isolate and identify probable bacteria antagonist from rhizosphere soil.
3. To determine the efficacy of antagonistic bacteria against seed borne fungal pathogens of rice.

# CHAPTER 2

# REVIEW OF LITERATUR

## 2.1 Rice seed borne fungal pathogen

Butt *et al*. (2011) studied seed borne mycoflora of different stored grain of rice varieties by using blotter method and its chemical control they reporeted varieties of rice (*Oryza sativa* L.) viz. KS-282, Basmati-385, Basmati-370, Basmati Kernal and Basmati-198 were investigated the occurrence of seed-borne mycoflora using blotter paper method, and 27%, 19%, 17%, 16% and 14% mycoflora was found associated with the seeds of Basmati kernel, Basmati-385, Basmati- 370, Basmati-198 and KS-282, respectively. Four fungal species namely *Fusarium moniliforme, Alternaria* sp., *Helminthosporium* sp. and *Curvularia* sp. were isolated from different test rice varieties. Four chemical fungicides namely antracal, topsin, mencozeb and derosal were used to investigate their effect on seed-borne mycoflora of rice. Seed treatment with different fungicides exhibited insignificant effect on the occurrence of *F. moniliforme* and *Alternaria* sp. Antracal completely stopped the growth of *Helminthosporium* sp. and *Curvularia* sp. The other three fungicides markedly suppressed the growth of *Helminthosporium by* up to 50%. Similarly, topsin and mencozeb suppressed the growth of *Curvularia* sp by up to 50%.

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

Tripathi and Dubey (2004) reported that the most destructive seed-borne fungi of rice are *Bipolaris oryzae, Pyricularia oryzae, Sarocladium oryzae, Rhizoctonia solani, Bipolaris oryzae, Sclerotium rolfsii, Fusarium* spp., *Curvularia oryzae* and *Nigrospora oryzae*. Natural plant extracts are important sources of new means and nonselective pesticide for control of plant diseases.

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

Javaid *et al. (*2002), Wahid *et al.* (1993 and 2001) and Khan (1999 and 2000) isolated *Alternaria alternata, A. padwickii, A. longissima, Aspergillus niger, Curvularia oryzae, C. lunata, Drchslera oryzae, Fusarium miniliforme, F. semitectum, F. oxysporum, F. soalni, Pyricularia oryzae,* and species of *Phoma, Cercospora, Chaetomium, Sclerotium, Pecicillium, Myrothecium* and *Colletotrichum* from seeds of different varieties of rice collected from different regions of the pakistan.

Fakir (2000) reported that rice suffers from more than 60 different diseases. In Bangladesh, 43 diseases are known to occur on the rice. Among these diseases, 27 are seed borne of which 14 are of major importance. Fungi are the principal organisms associated with seed in storage. Of the seed borne diseases of rice, 22 are caused by fungi.

Radha jeyalakshmi (1998) reported that totally 18 fungal species belonging to twelve genera were found to be associated with the rice field seed samples in Tamil Nadu. The frequency of evaluation of fungicides against fungi causing *S. oryzae* was 36.72 percent.

Riaz and Ahmed (1995) conducted an experiment on seed-borne fungi of rice collected from Pakistan and isolated *Helminthosporum* spp, *Curvularia, Fusarium* and *Aspergillus* from various seeds from North Southern provinces of Pakistan.

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

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

Imolehin (1987) studied rice seed multiplication centres in relation to seed- borne pathogens of rice: A case study of on do State Rice Multiplication Centers and stated that *Fusarium moniliforme* and *Drechslera oryzae* were the major pathogens that caused devastating seedling disease of rice in the field (*D. oryzae* 12%, *F. moniliforme* 40%). This work is a survey of the incidence of seed-borne fungi of rice associated with three varieties of rice: Faros 12, 15, and 29 popularly cultivated in Afikpo North local government area of Ebony State and isolated *B. oryzae* (*Drechslera oryzae*), *Curvularia lunata*, *Chaetomium* spp., *Trichoderma* spp., *Aspergillus* spp. and *Penicillium* spp. from twenty-two different rice cultivars from South West Nigeria.

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

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

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

Esuroso *et al*. (1975) studied on seed-borne fungi of rice (*Oryzae sativa* L.*)* in Nigeria and reported that wide range of fungi occurred on the seeds of rice in Nigeria.

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

## 2.2 Antifungal properties of bacteria

Nourozian *et al*. (2006), Moita *et al.* ( 2005) Siddiqui *et al.* (2005) Radheshyam *et al.* (1990) investigated that the use of bacteria like *Pseudomonas* sp., *Bacillus* sp. and their properties to produce antifungal metabolites and protect plants from fungal infection.

Bennett and Klich (2003) reported that fungi may produce several mycotoxins which can cause economic losses and may affect human health. Mycotoxins are toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp.

Kim *et al.* (2003) reported that *Bacillus* sp. have the characteristics of, being widely distributed in soils, having high thermal tolerance, showing rapid growth in liquid culture, and readily form resistant spores. It is considered safe biological agents and their potential as bio control agents is considered to be high.

Munimbazi and Bullerman (1998) reported that extracellular antifungal metabolites produced by *Bacillus pumilus* inhibited mycelial growth of many species of *Aspergillus, Penicillium* and *Fusarium.*

## 2.3 Bacterial antagonist

### 1. *Pseudomonas* sp

Palumbo *et al*. (2010) isolated *Pseudomonas chlororaphis* strain JP1015 and *P.fluorescens* strain JP2175 Mississippi cornfield soil samples and selected for their growth inhibition of *Aspergillus flavus* in laboratory culture. In this study was determined that growth of *A. flavus* was inhibited up to 100-fold by *P. chlororaphis*

strain JP1015 and up to 58-fold by *P. fluorescens* strain JP2175 within 3 days following soil co inoculation.

Goud and Muralikrishnan (2009) studied that in pour plate method, *Pseudomonas fluorescens* on co-inoculation with fugal pathogens decreased their growth rate. Maximum inhibition was observed in *Pythium ultimim* (80%) followed by *Macrophomina phaseolina* (70%) *Pyricularia oryzae* (50%).

Srivastava (2009) studied the antifungal activity of different strains of *Pseudomonas flurescens* against some plant pathogens such as *Alternaria cajani, curvularia lunata, Fusarium* sp., *Bipolaris* sp. and *Helminthosporium* sp. *in vitro* with diffrent concentrations (1000-2000, 3000, 4000 and 5000 ug/ml) of *Pseudomonas fluorescens* and concluded that all the strains of *Pseudomonas fluorescens* presented a most significant value against *Alternarja cajani and Curvularia lunata*. Out of the five strains studied, the best result was shown by A-5, which showed almost complete inhibition against pathogenic fungi such as *Curvularia lunata* and *Fusarium* spp. at 4000 and 5000 ug/ml while strain L-5 was resistant against *Fusarium* spp. *and Helminthosporium* sp. at 5000 ug/mL and bacterial strains C-03 and Pf4-1 were found to be more sensitive to *Fusarium* sp. and *Helminthosporium* sp.

Ganeshan and Kumar (2005) stated that *P. fluorescents* is a potential bio-pesticide for augmentative biological control of many diseases of agriculture and horticulture importance.

Jayaswal *et al.* (1993) studied two strains of *Pseudomonas capacia*, RJ3 and ATCC 52796, have been identified as potential antagonists of fungal plant pathogens. They compared the antagonistic activity of these two strains against various fungal pathogens. Although both strains displayed high levels of antagonism, ATCC 52796 was slightly more antagonistic than RJ3.

**ii) *Burkholderia* sp**

Omar *et al.* (2006) studied on two bacterial isolates, *Bacillus megaterium* (C96) and *Burkholderia cepacia* (c91) to find out the antagonistic efficacy against *Fusarium oxysporum* f.sp. *radicis-lycopersici*, the causal organism of Fusarium crown and root rot of tomato and reported that these isolates reduced disease incidence by 75 and 88% respectively.

Bevivino *et al.* (2005) reported that *Burkholderia cepacia* complex (Bcc) bacteria are naturally present in the rhizosphere of several crop plants and have been found to antagonize a wide range of important plant pathogens.

Reddy (1997) reported that *B. cepacia* can also antagonize and supress many fungal plant pathogens such as *Fusarium, Pythium, Rhizoctonia, Cylindrocarpum, Botrytis* and *Alternaria.*

Hebbar *et al.* (1997) used *Burkholderia capacia (syn Pseudomonas capacia)* strain PHQM100 applied as seed coating was tested its ability to suppress pre-emergence damping-off, and postemergence damping-off in corn inducted by *Pythium* and *Fusarium* spp.

Bevivino *et al.* (1998) reported that *Burkholderia cepacia* MCI 7 was able to determine an increased growth response ($P<0.05$) of the two corn cultivars in both un-infested soil and soil infested with *Fusarium moniliforme.* Moreover, *B. cepacia* MCI 7 was able to negatively affect the rhizoplane colonization of *F. moniliforme.*

Mao *et al.* (1998) studied on the bio-control of soil borne diseases of tomato caused by *Rhizoctonia solani* and *Pythium ultimum* alone or in combination with *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *lycopersici* in the greenhouse and field and reported that combined *Gliocladium virens-3* (G1-3) + *Burkholderia cepacia* - F(Bc-F) application resulted in greater fresh weight and lower DSI for pepper, and greater fruit yield for tomato than those obtained with either G1-3 or Be--F alone.

## 2.3 Isolation and purificatioin of antragonistic bacteria from soil

Goud and Muralikrishnan (2009) reported that *Pseudomonas fluorescens* is one of the most suitable bio-control agent in suppressing the phytopathogenic fungi and replace chemical fungicides. In this study, they conducted physiological and biochemical tests such as colony character, motility, gram stain nature, Production of fluorescent pigment, gelatin liquefaction, Starch hydrolysis and utilization of nitrate to authenticated the isolated organism as *P.fluorescens.*

Wang *et al.* (2009) studied on the antagonistic activity of *Bacillus subtilis* and they identified as *Bacillus subtilis* according to its morphological, physiological, and biochemical characteristics and i6SrDNA sequence analysis.

Filippi *et al.* (2008) isolated thirty two bacteria from soil samples that were antagonistic to a number of phyto-pathogenic fungi. One bacterial strain, designated as M 51, appeared to be particularly active towards *F. oxysporum , f. sp. idianthili in vitro* and *in vivo* and it was inhibitory *in vitro* to three other *Fusarium* spp. used.

Kazempour and Anvary (2008) evaluated the antagonistic activity of *Pseudomonas fluorescens* against *Fusarium fujikuroi in vitro* by using dual culture test, also by Demonstration of antibiotic activity and production of diffusible antibiotic.

Selvakumar *et al.* (2008) reported that *Exiguobacterium acetylicum* strain 1P (MTCC 8707) is a rhizospheric, Gram positive, rod shaped, yellow pigmented bacterium isolated from an apple orchard rhizospheric soil and in separate in-vitro compound produced by the bacterium was found to be the most potent in inhibiting the hyphal development of *Rhizoctonia solani, Sclerotium rolfsii, Pythium* and *Fusarium oxysporum* by 45.55, 41.38, 28.92 and 39.74% respectively.

Adesina *et al.* (2007) studied the antagonistic potential of soil bacteria towards *Rhizoctonia solani* AG3 and *Fusarium oxysporum* f.sp. lini (Foln3). For this purpose they screened 1,788 isolates from the soils and *in vitro* 327 isolates were found as antagonists.

Jha and Anjaiah (2007) studied on Metabolities of rhizobacteria antagonistic towards fungal plant pathogens and in this study double-layer technique was used for isolation of antagonistic bacteria from rhizosphere against plant pathogenic fungi. Four potential rhizobacteria was selected in dual culture plate method based on their antifungal activity against several soil-borne fungal plant pathogens.

Ramarathnam *et al.* (2007) reported that *Bacillus subtilis* DFH08, an antagonist of *Fusarium graminearum,* and other *Bacillus* spp. that are antagonists of common fungal pathogens of canola were screened for peptide synthetase biosynthetic genes of fengycin and bacillomycin D. Specific polymerase chain reaction (PCR) primers identified *B.subtilis* strains DFH08 and 49 for the presence of the fenD gene of the fengycinopero.

Aboelnaga (2006) evaluated the antagonistic activity of *Bacillus subtillis* i*n-vitro* by using dual culture test and pot assays for controlling damping off of sugar beet

Xie *et al.* (2003) reported 16 species or types of *Pseudomonas* and 17 genera of non-pseudomonas gram negative bacteria as antagonistic against major pathogens of rice from rice seed in the tropic environment and they were initially characterized on the basis of colony morphology and results of biochemical and pathogenicity tests. Then six hundred and fifty two strains were further identified by biology, from which 133 were selected for fatty acid methyl ester (FAME) analysis together with 80 standards reference Strains.

Brion and Genevieve (1999) tested the antagonistic activity of *Pseudomonas fluorescens* by using Cross streak assay and pour plate method and reported that *P.fluorescens* inhibit the growth of plant pathogens.

Ahmed and Ahmed (1963) screened out bacterial antagonists (*P. citrimum, Streptomyces sp.* and bacterial organism B-7 which found effective against 18 among 32 isolated fungus organisms from potted soil (collected from betel vine soil).

# CHAPTE 3

# MATERIALS AND METHODS

## 3.1 Experimental site

The experiments were conducted in the Plant Disease Diagnostic Laboratory and Seed Pathology Laboratory of Department of Plant Pathology of Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207**.**

### 3.2 Experimental period

The experiments were conducted during the period of June, 2011 to May, 2012.

### 3.3 Seed collection

Seeds of rice variety BRRI Dhan 46 were collected from Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur.

### 3.4 Detection and identification of seed borne fungi of rice

A composite sample of rice variety BRRI dhan 46 was assayed for the presence of seed borne fungi by Standard Blotter method (ISTA, 1996).

