ISOLATION AND IDENTIFICATION OF MYCOFLORA FROM MUSHROOM SUBSTRATES AND EVALUATION OF THEIR EFFICACY AGAINST THREE SELECTED PATHOGENIC FUNGI

MD. ASHIKUR RAHMAN SARKER

DEPARTMENT OF PLANT PATHOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA -1207

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BY

MD. ASHIKUR RAHMAN SARKER

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Approved by:

Dr. Nazneen Sultana Professor Department of Plant Pathology SAU, Dhaka **Supervisor**	**Khadija Akhter** Associate Professor Department of Plant Pathology SAU, Dhaka **Co-Supervisor**

.....

Assoc. Prof. Dr. Md. Belal Hossain
Chairman
Examination Committee
Department of Plant Pathology
Sher-e-Bangla Agricultural University, Dhaka

DEPARTMENT OF PLANT PATHOLOGY
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207

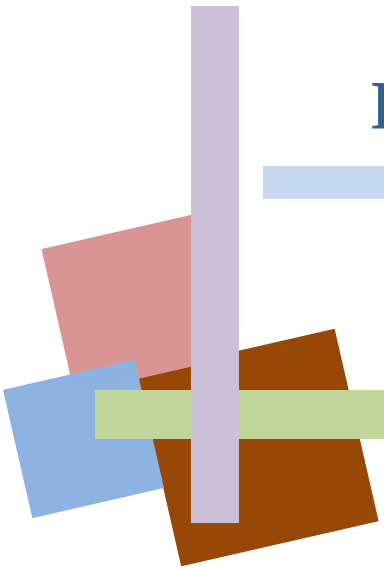
CERTIFICATE

This is to certify that the thesis entitled "***ISOLATION AND IDENTIFICATION OF MYCOFLORA FROM MUSHROOM SUBSTRATES AND EVALUATION OF THEIR EFFICACY AGAINST THREE SELECTED PATHOGENIC FUNGI"*** submitted to the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of ***MASTER OF SCIENCE*** in ***PLANT PATHOLOGY***, embodies the result of a piece of bona-fide research work carried out by ***MD. ASHIKUR RAHMAN SARKER***, **Registration No. 11-04670** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: 25th September,2017
Dhaka, Bangladesh

Dr. Nazneen Sultana
Supervisor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Dhaka-1207



Dedicated To

My Beloved Parents & Respected Research Supervisor

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The
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ABSTRACT

The study was carried out at the Molecular Plant Pathology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, (SAU), Dhaka-1207, during the period of January to June, 2017 to isolate the mycoflora from different mushroom substrates and to evaluate their biological activities against selected pathogens (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori).* For this purpose three types of mushroom substrates viz. sawdust, rice straw and newspaper were used. Four fungi were isolated and identified from mushroom substrates. These were *Aspergillus flavus, Aspergillus niger, Trichoderma harzianum,* and *Penicillium* sp. In *in-vitro* dual culture method *Trichoderma harzianum* showed the best performance in reducing radial mycelial growth of the selected pathogenic fungi, whereas *Penicillium* sp showed the least significant result against these pathogens. *T. harzianum* showed the highest inhibition in case of *Fusarium oxysporum* (71.62%) followed by *Sclerotium rolfsii* (61.64%) and *Colletotrichum corchori* (57%).

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CHAPTER I

INTRODUCTION

The first thing comes to our mind when we think of plants is green plants with colorful flowers and fruits. But there are some other plants which are not green. They do not produce flower or seeds and are often more or less conspicuous. They are associated with spoiling and decaying of food, wood etc. by their vegetative growth and thereby producing large fleshy masses. Mushrooms are the member of these groups of plants belonging to class Basidiomycotina. Mushrooms are non-green fungal plants occurring seasonally all over the world. They comprise a large heterogeneous group having various shapes, sizes, colors; appearance and edibility (Chang and Miles, l99l). There are 10,000 different types of mushrooms in the world of which only 2000 are edible. Among the edible mushrooms Oyster mushroom (*Pleurotus ostreatus)* is most suitable for the climatic condition of Bangladesh. At present Oyster mushroom ranks second among the cultivated mushrooms in the world (Chang and Miles 1991).The economic importance of the mushroom lies primarily in its consumption as food by human. It is good source of protein, vitamins and minerals (Khan *et al*., 1981). Mushroom is a tasty vegetable having medicinal values like strengthening immune system of human body, helping to cure high blood pressure, diabetes, gastric, jaundice and heart ailment.

Mushroom substrate refers to materials in which mycelium grows by inoculating it with spawn. Mushrooms are produced in different substrates. In mushroom cultivation substrates play the same role as soil executes in plant production (Kwon and Kim, 2004). The enormous amount of wastes prevailing in the agro and wood industry could be used in making different substrates for mushroom production. The overall production of fresh mushrooms can be resulted in 317 million metric tons (317 billion kg) by using only 25% of the yearly volume of burned cereal straws in the world (Chang & Miles, 1991). Different substrates can be recommended in different region as per local availability of agricultural wastes (Cohen *et al.,* 2002). Most commonly used substrates include sawdust,

cotton seed straw, cereal straw, corncob, sugar cane straw, newspaper, waste paper and other plant fibres showed promising result in case of cellulose content (Ragunathan *et al.*, 1996; Kwon and Kim 2004). Many species of *Pleurotus* are commonly grown on a wide range of lingo-cellulosic materials (Sanchez, 2004). Bughio (2001) successfully produced *Pleurotus ostreatus* on a combination of wheat straw, cotton boll straw, paddy straw, sugarcane and sorghum leaves. Besides shredded newspapers combining with rice bran or sawdust showed significant performance for *Pleurotus* production (Hashimoto, 1976). Paper waste, shredded paper can also be used for *Pleurotus* production (Poppe, 1995). Research assays that cereal straw provides higher production rate in compare to the other substrates though apparently sawdust is the most common type of substrate for both commercial and home cultivators. Presently, in Asia (including Taiwan), the main substrate used for the commercial cultivation of oyster mushroom is saw dust (Rizki *et al*., 2011).

In Bangladesh, about 30 million tons of agricultural wastes like paddy straw, wheat straw, saw dust and sugarcane bagasse are being lost by improper utilization (Ahmed, 2001). Oyster Mushroom are reported to be easily grown on different lignocellulose wastes such as banana leaves, cereal straw, paper wastes, sawdust, rice, wheat straw and other agro-wastes (Bhuyan, 2008). Rice straw, paper and saw dust are very available and cheap to use as a substrate for mushroom production in Bangladesh.

Considering its nutritional, medicinal and economic importance it is important to improve its production techniques using local low cost materials. Remarkable various factors are responsible for reducing production in Bangladesh. Among them, fungal disease and their antagonistic effect is one of the major influential factor. Mushroom crops can be attacked by several weed moulds or associated mycoflora, i.e. other fungi which act as competitors or antagonists of the mushrooms that are frequently responsible for crop loss. The substrates harbour many weed fungi causing serious problems during its cultivation. Most of them act as competitor moulds thereby spawn run is adversely affected either by

competition for food material or through production of toxic substances (Vijay & Sohi, 1987). Mushroom production is also greatly affected by *Trichodcrma* that is resistant to pasteurization. This is isolated from both fruit bodies and substrates of mushroom (Singh et al., 2006). According to Wickremasinghe *et al*., (1999) four species of fungi *Aspergillus fumigatus, Chetomiumthermophile, Mucor pusillus* and *T. harzianum* were are isolated and identified from straw and oyster compost substrates collected from the research station of Export Development Board, Ratmalana, Sri Lanka. Parvez, (2008) conducted a study in order to isolate mycoflora associated with *Pleurotus ostreatus* (Oyster mushroom) substrate during culture in the spawn packet. A total number of 50 spawn packets with colonized substrate of *Pleurotus ostreatus* were collected randomly at different growth stages from Mushroom Culture Center, Savor, Dhaka. Ten mycoflora including *Aspergillus flavus, A. fumigatus, A. niger, A. nidulance, A. terreus, Penicillium citrinum, P. thiersii, Penicillium.* sp., *Rhizopus stolonifer* and *T. harzianum* were isolated and identified as associated with *Pleurotus ostreatus* (Oyster mushroom) substrate. Different saprophytic and plant pathogenic fungi occurring in the substrate and competing with mushroom mycelium for space and nutrition are *Aspergillus niger*, *A.flavus, Alternaria alternata, Drechslera bicolor,Fusarium moniliforme, Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Rhizpus stolonifer, Sclerotium rolfsii,, Trichoderma viride* (Sharma *et al*., 2007; Sharma and Kumar, 2011). There might be an interaction between *Trichoderma* sp. and the mushroom due to the enzymatic action on substrate by mushroom that favors green mold fungal growth.

