Studies on diversity and physiological characterization of morels of the Western Himalayan region

A Thesis
Submitted in fulfilment of the requirements
for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

Harpreet Kaur Kanwal
(Regd. No. 90700001)

Department of Biotechnology and Environmental Sciences
Thapar University
Patiala-147004
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Certificate

It is hereby certified that the thesis **"Studies on diversity and physiological characterization of morels of the Western Himalayan region"** which is submitted by Ms. **Harpreet Kaur Kanwal, (Regd No. 90700001)**, in fulfilment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

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Declaration

I hereby declare that the work which is being presented in the thesis **"Studies on diversity and physiological characterization of morels of the Western Himalayan region"** submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the able supervision of Dr. M. Sudhakara Reddy, Professor and Head, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

Harpreet Kaur Kanwal.

(Harpreet Kaur Kanwal)

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The following publications are the outcome of the research work carried out in this Thesis.

- **Kanwal HK, Acharya K, Ramesh G, Reddy MS (2011) Molecular characterization of *Morchella* species from the Western Himalayan region of India. *Curr Microbiol.* 62(4):1245-1252**

- **Kanwal HK, Reddy MS (2010) Effect of carbon, nitrogen sources and inducers on ligninolytic enzyme production by *Morchella crassipes*. *World J Microbiol Biotech* 27:687–691**

- **Kanwal HK, Reddy MS (2011) The effect of carbon and nitrogen sources on the formation of sclerotia in *Morchella* spp. *Annals Microbiol* DOI:10.1007/S13213-01-0241-6**

- **Lignolytic enzyme production and substrate utilization by *Morchella crassipes* during sclerotia formation. (Communicated to *J Food Sci*)**

Conferences

- **Harpreet Kaur Kanwal** and M. Sudhakara Reddy (2011). Ligninolytic enzyme production and substrate utilization by Morel mushroom *Morchella crassipes* during sclerotia formation during its cultivation on different agrowastes. **National symposium on Fungal Biology and applications.** Department of Botany, Punjabi University, Patiala, Punjab, India.
- **Harpreet Kaur Kanwal** and M. Sudhakara Reddy (2010). Ligninolytic enzyme production by *Morchella crassipes*. **National Conference on Emerging Trends in Bio pharmaceuticals: Relevance to Human Health and 4th Annual Convention of Biotechnology and Pharmacy.** Department of Biotechnology and Environmental sciences, TIFAC-CORE, Thapar University, Patiala, Punjab, India.
- **Harpreet Kaur Kanwal** and M. Sudhakara Reddy (2010). Molecular diversity of *Morchella* spp. of western Himlayan region of India. **National Symposium on Botanical Researches: Present Scenario.** Department of Botany, Punjabi University, Patiala, Punjab, India.
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- **Harpreet Kaur Kanwal** and M. Sudhakara Reddy (2009). Effect of carbon/nitrogen sources on laccase production in *Morchella crassipes*. **National symposium on New vistas for Mycology in Meeting Global Challenges.** Centre for Advanced Studies in Botany, University of Madras, Chennai, India.

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Abbreviations

bp	Base pair

BLAST	Basic local alignment search tool
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
g	Gram
hrs	Hours
ITS	Internal transcribed Spacer
IPTG	Isopropyl--thiogalactoside
Kb	Kilo base
L	Litre
mg	Milligram
ml	Milliliter
MQ	Milli Q
NCBI	National centre for biotechnology and information
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
rpm	Revolution per minute
TBE	Tris Borate EDTA
Tris	Tris-(hydroxymethyl-) aminomethane
µg	Microgram
µl	Microlitre
mS cm-1	Millisiemens per cm
X-Gal	5-Bromo-4-chloro-3-indolyl--D-galactoside

Chapter I

INTRODUCTION

INTRODUCTION

Biodiversity of fungi

There are a large number of ecosystems per unit area on this earth out of which the Himalayan ecosystem in India is known to have one of the richest biodiversity. Biodiversity is often defined as the variety of all forms of life, from genes to species, and further, to ecosystems. Fungal biodiversity is thought to be the second largest group of organisms in the world. The variety and galaxy of fungi and their natural beauty occupy prime place in the biological world and India has been the cradle for such fungi (Manoharachary et al., 2005). A small portion of the total number of fungi has been recognized by the scientific community and still a large fraction remains unexplored. Out of 1.5 million of fungi, only 50% are characterized until now and only around 5–10% of fungi can be cultured artificially.

Fungi make many contributions to the good of humanity, and also have many ill effects. They have long been used as a direct source of food, such as mushrooms (*Agaricus* spp., *Morchell*a spp.), as a leavening agent for bread, and in fermentation of various food products (e.g. *Saccharomyces cerevisiae*). Many of the fungi have largely been used for the production of antibiotics (e.g. *Penicillium chrysogenum)*, and, more recently, various enzymes produced by fungi are used industrially and in detergents. These fungi, mostly, members of white rot fungi, perform an essential role in the decomposition of organic matter and have fundamental roles in nutrient cycling and exchange. Many species *(Aspergillus flavus, Fusarium graminearum),* produce bioactive compounds called mycotoxins such as alkaloids and polyketides that are toxic to animals including humans. The fruiting structures of a few species contain psychotropic compounds and are consumed recreationally or in traditional spiritual ceremonies. Some fungi can break down manufactured materials and buildings, and become significant pathogens of humans and other animals. Losses of crops due to fungal diseases or food spoilage (Dutch Elm Disease, caused by the closely related

species *Ophiostoma ulmi, Cryphonectria parasitica* is responsible for attacking *Castanea sativa*) can have a large impact on human food supplies and local economies. Several species are common model organisms in biology, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Neurospora crassa*. The genomes of a number of ascomycete fungi have been fully sequenced. Fungal biotechnology has become an integral part of the human welfare.

Morels

Morels (*Morchella sp*.), belonging to the family Helvellaceae, form a special part among the wild edible ascomycetous mushrooms of fungi. *Morchella*, the true morels, is a genus of edible mushrooms closely related to anatomically simpler cup fungi (Pegler, 2003) (Fig 1.1). These distinctive mushrooms appear honeycomb-like in that, the upper portion is composed of a network of ridges with pits between them (Fig 1.2). All over the world, morels have been called by many local names such as, dryland fish, hickory chickens, merkels, miracles, molly moochers, sponge mushroom. In India, the Himalayan morel is popularly known as **"Gucchi"** by the local people. Compared to many other Himalayan mushrooms, morels are considered as one of the costliest mushrooms. The dried morels fetch a fabulous price of Rs.5000-10000 per Kg in India (Prasad et al., 2002; Negi, 2006) whereas $750 a kilo in western countries. In India, morels grow abundantly in Jammu and Kashmir (Kaul, 1976; Waraitch, 1976), Himachal Pradesh (Sohi et al., 1965; Lakhanpal and Shad, 1986) and Uttaranchal during March to May and during August to September. Some *Morchella* species have been reported from Maharashtra (Wakode, 1983; Ghurde and Wakode, 1981), Uttarpradesh (Hennings, 1901; Ghosh and Pathak, 1962) and Punjab (Purkayastha and Chandra, 1985).

A total of 18 species of *Morchella* have been reported from 28 countries, wherein altogether 14 species are edible or used as food, and 5 to be used medicinally (FAO, 2004).

They have always attracted mycologists because of their commercial value, beautiful appearances, medicinal purposes and good taste, (like that of fish), which gives it a unique flavour. They are highly prized for their culinary uses, particularly as a gourment food and are used in gravies, sauces and soups. Morels are not only delicious; they are also considered as healthy and nutritious food. They contain 42% protein on a dry weight basis, are low in calories and rich in minerals. Mushroom metabolites are also used as adaptogens and immunostimulants, as well as antitumour agents for clinical use (Franz, 1989; Chang, 1991; Nitha et al., 2007). Morels are considered to be one of the highest non value timber forest products. Due to these rich properties, morels are harvested each year on a large scale. In India, it has been reported that in Himalayan region, a large number of families located in those hilly areas are actively involved in the harvesting of morels (Mcfarlane et al., 2005). On an average a person may collect about 200-300 (2-3 Kg on fresh weight basis) of *Morchella* fruiting bodies. An average family of 3-5 persons may collect 1.5 kg of *Morchella* (on air dry-weight basis) every year and sell them at a rate of Rs 5000 per kg, enabling an annual income of Rs 7500. Middlemen earn 35-40 % of the total profit (Prasad et al., 2002).

There ia a myth that forest fires enhance the occurrence of morels in the next season, therefore, local people set the ground on fire for the greed of large number of morels (Mcfarlane et al., 2005). Although these morels serve as a source of income for the poor families in the Himalayan region, yet the collection of morels and the traditional practice of setting the ground on fire have a negative impact on forest biodiversity and ecosystem of Himalayan region. Therefore there is an urgent need to document the diversity of Himalayan morels.

Fig 1.1: Fruiting bodies of *Morchella* spp. (a) Yellow morel (b) Black morel (c) Yellow morel.

(Source:www.mushroomthejournal.com;www.weatherpulse.info/2011/04/mushroomweather)

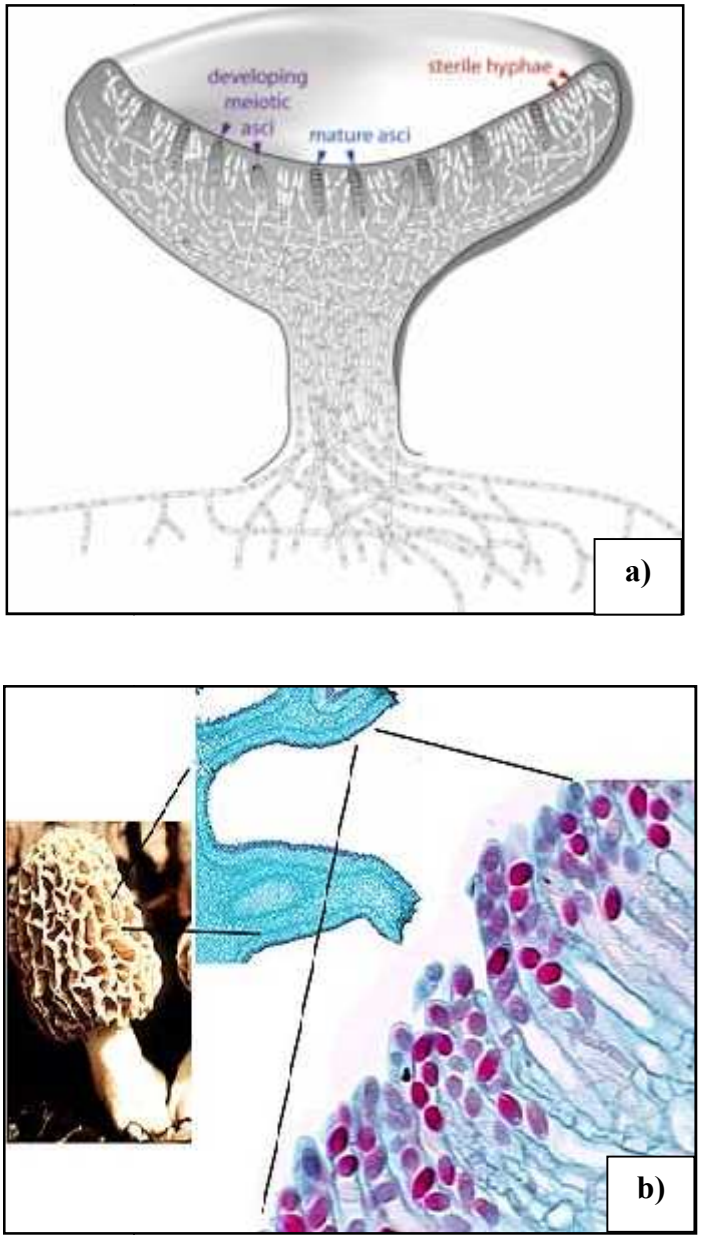


Fig 1.2: a) Internal structure of ascocarp of *Morchella* spp. b) Structure of ascus with ascospores of *Morchella* spp.

(Source:www.thefullwiki.org/Ascomycete;www.flickr.com/photos/52345239@N05/5226817921/in/photostream)

Diversity of Morels

Diversity of a place is based on phenotypic approach of observable characteristics (classical approach) as well as on genotypic approach (molecular approach). In the earlier times, taxonomy of fungi used to be based largely on morphological descriptions of the macroscopic appearance of sexual fruiting structures (Bruns et al., 1998). Some of the fungi produce beautiful fruiting structures and morphologies of these have enabled mycologists to identify some types very accurately. However, all fungi are not easily identified. Classical fungal taxonomy relies heavily on using the size and the shape of the fruiting bodies, spore morphologies, release mechanisms as coloration, and habitat to define taxa but the microscopic features throughout the taxon are quite homogeneous and ascocarp morphology is highly variable and most dependent on ecology. With time, it has become clear that these morphological features do not accurately reflect the diversity or species composition of all the fungal communities (Zhou et al., 2001). This may be because some fungi especially, members of Ascomycota, Corticiaceae and Thelephoraceae produce small or cryptic fruiting bodies that are overlooked during fruiting body surveys. Other fungi may be missed from such surveys because they produce hypogenous fruiting bodies or have no known sexual stage like *Cenococcum geophilum.*

Further, the production of fruiting bodies is dependent on environmental conditions such as moisture and temperature and thus fungi do not produce fruiting bodies each year. It has been suggested that morphological classification is not precise enough to accurately describe fungi (Mehmann et al., 1995), is too time consuming to learn and may be consistent amongst laboratories. In these situations it is very difficult to define the limits of a species or know for sure whether one morphological type is same as another.

Molecular approach

In recent years, morphological techniques have been influenced by modern procedures, which allow more reliable phenotypic studies to be performed. Since the distinguishing morphological characteristics of a fungus are frequently too limited to allow its identification, physiological and biochemical techniques are applied for characterising fungi. For studying the molecular diversity, Internal Transcribed Spacer (ITS), D1 & D2 regions of Large Subunit (LSU) of 28S rRNA, protein coding genes such as RNA Polymerase II Largest subunit (*RPB1, RPB2)* and Elongation factor (*EF-1α)* (Hansen et al., 2005; Rehner and Buckley, 2005; Spooner et al., 2010) are frequently used in fungi.

There are basically two important technical advances that have stimulated the use of molecular techniques - firstly, the advent of Polymerase Chain reaction (PCR) has allowed the analysis of small numbers of fungal cells or even single spores, dried herbarium material (Carlile, 1994), or extinct organisms (Golenberg et al., 1990) and secondly, the selection of universal oligonucleotide primers specific to fungi (White et al., 1990) that has provided easy access to nucleotide sequences. Besides these, numerical taxonomy, effective statistical packages, and the application of computer facilities to the development of identification keys offer some solutions and the possibility of a renaissance of morphological studies (Chapela, 1991; Seifert et al., 1995).

Sclerotia and Cultivation of morels

Morels are difficult to cultivate under controlled conditions; the answer to this fact lies in the understanding of the life cycle of morels. In morel's life cycle, plasmogamy between strain variants creates a heterokaryotic mycelium (secondary mycelium) which later on forms heterokaryotic sclerotia, which can either revert back to secondary mycelium or lead to ascocarp formation (Volk and Leonard, 1989; 1990). Sclerotium is a hard surfaced resting

body of fungal cells which acts as a nutrient reservoir and is resistant to unfavorable environmental conditions. As this sclerotium acts as an intermediate stage during fruiting body formation, so it plays an important part in their reproduction. Nutrients mainly neutral lipids in the form of triglycerides are stored in the sclerotia and during the sexual cycle, substantially all of the nutrients for fruiting body development are drawn from sclerotia (Ower et al., 1986). Sclerotia remain dormant for long periods of time and resume growth on the return of favorable environmental conditions (Volk and Leonard, 1990). The production of a fruiting body from sclerotia requires very specific environmental conditions of nutrition, humidity and temperature to do so (Sharma and Devi, 2006). *Morchella* will usually generate new mycelium because the environmental requirements for the formation of fruiting body are less specific due to which morels cannot be cultivated artificially.

Ligninolytic enzyme production

For commercial cultivation of mushrooms, compost prepared from wheat or rice straw along with manure, calcium sulphate and water serves as a substrate. As in the compost, the carbon sources utilized by fruiting body producing fungi are mostly provided by the lignin based substrates; so the fungi during vegetative growth produce a wide range of ligninolytic enzymes to degrade the substrates (De Groot et al., 1998) (Fig 1.3). Fungal ligninolytic enzymes are a diverse group of enzymes consisting of lignin peroxidase (EC 1.11.1.14), Manganese peroxidase (EC 1.11.1.13) and a copper containing phenoloxidase, laccase (EC 1.10.3.2). Fungal ligninolytic enzymes are economically important enzymes that play an important role in many physiological functions involved in lignin degradation (Ardon et al., 1998; Riva, 2006; Sharma and Kuhad, 2008), synthesis of melanin and other pigments (Leonowicz et al., 2001), formation of fruiting bodies (Langfelder et al., 1998), detoxification of phenolic compounds inhibitory to fungal growth (Vaiterbo et al., 1993) and bioremediation of persistent pollutants (Koroleva et al., 2002).

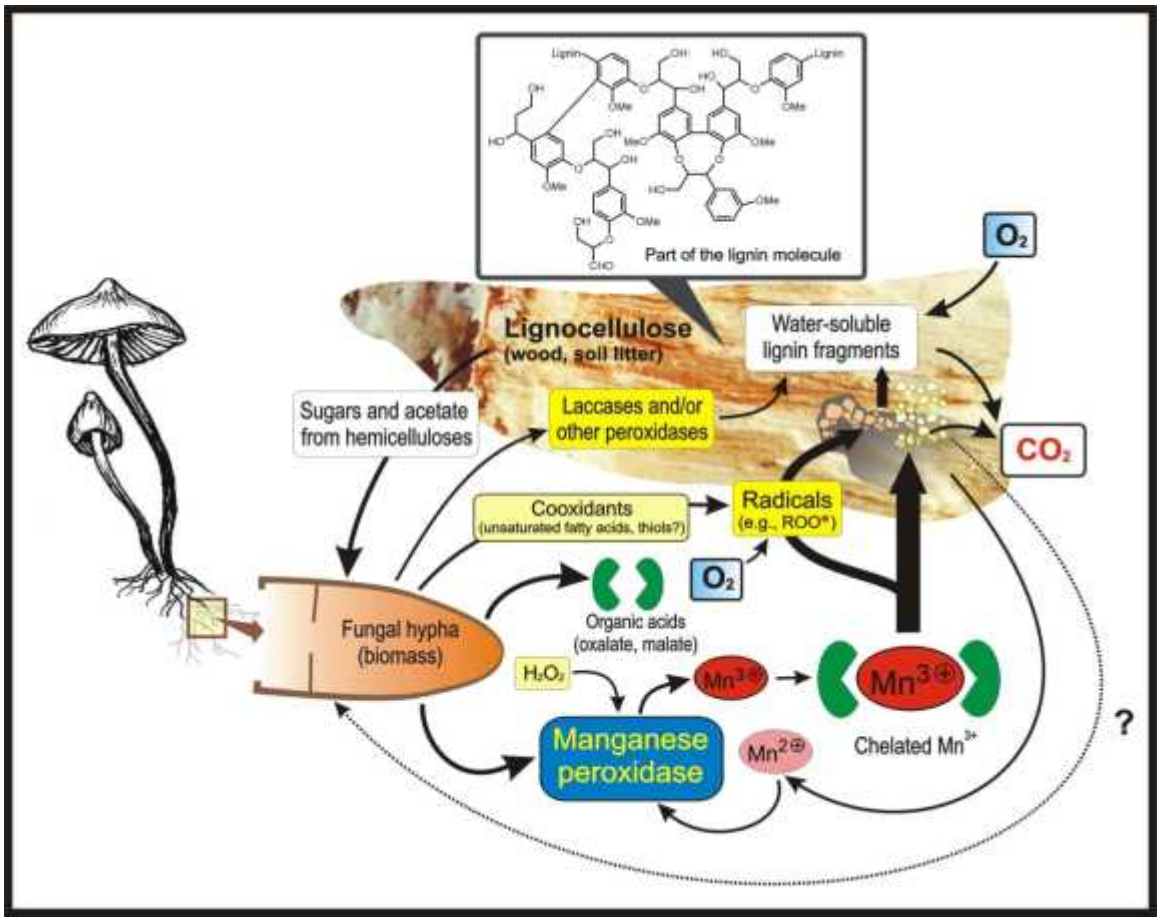


Fig 1.3: An example of lignin degradation by basidiomycetous fungi (e.g., *Nematoloma frowardii, Phlebia radiata*). Fungi utilize preferentially sugars and acetate residues from hemicelluloses as carbon and energy source, while lignin is unproductively degraded ("enzymatic combustion") and the major part of cellulose remains fairely unused giving white-rotted wood its characteristic appearance (Modified after Hofrichter, 2000).

Many researchers have established correlations between mushrooms production, ligninolytic enzyme synthesis and lignin degradation (Valmaseda et al., 1991). Variations have been found in the laccase enzyme levels of many mushrooms during vegetative growth

of fungal mycelium compared to that during fruiting body formation (Ohga, 1992, 1999; De Groot et al., 1998). Laccase has been found to be induced in response to heavy metals and substrates during sclerotia formation in many basiodiomycetous fungi (Wong and Willetts, 1973; Crowe and Olsson, 2001; Ryan et al., 2003; Gaita´n-Herna´ndez and Salmones, 2008). In the life cycle of morels, sclerotium is an intermediate stage during the fructification in morels (Volk and Leonard, 1990). These ligninolytic enzymes might play an important role during sclerotia formation and maturation in morels.

Aim of present study

At present, our knowledge about the diversity and the cultivation aspects of *Morchella* spp. in Western Himalayan region of India is rather limited. Collection of unopened fruiting bodies prevents the dispersal of spores (Pilz and Molina, 2002). Fruiting bodies are usually picked without causing damage since these are all above ground (Negi, 2006). If the soil is left compacted or the leaf litter is disturbed, this affects the production since the mycelium present in the upper layers of soil or the litter mass gets damaged. Due to extensive collection of morels and failed attempt of its domestication, there is a need to document the diversity and carry out its cultivation studies in order to conserve it. *Morchella* diversity has been studied in the western countries but not from India. No study has been carried out for the genetic diversity and identity of morels occurring in this region of India. Reports on considerable morphological variations in the physiology of *Morchella sp.* in different carbon (Winder, 2006) and nitrogen sources (Guler and Arkan, 1999) are available, whereas reports dealing with the growth studies and ligninolytic enzyme production by *Morchella sp.* with different nutritional parameters are scarce. Artificial commercial cultivation of morels on different substrates is problematic due to the occurrence of intermediate stage - sclerotia in their life cycle. For commercial cultivation of morels, large sized and abundant sclerotia are needed that can act as spawn cultures for fruiting body formation. Selection of carbon and

nitrogen sources and substrates to produce sclerotia is a necessary condition so that they can be used further for cultivation purposes. There is a need to explore the potential lignocellulolytic enzyme activities and lignin degradation ability of *Morchella spp.* on different lignocellulosic substrates so that they can be utilized further for the fructification in morels.

There exists a lot of variation among the *Morchella* spp. and the taxonomy of *Morchella* spp. has been revisited in many parts of the world by using molecular approaches to identify the different species of *Morchella* (Stefani et al., 2010; Spooner et al., 2010; O'Donnell et al., 2011). In the present study, an attempt has been made to study the diversity and taxonomic position of *Morchella* isolates occurring in the western Himalayan region of India. *Morchella* isolates collected from different parts of India were studied for variation among isolates by their morphological and physiological characteristics. In the present study, we have examined the diversity of *Morchella* spp. from the Western Himalayan region of India by collecting the fruiting bodies and studying the lengths of ITS/5.8S regions of rRNA. Inter and intraspecific polymorphism among these putative species of *Morchella* were observed based on the analysis of RFLP of ITS/5.8S rRNA gene regions and phylogenetic relationships of different *Morchella* spp. The physiological aspect of these *Morchella* isolates was also studied. This study, also, evaluates the ligninolytic enzyme activities and lignin degradation on different lignocellulosic substrates that can be utilized further for the fructification in morels. Also examined the role of ligninolytic enzymes during the phase of sclerotia formation and maturation in morels so as to get an insight in understanding the cultivation aspects in morels.

Cultivation of morels not only improves the socioeconomic conditions of the locals of the higher Himalayan region, but also helps in conserving Himalayan Biodiversity.

Chapter II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Fungal diversity

Fungi are among the most important organisms in the world due to their influence on humans and human-related activities as they play vital roles in ecosystem functions (Mueller et al., 2004). Most of the fungal species differ considerably in habitat, morphologies, ecologies, and life history strategies. Still, only limited and incomplete information is currently available for most species and current estimates of species numbers for fungi differ significantly. The total number of fungi from earth have been estimated as 1.5 million species (Hawksworth, 1991); whereas other estimates ranging from 0.5-9.9 million too have been hypothesized (Hawksworth, 2004). The estimated number of fungal species from world is based primarily on the observed ratio between flowering plant diversity and fungal diversity in countries where fungi have been sufficiently well studied to enable a reasonable estimate of true diversity (Hawksworth, 1991; 2004).

Hawksworth and Rossman (1997) identified the missing unexplored fungal categories as fungi in tropical forests, fungi in unexplored habitats and lost or hidden species. Little progress has been made in the exploration of fungi in tropical forests. Studies from tropical forests (Hyde, 2001; Aptroot et al., 1997; Henkel et al., 2002; Pandey et al., 2003; Beilharz and Cunnington, 2003) have resulted in an enormous increase in the total number of species. Fungi, in nature, occupy diverse habitats. There is a need to explore more habitats to study the fungi there. Lost or hidden species include the cryptic species, those lost within broadly circumscribed species, named but orphaned species, and those collected but yet unidentified. Almost any single species of fungus studied by molecular or incompatibility methods proves to comprise several biological species, many of them having different morphological features. Examples can be found in almost all groups of fungi, ranging from macromycete genera such as *Armillaria* (Pegler, 2001), *Cantharellus* (Dunham et al., 2003) and

Ganoderma (Hong and Jung, 2004), to micromycetes such as *Trichoderma* (Chaverri and Samuels, 2003) and lichen forming fungi (Kroken and Taylor, 2001; Molina et al., 2004). Lack of basic information regarding the taxonomic diversity has significant implications for many aspects of evolutionary biology, e.g., phylogenetic hypotheses, the role that biodiversity plays in providing resilience to perturbations, coevolutionary relationships and processes, interpretations of biogeographic patterns, natural products screening programs, etc. (Mueller et al., 2004; Hawksworth and Mueller, 2005). Having a stable and accepted estimate of taxonomic diversity for fungi is also necessary to enable fungi to be included in considerations of biodiversity conservation, land-use planning and management, and related subjects. Even so, there have been few attempts to compile available information on fungal diversity or to use these data to rigorously estimate global diversity for fungi.

Biodiversity of fungi in India

The total number of fungi recorded in India exceeds 27,000 species, which is considered as the largest biotic community after insects (Sarbhoy et al., 1996). The true fungi belong to kingdom Eukaryota which has four phyla, 103 orders, 484 families and 4979 genera. About 205 new genera, of the total new genera described from world, have been described from India.

Ascomycetes and their importance

Ascomycotina is the largest sub-division of the fungi encompassing 2700 genera and 28,500 species. Ascomyceteous fungi comprise a wide variety of fungi that differ in morphology, ontogeny, ascocarp details, ascus organization, nature of ascospores, ultrastructure and other characters besides occurrence in diversified habitats (Onions et al., 1981). Familiar examples of these fungi include morels, truffles, brewer's yeast and baker's yeast, Dead Man's Fingers, and cup fungi. The fungal symbionts in the majority of lichens such as *Cladonia* belong to

the Ascomycota. There are many plant-pathogenic ascomycetes, including apple scab, rice blast, the ergot fungi, black knot, and the powdery mildews.

Ascomycetes have attracted considerable attention as a source of new and novel metabolites with antibiotic, antiviral, phytotoxic and cytistatic activity. Ascomycetous yeasts are common in moist, sugar-rich environments like plant surfaces and fruits but are also prevalent in soil, and fresh and marine water bodies. Importance of yeasts in industrial fermentation, like brewing and baking, is well known. The mycelial members, *Chaetomium*, *Xylaria*, *Neurospora, Sordaria* and *Ascobolus* are common saprophytes in soil, plant and animal remains. Some fungi such as *Lulworthia* and others are common in estuarine environment (Sharma, 1989). All such fungal forms are important in decomposition processes, because of their ability to degrade cellulose and other plant polymers. Truffles and morels form ectomycorrhiza on forest trees and fruiting bodies of fungi such as *Morchella* are a delicacy and highly prized commodity (Prasad et al., 2002).

Morchella

The fruiting bodies of *Morchella* spp. (morels) are highly prized for their edibility and appearance, which is similar to "a sponge on a stick". The morels resemble mushrooms to the extent that they have a cap borne upon a central stem, while the truffles form solid, round balls, which grow underground. Six species, namely *Morchella esculenta*, *M. conica*, *M. deliciosa*, *M. angusticeps*, *M. arassipes* and *M. hybrid* (*M. semilibera*) have been reported from India (Wariatch, 1976).

Morchella mostly grows in soil, which is rich in organic matter usually in the dense deciduous forest litter. The epigeal fructifications (ascocarp) often appear in clusters in nature during spring in the cool areas of the temperate zone when soil temperature is about 10 °C. The habitats of *Morchella* spp. are distinguished by the dominance of tree species, viz. *Rhododendron arboreum*, *R. lepidotum*, *Taxus baccata*, *Pinus wallichiana*, *Cedrus deodara*,

Betula utilis, *Cupressus juniperus*, and important medicinal and aromatic plants, viz. *Podophyllum hexandrum*, *Dactylorrhyiza hatagirea*, *Picrorhiza kurrooa*, *Rheum emodi*, *Pleurospermum angelicoides*, *Angelica glauca*, *Arnebia benthamii*, *Saussurea costus*, *Megacarpea polyandra*, *Selinum wallichianum*, *Nardostachys jatamansi*, *Aconitum* species and *Polygonatum* spp. In India, it appears on a large scale during the month of March and its collection starts between April and June (Prasad et al., 2002).

Morels are known to produce fruiting bodies under two distinct ecological environments: undisturbed and disturbed. Stable undisturbed ecosystems produce a limited number of fruiting bodies each spring with production continuing over many years (Kaul, 1977; Scott and Mohammad, 2004). Disturbed habitats produce numerous fruiting bodies in the spring following the disturbance but production rapidly declines over following years (Kaul, 1977). This type of habitat is believed to have high nutrient availability and low levels of competition as a result of disturbance by mechanical or chemical means (Buscot and Kottke, 1990), fire damage (Stamets, 1993b; Pilz et al., 2004) or after volcanic eruption (Carpenter et al., 1981). *Morchella esculenta*, referred to as the natural morel, has been said to fruit in forests two or three years after disturbance caused by tree felling, as long as sufficient rain has occurred (Rowe, 1997).

