MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF MYCOFLORA ASSOCIATED WITH SELECTED SPAWN SUBSTRATES AND ITS EFFECT ON YIELD OF OYSTER MUSHROOM (*Pleurotus florida*)

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CERTIFICATE

This is to certify that the thesis entitled, *"MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF MYCOFLORA ASSOCIATED WITH SELECTED SPAWN SUBSTRATES AND ITS EFFECT ON YIELD OF OYSTER MUSHROOM (Pleurotus florida)"* submitted to the Department of Plant Pathology, 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 results of a piece of bona fide research work carried out by **RESHMA AKTER TONU** bearing Registration No**. 21-010089** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

Dated: August 10, 2023
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DEDICATED
TO
MY BELOVED PARENTS AND
TEACHERS

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ABSTRACT

An experiment was conducted to identify and characterize contaminants associated with the substrates of oyster mushrooms (*Pleurotus florida*) based on morphological and molecular characteristics, to assess the degree of contamination of various oyster mushroom substrates and to determine the effect of mycoflora on the growth and yield-contributing traits of *Pleurotus florida*. Five different types of mushroom substrates, including rice straw, sugarcane bagasse, waste paper, grass (*Cynodon dactylon*) and banana leaves were used for this purpose. By morphological and molecular analysis, three fungi were isolated and identified from mushroom substrates. These were *Exophiala spinifera*, *Trichoderma asperellum*, and *Rhizopus* sp. Among these fungal isolates, the DNA sequence of *Trichoderma asperellum* and *Exophiala spinifera* were detected by Basic Local Alignment Search Tool (BLAST) with the help of NCBI. DNA sequence of *Exophiala spinifera* and *Trichoderma asperellum* were matched by 99.83% (ITS1) and 100% (ITS4)as well as 65.60% (ITS1) and 99.50% (ITS4) with the nucleotide sequence of NCBI gene bank, respectively. Among the five mushroom substrates, waste paper showed the highest mycelium growth rate (0.48 cm), while banana leaves showed the lowest growth rate (0.41 cm). For mycelium running completion, sugarcane bagasse required the longest period of time (15.8 days), whereas waste paper required the shortest period of time (12.3 days). The maximum number of primordia (37.9), effective fruiting bodies (17.5), the highest biological yield (144.2 g), economical yield (113.3g) and biological efficiency (24.84%) per spawn packet were recorded from waste paper, while the minimum number of primordia (30.9), effective fruiting bodies (12), biological yield (119.7 g), economical yield (86.2 g) and biological efficiency (23.94%) were recorded in banana leaves. The maximum pileus diameter (5.03 cm) and stipe length (2.84 cm) were recorded from grass (*Cynodon dactylon*) whereas the minimum pileus diameter (3.61 cm) and stipe length (2.26 cm) were found in banana leaves. The maximum contamination severity was recorded in banana leaves (6.8%) whereas waste paper had the minimum level of contamination (0.4%). Among the selected substrates, waste paper showed the best performance in case of growth rate, mycelium running completion, yield and yield contributing characters.

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LIST OF SYMBOLS AND ABBREVIATIONS

% = Percentage

et al. = And others

spp. = Species

J. = Journal

No. = Number

viz. = Namely

df. = Degrees of freedom

& = And

etc. = Etcetera

^{0}C = Degree Celsius

@ = At the rate of

cm = Centimeter

cfu = Colony forming unit

ppm = Parts per million

NaCl = Sodium chloride

Kg = Kilogram

g = Gram

ml = Milliliter

WP = Wettable Powder

hr = Hour (s)

cv. = Cultivar (s)

i.e. = That is

T = Treatment

ft = Feet (s)

pv. = Pathovar

var. = Variety

mm = Millimeter

µl = Microliter

µm = Micrometer

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

SAU	=	Sher-e-Bangla Agricultural University
FAO	=	Food and Agriculture Organization
USA	=	United State of America
UK	=	United Kingdom
DAE	=	Department of Agricultural Extension
PDA	=	Potato Dextrose Agar (media)
CRD	=	Completely Randomized Design
ANOVA	=	Analysis of variances
LSD	=	Least Significant Difference
DMRT	=	Dunkan's Multiple Range Test
CV%	=	Percentages of Co-efficient of Variance
RAPD	=	Randomly Amplified Polymorphic DNA
DNA	=	Deoxyribonucleic acid
RFLP	=	Restriction Fragment Length Polymorphism
AFLP	=	Amplified Fragment Length Polymorphism
PCR	=	Polymerase Chain Reaction
rDNA	=	Ribosomal DNA
ITS	=	Internal Transcribed Spacer
CTAB	=	Cetyl Trimethylammonium Bromide
TAE	=	Tris Acetate EDTA
TE	=	Tris EDTA
EDTA	=	Ethylene Di-amino Tetra Acetic Acid
DNTP	=	Deoxynucleotide Tri-phosphate
NCBI	=	National Centre for Biotechnology Information
SMC	=	Substrate Moisture Content
SDS	=	Sodium Dodecyl Sulfate
EtOH	=	Ethyl Alcohol

CHAPTER I

INTRODUCTION

Oyster mushrooms (*P. florida*) are edible fungi belonging to the class Basidiomycetes and are increasingly becoming popular as protein-rich delicious vegetable. *Pleurotus* species are characterized by a white spore pint attached to recurrent gills, often with an eccentric (off center) stipe, or no stipe at all. The common name "oyster mushroom" comes from the white shell-like appearance of the fruiting body (Stanley, 2011). Mushroom cultivation is highly labour intensive, short duration crop and land saving, can be welcomed by the poor farmers. At present mushroom production is approximately 1.5 million tons in the world. Production of *Pleurotus* spp. accounted for 14.2% of the total world output (6,161,000 t) of edible mushrooms in 1997, the most recent year statistics were available (Chang, 1999). The Peoples' Republic of China is the major producer and consumer of oyster mushrooms, accounting for nearly 90% of total world production.

Mushrooms with their pleasant flavour, texture and high productivity per unit area have been recognized as an exceptional food source to alleviate malnutrition in developing countries. Stanley *et al*., (2011) reported a high nutritional values of oyster mushrooms with protein (25-50%), fat (2 - 5%), sugars (17 - 47%), mycocellulose (7 - 38%) and minerals (potassium, phosphorus, calcium, sodium) of about 8 - 12%. Edible mushrooms are also rich in vitamins such as niacin, riboflavin, vitamin D, C, B1, B5 and B6 (Syed *et al*., 2010). Various Pleurotus species have been shown to possess a number of medicinal properties, such as antitumour, immunomodulatory, antigenotoxic, antioxidant, anti-inflammatory, hypocholesterolaemic, antihypertensive, antiplatelet- aggregating, antihyperglycaemic, antimicrobial and antiviral activities (Gregori *et al*., 2007). These therapeutic activities are exhibited by extracts or isolated compounds from *Pleurotus* spp. fermentation broth, mycelia and fruiting bodies. In particular, polysaccharides appear to be potent antitumour and immuno-enhancing substances, besides possessing other beneficial activities (Zhanxi and Zhanahua, 1997; Taniguichi, 2000).

The oyster mushrooms are grown on organic substances termed as substrates. *Pleurotus ostreatus* can be grown on a wide range of agricultural byproducts and industrial wastes (Pani *et al*., 1997). Mushroom substrate has been defined as a ligno cellulose material which supports the growth, development and fruiting of mushroom (Chang and Miles, 1988). Mushroom spawn is the

mushroom mycelium growing on a given substrate and serves as the planting material (seed) in mushroom cultivation (Stanley and Awi-Waadu 2010). They have immense abilities to utilize various lignocellulose substrates with the aid of extracellular enzymes capable of degrading complex organic material (Martínez-Carrera, 2002). Mushrooms play an important role in managing organic wastes whose disposal have become a problem and are causing massive pollution to the environment as a result of dumping of agricultural wastes (Das and Mukherjee, 2007;Akinmusire *et al*., 2011). The wide range of plant waste includes sawdust, paddy straw, sugarcane baggage, corn stalk, corn cobs, waste cotton, leaves and pseudo stem of banana, water hyacinth, duck weed, rice straw etc. and does not require costly processing method and enrichment material (Modal 2010; Stanley, 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.

Many pests and diseases can cause yield losses in *P. ostreatus*. So far, the main diseases reported in the oyster mushrooms are green mold caused by *Trichoderma*, black-gray velvet caused by *Trichrus spiralis*, fire mold caused by *Neurospora* sp., brown spot of *Pseudomonas tolaassi*, and virus in Korea (Kim *et al*., 1995, 2000). 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., *Sclerotium rolfsii*, *Trichoderma viride* (Sharma *et al*., 2007; Sharma and Kumar, 2011). 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. The association of *Trichoderma* species with the cultivation substrate has long been known to limit production (Anonymous, 2005). 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. The most serious outbreak of *Trichoderma* species on mushroom crops was caused by biotype Th-2 of *T. harzianum*, in Ireland in 1985-86 and resulted in loss of about 3~4 million pounds in mushroom industries in UK and Ireland (Fletcher, 1990). Sharma & Vijay (1996) reported green mold of oyster mushroom caused by *Trichoderma viride* Pers. in North America, while severe cases of green mold of *P. ostreatus* were detected recently in South Korea (Park *et al*., 2004a–c), Italy (Woo *et al*., 2004), Hungary (Hatvani *et al*., 2007) and Romania (Kredics *et al*., 2006). The first serious attempts to morphologically distinguish *Trichoderma* species was made by Rifai (1969), who divided them into nine taxa on the basis of conidiophore branching and conidia shape. The most detailed morphological studies of the anamorphs were carried out by Bissett (1984, 1991a, b), who distinguished about 21 texa in sect. Pachybasium and seven in sect. Longibrachiatum. Recently, other taxonomic methods supplementary to morphology showed a great diversity of *Trichoderma*. Samuels (1994, 2002) has used isoenzyme profiles as a taxonomic technique. ITS and 5.8S rDNA sequences and fingerprinting techniques have revealed the intra-generic relationships amongst species of *Trichoderma* (Fujimori and Okuda, 1994; Kim *et al*., 2000).

The production of oyster mushroom is affected by several pests, including *Pseudomonas* spp. and flies, but in recent years the most severe crop losses have been caused by green mould infections worldwide. With this background and situation the present study was conducted for fulfilling the following objectives:

- To identify and characterize *Trichoderma* spp. and other contaminants present in the substrates of oyster mushroom based on morphological and molecular characteristics
- To determine the contamination severity of different substrates of oyster mushroom
- To find out the impact of mycoflora on growth and yield contributing characters of oyster mushroom

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. There were 19 different types of Deuteromycctes fungus species identified, with *Cladosporium oxysporum* being the most prevalent kind. When dry straw was chopped, the highest air mycoflora was detected at ground level, and *Trichoderma harzianum* levels significantly increased.

Jandaik *et al.,* (1993) reported that during 1989-93, some mushroom units in Himachal Pradesh, India, were affected by a severe form of the yellow blotch of *P. sajor–caju* caused by *P. agarici*. Only (850 g/bag) and the mixture (600 g/bag) had disease incidences that fell between 42 and 89% of the time.

Sharma *et al.,* (2007) reported that variations of the fungi that attack on mushroom substrates vary depending on the variety of substrates used, the method of the substrate being prepare, and the conditions of container used for cultivation.

Shah *et al*., (2011) examined green mold infecting substrate in poly bag and spawn bottles of *P. sajor-caju* and found that the fungus causing green mold was identified as *T. harzianum*.

Castle *et al*., (1998) found that *Trichoderma* species are common contaminants of spawn, compost, and wood. They analyzed 160 isolates of *Trichoderma* from mushroom farms based on morphological, cultural, and molecular characteristics and it was identified as a strain of *T. harzianum.*

Wickremasinghe (1999) showed that frequency of contaminants, *A. fumigates* and *T. harzianum* occurrence was 100% irrespective of the stage of processing of straw and oyster compost.

According to Xu and Wen (1994) of Ya'an Sichuan, China, the most prevalent contaminating fungus in *P ostreatus* were *Gliocladium* spp., whereas *Trichoderma harzianum* was the cause for *L. edodes*.

Thakur *et al.,* (2001) stated that there were three times as much mycoflora associated with paddy straw substrate during *pleurotus florida* production on untreated straw substrate as opposed to chemically treated paddy straw substrate. While only 8 fungal species from 5 genera were found on treated paddy straw, a total of 12 fungal species from 7 genera were found on untreated paddy straw. *Aspergillus flavus*, *Rhizopus* sp., *Aspergillus niger*, and *Trichoderma* sp. were the most prevalent among the isolated fungus 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) found that *Aspergillus flavus*, *Aspergillus niger*, and *Trichoderma harzianum* were the most significant pollutants during the cultivation of the oyster mushroom variety Aruppukotai-l (*Pleurotus eous*). These pollutants were removed from the beds and cultivated on a medium made of sorghum grain, which is used to produce spawn.

Mallesha and Shetty (l988) found that during the spawn running stage, 27 to 37% of oyster mushrooms were infected with Brown spot infection, which resulted in a 27–6% production reduction in 4 mushroom farms in India.

According to Monaco *et al*. (1991), *Trichoderma* sp. is a biocontrol agent for *Sclerotium rolfsii* and *Fusarium* sp. that exhibits efficacy through seed treatment.