#### 3.4.1 Prevalence of seed borne fungi in blotter method

In this method, 400 seeds were randomly taken from rice seed sample. The seeds were placed on water soaked three layered Whatman No.1 filter paper in plastic petridish. In each petridish, 25 seeds were plated at equal distance. All these petridishes were incubated at 20±2°C under 12 hours alternate cycle of Near Ultra Violet (NUV) light and darkness. After 7-10 days of incubation, petriplates containing incubated seeds were observed under stereomicroscope for detecting seed-borne fungi in rice seeds surface under stereomicroscope at 25X magnification. Where identification was difficult or doubtful under the stereomicroscope, temporary slide was prepared and examined under the compound microscope. Identification of pathogen was done following the keys of Burgess *et al*., (1994); Agarwal *et al*., (1989); Ellis (1993) and Mathur and Kongsdal (2003) and Barnett and Hunter (1992). Number of germinated seeds was recorded along with the seed-borne fungi after 7-10 days of incubation. The results were expressed in percentage.

$$\text{Frequency of occurrence (\%)} = \frac{\text{No. of seeds on which a fungal species occurs}}{\text{Total No. of seeds}} \times 100$$

### 3.4.2. Preparation of potato dextrose agar (PDA)

PDA (appendix-2.2) was prepared as described by Islam (2009). 200g peeled and sliced potato was boiled in 500ml water in a bowl for about half an hour. Then the extract of the potato was filtered through was cheese cloth. The other two ingredients viz. 20g dextrose and 20g agar were added in the extract and the volume was made up to 1L mark. Then the prepared standard PDA was poured in 1000ml conical flask and sterilized (121˚C, 15 psi for 15 min.) in an Autoclave.

### 3.4.3 Isolation and purification of seed borne fungal pathogen of rice

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

## 3.5 Isolation of probable antagonistic bacteria from Rhizosphere soil

Probable antagonistic Bacteria were isolated from rhizosphere soil flowing dilution plate method.

### 3.5.1 Collection of soil (rhizosphere) sample

The soil samples were collected from the field, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. Three soil samples were randomly collected from the rhizosphere of crop field at different growth stages. Soil collection was made with the help of an augur of one inch diameter at a depth of six inches. At the time of collection, the surface of the soil was scrapped to remove dry topsoil and other superficial plant debris. Each composite sample was kept in cellophane bag with proper labeling. After collection, the bags were kept under shade.

### 3.5.2 Preparation of nutrient agar (NA)

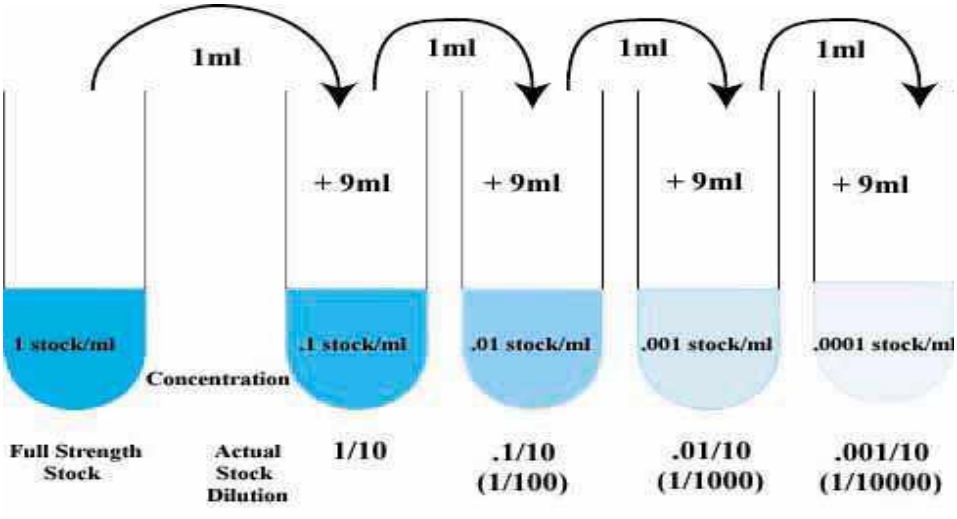
Nutrient agar Media (appendix-2.1) was prepared according to the method followed by Hossain (2006). 28g Nutrient agar mixed well in 1000 ml distilled water. It was then autoclaved at 121$^{o}$C under 15 PSI pressure for 15 minutes.

### 3.5.3 Isolation and purification of bacteria from soil by using soil dilution plate technique

Dilution plate technique was carried out as described by Islam (2009) for isolation of soil microbes (Flow chart. 1)

#### 3.5.3.1 Preparation of soil suspension

One gm of soil was placed in the test tube containing 9 ml of sterile water and stirred thoroughly for few minutes in order to obtain a uniform 1:10 dilute soil suspension. This was used as stock solution resulting $10^{-1}$ dilution. One ml of 1:10 stock suspension was transferred with the help of sterile pipette into the 2nd test tube containing 9 ml sterile water and shaken thoroughly resulting $10^{-2}$ dilution. Thus final dilution was made up to $10^{-4}$.




**Flow chart 1. Dilution plate technique for isolation of Bacteria.**

#### 3.5.3.2 Plating of bacteria on NA media by spread plate method

Each of the serial dilution was transferred into nutrient agar plate by using spread plate method. 0.1ml of an appropriately diluted culture was spread over the surface of agar using sterile glass spreader. The plate is then incubated until the colonies appear.

The surface of the plate was kept dry so that the spread liquid soaked. The step for spread plate method is given.

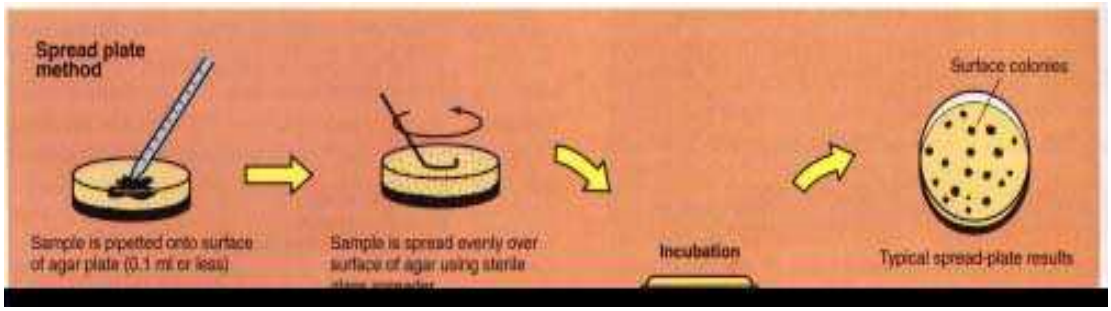



**Fig 1:** Spread Plate Method

**Source:** Adawiah (2008)

### 3.5.4 Isolation and purification of antagonistic bacteria

After incubation of the inoculated NA plates, bacteria appeared with various types of colony colors. Then the bacterial colonies were selected and isolated depending on their colony color and streaked on NA media separately. Again the streaked NA media plates were incubated at room temperature for 2 days. Purification was done by streaking NA plates from single colony.

## 3.6 Determination of efficacy of antagonistic bacteria

### 3.6.1 *In-vitro* bioassay of isolated bacteria

In-vitro bioassay of bacteria was done to cheek their antagonistic activity following dual culture method.

**Dual culture method**

The dual culture method was carried out following the description of Azadeh *et al.* (2010). A block cut of mycelium of each fungus (5 mm diameter) was plated at center of plate after that 48 hr old bacterial culture grown at room temperature on NA media was streaked by serial loop. 3 cm distance was maintained between two streaking. All the tested plates were incubated at $28^0$C for 5-7 days and antagonistic effect was determined by measuring the longest and shortest free growth zone between the isolated bacteria and the tested fungi. Control trial was done without streaking of

bacteria in the plate. Assessment was made when the fungi had achieved an equilibrium after which there was no further alternation in the growth. Since both of the organisms were mutually inhibited, the assessment was made for both organisms. The percentage inhibition of growth was calculation as follow:

$$\text{Percentage inhibition of growth} = \frac{r - r_1}{r} \times 100$$

r = growth of the fungus from the center of the colony towards the centre of the plate in the absence of antagonistic bacteria.

$r_1$ = growth of the fungus from center of the colony towards the antagonistic bacteria.

### 3.7 Identification of isolated bacteria by morphological test

#### 3.7.1 Study of colony color

For morphological characters, colony color and surface textures were carefully studied and recorded as bacteria developed after 24 hours of incubation in NA medium.

### 3.8 Identification of isolated bacteria by standard biochemical tests

The isolated bacteria were prepared by sub-culturing on nutrient agar plate incubating at $30^{o}$ C for 24 hours. Several biochemical tests were performed.

#### 3.8.1 Gram reaction

To determine gram positive or gram negative bacteria gram staining and reaction with 3% KOH were performed.

#### 3.8.1.1Gram staining

Gram stain was done on a clean slide, dried a thinly spreads bacterial film in air without heat. Then lightly flamed underside of the slide twice to fix the bacteria to the slide. Then the smear was flooded with Crystal violet solution for 60 seconds. It was washed with running tap water for a few seconds and excess water removed by air. Then the smear was flooded with Iodine solution (Lugol's Iodine) for 60 seconds and

then washed with running tap water for few seconds and excess water removed by air. After that the smear was decolorized with 95% Ethanol for 30 seconds and again washed with tap water and dried by air. Then the smear was counterstained with 0.5% Safranine for 10 seconds and washed briefly in tap water and excess water was removed by air. Finally it was examined under microscope at 40x oil emulsion objective.

**3.8.1.2. Reaction with 3% KOH**

On a sterile slide one loop bacterial colony of 24 hour old cultures was mixed with 1 drops of 3% KOH. It became gummy upon mixing with a loop (Suslow *et al.*1982).

**3.8.2 Oxidase test**

A portion of the test organism was picked up from the agar plate with a sterile wooden toothpick onto the filter paper soaked with oxidase reagent.

**3.8.3 Catalase test**

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

**3.8.4 Citrate utilization test**

A portion of the test organism was picked up from the agar plate with a sterile inoculating loop and streaked into Simmon's citrate agar slants and incubated at 30$^{o}$C for 24 hours.

**3.8.5 Gelatin liquefaction test**

One loop-full bacterial culture was inoculated with a sterile straight wire stabbed into the media and incubated at 30$^{o}$C for 24 hours.

**3.8.6 Pectolytic test**

Potato tubers were cut up into slices of about 5 mm thick, disinfected with 99% ethanol and made a nick in the centre of each slice. Then placed on moistened sterile filter paper in sterile petri dishes. Bacteria isolate were grown in nutrient broth media.

Approximately two loops of each bacterial isolate were uniformly spread onto the surface of the potato slices. One was disinfected for control. Development of rot on the slices was examined 24–48 hrs after incubation at 25°C. Observation was done for 5 days after inoculation. It was drown a inoculating loop across inoculated part to determine whether the slice had decayed beyond the point of inoculation.

### 3.9Treatments

$T_1$= Isolate 1 $T_5$= Isolate 7

$T_2$= Isolate 2 $T_6$= Isolate 10

$T_3$= Isolate 3 $T_7$=Control (without isolate)

$T_4$= Isolate 5

### 3.10 Determination the efficacy of antagonistic bacteria as seed treatment

Seeds were treated with bacterial suspension containing 10 cfu /ml. Seed were dipped in bacterial suspension for 2 hours. After that efficacy of antagonistic bacteria were done by different methods.

#### 3.10.1 Seed treatment with antagonistic bacteria and evaluation of their efficacy in controlling seed borne fungi and germination (Blotter method).

In this method seeds were treated following step (3.10). In this method 3 layers of blotter were soaked in sterilized water and placed at the bottom of the plastic petridish. 200 seeds were taken randomly and then 25 seeds were placed on the blotting paper in a petridish maintaining three replications. The petridish were incubated at 25±1$^{o}$C under 12/12 hrs light and darkness cycle for 7 days of incubation the seeds were observed for the presence of seed-borne pathogen of fungi of rice under stereo microscope following the key of Mathur and Kongsdal (2003).Germination (%) of seeds was recorded by this method.

#### 3.10.2 Effect of seed treatment with antagonistic bacteria on germination, normal and abnormal seedling and dead seed of rice (water agar test tube method).

This test was done by following methods of Khare *et al.* (1999). In these methods seeds were treated following 3.10 steps. In this technique, test tube slants were prepared by pouring 10 ml water agar in each test tube (2 cm in diameter and 15 cm in length) and then sterilized in autoclave for 15 minute under 15 lbs pressure at 121$^{o}$C. The water agar in the test tube was solidified at an angle of 60$^{o}$. One hundred seeds from each treatment was taken and one seed per test tube were placed on solidified water agar slant at the rate of one seed per tube. The seeded tubes were closed with cotton plugs and arranged in plastic racks. The tubes were then incubated at erect condition in an air cooled room (Tem 22$^{o}$C) under fluorescent day light tube. The cotton plugs were removed when the seedlings reached the rim of the test tube. Data were recorded on % seed germination, number of normal seedlings, number of abnormal seedlings and number of dead seeds.

### 3.10.3 Effect of seed treatment with antagonistic bacteria on seed infection germination , seedling growth and vigor index of rice (Rolled Paper Towel Method).

Rolled paper towel method was done following the method of Warham (1990). In this method, 200 treated seeds were randomly taken from each treatment with three replications and 50 seeds were placed uniformly between a pair of moist paper towels. The towels were rolled and the two ends were closed with rubber band as the moist could not remove easily. Then the rolled papers containing seeds were placed in an upright position for 7-10 days at room temperature under normal 12/12 light and darkness cycle. After incubation the shoot and root portions were blotted dry with fine tissue paper and fresh weight was taken before the materials could get desiccated. Length of shoot was measured from the base of the stem up to the growing point of the youngest leaf. Similarly, length of root was measured from the starting point of the root to the largest available lateral root apex. Germination%, % seed infection were also recorded. The seedling vigour index was calculated by using the formula as described by Baki and Anderson (1972):

**Vigor Index (VI) = (Mean of root length + Mean of Shoot length) × % Seed germination.**

### 3.11 Statistical analysis

The data of all tests were analyzed statistically for analysis of variance (ANOVA) using MSTAT-C computer program. The means were compared following Least Significant Difference (LSD) at 1% level of significance using same computer program. Whenever necessary the data were transformed before statistical analysis following appropriate method.

# CHAPTER 4

# RESULTS

### 4.1 Isolation and purification of fungi from rice seed

Five genera of fungi viz. *Fusarium* spp., *Bipolaris oryzae*., *Curvularia* spp., *Aspergillus flavus* and *Gliocladium* sp. (Plate 1-3) and a few unidentified isolates (data not shown) were isolated from rice seed using seed picking technique as described in materials and methods. Identification was done following the book "Illustrated Genera of Imperfect Fungi" (Barnett and Hunter, 1992).