Fungal taxonomy

Fungal taxonomy is a dynamic, progressive discipline that consequently requires changes in nomenclature. The basic rank in taxonomy is species. However, the name of species can vary widely according to mycologists and there are different approaches for delineating the species concept. Attempts to reach a consensus for a universal definition of species have been unsuccessful, and consequently several different concepts have been used.

Classical approach for studying taxonomy

The morphological (phenetic or phenotypic) concept is the classic approach used by mycologists; in this approach, units are defined on the basis of morphological characters and ideally by the differences among them. In case of fruiting body forming macro fungi, the type of fruiting body (basidioma in basidiomycetes, ascoma in ascomycetes), type of ascus (a microscopic, unicellular, frequently globose, saccate, or cylindrical structure which often contains eight ascospores produced after meiosis, and is usually developed in the cavity of fruiting bodies), shape, colour and the opening of ascomata are important features in the recognition of higher taxa (Ainsworth, 1973).

Morels have been attributed to different species, solely on the basis of classical taxonomy. The earliest record of *Morchella* from India is by Cooke (1969) as *M. gigaspora* Cooke but this species is a synonym of *M. bohemica* Krombh (Butler and Bisby, 1931) and is presently placed in genus *Verpa*. Berthet (1964) used the criterion of total number of nuclei per spore to separate Morchellaceae as the group with highest number of nuclei per spore (coenosity level). Based on the morphological features, some researchers divide the genus *Morchella* into as high as 50 (Jacquetant et al., 1984) and some divide that into 3-6 species (Weber et al., 1988). The report of *M. vulgaris* Boud. (a synonym of *M. esculenta* (L.) Pers. Ex Fr.) from Assam forests by Bhattacharya and Baruah (1953) has been doubted by Ghosh and Pathak (1962) who reported 5 species of this genus from Kashmir Hills. Presently, six species have been reported from India, *M. deliciosa* Fr. (delicious morels), *M. esculenta* (L) Pers. ex Fr. (Common morel), *M. conica* Pers. (conical morels), *M. crassipes* (thick stemmed morel), *M. angusticeps* Peck. (Black morel) and *M. hybrida* (hybrid morel) (Waraitch, 1976).

The phenotypic approach has been largely criticized for its lack of standardization and stable terminology and for its high subjectivity. Moreover, some phenotypic characteristics have been considered to be unstable and dependent on environmental conditions.

Molecular approach for studying the taxonomy

Since the distinguishing morphological characteristics of fungi are frequently too limited to allow its identification, molecular methods have now become the universally acceptable methods to study the diversity. The aim of molecular studies in biodiversity is fourfold: (i) phylogenetic studies, i.e., tracing back the most probable course of evolution and the historic coherence between groups at higher taxonomic ranks; (ii) taxonomic studies, mostly at the level of genera and species; (iii) diagnostic applications, i.e., recognition of defined taxonomic entities; and (iv) epidemiology and population genetics, i.e., monitoring outbreaks of subspecific entities with respect to the analysis of populations and their mode of reproduction (Guarro et al., 1999).

Eukaryotes possess four nuclear ribosomal RNA (rRNA) genes: 28-26S, 18S, 5.8S and 5S. These genes are repeated several hundred to a few thousand times in the genome and are nearly always arranged either in a single large tandem repeat array or in multiple tandem arrays found on one or a few chromosomes (Tilak et al., 2005; Chung et al., 2005). This complex has several domains that evolve at varying rates (Jorgenson and Cluster, 1988), and thus have different phylogenetic utilities. The 18S and 28S rRNA genes evolve relatively slowly and are useful in addressing broad phylogenetic hypotheses involving a broad range of organisms (Bruns et al., 1991; Cullings, 1994; Maidak et al., 1997). The large intergenic spacer, or IGS, which separates the 28S and 18S coding regions, contains signals for transcription initiation and termination. It often contains repetitive regions, which can vary in length due to variation in the number of repeated sequences. The internal transcribed spacers (ITS), which separate the 5.8S gene from the 18S and 28S genes on either side of it, contain motifs responsible for the correct splicing of the mature 18S, 28S and 5.8S rRNA molecules from the primary rRNA transcript (Fig 2.1). To obtain greater specificity, the analysis of amplification of ITS region is required (Viaud et al., 2000). The internal transcribed spacer

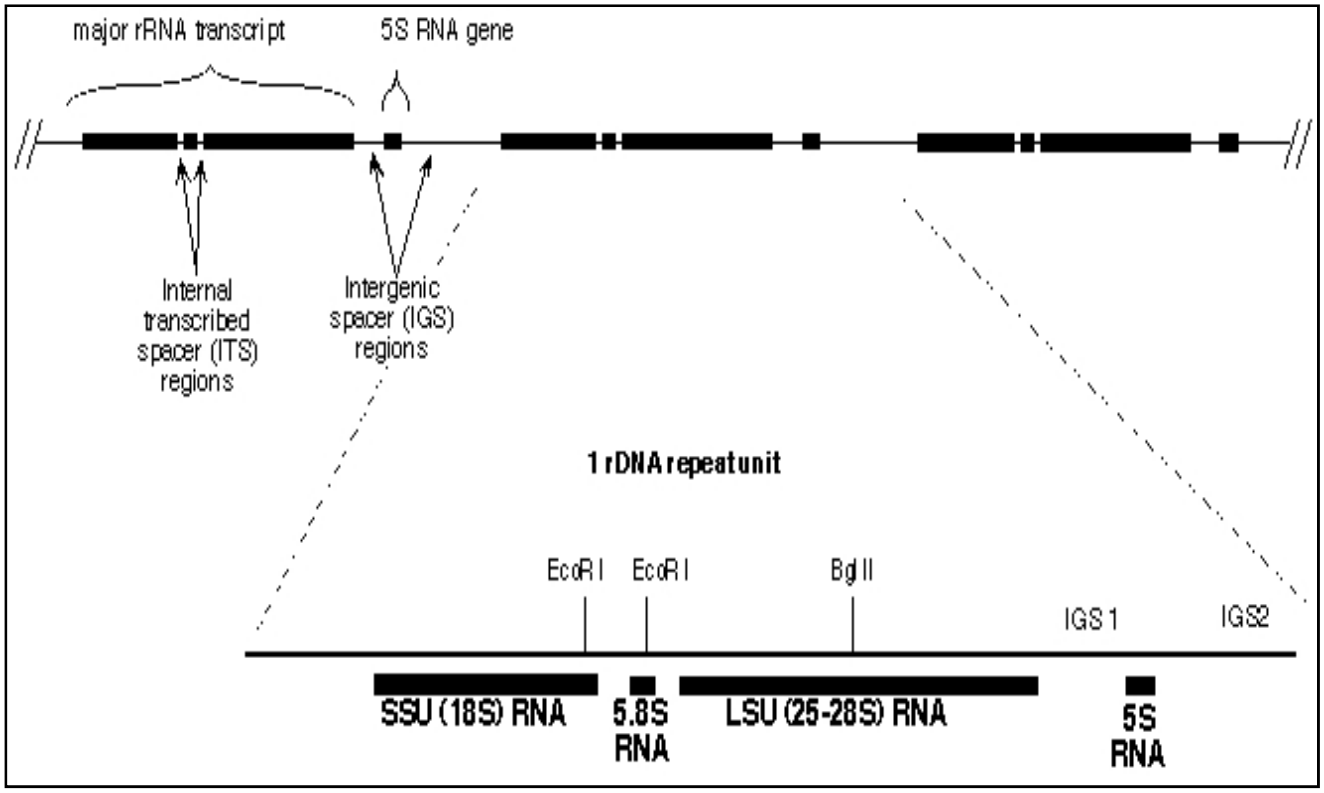


Fig 2.1: Structure of the ribosomal RNA gene cluster showing the positions of fungal PCR primers. The cluster is split into coding (18S, 5S and 28S genes) and non-coding (Inter-Genic Spacer or IGS and Internally Transcribed Spacer or ITS sequences) regions. Some of the non-coding areas are transcribed (Externally Transcribed Spacer or ETS and ITS) while others are not (Non-Transcribed Spacer or NTS sequence). The positions of the primers and their direction of replication are indicated by arrows.The IGS and ITS regions usually exhibit the most sequence variation while the coding areas are the most conserved (Source: Mitchell, 1995).

are noncoding regions of DNA sequence that separate genes coding for the 28S, 5.8S, and 18S ribosomal RNAs. These ribosomal RNA (rRNA) genes are highly conserved across taxa while the spacers between them may be species-specific. The variation in the spacers has proven useful for distinguishing among a wide diversity of difficult-to-identify taxa. The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g. to identify geographic races). Because of its higher degree of variation than other regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions.

Differentiating between the different species of morels has been the principal focus of genetic studies of morels (Buscot et al., 1996; Gessner et al., 1987; O'Donnell et al., 1997; Royse and May, 1990; Wipf et al., 1996; 1999; Yoon et al., 1990). According to Weber (1988, 1995, 1997) there are only three types of morels: yellow (*Sectio adnate*), black (*Sectio distantes*) and half-free. But Volk and Leonard (1989) suggested that black morels (*M. angusticeps*, *M. conica*, and *M. elata*) and yellow morels (*M. esculenta*, *M. crassipes* and *M. deliciosa*) are co-specific based on the phylogenetic tree developed from restriction data. Further Jung et al. (1993) confirmed that the different species of morels such as *M. deliciosa* and *M. crassipes* and *M. esculenta* are the same mushrooms and the difference in their morphology and genetic behavior is very minute and that may be due to geographical isolation and different growing conditions. Jung et al. (1993) also confirmed the separation of the half-morel, *M. semilibera*, from the yellow morel.

Royse and May (1990) based on genetic variability of 12 enzymes encoded by 12 loci proposed only two major species grouping *M. esculenta* as yellow morel and *M. angusticeps* as black morel group.

DNA research by Jung et al. (1993) confirmed that *M. deliciosa* and *M. crassipes* do not differ enough genetically from *M. esculenta* to merit species status, and that the difference observed among these mushrooms are likely due to development and environment. In short different growing conditions produce different looking yellow morels- but they are the same mushrooms and that more variation may occur between geographically isolated populations of the same species of *Morchella* than between two putatively distinct species. Similar results were noted by Yoon et al. (1990) among populations of *M. deliciosa* and *M. esculenta*.

Bunyard et al. (1995) amplified the large subunit (28S) of the ribosomal DNA repeat of *Morchella, Verpa, and Disciotis* and a closely related genus (*Gyromitra*) via the polymerase chain reaction. They found the Restriction Fragment Length Polymorphisms among the lines investigated. More variability was seen towards 5' end than toward the 3' end of the 28S rRNA gene. They used the RFLP banding pattern to assemble a phylogenetic tree for the taxonomic group. Based on the RFLP banding pattern, three black *Morchella* species isolated were found differed by approximately 0.5, 1.0 and 1.5% from all other isolates in the Morchellaceae which they examined in their study. They used *Gyromitra gigas* as an out-group and in this out-group they observed approximately 6.2% difference from all members of the Morchellaceae. In some cases, more genetic variation was observed intraspecifically than between putative species. Additionally, their findings supported the hypothesis that *Morchella* is composed of only a few (possibly three) polymorphic species. Researchers have also analyzed the ITS rDNA region between yellow and black morels and called for separation of genus *Morchella.*

Buscot et al. (1996) showed that polymorphism of ITS region of rDNA serves better for morel systematics. Slight variations and different restriction profiles allowed the identification of several species within the group. They sequenced the whole ITS region in

morels and classified morels as black and yellow morels exhibiting ITS lengths of 740 to 760 bp and 1150 to 1220 bp, respectively. Researchers have also analyzed the ITS rDNA region between yellow and black morels for separating the genus *Morchella.*

Wipf et al. (1999) worked on 66 strains belonging to 11 putative species of Morchellaceae and 3 species of the Discinaceae. They analyzed the ITS regions of rDNA genes of all the species by PCR and carried out RFLP of all amplified regions. All species were separated within *Morchella*. They could clearly distinguish four putative species in the yellow morels group. But no distinction could be obtained between the compared taxa in the group of black morels. Only 5.8S gene could be aligned in all sequenced samples. The sequence analysis confirmed that yellow morels were distinct and one putative taxon can be separated from the others in black morels. Both maximum parsimony and neighbor joining analyses confirmed the great distance between *Morchella* species groups and addressed the question of a separation in the genus *Morchella.*

Gargas and Taylor (1995) examined the evolutionary relationships of apothecial ascomycetes, commonly known as cup fungi, using nucleotide sequences of the small subunit ribosomal genes (SSU rDNA). A maximum parsimony tree (MPT) was developed on the basis of parsimony analysis of approximately 1750 aligned nucleotides of the SSU rDNA from representatives of 10 fungal genera from four orders: Pezizales (*Ascobolus lineolatus*, *Morchella elata*, *Peziza badia*); Leotiales (*Leotia lubrica, Sclerotinia sclerotium*);Caliciales (*Calicium tricolor, Mycocalicium albonigrum, Spherophorus globosus*); and Lecanorales (*Lecanora dispersa, Porpidia crustulata*). The most parsimonious tree (MPT) produced using this dataset supported the monophyletic nature of the orders Pezizales, Leotialesb and Lecanorales. However there was no support of the monophyletic nature of the representative Caliciales. *Spherophorus globosus* had affinities with the members of Lecanorales. The MPT

constructed by them provided foundation for understanding evolution of the ascomycetous fungi.

Buscot et al. (1996) showed that polymorphism of the ITS-rDNA is more adequate to improve morel systematics. They showed that both species groups recognized in all classifications, i.e., black (S*ectio distantes*) and yellow morels (S*ectio adanatae*), exhibited respective ITS lengths of 740 to 750bp and 1,150 to 1,220 bp. Wipf et al. (1996) further showed that slight length variations and different restriction profiles allowed the identification of several species within the group. The whole ITS regions of the black morel (*M. conica*) and the yellow morel (*M. esculenta*) was sequenced to identify the origin of the ITS length difference between the two species groups. The high homologies with the 5.8 S rDNAs of the other ascomycetes allowed localization of the 5.8S gene and ITS1 and ITS2 spacers in the morels. The non-coding ITS1 and ITS2 exhibited polymorphisms. Due to good homologies they could characterize the extremities of the spacers bordering the 5.8S gene and the 3' end of ITS2. Length discrepancy in ITS2 corresponded to several small additional fragments (up to 15 bases) between bases 793 and 1046 of *Morchella esculenta* ITS region. Length discrepancy between the ITS1 spacers of both morels was concentrated principally between bases 89 and 420 of the *M. esculenta* ITS. This region with insertions (40 to 68 bases) in *M. esculenta* showed only 21.5% homology between the two morels.

Kellner et al. (2005) carried out diversity analysis of *M. esculenta* group in Germany and France by using RFLP technique of ITS regions within the rDNA and sequences analysis of ITS regions. They indicated the presence of three distinct species of *M. esculenta*, *M. crassipes* and *M. spongiola* based on the RFLP patterns produced by different restriction enzymes. They further suggested that RFLP can be used as a criteria to separate yellow morels.

Dalgleish and Jacobson (2005) used RAPD PCR technique to examine genetic variation among *M. esculenta* population and found out higher levels of genetic polymorphism among 57 fruiting bodies. They also found the same evidence for inbreeding that can help in resolving important aspects of morel life cycle.

Singh et al. (2004) reported the interspecific polymorphism among many putative species of *Morchella* belonging to varied geographical regions, firstly through diversity analysis in ITS and the 5.8S rRNA gene region, secondly through random amplified polymorphic DNA (RAPD). DNA of 46 monospore cultures was amplified to generate ITS-5.8S gene profiles. The PCR-RAPD amplified profiles of different monospores were identical at intraspecifc levels. They found interspecific polymorphism among eight putative species namely *Morchella esculenta, M. angusticeps, M. crassipes, M. conica*, *M. semilibera, M. spongiola, M. vulgaris* and *Verpa* belonging to varied geographic regions. Thus, they showed that through RAPD markers, one could identify putative species of family Morchellaceae rather than resorting to sequencing DNA each time to improve morel systematics.

Stefani et al. (2010) reported a new clade of morels i.e. tomentosa clade comprising of *Morchella tomentosa* in a burned forest area of north of Fairbanks, Alaska, USA. They constructed bayesian and maximum parsimony trees based on ITS-rRNA regions and nLSU gene regions of all species of *Morchella* along with *M. tomentosa* that they had reported. Strong genetic divergence was observed between *M. tomentosa* and the other morels that suggested very different biological behaviour of tomentosa group. They further reported a peculiar belowground structure associated with *M. tomentosa* and named that as radiscisclerotium. Radiscisclerotium was reported for the first time by them. This radiscisclerotium was similar to the connective mycelium of *M. rotunda* and *Mitrophora semilibera* associated with *Fraxinus excelsior* and *Cornus sanguinea* roots (Buscot and Roux, 1987).

Recently, Taskin et al. (2010) studied the phylogenetic analyses of the individual and combined datasets (consisting of partial sequences from three nuclear protein-coding genes, RNA polymerase I, RNA polymerase II, translation elongation factor, and partial LSU rDNA gene sequences) using maximum parsimony and maximum likelihood approach. The results of their study fully resolved phylogenies that were highly concordant topologically. Genealogical concordance (GCPSR) analysis of the 62-taxon dataset resolved 15 putative phylogenetically distinct species. The early diverging Elata (black morels) and Esculenta Clades (yellow morels) were represented, respectively, by 13 and two species. Eight of the species within the Elata Clade were found to be new to world. Results of their study have provided essential data for ensuring the sustainability of morel harvests through the formulation of sound conservation policies.

Mycorrhizal status of morels

Morels form mycorrhizal association with tree roots in stable ecosystems and it is this association that ensures long-term annual fruiting (Lakhanpal et al., 1991; Buscot et al., 2000). Growth of *M. conica* and *Pinus sylvestris* has shown them to be ectomycorrhizal in nature (Mayr, 1982). Further, Buscot and Roux (1987) found that fruiting bodies of *M. rotunda* were connected by mycelial strands to subterranean compact mycelial masses surrounding the roots, which they termed "mycelial muffs". These muffs were always attached to the roots of living trees such as *Fraxinus excelsior, Ligustrum vulgare, Ulmus campestris, Quercus robur, Corylus avellana* or *Cornus sanguinea*, or herbaceous plants: *Equisetum hiemale, Illium ursinum* and occasionally *Taraxacum* spp. and *Eupatorium cannabinum* (Buscot and Kottke, 1990). These researchers further reported the possible ectomycorrhizal association between *Picea abies* and two species of morel (*M. esculenta* and *M. rotunda*). Sclerotia of *M. elata* have been reported to be associated with ectomycorrhizal root tips of *Picea abies* (L.) Karst (Norway spruce). Dahlstrom et al. (2000) made an

interesting observation that *Morchella* forms mycorrhiza with Pinaceae family such as *Arbutus menziesii, Larix occidentalis, Pinus contorta, Pinus ponderosa* and *Pseudotsuga menziesii*. A number of studies have supported the mycorrhizal nature of *Morchella sp*. with elm trees (Miller, 2005) and herbaceous or woody plants (Mihail et al., 2007).

Physiology

Cytology, Moprphology, anatomy, spore germination of morels has been well studied (Arkan, 1992; Amir et al., 1993; Buscot, 1993a; Scmidt, 1983) but there are a few reports about morel life cycle and cultivation of morels on synthetic media. Guler and Arkan (1999) did extensive studies on the growth and morphology of *M. esculenta* mycelium on Potato dextrose agar, Malt extract agar plates with different nitrogen sources, and found remarkable differences in colony morphology and pigmentation patterns of yellow-brown to brown with different parameters.

Devi and Sharma (1999) did extensive growth studies with different media, temperature, pH and nutritional parameters. Their studies revealed that Coons medium was the best for growth studies. Temperature optima of 20 °C and pH of 6.0 was observed to be the best for growth studies (Sud and Sharma, 1999). Metals such as Boron (10 ppm) and calcium (5 and 10 ppm) have been observed to enhance the mycelia growth in *Morchella sp*. Vitamins such as folic acid, ascorbic acid, nicotinic acid and biotin have been observed to be utilized well by *Morchella sp*. (Devi and Sharma, 1999).

Kalm and Kalyoncu (2008) studied growth rates of mycelium of six wild *Morchella* species (*M. costata*, *M. esculenta*, *M. esculenta* var. *rigida*, *M. esculenta* var. *rotunda*, *M. intermedia* and *M. pseudoumbrina*) on potato dextrose agar (PDA), Hagem medium (HM), Minimal medium (MM) and Malt Extract Agar (MEA). The results concluded that mycelial growth rates of all isolates decreased in MM and HM media, whereas, PDA and MEA were found to be the most suitable media for mycelial growth of *Morchella spp.*

Guler and Okazaya (2009) studied the development of mycelium of *M. conica* with different concentration of sucrose (0.25, 0.50, 0.75, 1.00 and 1.25%) to wheat agar, potato dextrose agar, malt extract agar and complete medium yeast agar. The colonization period of the mycelium was 7 days in potato dextrose agar, 5 days in malt extract agar and 5 days at complete medium yeast agar. Their study concluded dense and circular growth on potato dextrose agar and rhizomorphic growth on malt extract agar and completed medium yeast agar.

Winder (2006) carried out growth studies of *M. elata* on using different carbon sources, pH and temperature ranges, different spores from the same ascocarp and different substrates. Their study concluded carbon sources such as, sucrose, mannose and lactose to be the best carbon sources for growth. Temperature of 16-24 °C or above for a faster-growing isolate and 20-24 °C or above for a slow-growing isolate was found to be the best. Maximum growth was observed at pH 7-7.5. Growth in more acidic or alkaline pH conditions produced the least growth. Alkaline chemicals used for adjusting pH shifted the pH optima to 7.7 in their study. They further concluded that wood ash and other calcium compounds may not only stimulate growth in natural settings, but can also alter the optimal pH for proliferation of *M. elata*.

Cultivation

Due to rich nutritional properties of morels, human beings have always been interested in cultivating morels. The optimum conditions favorable for fruiting body formation have not been clearly determined and there is still a confusion regarding the commercial domestication of morels. A few researchers have succeeded in production of sclerotia under experimental conditions, yet no one has become fully successful in utilizing these resources for successful cultivation of these sclerotia for commercial scale.

Life cycle of morels

Morels being ascomycetous fungi have both asexual and sexual reproductive phases. Asexual reproduction occurs through conidia formation whereas sexual reproduction occurs by ascospore formation (Alexopoulos et al., 1996). Volk and Leonard (1990) proposed the developmental states in the life cycle of morels (Fig 2.2). There are two alternative pathways for the formation of fruiting bodies from primary mycelium i.e. formation of secondary mycelium or formation of sclerotia. In spring, the sclerotia can form fruiting body on the onset of favourable environmental conditions or can revert back to form secondary mycelium.

Sclerotia formation and development

Sclerotial structures in morels were first reported by Molliard (1905) who had observed them on sterile moistened bread. The importance of sclerotia has been described by Ower 1982, when they acted as nutrient sink under defined conditions. A patent regarding the cultivation of *Morchella* had been registered by Ower et al. (1986). The patent deals with the culturing of fruiting bodies of *Morchella* from mycelium. The mycelium and the sclerotia were initially grown in nutrient poor substrate (sand layer) and after the growth it was induced to form fruiting body under conditions of nutrient stress and excess of water. They further described the role of water acting as a triggering shock (changing the osmotic pressure) as an inducer for ascocarp formation.

Volk and Leonard (1989) studied the effect of various nutritional (media, carbon, nitrogen, agrowastes) and environmental factors for sclerotia formation. They tried jar method of Ower et al. (1986) and concluded the positive effect of addition of asparagine for sclerotia formation.

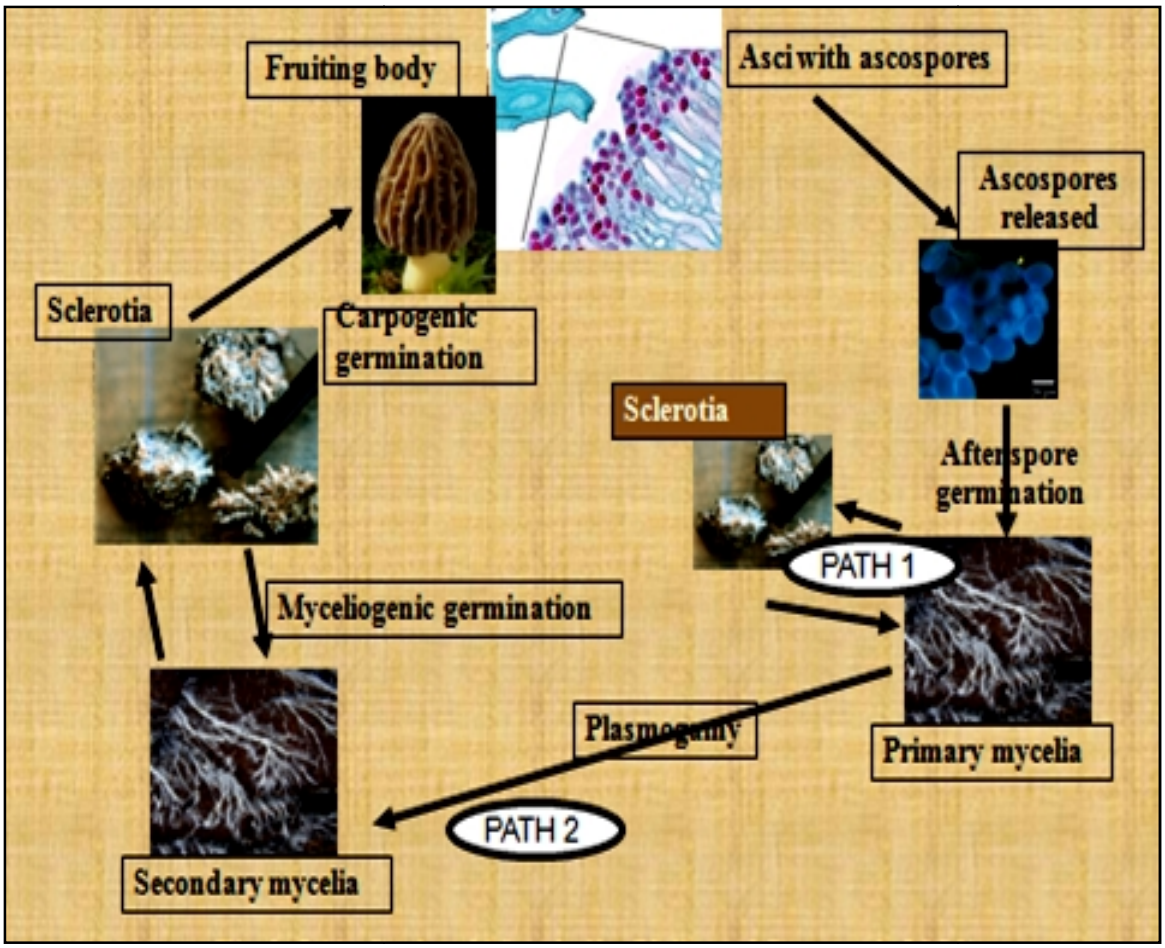


Figure 2.2: Schematic representation of the life cycle of morels (Modified after Ower, 1982; Volk and Leonard, 1989).

Buscot (1993b) identified two forms of sclerotia on Buscot medium A and Buscot medium B. These sclerotia were termed as early encrusting sclerotia (EES) and late isolated sclerotia (LIS), based on the morphogenetic and other characteristics. EES were formed when the hyphal growth was interrupted at the margins whereas LIS form natural subterranean mycelia masses connected to ascomycota. EES were 0.2-0.5 mm in diameter and rapidly aggregated into circular crusts that became pigmented. EES were formed at lower temperature and have similar types and quantities of mycosporins as the young fruiting bodies have (Buscot and Bernillon, 1991). LIS formation is dependent on the age of culture,

are lesser in number but larger in size (0.5-0.8 mm in diameter) than EES. Single ascospore cultures were grouped on the basis of presence of EES, presence of LIS only, or absence of sclerotia. Confrontation studies between ascospore cultures from different ascocarps formed a line of aerial mycelium in all non-self pairings both between isolates from the ascocarp and between isolates from different ascocarps (Hervey et al., 1978).

In a study by Singh et al. (1999), sclerotia of *M. esculenta* increased in number and weight by the addition of sand with substrates such as saw dust, wheat straw, paddy straw, sand, farmyard manure, garden soil or sand plus garden soil. More and heavier sclerotia were observed in the growth of different isolates of same species than with single isolate.

Masaphy (2005) discovered four main stages during the fruiting body development of *Morchella sp*., before the development of asci. First stage comprised of a disk-shaped knots of 0.5–1.5 mm on the surface of the substrate. Next stage consisted of the inflatation of the knot and formation of a primordial stem from its centre. Afterwards, in the third stage, the stem lengthened, oriented upward, and two types of hyphal elements were developed: long, straight and smooth basal hairy hyphae and short stem hyphae, some of which were inflated and projected out of a cohesive layer of tightly packed hyphal elements. Finally, in the last stage, when the stem was 2-3 mm long, pre-apothecia emerged in the apical end, with ridges and pits having distinguished types of paraphyses. Extracelluar mucilage covered the ridge layer and helped give the tissue its shape and rigidity.

Recently, Masaphy (2010) reported the successful fruiting body formation of *Morchella rufobrunnea* from sclerotia. In his study, the fungal mycelium was inoculated into a sterile layer of potting soil placed above the layer of nutritionally rich medium based on wheat grains. After incubation for 2-3 weeks at 18-25 °C, sclerotia were formed on the soil layer. The sclerotium-containing medium was subjected to continuous watering so as to induce fruiting body initiation and development. After 3-6 days, vegetative mycelium

containing conidia were observed in their study. Fruiting body primordia appeared after decline of the conidial layer. Scanning electron microscope analysis revealed four main stages of ascocarp development as described by Masaphy (2005) earlier. Following primordium initiation, the fruiting body gradually developed, resulting in mature fruiting bodies within 2–3 weeks of initiation.

Factors affecting sclerotia formation

Sclerotia formation is influenced by medium composition containing salts and sugars and water potentials. An experimental set up consisting of nutrient rich and nutrient poor medium in the same plate known as split plate (Amir et al., 1992) was used to study the effect. In their experiment, sclerotia were mainly formed on the nutrient poor medium. Sclerotia formation was inhibited by salts, whereas sugars enhanced the sclerotia formation. In their study, a positive correlation was found between the turgor potential generated in the mycelium and the quantity of sclerotia produced.

Amir et al. (1995) studied the phenomenon of translocation to play an important role in sclerotia formation. Sodium azide and nikkomycin resulted in an inhibition of sclerotia formation. They concluded that translocation to the sclerotia takes place by turgor driven mass flow, but is affected by activities in both the source and sink. The addition of potassium nitrate and starch to boiled wheat grain, 1:4:400 respectively, were found to produce high yields of *M. esculenta* sclerotia within 35 to 40 days when grown at a temperature of 25 ºC without light (Sharma et al., 1997).