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

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) examined the effectiveness of 14 isolates of *Trichoderma* spp. (*Trichoderma harzianum* and *T. viride*) in preventing *Fusarium oxysporum*-induced lentil foot and root rot. Studies are also conducted on the pathogenecity of 12 isolates of *F. oxysporum* and the mass generation of an isolate of *T. harzianum* on 25 substrates. *F. oxysporum* growth on agar plates was 92.07% suppressed by *trichoderma* isolates.

Recently, it was discovered that two new species of *Trichoderma*, *Trichoderma pleuroticola* S.H. Yu & M.S. Park and *Trichoderma pleurotum* S.H. Yu & M.S. Park, which are genetically related but phenotypically distinct, are responsible for the green mold of oyster mushrooms (Park *et al*., 2004a-c, 2006; Komon-Zelazowska'*et* al., 2007). Both species have been found on cultivated *P. ostreatus* and substrates on which the mushroom is grown in Europe, Iran and South Korea, but *T. pleuroticola* has also been isolated from soil and wood in Canada, the United States, Europe, Iran, New Zealand (Park *et al.,* 2004a–c, 2006; Komon-Zelazowska ´ *et al.,* 2007) and Hungary (Szekeres *et al.,* 2005).

Harman *et al*. (2004) observed that the genus *Trichoderma* is common in a variety of habitats, such as soil, forests, and root ecosystems. Harman *et al.,* (2004) also stated that *Trichoderma* spp. produce large numbers of conidia during asexual development, which can be transferred throughout mushroom farms by ventilation and workers because these species are airborne and have a high ability to adhere to clothes as described by Wellings *et al.,* (1987).

Some *Trichoderma* species have reportedly been employed to manufacture enzymes and inhibit other fungus as part of the biological management of plant diseases (Samuels, 1996).

Goltapeh and Dnaesh (2000) reported that the genus *Trichoderma* causes disease in oyster mushroom by competition for nutriments and lysis of the mushroom cell by secretion of hydrolytic enzymes.

Sinden and Houser (1953) were the first to recognize *Trichoderma* spp. as a potentially important pathogen and/or competitor that affects white button mushroom production.

Trichoderma species use a variety of substrates and thrive quickly in a variety of environments, according to Singh *et al*. (2006). Numerous T. species are commonly found in close proximity to mushrooms, including *T. harzianum*, *T. viride*, *T. virens*, *T. longibrachiatum*, and *T. polysporum*.

The most significant species among them and the possible source of losses has been identified as *T. harzianum* (Singh *et al*., 2006).

Hassan (2013) reported that the growth of competitions fungi such as *Penecillium*, *Aspergillus*, *Fusarium*, and others in mushroom media led to occupy large areas of the medium and deplete nutrients as well as their production of metabolites that inhibit the growth of mushroom, then decreased mycelium growth and consequently a decrease in the yield of mushrooms, while the pathogenic fungi form direct infections on the mycelium and fruit bodies of mushroom, such as green rot disease caused by *Trichoderm* as described by Mwangi and Wagara (2017), dry bubble disease caused by *Lecanicillium fungicola* and Cobweb disease caused by *Cladobotryum* sp which are the most destructive pathogens of the mushroom industry causes great losses in commercial production of mushrooms.

Sharma and Vijay (1996) reported green mould of oyster mushroom caused by *T. viride* in North America, but the first significant crop losses of cultivated *Pleurotus ostreatus* caused by this disease were reported in South Korea by Yu (2002).

Mesumbe (2006) reported on green mould disease of *Lentinula edodes* and *Pleurotus* spp. caused by *T. viride* in Cameroon.

Woo *et al.,* (2004) reported serious green mould infections of *P. ostreatus* in Italy, which have led to a crisis situation in *Pleurotus* cultivation there. Preliminary results obtained from the morphological and genetic characterisation of the infective agents have suggested that the isolates were of *T. harzianum*.

Chen and Moy (2004) have stated that certain mushroom cultivation factors, such as the sources of carbon and nitrogen, high relative humidity, warm temperatures, a fluctuation of these factors, and the lack of light during spawn run, are also favorable environmental conditions for moulds and can easily result in contamination. Molds develop quickly under these ideal circumstances, making them more effective competitors for resources and space than mushrooms. Additionally, they have the capacity to create volatile organic compounds, extracellular enzymes (such as glucanases), and poisonous secondary chemicals that can cause a sharp decline in productivity or even the eradication of whole crops. Emerging mushrooms may become heavily speckled and often deformed when pathogenic green moulds colonize the ground or develop on their surface.

No fruit bodies are generated during severe epidemics. During the spawn flow, *Trichoderma* spp. develop white mycelia that are difficult to differentiate from those of the mushrooms, making it challenging to detect the infection.

Hatvani *et al.,* (2007) developed a polymerase chain reaction (PCR)-based technique for the rapid detection of *T. pleurotum* and *T. pleuroticola*. Based on the tef1 gene, PCR primers were created that are distinct for both *T. pleurotum* and other *Pleurotus* pathogenic *Trichoderma* species. In this multiple PCR, 28 other *Trichoderma* species in addition to *T. pleurotum* and *T. pleuroticola* were examined. The outcomes showed that a three-primer set may be used to clearly differentiate *T. pleurotum* and *T. pleuroticola* from one another and from other fungi species. This PCR method allowed for the detection of *T. pleuroticola* in the natural substrate of wild-grown oyster mushrooms, indicating that this kind of mushroom may be a possible source of *T. pleuroticola* infection. Using ITS sequence analysis is not necessary to quickly identify the two recently discovered *Pleurotus* pathogenic *Trichoderma* species. This discovery might aid in the early detection and management of *P. ostreatus* green mould illness.

Yu (2002) and Woo *et al.,* (2004) have observed that *Trichoderma* species are present at the initial phase of substrate preparation, then disappear with pasteurisation, but they can be found again in the substrate after spawning (inoculation with *Pleurotus*), during spawn-run (incubation phase) and in the harvesting cycles.

Komoń-Zelazowska *et al.,* (2007) used an integrated method in order to fully characterize various *T. pleurotum* strains from Hungary, Italy, and Romania as well as *T. pleuroticola* isolates from Canada, the United States, Italy, Hungary, Romania, Iran, the Netherlands, Germany, and New Zealand. Both species belong to the *Harzianum* clade of *Hypocrea*/*Trichoderma*, together with *T. aggressivum*, which is the cause of *Agaricus* green mould disease. According to morphological research, *T. pleurotum* has conidiophore morphology resembling *gliocladiums*, but *T. pleuroticola* has pachybasium-like characteristics typical of the *Harzianum* clade.

Yu (2002) examined the impact of pasteurization at various temperatures, times, and substrate moisture content (SMC) levels on the growth of green mold infection caused by *Trichoderma* and *Hypocrea*. According to the findings, *Trichoderma* may be pasteurized at 60°C for 10 hours or more at both 50 and 70% SMC without any mycelial development continuing, whereas *Hypocrea* cannot live after being heated for 5 hours at 50°C. However, the heat conduction rate of the

substrate must be taken into account when determining the pasteurization duration (depending on the kind of material, substrate volume, and moisture content), since SMC might drop too low and allow the substrate to harbor harmful fungus. In this study, the impact of SMC on the development of *Pleurotus* and *Trichoderma* was also investigated. The oyster mushroom's growth was limited above 80%, and its optimal range was between 60 and 70%. The mycelial growth of green mould isolates, in contrast, was proportional to SMC and peaked at 80%. is crucial to understand since the SMC in mushroom growth rooms varies depending on the shelf height. Although the optimal temperature for oyster mushroom growth varies depending on the strain, room temperatures of around 25°C, 13–15°C, and 18°C are required for the spawn-run, induction of fruit body development, and fruiting, respectively. In order to reduce the likelihood of green mould infection developing and spreading, the substrate is primarily exposed to it during spawn-run.

Woo *et al*. (2004) studied the impact of temperature and pH on the mycelial development of isolates of *P. ostreatus* and green mold. ¬The ideal temperature for *Pleurotus* growth was 28°C, but *Trichoderma* could grow well throughout a larger range (20–28°C) and outgrew *Pleurotus* three times faster at 25°C. *Pleurotus* grew best in alkaline settings (pH 8–9), but *Trichoderma* favored acidic–neutral environments (pH 5-7). This data implies that bringing the pH of the substrate down to 8 or 9 could inhibit *Trichoderma* development and stop the infection from spreading. This study tested the inhibitory effects of several fungicides that are frequently used in agriculture, including prochloraz, thiabendazole, dichloran, benomyl, propiconazole, and thiofanatomethyl. Prochloraz and thiabendazole, the pesticides that are permitted in the production of edible mushrooms, were found to inhibit the growth of the aggressive *Trichoderma* isolates without negatively affecting *Pleurotus*.

L. Hatvani *et al.,* (unpublished data) tested the influence of a range of *Bacillus* sp. isolates on the growth of *T. pleurotum* and *P. ostreatus* in in vitro confrontation assays, and several of them have inhibited *Trichoderma* without affecting *Pleurotus* significantly. ese isolates might also have potential for use in biological control strategies.

CHAPTER III
MATERIALS AND METHODS

The experiment was carried out to isolate and identify mycoflora associated with selected mushroom substrates based on morphological and molecular characteristics and to estimate their impact on yield contributing parameters of oyster mushroom (*Pleurotus florida*). This chapter deals with a brief description on location and design of experiment, treatments, preparation of substrates, preparation of packets, cultivation of spawn packets, collection of produced mushrooms, data recording and their analysis as well as the molecular identification of the fungi under the following headings and sub-headings.

3.1 Experiment site

The field experiment was conducted at Mushroom Culture House (MCH) of Sher-e-Bangla Agricultural University, Dhaka. Lab experiment and the molecular study were conducted at Plant Pathology laboratory, Sher-e-Bangla Agricultural University and Central Dogma Lab, Dhaka, Bangladesh, respectively.

3.2 Duration of the experiment

The experiment was carried out during the period from April to October, 2022

3.3 Spawn production

3.3.1 Collection of materials for spawn production

Rice straw, sugarcane bagasse, banana leaves, and grass (*Cynodon dactylon*) were collected from the farm of Sher-e-Bangla Agricultural University and paper waste was collected from local paper stores. Mother culture of *Pleurotus florida*, neck and 7×11 inch polypropylene bag @ 500g were collected from Mushroom Development Institute (MDI), Savar, Dhaka.

3.3.2 Design and layout of the experiment

The experiment was laid out in a single factor Completely Randomized Design (CRD) and considered five treatments with ten replications (one packet under each replication).

3.3.3 Preparation of the substrate

The trial was started on 2nd April, 2022. In the first step rice straw, sugarcane bagasse, banana leaves, and grass (*Cynodon dactylon*) were chopped to 2-3 cm size and stored under a covered shade. Waste paper was fragmented into small pieces with a sickle. Chopped rice straw, sugarcane bagasse, banana leaves, and grass along with fragmented waste paper were dipped into water for 12 hours. Then the substrates are sun-dried until the moisture content reached around 65%.

Plate 1. **A.** Sugarcane bagasse, **B.** Rice straw, **C.** Banana leaves, **D.** Waste paper

3.3.4 Preparation of spawn packets

The measured substrates were taken in a plastic bowl and mixed thoroughly with sawdust by hand @ 1% on dry weight basis. Then substrates were filled into 7×11 inch polypropylene bag @ 500 g with 50 g mother spawn into three layers in each bag. The filled polypropylene bags were prepared by using plastic neck and plugged the neck with cotton and covered with brown paper placing rubber band to hold it tightly in place. Ten spawn packets were prepared for each treatment.

3.3.5 Incubation of spawn packets

After being prepared, spawn packets were filled and incubated in a dark space at a temperature between 22 and 25°C with a humidity level of 90% until the mycelium run was finished. The rubber band, brown paper, cotton plug, and plastic neck of the mouth of the spawn packet were removed after the straw was completely coated in white mycelium. Next, the bag was ripped open, and the mouth was firmly closed with rubber band. These spawn packets were then moved to the culture building.

3.3.6 Cultivation of spawn packets

The spawn packets were cut in "D" shape at both ends with a blade, then opened by removing the plastic sheet. The substrate's surface was then softly scraped with a tea spoon to remove the thin, white mycelial layer. On the iron shelves of the culture house, the packets of each sort were arranged side by side. Three times each day, water was sprayed into the culture house to keep it wet. The cultural house was kept between 22º and 25º C.

3.3.7 Harvesting of produced mushrooms

According to Amin (2002), the curial border of the cap served as a sign that a fruiting body was ripe. By twisting to uproot from the base, mushrooms were gathered. After performing the first harvest once again, the packets were scraped at the location of the 'D' shaped cut. The spawn packets were then immersed in water for 5 minutes, turned upside down to remove excess water for another 5 minutes, and water was sprayed on a regular basis. After the first and second harvests were completed, primordia started to form, and water spraying was resumed until the mushrooms were ready to be harvested.