## 4.2 Isolation and purification of bacteria from rhizopshere soil

Fifteen bacterial isolates were isolated from soil samples using dilution plate technique as described in materials and methods and purified on NA media. The bacterial isolates were named as Iso-1, Iso-2, Iso-3, Iso-4, Iso-5, Iso-6, Iso-7, Iso-8, Iso-9, Iso-10, Iso-11, Iso-12, Iso-13, Iso-14 and Iso-15 respectively.

**Fig 2.** Seed health test (blotter method)

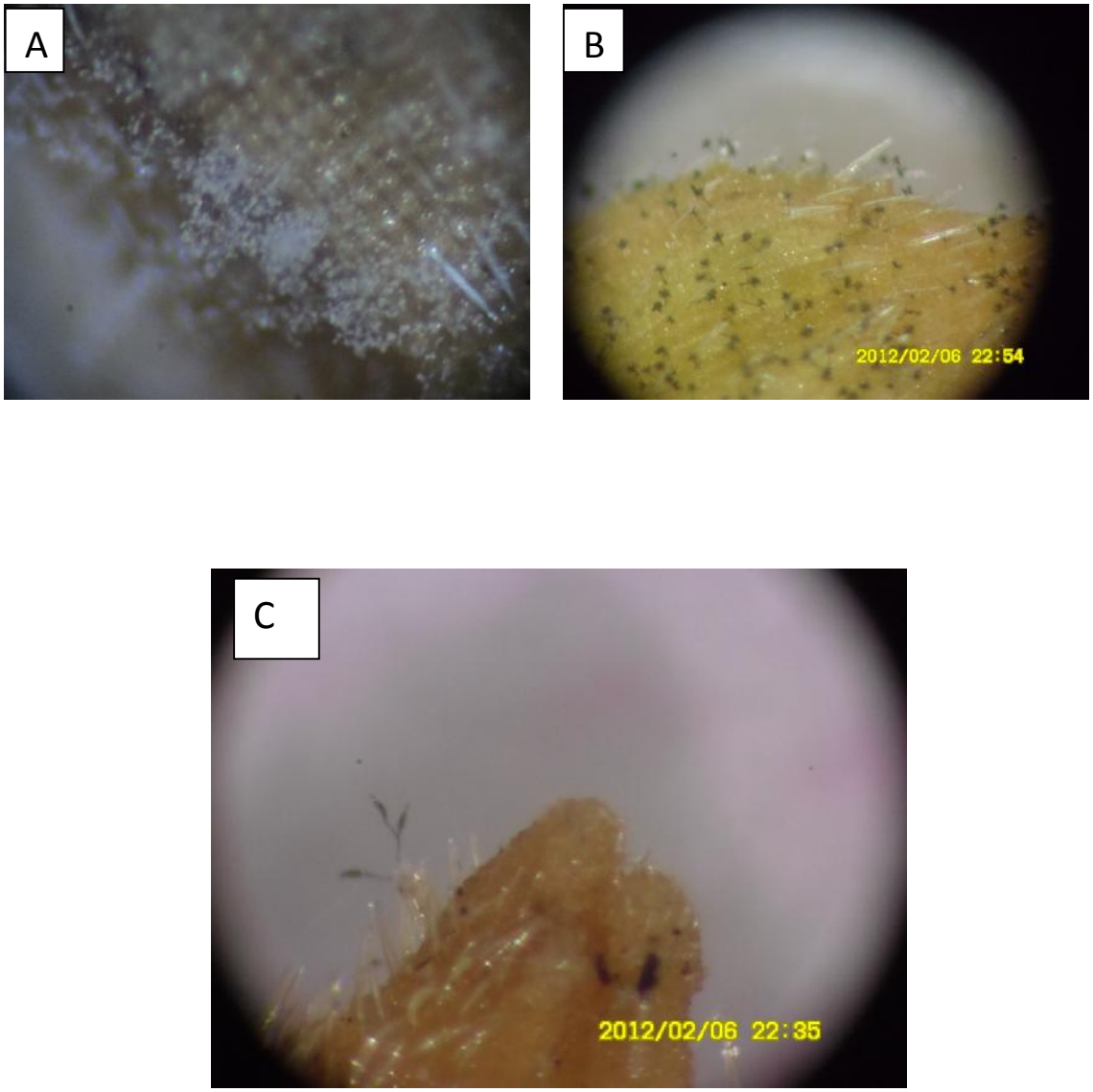



**Plate 1.** Incubated rice seed under stereo microscope showing growth of fungi over the surface of the seed.

Fig A. *Fusarium* spp.

Fig B. *Curvularia* sp

Fig C. *Bipolaris oryzae*

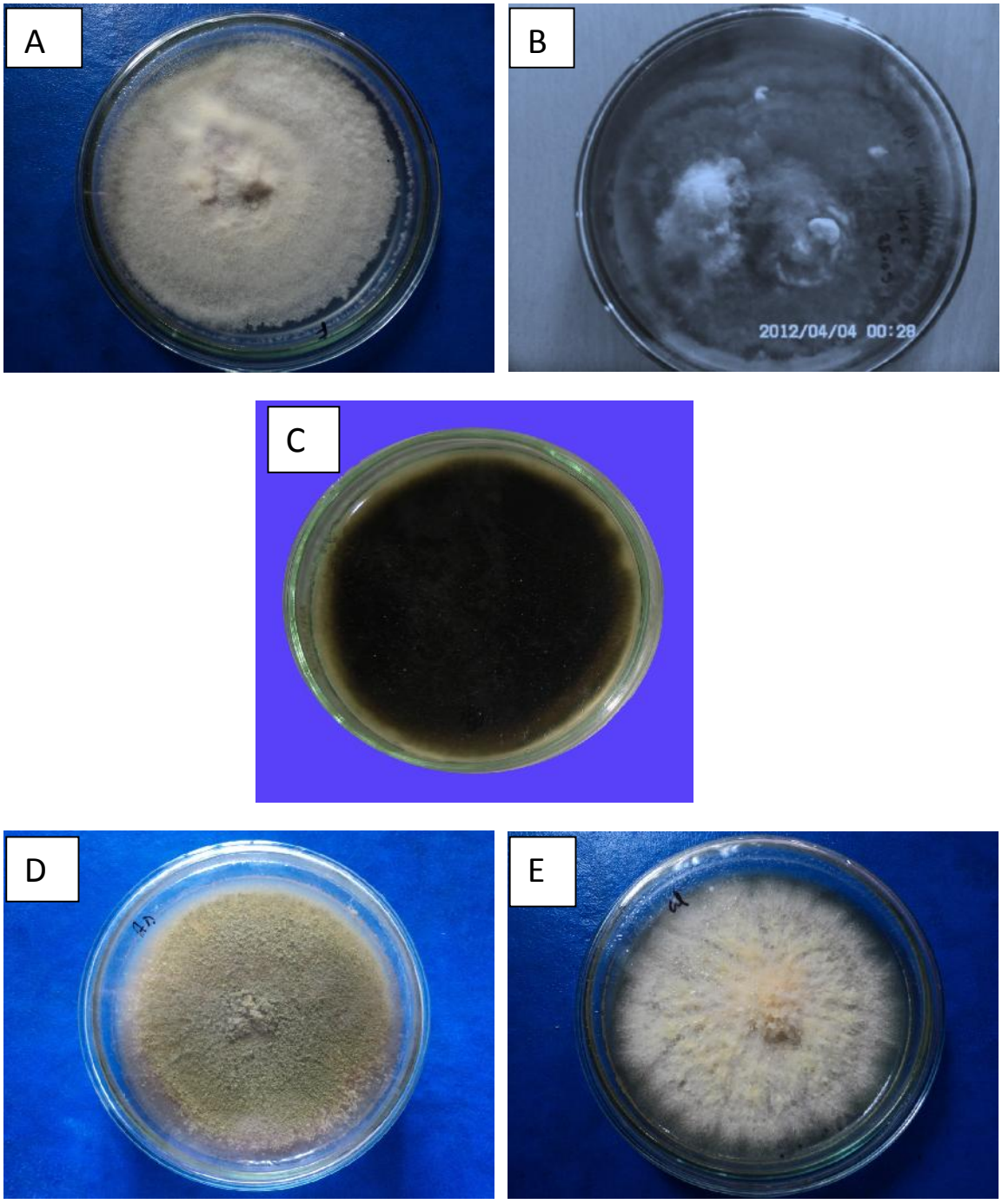



**Plate 2.** Pure culture of different fungi.

Fig A. *Fusarium sp*

Fig B. *Bipolaris oryzae*

Fig C. *Curvularia* spp.

Fig D. *Aspergillus flav*us

Fig E. *Gliocladium* sp

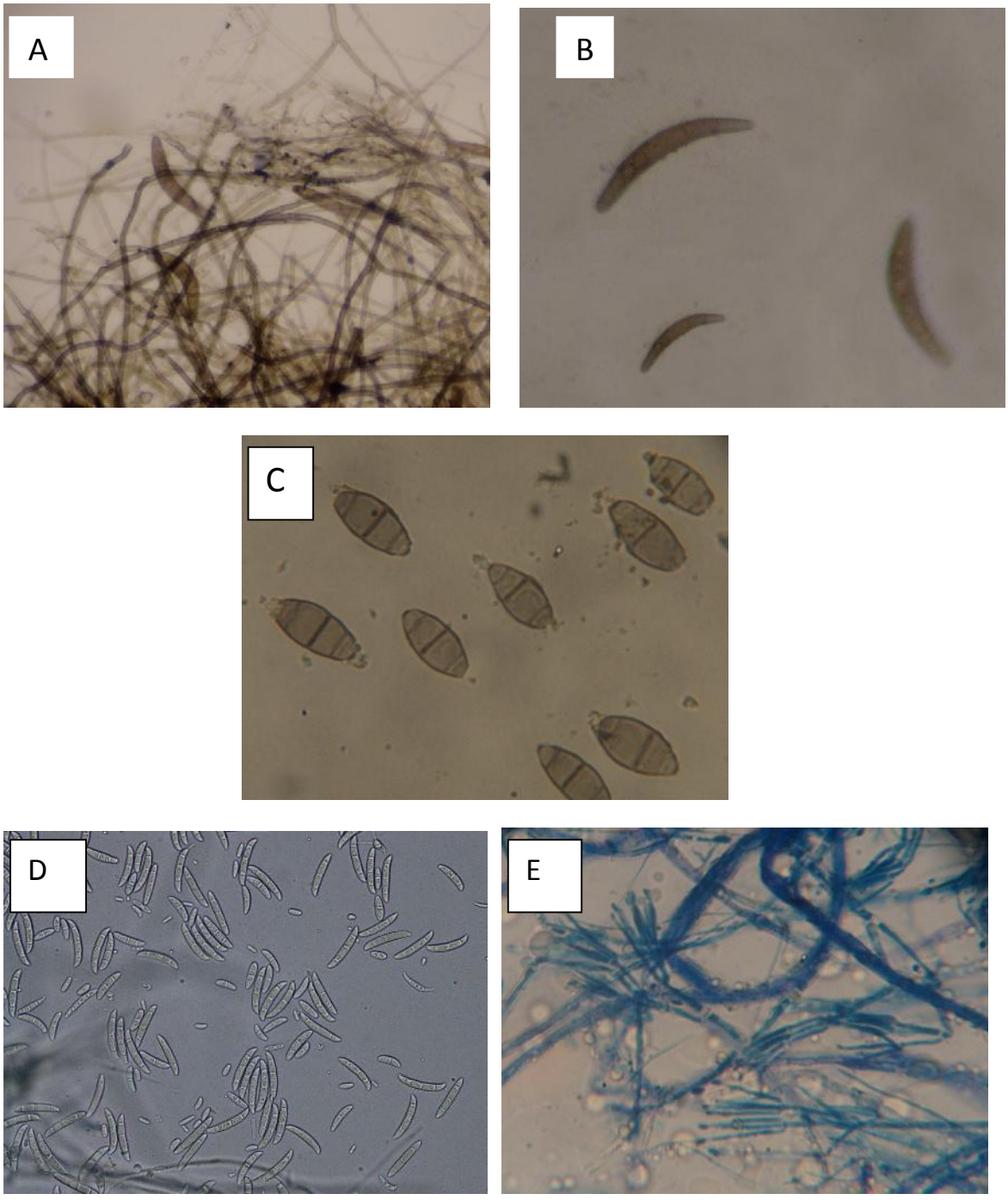



**Plate 3.** Rice seed borne fungal genera under compound microscope.

Fig A. Conidia and mycelium of *Bipolaris oryzae* at 10 ×

Fig B. Conidia of *Bipolaris oryzae* at 40 ×

Fig C. Conidia of *Curvularia sp* at 40 ×

Fig D. Macro and micro conidia of *Fusarium* spp.

Fig E. Conidia, conidiophore and mycelia of *Gliocladium* sp

### 4.3 Antibiosis of bacterial isolates with their antagonistic activity

Antibiosis of bacterial isolates with their antagonistic activity were studied and presented in Table 1. It was found that in dual culture methods Iso-1, Iso-2, Iso-3, Iso-5, Iso-7, Iso-10 isolates (Plate 4-7) were able to inhibit the growth of the isolated five fungi. In case of antagonism against *Fusarium* sp highest zone of inhibition (20.25%) observed in Isolate-2 followed by Isolate-1(16.25%) which was statistically identical with Isolate-10 (16.05%) and lowest inhibition zone observed in (2.38%) in Isolate-7 proceeded by Isolate-3 (4.50%) and Isolate-5 (9.75%).

In case of *Bipolaris oryzae* highest zone of inhibition (27.40%) observed in Isolate-2 which was statistically identical with Isolate-7 (26.30%) followed by Isolate-3(21.06%) , Isolare-1(15.09%) which was statistically identical with Isolate-5 (14.71) and lowest inhibition zone observed in (9.520%) in Isolate-10.

In case of *Curvularia* spp highest zone of inhibition (31.91%) observed in Isolate-2 followed by Isolate-1 (22.09%), Isolare-10 (17.37%) and Isolate-5 (12.47%) and lowest inhibition zone observed (4.05%) in Isolate-7 proceeded by Isolate-3 (9.41%).