Different substrates and soil amendments, too, have been found as the important factors affecting the sclerotia formation in *Morchella* sp. (Pal et al., 1999; Sharma and Devi, 2000). Sclerotia formation is influenced by different carbon sources. Guler and Ozkaya (2008) studied the effects of carbohydrate concentrations of 0.25%, 0.5%, 0.75%, 1% and 1.25% on sclerotia formation. Variations were observed in the pigmentation, time of sclerotia

formation, localization, structure and quantity of sclerotia. Vegetative hyphae were developed very well in agar media, containing malt extract, wheat, potato dextrose and complete medium yeast extract but moderate development occurred in the media containing glucose, sucrose, maltose and starch.

Sharma (2001) carried out extensive experimental studies on the soil collected from the morel bearing sites; no direct relationship between the soil pH and morel growth could be traced in their experiment, even though positive relation was observed between the growth of *M. esculenta* and nutrient supply (Sharma et al., 2001). The effects of propolis concentration on sclerotia formation of *Morchella conica* resulted in marked variations in formation of sclerotia, pigmentation, location and structure of sclerotia in the presence of different concentrations of propolis (Guler et al., 2005).

Fruiting body formation

According to Singer and Harris (1987), cultivation of the morel (*M. hortensis, M. esculenta* and *M. costata)* is most similar to the cultivation of *Volvariella volvacea*, the only difference being a great dependency on the presence of trees, a slight difference in nutritional requirements and very much lower temperature optima. *Morchella* fruiting bodies have been reported to be grown on cultivation was *Helianthus tuberosus*, Jerusalem Artichoke (Roze, 1883), apple compost (Molliard, 1905), burned leaves (Ramsbottom, 1953), Cymbidium orchids (Baker and Matkin, 1959), and *Begonia tuberosus* (Volk and Leonard, 1990, 1992).

Patents for the formation of fruiting bodies

In 1982, Ower published a method for cultivating morels (Ower, 1982). In conjunction with Neogen, a company affiliated with Michigan State University, Ower developed Patent no. 4,594,809 (Ower et al., 1986) and second patent no. 4,757,640 (Ower et al., 1989). Many researchers have tried the same method and produced sclerotia but ascocarp production has been unsuccessful.

In 2005, Miller registered a patent (patent no. 6907691 B2) for the cultivation of morels. The invention provided a process of cultivating *Morchella* ascocarps using mycelium and a tree seedling; the tree seedling having a root and shoot system. Further, the process involved inoculating the root system with mycelium to produce an inoculated tree seedling, stimulating the mycelium to form sclerotia and inducing the sclerotia to form ascocarps. Recently, Masaphy (2010) reported the successful initiation and development of fruiting bodies through different morphological developmental stages as sclerotium formation, scelerotium germination, asexual spore formation, formation of initial knots and development of the fruiting body in *Morchella rufobrunnea* under soilless conditions.

Ligninolytic enzymes

Lignocellulose is the major component of plant biomass, comprising around half of the total organic matter produced trough CO_2 fixation fueled by energy derived from photosynthesis (Sánchez, 2009). The degradation of lignocellulosic biomass in nature is mainly caused by ligninolytic enzymes produced by fungi, which represent the unique microorganisms that have evolved with the ability to break down lignin, the most recalcitrant component of plant biomass. Among fungi, the organisms predominantly responsible for lignocellulose degradation are basidiomycetes and ascomycetes (Ten Have and Teunissen, 2001). Ligninolytic enzymes are a complex group of enzymes consisting of Laccase, Lignin peroxidase and manganese peroxidase. Laccase has proved to be the dominant ligninolytic enzyme in relatively high concentration – compared to agricultural or meadow soils – in forest litter and soils in both broadleaves (Criquet et al., 2000) and coniferous forests (Ghosh et al., 2003). The ability to efficiently degrade lignocellulose efficiently is thought to be associated with a capillary hyphal growth habit that allows the fungus to delocalize scarce available compounds (e.g., nitrogen and iron) and enzymes (e.g., glucanases, peroxidases and

laccases) to a distance into the nutrient-poor lignocellulosic substrate that constitutes its carbon source (Hammel, 1997).

Sclerotia formation in relation to ligninolytic enzyme production

Sclerotia are asexual, multicellular, firm resting structures. Sclerotia of fungi develop from repeated localized vegetative hyphal branching followed by adhesion of the branches. Outer hyphae form a melanized rind while the inner tissue develops into a cortex of thick-walled cells and a central medulla. Fungal sclerotia are often irregular in shape, but the constituent cells tend to be more or less equal in diameter (Wong and Willetts, 1980). Sclerotia are usually compact bodies of aggregated hyphae, and the degree of their inter-tangling and the presence of a hard, melanised rind and organization of the internal hyphal tissue into different layers distinguishes four sclerotial types: terminal, loose, lateral-chained, and lateral-simple. In fungi, three stages in sclerotia formation are distinguished (1) distinct initials formed from highly proliferating interwoven hyphae, (2) development (SD state), an increase in size, (3) maturation (SM state), characterized by surface delimitation, internal consolidation, melanin pigmentation, and often associated with droplet excretion (exudate).

Onset of fruiting body development from sclerotia correlates with nutritional exhaustation of the growth substrates. Fruiting body development for commercial mushroom production is thus often induced by vegetative mycelium by nutrient poor layer (Scarse and Elliot, 1998). Moreover, the induction of sclerotia to form fruiting body is triggered by the presence of excess water and the removal of the exogenous nutrients; so that the sclerotia can utilize the stored nutrients to form fruiting body (Ower et al., 1986).

Further, for the induction of sclerotia to form fruiting body, it is important to keep a balance between carbon and nitrogen sources (Mandelin, 1956). Hyphal knots in fungi will easily form into sclerotia when C/N ratio becomes too high (Moore 1998a). Since the carbon sources utilized by fungi are usually of a lignocellulosic character, the fungi during vegetative

growth produce a wide range of enzymes to degrade the lignocellulosic substrates. The enzymes are peroxidises and laccases for lignin degradation and various types of glucanases, cellulases and xylanases for cellulose and hemicellulose degradation (De Groot et al., 1998).

Substrates used for mushroom cultivation normally contain organic nitrogen sources are less in concentration and in free ammonium, since excess can inhibit growth or fruiting of the organism. Studies by De Groot et al. (1998) have shown the presence of high affinity transport systems for amino acids and ammonium ions. In their study, when *Agaricus bisporus* and *Volvariella volvacea* were grown on protein as carbon source, excess nitrogen gained from protein degradation was found to be excreted as ammonium into growth medium. Ammonium release is thought to play a positive role for fruiting body initiation in fungi as it inhibits the competing process of sclerotia formation from hyphal knots (Moore 1998a).

Considerable changes in ligninolytic enzyme activities occur during fruiting, indicating a connection to the regulation of fruiting body development. In *Aspergillus bisporus* and *Lentinula edodes*, enzyme activities have been found to vary with mycelium and fruiting bodies. Laccase activities were the highest just before fruiting body initiation and decline rapidly with aggregate formation. Similarly, Eriksson et al. (1990) showed the positive influence of ligninolytic enzymes along with mushroom production. Georgiou (1997) reported that sclerotial formation in *Sclerotium rolfsii* was accompanied by the accumulation of high levels of lipid peroxidation products, a well-established oxidative stress indicator

Ryan et al. (2003) reported the presence of laccase enzyme during sclerotia formation in Sclerotium rolfsii. After treatment with a combination of chitinase and 1,3-glucanase, two different laccases were isolated from the sclerotia depending on the stage of sclerotia

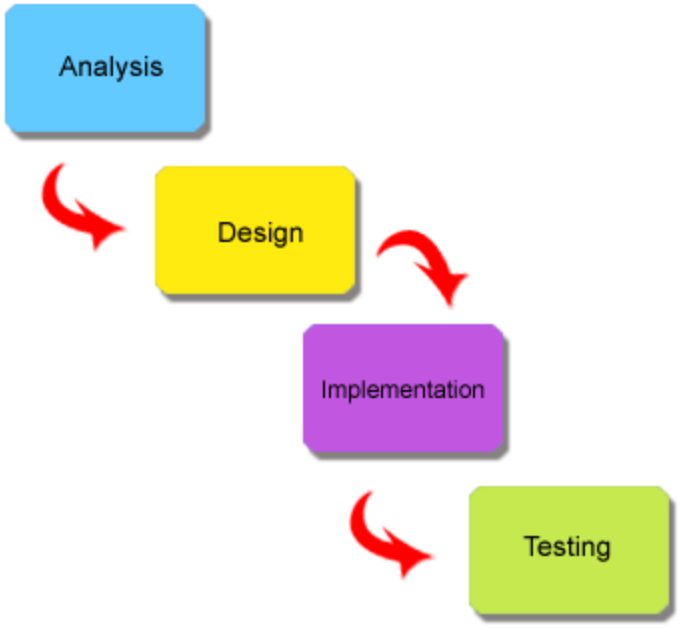
development. The laccases isolated from the fungi was utilized further for wool dye decolorization studies.

Recently, Papinutti and Lechner (2008) and Zhang et al. (2010) studied the ligninolytic enzyme activities in *Morchella esculenta* on different agricultural substrates. High laccase activity was observed with wheat bran as the substrate. Sclerotia were formed on the substrates but was not analysed quantitatively and also relationship between enzyme production and sclerotia number was not observed in their study.

Since there is an urgent need to conserve the diversity of morels, the present work is an attempt to study the diversity and physiology of morels along with carrying out the sclerotial studies for solving the problem of cultivation in morels.

Chapter III

MATERIALS AND METHODS



MATERIALS AND METHODS

3.1 Fungal cultures

Morels are the most desirable edible mushrooms, belonging to the family morchellaceae of class ascomycetes of fungi. Morels are usually found at an altitude of 2000 – 3500 meters above the mean sea level. Morels are widely distributed in the temperate zones of the world. Ecological studies have been conducted in Europe, North America and India (Kaul, 1981; Bartelli 1990; Sharma et al., 1997, Lakhanpal et al., 2010). *Morchella* mostly grows in soils rich in organic matter, usually in dense deciduous forest litter in the spring. Occasionally it is seen growing on stump and log of decaying wood, growing on humus soil taking nourishment from the dead organic matter.

Thirty-two distinct morphotypes of fruiting bodies of *Morchella* (MR1 to MR29, F1, F2 and FK) were collected during the period from March 2005 to October 2009 from different regions of Srinagar, Palampur, Narkanda, Solan and other regions of the Western Himalayas (Fig. 3.1). Some of the dried fruit bodies were also procured from the tribes local to these regions. These fruiting bodies were separated based on the shape, size and color. A few cultures of morels available at Directorate of Mushroom Research, ICAR, Solan, India were also procured and used in this study (Table 3.1).

Internal parts of fresh ascocarps were cultured on potato dextrose agar (PDA) plates with ampicillin (100 mg/ml) so as to form mycelia from fruiting bodies without bacterial contamination. The cultures were maintained further on potato dextrose agar and stored at 4 °C. The sun dried fruiting bodies were used as such for further experiments.

Table 3.1. A list of *Morchella* species and their collection locations from different regions of Himachal Pradesh and Jammu and Kashmir region of Western Himalayas, India.

S.No	Isolate Code	Location	State/province	Collection origin
1	MR1[f]	Sirmour	Himachal Pradesh	30.2230N, 77.0112E
2	MR2[c], MR5[c], MR6[f,c], MR7[c]	Rohru	Himachal Pradesh	31.2167N , 77.7500E
3	MR3[f,c], MR15[cp],MR16[cp], MR17[cp], MR18[cp]	Chambaghat	Himachal Pradesh	30.923506N, 77.098768E
4	MR4[c]	Mandi	Himachal Pradesh	31.71119N, 76.952362E
5	MR9[cp], MR10[c]	Solan	Himachal Pradesh	30.924532N, 77.121277E
6	MR19[cp], MR20[f]	Narkanda	Himachal Pradesh	31.257772N, 77.460158E
7	MR25[f], MR26[f], MR27[f], MR28[f], MR29[f]	Shimla	Himachal Pradesh	31.097473N, 77.184448E
8	MR8[f,c,] MR11[f], MR12[c], MR13[f], MR14[f], F1[f], F2[cp], FK[f]	Palampur	Himachal Pradesh	32.114528N, 76.567841E
9	MR21[f], MR22[cp], MR23[f], MR24[f]	Srinagar	Jammu and Kashmir	34.068436N, 74.833374E

* f-fruiting bodies, *c-cultures isolated, *cp-cultures procured

Fig. 3.1: Map of Himachal Pradesh and Jammu & Kashmir, India, showing the distribution of morel collection sites

The cultures were deposited at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India (Accession Nos. MTCC10160–MTCC10170) (Table 3.2).

Table 3.2: List of *Morchella* cultures along with their accession numbers (deposited at MTCC, IMTECH, Chandigarh, India)

S. No.	*Morchella* spp.	MTCC No.
1.	MR1	10160
2.	MR2	10161
3.	MR3	10162
4.	MR4	10163
5.	MR5	10164
6.	MR6	10165
7.	MR8	10166
8.	MR10	10167
9.	MR12	10168

3.2 Classical Taxonomy

Macroscopic morphological details such as size, shape, and color of the fresh *Morchella* fruiting bodies (MR1, MR3, MR6, MR8, MR11, MR13, MR14, MR20, MR21, MR23, MR24, MR25, MR26, MR27, MR28, MR29, F1, and FK) were recorded. The characteristics that were recorded included:

- Pileus: Shape, size, color of pits, arrangement and color of ribs.
- Stipe: Shape, size, color, smooth or lacunose, swollen at the base or not

Microscopic features were determined from rehydrated sections of fruiting bodies mounted in 5% KOH and stained with 2% congo red.

The spore measurements were recorded on twenty-five randomly selected matured spores using Nikon 50i eclipse microscope at a magnification of 40X. The macro and microscopic observations were performed according to the methods of Waraitch (1976). A scatter plot with the measurements of length and width of spores of different fruiting bodies was performed. Linear regressions were performed to predict the value of spore length and width of different species of *Morchella* examined in this study.

3.3 Molecular Methods

3.3.1 Isolation of genomic DNA

For genomic DNA isolation, sundried fruiting bodies and cultures of *Morchella* spp. were crushed in liquid nitrogen and grounded into fine powder using a mortar and pestle. For cultures, mycelial discs of *Morchella* spp. (MR1 MR2, MR3, MR4, MR5, MR6, MR8, MR10, MR12, MR17 and MR18) were inoculated in 50 ml of potato dextrose broth (PDB) medium and incubated at 25 °C for 14 days. After required incubation period, mycelia were harvested and rinsed with sterile distilled water. The harvested mycelia was dried completely and placed in liquid nitrogen and grounded into fine powder using a mortar and pestle. Genomic DNA was extracted from the ground mycelia by the method of Vankan et al. (1991). The pellet of DNA was dissolved in sterile MQ (Milli Q) water and stored at -20 ºC for future use.

Procedure for DNA isolation (Vankan et al., 1991)

a) A 1.5 ml eppendorf tube was filled 1/3 rd with freeze-dried mycelium powder.

b) To the mycelium in eppendorf tube, 0.5 ml of extraction buffer (Appendix I) was added, mixed well and kept for 15-20 minutes at 65 ºC.

c) Then 0.5 ml of equilibrated phenol was added and mixed well and incubated for 15 minutes at room temperature.

d) To this mixture, 0.5 ml of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. Then the tubes were incubated at room temperature for 15 minutes and centrifuged for 20 minutes at 12,000 g.

e) The upper aqueous layer was removed and transferred to new eppendorf tube.

f) To the aqueous layer, 400 µl of chloroform: isoamyl alcohol (24:1) was added and mixed by inverting gently and centrifuged for 10 minutes at 12,000 g and transferred the supernatant to a new centrifuge tube.

g) Then 0.54 volumes of isopropanol was added to precipitate DNA and incubated for 15 minutes and centrifuged for 10 minutes at 12,000 g.

h) The pellet was washed with 100 µl of 70% (v/v) ethanol.

i) DNA pellet was resuspended in 300 µl of 0.2 M ammonium acetate and incubated overnight at 4 ºC.

j) Next day, DNA was precipitated by adding 600 µl of ethanol and centrifuged at 10000 g for 15 minutes at 4 °C. The supernatant was discarded.

k) The pellet was washed with 300 µl of 70% ethanol.

l) The pellet was dissolved in 50 µl of TE buffer (pH 8) (Appendix II).

3.3.2 Molecular analysis of nucleic acids

Electrophoresis of nucleic acids on non-denaturing agarose gels

Nucleic acids were loaded on agarose gels (0.8%(w/v)) prepared in 0.5X TBE buffer pH 8.0 (Appendix I) using a 6X loading buffer (Appendix I). Ethidium bromide (EtBr) (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then migrated at 50 Volts for 45-60 minutes and visualized on U.V. transilluminator (312 nm).

3.3.3 Nucleic acid quantification by Nanodrop spectrophotometer

The concentration of DNA was determined by NanoDrop™ 1000 spectrophotometer (Thermo scientific, USA). The Thermo Scientific NanoDrop™ 1000 Spectrophotometer measures 1 ul sample with high accuracy and reproducibility. The full spectrum (220-750 nm) spectrophotometer utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place. This eliminates the need for cumbersome cuvettes and other sample containment devices and allows for clean up in seconds. In addition, the NanoDrop™ 1000 Spectrophotometer has the capability to measure highly concentrated samples without dilution (50X higher concentration than the samples measured by a standard cuvette spectrophotometer). In this spectrophotometer, 1 ul sample is pipetted onto the end of a fiber optic cable (the receiving fiber) and a second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1 mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a detector utilizing a 2048-element linear silicon CCD array is used to analyze the light after passing through the sample. The instrument is

controlled by computer based software, and the data is logged in an archive file on the computer. The quantification is based on the principle of absorbance at wavelength of 260 nm. The quality of the DNA was evaluated by measurement of the A_{260} and A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0. Ratios less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

Procedure for DNA quantification

The main steps for using the sample retention system are listed below:

a) The Nanodrop spectrophotometer and the computer attached to it were switched on.

b) The sampling arm of Nanodrop spectrophotometer was opened and the fiber optic cable (the receiving fiber) was wiped off with a soft laboratory wipe.

c) A 1 ul blank sample was pipetted onto the end of a fiber optic cable (the receiving fiber).

d) Closed the sampling arm and initiated a spectral measurement by setting as blank for DNA using the operating software on the computer.

e) Followed the procedure for blank so as to set reblank. This is to check that the instrument is working properly.

f) With the sampling arm open, pipetted 1 µl of the sample onto the lower measurement pedestal. Closed the sampling arm and initiated a spectral measurement using the operating software. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement made.

g) When the measurement was complete, opened the sampling arm and wiped the sample from both the upper and lower pedestals using a soft laboratory wipe.

h) The measured quantified values were used for further use.

3.3.4 DNA amplification by polymerase chain reaction (PCR)

The isolated and quantified DNA was used as a template to amplify ITS (Internal transcribed spacer) regions together with the 5.8S rDNA gene through PCR using Universal primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') (White et al., 1990). The 50 µl reaction mixture for PCR amplification contained: 10 ng DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 µM of each primer and 2.5 units of Taq DNA polymerase (Fermentas, USA). Amplifications were performed in a GeneAmp PCR systems (Applied Biosystems, USA) with an initial denaturaton step of 94^{o}C for 3 min followed by 35 cycles of 94 ^{o}C for 1 min, 50 ^{o}C for 1 min and 72 ^{o}C for 1.5 min and a final extension of 72 ^{o}C for 8 min. PCR products were run for 45 minutes through 1.2% agarose gel stained with ethidium bromide and visualized under UV transillumination and photographed using gel doc system.

3.3.5 Restriction digestion of ITS-PCR products

The ITS-PCR products were digested with restriction enzymes such as *TaqI, HinfI, AluI* and *MboI*.

Procedure:

a) Sterile water was added in a sterile microfuge tube containing ITS-PCR product and made up a volume of 17 µl (500 ng).
b) The appropriate 10X restriction enzyme assay buffer (Fermentas, USA) was added and mixed thoroughly by tapping the tube.

c) 1 µl (2-5 units) of the restriction enzyme was added, mixed by tapping the tube.

d) The mixture was incubated at the appropriate temperature for 3 hrs.

e) To stop the reaction, 4-5 µl gel loading buffer was added, mixed by vortexing briefly.

f) The products were then run on a 1.5% agarose/EtBr gel and visualized under UV transilluminator.

3.3.6 Purification of ITS-PCR products

The different sized ITS-PCR products were purified using PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Purified PCR products were eluted with 40 µl TE buffer (pH 8.0). This purified product is free from primers, nucleotides, and salts and can be used directly for ligation and further for cloning.

3.3.7 Quantification of purified PCR-products

The concentration of purified PCR products was determined by NanoDrop™ 1000 spectrophotometer (Thermo scientific, USA).

3.3.8 Ligation in T-vectors

The purified and quantified PCR products were ligated into pTZ57R/T vector using InsTA clone™ PCR cloning kit (Fermentas, USA).

Procedure:

a) The amount of PCR product equivalent and to 0.54 pmoles of ends was determined by referring to the table 3.2 (Manual of InsTAclone™ PCR Cloning Kit).

b) A well labelled 1.5 ml microcentrifuge tube was taken and water (nuclease free, up to 29µl) was added.

c) To this water, 6µl of 5X Ligation Buffer was added and mixed properly by tapping the microcentrifuge tubes.

d) To this mixture, 3µl of vector pTZ57R/T, (0.165µg, 0.18pmol ends) was added and mixed properly.

e) Purified PCR fragment (approx. 0.54pmol ends, calculated according to the table 3.2) was added to the solution and mixed thoroughly by pipetting.

f) 1µl (5U) of enzyme T4 DNA Ligase was then added. The components in the tube were mixed properly.

g) The reaction mixture was kept overnight at 22 °C and after that analyzed on 0.8% agarose gel to check the ligation

Table 3.2: Table for calculating the amount of a PCR fragment (µg) required per ligation reaction

Length of DNA fragment (bp)	**pmoles of ends per 1µg of DNA**	**Quantity of PCR fragments for ligation reaction in µg**
100	30.0	0.018
300	10.0	0.054
500	3.0	0.090
1000	3.0	0.180
2000	1.5	0.360
3000	1.0	0.540

3.3.9 Cloning of ITS-PCR products

The ligated products were transformed into *E.coli* DH5α cells.

Transformation of DH5α by heat shock method

a) *E. coli* DH5α cells were taken from glycerol stock and streaked on Luria Agar (LA) plates and incubated at 37 °C overnight.

b) Single colonies were isolated and inoculated in Luria Broth (LB) (Appendix I) and incubated overnight at 37 °C with shaking (180 rpm in a rotary shaker).

c) 100 µl of the overnight grown culture was inoculated in 50 ml LB and incubated in a shaker at 180 rpm for 5 hours and then the culture was centrifuged at 7500 g for 15 minutes at 5 °C in autoclaved 30 ml tubes.

d) The supernatant was discarded and 10 ml of filter sterilized 0.1 M $CaCl_2$ was added and the tubes were incubated in ice for 15 minutes.

e) The cells were again centrifuged at 5000 rpm for 10 minutes at 4 °C and the supernatant was discarded and 1 ml of 0.1 M $CaCl_2$ was added and the tubes were incubated in ice for 3 hours to make competent cells.

f) To 100 µl of competent cells, 5 µl of ligated product was added and mixed gently and kept in ice for 30 minutes for binding of the plasmid to the cells.

g) Then the cells were given a heat shock treatment for exactly 2 minutes at 42 °C in a still water bath.

h) The cells were rapidly transferred into ice and kept for 2-3 minutes. To each tube, 1 ml of LB + Ampicillin (50 µg/ml) was added and the tubes were incubated at 37 °C for 1 hr for expression of Amp^r gene of the transformed cells.

i) Luria agar supplemented with ampicillin (50 µg/ml) agar plates were spreaded with 100 µl of 100 mM IPTG (isopropyl beta-D-thiogalactopyranoside) and 20 µl of 50 mg/ml X-gal (5-bromo-4-chloro-3-indoyl β-D galactoside). 100 µl of the transformed cells were plated on LA + Amp + X-Gal + IPTG plates (Appendix I).

j) The plates were incubated at 37 ℃ for 16-20 hrs and observed for the appearance of white and blue colonies. The plates were kept in a refrigerator overnight to intensify the blue color of the colonies and differentiate between recombinants and nonrecombinants.

3.3.10 Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (LacZ) bacterial host cells were spreaded on Luria Agar medium containing 50 µg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T easy vector is located in the multiple cloning site (MCS) of the plasmid's lacZα gene; if insert was present, non-functional β-galactosidase is produced, and the transformed bacterial colony is white.

Bacterial plasmid DNA isolation by alkaline lysis method

a) The recombinant white colonies were picked from the plates with the help of autoclaved toothpicks and inoculated in 2 ml of LB + Amp (Appendix I) and incubated overnight at 37 ℃.

b) 1.5 ml of the culture was taken in a tube and centrifuged the cells at 8000 g for 10 minutes and discarded the broth.

c) To the pettet, 100 µl of Solution 1 (Appendix I) was added and the cells were dissolved in the solution by vortexing. They were placed at room temperature for 5 minutes.

d) 200 µl of freshly prepared Solution 2 (Appendix I) was added to the cells and mixed the contents by inverting the tubes 10-12 times rapidly and kept for 2 minutes.

e) 150 µl of Solution 3 (Appendix I) was then added and the tubes were inverted slowly 10-12 times and kept in ice for 10-15 minutes. Centrifuged at 12,000 g for 10 min at 4 °C in a microfuge. Carefully transferred the supernatant to a fresh tube.

f) 400 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added to the contents and inverted the tubes for 2 minutes and centrifuged at 12,000 g for 10 minutes.

g) The upper aqueous layer was transferred to a fresh tube and added 800 µl of 95% ethanol. Mixed the contents by vortexing.

h) After proper vortex, the tubes were kept at room temperature for 5 minutes and centrifuged at 12,000 g for 5 minutes for precipitation of DNA.

i) The supernatant was discarded and the pellet was washed with 400 µl of 70% ethanol and centrifuged at 7000 g for 5 minutes.

j) The supernatant was removed and the pellet was air-dried and dissolved in 50 µl of autoclaved MQ water

k) Stored the plasmid at -20 °C for further use.

3.3.11 Screening for positive clones

Plasmid DNAs of the recombinant bacterial cells were isolated to confirm the presence of the plasmid in the cells. These plasmids were used for PCR reaction by insert specific primes (ITS-1 and ITS-4) to know the plasmid DNA containing the insert.

3.3.12 Sequencing

The inserts in the plasmids were sequenced by chain termination method (Sanger et al., 1977) using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South campus, Delhi university, New Delhi, India).

3.3.13 Sequence analysis

Comparison of clone sequences was performed using BLAST program (Altschul et al., 1990) to reported nucleotide sequences in GenBank database accessible through NCBI (National Centre for Biotechnology Information – http://www.ncbi.nlm.nih.gov). Sequences having 98% and above similarity were retrieved and used further for analyses. The sequences were edited with BioEdit v5.0.9 (Hall, 1999). The sequences were aligned with the program MAFFT v6 (Katoh and Toh, 2008). Phylogenetic analysis was performed by a Maximum likelihood method. A Maximum likelihood Rapid Bootstrapping algorithm was implemented on the data set for 1000 replicates in the program RAxML v7.03 (Stamatakis 2006a,b; Stamatakis et al., 2008), using the GTRMIX model with parameters optimized for each partition. In addition, Bayesian phylogenetic analysis was carried out using the Metropolis-coupled Markov chain Monte Carlo method (MCMCMC), in MrBayes v3.04 (Ronquist and Huelsenbeck, 2003),

performing two runs each using four chains and 5,000,000 generations. To implement the optimal model in Bayesian analyses, the GTR model was specified. The substitution rate matrix, transition/transversion rate ratio, character state frequencies, gamma shape parameter α, and the proportion of invariant sites were unlinked across the ITS partitions. Trees were sampled every 100^{th} generation. The proportion of burn-in trees sampled before reaching equilibrium was estimated by plotting likelihood scores as a function of the number of generations. Only Bayesian posterior probabilities (PP) greater than or equal to 0.95 are considered significant and shown on tree. The output tree was evaluated with program TREEVIEW (www.taxonomy.zoology.gla.ac.uk/rod/rod.html).

3.4 Growth of *Morchella* species *in vitro*

Six *Morchella* cultures (representing four different *Morchella* species) such as *Morchella sp*. (MR2), *M. elata* (MR3), *M. elata* (MR6), *M. crassipes* (MR5), *M. crassipes* (MR8), and *M. spongiola* (MR17) were selected on the basis of availability of pure cultures to carry out further physiological studies.

3.4.1 Growth studies in different liquid media

Eleven different media, which are being widely used for studying growth pattern in different fungi, were used. Liquid media used in the study were Malt Extract (ME), Modified Melin Norkrans Media (MMN), Czapek Dox (CZD), Yeast Maltose Glucose (YMG), Sabourd's media (SB), Basal media (BS), Minimal Mineral media (MMM), Potato Dextrose broth (PDB), Mineral Salts broth (MSB), morel growth broth (MGB)

and pikovskaya's (PKV) media (Appendix II). pH of 5.6 was set with 1N HCl or 1N NaOH (as required) for each media.

Fifty ml of the above said media were dispensed in flasks and autoclaved at 121 °C for 15 minutes. Mycelial discs (5 mm in diameter) of different *Morchella* species, *Morchella sp*. (MR2), *M. elata* (MR3), *M. elata* (MR6), *M. crassipes* (MR5), *M. crassipes* (MR8), and *M. spongiola* (MR17) were inoculated into the medium. After inoculation, the flasks were incubated for 7 days at 25 ± 1 °C in dark under stationary conditions. After 7 days, the cultures were harvested and washed with sterile double distilled water. Cultures were oven dried at 60 °C. Biomass, pH, protein content and enzyme activities such as acid phosphatase, phytase, laccase, lignin peroxidase and manganese peroxidase were determined.

3.4.2 Growth studies with different pH

To study the effect of pH on the growth, mineral salts broth (MSB) was used. pH of media was set at 2.5, 3.5, 4.5, 5.0, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5 by using 1N HCl or 1N NaOH (as required). Fifty ml of MSB media with different pH was dispensed in flasks and the flasks were then autoclaved at 121 °C for 15 minutes. The autoclaved media was inoculated with two mycelial discs (each 5 mm in diameter) of *Morchella sp*. (MR2), *M. elata* (MR3), *M. elata* (MR6), *M. crassipes* (MR5), *M. crassipes* (MR8), and *M. spongiola* (MR17). The flasks were incubated for 7 days at 25 ± 1 °C under stationary conditions. After 7 days, the cultures were harvested. Biomass, pH and protein content of culture filtrate were determined.

3.4.3 Growth studies with temperature variations

To study the effect of temperature on the growth of *Morchella* spp., cultures of *Morchella sp*. (MR2), *M. elata* (MR3), *M. elata* (MR6), *M. crassipes* (MR5), *M. crassipes* (MR8), and *M. spongiola* (MR17) were grown in temperature stress. Autoclaved MSB media (50 ml) with pH of 5.6 was inoculated with two mycelial discs (each 5 mm in diameter) of each culture. The flasks were then kept in dark under different temperature conditions of 4, 10, 15, 25, 30, 35, 40, and 50 °C.