Plate 2. A. Mycelium running in spawn packet, **B.** Primordia initiation in spawn packet, **C.** Mature fruiting body in spawn, **D.** Harvested mushroom from spawn packet

3.4. Isolation of fungi on PDA media

3.4.1. Preparation of potato dextrose agar (PDA)

PDA was prepared as described by Islam (2009). 500 ml of water and 200g of peeled, sliced potatoes were cooked in a dish for around 30 minutes. Next, cheese cloth was used to filter the potato extract. 20g of dextrose and 20g of agar, the last two components, were mixed into the extract to bring the volume up to the 1L threshold. The produced standard PDA was then transferred to a 1000 ml conical flask and sterilized in an autoclave at 121 °C and 15 pressure for 15 minutes.

3.4.2 Isolation and purification of mycoflora from contaminated spawn

10g samples were collected from the contaminated packages and combined with 100 ml of sterile, distilled water. To create a series of dilutions, 1 ml from the stock solution was added to 9 ml of sterile water and vigorously shaken to create a $10^{-}1$ dilution. Similarly $10^{-}2$, $10^{-}3$ and $10^{-}4$ dilutions of the substrate suspension were prepared (Dhingra and Sinclair 1985). 0.5 ml volumes were pipetted onto PDA medium from each of the substrate dilutions and incubated for 3–4 days at 27ºC (±2)ºC. Individual culture plates of substrate samples were used to separate the pathogen from the mixed colony it had developed in. A sufficient number of subcultures using the hyphal tip approach were carried out to create pure culture (Hyakumachi, 1994).All the pure cultures were kept in refrigerator at 4ºC for preservation.

3.4.3 Identification of pathogens

By studying the cultural and morphological characteristics of the diseases, the pathogens were identified. From a pathogen culture that had been growing for 10 days, the morphological characteristics were studied under low (10X) and high (40X) power magnification, and they were compared to those described in the literature. A microscope was also used to obtain a microphotograph of the contaminants. Each fungus was identified by its morphological traits, which were noted and compared with the relevant key books like the CMI description of fungi (Barnett, 1972).

3.5 Data collection

Data were taken on the following parameters

3.5.1 Days required for completing mycelium running

Days required from inoculation in spawn packets to completion of mycelium running were recorded.

3.5.2 Days required for the primordia formation

Days required from completion of mycelium to pin head formation were recorded.

3.5.3 Days required from primordia initiation to final harvest

Days required from primordia formation to final harvest were recorded.

3.5.4 Data on yield contributing parameters

Number of primordia and well-developed fruiting body was recorded. Dry fruiting bodies were discarded. Average weight of individual fruiting body was calculated by dividing the total weight of fruiting body per packet by the total number of fruiting body per packet.

a) Number of primordia per packets

b) Number of fruiting body

c) Number of effective fruiting body

d) Weight of individual fruiting body (g)

3.5.5 Dimension of fruiting body (stipe and pileus)

Length and diameter of stipes of three randomly selected fruiting bodies was measured using a slide calipers. Diameter and thickness of pileus were also measured.

a) Length of stipe (cm)

b) Diameter of pileus (cm)

c) Diameter of stipe (cm)

3.5.6 Biological yield (g)

Biological yield per spawn packet was measured by weighing the whole cluster of fruiting body without removing the lower hard and dirty portion.

3.5.7 Economic yield (g)

Economic yield per 500 g packet was recorded by weighing all the fruiting bodies in a packet after removing the lower hard and dirty portion.

3.5.8 Percent contamination (%)

Contamination severity was calculated for the test and control beds depended upon the following scale Grade 0: 0% – Free from infection

Grade 1: >0 – 20% Spawn area coverage by the contaminants

Grade 2: >20 – 40% Spawn area coverage by the contaminants

Grade 3: >40 – 60% Spawn area coverage by the contaminants

Grade 4: >60 – 80% Spawn area coverage by the contaminants

Grade 5: >80 – 100% Spawn area coverage by the contaminants

$$\text{Severity of contamination}(\%) = \frac{\text{Sum of total score}}{\text{Total no. of observation} \times \text{Maximum grade of the scale}} \times 100$$

3.5.9 Biological efficiency:

Biological efficiency was determined by the following formula:

$$\text{Biological efficiency} = \frac{\text{Total biological weight (g)}}{\text{Total dry weight of substrate used (g)}} \times 100$$

3.6 Analysis of data

The data was recorded for each character from the experiment was analyzed statistically using Statistix 10 computer program. The mean from all the treatment were calculated and analysis of variance (ANOVA) of characters under study was performed by LSD variance test.

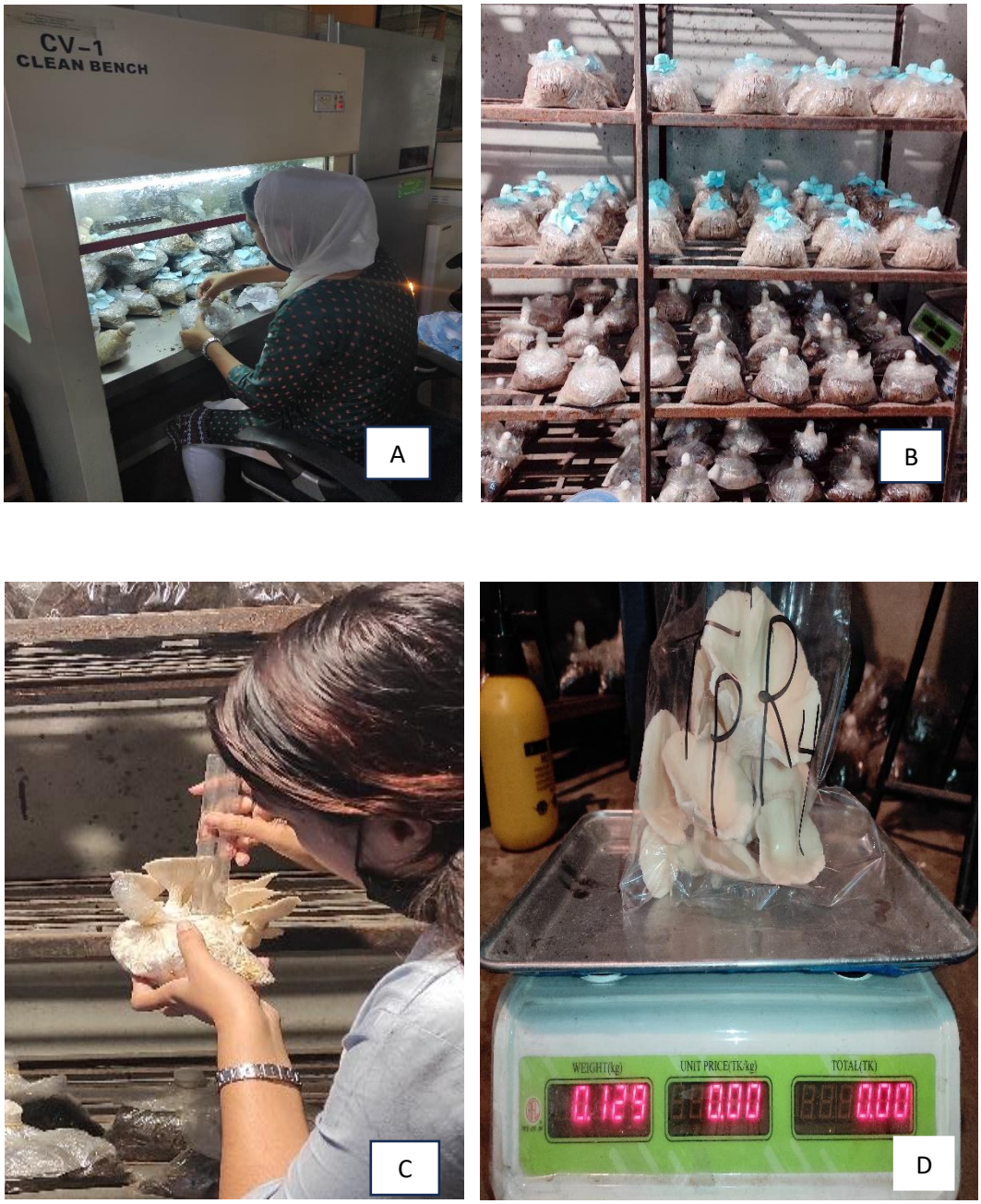

Plate 3 . **A.** Inoculation of mother in spawn packets, **B.** Prepared spawn packets, **C.** Measurement of stalk length, **D.** Measurement of the weight of fruiting bodies

3.7 Molecular identification of fungi

3.7.1 Collection of fungal isolates

Trichoderma asperellum, Rhizopus sp. and *Exophiala spinifera* were isolated from *Pleurotus florida* spawn having base materials viz; rice straw, grass, waste paper, sugarcane bagasse and banana leaves from Sher-e-Bangla Agricultural University (SAU) mushroom culture house.

3.7.2 Morphological observations

For the isolation of *Trichoderma asperellum*, *Rhizopus* sp. and *Exophiala spinifera* from the contaminated samples collected from SAU mushroom culture house, mycelia with green, black, and grey color were picked up with help of needles from the symptom part of the spawn. These isolates were grown on PDA (potato dextrose agar, difco), and they were kept at 25° C for two to three days under regular light. Compound microscope investigations on the morphology of conidiophores, phialides, and conidia were conducted for the morphological characterization of *Trichoderma asperellum*, *Rhizopus* sp., and *Exophiala spinifera*. The data includes information on the degree and type of conidiophore aggregation, the branching pattern of the conidiophores, the placement of phialides, the size and shape of phialides, the size and shape of conidia, and the kind of conidiophore.

3.7.3 DNA extraction protocol for fungi

The technique of Zolan and Pukkila (1986) was used to extract genomic DNAs from several isolates of *Trichoderma* spp., *Rhizopus* sp., and *Exophiala spinifera*. Potato dextrose agar (PDA) cultures were used to develop fungus isolates for two weeks at 25º C. The mycelial mats were dried by freeze-drying after being harvested from several isolates. Mycelial mats were ground with a micropestle for DNA extraction in 1.5 ml microcentrifuge tubes, and 400 µl of lysis buffer containing 3% Sodium dodecyl sulfate, 50 mM EDTA, 50 mM Tris-HCl (pH 7.2), and 1% 2-mercaptoethanol were then incubated at 68º C for an hour. The mixture was then gently extracted with a solution of chloroform: isoamylalcohol (24: 1) containing 5% phenol, and centrifuged at 12,000 rpm for one minute at room temperature. A fresh tube was filled with an equivalent volume of pure EtOH solution, and the supernatant was added before being centrifuged to remove the DNA. After being cleaned with 70% ethanol, the pellet was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

3.7.4 Procedure and Preparation of the PCR Reaction

The ITS region of DNA was amplified using the PCR technique using the following components, depending on the Premix reaction mix: i-Taq DNA Polymerase at 5U/µl, DNTPS at 2.5Mm, Reaction buffer (10X) at 1X, and Gel loading buffer at 1X.. The components of the reaction mixture with their concentrations were final volume of 25 µl as follows: Taq PCR PreMix at a concentration of 12.5 µl, Forward primer at a concentration of 10-20 picomol/ml (1 µl), Reverse primer at a concentration of 10-20 picomol/ml (1 µl), DNA at a concentration of 1.0 µl, Distilled water (9.5) µl).

3.7.5 Agarose Gel Electrophoresis

Alpha imager MINI gel documentation system was used in the process of amplification of the mentioned gene. The PCR product was separated using electrophoresis on agarose gel (1.5%), then the genomic DNA bands were shown using UV rays with a length of wavelength (302nm) after treatment with the dye.