In case of *Gliocladium* sp highest zone of inhibition (35.70%) observed in Isolate-2 followed by Isolate-1 (34.88%) , Isolare-3 (25.70%) and Isolate-7 (20.50%) and lowest inhibition zone observed (13.75%) in Isolat-7 proceeded by Isolate-5 (16.41%)

In case of *Aspergillus flavus* highest zone of inhibition (43.58%) observed in Isolate-1 followed by Isolate-2 (33.59%) , Isolare-3 (31.63%) which was statistically identical with Isolate-7 (30.15) and lowest inhibition zone observed in (19.05) % Isolate-10 proceeded by Isolate-5 (26.05%).

**Table 1. Anti-fungal activity of isolates of bacteria against isolated**

**seed borne fungi in dual culture method.**

| Isolate of Bacteria | % Inhibition of mycelium growth (mm) | | | | |
|---|---|---|---|---|---|
| | *Fusarium spp.* | *Bipolaris oryzae* | *Curvularia spp.* | *Gliocladium* | *Aspergillus flavus* |
| Iso-1 | 16.25 b | 15.09 c | 22.90 b | 34.88 b | 43.58 a |
| Iso-2 | 20.25 a | 27.40 a | 31.91 a | 35.70 a | 33.59 b |
| Iso-3 | 4.500 d | 21.06 b | 9.410 e | 25.70 c | 31.63 c |
| Iso-5 | 9.750 c | 14.71 c | 12.47 d | 16.41 e | 26.05 d |
| Iso-7 | 2.383 e | 26.30 a | 4.050 f | 20.50 d | 30.15 c |
| Iso-10 | 16.05 b | 9.520 d | 17.37 c | 13.75 f | 19.05 e |
| Control | 00.00 f | 00.00 e | 00.00 g | 00.00 g | 00.00 f |
| $LSD_{(0.05)}$ | 1.135 | 1.486 | 1.993 | 0.6684 | 1.486 |

In a column figure having same letter(s) do not differ significantly at 5% or 1% level by DMRT

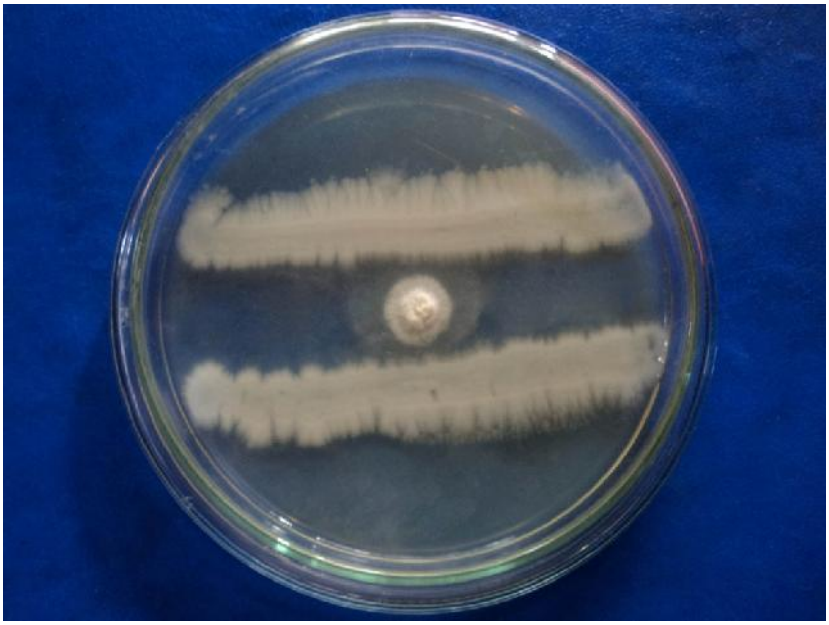


**Plate 4.** A. Growth of *Bipolaris oryzae* on NA medium without bacteria (Control) after 7 days.

B. Inhibition of mycelial growth of *Bipolaris oryzae* on NA medium in dual culture method after 7 days.

**Plate 5.** A. Growth of *Gliocladium* on NA medium without bacteria (Control) after 7 days

B. Inhibition of mycelial growth of *Gliocladium* on NA medium in dual culture method after 7 days.

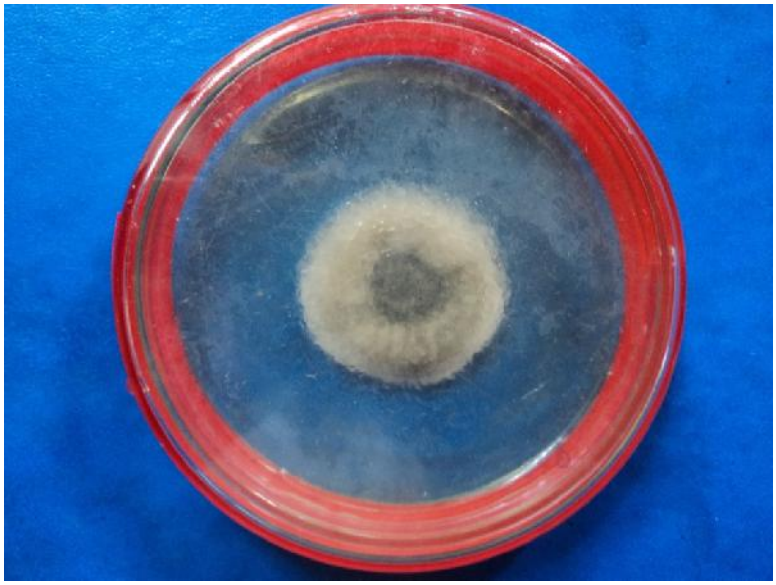
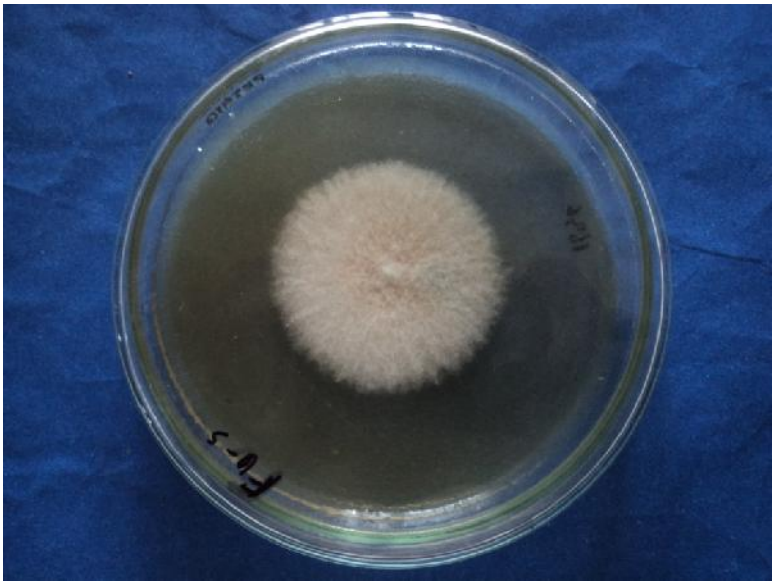



**Plate 6.** A. Growth of *Curvularia* spp. on NA medium without bacteria (Control) after 7 days.

B. Inhibition of mycelial growth of *Curvularia* spp. on NA medium in dual culture method after 7 days.



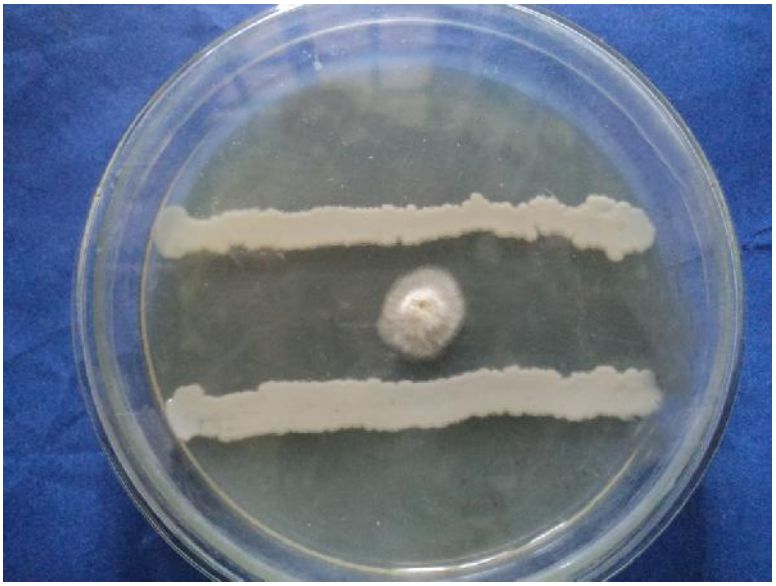


**Plate 7.** A. Growth of *Fusarium* spp. on NA medium without bacteria (Control) after 7days

B. Inhibition of mycelial growth of *Fusarium* spp. on NA medium in dual culture method after 7 days.

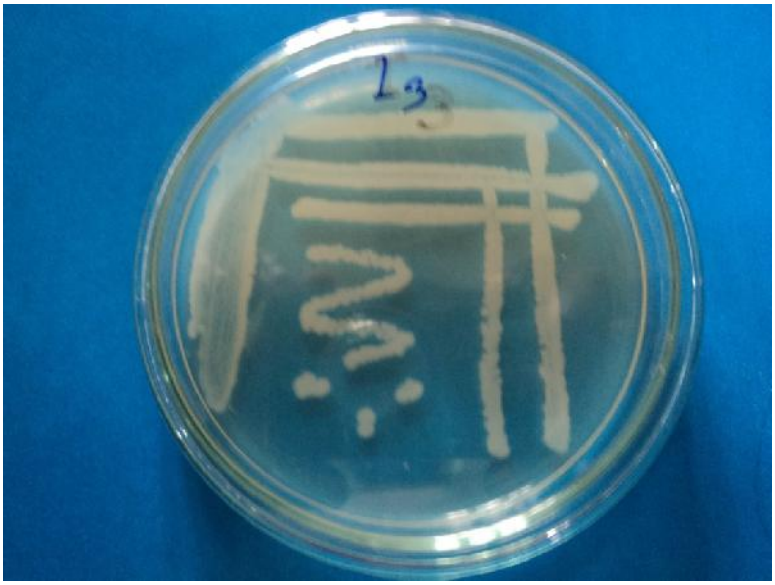
### 4.4 Identification of isolated bacteria by morphological test

Cultural and morphological characteristic of different isolates were studied. Morphological test was carried out on the basis of colony color on NA medium (Table 2, Plate 8) The all isolate except Iso-10 produced cream color colony and Iso-10 produce light yellow color on NA media after 24 hours of incubation.

Based on the antagonistic potential and other characteristics, six isolates were studied in detail for colony, color, growth type, and cell shape. It was evident from the observations that all the six isolates viz., Iso-1, Iso-2, Iso-3, Iso-5, Iso-7 and Iso-10 produced round shaped colonies and rod shaped cells (Table 2).

**Table 2. Cultural and morphological characteristic of different isolated antagonistic bacteria.**

| Isolates | Cell shape | Colony type | Colony color | Type of growth |
|---|---|---|---|---|
| Iso-1 | Rod | Round | Cream | Fast |
| Iso-2 | Rod | Round | Cream | Fast |
| Iso-3 | Rod | Round | Cream | Fast |
| Iso-5 | Rod | Round | Cream | Fast |
| Iso-7 | Rod | Round | Cream | Fast |
| Iso-10 | Rod | Round | Light yellow | Medium fast |



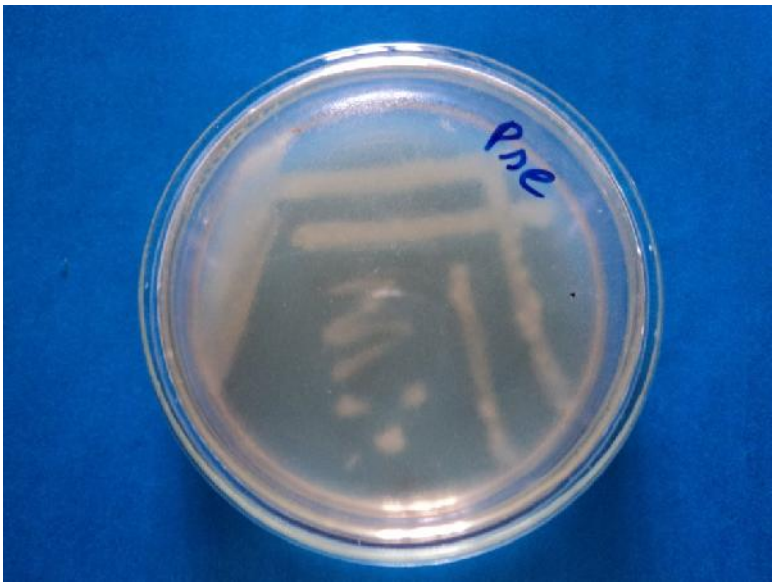


**Plate 8.** A. Iso-3 on NA medium (Cream color colony)

B. Iso-10 on NA medium (light yellow color colony)

## 4.5 Identification of isolated bacteria by standard biochemical tests

The biochemical tests performed for the identification of the effective native isolates and different test result presented in Table 3.

#### 4.5.1.1 Gram staining test

In Gram staining, all isolate yielded negative reaction and under microscope produced pink color, straight and curved rod (Plate 9) with no particular arrangement.

#### 4.5.1.2 Reaction with 3% KOH

All Isolates were treated with 3% KOH reagent as described in materials and methods. It was observed that all Isolate of Bacteria was able to raise viscid materials after several strokes by a loop few centimeters from the glass slides. This result indicates that all isolate were gram negative bacteria.

### 4.5.2 Oxidase test

The all isolates was tested in Oxidase where the filter paper soaked with oxidase reagent it gave positive reaction. After 5-10 seconds dark purple color (Plate 10) was developed in the filter paper.

### 4.5.3 Catalase test

The Isolates of Bacteria were tested with $H_2O_2$ (Hydrogen Peroxide) as described in materials methods. It was observed that all isolate of Bacteria were formed bubbles within a few seconds indicted a positive (Plate 10) test for catalase.