3.4.4 Growth and enzymatic studies with different carbon and nitrogen sources

To study the effect of different carbon sources on growth and enzyme activities, glucose in mineral salts broth (50 ml, pH 5.6) was replaced with equal amounts (10 g/L) of other carbon sources as fructose, sucrose, mannose, cellobiose, ribose, rhamnose, galactose, arabinose, xylose, sorbose and mannitol. Nitrogen sources were evaluated similarly by replacing sodium nitrate ($NaNO_3$) (0.2 g/L) with other nitrogen sources such as, ammonium nitrate (NH_4NO_3), sodium nitrite ($NaNO_2$), peptone, yeast extract, casein and tryptone. *Morchella sp*. (MR2), *M. elata* (MR3), *M. elata* (MR6), *M. crassipes* (MR5), *M. crassipes* (MR8), and *M. spongiola* (MR17) were inoculated into the media. The flasks were incubated for 7 days at 25 ± 1 °C. After 7 days, mycelia were harvested, washed with double distilled water and dried at 70 °C for 48 hrs. Biomass, pH and enzyme activities such as acid phosphatase, phytase, laccase, lignin peroxidase and manganese peroxidase after 7 days were studied.

3.4.5 Growth and enzymatic studies with different inducers

To study the effect of inducers on growth and enzyme activities, 50 ml of mineral salts broth with glucose as carbon source and sodium nitrate as nitrogen source and pH 5.6 was inoculated with two mycelial discs of *Morchella* cultures (*Morchella sp*. (MR2), *M. elata* (MR3), *M. elata* (MR6), *M. crassipes* (MR5), *M. crassipes* (MR8), and *M. spongiola* MR17). The flasks were incubated at 25 ℃ in dark for 24 hrs. In order to avoid inducer stress, when the mycelia had just initiated growth after 24 hrs, different chemical inducers such as DNBA (DiNitrobenzoic acid), DOPA (3,4-Dihydroxyphenylalanine), pyrogallol (PG), 8-Hydroxyquinoline (8-OHQ), p-Nitroaniline (NA), ferulic acid (FA), veratryl alcohol (VA), phenol red (PR), resorcinol (RC) and 2,2-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) (at a concentration of 1 mM) as well as natural substrates as finely crushed eucalyptus leaves (EL), leaf litter (LL), saw dust (SD), pine needles (PN), groundnut shell (GS), wheat straw (WS) and rice straw (RS) (at a concentration of 1%) were added. The flasks were incubated again at 25 ℃ for 7 days in dark under stationary conditions. Mycelia were harvested and dried after 7 days. Growth and ligninolytic enzyme activities such as laccase, lignin peroxidase and manganese peroxidase were determined.

3.4.5.1 Bradford Assay for protein estimation (Bradford, 1976)

Reagents

1. Bradford solution: Dissolved 100 mg of Coomassie Blue G in 50 ml of 95% ethanol. To this solution, added 100 ml of 85% H_3PO_4, and diluted to 1 Litre with water.
2. Bovine Serum Albumin (BSA): 1 mg/ml of BSA in H_2O

Procedure:

a) To the test tube, added 1 ml of culture filtrate.

b) To this, added 5 ml of Bradford reagent.

c) Measured the absorbance at λ=595 nm against blank containing distilled water and reagent only.

d) For standard curve, pipetted out duplicate volumes of 5, 10, 15, 20, 25, 25, 30, 35, 40, 45, and 50 µl of BSA (1mg/ml) in test tubes. The final volume was made up to 1 ml with water. Added 5 ml of Bradford reagent to this solution. Measured the absorbance at λ=595 nm and plot the standard graph between concentration and absorbance. The protein concentration was determined from the standard graph.

3.4.5.2 Acid Phosphatase activity

Acid phosphatase activity from mycelium was determined by the method of Tabatabai and Bremner (1969). Acid phosphatase from culture filtrate was determined by the method of Dorn and Riviera (1966).

3.4.5.2.1. Determination of acid phosphatase activity from mycelium (Tabatabai and Bremner, 1969)

Reagents:

1. Modified Universal buffer 5X (pH 5.5)

Tris (hydroxyl methyl) amino methane	3.03 g
Maleic acid	2.90 g
Citric acid	3.50 g
Boric acid	1.57 g
NaOH (1 N)	122 ml

Water to make volume 250 ml.

2. 0.5 N NaOH: Dissolved 20 g NaOH pellets in distilled water and made the volume to one litre.
3. 0.115 M p-nitro phenyl phosphate solution: Dissolved 4.268 g of p-nitro phenylphosphate disodium salt hexahydrate in 100 ml of modified universal buffer (1X).
4. 1 mg/ml p- nitrophenol (PNP): 1 mg/ml p- nitrophenol (PNP) solution in modified universal buffer (1X).

Procedure:

a) The mycelium previously was filtered and washed aseptically with sterile distilled water followed by a rinse with filter sterilized modified universal buffer (1X).
b) After an aseptic rinse with sterile modified universal buffer, mycelium was placed in sterile 30 ml screw cap tubes with 4 ml of sterile (1X) buffer solution.
c) Added 1 ml of filter sterilized 0.115 M disodium p- nitro phenyl phosphate solution.
d) The contents were incubated at 30 °C in a water bath for 2 hours in the dark.
e) After 2 hrs of incubation, 5 ml of 0.5 N NaOH was added to stop the reaction.
f) The mixture was swirled and filtered through Whatman No 1 filter paper.
g) Transferred the filtrate to glass cuvette and measured the yellow color intensity with UV-VIS spectrophotometer (Hitachi U-2001) at 410 nm.
h) The filtered out mycelium from the each vial was dried for 48 hrs at 70 °C and the dry weight was recorded separately.

i) Phosphatase activity was indicated as the amount of p-nitrophenol released in the filtrate from the p-nitro phenyl phosphate substrate per gram of the mycelium. The p- nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.

j) To perform controls, followed the procedure described for the assay but made the addition of 1 ml of p-nitro phenyl phosphate after the addition of 0.5 N NaOH (i.e. immediately before filtration).

Calculation:

$$\text{Acid phosphatase activity} = \frac{\text{Concentration of PNP } (\mu M)}{2 \times \text{weight of mycelium}}$$

3.4.5.2.2. Acid phosphatase activity in culture filtrate (Dorn and Riviera 1966)

Reagents:

1. 0.6 M acetate buffer, pH 4.8
2. p-nitro phenyl phosphate solution (1.25 mg/ ml in 0.6 M acetate buffer)
3. p-nitrophenol (PNP) : (1 mg/ml) solution in 0.6 M acetate buffer.
4. NaOH (1 N): 40 g NaOH was dissolved in distilled water and volume was made to 1litre.

Procedure:

a) To 0.2 ml of culture filtrate, 0.8 ml of substrate (5mg/4ml of p-nitro phenyl phosphate in 0.6 M acetate buffer) was added.

b) Mix the contents and incubated them at 30 °C in dark for one hour.

c) After incubation, added 2 ml of 1 N NaOH to stop the rection.

d) Measured the yellow color intensity with UV-VIS spectrophotometer (Hitachi U-2001) at 410 nm.

e) The p-nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standard containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.

Enzyme activity was expressed as micromoles of p-nitrophenol released per hour per millilitre of culture filtrate.

3.4.5.3 Phytase activity (Kim and Lei, 2005)

Reagents:

1. Citrate buffer (0.2M*)*, pH 5.5

2. Sodium phytate 1% (9 mM) in 0.2 M citrate buffer, pH 5.5

3. Trichloroacetic acid (TCA) 15% (w/v) (room temperature)

4. Color reagent (prepared fresh daily): mixed three volumes of 1 M sulfuric acid and one volume of 2.5% ammonium molybdate, one volume of 10% ascorbic acid (w/v) and mixed well.

Procedure:

a) Added 0.2 ml of culture filtrate into 10-ml test tubes.

b) Incubate the test tubes in 37°C water bath for 5 min.

c) Added 0.2 ml of 1% (wt/vol) sodium phytate to start the enzymatic hydrolysis of phytate.

d) Incubated them for 15 min at 37°C.

e) Stopped the reaction by adding 0.4 ml of 15% trichloroacetic acid (room temperature).

f) Mixed 0.2 ml of the reaction mixture with 1.8 ml of MQ water.

g) Added 2.0 ml of fresh color reagent (Appendix II) to each tube and mixed well.

h) Incubated the mixture at 50 °C for 15 min and then cooled the tubes to room temperature.

i) Read the absorbance of each sample solution at 820 nm.

j) The serially diluted potassium phosphate solution was used as standards.

One unit of phytase is defined as the amount of enzyme required to release 1µmol of inorganic P/min from sodium phytate at 37°C.

3.4.5.4 Laccase enzyme assay (Eggert et al., 1996)

Reagents:

1. 0.1 M sodium acetate buffer (pH 5)
2. 500 µM ABTS (2,2′-azino-bis-[3-ethyl benzothiazoline-6-sulfonic acid])

Procedure:

a) To the test tube, added 2.5 ml of 0.1 M sodium acetate buffer (pH 5)

b) To this buffer, added 330 µl of 500 µM ABTS (2,2′-azino-bis-[3-ethyl benzothiazoline-6-sulfonic acid])

c) The solution was incubated at 25 °C for 5 minutes.

d) To start the reaction 0.17 ml of culture filtrate was added to the above mixture of buffer and substrate. Initial absorbance at time t=0 was observed at λ=420 nm spectrophotometrically.

e) The test tubes were then incubated at 25 °C in dark for 15 minutes.

f) Final absorbance at t=15 min was observed λ=420 nm.

g) Average of the two determinations was calculated to get ΔA, increase in absorbance/min.

$$\Delta A = (A_{15} - A_1)/15$$

One unit of the enzyme is amount of enzyme that converts 1 mM substrate in one minute under the described conditions.

1U/ml = 1 mM/ml.min = ΔA * (1000/ε420 * d) * V/v * F

Where

ΔA -- Increase in absorbance/min at 420 nm

(1000/ε420 * d) -- Conversion to mmol converted substrate/ml using the molar extinction coefficient (εABTS, 420 = 3,600[l * mol-1 * mm])

d -- Length of the light path in mm (10 mm)

V -- Total volume in ml (3000 µl)

v -- Volume of sample ml (170 µl)

V/v -- Dilution in assay (17.647)

F -- Dilution factor of stock

The resulting equation is: = ΔA * 0.490 * F

3.4.5.5 Manganese peroxidase (MnP) activity (Warishi et al., 1992)

Reagents:

1. 500 mM sodium malonate buffer (pH 4.5)
2. 5 mM $MnCl_2$
3. 2 mM of H_2O_2

Procedure:

a) To 2 ml of eppendorf, added 200 µl of 500 mM sodium malonate buffer (pH 4.5).

b) To this buffer, added 200 µl of 5 mM $MnCl_2$ and 1.4 ml of culture filtrate

c) Incubated them at 25 °C for 5 minutes.

d) Added 200 µl of 2 mM of H_2O_2 and observed the absorbance at λ= 270 nm at t=0.

e) Incubated the eppendorfs at 25 °C in dark for 15 minutes.

f) Measured the final absorbance at t = 15 at λ = 270 nm

g) MnP activity was measured by monitoring the rate of Mn(III)-Malonate complex formation at 270 nm

One unit of enzyme activity was defined as 1 µM of substrate oxidized per min.

1U/ml = 1 mM/ml.min = ΔA * (1000/ε270 * d) * V/v * F

$$\frac{U}{L} = \frac{\Delta A \times 1000 \times 3000}{11590 \times 200}$$

Where : $\Delta A = (A_{15} - A_1)/15$, ε270 nm=11590$M^{-1}cm^{-1}$

3.4.5.6 Lignin peroxidase (LiP) activity (Tien and Kirk, 1984)

Reagents:

1. 125 mM tartarate buffer, pH 2.5
2. 20 mM veratrylalcohol
3. 10 mM H_2O_2

Procedure:

a) LiP was determined by H_2O_2-dependent veratrylaldehyde formation from 2 mM veratrylalcohol.

b) To the test tube, added 2 ml of water and 380 µl of culture filtrate.

c) To this, 1.2 ml of 125 mM tartaric acid was added.

d) 300 µl of substrate 20 mM veratrylalcohol was added to this buffer and incubated at 25 °C for 5 minutes.

e) After incubation, added 120 µl of 10 mM H_2O_2 to start the reaction and measured the increase in absorbance from time t = 0 to t = 2 minutes at λ=310 nm.

One unit of enzyme activity was defined as 1 µM of substrate oxidized per min.

1U/ml = 1 mM/ml.min = ΔA * (1000/ε310 * d) * V/v * F

$$\frac{U}{L} = \frac{\Delta A \times 1000 \times 3000}{9300 \times 380}$$

Where: $\Delta A = (A_{15} - A_1)/15$, ε270 nm=9300$M^{-1}cm^{-1}$

3.6 Sclerotial studies of *Morchella* spp.

3.6.1 Sclerotial studies in different media

Sclerotium is a big structure having large cells and thick cell walls. Role of sclerotium is that it allows the organism to survive in adverse conditions and it acts as an intermediate stage in the formation of fruiting body. Sclerotia formation requires definite conditions of nutrition and environment and is dependent on a number of factors.

3.6.2 Sclerotia formation in different media in petriplates

To study the effect of different media on sclerotia formation, different *Morchella* spp. (*Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) were inoculated in different agar media in petriplates. Media used were malt extract (ME), modified melin norkrans media (MMN), czapek dox (CZD), yeast maltose glucose (YMG), sabourd's media (SB), basal media (BS), minimal mineral media (MMM), potato dextrose broth (PDB), mineral salts broth (MSB), morel growth broth (MGB) and pikovskaya's media (PKV). The media were autoclaved and dispensed into sterilized 90 mm petriplates. Mycelial discs (5 mm diameter) were placed in the centre of petriplates containing media. Petriplates were incubated at 25 °C in dark and observed daily for mycelia and sclerotia formation. When the petriplates were fully covered with mycelia, they were placed at 4 °C in dark. The media in which sclerotia were formed were selected for carrying out further studies.

3.6.3 Sclerotia formation in split plates

To study the influence of two different media (nutrient rich and nutrient poor media) on sclerotia formation; different *Morchella* spp. (*Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) were grown in split plates (Amir et al., 1993; 1995) containing both the media.

Split plates are the plates in which a smaller 90-mm petriplate is placed in the centre of a larger 150-mm petriplate and both the petriplates are filled with two different media (Fig 3.2). One of the media is nutrient rich while the other is nutrient poor. Mycelium is inoculated in the centre of the smaller petriplate containing media A and after its growth in this medium; it grows towards the other medium B.

The experiment was performed so as to study the effect of excess of nutrients and nutrient deficiency on sclerotia formation. The two different media used were Buscot media (Buscot medium A and B) (Buscot, 1993b) and nutrient media (nutrient rich medium A and nutrient poor distilled water agar B). All the media alone in single petriplates were also inoculated with each isolate tested so as to verify whether sclerotia are formed alone in each media tested or not. Mycelial discs (5 mm diameter), taken from the growing edges of *Morchella* spp. (*Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) were placed, with the hyphal surface in contact with the agar. The plates were incubated at 25 °C in dark. Mycelial growth and pigmentation were observed. Formation, location and type of sclerotia were assessed at weekly intervals.

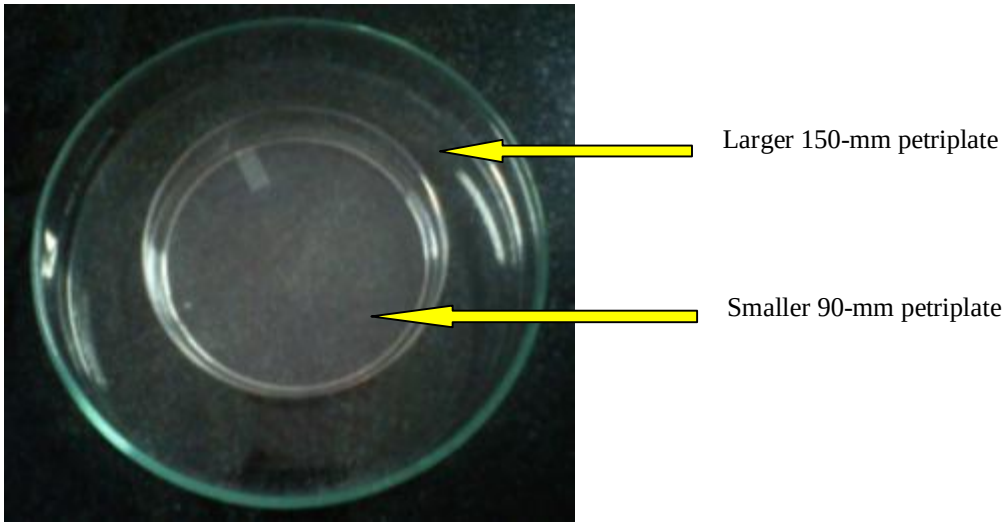


Fig 3.2: Split plate having used for two different media A and B. (Amir et al., 1993, 1995)

3.6.4 Dual culture studies for sclerotia formation

To study the effect of interactions between two different *Morchella* spp. on sclerotia formation; dual culture tests were carried out. In dual culture tests, two different cultures are grown simultaneously on the same plate at its perimeter and the interactions between the two cultures are observed. Pure cultures of *Morchella* isolates (*Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) in combinations were inoculated on the perimeter of petriplates on malt extract agar and potato dextrose agar media. Mycelial discs of 5 mm diameter were cut using cork borer from actively growing pure cultures. Two mycelial discs of different *Morchella* isolates were placed near periphery of the petriplates. These petriplates were incubated at 25 °C in dark. Mycelial growth, pigmentation, and sclerotia formation were observed daily.

3.6.5 Sclerotia formation in jars

To test the effect of volume (air space) on sclerotia formation, tissue culture jars were used. Fifty ml of modified Buscot media (Buscot 1993b) (starch (15 g/L), yeast extract (4 g/L), KH_2PO_4 (1 g/L), $MgSO_4.7H_2O$ (500 mg/L), agar 15 g/L) supplemented with glucose (10 g/L) and sodium nitrate (250 mg/L) was dispensed into jars. The jars (Kasablansky, Mumbai, India) (used for plant tissue culture) had the capacity of 300 ml of length 13 cm. The bottles were constricted with a closed enclosure. The bottles were made up of glass whereas the caps were made up of plastic. The jars were autoclaved for 15 minutes at 121 °C. After cooling, *Morchella* spp. (*Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) were inoculated in the centre of jars and incubated at 25 °C in dark. The resting fungal colonies were observed till the formation of sclerotia.

3.6.6 Effect of carbon and nitrogen sources on sclerotia formation

Nutritional parameters such as carbon and nitrogen sources are the main factors that influence sclerotia formation. To study which of the carbon/nitrogen source influence the best for sclerotia formation in *Morchella* spp.; different cultures of *Morchella* spp. (*Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) were cultured on modified Buscot media (Buscot 1993b) supplemented with glucose (10 g/L) and sodium nitrate (250 mg/L). Glucose in the media was replaced by equal amounts of other carbon sources such as ribose, arabinose, xylose, mannose, galactose, fructose, sorbose, rhamnose, sucrose, cellobiose, starch soluble, and mannitol. Similarly, for studying the effect of nitrogen sources, sodium nitrate (250 mg/L) was replaced by other nitrogen sources such as, casein, peptone, yeast extract, tryptone, sodium nitrite

($NaNO_2$), ammonium chloride (NH_4Cl), and ammonium nitrate (NH_4NO_3). Mycelial discs (5 mm-diam.) cut from the edge of actively growing 6-day old cultures were placed with the hyphal surface in contact with the agar at the centre of glass jars. Jars were then incubated at 25 °C in dark. Fifty ml of the above said media with different carbon/nitrogen sources and containing 1.5% agar was dispensed in jars and autoclaved. Mycelial discs of *Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) were inoculated in the centre of jars and incubated at 25 °C in dark. The jars were observed daily till sclerotia formation. Mycelial pigmentation and sclerotia number were assessed at weekly intervals.

3.6.7 Effect of substrates on enzyme activity

Mushrooms are cultivated on substrates, particularly, agricultural substrates, that are rich in carbon and nitrogen sources. In an attempt to cultivate morels, different *Morchella* spp., such as, *Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17) were inoculated on substrates. The substrates used in the study were 62 gm of wheat straw (WS), 25 gm of rice straw (RS), 25 gm of saw dust (SD), 50 gm of apple leaves (AL), 20 gm of groundnuts shell (GS), 35 gm of eucalyptus leaves (EL), 15 gm of bamboo leaves (BL), 45 gm of orange peels (OP), 65 gm of pine needles (PN), 98 gm of wheat bran (WB), 65 gm of wheat grains (WG), and 23 gm of carrot peels (CP). These substrates were chopped into smaller pieces in a grinder and soaked overnight in water. The next day, excess water was drained and the substrates were then filled in glass jars (one forth volume of jars). Fifty ml of water was added to them. They were then autoclaved at 121 °C for 1 hr. After cooling, these substrates were then inoculated with

discs of mycelia of *Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8), and *M. spongiola* (MR17). Control groups without any mycelia too were placed along with treatment groups. These were incubated at 25 ℃ and 60 % relative humidity in dark till the mycelia had grown fully on the substrates and sclerotia were formed.

Preparation of Culture extract (Papinutti and Lechner, 2008)

a) After the required incubation period, the fungal mass grown on substrates was soaked with 50 ml of 0.1 M sodium acetate buffer (50mM, pH 4.5).
b) The jars were then kept on shaker for shaking at 120 rpm at room temperature for one hour.
c) After one hour, the crude solution was centrifuged at 8000 rpm for 10 minutes at room temperature.
d) The supernatant was collected in vials and used further for studying enzyme activities such as acid phosphatase, phytase, cellulase, laccase, lignin peroxidase and manganese peroxidase.
e) The substrates left out as the pellet were dried completely and used for their chemical characterization such as lignin, cellulose and ash content.
f) For pH determination, mushroom compost was soaked with water and kept for one hour for shaking at room temperature at 120 rpm.

3.6.7.1 Acid phosphatase activity

Acid phosphatase was determined with the supernatant by the method of Dorn and Riviera (1966) as described previously in section 3.4.5.2, by using p-nitro phenyl phosphate as substrate.

3.6.7.2 Phytase activity

Phytase activity was determined by the method of Kim and Lei (2005) as described previously in section 3.4.5.3 by using sodium phytate as substrate.

3.6.7.3 Laccase enzyme assay

Laccase activity was measured by monitoring the oxidation of 500 μMABTS (2,2′-azino-bis-[3-ethyl benzothiazoline-6-sulfonic acid]) buffered with 0.1 M sodium acetate buffer (pH 5) at 420 nm (Eggert et al., 1996) (as described previously in section 3.4.5.4). The reaction mixture (3 ml) contained 0.17 ml of culture filtrate. One unit of enzyme activity was defined as 1 μM of substrate oxidized per min.

3.6.7.4 Manganese peroxidase (MnP) activity

MnP activity was measured by monitoring the rate of Mn(III)-Malonate complex formation at 270 nm (Warishi et al., 1992) (as described previously in section 3.4.5.5). The reaction mixture contained 50mM sodium malonate (pH 4.5), 0.5mM $MnCl_2$ and the reaction was initiated by adding 0.2mM of H_2O_2. One unit of enzyme activity was defined as 1 μM of substrate oxidized per min.

3.6.7.5 Lignin peroxidase (LiP) activity

LiP was determined by the H_2O_2-dependent veratrylaldehyde formation (Tien and Kirk, 1984) from 2mM veratrylalcohol in 0.1M sodium tartrate buffer (pH 3), reactions were started by the addition of 0.4mM H_2O_2 (as described previously in section 3.4.5.6). One unit of enzyme activity was defined as 1 μM of substrate oxidized per min.

3.6.7.6 Cellulase activity

Cellulase activity was determined by the method of Ghosh (1987)

Reagents:

1. Carboxy Methyl Cellulose (2 %) in 0.05 M sodium citrate buffer, pH 4.8
2. Glucose stock: 1 mg/ml of glucose in sterile double distilled water
3. Dinitrosalicylic Acid Reagent Solution, 1% for 1 Litre:

Dinitrosalicylic acid:	10.0 g
Phenol:	2.0 g
Sodium sulfite:	0.5 g
Sodium hydroxide:	10.0 g
Potassium sodium tartrate solution:	40%

Procedure:

a) To the test tube, added 0.5 ml of supernatant

b) To this solution, added 0.5 ml of 2% Carboxy Methyl Cellulose in 0.05 M sodium citrate buffer, pH 4.8

c) Incubated the mixture for 30 min at room temperature (25 °C)

d) After incubation, 3 ml of dinitrosalicyclic (DNS) reagent was added

e) The solution was then boiled for 5 min and the absorbance was measured at 540 nm.

f) The concentration of the sample was measured by analyzing the standard curve of 1 mg/ml glucose used as a standard.

3.6.7.7 Chemical characterization of substrates

The substrates used were mostly rich in lignin and it was important to find out the influence of these substrates on sclerotia formation and also on ligninolytic enzymes. The chemical characterization was carried out in order to study the rate of degradation of components in substrate. The chemical characterization of substrates (lignin, cellulose and ash content) was estimated by following methods.

3.6.7.7.1 Lignin estimation (TAPPI T-222)

Lignin is a polymer rich in aromatic hydrocarbons. Ligninolytic enzymes are resoponsible for breaking down the lignin polmer into its subunits.

Reagents used:

Conc. H_2SO_4 reagent (72 %)

Procedure:

a) One gm of substrate sample was taken in a 100 ml beaker and kept at water bath at 20 ℃.
b) To the beaker, 15 ml of ice cold (10 ℃ approx.) H_2SO_4 reagent (72 %) was added to it.
c) The beaker was kept as such at 20 ℃ in a water bath for 2 hrs with frequent stirring.
d) The material was then transferred to 1000 ml flask with 300 ml of water and the final volume was made up to 575 ml with water.
e) The solution was then boiled for 4 hrs. The insoluble material (lignin) was allowed to settle by keeping the beaker inclined. The supernatant was decanted.

f) The insoluble material (lignin) was dried at 105 ± 3 °C to a constant weight.

g) Cooled the material and weighed the substance.

h) The substrates of control and treatment groups were characterized for lignin content and % lignin degradation was calculated.

i) The %lignin was calculated by using the formula

$$\text{Lignin \%} = \frac{A \times 100}{W}$$

Where A= weight of lignin (g)

W= weight of test specimen (g)

3.6.7.7.2 Cellulose estimation (Updegraff method, 1969)

Reagents

1. Acetic/nitric reagent: Mixed 150 ml 0f 80% acetic acid and 15 ml of conc. HNO_3.
2. Anthrones reagent: Dissolved 200 mg of anthrone in 100 ml of conc. H_2SO_4.
3. Conc. H_2SO_4 reagent (67 %)
4. Carboxy methyl cellulose (CMC): 1 mg/ml in 0.1 M Sodium acetate buffer pH 4.5

Procedure:

a) Sample (0.5 g) was homogenized in a vortex mixture in 50 ml of double distilled water.

b) 10 ml of the above mixture was centrifuged for 5 min at 2000 rpm.

c) Discarded the supernatant and added 3 ml of acetic acid/nitric acid reagent.

d) Placed the tubes in boiling water bath for 30 min.

e) Cooled and centrifuged for 10 min at 350 rpm.

f) Discarded the supernatant and washed the residue with double distilled water. Added 10 ml of 67% (v/v) H_2SO_4 and allowed to stand for 1 hr.

g) Dilute 0.1 ml of this solution to 100 ml and 1 ml of this diluted to 10 ml of anthrone reagent and mixed well.

h) Placed the tubes in boiling water bath for 10 min.

i) Cooled and measured the colour at 620 nm. Blank was set with the anthrone reagent and double distilled water.

j) CMC (1 mg/ml) was prepared by mild heating and final volume was made up to 500 ml with water.

k) Taken 40-200 µg of CMC as cellulose standard. To 1 ml of this solution, added 10 ml of anthrone reagent and mixed well.

l) Placed the tubes in boiling water bath for 10 min. Cooled and measured the colour at 620 nm.

m) The concentration of sample was measured by analyzing the concentration of standard CMC from the graph.

3.6.7.7.3 Ash content of substrates

Procedure:

a) The digestion tubes were washed properly and dried completely.

b) To the digestion tubes, 200 mg of substrate sample was added.

c) The digestion tubes were kept on the digestion chamber.

d) The substrates were heated for 3 hours. The substrates were heated so that all the material became carbonized without flaming.

e) Cooled the digestion tubes and the weight of the left over material was recorded.

$$\text{Ash } \% = \frac{A \times 100}{B}$$

Where, A= weight of ash in grams

B=weight of test specimens in grams

3.6.8 Effect of substrates on enzyme activities during sclerotia formation

To study the relationship of sclerotia and ligninolytic enzymes produced during the formation and maturation of sclerotia, the mycelia of *Morchella crassipes* (MR8), was grown in the soil and substrate combination by the method described by Ower et al. (1986). The culture that produced the highest ligninolytic activity (*M. crassipes* (MR8)) was selected for carrying out this study. Briefly, the bottom one-fourth of the jar was filled with (overnight soaked) wheat straw (190 g)/wheat grains (175 g) and the other one-forth was filled with moistened garden soil (25 g). These two layers were separated from one another by placing an aluminium foil, having holes in it. The jars were autoclaved at 121 °C for one hour. Weight of jars before inoculation was recorded. Mycelial discs (two discs of 5 mm sized diameter) of *M. crassipes* (MR8) were inoculated on the soil layer. Jars were incubated at 25 °C in dark and observed for sclerotia formation at weekly intervals.

Preparation of culture extract (Papinutti and Lechner, 2008)

a) The sclerotia formed after 15, 18, 21, 24 and 27 days were counted.

b) To the jars, 100 ml of 0.1 M sodium acetate buffer (50 mM, pH 4.5) was added and kept for one hour on a shaker for shaking at 120 rpm at room temperature.

c) After one hour, the crude solution was centrifuged at 8000 rpm for 10 minutes at room temperature.

d) The supernatant was collected in vials and stored at -20 C for furthe use.

e) Ligninolytic enzyme activities such as laccase, lignin peroxidase and manganese peroxidase along with cellulose activity were determined in the extract as described previously (in section 3.4.5.4 - 3.4.5.6 and 3.6.7.6).