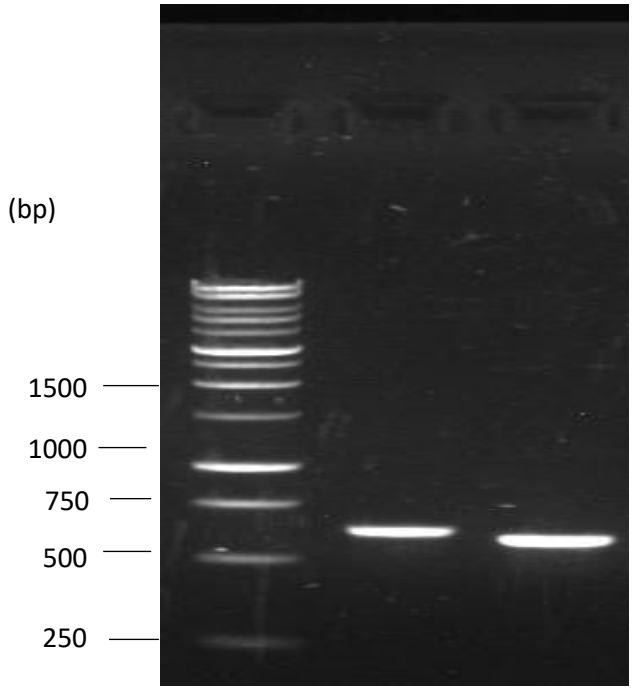

Figure 1. Band image of *Exophiala spinifera* and *T. asperellum*

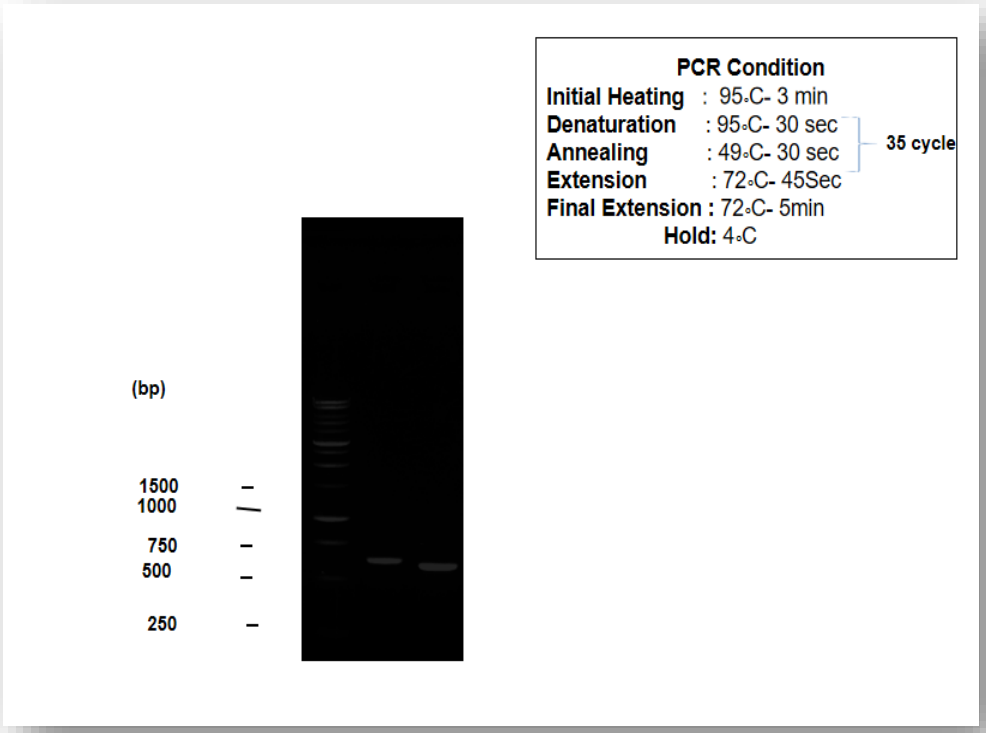

Figure 2. ITS profile of ITS1 and ITS4 primer generated from fungal isolates M: denotes 1 kb DNA ladder (marker)

3.7.6 Nucleotide Sequencing Analysis

The nucleotide sequences of the PCR amplified gene were determined after obtaining the 5.8S rRNA amplifications directly by sending a volume of 25 µl of PCR product and a volume of 10 µl (10 picomole concentration) of each primer to Central Dogma Lab, DNA Sequencer device used). The results were compared using a web-based computer program (the basic in-situ nucleotide sequence search tool). Basic Local Alignment Search Tool (BLAST) with the database at the National Center for Biotechnology Information (NCBI), which matches the nucleotide sequences of the studied genes with respect to the fungal isolates placed in the search and knows their species according to the match in the aforementioned database.

CHAPTER IV

RESULTS

4.1 Isolation and identification of microbial contamination from spawn

The infected spawn packets were taken for isolation of pathogens infecting substrate. They were isolated on potato dextrose agar. After purification of the fungus as described under materials and methods, morphological characters of the fungus on potato dextrose agar were studied for the purpose of identification and compared with those described in the literature. Based on the morphological characters commonly three pathogens were identified. These are *Trichoderma asperellum* , *Rhizopus* sp., and *Exophiala spinifera.*

Plate 4. A-B. *Trichoderma* contaminated spawn packet, **C.** *Rhizopus* contaminated spawn packet, **D.** *Exophiala spinifera* contaminated spawn packet

4.2 Morphological characterization of isolated fungal contaminants from spawn

4.2.1 *Trichoderma asperellum*

Due to intense sporulation of the causative agent, green-colored mycelium development was seen in the contaminated spawn packet (Plate 4 A-B). Colonies often develop quickly and start off yellowish before changing to a vivid green color (plate 5 A-B). *T. asperellum* was characterized by finer conidial ornamentation, slightly ovoidal conidia, mostly paired branches, ampulliform phialides, and consistent presence of chlamydospores.

4.2.2 *Rhizopus* sp.

The growth of *Rhizopus* species is filamentous, with branching, coenocytic (multinucleated) hyphae. Three different forms of branching hyphae exist: stolons, pigmented rhizoids, and unbranched sporangiophores. They create a fast-growing, white colony (Plate 5 C-D). Sporangiospores, the result of asexual reproduction, are generated in a spherical organ called a sporangium. The sporangiospores are globose to ovoid in shape, single-celled, hyaline to brown, and striate. A long stalk conidiophore known as a sporangiophore is where the many, black sporangia are created. The sporangiophores grow from rhizoids that resemble roots; they are spherical, generating a large number of multinucleated spores.

4.2.3 *Exophiala spinifera*

E. spinifera initially forms mucoid, yeast-like, black colonies, then grows tufts of mycelium (long, brown, septate, and annellophores), before changing texture to something between suede-like and downy (Plate 5 E-F). Conidiophores can be simple or branching, upright or sub-erect, spine-like, and have quite thick brown pigmented walls. They are between one and three millimeters long, slightly tapered, and barely annellate. On lateral pegs, conidia are produced in basipetal succession; they are one-celled, subhyaline, smooth, thin-walled, sub-globose to ellipsoidal organisms. Toruloid hyphae and yeast-like cells with secondary are features of *E. spinifera*. They are slow-growing and frequently cause human skin diseases.

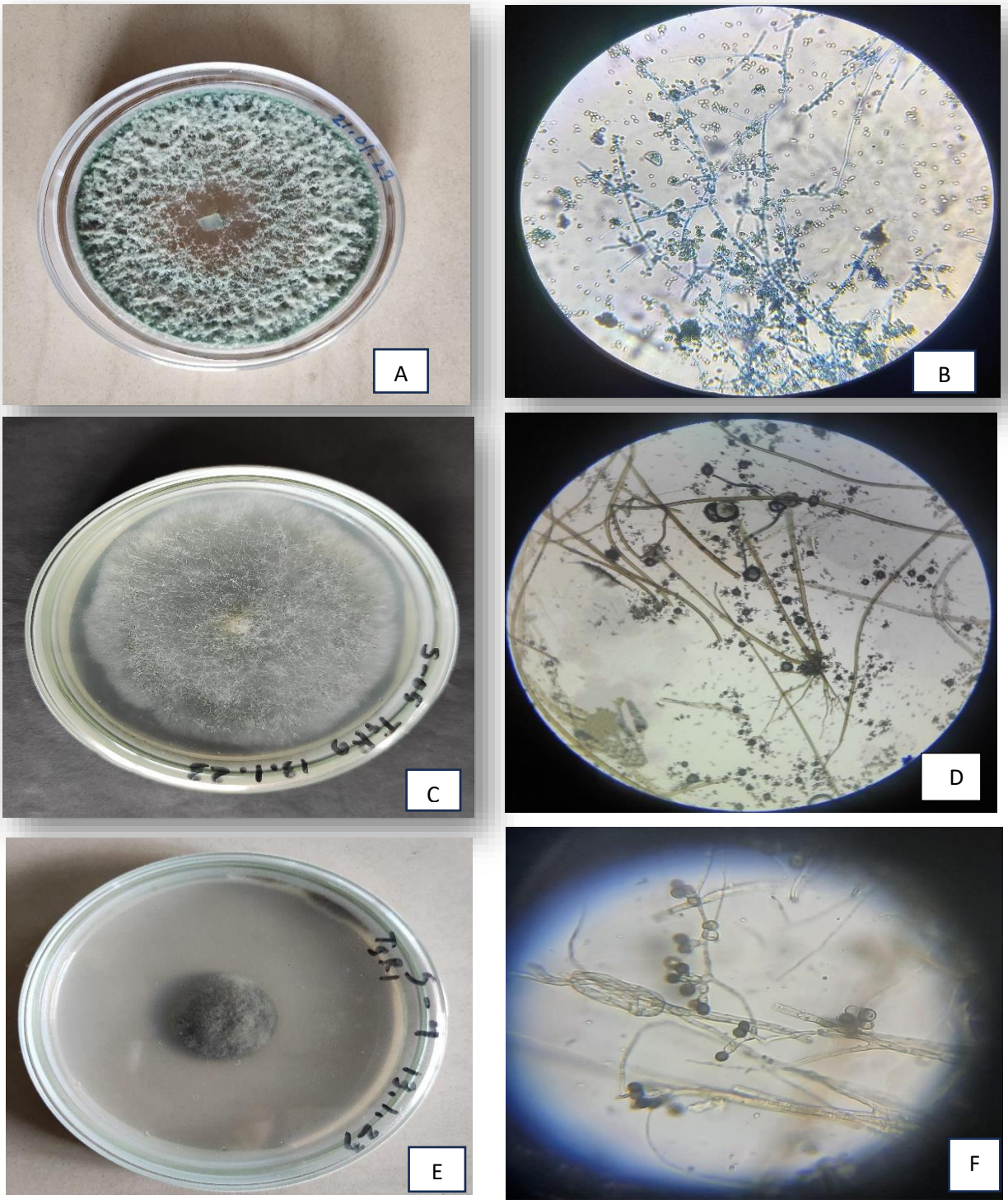

Plate 5. A. Pure culture of *Trichoderma asperellum*, **B.** Microscopic structure of *Trichoderma asperellum*, **C.** Pure culture of *Rhizopus* sp., **D.** Microscopic structure of *Rhizopus* sp., **E.** Pure culture of *Exophiala spinifera*, **F.** Microscopic structure of *E. spinifera*

4.3 Effect of different substrates on growth and yield contributing characters of oyster mushroom (*Pleurotus florida*)

4.3.1 Mycelium growth rate (cm/day)

Mycelium growth rate of oyster mushroom (*Pleurotus florida*) varied significantly due to different substrates (Table 1). The highest mycelial growth rate (0.48 cm) was recorded from T_3 whereas the lowest mycelial growth rate (0.41 cm) was found in T_5. The second highest mycelium growth rate (0.46 cm) was observed in T_2, which was closely followed by T_4 (0.44 cm) and T_1 (0.42 cm) respectively. It was observed that T_1 (0.42 cm) and T_5 (0.41) had statistically similar mycelial growth rate over the time in case of oyster mushroom (*Pleurotus florida*).

4.3.2 Days required to completion of mycelium running

Days required for mycelium running of oyster mushroom varied significantly due to different substrates (Table 1). The highest days (15.8 days) required for mycelium running was recorded from T_2 which was closely followed by T_4 (14.8 days), T_5 (14.2 days) and T_1 (13.7) . On the other hand, the lowest time (12.3 days) for mycelium running was found in T_3.

4.3.3 Days required for primordia formation

Statistically significant variation was recorded in terms of days required for primordia formation of oyster mushroom (*Pleurotus florida*) due to the use of different substrates (Table 2). The highest days (7.8 days) required for primordia formation was recorded from T_5. The second highest days (7.6 days) required for primordia formation was recorded from T_1 which was statistically similar to T_4 (7.3 days) and closely followed by T_2 (7.1 days), while the lowest time (6.6 days) was found in T_3.

4.3.4 Days required from primordia initiation to harvest

Different substrate showed statistically significant differences in terms of days required for primordia initiation to harvest of oyster mushroom (Table 2). The highest days (15.3 days) required for primordia initiation to harvest was observed from T_5 which was statistically similar to T_1 (14.9). The third highest days (13.6) required for primordia initiation to harvest was recorded from T_4 which was statistically similar to T_2 (12.8) days), while the lowest time (11.3 days) was found in T_3.

Table 1. Effect of substrates containing mycoflora on mycelium growth rate (cm/day) and days required to completion of mycelium running

Treatment	Mycelium growth rate (cm/day)	Days required to completion of mycelium running
T_1	0.42 c	13.7 c
T_2	0.46 ab	15.8 a
T_3	0.48 a	12.3 d
T_4	0.44 bc	14.8 b
T_5	0.41 c	14.2 bc
CV (%)	8.19	7.66
LSD value	0.03	0.98
Level of Significance	**	**

**- Significant at both 1% and 5%

In a column means having similar letter (s) are statistically similar and those having dissimilar letter (s) differ significantly at 0.05 level of probability

Legends

T_1 : Rice straw

T_2 : Sugarcane bagasse

T_3 : Paper waste

T_4 : Grass (*Cynodon dactylon*)

T_5 : Banana leaves

Table 2. Effect of mycoflora associated with mushroom substrates on days required for primordia formation and primordia initiation to harvest

Treatment	Days required for primordia formation	Days required from primordia initiation to first harvest
T_1	7.6 ab	14.9 a
T_2	7.1 bc	12.8 b
T_3	6.6 c	11.3 c
T_4	7.3 ab	13.6 b
T_5	7.8 a	15.3 a
CV (%)	9.47	7.53
LSD value	0.62	0.92
Level of Significance	**	**

**- Significant at both 1% and 5%

In a column means having similar letter (s) are statistically similar and those having dissimilar letter (s) differ significantly at 0.05 level of probability

Legends

T_1 : Rice straw

T_2 : Sugarcane bagasse

T_3 : Paper waste

T_4 : Grass (*Cynodon dactylon*)

T_5 : Banana leaves

4.3.5 Number of primordia

Statistically significant variation was recorded due to the effect of different substrates in terms of number of primordia/packet of oyster mushroom (Table 3). The maximum number of primordia/packet (37.9) was observed from T_3 which was closely followed by T_2 (36.2) and T_4 (34.2) and T_1 (32.6) . On the other hand, the minimum number of primordia/packet (30.9) was found in T_5.