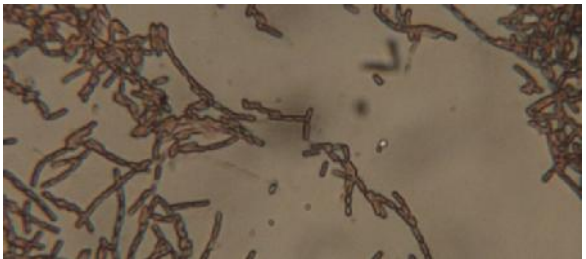
### 4.5.4 Citrate utilization test

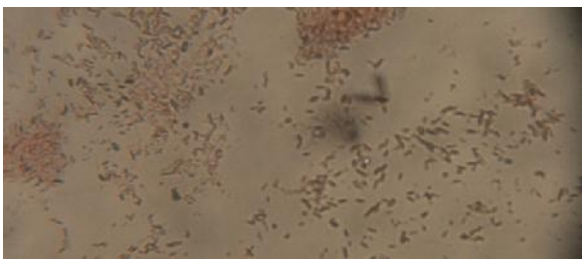
The Isolates of bacteria were tested Simmon's citrate agar slants as described in materials methods. After incubation at $30^oC$ for 24 hours only Isolate-10 showed (Plate 10) positive results by changing of the green bromothymol blue.

**Table 3. Morphological and biochemical characteristics of isolated**

**bacteria (antagonistic).**

| Biochemical Test | Isolate of Bacteria | | | | | |
|---|---|---|---|---|---|---|
| | Iso-1 | Iso-2 | Iso-3 | Iso-5 | Iso-7 | Iso-10 |
| Gram staining reaction | – | – | – | – | – | – |
| KOH solubility test | – | – | – | – | – | – |
| Oxidase test | + | + | + | + | + | + |
| Catalase test | + | + | + | + | + | + |
| Gelatin liquefaction test | + | + | + | + | + | + |
| Citrate utilization test | – | – | – | – | – | + |
| Pecteolytic test | – | – | – | – | – | – |





**A** **B**

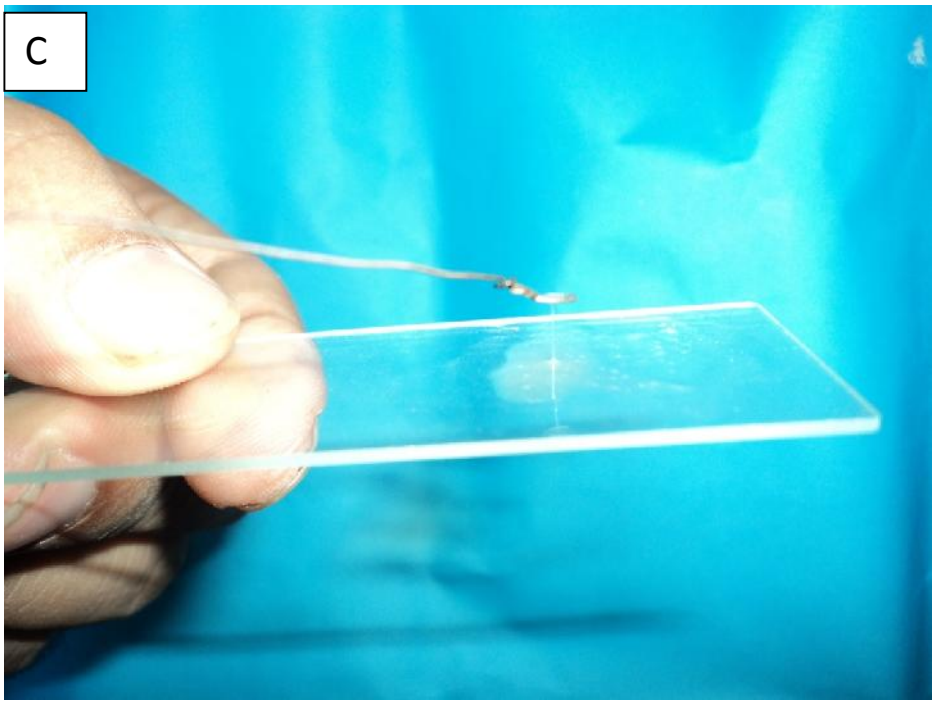
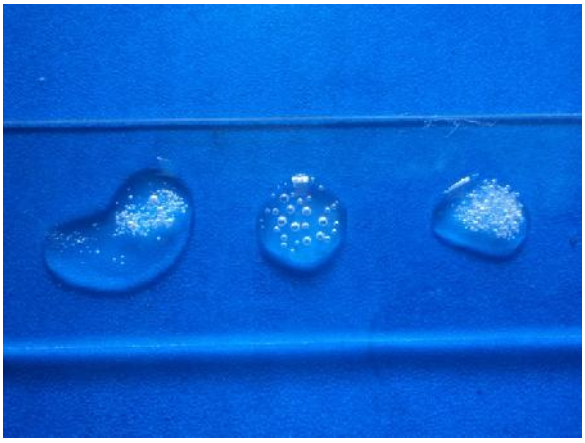



**Plate 9.** A. Gram staining of Iso-2 showing gram negative, pink color, chained rod shaped organism.

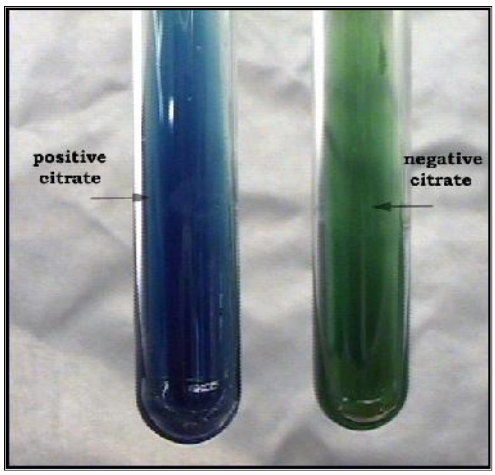
B. Iso-10 showing gram negative, pink color, single rod.

C. Gram reaction with 3% KOH test showing positive reaction.

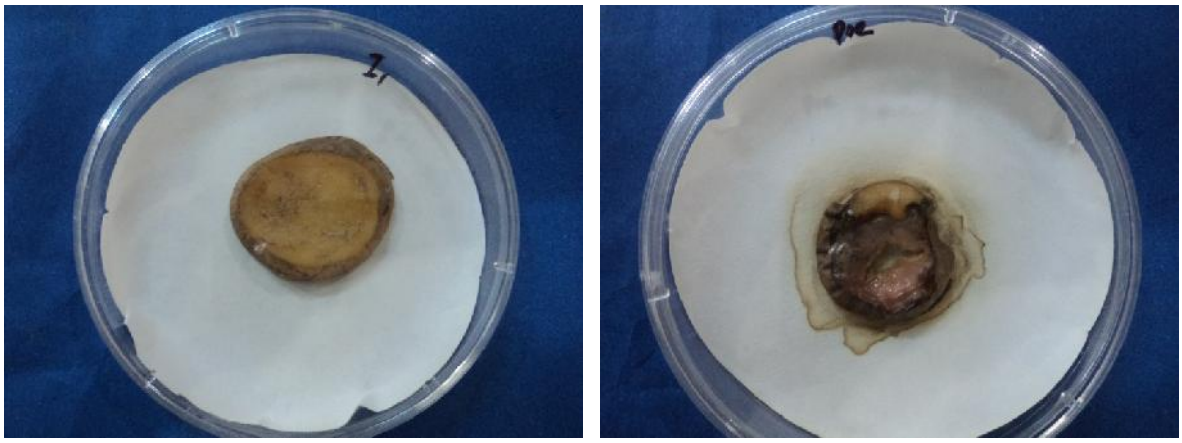
Oxidase test



Catalase test




Simmon's citrate agar test



Pectiolytic test

**Plate 10.** Different biochemical test.

### 4.5.5 Gelatin liquefaction test

All the six isolates showed positive result in Gelatin liquefaction test. All the isolates formed liquid culture in the presence of $4^{o}$C refrigerator. Thus, there was no variation among all the isolates in case of Gelatin liquefaction test (Table 3).

### 4.5.6 Pecteolytic test

In pecteolytic test, all the bacterial isolate showed negative result.

## 4.6. Effect of seed treatment with antagonistic bacteria and evolution their efficacy by different method

Different methods were used to evaluate the efficacy of antagonistic bacteria viz. blotter method, water agar test tube method, rolled paper towel method. Among them blotter method was best for evalution the efficacy of antagonistic bacteria.

### 4.6.1 Effect of seed treatment with antagonistic bacteria on the prevalence of seed borne fungi of rice (Blotter Method)

Germination varied from 91% to 98.33% and $T_1$, $T_2$, $T_3$, $T_4$ and $T_5$ gave statistically insignificant result, where highest value (98.33%) found in $T_1$ and $T_2$ treatment and lowest value in $T_7$ (control).

Significant variation among the treatment in respect of percent seed borne fungi was observed (Table 4). Rice seed yielded *Fusarium* spp., *Bipolaris oryzae, Curvularia* sp., *Gliocladium* sp. and *Aspergillus flavus.* It was observed that none of the treatments could completely inhibit the growth of seed borne *Fusarium* spp. $T_2$ (Isolate 2) and $T_4$ (Isolate 5) showed significant effect in reducing seed borne *Fusarium* spp., where $T_2$ (6.50%) comparatively gave the better result in reducing seed borne *Fusarium* spp., followed by $T_4$ (8.5%), $T_1$ (9.0%) and $T_6$ (10.50%) and $T_5$ (13.50%) and $T_6$ (16.50%). In $T_7$ (control) highest prevalence (21.5%) was recorded. The growth of *Bipolaris oryzae* was completely controlled in $T_1$, $T_2$

**Table 4. Effect of seed treatment with some antagonistic bacteria on prevalence of seed borne fungi of rice and germination (Blotter method).**

| Treatments | % Germination | Prevalence of seed borne fungi | | | | |
|---|---|---|---|---|---|---|
| | | *Bipolaris oryzae* | *Fusarium spp.* | *Curvularia spp.* | *Gliocladium* | *Aspergillus flavus* |
| $T_1$ | 98.00a | 0.00c | 9.00de | 1.50bcd | 0.00a | 0.00c |
| $T_2$ | 98.33a | 0.00c | 6.50e | 0.50de | 0.00a | 0.00c |
| $T_3$ | 97.00a | 0.50c | 13.50c | 2.50b | 0.00a | 0.00c |
| $T_4$ | 97.00a | 1.50b | 8.50de | 2.00bc | 0.00a | 0.50c |
| $T_5$ | 96.00a | 0.00c | 16.50b | 1.00cde | 0.00a | 1.50b |
| $T_6$ | 90.00b | 0.00c | 10.50d | 0.00e | 0.00a | 0.50c |
| $T_7$(Contol) | 91.00b | 6.25a | 21.50a | 4.750a | 0.25a | 3.50a |
| $LSD_{(0.05)}$ | 3.515 | 0.8113 | 2.665 | 1.140 | 0.3898 | 0.8457 |

In a column figure having same letter(s) do not differ significantly at 5% or 1% level by DMRT

$T_1$= Isolate 1

$T_2$= Isolate 2

$T_3$= Isolate 3

$T_4$= Isolate 5

$T_5$= Isolate 7

$T_6$= Isolate 10

$T_7$=Control (without isolate)

and $T_6$ treatments. But highest prevalence (6.25%) was observed in treatment $T_7$, followed by $T_4$ (1.50%) and $T_3$ (0.5%). The growth of *Curvularia* was completely controlled in $T_6$ and highest prevalence (4.75%) was observed on treatment $T_7$ (control) flowed by $T_3$, (2.50%), $T_4$ (2.00%), $T_1$ (1.50%), $T_2$ (0.50%). The growth of *Gliocladium* was completely controlled by all treatments except $T_7$ (0.25%). The growth of *Aspergillus flavus* was completely controlled in $T_1$, $T_2$ and $T_3$ and highest prevalence (3.50%) was observed in treatment $T_7$ followed by $T_5$ (1.50%).$T_4$, $T_6$ gave statistically similar result (0.5%).

### 4.6.2 Effect of seed treatment with antagonistic bacteria on germination, normal and abnormal seedling and dead seed of rice (water agar test tube method).

Effect of seed treatment with antagonistic bacteria on % germination, % normal seedling , % abnormal seedling dead seed % of rice were determined and the result were presented in table-4. The highest (96.67%) germination was recorded in treatment $T_2$ which was statistically insignificant with other treatment except $T_7$ (control). The lowest germination (89.00%) was recorded in Control.

The treatments showed variation from one another regarding percent normal seedling and the results for all the treatments ranged from 85.33-74%, where the maximum counts (85.33%) was found in $T_1$ (Isolate-1) and minimum (74.00%) in $T_7$ (Control).$T_1$, $T_2$ , $T_3$, $T_4$ and $T_5$ have insignificant effect in producing normal seedling.

In case of present abnormal seedling all treatment showed statistically non significant result. The ranged of abnormal seedling varied from 9.67-15.00%, where the highest (15.00%) percent was observed in $T_7$ (Control) followed by $T_6$ (14.67%) as well as $T_2$ (13%) and the lowest (9.67%) percent was found in $T_1$ (Isolate-1).

**Table 5. Effect of seed treatment with some antagonistic bacteria on germination, normal and abnormal seedling and dead seed of rice (Water agar test tube method).**

| Treatments | %Germination | %Normal Seedling | %Abnormal Seedling | %Dead Seed |
|---|---|---|---|---|
| $T_1$ | 95.00ab | 85.33a | 9.667c | 5.00bc |
| $T_2$ | 96.67a | 83.67a | 13.00ab | 3.33c |
| $T_3$ | 93.33b | 83.33a | 10.00c | 6.66b |
| $T_4$ | 94.00ab | 83.33a | 10.67bc | 6.00bc |
| $T_5$ | 96.00ab | 85.33a | 10.67bc | 4.00bc |
| $T_6$ | 93.67b | 79.00b | 14.67a | 6.33b |
| Control | 89.00c | 74.00c | 15.00a | 11.00a |
| $LSD_{(0.05)}$ | 2.486 | 2.546 | 2.424 | 2.486 |

In a column, figure having same letter(s) do not differ significantly at 5% or 1% level by DMRT.