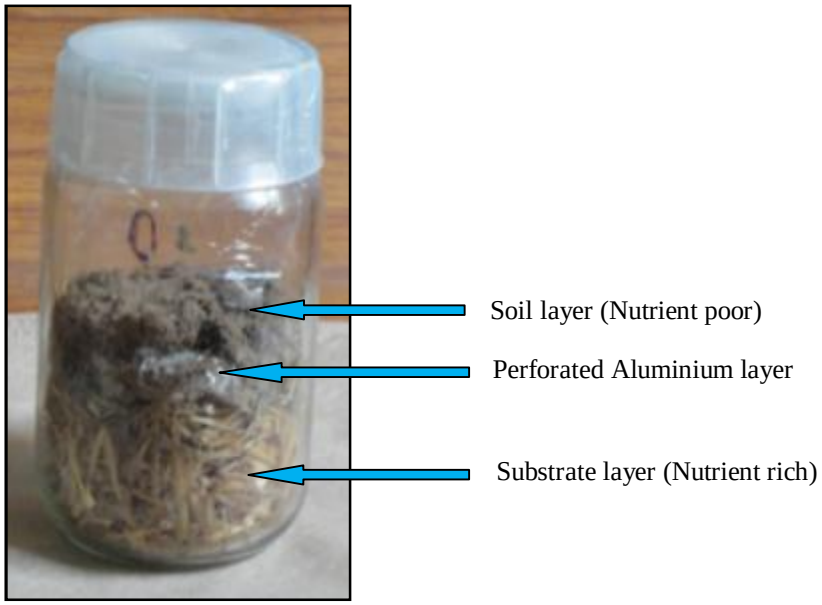


Fig 3.3: Open jar method containing nutrient rich and nutrient poor substrates used for sclerotia formation (Ower et al., 1986)

3.6.9 Statistical analysis

Three replicates were used for each experiment. Data were subjected to analysis of variance (ANOVA) and means were compared with Tukey's test at $P<0.05$. All the analysis was performed by using GraphPad prism 5 software (GraphPad Software, Inc, San Diego, 2007).

Chapter IV

Diversity of Morchella species

4.0 Survey and study the diversity of *Morchella* species of the Western Himalayan region

4.1 Survey results

Different geographical areas (mentioned in Table 3.1) were surveyed for *Morchella* fruiting bodies and thirty-two distinct morphotypes of fruiting bodies of *Morchella* (MR1 to MR29, F1, F2 and FK) were collected/procured from the western Himalayan region. The survey results showed that morels are usually found in cold places at an altitude of 2000 – 3500 meters above the mean sea level. *Morchella* spp. were mostly seen growing in soils that was rich in organic matter. In some places, it was seen growing on stumps and log of dead and decaying wood and rotten leaves in soil. Fruiting bodies were also seen growing on humus soil taking nourishment from the dead organic matter. The fruiting bodies were dried and their macroscopic and microscopic features were recorded so as to identify them.

4.2 Classical taxonomy to study the diversity

Macroscopic morphological details of specimens (MR1, MR3, MR6, MR8, MR11, MR13, MR14, MR20, MR21, MR23, MR24, MR25, MR26, MR27, MR28, MR29, F1, and FK) were recorded. Characteristics such as size, shape and color of apothecia, stipe, pileus, and spores were observed. Considerable variations were found in different *Morchella* species in the present study.

Color of fruiting bodies ranged from yellow, brown to black colored, and accordingly, they were classified as yellow or black morels. Apothecia of *Morchella* fruiting bodies such as MR1, MR8, MR21, MR25, MR26, MR27, MR28, MR29, MR11, MR13, MR14, and MR20 appeared to be yellow colored; while MR3, MR6, MR23, MR24, FK, and F1 were black in color (Fig 4.1). Apothecia were found to be terrestrial, stipitate, hollow and a clear

Fig 4.1: Morphological diversity of *Morchella* ascocarps a) *M. crassipes* (MR1) b) *M. elata* (MR3) c) *M. elata* (FK) and d) *M. crassipes* (MR20) in natural habitat of the forest areas of Himachal Pradesh.

differentiation into pileus and stipe was observed. Variations in the shape and size of pileus and stipe of these fruiting bodies were observed (Table 4.1). The spore measurements were recorded on twenty-five randomly selected spores using Nikon *50i* eclipse microscope (Fig 4.2-4.7).

Morphological characteristics of different fruiting bodies of *Morchella* have been summarised in Table 4.1. Based on the morphological features of fruiting bodies two different groups- one group of yellow morels, comprising of *M.crassipes/M. esculenta* (MR1, MR8, MR21, MR25, MR26, MR27, MR28, MR29, MR11, MR13, MR14, and MR20) and the other group of black morels, comprising of *M. elata/M. conica* (MR3, MR6, MR23, MR24, FK, and F1) were observed. A detailed view of the characteristics of the groups are mentioned below:

Yellow morels - *M. crassipes/M. esculenta*: Fruiting bodies of *Morchella* isolates such as, MR1, MR8, MR21, MR25, MR26, MR27, MR28, MR29, MR11, MR13, MR14, and MR20 had similar morphological characteristics; so they were grouped together into one group of yellow morels.

Fruiting bodies of yellow morels had yellow colored apothecium (15.0-18.5 cm), which was scattered, solitary, pileate, hollow and stipitate, clearly differentiated into pileus and stipe. Color of apothecia changed to dirty white in color after drying. Pileus was elongated, measured 10.3-11.7 × 3.8-5.5 cm, attached at the base to stipe, ovate, apex obtuse, pitted. Pits, 15 × 1 cm, irregular to rounded, up to 8 mm deep, yellowish in color on the surface and brownish on the inner side. Ribs were regularly anastamosed and light yellowish in color, edges obtuse, up to 1.5 mm thick, concolorous with the interior of pits. Stipe

measured 9.0-12.5 × 1.4-2.4 cm, swollen at the base, hollow and cylindrical, creamish in color, irregularly lacunose at the base but nearly even above, pubescent; pubescent hairs subhyaline, in conical fascicles, composed of 8 cells, which are thin walled. Asci were cylindrical, measured 245-350 × 16.8-26.2 µm, 8-spored, cylindrical, apex obtuse, J-ve. Spores had the size of 21-28 × 11-15 µm, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end. Paraphyses up to 9 µm wide below and 14.5 µm at the top, stout, thin walled, septate, simple or branched below, subhyaline individually, yellowish in mass, slightly projecting beyond the ascal tips.

These macroscopic and microscopic features were analysed and compared with the classical features reported by Waraitch (1976). Based on these features, the fruiting bodies were identified as *M. crassipes* or *M. esculenta.*

Among the yellow morels, minute variations were observed in the morphological features of test specimens.

Morphological variations in MR1: Fruiting body of MR1 had yellow colored apothecium (15.2-18.5 cm), which was stipitate, hollow and clearly differentiated into pileus and stipe (Fig 4.2). Minute variations were observed in the size of pileus (10.3-11.6 × 4.8-5.5 cm), stipe (9-11.5 × 1.9-2.4 cm), and asci (245-350 × 17.3-22.2 µm). Spores had the size of 13-28 × 11.95-15.5 µm (mean value, 24.39 × 13.82 µm), *Q* (length/width ratio) = 1.76 with uniseriate wall and were ellipsoidal in shape. In clusters, they appeared yellowish in color, smooth, eguttulate, polar oil droplets present at the ends. Paraphyses were present. Paraphyses stout, thin walled with septa, measured 356-426 µm and 7-11 µm wide and yellowish in color (Fig 4.2). Voucher specimen of the fruiting body was deposited in the Herbarium of Department of Botany, Punjabi University, Patiala, Punjab, India, with voucher no. PUN4117.

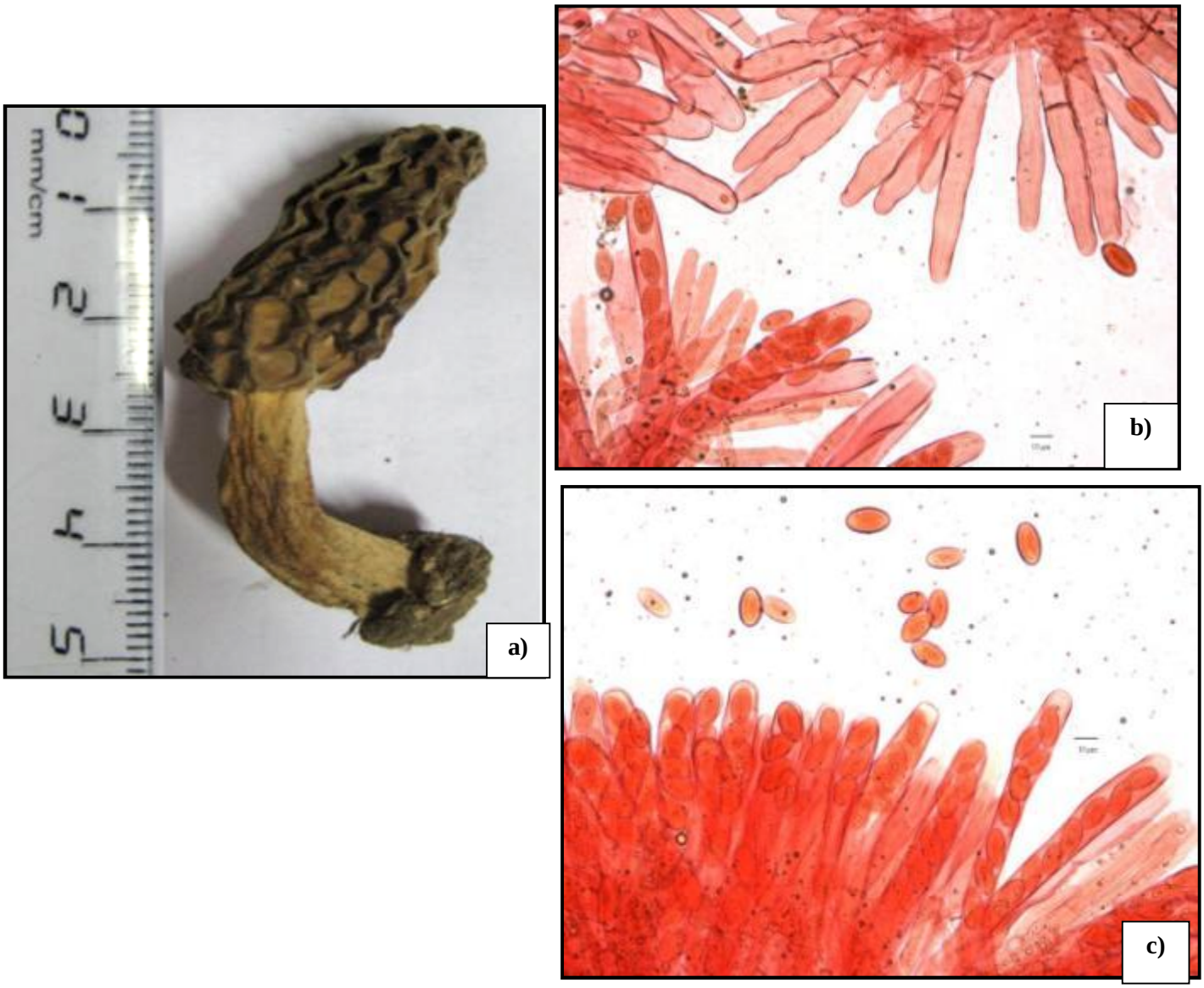


Fig 4.2: *Morchella crassipes* (MR1): a) Dried fruiting body along with scale, showing the size b) Congo red stained ascus containing ascospores d) Ascospores and paraphyses stained with congo red (40X magnification)

Morphological variations in MR8:

Fruiting body of MR8 had yellow colored apothecium measuring 15.0-17.8 cm (Fig 4.3). Pileus measured 10.3-11.7 × 4.0-5.0 cm, attached at the base to stipe, ovate, apex obtuse, pitted. Stipe was swollen at the base and measured 10.35-12.5 × 1.4-2.1 cm. Asci were cylindrical, 8-spored, and measured 255-289 × 16.8-26.2 µm. Ribs were regularly anastamosed and light yellowish in color, edges obtuse, up to 1.5 mm thick, concolorous with the interior of pits. Stipe measured 9.0-12.5 × 1.4-2.4 cm, swollen at the base, hollow and cylindrical, creamish in color, irregularly lacunose at the base but nearly even above, pubescent; pubescent hairs subhyaline, in conical fascicles, composed of 8 cells, which are thin walled.

Spores had the size of 21-27.49 × 12.95-15.32 µm (mean value, 24.12 × 14.47 µm), *Q* = 1.67, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end. Paraphyses up to 9 µm wide below and 14.5 µm at the top, stout, thin walled, septate, simple or branched below, subhyaline individually, yellowish in mass, slightly projecting beyond the ascal tips.

Voucher specimen of the fruiting body was deposited in the Herbarium of Department of Botany, Punjabi University, Patiala, Punjab, India, with voucher no. PUN4118.

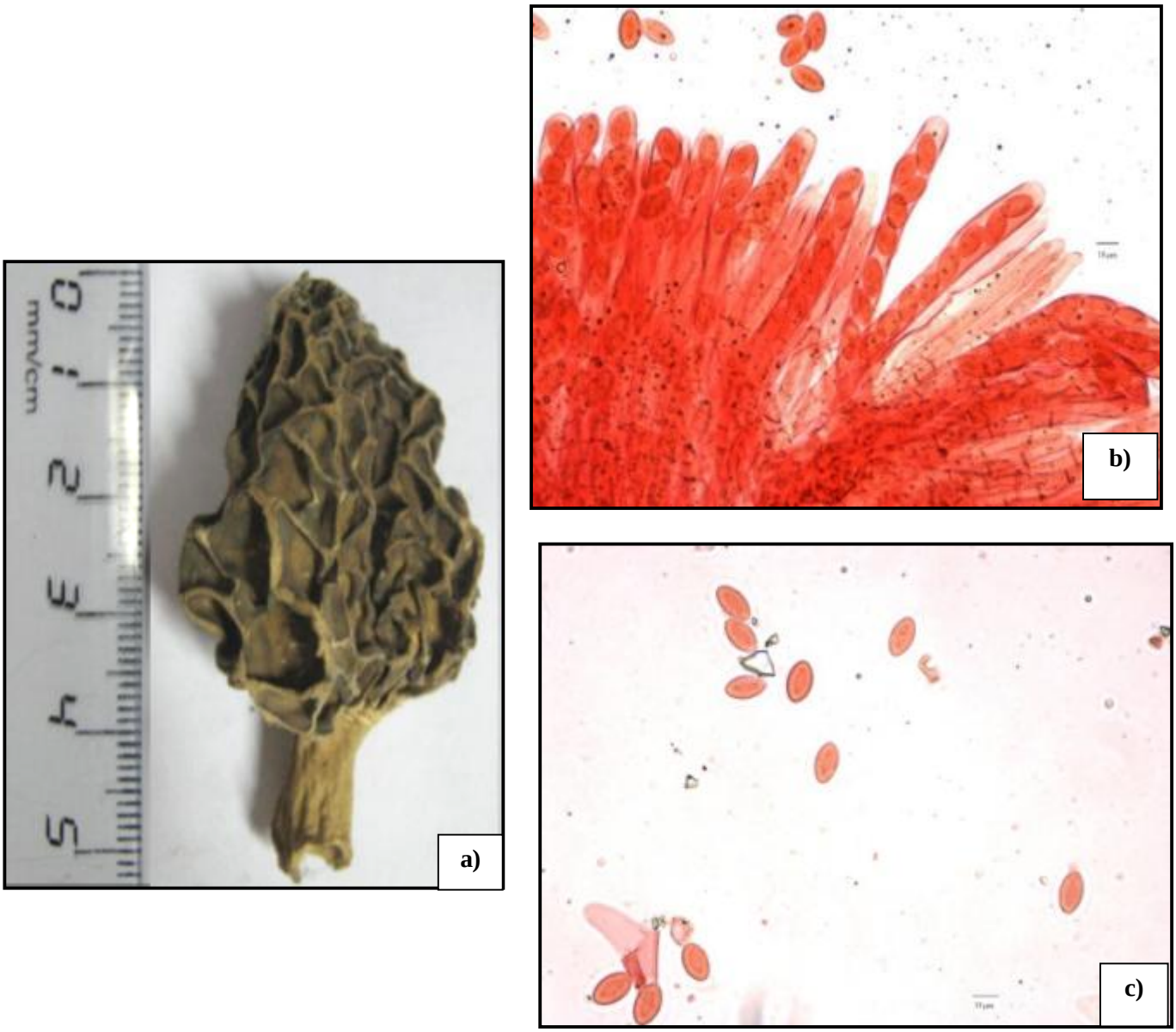


Fig 4.3: *Morchella crassipes* (MR8): a) Dried fruiting body along with scale, showing the size b) microscopic congo red stained slides of ascospores and ascus c) Ellipsoidal ascospores stained in congo red (40X magnification)

Morphological variations in MR20:

Fruiting body of MR20 had yellow colored apothecium (15-18.38 cm), which was scattered, solitary, hollow and stipitate, clearly differentiated into pileus (measuring 9.5-11.39 × 3.8-5.21 cm), and stipe (measuring 10-12 × 1.8-2 cm) (Fig 4.4). Ribs were regularly anastamosed and light yellowish in color, edges obtuse, up to 1.5 mm thick, concolorous with the interior of pits.

Asci were cylindrical, measured 263-338 × 17.7-25.34 µm, 8-spored, cylindrical, J-ve. Pits up to 4 × 1 cm, elongated, divided by low cross ribs, ochraceous, becoming brown towards margins, darker on drying. Ribs were irregularly anastamosed, less than 1 mm in thickness; edges sharp to obtuse, up to2 mm broad, concolorous with interior of pits, later blackish, giving rise to pubescent hairs.

Spores had the size of 22.0-27.5 × 11.05-14.58 µm (mean value, 25.21 × 12.8), *Q* = 1.96, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end. Paraphyses stout, thin walled with septa, measured 356-426 µm and 7-11 µm wide and yellowish in color.

Voucher specimen of the fruiting body was deposited in the Herbarium of Department of Botany, Punjabi University, Patiala, Punjab, India, with voucher no. PUN4119.

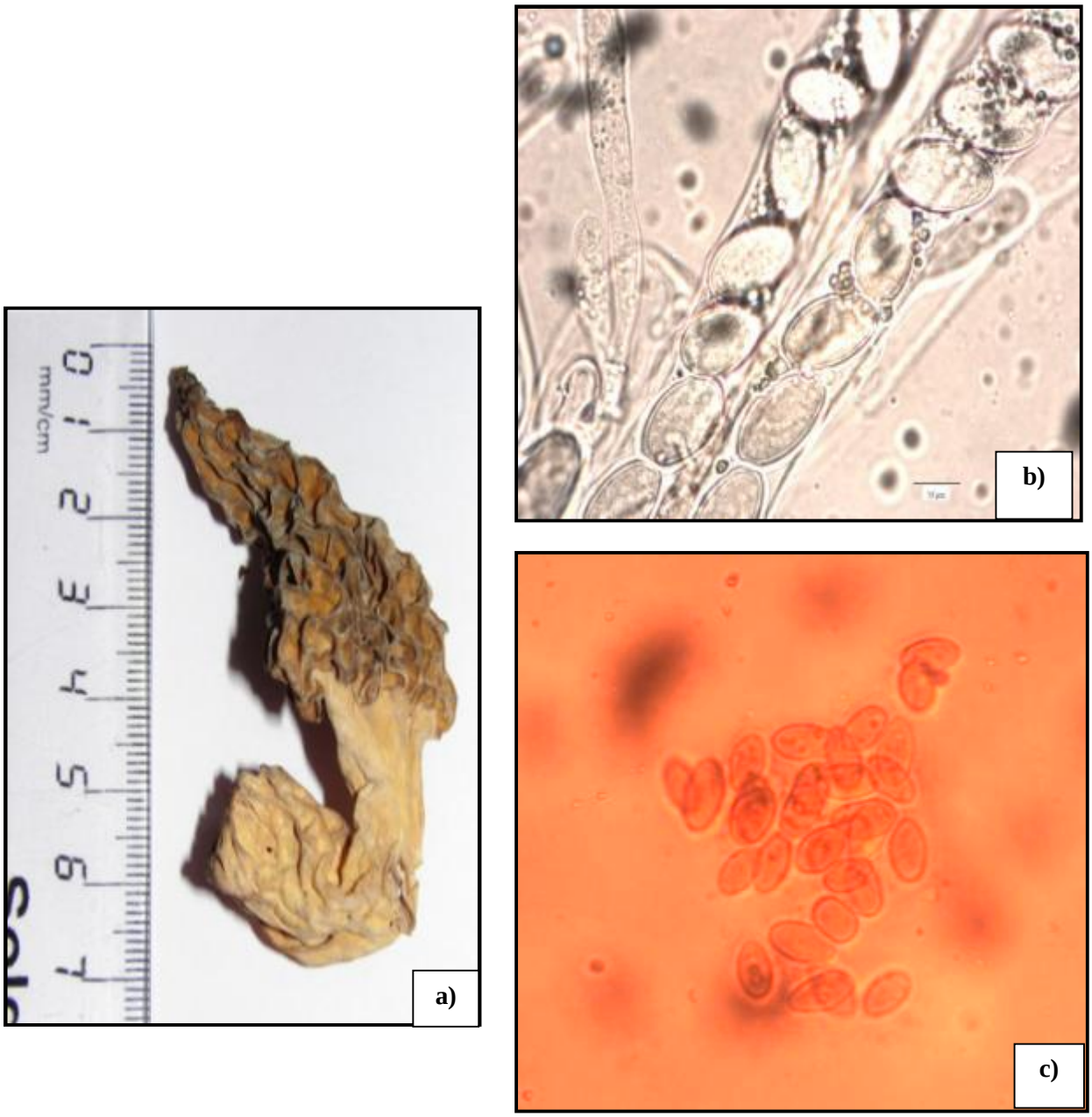


Fig 4.4: *M. crassipes* (MR20): a) Dried fruiting body along with scale, showing the size b) rehydrated sections of ascus containing ascospores (100X magnification c) Congo red stained ascospores in groups (40X magnification)

Black morels – *M. elata/M.conica*: Fruiting bodies of *Morchella* isolates such as, MR3, MR6, MR23, MR24, FK, and F1 had similar morphological characteristics; so they were grouped together into one group of black morels.

Fruiting bodies of black morels had black colored apothecium (5.5-12.5 cm), which was stipitate, hollow and clearly differentiated into pileus and stipe. Pileus, measured 6.3-10.0 × 4.5-5.5 cm, elongated, conical in shape, acute at the apex, conical in shape and adnate at the base. Pits were dark brown in color on the surface and light brownish on the inner side, connected by cross ribs. Ribs more or less longitudinally disposed, anastomosing or connected by few crossribs, less than 1mm in thickness; edges up to 2 mm thick, obtuse to rounded, concolorous with the interior of pits, giving rise to pubescent hairs. Stipe, 6.3-8.5 × 2.1-4.0 cm, almost of the same width throughout or swollen up to 3.5 cm at the base, stout, hollow, attenuated at the base and measured hollow and cylindrical, creamish in color, irregularly lacunose near the base, even above, pubescent; pubescent hairs subhyaline, in fascicles, composed of up to 8 cells which are thin walled and of various shapes. Asci were cylindrical, measured 250-380 × 17.4-26.0 µm, 8-spored, ascospores were eight in number.

Fruiting bodies had spore sizes of 21-28 × 10.47-19.22 µm, having uniseriate wall and ellipsoidal in shape. Spores were smooth, eguttulate, and polar oil droplets were present at the ends. Paraphyses up to 12 µm wide below and 14 µm at the top, stout, thin walled, septate, simple or branched below, subhyaline individually, faint brown in mass, almost as long as asci. These macroscopic and microscopic features were analysed and compared with the

classical features reported by Waraitch (1976). Based on these features, the fruiting bodies were identified as *M. elata* or *M. conica.*

Slight variations in the morphological characteristics of different fruiting bodies were observed among black morels.

Morphological variations in MR3:

Fruiting body of MR3 had black colored apothecium (15.2-18.5 cm), which was scattered, solitary to caespitose, stipitate, hollow and clearly differentiated into pileus and stipe (Fig 4.5). Pileus, 7.5-10.0 × 4.8-5.2 cm, was elongated, attached at the base to stipe. Stipe stout, hollow, attenuated at the base and measured 6.3-8.0 × 2.5-3.8 cm. Asci measured 250-380 × 18-26 µm, 8-spored, cylindrical to subcylindrical, apex obtuse, J-ve.

Spores measured 22.4-28.0 × 10.47-15.25 µm (mean value, 25.96 × 12.9 µm), Q = 1.9 with uniseriate wall and ellipsoidal in shape. Spores were smooth, eguttulate, and polar oil droplets were present at the ends. Paraphyses up to 12 µm wide below and 14 µm at the top, stout, thin walled, septate, simple or branched below, subhyaline individually, faint brown in mass, almost as long as asci.

Voucher specimen of the fruiting body was deposited in the Herbarium of Department of Botany, Punjabi University, Patiala, Punjab, India, with voucher no. PUN4120.

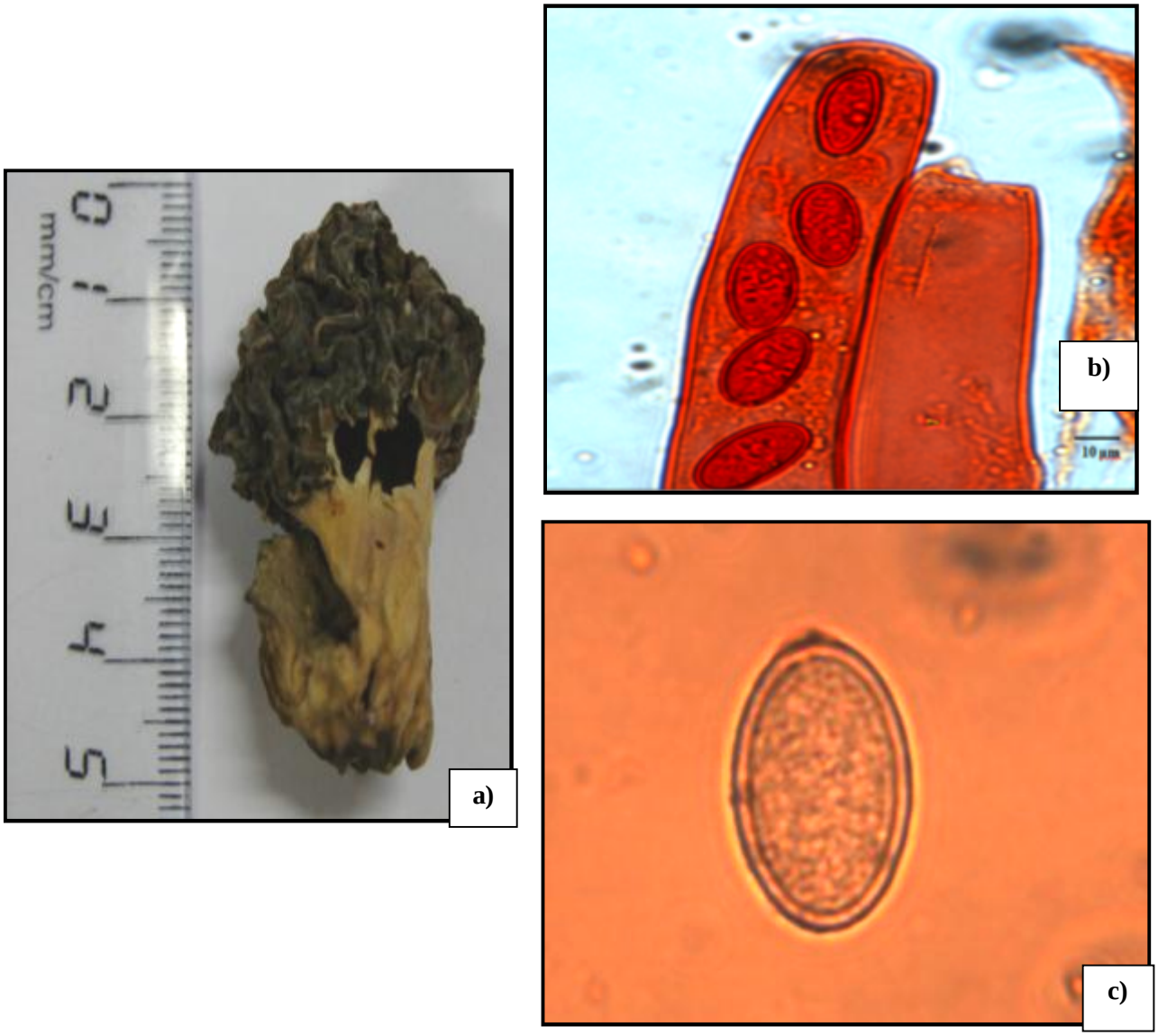


Fig 4.5: *Morchella elata* (MR3): a) Dried fruiting body along with scale, showing the size b) Congo red stained microscopic slides of cylindrical ascus with ascospores (100X magnification) c) Uniseriate, smooth, ellipsoidal ascospore containing oil droplets present at each end (100X magnification)

Morphological variations in MR6

Fruiting body of MR6 had black colored apothecium (6.3-10.6 cm), which was stipitate, hollow and clearly differentiated into pileus and stipe (Fig 4.6). Pileus measured 6.3-9.4 × 4.5-5.5 cm and pitted. Pits were dark brown in color on the surface and light brownish on the inner side, connected by cross ribs. Ribs more or less longitudinally disposed, anastomosing or connected by few crossribs, less than 2 mm in thickness; edges up to 2.5 mm thick, obtuse to rounded, concolorous with the interior of pits, giving rise to pubescent hairs.Stipe measured 7.0-8.2 × 2.1-4.0 cm. Asci were cylindrical, measured 272-363 × 18.35-26 µm, 8-spored, ascospores were eight in number.

Fruiting bodies had spore sizes of 21-28 × 12.22-19.22 µm (mean value, 25.1 × 16.7 µm), Q = 1.5 having uniseriate wall and ellipsoidal in shape. Spores were smooth, eguttulate, and polar oil droplets were present at the ends. Paraphyses up to 12 µm wide below and 14 µm at the top, stout, thin walled, septate, simple or branched below, subhyaline individually, faint brown in mass, almost as long as asci.

Voucher specimen of the fruiting body was deposited in the Herbarium of Department of Botany, Punjabi University, Patiala, Punjab, India, with voucher no. PUN4121.