Table 3. Effect of mushroom substrates and their contaminants on the number of primordia and fruiting body of oyster mushroom (*Pleurotus florida*)

Treatment	Number of primordia/ packet	Number of effective fruiting bodies/packet
T_1	32.6 d	13.2 d
T_2	36.2 b	15.6 b
T_3	37.9 a	17.5 a
T_4	34.2 c	14.3 c
T_5	30.9 e	12 e
CV (%)	4.37	8.25
LSD value	1.35	1.07
Level of Significance	**	**

**- Significant at both 1% and 5%

In a column means having similar letter (s) are statistically similar and those having dissimilar letter (s) differ significantly at 0.05 level of probability

Legends

T_1 : Rice straw

T_2 : Sugarcane bagasse

T_3 : Paper waste

T_4 : Grass (*Cynodon dactylon*)

T_5 : Banana leaves

4.3.6 Number of effective fruiting body/packet

The maximum number of effective fruiting body/packet (17.5) was observed from T_3 (Table 3) which was closely followed by T_2 (15.6). The third highest number of fruiting body/packet (14.3) was found in T_4 which was followed by T_1 (13.2) while the minimum number (12) was recorded in T_5.

4.3.7 Biological yield (g)

Statistically significant variation was recorded in terms of biological yield of oyster mushroom (Table 4). The highest of biological yield (144.2 g) was found from T_3 which was statistically similar to T_2 (140.6 g). The third highest biological yield was observed in T_4 (134 g) which was followed by T_1 (128.8) while the lowest biological yield (119.7g) was recorded in T_5.

4.3.8 Economical yield (g)

Economical yield of oyster mushroom showed statistically significant variation due to use of different substrates (Table 4). The highest economical yield (113.3 g) was recorded from T_3 which was followed by T_2 (107 g), T_4 (100.6 g) and T_1 (93.2 g) whereas the lowest economical yield (86.2 g) was found in T_5.

4.3.9 Diameter of pileus (cm)

Diameter of pileus showed statistically significant differences due to the effect of substrates in terms of diameter of pileus of oyster mushroom (Table 5). The highest diameter (5.03 cm) of pileus was found from T_4 which was followed by T_3 (4.94 cm)T_2 (4.90 cm) and T_1 (4.82 cm) respectively. On the other hand the minimum diameter (4.74 cm) of pileus was observed in T_5.

4.3.10 Length of stipe (cm)

Length of stipe of oyster mushroom varied significantly due to different substrates (Table 5). The highest length (2.84 cm) of stipe was recorded from T_4 whereas the second highest length (2.76 cm) was observed in T_2 which was closely followed by T_3 (2.69 cm) and T_1 (2.62 cm). However, T_5 showed minimum length (2.26 cm) of stipe for all the time.

Table 4. Effect of mycoflora associated with substrates on different yield contributing parameters of oyster mushroom (*Pleurotus florida*)

Treatment	Biological yield (g)	Economical yield (g)
T_1	126.8 c	93.2 d
T_2	140.6 a	107 b
T_3	144.2 a	113.3 a
T_4	134 b	100.6 c
T_5	119.7 d	86.2 e
CV (%)	4.33	2.86
LSD value	5.18	2.57
Level of Significance	**	**

**- Significant at both 1% and 5%

In a column means having similar letter (s) are statistically similar and those having dissimilar letter (s) differ significantly at 0.05 level of probability

Legends

T_1 : Rice straw

T_2 : Sugarcane bagasse

T_3 : Paper waste

T_4 : Grass (*Cynodon dactylon*)

T_5 : Banana leaves

Table 5. Effect of oyster mushroom (*Pleurotus florida*) substrates and their contaminants on the pileus diameter and stipe length

Treatment	Pileus diameter (cm)	Stipe length (cm)
T_1	4.82 bc	2.62 cd
T_2	4.90 b	2.76 ab
T_3	4.94 ab	2.69 bc
T_4	5.03 a	2.84 a
T_5	4.74 c	2.58 d
CV (%)	2.89	4.42
LSD value	0.127	0.107
Level of Significance	**	**

**- Significant at both 1% and 5%

In a column means having similar letter (s) are statistically similar and those having dissimilar letter (s) differ significantly at 0.05 level of probability

Legends

T_1 : Rice straw

T_2 : Sugarcane bagasse

T_3 : Paper waste

T_4 : Grass (*Cynodon dactylon*)

T_5 : Banana leaves

4.3.11 Contamination severity

The contamination severity was varied from 0.4 to 6.8% where the maximum contamination was found in banana leaves and the minimum contamination was recorded in waste paper . The contamination severity was 4% in case of rice straw which was closely followed by grass and sugarcane bagasse where the severity was 3.2% and 2.6% respectively (Figure 3).

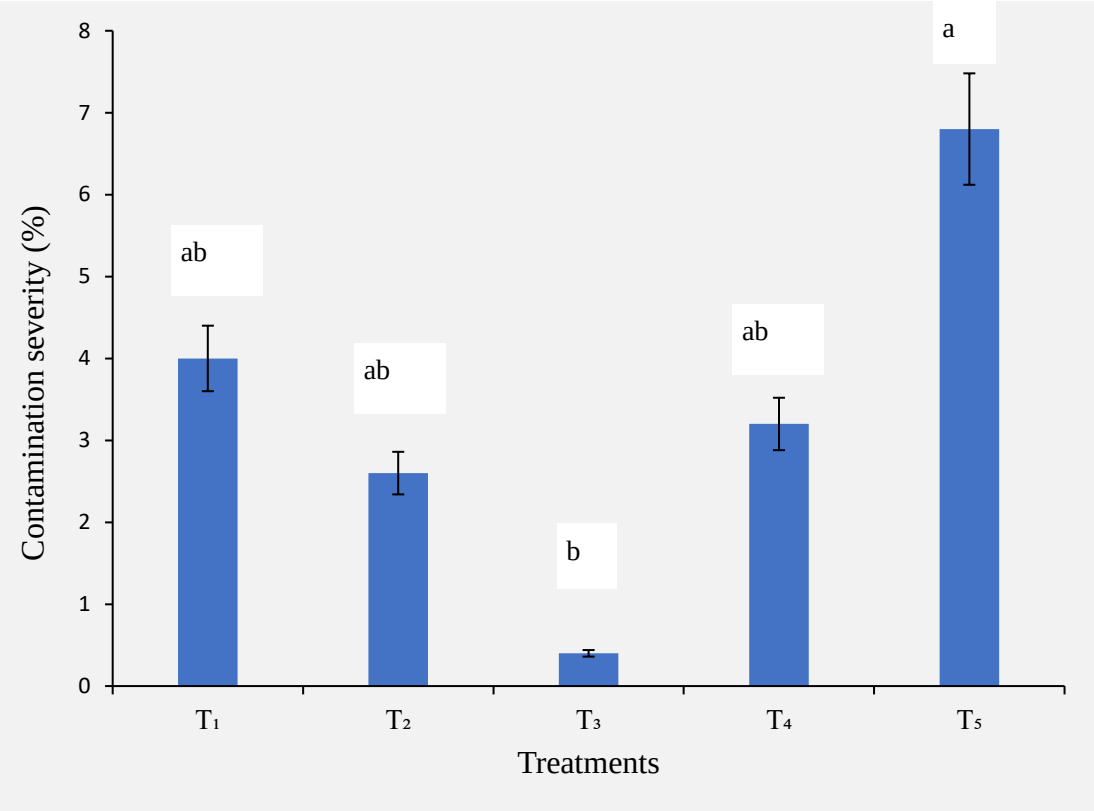

Figure 3. Effect of mycoflora associated with substrates of oyster mushroom (*Pleurotus florida*) on contamination severity

Legends

T_1 : Rice straw

T_2 : Sugarcane bagasse

T_3 : Paper waste

T_4 : Grass (*Cynodon dactylon*)

T_5 : Banana leaves

4.3.12 Biological efficiency

The maximum of biological efficiency (28.84%) was observed from T_3 followed by T_2 (28.12%), while the minimum (23.94%) biological efficiency was recorded in T_5. So, it was noted that waste paper was among the used substrates for cultivation of *Pleurotus florida* (Figure 4).

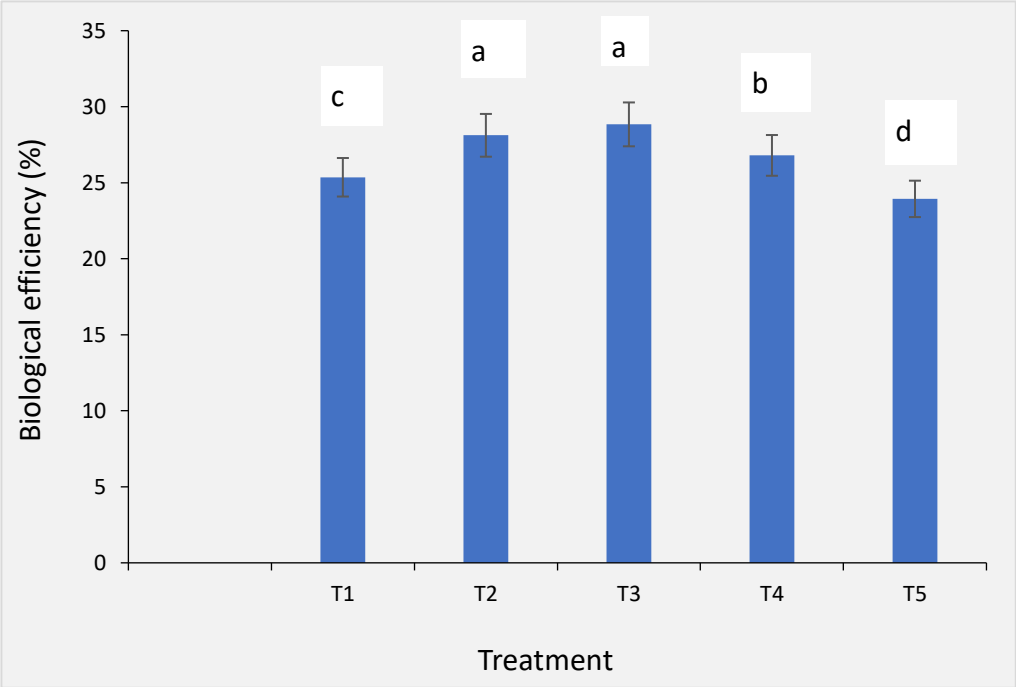

Figure 4. Effect of substrates and their contaminants on biological efficiency of oyster mushroom (*Pleurotus florida*)

Legends

T_1 : Rice straw

T_2 : Sugarcane bagasse

T_3 : Paper waste

T_4 : Grass (*Cynodon dactylon*)

T_5 : Banana leaves

4.3.13 Functional relationship between economic yield and biological efficiency, contamination severity and biological efficiency and effective fruiting body and biological efficiency

The biological efficiency of oyster mushroom was correlated positively with economic yield, and number of effective fruiting body. The value of correlation ($R^2 = 0.9926$) was linear and could be expressed by the regression equation by $y = 0.1851x + 8.0902$ (Figure 10 A). The relationship of biological efficiency and contamination severity are shown in Figure 9 B. The relationship was also linear but negatively correlated, where the biological efficiency decreased gradually when the contamination severity increased. The relationship might be expressed by the equation $y = -0.8224x + 29.408$ ($R^2 = 0.9142$) . Strong linear correlation ($R^2 = 0.9495$) was also observed between effective fruiting body and biological efficiency, where the equation was $y = 0.9134x + 13.35$, stated that the biological efficiency increased gradually at the rate of 0.9134% (Figure 10 C).