$T_1$= Isolate 1

$T_2$= Isolate 2

$T_3$= Isolate 3

$T_4$= Isolate 5

$T_5$= Isolate 7

$T_6$= Isolate 10

$T_7$=Control (without isolate)

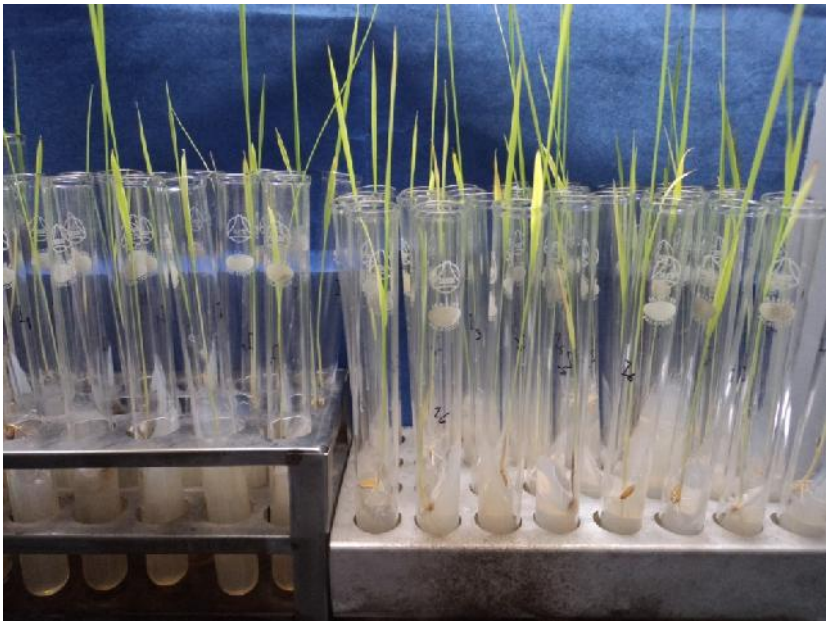


**Fig 3.** Seedling symptom test on water ager test tube method.

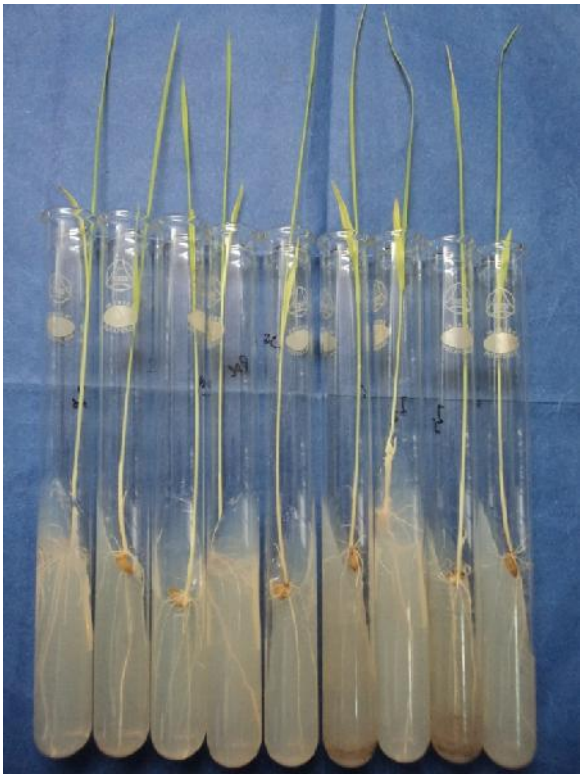


**Fig 4.** Normal seedling in water agar test tube.

**Fig 5.** Normal seedling.

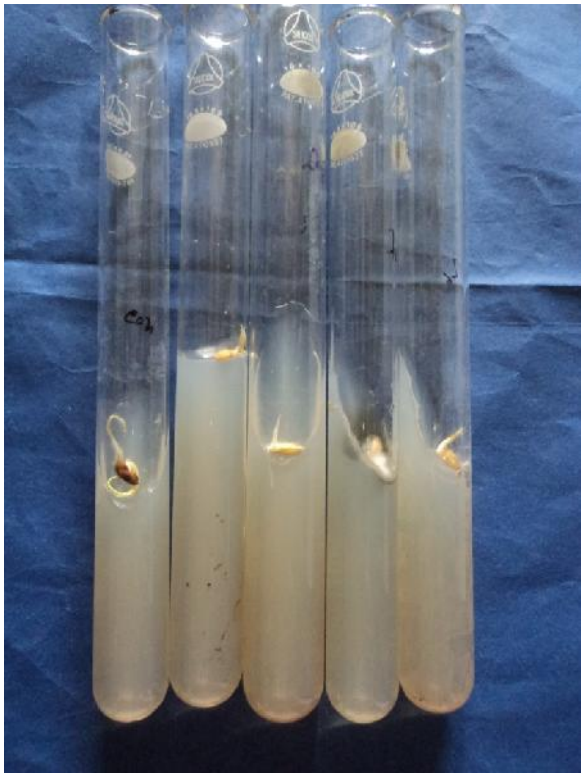


**Fig 6.** Dead seeds in water agar test tube.

Similar trend was found in case of percent dead seed. The results varied from 3.33-6.67%. The minimum (3.33%) dead seed were found in $T_2$ (Isolate-2) followed by $T_5$, $T_1$ and $T_4$, thus were statistically similar. The maximum counts (11%) were found in $T_7$ (Control).

### 4.6.3 Effect of seed treatment with some antagonistic bacteria on germination, root length, shoots length, vigor index and seedling infection of Rice (Rolled paper towel method).

Effect of seed treatment with some antagonistic bacteria on germination, root length, shoots length, vigor index and seedling infection were recorded and showed in Table 6 and plate. Significant variation was not found in germination. Germination varied form 96%-93.67%, where the highest germination (96%) was recorded in $T_2$ and $T_6$ (Isolate-2 and Isolate-7) and the lowest germination (93.67%) was observed in Control.

Different antagonist treated showed variation in respect of root length (cm), shoot length (cm), 100 seedling weight (g) and vigor index. In case of shoot length all treatment were statistically non significant except $T_7$ (control). Shoot length varied from 4.92 to 3.25 cm. The maximum shoot length (4.92cm) was recorded in $T_5$ and the minimum (3.25 cm) shoot length was found in $T_7$ (Control).

The root length varied from 5.36 to 10.50 cm. The maximum root length (10.50 cm) was recorded in $T_2$ followed by $T_6$ (8.97 cm), $T_1$ (8.83 cm) and $T_4$ (8.29 cm). And the minimum (5.37 cm) root length was found in $T_7$ (Control) which was statistically similar to $T_5$ as well as proceeded by $T_3$ (7.58 cm).

In case of vigor index significant variation were found among the treatments. Vigor index varied from 753 to1424. The higher vigor index (1424) obtained from $T_2$ (Isolate-2) treated seeds followed by $T_6$ (1319), $T_1$ (1230) and $T_4$ (1164) and lower vigor index (753) from $T_7$ (Control) proceeded by $T_5$ (989.6) and $T_3$ (753).

**Table 6. Effect of seed treatment with some antagonistic bacteria on germination, seedling growth, vigor index and seedling infection of rice (Rolled paper towel method).**

| Treatments | % Germination | Shoot Length (cm) | Root Length (cm) | Vigor Index | Seedling Weight (gm) | Seedling Infection (%) |
|---|---|---|---|---|---|---|
| $T_1$ | 94.00a | 4.25abc | 8.83b | 1230c | 6.80ab | 16.00b |
| $T_2$ | 96.00a | 4.33abc | 10.50a | 1424a | 7.50a | 12.33d |
| $T_3$ | 95.00a | 4.00c | 7.583b | 1101e | 6.50ab | 16.00b |
| $T_4$ | 94.00a | 4.10bc | 8.29b | 1164d | 7.00ab | 12.67d |
| $T_5$ | 95.00a | 4.92a | 5.50c | 989.6f | 7.00ab | 14.33c |
| $T_6$ | 96.00a | 4.78ab | 8.97b | 1319b | 6.100 b | 14.67bc |
| $T_7$ (Control) | 95.00a | 3.25d | 5.37c | 753.0g | 6.25ab | 19.00a |
| $LSD_{(0.05)}$ | 2.144 | 0.7116 | 0.4378 | 7.934 | 1.143 | 1.409 |
| CV % | 1.27 | 9.44 | 9.64 | 6.73 | 9.54 | 5.28 |

In a column, figure having same letter(s) do not differ significantly at 5% or 1% level by DMRT.

$T_1$= Isolate 1

$T_2$= Isolate 2

$T_3$= Isolate 3

$T_4$= Isolate 5

$T_5$= Isolate 7

$T_6$= Isolate 10

$T_7$=Control (without isolate)

**Fig 7.** Rolled paper towel method in laboratory.

**Fig 8.** Seedling raised on paper towel.

**Fig 9.** Fungi infested seedling on rolled paper towel method.

**Fig 10.** Un-germinated seed on paper towel method.

The higher value of vigor index refers to the high vigor of seeds. Increase in seedling vigor of seed samples occur mostly because of increase in 100-seed weight.

In case of seedling weight all treatment were statistically non significant. 100 seedling weights varied from 6.10 to7.50. The maximum seedling weight (7.50) was recorded in $T_2$ and the minimum in (6.10) Control.

Significant variation was found among the treatments in case of seedling infection and the results varied from 19 to12.33. The maximum (19%) seedling incidence was found in $T_7$ (Control) followed by $T_1$ and $T_3$ (16%), $T_6$ (14 67%). The minimum (12.33%) seedling incidence was recorded in $T_2$ (Isolate-2) preceded by $T_4$ (12.67) and $T_5$ (14.33).

# CHAPTER-5

# DISCUSSION

This study was conducted to determine the prevalence of seed borne fungi of rice and to isolate promising antagonist bacteria from rhizosphere soil to control of rice seed borne fungi. Seed health test in blotter method yielded five different fungi viz., *Curvularia* spp., *Bipolaris oryzae, Fusarium moniliforme, Gliocladium* sp, *Aspergillus flovus*. A considerable number of seed borne fungi belonging to the genera *Aspergillus, Bipolaris, Curvularia* and *Fusarium* have also been detected in rice seed by a number of researchers (Main and Fakir, 1989; Riaz and Ahmed, 1995; Gopalakrishnan *et al*. 2010; Tripathi and Dubey, 2004). Sharma *et al*. (1987) detected 10 fungal species of fungi from the rice seeds where *Fusarium moniliforme (Gibberella fujikuroi), Curvularia lunata (Cochliobolus lunata), Aspergillus flavus* and *Rhizopus* spp. were the most common. The association of fungi with rice seed has also been reported by quite a good number of workers (Esuruoso *et al*., 1975; Mia and Mathur, 1983; Khan *et al*., 2000; Hossain *et al*., 2000).

In the present study, fifteen Bacterial isolates were isolated from the soil sample using dilution plate technique and their biocontrol potency were tested against the seed borne fungi of rice. Among them six isolates showed antagonistic activity. *Burkholderia cepacia* produces pyrrolnitrin and other volatile compounds, which were able to inhibit a wide range of pathogen including *Fusarium* spp. (Chen and Wu, 1999; Larkin and Fravel, 1998). *Burkholderia cepacia* suppress many fungal plant pathogens such as *Fusarium, Pythium, Rhizoctonia, Cylindrocarpum, Botrytis and Alternaria* (Reddy, 1997). Adesina *et al*. (2007) isolated 1788 isolate from soils among them 322 isolate were found as antagonistic. Extracellular antifungal metabolites produced by Bacillus pumilus inhibited mycelial growth of many species of *Aspergillus, Penicillium* and *Fusarium.* (Munimbazi and Bullerman, 1998). The biocontrol mechanism to suppress fungal pathogens by *Pseudomonas* spp. normally involves the production of antibiotics and *P. fluorescens* has a gene cluster that produces a suite of antibiotics, including compounds such as 2,4-diacetylphloroglucinol (DAPG), phenazine, pyrrolnitrin, pyoluteorin and biosurfactant antibiotics (Angayarkanni *et al*., 2005).

Effect of seed treatment with antagonist bacteria on prevalence of seed borne fungi were done in blotter method. It was observed that all the isolated bacteria inhabited the growth of seed borne fungi viz. *Bipolaris oryzae*, *Fusarium* spp., *Aspergillus flavus., curvularia* spp., *Gliocladium*. This result of biological control of seed borne fungi is supported by Parveen and Gaffer (1991). Numerous antagonistic microorganism with the potential for bio-control of plant diseases has been reported and identified over the past few years ( Fereid *et al*., 1991). The possibility of controlling pathogenic fungi by antagonistic microorganism has been explored by various workers (Blakeman, 1985; Pandey *et al*, 1993 and Agarry, 2005). The mechanism of biological control of plant pathogens by antagonistic bacteria and fungi have been the subject of studies in the past two decades (Janisiewicz *et al* 2000). Most of the studies were on the control of root and soil borne plant pathogens and to lesser extent such as bacteria, fungi, actinomycetes, viruses and nematods that reduced the number of disease producing activities of the pathogens (Whipps and Lumsden, 2001). Mechanism of bio-control of root and soil borne pathogens are as a result of the direct action of predation or parasitism, induced resistance of the host plant and direct competition for space and limited resources (Janisiewicz *et al*., 2000). These mechanisms reduce the infection level and bring about the desired result, Linderman (2000). Many bio-control agents have been successfully used in laboratory and greenhouse experiments to control root/soil borne fungal and bacterial pathogens. Bacteria are important as antagonists of soil pathogens such as *Fusarium* spp and certain other pathogens that attack root rapidly through multiple infection. Example of such bacteria are *Pseudomonas fluorescens* (Georgakopoulos *et al.*, 2002), *P. putida* (Scher and Baker, 1982), *Pseudomonas* spp. (Whipps and Lumsden, 2001).

Effect of seed treatment with antagonistic bacteria on germination, normal seedling, abnormal seedling and dead seed were done in water ager test tube method. In this method significant variation were not recorded but showed better result than control regarding seed germination, abnormal seedling, normal seedling and dead seed. Effect of seed treatment with antagonistic bacteria on germination, shoot length, root length,

vigor index, 100 seed weight and seedling incidence were recorded in rolled paper towel method. In this method the highest germination (96) was recorded in $T_2$ and $T_6$ (Isolate-2 and Isolate-7) and the lowest germination (93.67%) was observed in $T_7$ (Control). higher vigor index (1424) obtained from $T_2$ (Isolate-2) treated seeds and lower vigor index (753) from $T_7$ (Control). No related works has been found related to the work from the previous study. Further works to be needed in this area.

# CHAPTER 6
# SUMMARY AND CONCLUSION

This study was carried out to identify bacterial antagonist from the soil rhizosphere and to control seed borne fungal pathogen of rice as we have no formulated bacterial

antagonist. Five seed borne fungi such as *Bipolaris oryzae, Fusarium* spp., *Curvularia* spp. *Aspergillus flav*us, *Gliocladium* sp were isolated from incubated rice seeds. Fifteen probable bacterial isolates were isolated from the rhizosphere soil. Isolation was carried out following dilution plate method.