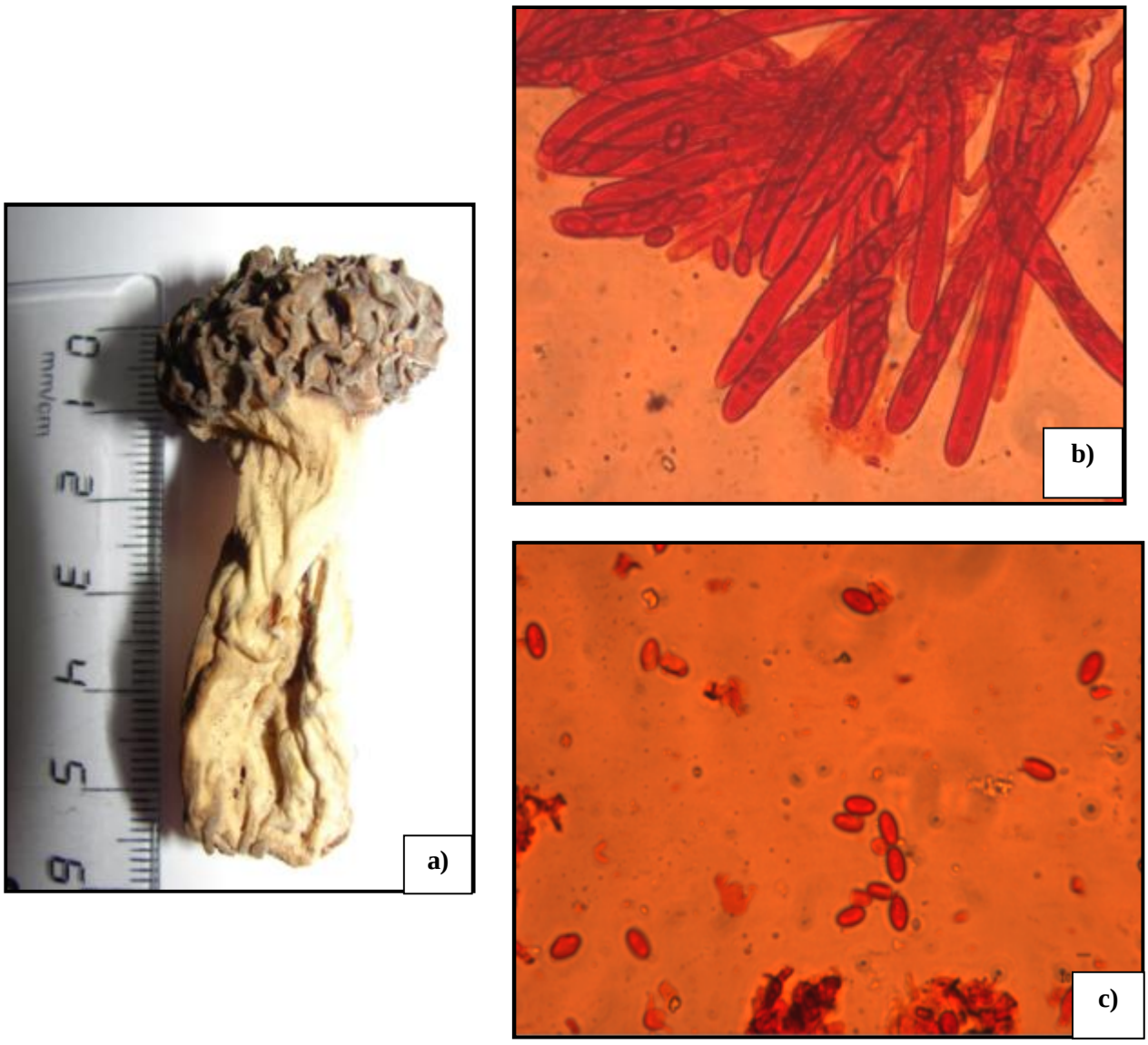


Fig 4.6: *Morchella elata* (MR6): a) Dried fruiting body along with scale, showing the size dried fruiting body b) Congo red stained microscopic slides of ascus along with ascospores (40X magnification) c) ascospores (40X magnification)

Morphological variations in FK:

Fruiting body had black colored apothecium (5.5-11.9 cm), which was scattered, solitary, stipitate, hollow and clearly differentiated into pileus and stipe (Fig 4.7). Pileus measured 7.2-9.8 × 4.2-5.5 cm, was elongated attached at the base to stipe, conical in shape, acute at the apex suboptuse, conical in shape and adnate at the base.

Pits were dark brown in color on the surface and light brownish on the inner side, connected by cross ribs. Ribs more or less longitudinally disposed, anastomosing or connected by few crossribs, less than 1mm in thickness; edges up to 2 mm thick, obtuse to rounded, concolorous with the interior of pits, giving rise to pubescent hairs.Stipe stout, hollow, attenuated at the base and measured 6.5-8.5 × 2.3-3.5 cm, hollow and cylindrical, creamish in color. Asci were cylindrical, measured 262-371 × 17.4-25.4 μm, 8-spored, J-ve.

FK fruiting bodies had spore sizes of 21-27.94 × 11.58-17.3 μm (mean value, 25.13 × 15 μm), Q = 1.67 with uniseriate wall and were ellipsoidal in shape. Spores were smooth, eguttulate, and polar oil droplets were present at the ends

Voucher specimen of the fruiting body was deposited in the Herbarium of Department of Botany, Punjabi University, Patiala, Punjab, India, with voucher no. PUN4122.

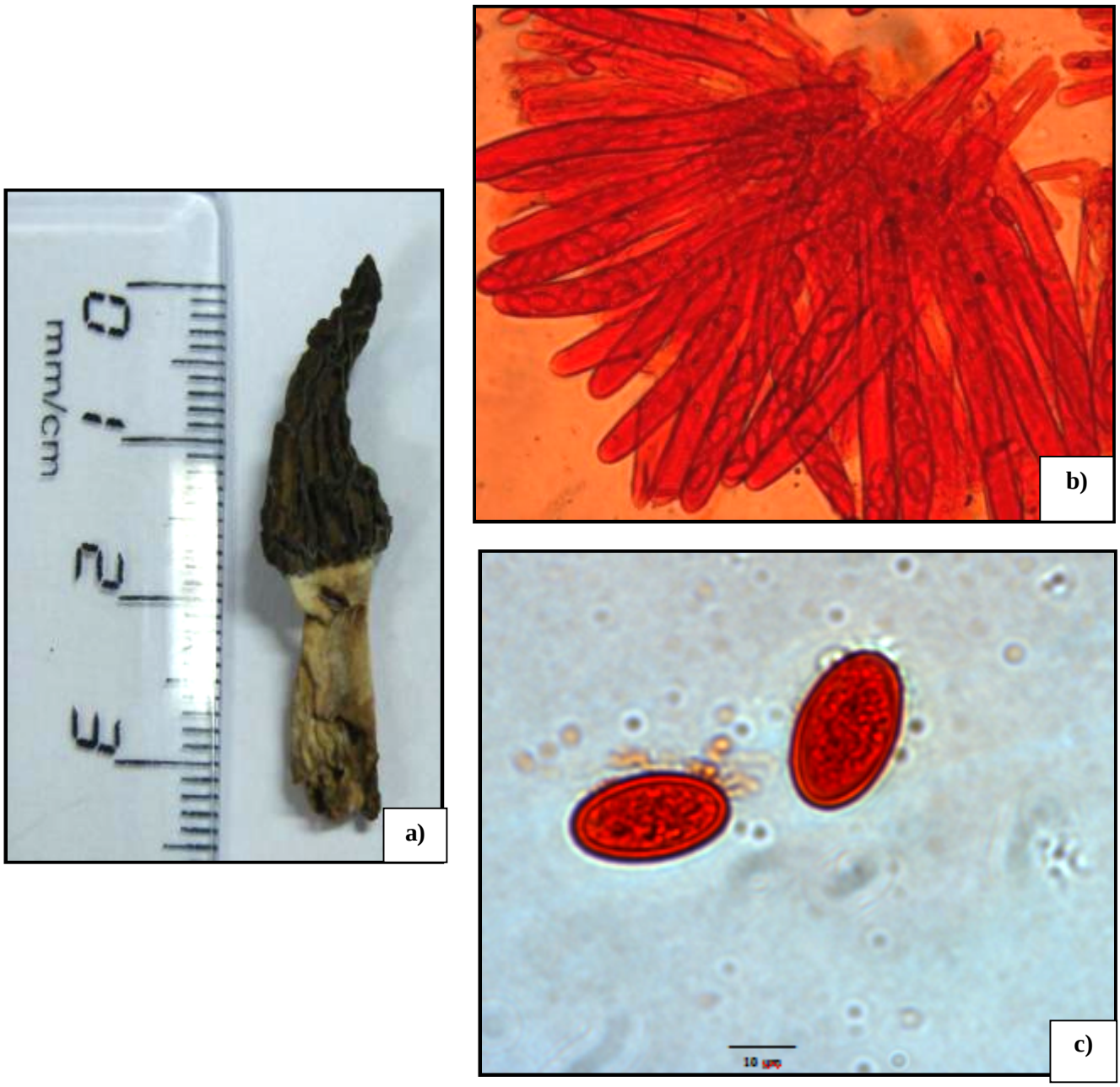


Fig 4.7: FK: a) Dried fruiting body along with scale, showing the size b) Congo red stained microscopic slides of ascus (40X magnification) c) Uniseriate, smooth and ellipsoidal spores containing polar oil droplets (100X magnification)

Table 4.1: Morphological characteristics of *Morchella* spp. collected from different geographical regions of Western Himalayas of India.

Isolate code	Nearest Genus name	Color of fresh fruiting body	Color of dried fruiting bodies	Apothecia (cm)	Pileus (cm)	Stipe (cm)	Ascus length/width (µm)	Spore morpholgy/size (µm)
MR1	*M. crassipes/ M. esculenta*	Yellow	Dirty White	15.2-18.5	10.3-11.6 × 4.8-5.5	9.0-11.5 × 1.9-2.4	245-350 × 17.3-22.2, 8-spored	21.1-28.0 × 11.9-15.5, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end
MR8	*M. crassipes/ M. esculenta*	Yellow	Dirty White	15.0-17.8	10.3-11.7 × 4.0-5.0	10.4-12.5 × 1.4-2.1	255-289 × 16.8-26.2, 8-spored	21.0-27.5 × 12.9-15.3, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end
MR20	*M. crassipes/ M. esculenta*	Yellow	Dirty White	15.0-18.4	9.5-11.4 × 3.8-5.2	10.0-12.0 × 1.8-2.0	263-338 × 17.7-25.3, 8-spored	22.0-27.5 × 11.1-14.6, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end
MR3	*M. elata/ M. conica*	Black	Black	7.5-12.5	7.5-10.0 × 4.8-5.2	6.3-8.0 × 2.5-3.8	250-380 × 18.0-26.0, 8 spored	22.4-28.0 × 10.5-15.3, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end
FK	*M. elata/ M. conica*	Black	Black	5.5-11.9	7.2-9.8 × 4.2-5.5	6.5-8.5 × 2.3-3.5	262-371 × 17.4-25.4, 8 spored	21.0-27.9 × 11.6-17.3, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end
MR6	*M. elata/ M. conica*	Black	Black	6.3-10.6	6.3-9.4 × 4.5-5.5	7.0-8.2 × 2.1-4.0	272-363 × 18.4-26.0, 8-spored	21.0-28.0 × 12.2-19.2, uniseriate, subhyaline, ellipsoid, smooth, oil droplets present at each end

Variations were observed in the spore sizes of different *Morchella* spp. Scatter plot analysis of spores of different fruiting bodies with the spore sizes clearly revealed that there are variations in the spores of different fruiting bodies (Fig 4.8).

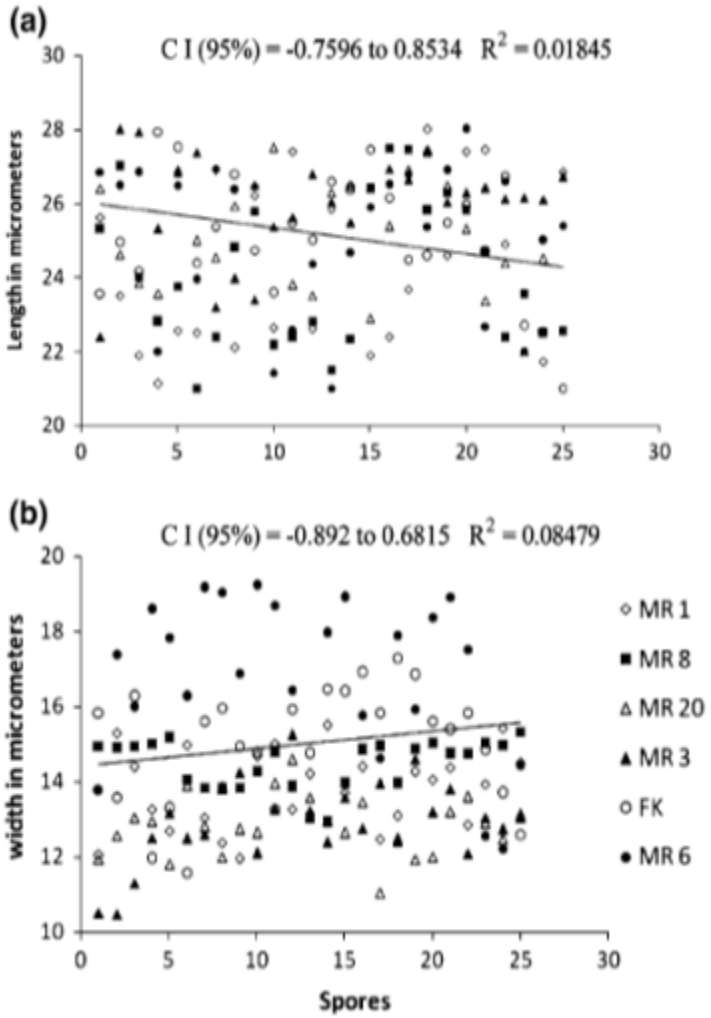


Fig 4.8: Scatter plot and line of best fit showing the relationship between a) length and b) width of spores of different *Morchella* spp.

Based on these features, the fruiting bodies could be grouped together into two groups - one consisting of yellow morels with *M. crassipes or M. esculenta* (MR1, MR8, MR21, MR25, MR26, MR27, MR28, MR29, MR11, MR13, MR14, and MR20) the other as black morels with *M. elata or M. conica* (MR3, MR6, MR23, MR24, FK, and F1).

4.3 Molecular approach to study the diversity

Thirty-two fruiting bodies/cultures of *Morchella* spp. (MR1 to MR29, F1, F2 and FK) were analyzed to see the genetic variations among them.

4.3.1 DNA isolation and PCR

Genomic DNA was isolated from the fruiting bodies/cultures by the method of Vankan et al. (1991). ITS region of rDNA from genomic DNA was amplified by the PCR (Polymerase chain reaction) using ITS1 and ITS4 primers. Resultant PCR products were viewed after electrophoresis in agarose gel. Amplification of ITS/5.8S rRNA region of morels using ITS1 and ITS4 primers resulted in different size products ranging from 700 bp to 1200 bp. Out of total thirty two isolates, sixteen isolates showed ITS size of ~1.2 kbp (MR1, MR5, MR8, MR9, MR11, MR12, MR13, MR14, MR18, MR20, MR21, MR25, MR26, MR27, MR28 and MR29), eleven isolates showed a size of ~ 750 bp (MR3, MR4, MR6, MR10, MR15, MR16, MR19, MR23, MR24, F1 and FK), two showed a size of 650 bp (MR22 and FK) and others showed sizes of ~1000 bp (MR17), ~ 950 bp (MR2), and ~ 800 bp (MR7), respectively (Fig 4.9 and 4.10).

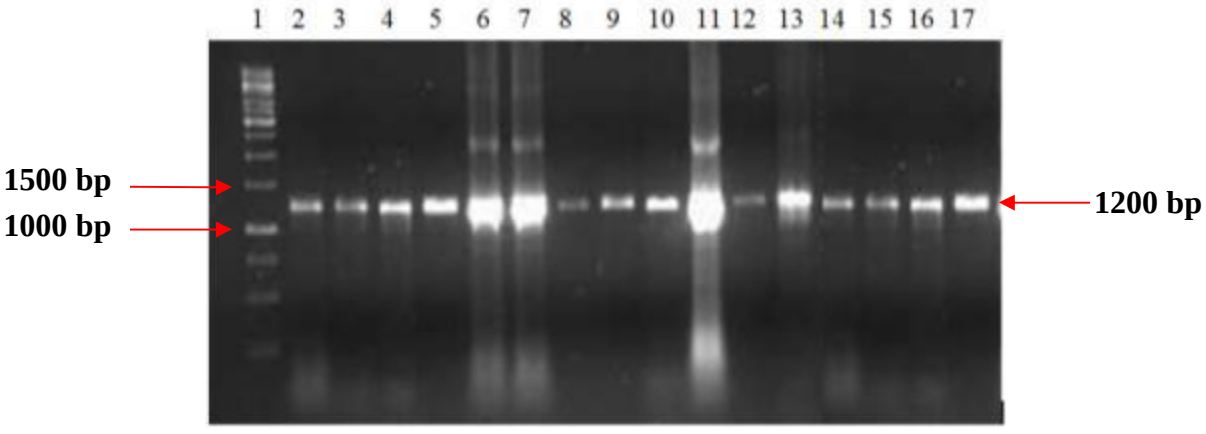


Fig 4.9: ITS-PCR products of *Morchella* spp. amplified with ITS1 and ITS4 primers. Lane 1: 1 Kb Generuler ladder, Lane 2-17: MR1, MR5, MR8, MR9, MR11, MR12, MR13, MR14, MR18, MR20, MR21, MR25, MR26, MR27, MR28, MR29

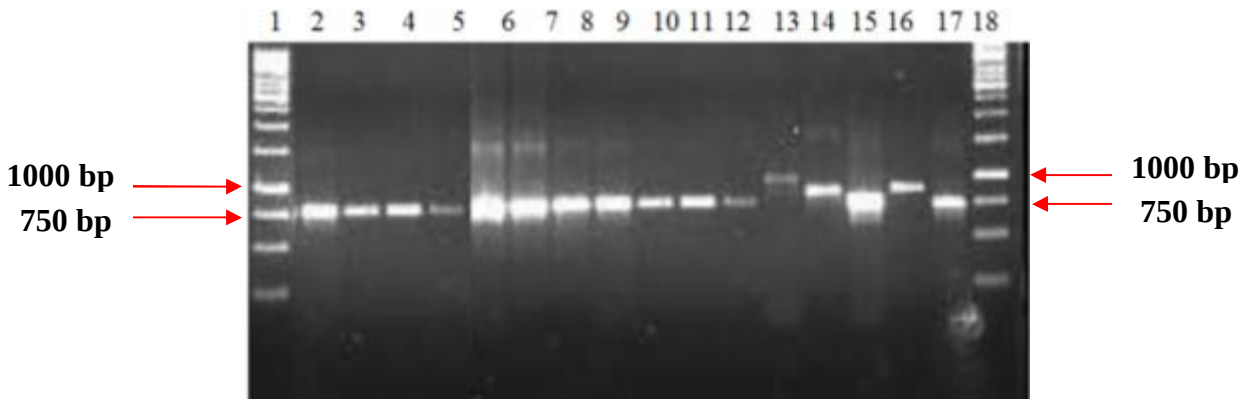


Fig 4.10: ITS-PCR products of *Morchella* spp. amplified with ITS1 and ITS4 primers.

Lane 1: 1 Kb Generuler ladder, Lane 2-17: MR3, MR4, MR6, MR10, MR15, MR16, MR24, F1, F2, FK, MR2, MR7, MR22, MR23, F2, MR19

4.3.2 Restriction enzyme analyses

ITS-PCR products obtained after PCR amplification were subjected for Restriction Fragment Length Polymorphism analysis with restriction enzymes *TaqI, HinfI, AluI and MboI*. RFLP analyses of all 16 isolates using the 1.2 kbp ITS region with restriction enzymes showed 5 different restriction patterns (Fig 4.11, 4.12 and 4.13) comprising Group I with MR1, MR9, MR11, MR12, MR13 and MR14; group II with MR5, MR25, MR26, MR27, MR28 and MR29; group III with MR8; group IV with MR18, MR20 and group V with MR21. Restriction digestion of 11 isolates having ITS size of 750 bp showed similar banding patterns (Fig 4.14, 4.15 and 4.16).

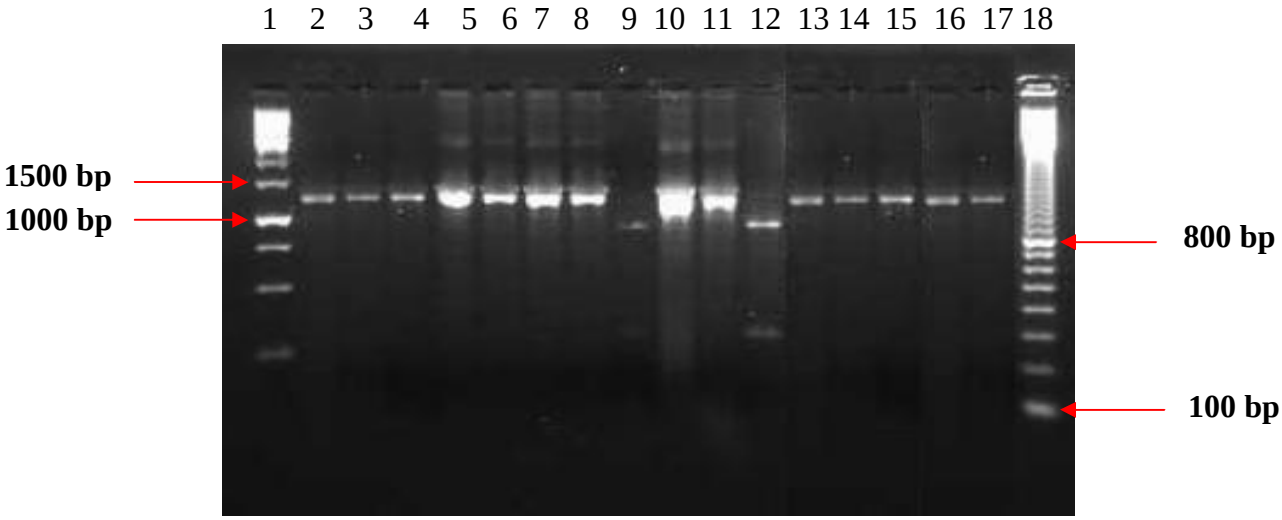


Fig 4.11: ITS – RFLP analysis of *Morchella* isolates having 1.2 Kb ITS band digested with *Alu I*. Lane 1: 1 Kb ladder, Lane 18: 100 bp ladder Lane 2-17: MR1, MR5, MR8, MR9, MR11, MR14, MR13, MR12, MR18, MR20, MR21, MR25, MR26, MR27, MR28, MR29

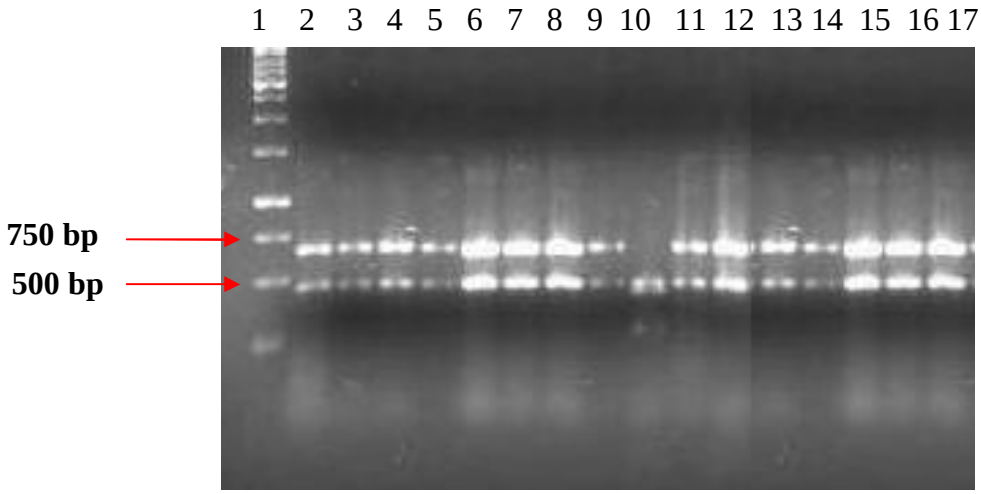


Fig 4.12: ITS – RFLP analysis of *Morchella* spp. having 1.2 Kb ITS band digested with *Taq I*. Lane 1: 1 Kb ladder, Lane 2-16: MR1, MR5, MR8, MR9, MR11, MR12, MR13, MR14, MR18, MR20, MR25, MR21, MR26, MR27, MR28, MR29

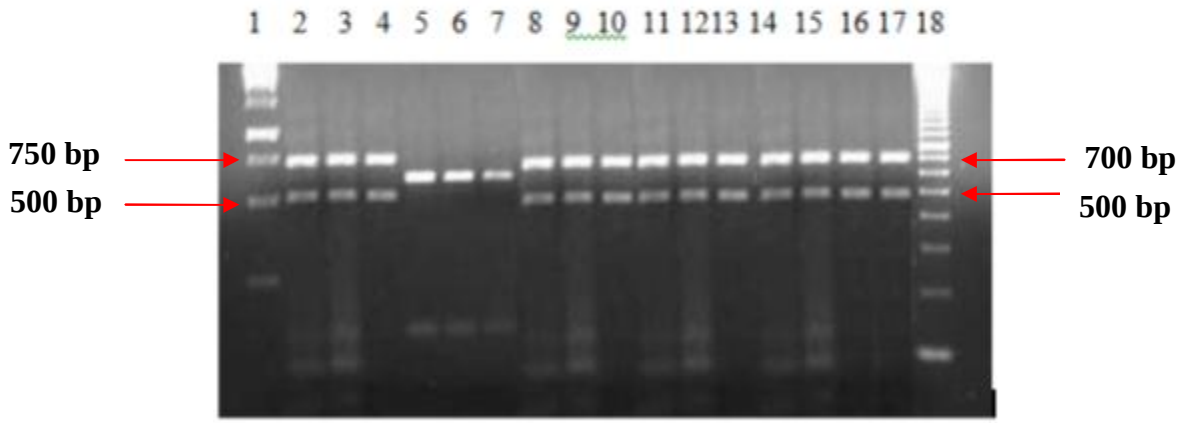


Fig 4.13: ITS – RFLP analysis of *Morchella* spp. having 1.2 Kb ITS band digested with *Rsa I*. Lane 1, 18: 1 Kb ladder, Lane 2-17: MR1, MR5, MR8, MR9, MR11, MR12, MR13, MR14, MR18, MR20, MR21, MR25, MR26, MR27, MR28, MR29

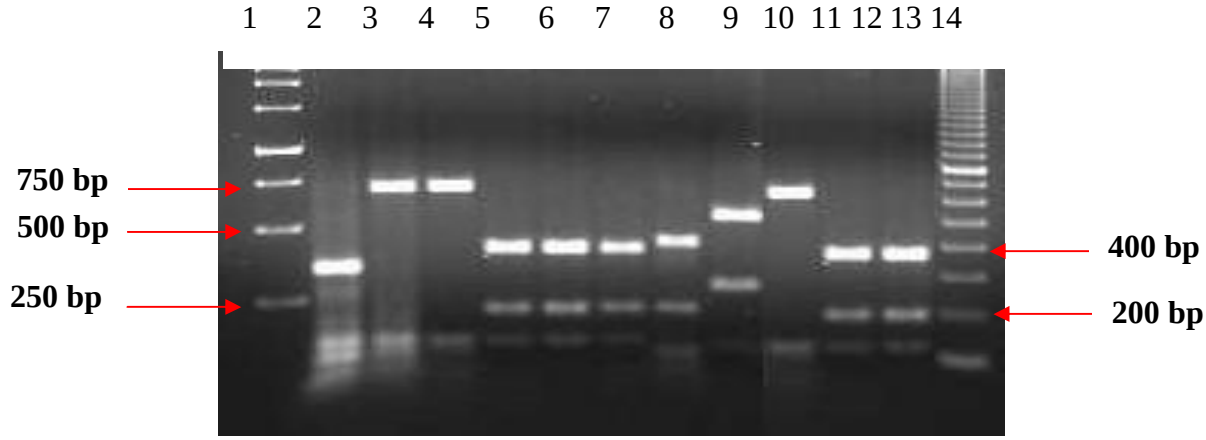


Fig 4.14: ITS – RFLP analysis of *Morchella* spp. having 750 bp ITS band digested with *Hinf I*. Lane 1: 1 Kb ladder, Lane 13: 100 bp ladder, Lane 2-12: MR3, MR4, MR6, MR10, MR15, MR16, MR24, F1, F2, FK, MR22

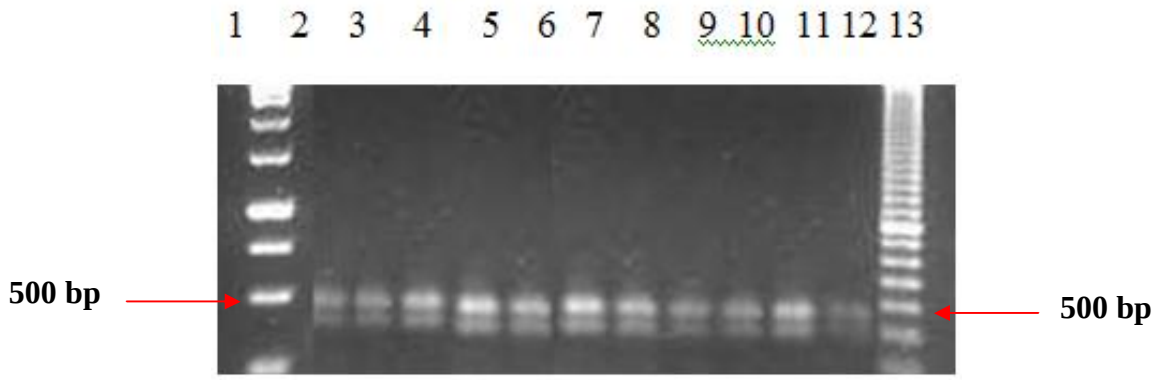


Fig 4.15: ITS – RFLP analysis of *Morchella* spp. having 750 bp ITS band digested with *Taq I.* Lane 1, 13: 1 Kb ladder, Lane 2-12: MR3, MR4, MR6, MR10, MR15, MR16, MR24, F1, F2, FK, MR22

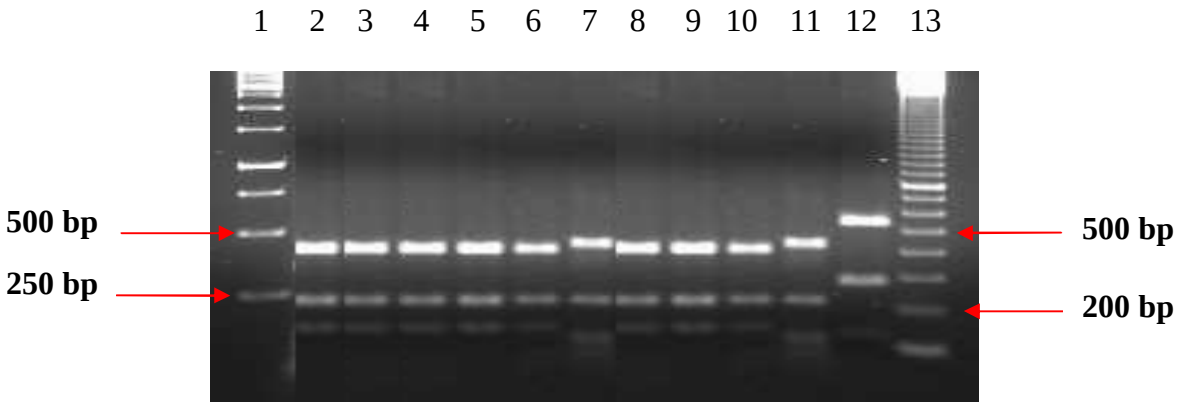


Fig 4.16: ITS – RFLP analysis of *Morchella* spp. having 750 bp ITS band digested with *Rsa I.* Lane 1, 13: 1 Kb ladder, Lane 2-12: MR3, MR4, MR6, MR10, MR15, MR16, MR24, F1, F2, FK, MR22

Table 4.2: RFLP pattern of *Morchella* spp. digested with different restriction enzymes. Exact size of fragment was determined using web programme Restriction mapper version 3 (www.restrictionmapper.org)

S. No.	Restriction enzymes	MR1	MR5	MR8	MR12	MR18	MR20	MR21
1	*AluI*	No restriction site	No restriction site	No restriction site	No restriction site	No restriction site	No restriction site	911, 302
2	*TaqI*	651, 445, 59, 53	651, 443, 59, 53	654, 443, 59, 53	652,446, 59,53	652, 443, 59, 53	653, 443, 59, 53	445, 441,215, 59, 53
3	*RsaI*	708, 500	709, 479	712, 497	710, 500	711, 496	711, 497	704, 401, 108

S. No.	Restriction enzymes	MR3	MR4	MR6	MR10	MR23	MR24	F1	FK
1	*HinfI*	398,204,127, 8	398,205,127, 8	397, 206,128, 8	399,205, 127, 8	598, 143	398 205, 128, 8	398, 204, 127, 8	398,204, 127, 8
2	*TaqI*	541, 127, 69	347,279,59, 53	346, 281, 59, 53	348, 279, 59, 53	347,282, 59, 53	347, 280, 59, 53	347, 278, 59, 53	278, 210, 137, 59, 53
3	*RsaI*	571, 40	598, 140	597, 142	598, 141	598, 143	597, 142	597, 140	597, 140

4.3.3 DNA sequence analyses

The ITS products of the representative fruiting bodies from each group of 1.2 kbp (MR1, MR5, MR8, MR12, MR18, MR20 and MR21) and 750 bp (MR3, MR4, MR6, MR10, MR23, MR24, F1 and FK) were purified from agarose gel by using QIAquick columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and cloned into the pGEM-T Easy vector system (Promega, USA). The ligated products were transformed into *E. coli* DH5α cells. The inserts in the plasmids were sequenced by chain termination method (Sanger *et al.*,1977) using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of biochemistry, South campus, Delhi university, New Delhi, India). Similarly, different-sized ITS products of the fruiting bodies (MR2, MR7 and MR17) were also cloned and sequenced.