Sclerotium rolfsii Sacc. is a soil borne pathogenic fungus and harmful to many crops which are economically valuable in most of the tropical and subtropical region of the world (Aycock, 1966). The fungus is a facultative parasite and can maintain continuity of generation under adverse situation by the formation of sclerotia (Ahmed, 1980). It is very difficult to control even by the use of chemical fungicide. Many species of the fungal genera *Colletotrichum* cause a variety of diseases in a wide range of economically important plants around the world (Cannon *et al*., 2012 & Phoulivong, 2011). Stefańczyk *et al.*, (2016) surveyed

and stated that Several *Fusarium* spp. have been associated with potato dry rot. The most frequent and devastating of these species were *F. sambucinum*, *F. solani* and *F. oxysporum*, depending on the geographic location and the season. The *Fusarium* spp. that colonize potato tubers vary depending on geographic location. Dry rot in potato not only reduces the crop yield but can also contaminate the tubers with mycotoxins that cause cyto-, geno-, neuro- and hepatotoxic effects in animals and humans and thus threaten health when eaten directly (in *Fusarium*-infected plants) or indirectly (in the milk or meat of animals that have been fed with infected feed).

With the circumstances stated above, the present study was undertaken with a view to achieving the following objectives.

1. To isolate, identify and characterize the fungi associated with selected mushroom substrate.
2. To evaluate their efficacy against three selective pathogenic fungi.

CHAPTER II
REVIEW OF LITERATURE

Houdeau and Olivier (1989) studied that diseases and disorders affecting cultivated *Pleurotus* spp.: antagonism and mycoparasitism by (a) the green moulds *Trichoderma*, *Gliocladium*, *Penicillum*, *Aspergillus* spp, *Chaetomium olivaceum* (b) the black moulds *Stysanus*, *Doratomyces* and *Trichurus* spp., and (c) other moulds (*Neurospora*, *Mucor*, *Ostracoderma* spp. and myxomycetes).

Meera *et al.,* (1989) conducted an experiment that petri dishes containing malt extract agar were used to determine the air mycoflora before and after chopping of dry and wet straw. A total of 19 fungal species recorded were Deuteromycctes and *Cladosporium oxysporum* was the dominant species. Maximum air mycoflora was recorded at ground level during dry straw chopping and a substantial increase in *Trichoderma harzianum* was recorded.

Jandaik *et al.,* (1993) reported that during 1989-93, a severe form of yellow blotch of *P. sajor-caju* caused by *P. agarici* was observed at various mushroom units in Himachal Pradesh, India. Disease incidence was in the range 42-89%.only (850 g/bag) and the mixture (600 g/bag).

The most predominant contaminative fungi in *P ostreatus* were *Gliocladium* spp., while it was *Trichoderma harzianum* for *L. edodes* as described by Xu and Wen (1994) of Ya'an Sichuan, China.

Thakur *et all.,* (2001) stated that frequency and total number of mycoflora associated with paddy straw substrate during *pleurotus florida* cultivation was three fold higher on untreated straw substrate as compared to chemically treated paddy straw substrate. A sum of 12 fungal species belonging to 7 genera were associated with untreated paddy straw compared to only 8 fungal species belonging to 5 genera with treated paddy straw substrate. Among the isolated fungi, *Aspergillus flavus, Rhizopus* sp, *A. niger* and *Trichoderma* sp were most predominant with untreated and treated straw substrate.

Nussbaum *et al.,* (l997) observed that *Trichoderma* sp. were the most abundant contaminant in Oyster mushrooms (*Pleurotus ostreatus*) were grown in wheat straw substrate or wheat straw substrate + supplementary components (sawdust and lucerne hay) using different rates of mushroom spawn (0.9-3.6% fresh weight per substrate weight).

Anandh *et al.,* (1999) observed that during the cultivation of oyster mushroom variety Aruppukotai-l (*Pleurotus eous*); the important contaminants observed were *Aspergillus flavus, A. niger* and *Trichoderma harzianum*. These contaminants were isolated from beds and cultured on sorghum grain medium being used for spawn production.

Mallesha and Shetty (l988) observed that 27 to 37% of the oyster mushroom were infected from Brown spot infection during the spawn running stage caused a yield loss of 27-6l% in 4 mushroom farms in India.

Monaco *et al*., (1991) reported that *Trichoderma* sp. is a biocontrol agent of *Fusarium* sp. and *Sclerotium rolfsii* which shows effectiveness through seed treatment.

Sunder *et al.,* (1995) found that *T. viride* was most effective among the tested five *Trichoderma* spp. against *Macrophomina phaseolina* and *F. oxysporum* of castor in dual culture.

Katragadda and Murugesan (1996) found that in antagonism test between the mycoparasite *T. harzianum* and the cotton wilt pathogen *F. oxysporum* f. sp. *vasinfectum* it was determined that *T. harzianum* potentially reduced the radial growth *F. oxysporum* f. sp. *vasinfectum.*

Kashem *et al.,* (2011) conducted a series of experiments to assess the effect of 14 isolates of *Trichoderma* spp. *(Trichoderma harzianum* and *T. viride)* for controlling foot and root rot of lentil caused by *Fusarium oxysporum*. The pathogenecity of 12 isolates of *F. oxysporum* and the mass production of an

isolate of *T. harzianum* on 25 substrates are also studied. *Trichoderma* isolates inhibited the growth of *F. oxysporum* up to 92.07 % on agar plates.

Amin *et al.,* (2010) carried out a study on six isolates of *Trichoderma* spp. To evaluate their ability to inhibit soil borne pathogens of different vegetables viz., *Rhizoctonia solani* (isolates from tomato), *Sclerotium rolfsii* (causing collar rot of tomato) and *Sclerotinia sclerotiorum* under *in vitro* conditions. Dual culture of pathogens and *Trichoderma* spp. revealed that *T. viride* highly inhibited (65.71%) mycelial growth of *Rhizoctonia solani* over control. In case of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, *T. viride* proved to be potential inhibiting mycelial growth of the pathogens.

Chandrasehar *et al.,* (2005) conducted lab and green house experiments to determine the antagonistic effect of *Trichoderma harzianum* against *S. rolfsii* that caused tomato collar rot. They found that *Trichoderma harzianum* in *in vitro* condition completely suppressed the growth of *S. rolfsii* and in greenhouse condition in pot culture increased the percent survival of treated seedling applied as seed treatment and soil drenching.

Chowdhury *et al.,* (2000) reported that seed treatment with *Trichoderma harzianum* and *Gliocladium viride* against *Sclerotium rolfsii* resulted up to 21.61% and 48.43% increase in germination in mungbean, black gram, pigeon pea and tomato, respectively and showed good effect on seed born mycoflora.

Jabos and Kamoen (1986) found that *Trichoderma harzianum* produced cell wall lysine enzymes which developed antagonism against plant pathogens and improved biological control.

Mirkova (1982) studied the antagonistic activity of *Trichoderma* spp. against some pathogens and reported that among 5 *Trichoderma* spp, 3 isolates of *Trichoderma harzianum* were found most antagonistic.

Wells *et al.*, (1972) found *Trichoderma harzianum* was pathogenic to *Sclerotium rolfsii* in agar medium. They observed that *Trichoderma harzianum* effectively

controled *Sclerotium rolfsii* on peanuts, tomatoes and blue lupins under greenhouse condition.

Sultana and Hossain (1999) evaluated *Trichoderma harzianum* for controlling foot and root rot (*Fusarium oxysporum* and *Sclerotium rolfsii*) of Lentil cv. BARI Masur-1 under field condition. Seeds of lentil treated with *Trichoderma harzianum* contributed 47.85% to 112.49% reduced of foot and root rot diseased plants over control.

Wickremasinghe *et al.,* (1999) reported that when antifungal activity of crude extracts of *A. fumigatus* and *Trichoderma harzianum* was checked using the disc diffusion bioassay, inhibitory effect on the colony growth of the 3 test fungi- *Rhizoctonia solani, Fusarium oxysporum* and *Colletotrichum lindemuthianum* was observed at fairly high levels of 5000 and 10,000 µg. The effect of *A. fumigatus and T. harzianum* crude extracts on *Fusarium oxysporum* was more prominent at 5000 and 10,000 µg levels. Results indicated a 16.7% and 30% mean reduction of colony radius (cm) of *F. oxysporum* exposed to 5000 and 10,000 µg levels of crude extracts of *A. fumigatus* respectively. The mean reduction of the colony radius of the same fungus exposed to the crude extracts of *T. harzianum* was 13.4% and 50% at 5000 and 10,000 µg levels respectively. However, the same test fungi were insensitive to lower levels (100-1000 ug) of crude extracts from both *A. fumigatus* and *T. harzianum.*

Aydi Ben Abdallah *et al.,* (2015) reported that nine isolates of *Aspergillus* sp., isolated from soil and compost were tested *in vitro* for their antifungal activity against *Fusarium sambucinum* and *Phytophthora erythroseptica,* the causal agents of the *Fusarium* dry rot and pink rot of potato tubers. In dual culture method, the pathogen growth of *F. sambucinum* and *P. erythroseptica* was inhibited by 27 to 68% and 16 to 25% by all *Aspergillus* species, respectively. The highest inhibitory activity against both pathogens was induced by the isolate CH12 of *A. niger*. A significant reduction of the mycelial growth of both pathogens tested using the inverse double culture method involves the presence of volatile antifungal metabolites. This study reveals that *Aspergillus* sp. isolated

from compost and soil, exhibits an interesting antifungal activity toward F. *sambucinum* and *P. erythroseptica* and may represent a potential source of biopesticide. Testing of their culture filtrates, their organic extracts and their toxicity may give additional information on their safe use as biocontrol agents.