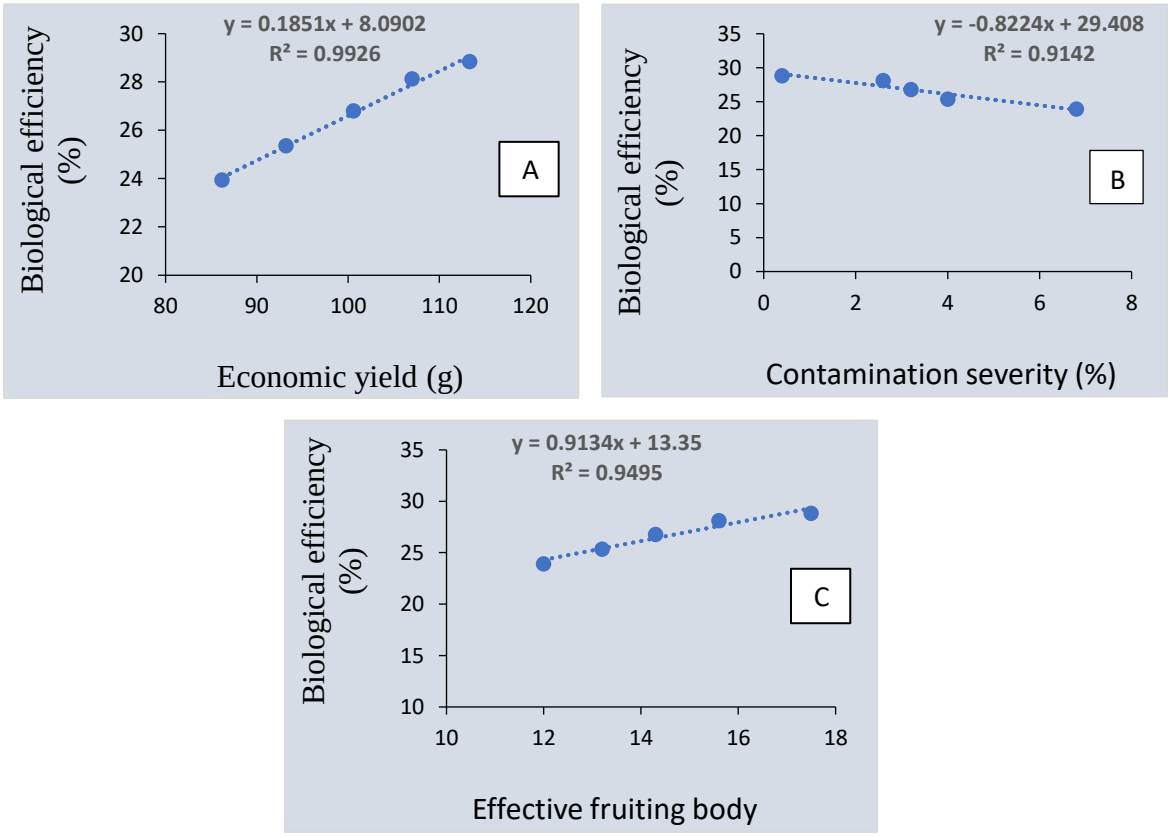

Figure 5. A. Relationship between biological efficiency and economic yield.

B. Relationship between biological efficiency and contamination severity

C. Relationship between biological efficiency and effective fruiting body

4.4 DNA sequencing of fungal isolates

DNA sequence of isolated fungi were submitted to National Center for Biotechnology (NCBI) for deposition in the Gene Bank and obtain accession number. The fungal isolates which matches the nucleotide sequences of the studied genes with respect to the fungal isolates were placed in the search and their species were identified according to the match in the aforementioned database. Three fungal isolates; *Trichoderma* sp., *Rhizopus* sp., and *Exophiala spinifera* isolates were used for DNA sequencing. Among the three fungal isolates, the DNA sequence of *Trichoderma* and *Exophiala* spp. were found in the Basic Local Alignment Search Tool (BLAST) with the database at the National Center for Biotechnology Information (NCBI). Nucleotide sequences of *Exophiala* spp. had a 99% match with *Exophialia spinifera* whereas *Trichoderma* sp. has 65% and 99%nucleotide identities with *Trichoderma asperellum* in case of the use of forward (ITS1) and reverse primers (ITS4), respectively. However, the molecular identification of *Rhizopus* sp. was not possible even after two trials.

4.4.1 DNA sequence of *Exophialia spinifera* by ITS1 and ITS4 primers

GGTCCTCACAGGCCCGACCTCCCAACCCATTGTTTATGATACTCAGTGTTGCTTCGGCAGGCC
TGGTCTTTGACCTGCCGGAGGGCCGTAACACGCCCCCCGGAGAGCGCCTGCCGACGGCCCCA
ACTTCAAAATTCTTAACTAAACATGTCTTTGTCTAAGTAAAGTCTTTAATAAAAGCAAAACTTT
CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGCG
AATTGCAGAATTCTCGTGAGTCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGAA
GGGCATGCCTGTTCGAGCGTCATTTTCACCCCTCAAGCCCCCGGCTTGGTGTTGGACGGTTTG
GTCCCGGGACCCCCCTGGACCCCTCCCAAAGACAATGACGGCGGGCTGTCGCACCCCCGGTA
CACTGAGCATCTTCACGGAGCACGTACCGGTCTTCAGGGACGACGGCACCCGGTCTCACACC
TATATCTTTCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAA
GCCGGAGGAA

CTACCTGATCCGAGGTCAACCTTGAAAGATATAGGTGTGAGACCGGGTGCCGTCGTCCCTGAA
GACCGGTACGTGCTCCGTGAAGATGCTCAGTGTACCGGGGGTGCGACAGCCCGCCGTCATTG
TCTTTGGGAGGGGTCCAGGGGGGTCCCGGGACCAAACCGTCCAACACCAAGCCGGGGGCTT
GAGGGGTGAAAATGACGCTCGAACAGGCATGCCCTTCGGAATACCAAAGGGCGCAATGTGCG
TTCAAAGATTCGATGACTCACGAGAATTCTGCAATTCGCATTACTTATCGCATTTCGCTGCGTT
CTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGCTTTTATTAAAGACTTTACT
TAGACAAAGACATGTTTAGTTAAGAATTTTGAAGTTGGGGCCGTCGGCAGGCGCTCTCCGGG
GGGCGTGTTACGGCCCTCCGGCAGGTCAAAGACCAGGCCTGCCGAAGCAACACTGAGTATCA
TAAACAATGGGTTGGGAGGTCGGGCCTGTGAGGACCCTAACTCGGTAATGATCCCTCCGCAG
GTTCACCTACGAAGACCTTGTTACGACTTTTACTTCCTCTAATGGACCAAG

4.4.2 DNA sequencing of *Trichoderma asperellum* by ITS1 and ITS4 primers

GGTTGGAATGTCTTTGTTCCTGACAGGGGACGTCCTAACGGGGGACTACTCCGGACCCGTCC
CGCCGGCCTCCCAGTGCCGGTTCCGCGGGTACTGCCCACGGGAGGCTGCCATCATACCAACC
CTGCATCTCGGGGACGAACCCCCCGTGAGGGGTCCGAATTCCCCACGCAGACCCACCTCTTG
GGTTTCTGGGTTGAAATGATGGCCTGAATGAACAGCCCACCACAATTTGTTAGGCGTAATGTG
TGCGAAAAATTCAATGATATGCTAAAATTTATGTTACTCATTACTTACCGTATTTCTTTGCGTCTT
TCATCGCTGATTGAACCCGCAAATCTTCTGGCGAACCTGCCGATCCGAGTTGAATATCAGCCC
TCCAACCCCTCCGGGCGACCCGGAGGAGGAACGGAACCCCTCTGGGGGTGCCCTCCCCCGG
ATACCGGGGTCCTGGGCTGCGAACCTCCCCGGGCCTGAACCCGGCAAAGCTCCAACTGGGAG
ACCTGCACATCGGCTTTCGGAGTAGACCCCTCTTTCATAATTGTTCAGCTGGTATCAGGAAGG
AATACCTTGTTGAACTTTTTACTATCCTTAAATGAAGGAG

CTACCTGNTCCGAGGTCACATTTCAGAAAGTTGGGTGTTTTACGGACGTGGACGCGCCGCGC
TCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATT
TCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATCCCCCGGAGGGGTTCGA
GGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTC
AAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCA
TCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGA
GCTGTAAGAAATACGTCCGCGAGGGGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGC
GCCTGGTTCCGGGGCTGCGACGCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGTAAC
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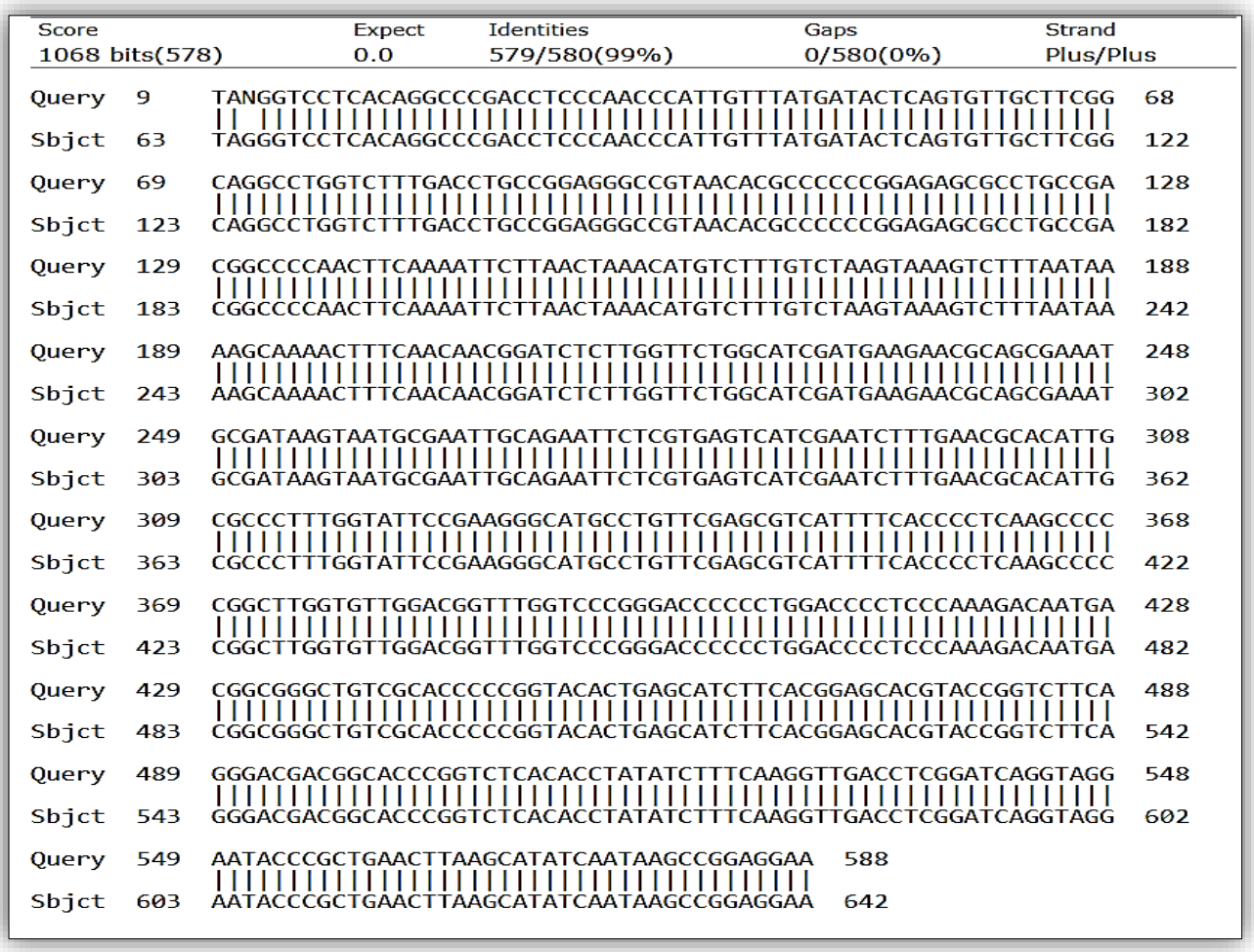
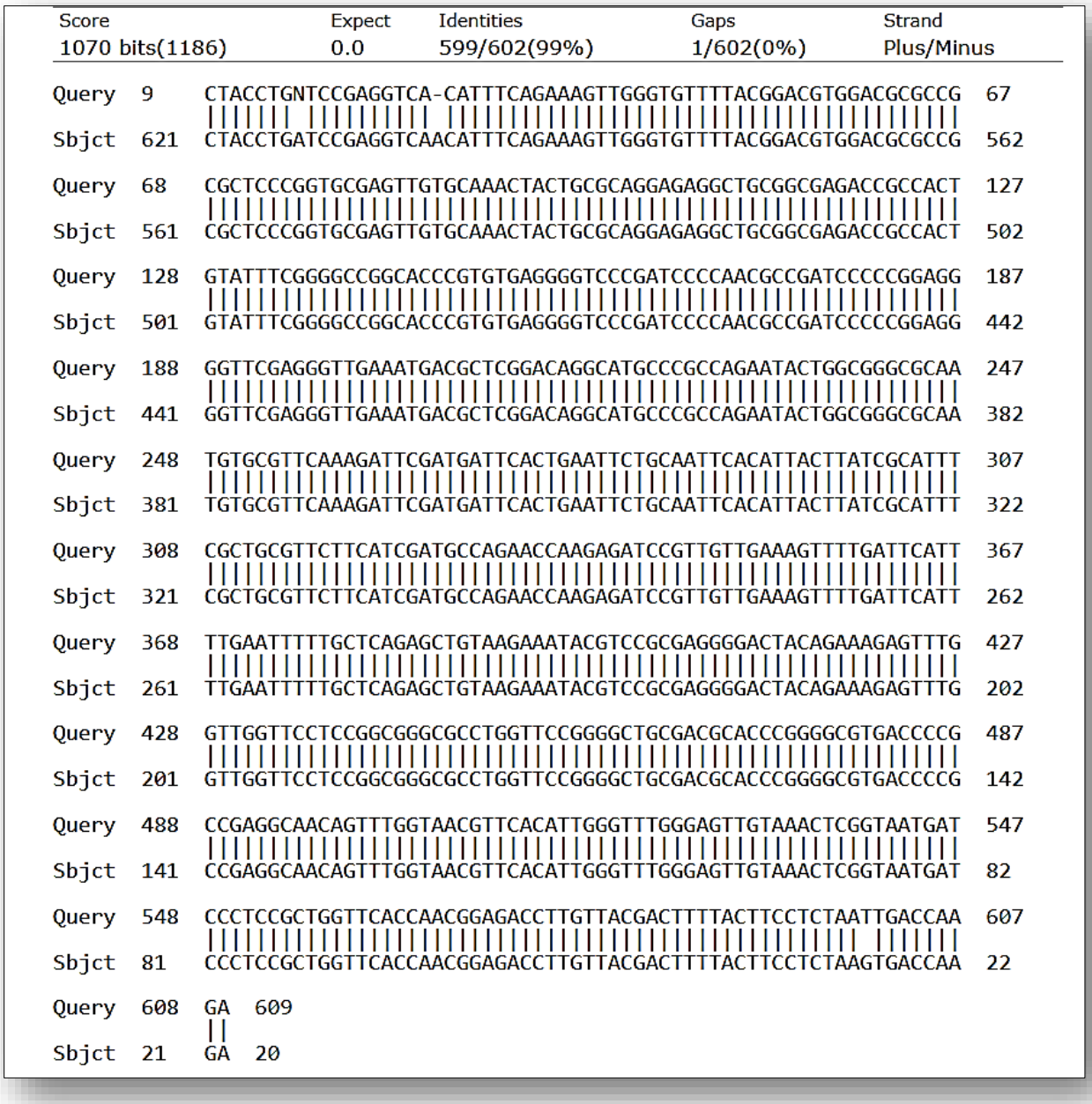