BLAST search was performed to find the similarity of sequences in NCBI database (Altschul et al., 1997). Sequence analysis using BLASTN revealed that the sequenc es of seven fruiting bodies (MR1, MR5, MR8, MR12, MR18, MR20 and MR21) having an ITS size of 1.2 kbp showed close similarity (97-99%) with *M. crassipes* (Table 4.3). The sequence of MR 17 showed very close similarity (99%) with *M. spongiola*. The sequences of MR3, MR4, MR6, MR10, MR23, F1 and FK having an ITS size of 750 bp showed close similarity (98-99%) with *M. elata* and MR24 showed close similarity (99%) with *M. angusticeps*. Sequence analysis of MR7 showed similarity to *M. gigas*; MR2 (ITS of 955 bp) did not correspond to any similar sequence in the NCBI databases indicating this might be a new species of *Morchella*. The sequences obtained after BLASTN having 98% and above similarity along with all Morchella spp. reported in NCBI database were used further for analyses. The sequences were edited with BioEdit v5.0.9. Because large insertions/deletions occur within *Morchella*, *Verpa* and *Gyromitra* ITS sequences (Stefani et al., 2010), phylogenetic analyses were performed based on the last part of ITS1 section (154 bp), the

complete 5.8S (155 bp) and the first and last parts of the ITS2 section (256 bp). The sequences were aligned using MAFFT v6 (Katoh and Toh 2008). The alignment acquired from MAFFT was submitted to TreeBASE (http://www.treebase.org) and obtained the submission ID of 10765. Sequences of *Morchella* spp. and *Verpa bohemica* in the present study were submitted to the GenBank nucleotide database and obtained the Accession numbers as mentioned in table 4.4.

Table 4.3: List of *Morchella spp* with ITS lengths and the cultures showing % similarity (BLAST results) from Genbank accession number

S. No.	Isolate code showing homology from Genbank	Homologous culture from Genbank	Genbank Accession no	% Identity	Query Coverage (%)	ΔE value
1.	MR1, MR5, MR8, MR12, MR18, MR20, MR21	*M. crassipes*	EU701002 AJ539479	97-99	99	0
2.	MR17	*M. spongiola*	AJ539478 AJ539477	99	100	0
3.	MR2	*M.conica*	AM269501	83	70	2.00E-151
4.	MR7	*M. gigas*	AJ543743 AJ543742	99	99	0
5.	MR3, MR4, MR6, MR10, MR23, MR24, F1, FK	*M. elata*	EF081000 AJ698477	97-99	100	0
6.	MR24	*M. angusticeps*	AJ544203	99	99	0
7.	MR22, F2	*Verpa bohemica*	AJ698479	83	99	0

Table 4.4: Sequences of *Morchella* spp. (cloned isolates) submitted to NCBI along with GenBank accession number

S. No.	Isolate	*Morchella* spp.	GenBank No.
1.	MR1	*M. crassipes*	GQ 228461
2.	MR5	*M. crassipes*	GQ 228462
3.	MR12	*M. crassipes*	GQ 228463
4.	MR18	*M. crassipes*	GQ 228464
5.	MR21	*M. crassipes*	GQ 228465
6.	MR8	*M. crassipes*	GQ 228466
7.	MR20	*M. crassipes*	GQ 228467
8.	MR3	*M. elata*	GQ 228468
9.	MR4	*M. elata*	GQ 228469
10.	MR6	*M. elata*	GQ 228470
11.	MR10	*M. elata*	GQ 228471
12.	MR23	*M. elata*	GQ 228472
13.	F1	*M. elata*	GQ 228473
14.	MR2	*Morchella sp.*	GQ 228474
15.	MR7	*M. gigas*	GQ 228475
16.	MR17	*M. spongiola*	GQ 228476
17.	MR24	*M. elata*	GQ 228477
18.	F2	*Verpa sp.*	GQ 281276
19.	MR22	*Verpa sp.*	GQ 281277

4.3.4 Phylogenetic analyses

To study the diversity of morels, two phylogenetic trees were constructed. One of the phylogenetic tree was constructed by using different *Morchella* spp. reported in our study along with *Verpa sp*. (closely related to *Morchella* sp.) as an outgroup taxon. A second tree was constructed by using all *Verpa* spp. reported in our study along with *Morchella sp*. as an outgroup taxon.

Phylogenetic analysis was performed by a Maximum likelihood method. A Maximum likelihood Rapid Bootstrapping algorithm was implemented on the data set for 1000 replicates in the program RAxML v7.03 (Stamatakis 2006a,b; Stamatakis et al., 2008), using the GTRMIX model with parameters optimized for each partition. In addition, Bayesian phylogenetic analysis was carried out using the Metropolis-coupled Markov chain Monte Carlo method (MCMCMC), in MrBayes v3.04 (Ronquist and Huelsenbeck 2003), performing two runs each using four chains and 5,000,000 generations. To implement the optimal model in Bayesian analyses, the GTR model was specified. The substitution rate matrix, transition/transversion rate ratio, character state frequencies, gamma shape parameter α, and the proportion of invariant sites were unlinked across the ITS partitions. Trees were sampled every 100^{th} generation. The proportion of burn-in trees sampled before reaching equilibrium was estimated by plotting likelihood scores as a function of the number of generations. Only Bayesian posterior probabilities (PP) greater than or equal to 0.95 are considered significant and shown on tree. The output tree was evaluated with the program TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

In case of *Morchella* diversity, the Maximum likelihood and Baseyian analyses resulted in nearly identical phylogenetic trees (Fig. 4.17). The phylogenetic reconstructions clustered all *Morchella* sequences into two clades differentiating yellow and black morles. *Verpa* spp. (MR22 and FK) of the present study were excluded while analyzing. Sequences of

Verpa bohemica (GQ304945, AJ698479) were used as an outgroup taxon to root the tree. The clade of yellow morels was further divided into different groups; crassipes group, spongiola group, rufobrunnea and esculenta group, whereas the black morels were divided into elata, conica, gigas, tomentosa, costata and angusticeps groups.

The tree showed the yellow morels- *M. spongiola*, *M. esculenta*, *M. rufobrunnea* and *M. crassipes* grouped into a cluster and supported by high bootstrap (BP) and posterior probability (PP) values. Several sequences such as, MR1, MR5, MR8, MR12, MR18, MR20 and MR21, belonged to crassipes group, while MR17 belonged to spongiola group.

Black morels, namely, *M. elata*, *M. angusticeps*, *M. costata*, *M. tomentosa* and *M. gigas* clustered into one clade. Sequences such as F1, FK, MR4, MR3, MR10, MR6 and MR23 were grouped with *M. elata*, MR24 was clustered with angusticeps group, and MR7 clustered to the gigas group. All of the groups were strongly supported by high bootstrap and posterior probability values. The sequence of MR2 branched separately from the black morel cluster, (supported by high PP and BP values), and clustered with *M. tomentosa*, indicating a new species from India (Fig 4.17).

For studying the *Verpa* diversity, the Maximum likelihood and Baseyian analyses were constructed and both analysis resulted in nearly identical phylogenetic trees (Fig. 4.18). Sequences of *Morchella elata* (AM269501) and *Morchella conica* (AM269501) used as an outgroup taxa to root the tree. The phylogenetic reconstructions clustered *Verpa* sequences into two clades comprising of bohemica and conica groups. The tree clearly showed isolates MR22 and F2 grouped into bohemica clade and supported by high bootstrap and PP values. So, based on the phylogenetic analysis, MR22 and F2 were designated as *Verpa bohemica*. Conica clade was clearly separated from bohemica clade and was supported by high PP and BP values (Fig 4.18).

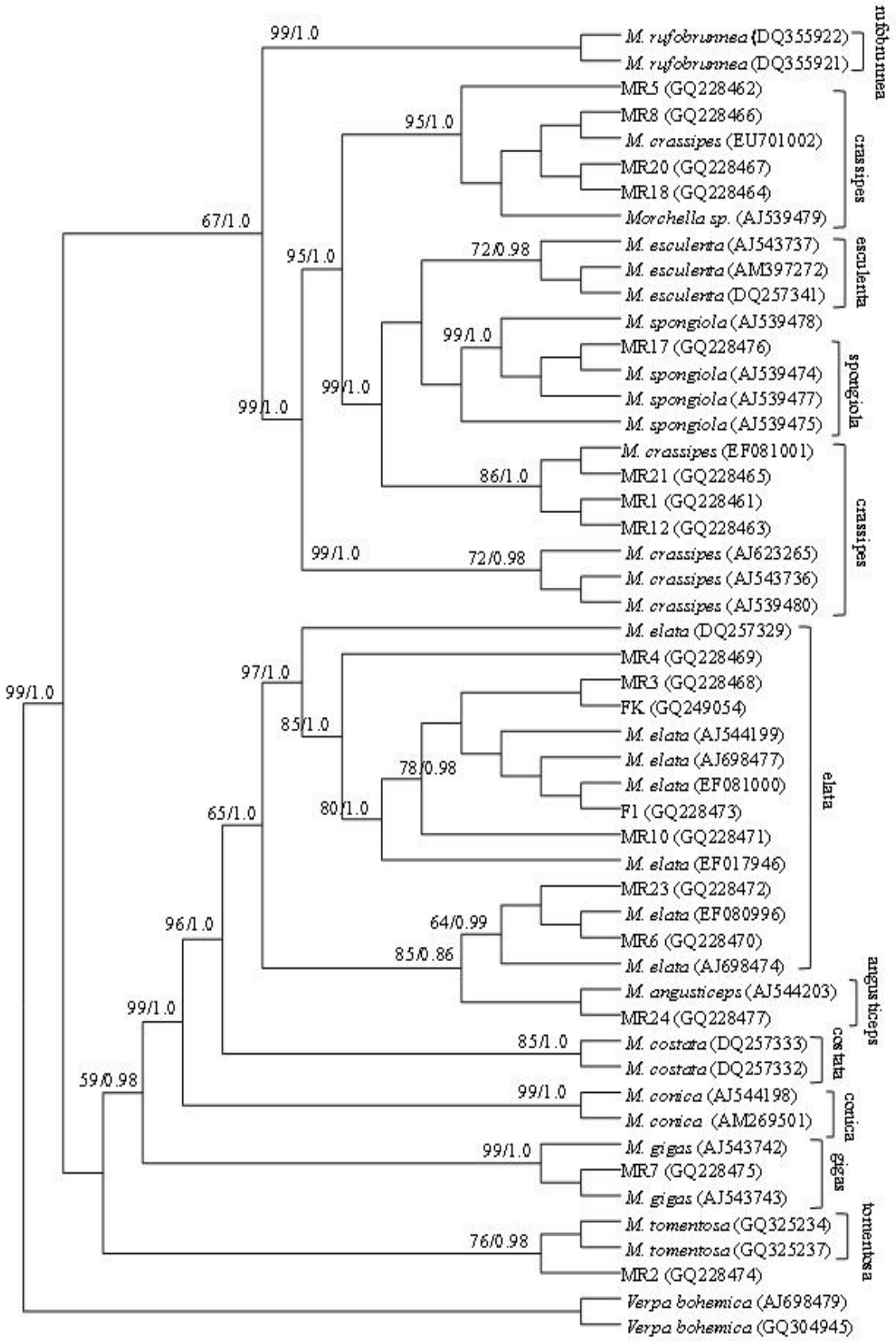

M. rufobrunnea (DQ355922)
M. rufobrunnea (DQ355921)
rufobrunnea
MR5 (GQ228462)
MR8 (GQ228466)
M. crassipes (EU701002)
MR20 (GQ228467)
MR18 (GQ228464)
Morchella sp. (AJ539479)
crassipes
M. esculenta (AJ543737)
M. esculenta (AM397272)
M. esculenta (DQ257341)
esculenta
M. spongiola (AJ539478)
MR17 (GQ228476)
M. spongiola (AJ539474)
M. spongiola (AJ539477)
M. spongiola (AJ539475)
spongiola
M. crassipes (EF081001)
MR21 (GQ228465)
MR1 (GQ228461)
MR12 (GQ228463)
M. crassipes (AJ623265)
M. crassipes (AJ543736)
M. crassipes (AJ539480)
crassipes
M. elata (DQ257329)
MR4 (GQ228469)
MR3 (GQ228468)
FK (GQ249054)
M. elata (AJ544199)
M. elata (AJ698477)
M. elata (EF081000)
F1 (GQ228473)
MR10 (GQ228471)
M. elata (EF017946)
MR23 (GQ228472)
M. elata (EF080996)
MR6 (GQ228470)
M. elata (AJ698474)
elata
M. angusticeps (AJ544203)
MR24 (GQ228477)
angusticeps
M. costata (DQ257333)
M. costata (DQ257332)
costata
M. conica (AJ544198)
M. conica (AM269501)
conica
M. gigas (AJ543742)
MR7 (GQ228475)
M. gigas (AJ543743)
gigas
M. tomentosa (GQ325234)
M. tomentosa (GQ325237)
MR2 (GQ228474)
tomentosa
Verpa bohemica (AJ698479)
Verpa bohemica (GQ304945)
99/1.0
95/1.0
67/1.0
72/0.98
99/1.0
95/1.0
99/1.0
99/1.0
86/1.0
99/1.0
72/0.98
97/1.0
99/1.0
85/1.0
78/0.98
65/1.0
80/1.0
96/1.0
64/0.99
85/0.86
99/1.0
85/1.0
59/0.98
99/1.0
99/1.0
76/0.98

Fig. 4.17: The best ML tree for phylogenetic analysis of *Morchella* spp. based on the analyses of ITS/5.8S rRNA gene sequences, resulting from a 1000 replicates Rapid Bootstrapping algorithm and a ML search in RAxML.*Verpa bohemica* was used as outgroup. The number within parentheses indicates the GenBank accession number. Different clades and groups have been marked and their significance is referred to in the text. BP value followed by PP values is shown at the nodes.

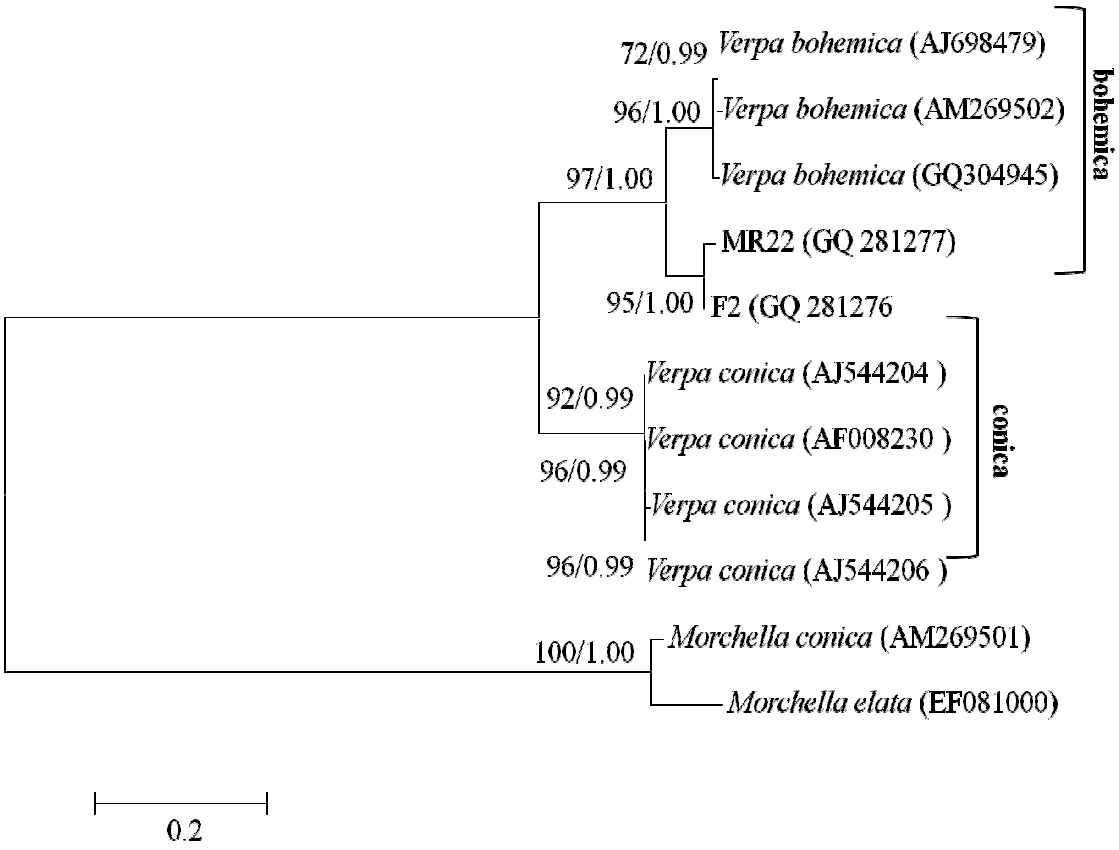


Fig. 4.18: The best ML tree for phylogenetic analysis of *Verpa* spp. based on the analyses of ITS/5.8S rRNA gene sequences, resulting from a 1000 replicates Rapid Bootstrapping algorithm and a ML search in RAxML. *Morchella* spp. has been used as outgroup taxon. The number within parentheses indicates the GenBank accession number. Different clades and groups have been marked and their significance is referred to in the text. BP value followed by PP values is shown at the nodes.

Both the classical and molecular taxonomy revealed that in the Western Himalayan region of India, there are mainly two types of morels; yellow morels comprising *M. crassipes* and *M. spongiola*; and black morels comprising *M. elata*, *M. angusticeps*, *Morchella sp.*, and *M. gigas*. Sequence analysis in this study showed that out of thirty-two morel species studied, thirty belonged to *Morchella* species and two were found to be of false morels i.e. *Verpa* species.

Chapter V

Physiological studies of Morchella spp.

5.0 Physiological characterization of a few *Morchella* species

Four different *Morchella* spp. (*Morchella sp*. (MR2), *M. elata* (MR3) and (MR6), *M. crassipes* (MR5) and (MR8), and *M. spongiola* (MR17)), representing six *Morchella* cultures, were selected on the basis of availability of pure cultures, for physiological characterization.

Different physiological parameters such as growth in different media, nutritional parameters such as carbon and nitrogen sources, pH, temperature, enzyme activities such as acid phosphatase activity, phytase activity, laccase, lignin peroxidase, manganese peroxidase and cellulase activity were determined.

5.1 Growth studies in different liquid media

Morchella spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17)) were inoculated in ten different media (Malt Extract (ME), Modified Melin Norkrans Media (MMN), Czapek Dox (CZD), Yeast Maltose Glucose (YMG), Sabourd's media (SB), Basal media (BS), Minimal Mineral media (MMM), Potato Dextrose broth (PDB), Mineral Salts broth (MSB), morel growth broth (MGB) and pikovskaya's (PKV) media). After 7 days of growth, cultures were harvested and biomass, pH, protein content, enzyme activities such as acid phosphatase, phytase, laccase, lignin peroxidase and Manganese peroxidase were determined with the culture filtrates.

5.1.1. Biomass

Analysis of dried biomass showed that all the *Morchella* spp. (*Morchella sp.* (MR2), *M. elata* (MR3 and MR6) and *M. crassipes* (MR5 and MR8) were able to grow well in all the media

tested with malt extract, mineral salts broth and potato dextrose broth serving as the best media (Table 5.1 and Fig 5.1). Significant differences in the biomass yield were found among the other media tested. Poor growth was observed in MMN, CZD, YMG, SB, PKV and MGB media.

Maximum biomass was observed in *M. elata* (MR3 and MR6) in ME, followed by MMN and PDB media (Fig 5.1 b and d). Moderate growth was observed in MSB, MGB, BS and MMN media. Least biomass was obtained in YMG and CZD media. *Morchella* sp. (MR2) and *M. spongiola* (MR17) produced less biomass compared to other fungi. *Morchella sp.* (MR2) produced maximum biomass in ME media, followed by MSB and MMM (Fig 5.1 a). Biomass was very less in CZD, YMG and SB media. Media such as MGB, PKV, and BS media served as the optimum media for biomass production.

For *M. crassipes* (MR8) ME and MSB served as the best media for growth studies as maximum biomass was produced in them (Fig 5.1 e). For *M. crassipes* (MR5 and MR8), the mycelium grew well and produced higher biomass in PDB, MMM, PKV and MGB media. In *M. crassipes* (MR8), significantly least biomass was observed in BS media; whereas maximum biomass was recorded in *M. spongiola* (MR17). Media such as ME, MMM, MSB, MMN, MGB and PKV were found moderate for the growth of *M. spongiola* (MR17) and significantly least biomass was obtained in SB media (Fig 5.1 f).

Table 5.1: Influence of different media on the biomass (mg/50ml) of different *Morchella* spp.

Media	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR5)	*M. elata* (MR6)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
ME	**50.8 ± 4.2c A**	**82.6 ± 18.0a A**	**62.0 ± 1.9d A**	**71.5 ± 3.8 b A**	**67.5 ± 5.5b A**	**42.8 ± 5.2c A**
MMN	19.1 ± 2.0c D	23.0 ± 0.9b D	17.1 ± 3.0d D	16.0 ± 2.0d C	19.7 ± 4.0c CD	29.5 ± 3.0a B
CZD	10.9 ± 2.3c E	15.0 ± 1.0ab E	13.1 ± 1.0b DE	10.5 ± 1.3c CD	17.1 ± 1.0a D	17.6 ± 1.2a C
YMG	4.8 ± 1.6c F	10.3 ± 1.0b F	9.9 ± 1.7b E	15.8 ± 2.0ab C	16.0 ± 2.5ab D	18.8 ± 1.8aC
SB	7.1 ± 1.5c EF	14.3 ± 1.3a	16.4 ± 2.3a D	7.6 ± 1.3c D	12.8 ± 1.5b DE	4.7 ± 0.6d D
BS	25.1 ± 1.7b C	25.7 ± 2.4b D	6.8 ± 1.0c F	7.6 ± 1.3c D	8.8 ± 2.0c E	45.5 ± 6.3a A
MMM	32.6 ± 1.2b B	66.1 ± 7.0a B	16.1 ± 1.7d D	22.6 ± 3.8c BC	29.0 ± 4.4c C	35.1 ± 2.3b B
PDB	17.4 ± 3.5c D	62.8 ± 7.5a B	32.8 ± 8.0b B	24.2 ± 1.3bc BC	34.6 ± 2.4b C	10.0 ± 1.4d
MSB	45.1 ± 7.0c AB	31.7 ± 4.0d C	**69.4 ± 5.2a A**	32.7 ± 1.0d B	**52.4 ± 4.3b B**	30.3 ± 3.0d B
PKV	24.5 ± 3.0b C	17.7 ± 2.5c E	33.8 ± 1.5a B	19.9 ± 2.7bc C	23.7 ± 7.5b CD	21.3 ± 0.74b
MGB	28.0 ± 3.8b C	31.8 ± 3.0a C	23.7 ± 0.7b C	32.2 ± 1.2a B	21.5 ± 0.5b CD	29.2 ± 2.5b B

ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media. Values sharing a common lower case letter within the row and uppercase letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3)**.** Values in bold indicate significant ones.

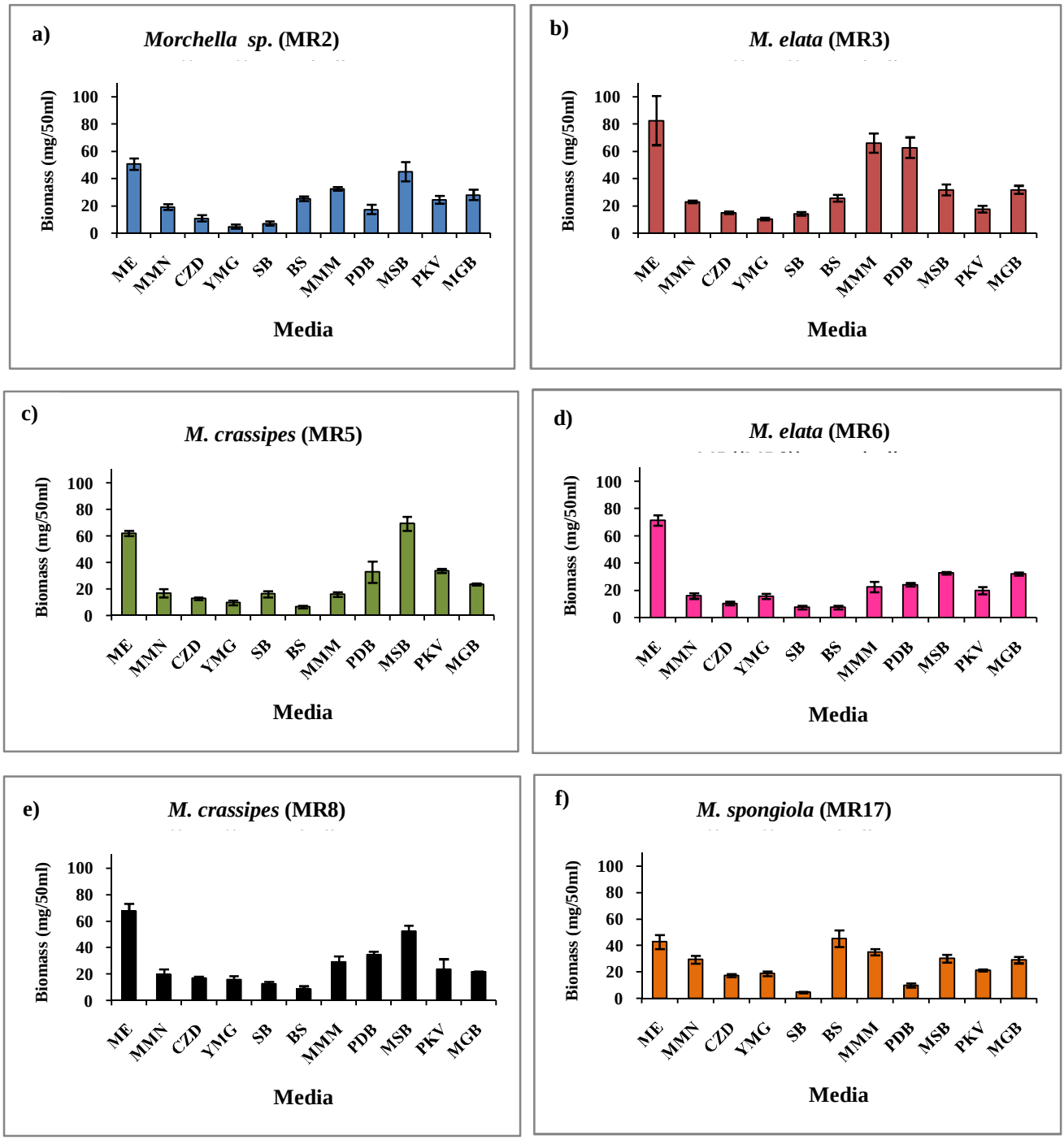


Fig 5.1: Effect of different media on biomass (mg/50ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media.Values are Mean ± SEM (n=3).

5.1.2. pH

The pH of the culture filtrates were recorded and compared with the control pH of 5.6. The pH of control set was set at 5.6. In *Morchella sp.* (MR2), a slight increase in pH was observed in the media used such as ME and CZD, where pH was increased to a small extent as compared to the control (Tab 5.2). Not much change in pH was observed in the remaining media used. The same pattern of change of increase in pH was observed in CZD media in the culture filtrates of *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17). Increase in pH in ME and CZD was observed in *M. elata* (MR6) and *M. spongiola* (MR17). Not much significant difference was found in the pH profiles of different *Morchella* spp. with different media (Tab 5.2, Fig 5.2).

Table 5.2: Influence of different media on pH of different *Morchella* spp.

Media	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR5)	*M. elata* (MR6)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
ME	6.3 ± 0.1a A	5.7 ± 0.1b AB	5.3 ± 0.2b B	5.9 ± 0.2b A	5.4 ± 0.1b B	**6.3 ± 0.2a A**
MMN	5.5 ± 0.1a B	5.5 ± 0.0a AB	5.5 ± 0.1a B	5.5 ± 0.2a A	5.4 ± 0.0a B	5.8 ± 0.3a B
CZD	**6.1 ± 0.2a A**	**6.2 ± 0.1a A**	**6.7 ± 0.1a A**	**6.2 ± 0.1a A**	**6.2 ± 0.1a A**	**6.5 ± 0.2a A**
YMG	5.4 ± 0.1a B	5.5 ± 0.2a AB	5.3 ± 0.1a B	5.4 ± 0.1a A	5.5 ± 0.2a B	5.7 ± 0.2a B
SB	5.6 ± 0.1a B	5.7 ± 0.2a AB	5.6 ± 0.2a B	5.6 ± 0.1a A	5.5 ± 0.2a B	5.5 ± 0.1a B
BS	5.5 ± 0.1aB	5.6 ± 0.1a AB	5.5 ± 0.1a B	5.8 ± 0.1a A	5.7 ± 0.0a B	5.6 ± 0.1a B
MMM	5.7 ± 0.1a B	5.5 ± 0.2a AB	5.9 ± 0.1a AB	5.5 ± 0.1a A	5.8 ± 0.1a B	5.6 ± 0.1a B
PDB	5.4 ± 0.1a B	5.3 ± 0.0a B	5.4 ± 0.1a B	5.5 ± 0.1a A	5.5 ± 0.1a B	5.8 ± 0.0a B
MSB	5.7 ± 0.1a B	5.7 ± 0.1a AB	5.7 ± 0.2a B	5.5 ± 0.1a A	5.3 ± 0.1a B	5.7 ± 0.1a B
PKV	5.7 ± 0.1a B	5.5 ± 0.2a AB	5.4 ± 0.1aB	5.5 ± 0.1a A	5.8 ± 0.3a B	5.6 ± 0.1a B
MGB	5.5 ± 0.0a B	5.8 ± 0.2a AB	5.7 ± 0.1a B	5.5 ± 0.1a A	5.7 ± 0.2a B	5.6 ± 0.0a B

ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media. Values sharing a common lower case letter within the row and uppercase letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3)**.** Values in bold indicate significant ones.