Kumar and Kumar (2014) evaluated the efficacy of five *Aspergillus* species as biocontrol agent against the *Fusarium oxysporum* f. sp. *lycopersici,* the causal organism of tomato wilt disease, in *in vitro* condition. Isolation and identification of *Fusarium oxysporum* f. sp. *lycopersici* was done form tomato cultivated soil from different areas in and around Mysore district, Karnataka state, India. Five *Aspergillus* isolates, isolated from indigenous soil, displayed significant antagonistic activity and suppressed the growth of the pathogen in dual culture technique *in vitro*. Among five, *A. niger* inhibited 80.33% growth of the pathogen, *A. flavus* and *A. tamarii* inhibited 77.03% growth of the pathogen when compared to control.

Dwivedi and Enespa (2013) conducted a research to find out the possibility of controlling the disease wilt of *Solanum melongena* and *Lycopersicon esculantum* caused by *Fusarium solani* and *Fusarium oxysporum* f. sp. *lycopersici* using eight biocontrol agents viz., four species of *Aspergillus (A. niger, A. flavus, A. sulphureus, A. luchuensis),* two species of *Trichoderma (T. viride, T. koningii)* and two species of *Penicillium (P. citrinum, P. italicum).* The assessment of fungitoxicity was carried out by poisoned food technique at three different concentrations i.e., 25, 50, 75% (v/v) against the test fungi. Assessment was carried out in terms of percent mycelial growth inhibition. All the bioagents showed significant reduction in the growth of the pathogens. Among different bioagents, *Aspergillus luchuensis* against *Fusarium oxysporum* f. sp. *lycopersici* was found significantly superior to the rest in checking the growth of pathogen and showed 100% inhibition at all the concentrations, while *Aspergillus niger* against *Fusarium solani* and *Fusarium oxysporum* f. sp. *lycopersici* was most effective and completly inhibited the mycelial growth at 50 and 75% concentration. On the other hand, *Aspergillus luchuensis* against *Fusarium*

solani and *Aspergillus sulphureus* against *Fusarium oxysporum* f. sp. *lycopersici* was most effective and completly inhibited the mycelial growth at 75% concentration followed by *A. flavus, T. koningii, T. viride, P. italicum and P. citrinum.*

Bosah *et al., (2010)* conducted an experiment on the pure cultures of three antagonistic fungi, *Trichoderma*, *Penicillium* and *Aspergillus* species and a fungal pathogen, *Sclerotium* sp. They were obtained after inoculation on potato dextrose agar (PDA) fortified with antibiotics to prevent bacterial contamination. Pathogenicity test was carried out when the antagonistic isolates were inoculated on PDA 24 h before and after Sclerotium inoculation. Of the three fungal antagonists evaluated for inhibitory efficacy, *Trichoderma* sp. proved to be the most effective as it exhibited the greatest inhibition to *Sclerotium* sp. both at the initial and final tests. This was closely followed by *Aspergillus* sp. with inhibitory effect on the pathogen at both trials. However *Penicillium* sp. was slightly inhibitory against *Sclerotium.* Percentage inhibitions of the antagonists on *Sclerotium* by *Trichoderma*, *Aspergillus*, and *Penicillium*, were up to 81.36-80.29%, 88.35-73.12% and 56.98-46.24%, at the 6th day of inhibition at both trials, respectively. The result implied that the extent of inhibition by the fungi provides the use of potential antagonists capable of controlling the pathogenicity of *Sclerotium* sp. in crops for sustainable agriculture.

Park *et al.,* (2014) found the diversity of marine-derived *Penicillium* from Korea using morphological and multigene phylogenetic approaches, analyzing sequences of the internal transcribed spacer region, β-tubulin gene, and RNA polymerase subunit II gene. In addition, the biological activity of all isolated strains was evaluated and tested for the extracellular enzyme activity of alginase, endoglucanase, and β-glucosidase, and antifungal activity against two plant pathogens (*Colletotrichum acutatum* and *Fusarium oxysporum*). A total of 184 strains of 36 *Penicillium* species were isolated, with 27 species being identified. The most common species were *Penicillium polonicum* (19.6 %), *P. rubens* (11.4 %), *P. chrysogenum* (11.4 %), and *P. crustosum* (10.9 %). The diversity of

Penicillium strains isolated from soil (foreshore soil and sand) and marine macroorganisms was higher than the diversity of strains isolated from seawater. More than half the strains (50.5 %) showed antifungal activity against at least one of the plant pathogens tested. Compared with other strains in this study, *P. citrinum* (strain SFC20140101-M662) showed high antifungal activity against both plant pathogens.

Cal *et al.,* (1995) reported that fungi known to produce lytic enzymes were used in an attempt to control wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Some of the fungal species (*Penicillium oxalicum, Penicillium purpurogenum and Aspergillus nidulans*) damaged hyphae of FOL in vitro and reduced the numbers of microconidia in the soil. Treatments with fungi did not result in a reduction in either chlamydospores of FOL in soil or populations of FOL in the rhizosphere of tomato. *P. oxalicum* was the most effective agent of biocontrol, and it reduced disease severity in both non-autoclaved (20% decrease) and sterile soil. In sterile soil, *P. oxalicum* reduced disease with different levels of severity (27% decrease at high levels and 50% decrease at low levels). Disease control by *A. nidulans* and *P. purpurogenum* was only achieved when disease severity was low in sterile soil (55% and 45%, respectively).

Rai *et al.,* (1975) observed the effect of sclerotial mycoflora on the survival and germination of sclerotia of *Sclerotinia sclerotiorum*. Of the twenty four fungal forms isolated from sclerotial surface, *Penicillium* sp. were dominant which played a significant role in their destruction. *Aspergillus ustus* and *A. flavus* also showed similar effects. Of the total isolates, nine forms viz.*A. flavus, A. niger, A. ustus, Penicillium sp.,P. citrinum, P. pallidum, P. funiculosum, P. steckii, Stachybotrys atra* var. *microspora* showed antagonistic activity in *in vitro* against *S. sclerotiorum*.

Rahman *et al., (*2013) studied the antagonist activities of different *Trichoderma* strains *in vitro* against *Colletotrichum capsici*, a causal agent of anthracnose fruit rot of chili. Dual culture test showed that *Trichoderma* strains effectively

inhibited mycelia growth of the pathogen. *T. harzianum* IMI-392433 showed the highest inhibition (81.96 %) and mycelial overgrowth (78.98%). Also, metabolites having 80% concentration extracted from 30-day-old *T. harzianum* IMI-392433 revealed the highest PIRG (percentage inhibition of radial growth) value of 85.16 and 87.18% by using normal poison and modified bilayer poison agar technique, respectively.

CHAPTER III

MATERIALS AND METHODS

3.1. Experimental Site

The experiment was conducted at the Molecular Plant Pathology Laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, and Biochemistry laboratory and Mushroom Culture House (MCH) of Biochemistry Department, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh.

3.2. Experimental Period

This study was conducted during the period of January, 2017 to June, 2017.

3.3. Preparation of mushroom substrate

3.3.1. Selection of substrate for spawn production

Consultation with advisors and checking the local availability resulted in a general consensus that three types of substrates viz. rice straw, newspaper and saw dust are highly appropriate for this experiment. Major consideration involved were availability of the materials, cost and easy obtaining. Sawdust, rice straw and newspaper were used to make three different types of spawn packets.

3.3.2. Preparation of saw dust

The saw dust were collected from Ahammed Tember Complex and Sawmill. After sun drying the moisture was increased by adding water. Then polypropylene bags (25×18 cm) were filled with 500 g prepared substrate and packed tightly. A hole of 3 to 5 cm was made with pointed steel at the center for space to put the inoculums. The packets were plugged with cotton and bind with rubber band to prevent the displacement of colored paper. The packets were sterilized in the autoclave for 15 minutes at 120°C with 15 psi atmospheric

pressure and were kept 24 hours for cooling. A total number of 20 packets were made following this procedure. One teaspoonful of mother culture materials containing mycelia was placed aseptically through the hole of the packet (Figure 1). The packets were then kept on the shelf in an incubation room at 25±1°C under 80% to 85% relative humidity and were allowed to complete the whitish mycelia growth (Parvez, 2008).

3.3.3. Preparation of rice straw

Rice straw were collected from the SAU Central farm and were sun dried. After that they were fragmented into small pieces (less than three centimeter particle size) with a sickle. The substrate consisted of rice straw only, nothing was supplemented. The materials were mixed thoroughly with mixture machine and the moisture was increased by adding water until it reached around 65% moisture content. Then polypropylene bags (25×18 cm) were filled with 500 g prepared substrate and packed tightly. A hole of 3 to 5 cm was made with pointed steel at the center for space to put the inoculums. The packets were plugged with cotton and bind with rubber band to prevent the displacement of colored paper. The packets were sterilized in the autoclave for 15 minutes at 120°C with 15 psi atmospheric pressure and were kept 24 hours for cooling. A total number of 20 packets were made following this procedure (Figure 2). One teaspoonful of mother culture materials containing mycelia was placed aseptically through the hole of the packet. The packets were then kept on the shelf in an incubation room at 25±1°C under 80% to 85% relative humidity and were allowed to complete the whitish mycelia growth (Parvez, 2008).