Figure 6. Variation pattern in ITS region of rDNA of representative strains of *Exophiala spinifera*



Score	Expect	Identities	Gaps	Strand
1070 bits(1186)	0.0	599/602(99%)	1/602(0%)	Plus/Minus

```
Query  9    CTACCTGNTCCGAGGTCA-CATTTCAGAAAGTTGGGTGTTTTACGGACGTGGACGCGCCG  67
            ||||||| |||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  621  CTACCTGATCCGAGGTCAACATTTCAGAAAGTTGGGTGTTTTACGGACGTGGACGCGCCG  562

Query  68   CGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACT  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561  CGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACT  502

Query  128  GTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATCCCCCGGAGG  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501  GTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATCCCCCGGAGG  442

Query  188  GGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAA  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  GGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAA  382

Query  248  TGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTT  307
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Sbjct  381  TGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTT  322

Query  308  CGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATT  367
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Query  368  TTGAATTTTTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACTACAGAAAGAGTTTG  427
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Query  428  GTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCGGGGCGTGACCCCG  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  GTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCGGGGCGTGACCCCG  142

Query  488  CCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTGTAAACTCGGTAATGAT  547
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Sbjct  141  CCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTGTAAACTCGGTAATGAT  82

Query  548  CCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTTTACTTCCTCTAATTGACCAA  607
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  81   CCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTTTACTTCCTCTAAGTGACCAA  22

Query  608  GA  609
            ||
Sbjct  21   GA  20
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Figure 7. Variation pattern in ITS region of rDNA of representative strains of *Trichoderma asperellum*

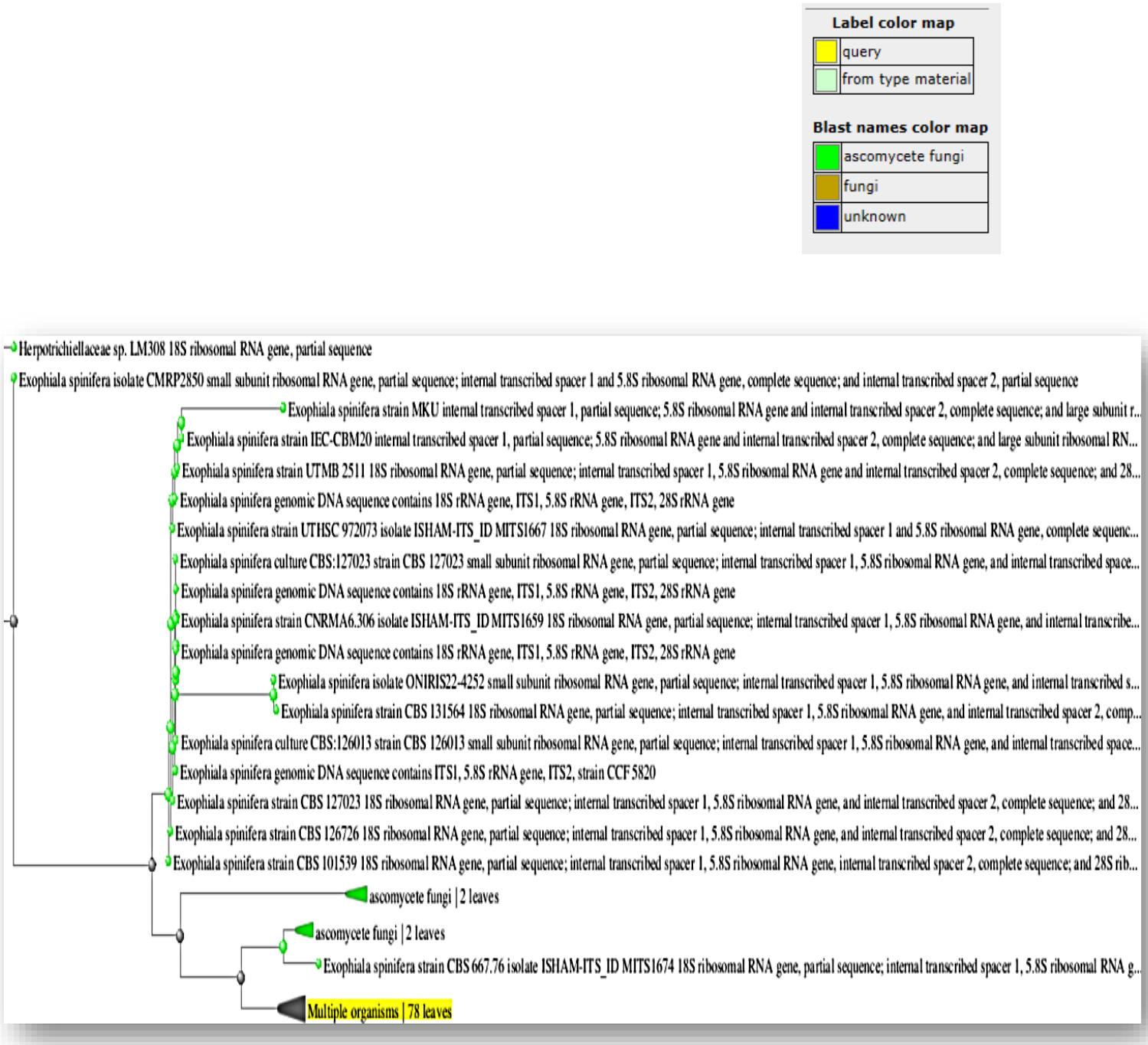

Figure 8. BLAST distance tree between the two sequence of *Exophiala spinifera*. Only the higher scoring sequence is included in the tree.

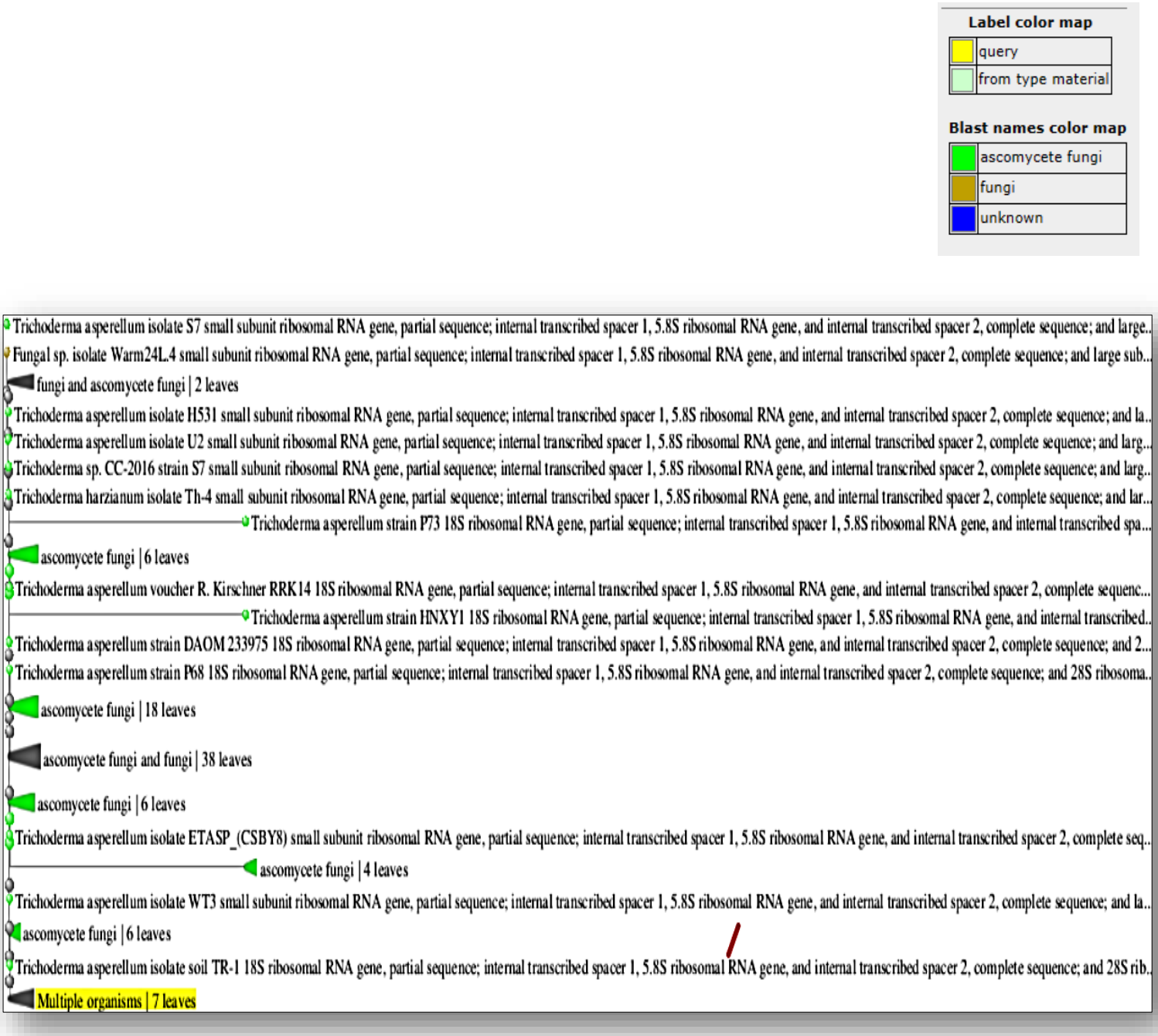

Figure 9. BLAST distance tree between the two sequence of *Trichoderma asperellum*. Only the higher scoring sequence is included in the tree.

Table 6. Percent identities of nucleotide of *Exophiala spinifera* forward and reverse primer (ITS 1 and ITS 4) collected from NCBI database

Accession	Description	Max score	Total score	Query coverage	E value	Identity
MH511829.1	*Exophiala spinifera* culture NCCPF:106024 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.	1068	1068	98%	0.0	**99.83%**
MH864216	*Exophiala spinifera* culture CBS:126742strainCBS126742 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.	1116	1116	96%	0.0	**100%**

Table 7. Percent identities of nucleotide of *Trichoderma asperellum* for forward and reverse primer (ITS 1 and ITS 4) collected from NCBI database

Accession	Description	Max score	Total score	Query coverage	E value	Identity
KU170986	*Trichoderma asperellum* isolate Tasp19 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence.	65.3	65.3	46%	3e-05	**65.60%**
KF815050.1	*Trichoderma asperellum* strain LAHC-FFPK-M16 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	1096	1096	98%	0.0	**99.50%**

CHAPTER V
DISCUSSIONS

The present experiment was conducted to identify and characterize *Trichoderma* spp. and other pathogens present in substrates of oyster mushroom (*Pleurotus florida*) based on morphological and molecular characteristics. In the present study the fungi were identified by investigating their cultural and morphological characters under microscope and then their molecular study was done. Three contaminants namely *Rhizopus* sp, *Trichoderma asperellum*, and *Exophialia spinifera* were isolated and identified from contaminated substrates and more or less similar findings have been reported by previous scientists. According to Pervez *et al*., (2010) weed mycoflora namely *Aspergillus* spp, *Penicillium* spp., *Rhizopus stolonifer* and *Trichoderma harzianum* were found to be associated with the substrate of oyster mushroom at different growth stages. However, in our present findings, we found *Trichoderma asperellum* associated with the substrates of oyster mushroom. Spillman (2002) recognized *Trichoderma* as green mould on the production bed of oyster mushroom. On oyster mushroom beds, *Trichoderma* sp., *Aspergillus* sp., and *Rhizopus* sp. were the most common microorganisms; their presence was particularly severe in the summer and spring seasons as opposed to the fall and winter (Jaivel and Marimuthu 2010). The airborne microflora in the incubation chamber, according to reports by Oxaley (1985) and Earanna (1991), is thought to be the cause of the spawn contamination. Air containing air-borne microorganisms may enter the autoclaved bags during the mother spawn transfer and contaminate the spawn during incubation. In the current investigation, similar kinds of findings have been noted.

The present findings of the experiment differed with the findings of Biswas and Kuiry (2013) reported that *Aspergillus niger*, *Coprinus sp*, *Penecillium* sp and *Sclerotium rolfsii* were the most predominant fungal contaminant of mushroom beds of *P. florida*. However, in our present findings, we found *Trichoderma asperellum, Rhizopus* sp. are more common *fungi* associated with the substrates of *pleurotus florida*.

Thick green sporulation on the substrate surface is a defining feature of *Trichoderma*-caused green mold infections (Hatvani *et al*., 2007). *Trichoderma* produce conidia rapidly and abundantly, and because of their little weight, they may swiftly disperse in the air. These floating conidia may adhere to the fungal cell wall and be identified by lectin (Barak *et al*., 1986). In earlier research,

the isolation and identification of pathogenic fungi responsible for green mold diseases of *Pleurotus* was the main emphasis (Choi *et al*., 2003 and Woo *et al*., 2004). In the current work, we carried out molecular identification to characterize *Trichoderma* spp. According to Hatvani *et al*. (2007), *Trichoderma asperellum* is one of the species that causes green mold illness in *Pleurotus* substrates. The culture of *P. ostreatus* revealed that substrates exposed to high temperatures were more susceptible to *Trichoderma* infection. From contaminated oyster mushroom substrates, several *Trichoderma* species have been isolated (Kredics *at al*., 2009; Choi *et al*., 2003).

In our present findings, *T. asperellum* was one of the most common strains in green molded substrate. High temperature can cause *T. asperellum* mycelia to produce a large number of conidia in a very short period of time and speed up the spread of conidia, resulting in a large area of affected by green mold disease. *Trichoderma asperellum* is morphologically indistinguishable from its cryptic species *T. asperelloides* (Samuels *et al*., 2010). *T. asperellum* is a successful biocontrol agent against *F. oxysporum*. f. sp. *lycopersici* (El Komy *et al*., 2015), *R. solani, S. rolfsii* (Hamed et al., 2015), and *Gibberella fugikorai (*Watanabe *et al*., 2007).

Green mold, which was mostly caused by several species of *Trichoderma*, *Rhizopus*, *Penicillium*, and *Aspergillus*, was identified by Sharma *et al*. (2007) as one of the most prevalent and devastating diseases in the mushroom production industry. In this experiment, a comparable outcome was seen. Choi *et al*. (2003) stated that *Trichoderma* spp. conidia are ellipsoidal and ovoid, while the phialides are lageniform and bowling pin shaped. Komon-Zelazowska *et al*. (2007) examined that infections with *Trichoderma* sp. in edible basidiomycetes have long been recognized. From contaminated spawn, *T. harzianum* was shown to be the main cause for the most serious issues. According to Hatvani *et al*. (2007), green mold disease of *P. ostreatus* have been found in South Korea, Italy, Hungary, and Romania in mushroom farms. The results of our study were more or less consistent with the findings of Won (2000) and Largeteau-Mamoun *et al*. (2002) who stated that it is challenging to identify the infection of *Trichoderma* at the stage when *Trichoderma* produces white mycelia that are identical to those of mushrooms during the spawn phase.

In Korea, oyster mushrooms are seriously affected by green mold disease, which is caused by *Trichoderma* spp. For the mushroom producers, it results in significant financial losses. In the article, efforts have been made to identify green mold using both morphological and molecular

features, as the identification based on morphological attributes alone is deceptive (Kim *et al*., 2000). Similar to button mushrooms, oyster mushrooms are susceptible to the same green mold disease symptoms and causative organisms. Danesh *et al*. (2000) also recognized *Trichoderma* sp., *T. virens*, *T. longibrachiatum*, and *T. harzianum* as the causative agents for green mold disease in *Agaricus bisporus* beds. The mushroom substrates are heavily covered in the mold, the cell walls of the mushrooms are lysed, and the mycelia of the mushrooms are able to access nutrients. These are indications of the green mold illness. According to Goltapeh and Danesh (2000), *Trichoderma* has the ability to secrete hydrolytic enzymes like cellulases, chitinases, and -glucanases that lyse the cell walls of mushrooms and are thought to be fundamental to the fungus' ability to mycoparasitically consume other organisms.