*In-vitro* interaction between seed borne fungi and isolated bacteria revealed antagonistic effect of isolated bacteria over seed borne fungi. In dual culture method that Iso-1, Iso-2, Iso-3, Iso-5, Iso-7 and Iso-10 reduced the growth of *Bipolaris oryzae, Fusarium* spp., *Curvularia* spp., *Aspergillus flav*us, *Gliocladium* during this study.

In morphological test all isolate except Iso-10 produced cream color colony and Iso-10 produce light yellow color on NA medium after 24 hours of incubation. In Gram staining and KOH test all antagonistic bacteria gave gram negative reaction. Under compound microscope produced pink color, straight and curved rod with no particular arrangement. All the isolates were positive in catalase and oxidase test and negative in pectiolysis test. Iso-10 showed the positive result in utilization of citrate where other isolates showed negative in utilization of citrate. All the six isolates showed positive result in Gelatin liquefaction test.

In this study different methods were used to determine the efficacy of bacterial isolates. The effect of seed treatment with bacterial isolates was studied in blotter method. In blotter method it was found that all the isolated bacteria showed significant effect in reducing the prevalence of seed borne fungi of rice. Iso-2 completely inhibit the growth of *Bipolaris oryzae, Aspergillus flavus* and *Gliocladium* sp. Iso-2, Iso-5 and Iso-6 completely inhibit the growth of *Bipolaris oryzae , Gliocladium* sp. Iso-2 also found better performance than other in respect germination (97%).

The effect of seed treatment with bacterial isolates was studied in water agar test tube method. Significant variation was recorded in regarding germination, normal

seedling, abnormal seedling and dead seed. Highest % of normal seedling was recorded in $T_1$ &$T_5$ (85.33) treatment and lowest % normal seedling was recorded in $T_7$ (74%). And $T_2$ showed better result than other treatments.

The effect of seed treatment with bacterial isolates was studied in rolled paper towel method. Significant variation were recorded in regarding germination, vigor index and seedling incidence. The highest germination (96) was recorded in $T_2$ and $T_6$ (Isolate-2 and Isolate-6) and the lowest germination (93.67%) was observed in $T_7$ (Control). The maximum (19%) seedling incidence was found in $T_7$ (Control) and the minimum (12.33%) seedling incidence was recorded in $T_2$ (Isolate-2). higher vigor index (1424) obtained from $T_2$ (Isolate-2) treated seeds and lower vigor index (753) from $T_7$ (Control).

Base on the finding of the study the following conclusion may be drawn

Rhizosphere soil is known to harbor a wide range of microorganisms. A number of antagonistic microorganisms inhabited in rhizosphere soil. This microbial antagonistic play an imprtent role in controlling or suppressing the growth of other pathogen present in soil and seeds. Among them *Pseudomonas* spp., *Bacillus* sp, *Burkholderia* sp and *Rhizobium* sp have found potential role against pathogenic fungi.

In this study bacterial antagonistic were isolated and tested against the seed borne fungi of rice. Among the isolates iso-2 have potential effect in controlling seed borne fungi of rice. So this could be applied as seed treatment. Further investigation and characterization of these bacterial isolates may be undertaken to determine its efficacy against rice seed borne fungi.

# CHAPTER 7

# REFERENCES

Aboelnaga, H. I. G. (2006). *Bacillus subtilis* as a Biocontrol Agent for Controlling Sugar Beet. Damping-off Disease. *Egypt. Journal of Phytopathology*. **34**(1): 51-59.

Adawiah, B. I. (2008). Isolation, Characterization and Identification of Microorganisms from Soil Contaminated with Pesticide. Bachelor degree thesis submitted to the Faculty of Chemical & Natural Resources Engineering University Malaysia Pahang, Malaysia. 18-19 pp.

Adesina, M. F., Lembke, A., Costa, R., Speksnijder, A. and Smalla, K. (2007). Screening of bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum:* Site-dependent composition and diversity revealed. *Soil Biology and Biochemistry*. **39**(11):818-828.

Agarry, O. O. (2005). Amesment of Microorganisms isolates from cassava (Manihot esculenta erantz) products as biocontrol agents, ph. D thesis, Federal University of Technology, Akune.

Agarwal, P. C., Carmen, N. and Mathur, S. B. (1989). Seedborne diseases and seed health testing of rice. Danish Government Institute of Seed Pathology for Developing Countries, Copenhagen, Denmark. 106 pp.

Ahmed, N. and Ahmed, Q. A. (1963). Selection of antagonistic soil microbes against a few fungal pathogens of piper betle L. *Mycopathologia.* **21**(3- 4): 334-340.

Angayarkanni, T., Kamalakannan, A., Santhini, E. and Predeepa, D. (2005). Identification of biochemical markers for the selection of *Pseudomonas fluorescens* against *Pythium* spp. In: Asian conference on Emerging Trends in Plant-Miceobial Interactions. University of Madras, Chennai. 295-303 pp.

Anonymous, (2011). Food and Agricultural Organization of the United Nations Production: FAOSTAT Available: http:/ faostat. fao. org. / site / 566 / defaults. aspx # ancor.

Azadeh, B. F., Sariah, M. and Wong, M. Y. (2010). Characterization of *Burkholderia cepacia* genomover as a potential biocontrol agent of *Ganoderma boninense* in oil palm. *African Journal of Biotechnology*. **9**(24): 3542-3548.

Baki, A. A., and Anderson, J. D. (1972). Physiological and Biological deterioration of seeds. In: Seed Biology. Vol. **11**, Academic Press, New York. 283-315 pp.

Barnett, H. L., and Hunter, B. B. (1992). *Illustrated Genera of Imperfect Fungi* Minneapolis:Burgess publishing Co. 241pp.

BBS (Bangladesh Bureau of Statistics), (2010). Statistical year book of Bangladesh. Ministry of Planning, Government of People's Republic of Bangladesh.

Bennett J. W. and Klich, M. (2003). Mycotoxins, Clinical Microbiol. Rev., 16, 497.

Bevivino, A., Peggion, V., Chiarini, L., Tabacchioni, S., Cantale, C. and Dalmastri, C. (2005). Effect of *Fusarium Verticillioides* on Maize-root-associated *Burkholderia cenocepacia* populations. Research in Microbiology. **156**(10):974-983.

Bevivino, A., Sarrocco, S., Dalmastri, C., Tabacchioni, S., Cantale, C. and Chiarini, L. (1998). Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia:* effect of seed treatment on disease suppression and growth promotion of maize. FEMS Microbiology Ecology. **27**:225-237.

Bhuiyan, N. I., Paul, D. N. R., and Jabber, M. A. (2002). Feeding the extra millions by 2025-Challenges for Rice Research and Extension in Bangladesh. A key

note paper presented in the National Workshop on Rice Research and Extension-2002 held at BRRI, Gazipur, Bangladesh.

Blakeman, J. P. (1985). Ecological succession of leaf surface Microorganisms in relation to biological control. *Phytopathol. Soc*. **935**:6.

Blakeman, J. P. and Brodie, I. D. S. (1977). Competition for nutrients between epiphytic microorganisms and germination of spores of plant pathogens on beetroot leaves. *Physiological Plant Pathology*. **10**: 29 - 42.

Brion, K. D. and Genevieve, D. (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by Pseudomonas fluorescens biocontrol strains. *Applied and Environmental Microbiology*. **65**:2429-2438. (s).

Burgess, L. W., Summerell B. A., Bullock S., Gott K. P. and Backhouse D. (1994). Laboratory Manual for Fusarium Research. University of Sydney, 3rd Ed. 133pp.

Butt, A. R. Yaseen, S. I. and Javaid, A. (2011). SEED-BORNE MYCOFLORA OF STORED RICE GRAINS AND ITS CHEMICAL CONTROL. *The Journal of Animal & Plant Sciences*, **21**(2):193-196 pp.

Chen, T. W. and Wu, W. S. (1999). Biological Control of Carrot Black Rot. *J. Phytopathology*. **147**: 99-104.

Cook, R. J. (2000). Advances in plant health management in 20th century. *Annual Review of Phytopatholog*. **38**: 95-116.

Ellis, M. B. (1993). More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute. 507 pp.

Esuroso, O. F., Komolafe, C. O. and Aluko, O. (1975). Seed-borne fungi of rice (*Oryzae sativa* L*)* in Nigeria. *Seed Science and Technology*. **3**:661-666.

Fakir, G. A., Hossain, I., Ahmed, M. U., Asad-ud-Doullah, M. and Alam, M. (2002). Quality of farmers Boro and T. Aman rice seeds collected before sowing from Bogra, Rajshahi and Rangpur districts of Bangladesh. A pappresented in the review and planning meeting of the Rice Seed Health Improvement (SHIP), PETRRA project held on 17-18 April at BRRI, Gazipur, Bangladesh.

Fakir, G. A. (2000). An Annotated List of Seed-Borne Disease in Bangladesh. Seed Pathology Centre, Dept. of Plant Pathology, Bangladesh Agricultural field. *Bangladesh Journal of Plant pathology*. **17** (1-2): 59-62.

Fakir, G. A. (1982). An annotated list of seed borne diseases in Bangladesh. Agricultural Information Service, Dhaka, Bangladesh 15p.

Fereid, J. H. S., Matthen F. N. and Thomas A. C. (1991). Biological controls of Eutypatala on gravepine by an antagonistic strain of Bacillus subtilis. *Phytopathology*. **81**:283-287

Filippi, C., Bagnoli, G., Treggi, G. and Picci, G. (2008). Antagonistic effects of soil bacteria on *Fusarium oxysporum* Schleeht f.sp. *dianthii (*Prill and Del.) Snyd and Hans. I.*In vitro* experiments and preliminary assays on Carnation (*Dianthus caryophyllus* L.). *Plant and Soil*. **80**(1):119-125.

Ganeshan, G. A. and Kumar, A. M. (2005). Pseudomonas fluorescens, a potential bacterial antagonist to control plant diseases. *Journal of Plant Infection*. **1**:123-131(s).

Georgakopoulos, D. G., Fiddaman, P., Leifert, C. and Malathrakis, N. E. (2002). Biological control of cucumber sugar beet damping off cauesed by *Pythium*

*ultimum* with bacterial and fungal antagonists . *Journal of Applied Microbiology*. **92**:1078-86.

Gilreath, P. (2002). *Manatee Vegetable Newsletter,* University of Florida, Manatee County Extension Service.

Gopalakrishnan, C. and Valluvaparidasan, V. (2007). Survival of *Sarocladium oryzae* in rice seeds as health management is a prerequisite for successful rice affected by storage periods. International Rice cultivation Research News Letter, **32**(1): 20.

Gopalakrishnan, G. Kamalakannan, A. and Valluvaparidasan. V. (2010). Survey of Seed-Borne Fungi Associated with Rice Seeds in Tamil Nadu, India. *Libyan Agriculture Research Center Journal Inter nation* **1** (5): 307-309.

Goud, M. J. and Muralikrishnan, V. (2009). Biological control of three phytopathogenic fungi by Pseudomonas fluorescens isolated from rhizosphere. *The Internet Journal of Microbiology*. **7**(2):62-64.

Guerrero, F. C., Mathur, S. B. and Neergaard, P. (1972). Seed health testing of rice. Seed borne fungi associated with abnormal seedlings of rice. *Proc. Int. Seed Test. Assoc*. **37**: 985-997.

Hebbar, K. P., Martel, M. H. and Heulin, T. (1997). Suppression of pre-and postemergence damping-off in corn by Burkholderia cepacia *European Journal of Plant Pathology*. **104**(1):29-36.

Hossain, M. A. (2006). Isolation and Analysis of Plasmid Profile from *Ralstonia solanacearum* and *Pseudomonas* spp. and its reaction to antibiotics. MS thesis, Dept. of Plant Pathology. Bangladesh Agricultural University, Mymensingh, Bangladesh. 24-47 pp.

Hossain, M. A., Huelma, C. C., Gonzales, P. G. and Mew, T. W. (2000). Effect of seed treatment on seed borne mycoflora and germination of rice seed collected from farmers of different locations in the Philippines. *Bangladesh Journal of Plant Pathology*. **16** (1 & 2): 13-15.

Imolehin, E. D. (1987). The rice seed multiplication centres in relation to seed- borne pathogens of rice: A case study of Ondo State Rice Multiplication Centers. *Nigerian Journal of Plant Protection*. **11**:37-42.

Imolehin, E. D. (1983). Rice seed-borne fungi and their effect on seed germination. *Plant Disease*. **67**: 1334-1336.

International Seed Testing Association (I.S.T.A) (1976). International Rules for Seed Testing. *Seed Science and Technology* **4**: 51-177.

IRRI (International Rice Research Institute), (2006). World Rice Statistics. Intl. Rice Res. Inst. http://www.irri.org/science/wrs.

Islam, T. (2009). Population dynamics of *phomopsis vexans, sclerotium rolfsii, fusarium oxysporum* f. sp *lycopersici* and *trichoderma* in the soil of eggplant field. An M.S. thesis submitted to the Dept. of Plant Pathology, Bangladesh Agricultural University, Mymensingh. 48-57 pp.

Islam, M. S., Jahan, Q. S. A., Bunnarith, K., Viangkum, S. and Merca, S. D. (2000). Evaluation of seed health of some rice varieties under different conditions. *Bot. Bull. Acad. Sin*. **41**: 293-297.

ISTA, (1996). International Rules for Seed Testing. International Seed Testing Association. *Seed Sci. Tech*. **24** (supplement): 29-72.

Janisiewicz, W. J., Tworkaski, T. J. and Sharer, C. (2000). Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients, *Phytopathology*. **90** (IV):1196-2000.

Javaid, M. S., Wahid, A., Idrees. A., Gill M. A. and Saleem, A. (2002). Seed mycoflora studies in rice. *Pakistan J. Phytopathol*. **14**: 132-134.

Jayaswal, R. K., Fernandez, M., Upadhyay, R. S., Visintin, L., Kurz, M., Webb, M. and Rinehart, K. (1993). Antagonism of *Pseudomonas cepacia* against phytopathogenic fungi. *Current Microbiology*. **26** (1)17- 22.

Jha, G. and Anjaiah, V. (2007). Metabolites of rhizobacteria antagonistic towards fungal plant pathogens. *Annals of Microbiology*. **57**(1):127-130.