3.3.4. Preparation of newspaper packets

Waste paper was fragmented into small pieces with a sickle. Moisture was added until it reached around 65% moisture content. Then polypropylene bags (25×18 cm) were filled with 500 g prepared substrate and packed tightly. A hole of 3 to 5 cm was made with pointed steel at the center for space to put the inoculums. The packets were plugged with cotton and bind with rubber band to prevent the

displacement of colored paper. The packets were sterilized in the autoclave for 15 minutes at 120°C with 15 psi atmospheric pressure and were kept 24 hours for cooling (Figure 3). A total number of 20 packets were made following this procedure. One teaspoonful of mother culture materials containing mycelia was placed aseptically through the hole of the packet. The packets were then and were kept on the shelf in an incubation room at 25±1°C under 80% to 85% relative humidity and were allowed to complete the whitish mycelia growth (Poppe, 1995).

Figure 1. Sawdust packet

Figure 2. Rice straw packet

Figure 3. Newspaper packet

3.4. Inoculation of spawn

The spawn packets with complete mycelium were transferred to the culture house and the colored paper, rubber bands, cotton plug and plastic neck of the spawn packets were removed and the mouths of the polypropylene bags were wrapped and tied with rubber bands. The plastic bags were opened by "D" shaped cut on the shoulder side and removed the sheet. The opened surface of substrate was scraped slightly with a blade for removing the thin whitish mycelial layer. The packets were placed separately side by side on the rack in the culture house. The relative humidity (RH %) was maintained by watering four times daily. The average temperature (22-27^{0}C) and relative humidity (70-85%) were measured.

3.5. Selection of spawn packet

Three packets were randomly selected from each (rice straw, saw dust, waste paper) substrate that were previously inoculated with oyster mushroom.

3.6. Isolation of fungi on PDA media

3.6.1. Preparation of potato dextrose agar (PDA)

PDA was prepared as described by Islam (2009). 200g peeled and sliced potato was boiled in 500 ml 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 (Figure 4).

3.6.2. Isolation procedure

Isolation was done from three kind of substrates samples (rice straw, saw dust, waste paper). The substrates were prepared in Mushroom Culture House (MCH) of department of Biochemistry, Sher-e-Bangla Agricultural University, Dhaka. Three polybags of rice straw was randomly selected from the inoculated polybags. The stalk solution was made by taking 1 gm of substrate from each polybag and mixing them with 100 ml sterile distilled water in a conical flask. Then dilution plate technique was followed as described (In 3.6.3). The same process was also followed in case of saw dust and newspaper poly bags.

3.6.3. Dilution plate technique

Dilution plate technique was carried out as described by (Dhingra and Sinclair, 1985) for isolation of mycoflora:

One ml of stock suspension 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^{-3} (Figure 5). After finishing the preparation of different dilution, 0.1 ml of 10^{-2} and 10^{-3} dilution was spread over PDA plate previously dried (to remove excess surface moisture) at three replications as described by Goszczynska and Serfontein (1998). The solution was spread with the help of alcohol flame sterilized glass-rod. The inoculated PDA plates were kept in incubation chamber at 25 ± 1^{0}C.The colonies grown over on PDA were recorded after 3-5 days of incubation. Sub cultures were made by transferring a bit of mycelia from the spread plate and transferred on PDA plates using the hyphal tip culture techniques (Tuite, 1969; Mian, 1995) to a new petri dish on the basis of color and morphology of the colony.

Figure 4. 250ml PDA media

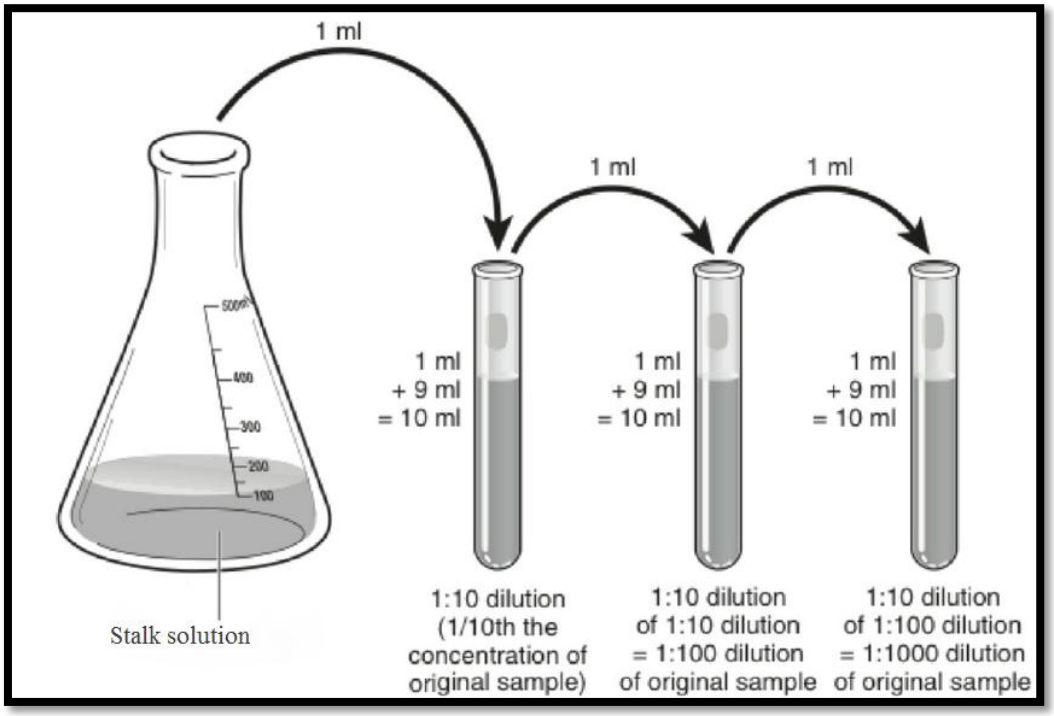

Figure 5. Dilution plate technique

[Source: http://ttktamop.elte.hu]

3.7. Identification of fungi

Identification was done with the help of different books, manuals and publications following the keys suggested by Barnet (1980), Barnett and Hunter (1992), Watanabe (2000), Mathur and Kongsdal (2003), Malone and Musket (1964), Ellis (1971).

3.8. Purification of fungi

Purification was carried out by reculturing the fungi by transferring single hyphal tip of the each fungus on PDA media and incubated at 22 ± 2^0C for 7 days.The contaminated plates were discarded.

3.9. Bioassay of isolated fungi

3.9.1. Collection of pathogenic fungi

Three pathogenic fungi *Fusarium oxysporum (*the causal agent of dry rot of potato), *Sclerotium rolfsii* (the causal agent of foot and root rot of betel vine), *Colletotrichum corchori* (the causal agent of anthracnose of jute) were collected from MS Laboratory Department of Plant Pathology of Sher-e-Bangla Agricultural University. The fungi were cultured in Potato Dextrose Agar (PDA) medium.

3.9.2. Dual culture method

PDA media was prepared and sterilized in an autoclave at 121°C for 15 minutes then the medium was poured into sterilized petri dish (90 mm diameter). Then the medium was allowed it to solidify at room temperature. The culture discs of the isolate and pathogen was cut separately with the help of sterilized cork borer. The culture discs of isolates and pathogen was aseptically transferred and placed at periphery of the petri dish containing the PDA medium (Care should be taken to place the both discs of isolates and pathogen at equidistance i.e. 20 mm apart from the periphery from the petri dish in opposite direction). Inoculated with culture discs of the pathogens alone in the petri dish containing PDA, which served as control. The inoculated petri dishes were transferred into the incubator

and incubated at 25°C. The growth of the fungus was observed periodically in petri-plates and measured the mycelial growth (diameter) in each petri dishs were taken (Parvin *et al.,* 2016).

3.9.3. Measurement of radial growth (mm) and determination of percent inhibition

After seven days of inoculation (7DAI), radial mycelial growth (mm) of fungus was recorded. The radial mycelial growth (mm) of of each fungi (pathogen and antagonist) was measured by taking average of the three diameters taken for each colony (Parvin *et al.*, 2016). Percent inhibition zone was estimated by the following formula as suggested by Johnson and Sekhar (2012).

$$\text{Percentage inhibition of growth} = \frac{C-T}{C} \times 100$$

C = Growth of fungus in control

T = Growth of fungus in treatment

3.10. Statistical analysis of data

Data collected during experiment period were tabulated and analyzed following Statistical package MSTAT-C. Treatment means were compared with Duncan's Multiple Range Test (DMRT)

CHAPTER IV

RESULTS

This chapter comprises the explanation and presentation of the results obtained from the experiment on isolation and identification of mycoflora from mushroom substrates and evaluation of their efficacy against three selected pathogenic fungi.

4.1. Isolation of fungi

Four fungal species were isolated from different mushroom substrates viz. *Aspergillus flavus, Aspergillus niger, Trichoderma harzianum, Penicillium* sp.

4.2. Identification of fungi

Identification was done on the basis of cultural and the morphological characteristics and studying microscopic structural characteristics of the isolated fungi.