The morphological characterization of *Trichoderma* spp. isolated from oyster mushroom growing substrates was done before based on morphology such as colonies, hyphae, conidiophores, phialides and conidia. In the present study, the emphasis was given on conidiophores spread type i.e gathering or non-gathering type and phialides-type fertile or non-fertile. *T. asperellum* was characterized by finer conidial ornamentation, slightly ovoidal conidia, mostly paired branches ampulliform phialides, and consistent presence of chlamydospores.

For taxonomic findings in a variety of taxa, including *Trichoderma* spp. and oyster mushrooms, molecular approaches like RAPD and rDNA sequencing have been widely applied (Bae *et al*., 1996; Kang *et al*., 2001; Kim *et al*., 2000; Samuels *et al*., 1994, 2002). Correlating molecular phylogeny with morphological and other biochemical and physiological features is so essential. *Trichoderma* species, the Ascomycetes *Hypocrea jecorina*, and *T. harzianum* strains that were harmful to the commercial production of mushrooms were all categorized using RAPD fingerprinting and rDNA sequencing (Muth-umeenakshi *et al*., 1994), as well (Kubicek and Harman, 1998). In the present study, we observed genetic diversity of *T. asperellum* from the other *Trichoderma* spp. isolates based on nucleotide patterns. Both the primers (ITS1 and ITS4) were used in classifying and grouping of *Trichoderma* isolates. ITS1 and ITS4 primers were helpful to identify the molecular characteristics of *Trichoderma asperellum*.

The *Exophiala spinifera* clade was first defined by (Haase *et al*., 1999) based on an analysis of SSU rDNA of the Herpotrichiellaceae *Exophiala* species are dematiaceous (black-pigmented)

saprophytic molds that are frequently isolated from hot and humid environments that are nutrient deficient but rich in hydrocarbons (Lian and De Hoog, 2010; Zhao *et al*., 2010; Woo *et al*., 2013).

Scanning electron microscopy (SEM) makes it easy to see that *Exophiala spinifera* is especially distinctive for its conidiophores and annellated zones, which are long cylindrical structures with multiple annellations (Nishimura *et al*., 1985). This genus contains more than 30 species and is a part of the Herpotrichiellaceae family. Due to the early morphological characteristics of their colonies, they are sometimes referred to as "black yeasts" (Matsumoto *et al*., 1987; Woo *et al*., 2013). In addition to colonizing the human intestinal and respiratory tract, notably in the airways of individuals with cystic fibrosis (De Hoog *et al*., 2005; Kondori *et al*., 2011), they are also isolated from environmental samples.

Exophiala species are challenging to identify using phenotypic testing, and many molecular approaches have been proposed for reliable species identification. These findings are supported by (Zeng *et al*., 2007; Woo *et al*., 2013; Silva *et al*., 2017). For this species, disagreements between morphological and genetic identification techniques have been documented in the past (Zeng *et al*., 2007). In the current work, we extract *Exophiala* spp. from the substrate of mushroom and describe it phenotypically and by ITS sequencing for precise species-level identification. In our present experiment, *Exophiala spinifera* was found on sugarcane bagasse which indicates that *E spinifera* might cause disease on mushroom substrate though the findings has not been demonstrated yet because human skin diseases are often caused by this fungus.

In this study, among the five substrate namely rice straw, sugarcane bagasse, waste paper, grass, and banana leaves, waste paper showed the best performance in all cases. The maximum growth rate (0.48 cm) was recorded from waste paper whereas the minimum growth rate (0.41 cm) found in banana leaves. The maximum days (15.8 days) required for mycelium running was recorded from sugarcane bagasse while the lowest time (12.3 days) was observed in waste paper. The present study is an agreement with Kumari and Achal (2008) who noted that colonization of the substrate with *P. ostreatus* was completed within 20 days of inoculation. Girmay *et al*. (2016) reported that mycelia running on waste paper took 14 days. Sharma *et al*. (2013) reported shorter pining period (26.40–31.60 days of incubation) on various substrates which is differed from our present findings. In our study, shorter pinning period was 19 days for waste paper from the inoculation. The maximum number of primordia was observed in waste paper (37.9) whilst

minimum primordia was found in banana leaves (30.9) . Waste paper showed significantly best performance with the total number of effective fruiting body and yield per spawn packets and this findings is similar with the findings of Sarker *et al*., (2007 a) who reported that the highest economic yield was estimated from the waste paper. The highest length (2.84 cm) of stipe was recorded from grass whereas the minimum length (2.26) of stipe was found in banana leaves. The highest of biological yield (144.2 g) was found from waste paper which was followed by sugarcane bagasse (140.6) g), while the lowest biological yield (119.7 g) was recorded in banana leaves which is supported by the previous study of Sarker *et al*., (2007a). Biological efficiency varies from substrate to substrate. Similar results were found by scientist previously. According to Bernardi *et al*., (2007) the productivity and biological efficiency will vary according to different strains and various kinds of substrates used. This confirms the finding of Mandeel *et al*., (2005) that B.E is highly affected by the quality of the spawn of the cultivated mushroom strain. Different substrates have been used to grow *Pleurotus* sp. with BE values varying from 32.10 - 79.18% (Dhanda *et al*., 1994).

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 April to October, 2022. The study was conducted to isolate, identify and characterize *Trichoderma* spp. and other contaminants present in substrates of oyster mushroom based on morphological and molecular characteristics, determine the contamination severity of different substrates of oyster mushroom and to find out the impact of mycoflora on the growth and yield contributing characters of oyster mushroom.

For this purpose, five types of mushroom substrates were prepared and total three fungal contaminants viz., *Trichoderma asperellum*, *Rhizopus* sp., *Exophialia spinifera* were isolated from different substrates of oyster mushroom (*Pleurotus florida*) by studying the cultural and morphological characteristics. *Trichoderma asperellum* was identified by finer conidial ornamentation, slightly ovoidal conidia, paired branches and ampulliform phialides whereas *Exophiala spinifera* was characterized by its black and budding yeast like conidia. To perform the molecular study, pure culture of fungal isolates were sent to the Central Dogma Laboratory, Banani, Dhaka. After the DNA extraction, PCR, and gel electrophoresis, DNA sequencing result analysis of *Trichoderma asperellum and Exophialia spinifera* was done by Basic Local Alignment Search Tool (BLAST) with the help of NCBI. DNA sequence of *Exophiala spinifera* and *Trichoderma asperellum* were matched by 99.83% (ITS1) and 100% (ITS4) as well as 65.60% (ITS1) and 99.50% (ITS4) with the nucleotide sequence of NCBI gene bank, respectively. The molecular identification of *Rhizopus* spp. was not possible even after two trials.

The maximum contamination with fungi was found in banana leaves where the severity was 6.8%. The contamination severity was 4% in case of rice straw which was closely followed by grass and sugarcane bagasse where the severity was 3.2% and 2.6%, respectively. The minimum contamination with fungi was observed in waste paper where the severity was 0.4%.

The rate of mycelium growth (cm/d) varied from substrate to substrate due to the impact of different mycoflora associated with them. The maximum growth rate (0.4826 cm) was recorded from waste paper whereas the minimum growth rate (0.4181 cm) found in banana leaves. The

maximum days (15.8 days) required for mycelium running was recorded from sugarcane bagasse while the lowest time (12.3 days) was observed in waste paper. The maximum number of primordia was observed in waste paper (37.9) whilst minimum primordia was found in banana leaves (30.9). Waste paper showed significantly best performance with the total number of effective fruiting body and yield per spawn packets. The maximum pileus diameter (5.03) and stipe length (2.84 cm) were recorded from grass whereas the minimum pileus diameter (3.61) and stipe length (2.26) were found in banana leaves. The maximum biological yield (144.2 g), economic yield (113.3) and biological efficiency (28.84%) were found from waste paper which was followed by sugarcane bagasse, while the minimum biological yield (119.7 g), economical yield (86.2 g) and biological efficiency (23.94%) were recorded in banana leaves.

From the present research work it may be concluded that,

- Total three fungal contaminants viz., *Trichoderma* sp., *Rhizopus* sp. and *Exophiala* sp. were isolated from different contaminated spawn of oyster mushroom morphologically.

    - *Trichoderma asperellum* and *Exophiala spinifera* were identified by the Basic Local Alignment Search Tool (BLAST) in the database of National Center for Biotechnology Information (NCBI) through molecular characterization.

    - DNA Nucleotide sequences of *Exophiala* sp. of this experiment was matched by 99.83% (ITS1) and 100% (ITS4) with *Exophiala spinifera* of BLAST.

    - However, the nucleotide sequence of *Trichoderma* sp. was matched with *Trichoderma asperellum* of BLAST by 65.60% and 99.50% in case of forward (ITS1) and reverse primers (ITS4), respectively.

- Minimum contamination severity of spawn packets was observed in waste paper whilst the maximum was found in banana leaves.
- Waste paper showed the best performance on yield contributing parameters of oyster mushroom (*Pleurotus florida*) whereas banana leaves showed the lowest performance in all cases.

CHAPTER VII
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