Kazempour, M. N. and Anvary, M. (2008). Isolation of *Fusarium fujikuroi* antagonistic bacteria and cloning of its phenazine carboxylic acid genes. *African Journal of Biotechnology*. **8**(23):6506-6510.

Khan, M. A., Ahmad, K. and Ahmad, J. (1999). Effect of potassium levels on the yield of sunflower (*Helianthus annuus* L.). *Pakistan J. Biol. Sci.* **2**(2): 402-403.

Khan, T. Z., Gill, M. A. and Khan M. G. (2000). Seed borne fungi of rice from central Punjab and their control. *Pakistan J. Phytopathol*. **12**: 12-14.

Khare, M. N. (1999). Seed health care in seed quality control. *Indian Phytopathology*. **52** (3): 305.

Kim, H. S., Park, J. S W., Choi, K. H., Choi, G. L., Ban, S. J., Lee, C. H. and Kim, C. S. (2003). Isolation and characterization of *Bacillus* strains for biological control. *J. Microbiol.,* **41**(3), 196.

Larkin, R. P. and Fravel, D. R. (1998). Efficacy of Various Fungal and Bacterial Biocontrol Organisms for Control of Fusarium Wilt of Tomato. *Plant Disease* **82**: 1022-1028.

Li, J. G., Jiang, Z. Q., Xu, L. P., Sun, F. F., Guo, J. H. (2008). Characterization of chitinase secreted by *Bacillus cereus* strain CH2 and evaluation of its efficacy against *Verticillium* wilt of eggplant. *Biocontrol*. **53**:931-944.

Mao, W., Lewis, J. A., Lumsden, R. D. and Hebbar, K. P. (1998). Biocontrol of selected soilborne diseases of tomato and pepper plants. *Corp Protection*. **17**(6):535-542.

Marthur, S. B. and Neergaard, P. (1970). Seed-borne fungi of rice in Phillipines, India, Portugal and Egypt:Investigation on *Trichoconis padwikii*. Proc.of First Int. Symposium on Plant Pathology, New Dehli 69- 81pp.

Matar, S. M., El-Kazzaz, S. A., Wagih, E. E., El-Diwany, A. l. and Moustafa, H. E. (2009). Bioprocessing and scaling -up cultivation of *Bacillus subtilis* as a potential antagonist to certain plant pathogenic fungi, III. *Biotechnology*. **8**:138-143.

Mathur and Kongsdal (2003). To evaluate the transmission of fungi from seed to seedling. *J. Phytophathol*. **150**:209-212.

Mew, T. W., Cottyn, B., Pamplona, R., Barrios, H., Li, X., Chen, Z., Lu, F., Arunyanart, P., Nilpanit, N., Rasamee, D., Kim, P. V. and Du, P. V. (2003). Applying rice seed-associated antagonistic bacteria to manage rice sheath blight in developing countries. *Plant Dis*.47 49 pp.

Mew, T. W. and Gonzales, (2002). A hand book of rice seed-borne fungi. IRRI Science Publishers. 83 pp.

Mia, M. A. T. and Mathur, S. B. (1983). Study on seed mycoflora of rice in Bangladesh. *Seed Research*. **11** (2): 254-257.

Mia, M. A. T., Rahman, M., Pearce, D. and Holderness, M. (2002). Effect of seed field. *Bangladesh Journal of Plant pathology*. **17** (1-2): 59-62.

Mian, I. H. and Fakir, G. A. (1989). Fungi, Moisture Content and Germinability of Rough Rice Grains during Storage. *Seed Res*. **17**: 169-173.

Mishra, A. K. and Dharam, V. I. R. (1992). Field evaluation of fungicides against fungi causing discolouration of paddy seeds. *Indian Phytopathology*. **45**: 49- 54.

Moita, C., Feio, S. S., Nunes, L. Curto, M. J. M. and Roseiro, J. C. (2005). Optimization of physical factors on the production of active metabolites by *Bacillus subtilis* 355 against wood surface contaminant fungi, International Biodeterioration & Biodegradation. 55 and 261 pp.

Munimbazi, C. and Bullerman L. B. (1998). Isolation and partial characterization on antifungal metabolites of *Bacillus pumilus, J. Appl. Microbiol*. **84**: 959 .

Neergaard, P. (1977). Seed Pathology. The Macmillan Press Ltd., London. 1187 p.

Neergaard, P., Lambat, A. K. and Marthur, S. B. (1970). Seed Health Testing of Rice.111.Testing procedures for detection of *Pyricularia oryzae*. Proc. Int. Seed Testing Association. **35**:157-163.

Nourozian J., Etebarian, H. R. and Khodakaramian, G. (2006). Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria, Nutraceutical and Functional Food. 28- 29 pp.

Odebunmi and Osikanlu, Y. K. (1989). Seed-borne pathogens of rice. Survival underexperimental and field conditions. *Nigeria Journal of Plant Protection.* **11**:1-8.

Omar, I., O'Neill, T. M. and Rossall, S. (2006). Biological control of fusarium crown and root rot of tomato with antagonistic bacteria and integrated control when combined with the fungicides carbendazim. *Plant Pathology*. **55**:92-99.

Ou, S. H. (1985). Rice diseases. CAB International Mycological Institute,Kew, Surrey, U.K. 39 p.

Palumbo, J. D., O'Keeffe, T. L., Kattan, A., Abbas, H. K., and Johnson, B. J (2010). Inhibition of *Aspergillus flavus* in Soil by Antagonistic *Pseudomonas* Strains Reduces the potential for Airborne Spore Dispersal. *The American Phytopathological Society*. **100**(6):532-538.

Pandey, R. R., Arond, D. K. and Dubey, R. C. (1993). Antagonistic interaction between fungal pathogens and phytoplane fungy of guava micopathology; **124**:31-39.

Parveen, S. and Gaffar, A. (1991). Effect of microbial antagonistic in the control of root rot of tomato. *Pakistan J. Botany*. **23** (2):179-182.

Powell, K. A., Faull, J. L. and Renwiek, A. (1990). The commercial and regulatory challenge, In: Biological control of soil-borne plant pathogens. Edited by Hornby D. C. A. B. International wallingford united kingdom. 445-64 pp.

Radha jeyalakshmi, R. (1998). Biological control of seed-borne diseases of rice. M.Sc. (Ag.) Thesis, Tamil Nadu Agricultural University, Coimbatore, developed from discolored seeds might serve as India.

Radheshyam, K., Jayaswal, M. A., Fernandez, and Schroeder, R. G. (1990). Isolation an characterization of a Pseudomonas strain that restrict growth of various phytopathogenic fungi. *Appl. Environ. Microbiol*. **56**(4): 1053.

Ramanathan, A., Shanmugam, V., Raghuchander, T. and Samiyappan, R. (2002). Induction of systemic resistance in ragi against blast disease by *Pseudomonas flourescens*. *Ann. P C Prot. Soc*. **10**: 313-318.

Ramarathnam, R., Bo, S., Chen, Y., Fernando, W. G. D., Xuewen, G. and Kievit, T. D. (2007). Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic tofungal pathogens of canola and wheat. *Canadian Journal of Microbiology*. **53**(7): 901-911.

Reddy, M. (1997). Status on commercial development of *Burkholderia cepacia* for biological control of fungal pathogens and growth enhancement of conifer seedling for a global market. Washington : US forest service general technical report PNW; Report no. **389**:235-244.

Riaz, M. and Ahmed, Z. (1995). Seed-borne fungi of rice collected from Pakistan. *Plant Genetic Resources Newsletter* **103**: 39-40.

Scher, F. M. and Baker, R. (1982). Effect of pseudomonas putida and synthetic iron chelaton on induction of soil suppresiveness to *Fusarium* wilt pathogens [compelition for iron as abiological control mechanism] *Phytopathology*. **72**(12):1567-73.

Selvakumar, G., Joshi, P., Nazim, S., Mishra P. K., Kundu S., and Gupta, H. S. (2008). *Exiguobacterium acetylicum* strain IP (MTCC 8707) a novel bacterial antagonist from the North Western Indian Himalays. *World Journal of Microbiology and Biotechnology*. **25**(1): 131-137.

Sharma, H. L., Randhawa, H. S., Kapur, A. and Singh, S. (1987). Seed Discoloration in Rice. Seed Research Associated wi Production Unit, **24**(1): 37-41.

Siddiqui S., Siddiqui, Z. A. and Ahmad, I. (2005). Evaluation of fluorescent *Pseudomonas* and *Bacillus* isolates for the biocontrol of a wilt complex of pigeonpea, *World J. Microbiol. & Biotechnol*. 21and 729 pp.

Srivastava, R. (2009). Antifungal Activity of Pseudomonas fluorescens against diffrent plant Pathogenic Fungi. *The Internet Journal of Icrobiology*. **7**(2).

Suslow, T. V., Schroth, M. N., and Isaka, M. (1982). Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. *Phytopathology*. **72**:917-918.

Tripathi, P. and Dubey, N. K. (2004). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*. **32**:235-245.

Wahid, A., Javaid, M. S., Idrees, M. S. and Gill, M. A. (2001). Studies on the association and effect of *Fusarium solani* on rice seeds in Punjab, Pakistan. In: Proc. 3rd National Conf. of Plant Pathology, Oct. 1-3, 2001. NARC Islamabad. 70-72 pp.

Wahid, A., Saleem, M., Khan, M. U., Tariq, H.and Saleem, A. (1993). Seed-borne mycoflora of rice. *J. Agric. Res*. **31**: 95-100.

Wang, Y., Guo, L.D. and Hyde, K.D. (2009). Taxonomic placement of sterile morphotypes of endophytic fungi from Pinus tabulaeformis (Pinaceae) in northeast China based on rDNA sequence. Fungal Diversity. **20**:235-260.

Warham, E. J. (1990). Effect of *Tilletia indica* infection on viability, germination and vigor of Rice Seed. *Plant Disease*. **74**: 130-135.

Whipps, J. M. and Lumsden, R. D. (2001). Commercial use of fungi as plant disease biological control agents status and prospects. In: Funji as biocontrol agents progress, problems and potential Eds. Butt. T. M. Jackson, C. Magan N. Wallingford, UK :CABI publishing. 9-22 pp.

Xie, G., Soad, A., Swings, J., and Mew, T. W. (2003). Diversity of Gram negative bacteria antagonistic against major pathogens of rice from rice seed in the tropic environment. *Journal of Zhejiang University Science*. **A**(4):463-468.

# Appendix 1

**Composition of Media:**

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

**1. Nutrient Agar (NA)**

| **Ingredients** | **g/L** |
|---|---|
| Beef extract | 3.0 |
| Peptone | 5.0 |
| Sodium chloride | 5.0 |
| Agar | 20.0 |

**2. Potato Dextrose Agar (PDA)**

| **Ingredients** | **g/L** |
|---|---|
| Peeled Potato | 200g |
| Dextrose | 20g |
| Agar | 17g |

| | |
|---|---|
| Water | 1000ml |

**3. Motility Indole Urease agar (MIU)**

| | |
|---|---|
| Peptone | 30.0 gm |
| NaCl | 5.0 gm |
| Urea | 20.0 gm |
| Monopotassium Phosphate | 2.0gm |
| Phenol red | 0.005 gm |
| Agar | 4.0 gm |
| Distilled water | 1000ml |
| pH | 7.0 gm |

All ingredients except urea were dissolved in 900ml distilled water sterilized at 121$^{o}$C under 15lbs/in pressure. Urea solution 20% was filter - sterilized using mili-pore filter and was added to the autoclaved medium.

**4. Simmon's citrate agar**

| | |
|---|---|
| Magnesium Sulfate | 0.2 gm |
| Sodium citrate | 2.0 gm |
| NaCl | 5.0 gm |
| Dipotassium Phosphate | 1.0 gm |
| Monopotassium Phosphate | 1.0 gm |
| Bromothymol Blue | 0.08 gm |
| Distilled Water | 1000ml |
| Agar | 20.0 gm |

Sterilized at 121oC under 15lbs/in pressure for 15 minutes.

# Apendix-2

**Preparation of Reagents**

**1. Gram Stain Solution**

a. Stock Crystal violet

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

b. Stock oxalate

| | |
|---|---|
| Ammonium Oxalate | 1gm |
| Distilled Water | 1000ml |

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

c. Lugol's Iodine Solution

| | |
|---|---|
| Iodine Crystal | 1gm |
| Potassium Iodide | 2gm |

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

| | |
|---|---|
| d. Ethyl alcohol (≥70%): Stored in $-20^0$c | 250ml |
| e. Acctone | 250ml |

f. Counterstain

| | |
|---|---|
| Safranine | 2.5ml |
| Ethyl alcohol (95%) | 100ml |

2. Phenol-red solution

0.2% aqueous solution of phenol red.

3. STE buffer:

| | | |
|---|---|---|
| Tris-Hcl | 10mM (pH-8.0) | 60.55gm/500ml |
| EDTA | 1mM (pH-.8.0) | 18.612gm/500ml |
| NaCI | 100mM | 1oom |

Preparation : (100X stock solution)

Tris-HcI +EDTA +NaCI
↓
Add 300ml distilled water
↓
Stirring and adjust pH to 8 with conc. HcI
↓
Volume up to 500ml

4. TE buffer (Tris-EDTA):100X

| | | |
|---|---|---|
| Tris-Hcl | 10mM (pH-8.0) | 60.55gm/500ml |
| EDTA | 1mM (pH-.8.0) | 18.612gm/500ml |

Preparation : (100X stock solution)

Tris-HcI +EDTA +NaCI
↓
Add 300ml distilled water
↓
Stirring and adjust pH to 8 with conc. HcI
↓
Volume up to 500ml

5. 20% SDS (Sodium dodecyl Sulphate)

6. CTAB (Cethyltrimethyl Ammonium Bromide): (10% CTAB)

| | |
|---|---|
| CTAB | 10g |
| 0.7M Nacl | 100ml |

Preparation:

CTAB+0.7M Nacl
↓
Stirring and shaking vigorously

7. Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

Preparation : For 50ml

a. 25 ml Phenol
b. 24 ml chloroform
c. 1 ml isoamyl alcohol

8. Ethyl alcohol (≥70%): Stored in $-20^0$c

9. Loading dye

- 0.25% xylene eyanol
- 0.25% bromophenol blue
- 30% glycerol
- 1 mM EDTA