4.2.1. Identification of *Aspergillus flavus*

Morphological characteristics of *Aspergillus flavus*

Aspergillus flavus grew rapidly producing colonies yellow green to green color with white mycelia at the edges (Figure 6.a). The conidia were finely rough, conidia heads were radiate to columnar with loosely packed phialides; the unisertiate conidia heads had radiate vesicle with the philiades covering upto three quarter of the vesicle; while bisertiate the vesicles were spherical to globose (Figure 6.b).

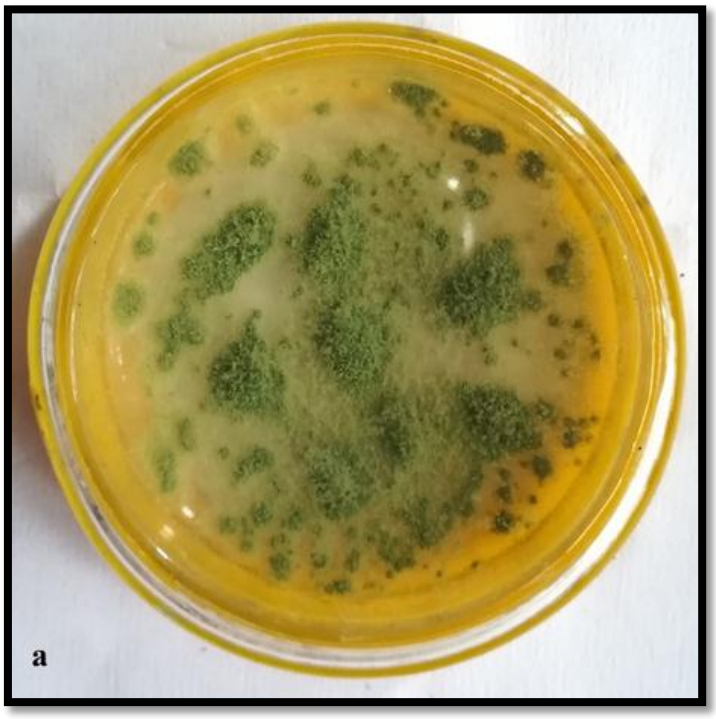

Figure 6. a. *Aspergillus flavus* on PDA medium

b. Microscopic appearance of *A. flavus* at 40X

4.2.2. Identification of *Aspergillus niger*

Morphological characteristics of *Aspergillus niger*

Aspergillus niger was fast growing producing black conidial head at the center and white mycelia towards the edge which changed color to brown with age (Figure 7.a). The colonies had thick mat of floccose mycelia beneath the colonies and at the edges. It formed radial furrows very close to each other. Conidia heads were biseriate and globose in shape with wide spherical to globose vesicle. The stipe was large and wide with smooth and slightly brown color. Conidiophores were smooth walled, hyaline or turning dark towards the vesicle. Conidia was globose to subglobose in shape (Figure 7.b).

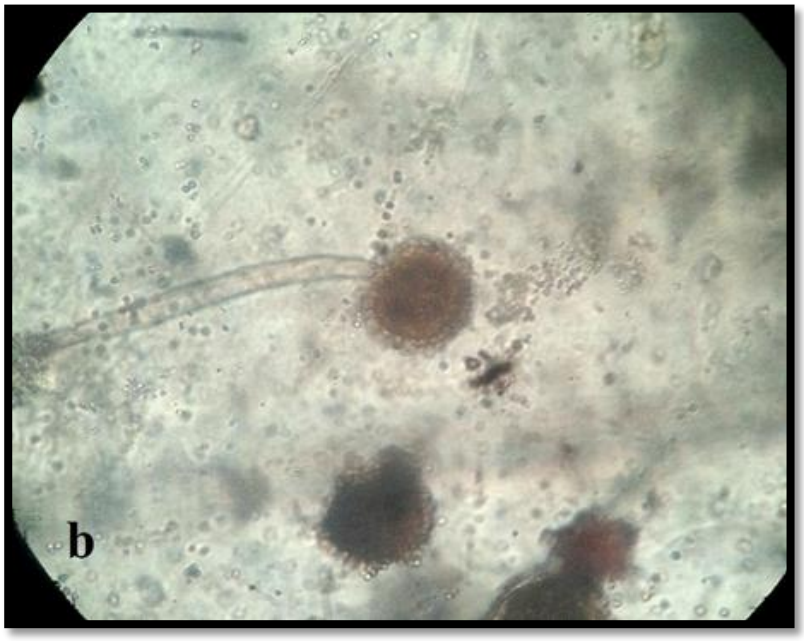

Figure 7. a. *Aspergillus niger* on PDA medium

b. Microscopic appearance of *A. niger* at 40X

4.2.3. Identification of *Trichoderma harzianum*

Morphological characteristics of *Trichoderma harzianum*

Trichoderma harzianum produced whitish to greenish colored mycelia in the beginning. Later a deep green color developed in central part and gradually extended to the periphery. Finally, it appeared a whitish green color (Figure 8.a). Mostly globose to subglobose conidia developed on phialides produced in the opposite direction in each point which observed under compound microscope. Conidiophores were septate, hyaline and loosely branched. Main branches produced lateral side branches. All primary and secondary branches arise at 90° angles with respect to the main axis (Figure 8.b).

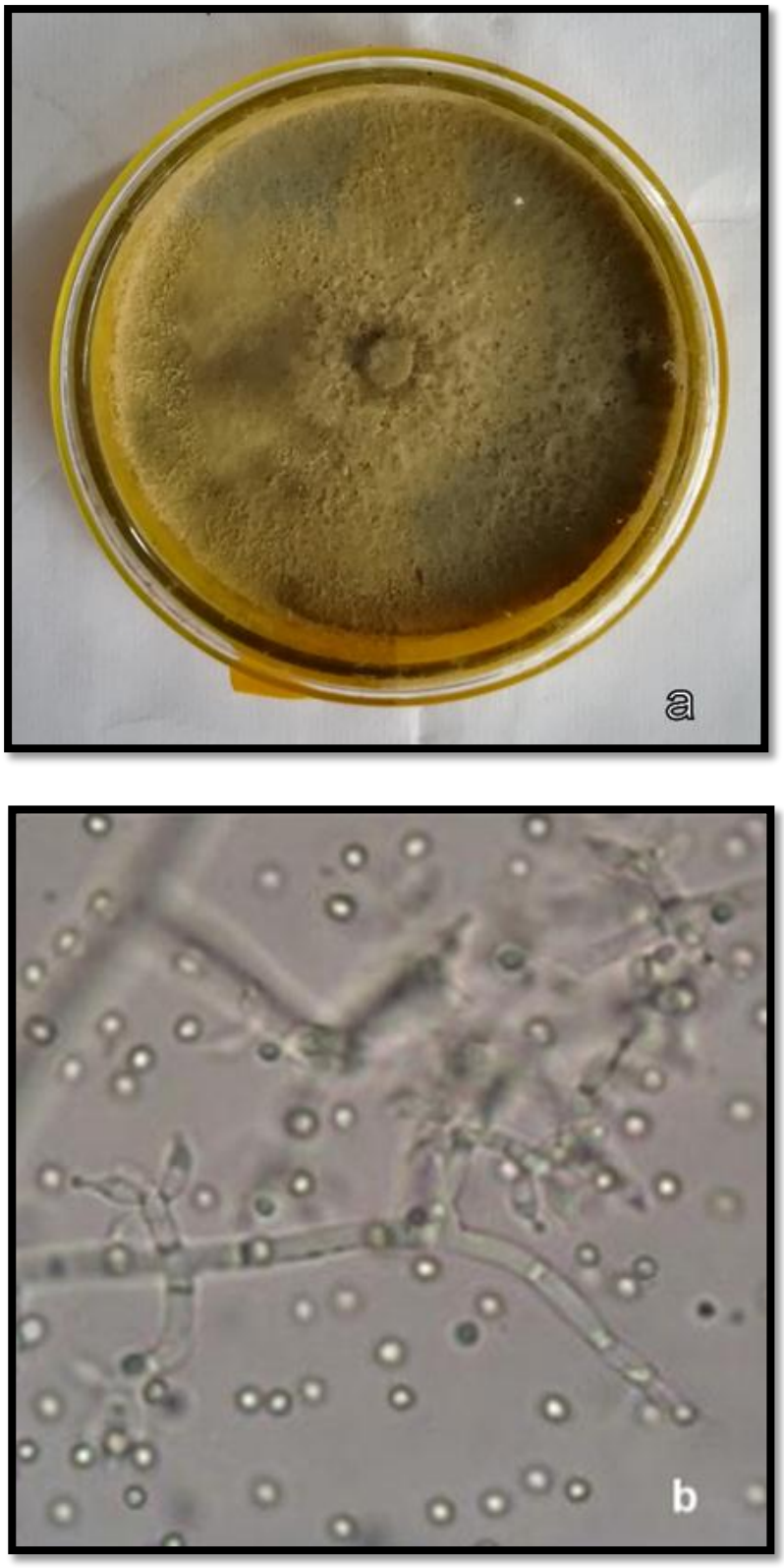

Figure 8. a. *Trichoderma harzianum* on PDA medium

b. Microscopic appearance of *T. harzianum* at 40X

4.2.4. Identification of *Penicillium* sp

Morphological characteristics of *Penicillium* sp

The mycelial mass of *Penicillium sp* initially appeared white and then gradually turned green with sterile white margin (Figure 9.a). The texture was powdery, mycelia was radially furrowed with heavy sporulation. Hyphae was hyaline and septated. Conidiophores were erect, septate, and branched. Phialides were grouped in brush-like clusters (penicilli) at the ends of the conidiophores (Figure 9.b). Conidia was in long dry chains, round to ovoid, hyaline or greenish, smooth or rough-walled.

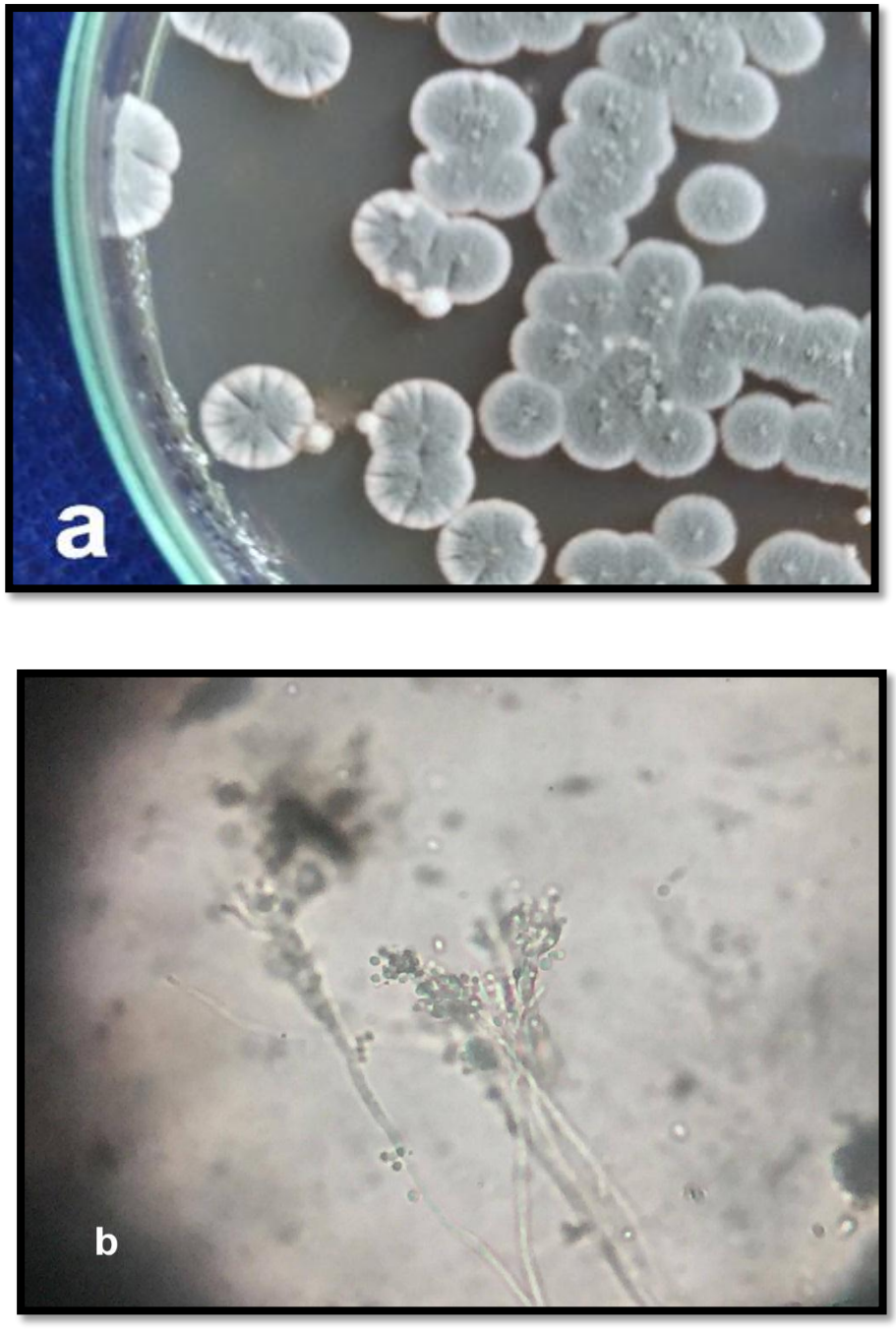

Figure 9. a. *Penicillium* sp on PDA medium

b. Microscopic appearance of *Penicillium* sp at 40X

4.3. Biological efficacy of isolated fungi against three pathogenic fungi

Table 1. Biological efficacy of fungi isolated from mushroom substrate against *Sclerotium rolfsii*

Treatment	Radial mycelial growth (mm) at 7 DAI*	% Inhibition of mycelial growth at 7 DAI*
Penicillium sp.	67.00	19.27
Aspergillus flavus	35.19	57.60
Aspergillus niger	45.00	45.78
Trichoderma harzianum	31.70	61.80
Control	83.00	-

*In column, DAI = Days after inoculation

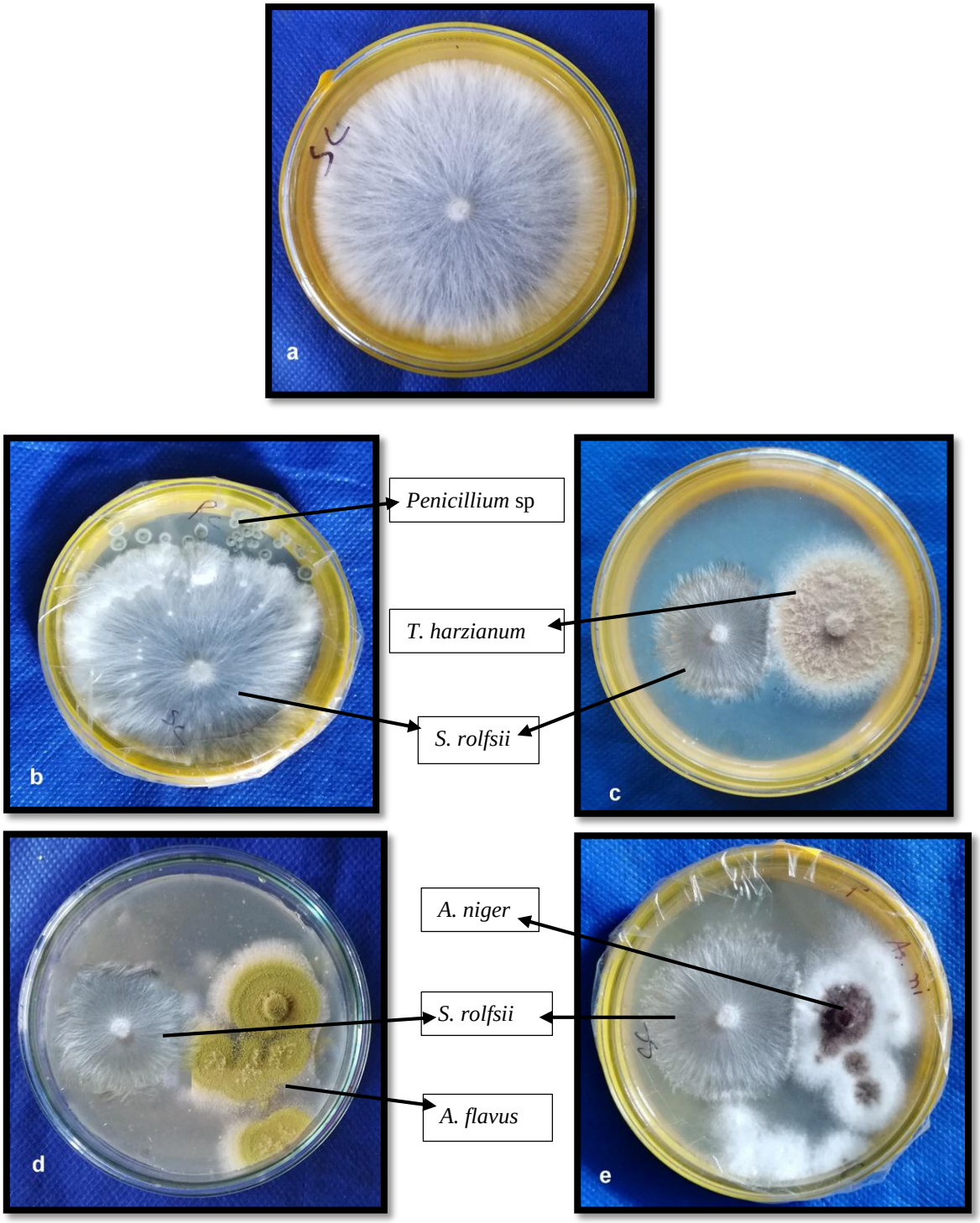

Figure 10. a. Growth of *S. rolfsii* at 7DAI

b. Growth of *S. rolfsii* against *Penicillium* sp. at 7DAI

c. Growth of *S. rolfsii* against *T. harzianum* at 7DAI

d. Growth of *S. rolfsii* against *A. flavus* at 7DA

e. Growth of *S. rolfsii* against *A. niger* at 7DAI

Table 2. Biological efficacy of fungi isolated from mushroom substrate against *Fusarium oxysporum*

Treatment	Radial mycelial growth (mm) at 7 DAI*	% Inhibition of mycelial growth at 7 DAI*
Penicillium sp.	30.75	28.81
Aspergillus flavus	28.90	33.10
Aspergillus niger	22.00	49.07
Trichoderma harzianum	12.20	71.75
Control	43.2	-

*In column, DAI = Days after inoculation

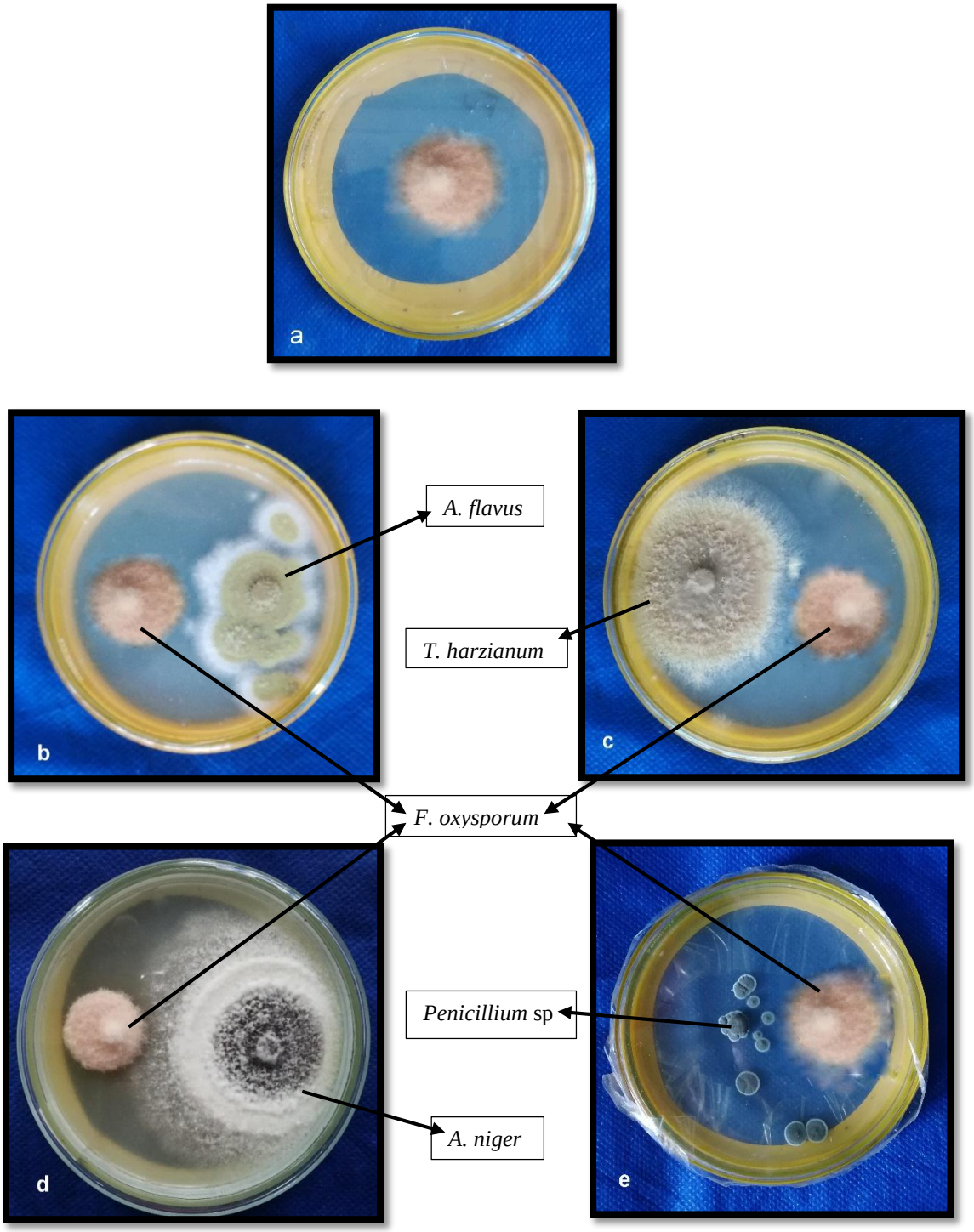

Figure 11. a. Growth of *Fusarium oxysporum* at 7DAI

b. Growth of *F. oxysporum* against *Aspergillus flavus* at 7DAI

c. Growth of *F. oxysporum* against *T. harzianum* at 7DAI

d. Growth of *F. oxysporum* against *Aspergillus niger* at 7DAI

e. Growth of *F. oxysporum* against *Penicillium* sp. at 7DAI

Table 3. Biological efficacy of fungi isolated from mushroom substrate against *Colletotrichum corchori*

Treatment	Radial mycelial growth (mm) at 7 DAI*	% Inhibition of mycelial growth at 7 DAI*
Penicillium sp.	32.30	39.96
Aspergillus flavus	28.00	47.95
Aspergillus niger	31.00	42.37
Trichoderma harzianum	23.00	57.24
Control	53.8	-

*In column, DAI = Days after inoculation

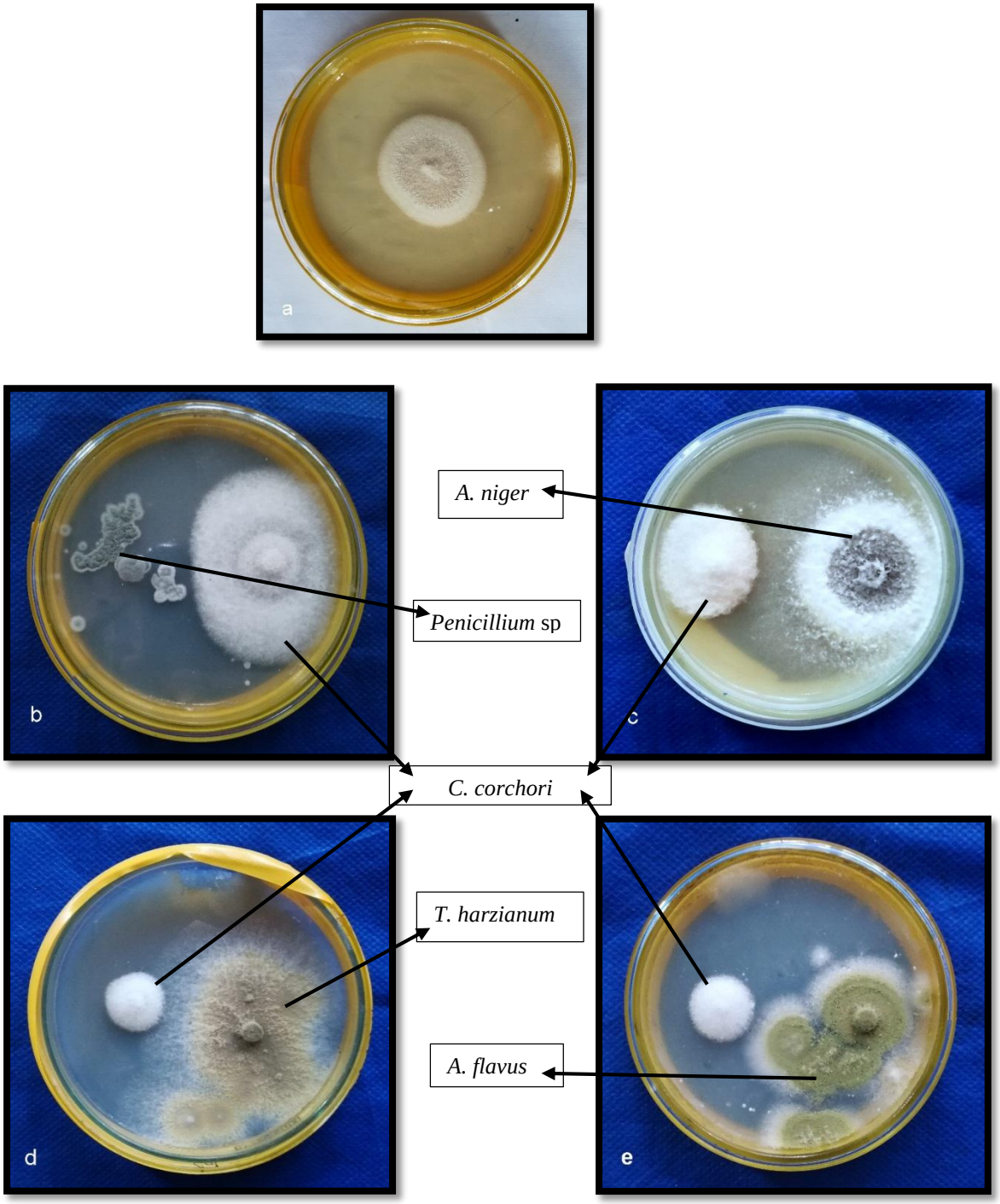

Figure 12. a. Growth of *Colletotrichum corchori* at 7DAI

b. Growth of *C. corchori* against *Penicillium* sp. at 7DAI

c. Growth of *C. corchori* against *Aspergillus niger* at 7DAI

d. Growth of *C. corchori* against *T. harzianum* at 7DAI

e. Growth of *C. corchori* against *Aspergillus flavus* at 7DAI

4.4. Antagonistic effect of isolated fungi against three selected pathogenic fungi

Biological efficacy of fungal isolated from mushroom substrates against three pathogenic fungi were studied and found significant variation in terms of percent inhibition of radial mycelial growth of pathogenic fungi. In case of *Fusarium oxysporum* the highest inhibition was observed against *T. harzianum* (71.62%) and the lowest against *Penicillium* sp (28.58%). In case of *Sclerotium rolfsii* highest inhibition observed against *T. harzianum* (61.64%) and the lowest against *Penicillium* sp (19.55%). In case of *Colletotrichum corchori* highest inhibition observed against *T. harzianum* (57%) and the lowest against *Penicillium* sp (39.59%). In this table (Table 4) it has been observed that among the fungal antagonist used against pathogenic fungi the most effective was *T. harzianum.*

Table 4. Biological efficacy of fungi (isolated from mushroom substrate) against three pathogenic fungi

Fungal Isolates	% Inhibition of mycelial growth		
	Fusarium oxysporum	*Sclerotium rolfsii*	*Colletotrichum corchori*
Penicillium sp	28.58 d	19.55 d	39.59 c
Aspergillus flavus	33.27 c	57.67 b	42.69 c
Aspergillus niger	49.23 b	45.63 c	47.69 b
Trichoderma harzianum	71.62 a	61.64 a	57.00 a
LSD (0.50)	2.62	2.39	3.32

CHAPTER V
DISCUSSION

The present study was conducted to isolate mycoflora from different kinds of mushroom substrate and to evaluate their biological activities against selective pathogen. *Aspergillus flavus, Aspergillus niger, Trichoderma harzianum, Penicillium* sp. were found most common types of fungi that existed on mushroom substrates. In the present study the fungi were identified by investigating their cultural and morphological characters under microscope. Then dual culture method was conducted to evaluate the antagonistic effect of isolated fungi against three selected fungi (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori).*

Aspergillus flavus produced yellow green to green color colonies with white mycelia at the edges. It was confirmed according to the description of. The conidia was rough, conidia heads were radiate to columnar with loosely packed phialides; while bisertiate type vesicles were spherical to globose. Whereas *Aspergillus niger* produced black conidia with white mycelia towards the edge which changed to brown color with age. Conidia heads were biseriate and globose with wide spherical to globose vesicle. Similar structure was observed by Nyongesa *et al.,* 2015, Wang *et al.*, 1990 and Thilagam *et al.,* 2016. *Trichoderma harzianum* produced whitish to greenish colored mycelia; globose to subglobose conidia developed on phialides produced in the opposite direction in each point which observed under compound microscope. Similar features were found by Jahan *et al*., 2013, Samuels *et al.* 2002, Shah *et al.,* 2012, and Environmental Reporter. 2010. *Penicillium* sp. is distinguished by its frequently greenish colonies and its branching or simple conidiophores supporting phialides in brush-like clusters known as penicillin (Larone and Davise, 1995) (Biotechnology Research International 2013).

In this study all of the identified fungi (*Aspergillus flavus, Aspergillus niger, Trichoderma harzianum, Penicillium* sp.) showed antagonistic activity against *Fusarium oxysporum, Sclerotium rolfsii* and *Colletotrichum corchori.*

Trichoderma harzianum showed the best performance by reducing the growth of *Sclerotium rolfsii* in duel culture. The inhibition was 61.80%, which was the highest inhibition percentage. *Aspergillus flavus, Aspergillus niger* and *Penicillium* sp. showed inhition 57.60%, 45.78% and 19.27%, respectively. The present findings agreed with the findings of Bosah *et al., (2010).* They conducted an experiment on the pure cultures of three antagonistic fungi, *Trichoderma, Penicillium* and *Aspergillus* species against *Sclerotium* sp. Among the three fungal antagonists evaluated for inhibitory efficacy, *Trichoderma* sp. proved to be the most effective as it exhibited the greatest inhibition to *Sclerotium* sp. both at the initial and final tests. This was closely followed by *Aspergillus* sp. with inhibitory effect on the pathogen at. However *Penicillium* sp. was slightly inhibitory against *Sclerotium.*

In *in-vitro* screening, *Trichoderma harzianum* showed best performance by reducing the growth of *Fusarium oxysporum* upto 71.75%, which is the highest inhibition. The lowest result was found in *Penicillium* sp (28.81%). *Aspergillus niger* and *Aspergillus flavus* showed 49.07% and 33.10%, respectively. The present findings is partially supported by findings of Kashem *et al.*, (2011). They conducted a series of experiments to assess the effect of 14 isolates of *Trichoderma* spp. *(Trichoderma harzianum* and *T. viride)* for controlling foot and root rot of lentil caused by *Fusarium oxysporum*. The pathogenecity of 12 isolates of *F. oxysporum* and the mass production of an isolate of *T. harzianum* on 25 substrates are also studied. *Trichoderma* isolates inhibited the growth of *F. oxysporum* up to 92.07 % on agar plates.

Mycelial growth of *Colletotrichum corchori* in dual culture method was mostly affected by *Trichoderma harzianum* (57.24%) followed by *Aspergillus niger* (47.95%), *Aspergillus flavus* (42.37%) and *Penicillium* sp. (39.96%). The present findings is partially supported by Fitsum *et al.*, 2014. They conducted an

experiment on bean anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara is one of the most devastating seed-borne diseases of common bean (*Phaseolus vulgaris L.*) in Ethiopia. Three fungicides viz., Mancozeb, Folpan and Mancolaxyl, and three bioagents viz., *Trichoderma harzianum* Rifai, *Trichoderma viride* Pers. Fr. and *Pseudomonas fluorescens* Migula, were screened *in vitro* for their antifungal activities against common bean anthracnose, using the dual culture and microtitre double-dilution techniques. The highest percentage of inhibition of the mycelia germination (80.39%) was obtained from *T. viride*, followed by 75.49% from *T. harzianum* and 40.2% from *P. fluorescens*.

Under this study all four isolated fungi showed statistically significant effect on the selected pathogenic fungi except in *Colletotrichum corchori.* Here *Penicillium* sp and *Aspergillus flavus* had statistically similar effect over *Colletotrichum corchori*. Among all the identified fungi *Trichoderma harzianum* showed the best significant result against all pathogenic fungi that was 71.62%. Furthermore *Trichoderma harzianum* showed inhibition percentage of mycelial growth in case of *Sclerotium rolfsii* 61.64% and *Colletotrichum corchori* 57%. The lowest antagonistic activity against *Fusarium oxysporum* (28.58%), *Sclerotium rolfsii* (19.55%) and *Colletotrichum corchori* (39.59%) were observed in case of *Penicillium* sp.

This result clearly indicates that the *Trichoderma harzianum* have the strong antagonistic effect and could be used as an eco-friendly management against *Fusarium oxysporum, Sclerotium rolfsii and Colletotrichum corchori.*

CHAPTER VI
SUMMARY AND CONCLUSION

The experiment was conducted in the Molecular Plant Pathology Laboratory of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, (SAU), Sher-e-Bangla Nagar, Dhaka, during the period on January to June, 2017 to isolate the mycoflora from different mushroom substrate and to evaluate the biological activities of isolated fungi against selected pathogen (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori).* For this purpose three types of mushroom substrate were prepared and four fungi isolates were found. They were *Aspergillus flavus, Aspergillus niger, Trichoderma harzianum, Penicillium* sp. Identification of the fungus were done by studying the cultural and morphological characteristics.

To evaluate the antagonistic effect of four isolated fungi against three selected pathogen (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori)* a dual culture method was conducted. Among the fungal isolates, *Trichoderma harzianum* has showed the most antagonistic activity against all three pathogen, where else *Penicillium* sp has the lowest antagonistic activity against the pathogens.

Trichoderma harzianum was most effective to inhibit the growth of *F. oxysporum* and its about 71.75%, *Aspergillus niger* also provided the capability to inhibit the growth of *F. oxysporum* after than *Aspergillus flavus* and *Penicillium* sp has lower capability to inhibit the growth of *F. oxysporum* its only 28.81%.

Against *Sclerotium rolfsii*, *Trichoderma harzianum* had the highest inhibition effect on the growth of mycelia which is around 62%, where *Penicillium* sp has the less capability to inhibihit the growth, its only 19.27%.

In case of *Colletotrichum corchori*, *Trichoderma harzianum* has the highest percent of inhibition of growth of mycelia which is 57.24% and *Penicillium* sp has the less capability to inhibihit the growth, its only 39.96%.

On a comparative study among the four isolated fungi *Trichoderma harzianum* showed the best significant antagonistic activity against all the selected pathogenic fungi. After *Trichoderma harzianum*, *Aspergillus* spp. had the most significant antagonistic activity. *Penicillium* sp showed less significant antagonistic activity than *Aspergillus niger* and *Aspergillus flavus.*

The inhibitory effect of the isolated mycoflora against the selected pathogenic fungi were not found up to the mark except *Trichoderma harzianum.* So, we can conclude from the present study that *Trichoderma harzianum* isolated from different mushroom substrate was found effective against the selected pathogenic fungi. Thus, *Trichoderma harzianum* could be used as bio-control agent against those pathogenic fungi. Further works need to be conducted to research the method of application of *Trichoderma harzianum* against those fungi.

CHAPTER VII

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