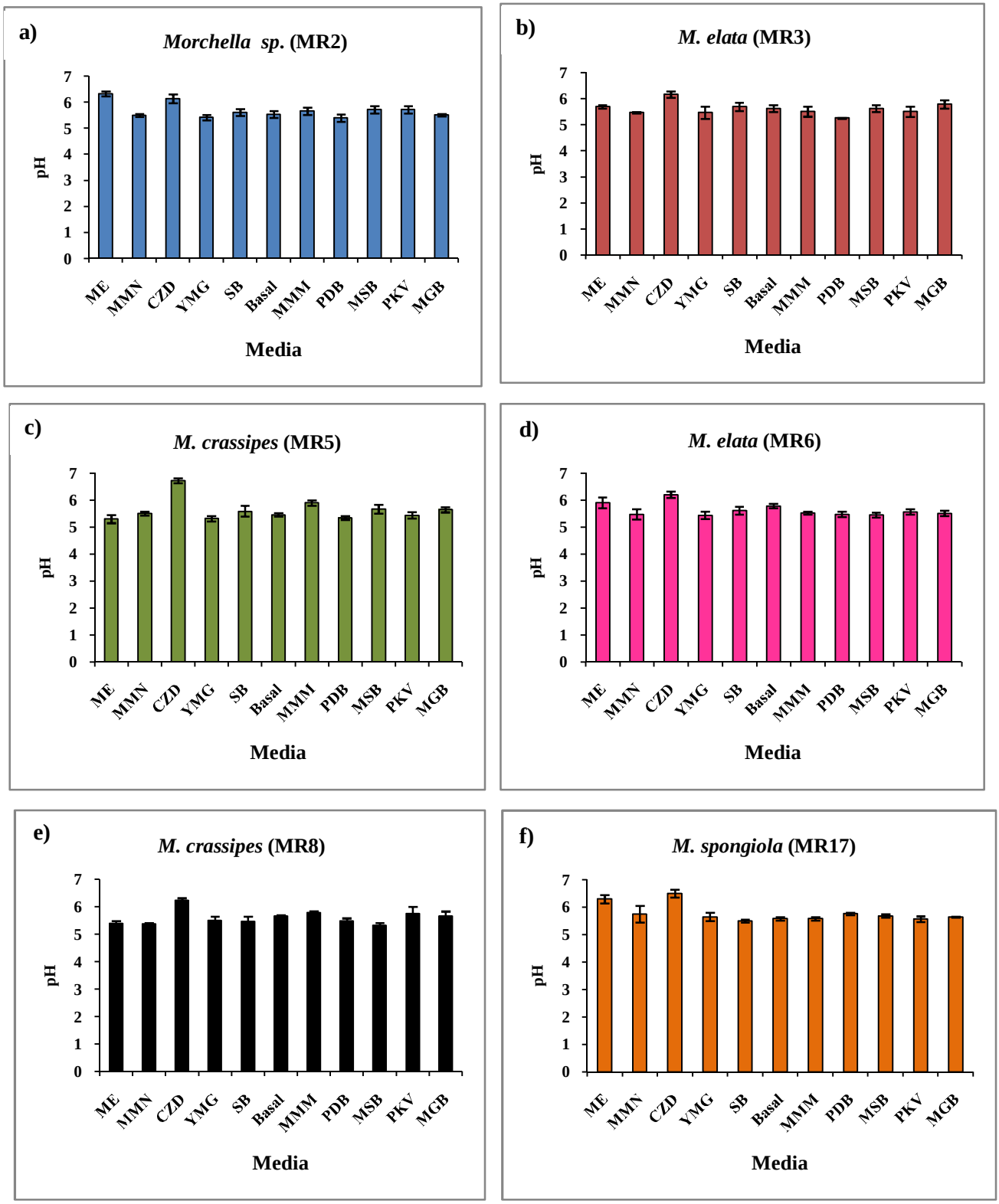


Fig 5.2: Influence of different media on pH of culture filtrate of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). ME, Malt Extract; MMN, Modified Melin Norkrans; CZD, CzapekDox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media.Values are Mean ± SEM (n=3).

5.1.3. Protein content

Extracellular protein content was observed in the culture filtrates of different *Morchella* spp. **(***Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17)) in different media. Extracellular protein content was found to vary with different *Morchella* spp. and media used in experiment.

Maximum protein content was observed by *M. crassipes* (MR5) in PDB medium, followed by *M. crassipes* (MR8) in ME medium (Tab 5.3). Other *Morchella* cultures such as *M. elata* (MR3 and MR6) and *M. spongiola* (MR17) produced optimum protein content as compared to *M. crassipes* (MR5) and *M. crassipes* (MR8).

Maximum protein content in *Morchella sp.* (MR2) was observed in PDB medium. Other media such as, MMN, MMM, too, were found to contain considerable amounts of protein content. As YMG and SB media served as poor media for growth as described previously, therefore, significantly less amount of protein was detected in the culture filtrate of these media.

Maximum protein content in *M. elata* (MR3), was observed in ME media followed by PDB. In other media, protein content was less. Maximum protein content was observed in PDB in *M. crassipes* (MR5). Other media such as ME and CZD, yielded optimum levels of protein content. Protein content was less in SB, MMN and MGB media.

Extracellular protein content levels in *M. elata* (MR6) are higher in ME medium, followed by MMN, CZD, YMG, PKV. Less protein content was observed in MMM and MSB media.

Maximum protein content was observed in MSB, followed by ME and BS in *M. spongiola* (MR17). Optimum amounts of protein content was observed in MMN, CZD, BS, and MMM media. SB and YMG produced less protein content. Significant difference was

found in protein content among different *Morchella* cultures and different media used in the study (Table 5.3, Fig 5.3).

Table 5.3: Effect of different media on the protein content (mg/ml) of different *Morchella* spp.

Media	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR5)	*M. elata* (MR6)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
ME	0.40 ± 0.1e D	**0.93 ± 0.1b A**	0.86 ± 0.0c C	**0.83 ± 0.1c A**	**1.16 ± 0.1a A**	0.52 ± 0.2d AB
MMN	0.68 ± 0.3a B	0.58 ± 0.2ab D	0.18 ± 0.1c G	0.70 ± 0.1a AB	0.24 ± 0.0c D	0.44 ± 0.2b B
CZD	0.30 ± 0.1c D	0.79 ± 0.2b C	0.96 ± 0.1a B	0.50 ± 0.1c B	0.34 ± 0.1c C	0.46 ± 0.1c B
YMG	0.17 ± 0.0b E	0.38 ± 0.2a E	0.39 ± 0.1a E	0.42 ± 0.2a B	0.39 ± 0.0a C	0.10 ± 0.0b E
SB	0.11 ± 0.1c F	0.20 ± 0.0a F	0.16 ± 0.0b G	0.20 ± 0.1a C	0.26 ± 0.0a D	0.09 ± 0.0c E
BS	0.36 ± 0.1b D	0.34 ± 0.1b E	0.41 ± 0.0ab E	0.30 ± 0.1b BC	0.18 ± 0.1c E	0.50 ± 0.0a AB
MMM	0.53 ± 0.1b C	0.24 ± 0.1c EF	0.52 ± 0.2b D	0.12 ± 0.1d D	0.91 ± 0.1a B	0.33 ± 0.1c C
PDB	**0.87 ± 0.1b A**	0.87 ± 0.1b B	**1.2 ± 0.1a A**	0.60 ± 0.1d AB	0.36 ± 0.1c B	0.23 ± 0.0c D
MSB	0.39 ± 0.1b D	0.28 ± 0.0c E	0.53 ± 0.1ab D	0.14 ± 0.1d D	0.41 ± 0.1b C	**0.67 ± 0.1a A**
PKV	0.20 ± 0.1b DE	0.34 ± 0.1a E	0.38 ± 0.1a E	0.40 ± 0.1a B	0.24 ± 0.0b D	0.36 ± 0.1a C
MGB	0.28 ± 0.0ab D	0.28 ± 0.0ab E	0.27 ± 0.1ab F	0.30 ± 0.0a BC	0.32 ± 0.1a C	0.12 ± 0.1b E

ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media. Values sharing a common lower case letter within the row and uppercase letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3)**.** Values in bold indicate significant ones.

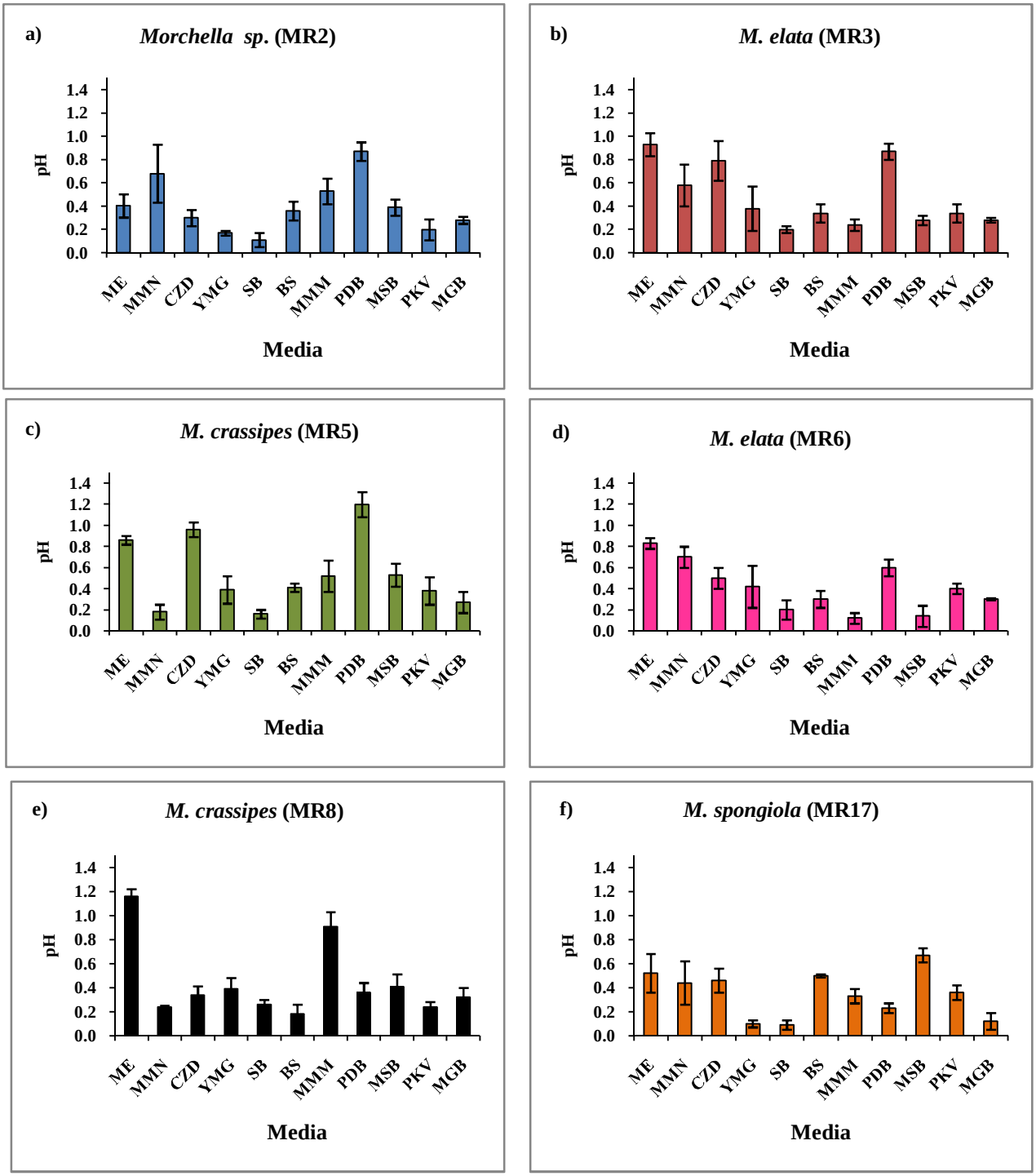


Fig 5.3: Effect of different media on protein concentration (mg/ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). ME, Malt Extract; MMN, Modified Melin Norkrans; CZD, CzapekDox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media.Values are Mean ± SEM (n=3).

5.1.4. Acid phosphatase activity

Acid phosphatase activity was determined in mycelia and culture filtrates. The acid phosphatase activity varied among the *Morchella* spp.

Acid phosphatase activity by mycelia

PKV was found to be the best media for high production of phosphatase activity by the mycelia of most of the *Morchella* cultures (Tab 5.4). Maximum amount of phosphatase activity was observed by mycelia of *M. crassipes* (MR5) in MMM medium followed by *Morchella sp.* (MR2) in PDB and SB. Mycelium of *Morchella sp.* (MR2) produced high phosphatase activity in different media. Maximum acid phosphatase activity was observed in SB medium in *Morchella sp.* (MR2). High level of acid phosphatase activity was observed in PDB, PKV, and BS media. Optimum amounts of acid phosphatase activity was also detected in MSB and MMM. In *M. elata* (MR3), maximum acid phosphatase activity was observed in MSB, followed by PKV media. Considerable amounts of acid phosphatase activity were also detected in YMG, SB, BS, and PDB media. MMN was found to be poor media for acid phosphatase activity. In *M. crassipes* (MR5), MMM served as the best media for acid phosphatase activity; while in *M. crassipes* (MR8), PKV and MSB served as the best media. Considerable amounts of acid phosphatase activity were also observed in BS and PDB media. Lesser acid phosphatase activity was detected in MMN.

PKV and MSB media were found to act as good media for acid phosphatase activity in *M. elata* (MR6). Optimum media for acid phosphatase activity was observed to be PDB and BS media. In *M. spongiola* (MR17), maximum acid phosphatase activity was observed in SB. Other media such as, PKV, MSB and MSB, too, yielded considerable amounts of acid phosphatase activity. Significant differences were found in the enzyme production among the different species and media (Tab 5.4, Fig 5.4).

Table 5.4: Effect of different media on the acid phosphatase activity (µM PNP/g mycelium/hour) by mycelia of different *Morchella* spp.

Media	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
ME	2004 ± 259b E	1391 ± 296c E	9402 ± 339a B	1290 ± 84c C	1451 ± 162c E	2365 ± 149b B
MMN	194 ± 50a F	127 ± 20ab F	225 ± 83a G	197 ± 35a D	165 ± 22a F	83 ± 17b D
CZD	3213 ± 640a D	2142 ± 293b D	2316 ± 150b F	2843 ± 495b B	1517 ± 348c E	1555 ± 67c B
YMG	5240 ± 594a C	3066 ± 69ab C	3118 ± 950ab E	2668 ± 99c B	1694 ± 123d E	1806 ± 348d C
SB	**13111 ± 3102a A**	3345 ± 198cd C	4689 ± 51c DE	1221 ± 412e C	2655 ± 478d D	**9817 ± 185b A**
BS	11284 ± 1179a A	3156 ± 223bc C	8545 ± 911b C	4577 ± 687b B	4120 ± 422b B	1591 ± 79c C
MMM	7054 ± 829b B	1379 ± 235c E	**15112 ± 4059a A**	1991 ± 398c C	1674 ± 429c E	1405 ± 153c C
PDB	12785 ± 1465a A	3985 ± 405c C	8289 ± 1156b C	4009 ± 154 c B	2028 ± 445d D	8887 ± 297b A
MSB	8063 ± 1750ab B	**13027 ± 2257a A**	3664 ± 435b E	8596 ± 910ab A	4795 ± 828b B	9085 ± 176ab A
PKV	11268 ± 824a A	8597 ± 1083b B	5694 ± 803c D	**9993 ± 2498a A**	**10202 ± 1209a A**	8173 ± 1117b A
MGB	2338 ± 195b E	1258 ± 298c E	3070 ± 231a E	1845 ± 221c C	3159 ± 242a C	2572 ± 206b B

ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media. Values sharing a common lower case letter within the row and uppercase letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

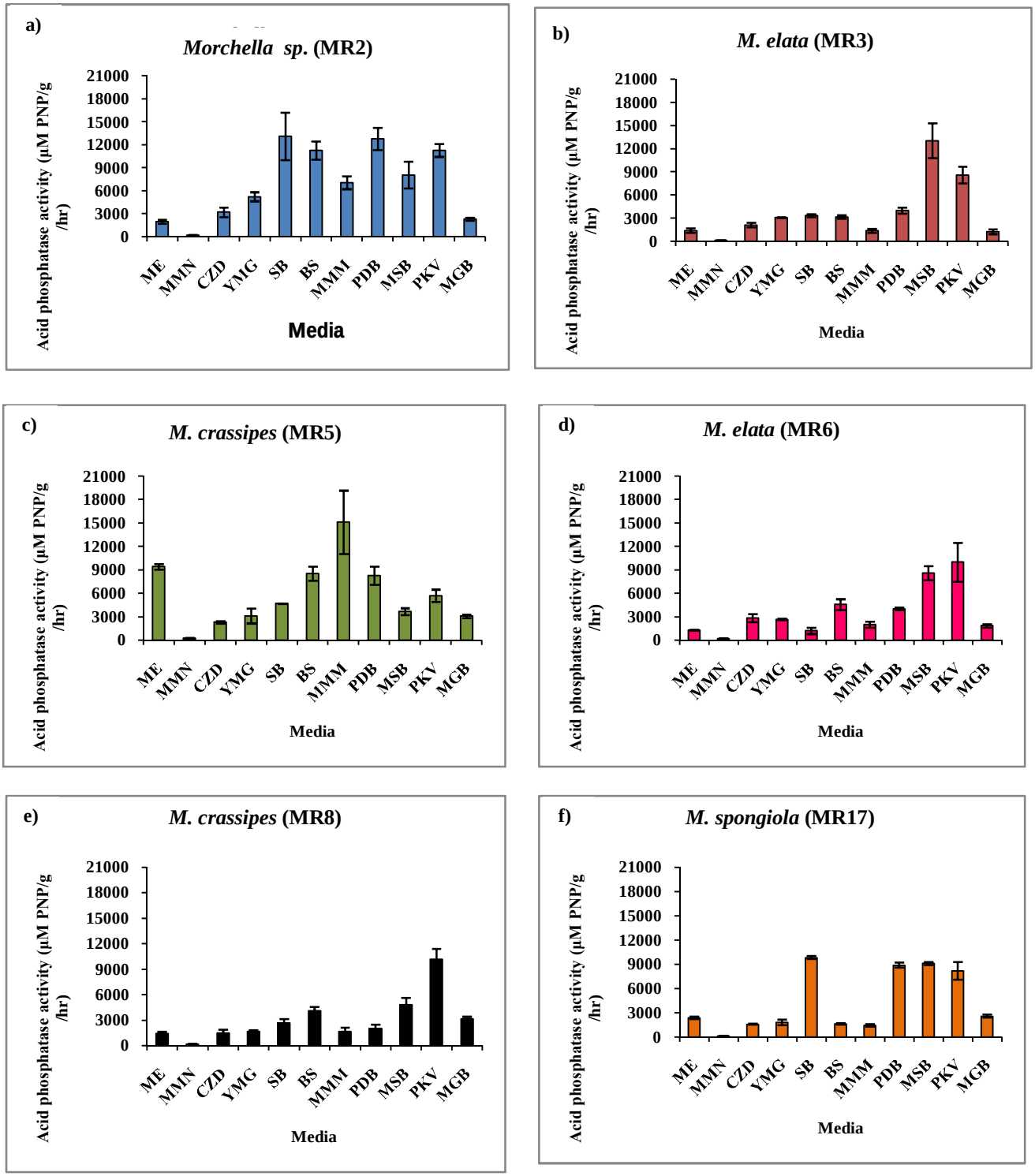


Fig 5.4: Effect of different media on acid phosphatase activity of mycelia of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). ME, Malt Extract; MMN, Modified Melin Norkrans; CZD, CzapekDox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media.Values are Mean ± SEM (n=3).

Acid phosphatase activity in culture filtrate

Acid phosphatase production in culture filtrate was found be very low as compared to acid phosphatase activity by mycelia. In case of extracellular acid phosphatase in the culture filtrate, MMM medium was found to be the best for high phosphatase production by most of the *Morchella* spp (Tab 5.5). Maximum enzyme activity was observed in the culture filtrate of *M. elata* (MR3) in MMM, followed by *Morchella sp.* (MR2) and *M. elata* (MR6).

Maximum acid phosphatase activity was observed in MMM, followed by SB and MSB media. Considerable amount of acid phosphatase activity was also observed in the culture filtrates of CZD, YMG, PDB and PKV media. Least acid phosphatase activity was detected in the culture filtrate of MMN.

In *M. elata* (MR3 and MR6), maximum acid phosphatase activity was observed in MMM. Media such as, PDB, MSB, and SB served as optimum media for acid phosphatase activity in *M. elata* (MR3) while in *M. elata* (MR6), SB served the same.

Maximum extracellular acid phosphatase activity was observed in MMM and MSB, in *M. crassipes* (MR5 and MR8). Optimum levels of acid phosphatase activity were detected in PDB, MSB and SB. Least acid phosphatase activity was observed in MGB and PKV.

For *M. spongiola* (MR17), maximum acid phosphatase activity was observed in MMM. Considerable level of acid phosphatase activity was detected in the culture filtrates of PDB. MGB served as the poorest media for acid phosphatase activity in *M. spongiola* (MR17) (Table 5.5, Fig 5.5).

Table 5.5: Effect of different media on acid phosphatase activity (µM PNP/ml/hour) in the culture filtrate of different *Morchella* spp.

Media	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
ME	22.30 ± 1.9d D	48.56 ± 3.1b BC	50.75 ± 3.4b B	51.69 ± 2.5b BC	69.18 ± 3.8a AB	38.25 ± 2.2bc CD
MMN	23.25 ± 2.2b D	30.43 ± 6.8b C	40.00 ± 3.8ab BC	29.18 ± 3.1b D	46.69 ± 3.6ab B	72.31 ± 4.4a B
CZD	40.44 ± 4.7b C	39.50 ± 1.6b BC	26.38 ± 2.2d C	37.94 ± 2.5b CD	32.94 ± 1.0bc BC	76.06 ± 3.1a B
YMG	33.88 ± 2.2a CD	45.44 ± 5.0a BC	32.00 ± 1.6a BC	42.62 ± 5.9a C	44.50 ± 1.6 a B	47.00 ± 2.2a C
SB	61.06 ± 1.9b B	52.64 ± 2.5b B	58.88 ± 6.6b B	67.00 ± 6.5b B	50.12 ± 5.3b B	83.56 ± 2.5a AB
BS	29.00 ± 2.5b CD	31.38 ± 2.2b C	30.44 ± 3.1b C	33.88 ± 2.2b CD	37.00 ± 3.4a BC	42.00 ± 1.6a C
MMM	**95.45 ± 5.0a A**	**100.75 ± 1.6a A**	**83.88 ± 1.6ab A**	**95.13 ± 4.0a A**	**73.25 ± 2.2b A**	**90.13 ± 1.6a A**
PDB	37.31 ± 1.9c C	54.81 ± 4.4ab B	59.19 ± 1.3ab B	35.75 ± 2.8c CD	49.50 ± 2.8 b B	68.88 ± 7.8a B
MSB	58.56 ± 6.9a B	52.94 ± 3.8a B	33.56 ± 4.4b C	40.13 ± 4.7B C	29.19 ± 3.1b C	34.81 ± 5.6b CD
PKV	30.43 ± 1.3a CD	22.63 ± 0.3a D	26.06 ± 0.6a C	28.56 ± 0.6a D	27.00 ± 0.3a C	27.63 ± 1.6a D
MGB	31.06 ± 1.9a CD	28.56 ± 3.1a D	27.94 ± 1.9a C	28.88 ± 0.3a D	21.68 ± 0.6a C	23.56 ± 2.5a D

ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media. Values sharing a common lower case letter within the row and uppercase letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

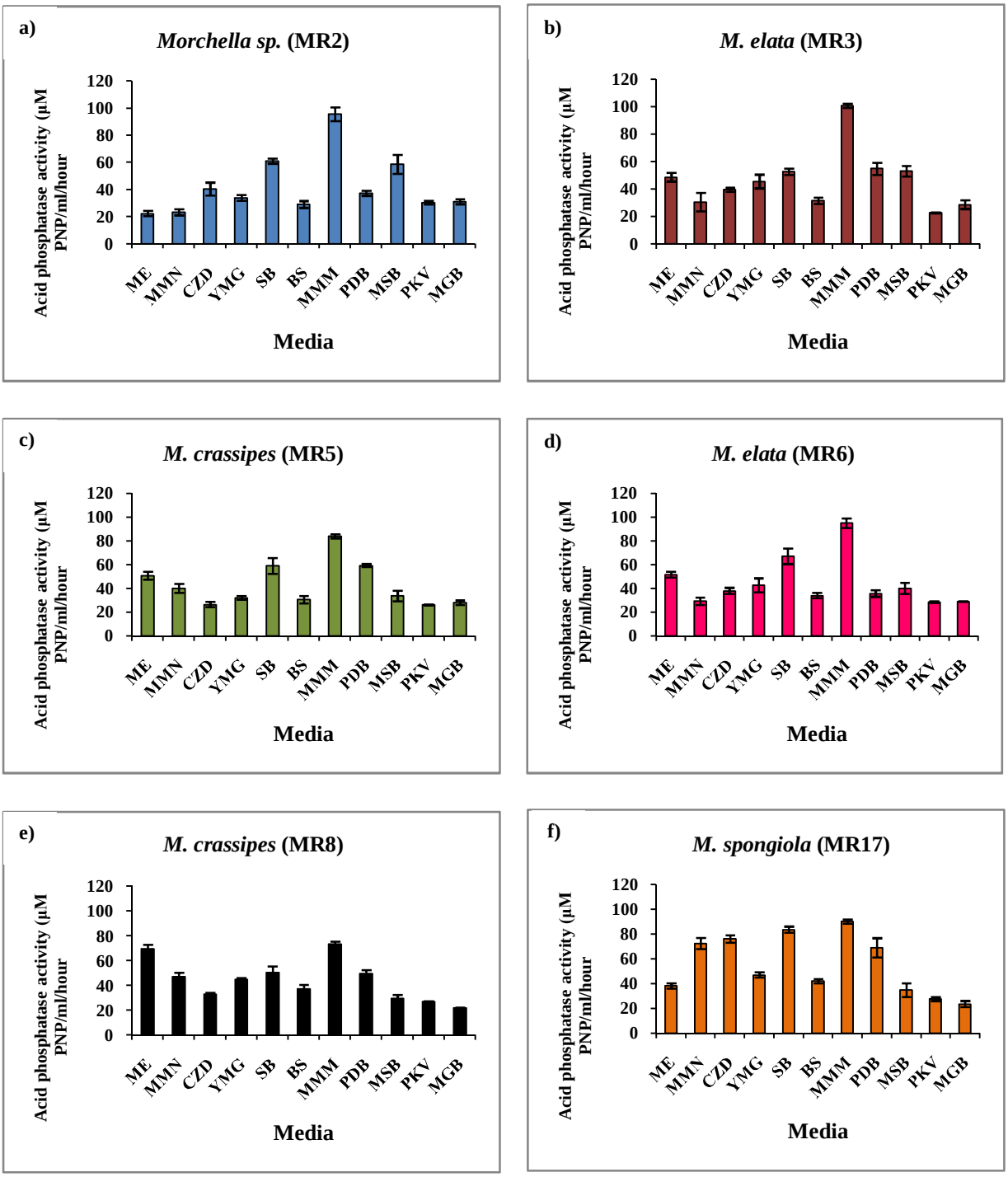


Fig 5.5: Effect of different media on the acid phosphatase activity in the culture filtrate of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). ME, Malt Extract; MMN, Modified Melin Norkrans; CZD, CzapekDox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media.Values are Mean ± SEM (n=3).

5.1.5 Phytase activity

The results showed that there were variations among different *Morchella* species in relation to phytase production in different media. *Morchella crassipes* (MR5) showed the highest amount of phytase activity in MMM medium. Other *Morchella* spp., *Morchella sp.* (MR2), *M. elata* (MR3), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17), too produced considerable amounts of phytase with least significant differences among them. MMN medium was found to be the best for phytase production than all the other media. Least phytase activity was detected by *Morchella sp.* (MR2) in YMG (Table 5.6, Fig 5.6).

Table 5.6: Effect of different media on phytase activity of (µM of iP/min) of different *Morchella* spp.

Media	***Morchella sp.*** **(MR2)**	***M. elata*** **(MR3)**	***M. crassipes*** **(MR5)**	***M. elata*** **(MR6)**	***M. crassipes*** **(MR8)**	***M. spongiola*** **(MR17)**
ME	112.3 ± 2a AB	31.7 ± 6c C	46.3 ± 4b D	53.3 ± 3b C	86.8 ± 15ab B	112.8 ± 7a AB
MMN	**136.0 ± 3a A**	**106.3 ± 8b A**	**141.6 ± 5a A**	**141.1 ± 2a A**	**134.9 ± 2a A**	**133.5 ± 1a A**
YMG	1.0 ± 1c G	19.4 ± 7ab D	25.5 ± 5a E	15.5 ± 2ab E	11.8 ± 2b E	10.6 ± 1b F
CZD	56.4 ± 1a CD	56.9 ± 3a BC	45.4 ± 7b D	53.9 ± 2a C	44.7 ± 2b C	40.8 ± 11b D
SB	43.5 ± 5a D	39.8 ± 2a C	42.8 ± 2a D	33.2 ± 7a D	24.3 ± 2b D	47.2 ± 5a D
BS	68.5 ± 2b CD	78.2 ± 10a B	74.4 ± 3a C	80.9 ± 5a B	81.2 ± 3a B	82.2 ± 9a C
MMM	73.5 ± 2b CD	71.9 ± 3b B	90.4 ± 18a B	77.2 ± 2b B	81.0 ± 7ab B	99.9 ± 5a B
PDB	82.9 ± 14a B	47.7 ± 2c BC	9.0 ± 2d F	5.2 ± 2e F	9.1 ± 1d E	52.1 ± 6b D
MSB	83.9 ± 19ab B	71.0 ± 11b B	91.9 ± 7a B	79.1 ± 5b B	77.2 ± 5b B	81.9 ± 3ab C
PKV	16.7 ± 2a E	19.7 ± 2a D	15.3 ± 2a EF	16.7 ± 1a E	18.1 ± 2a DE	19.2 ± 3a E
MGB	6.5 ± 1b F	10.9 ± 1ab E	10.0 ± 1ab F	15.6 ± 3a E	9.7 ± 1ab E	17.0 ± 1a E

ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media. Values sharing a common lower case letter within the row and uppercase letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3)**.** Values in bold indicate significant ones.

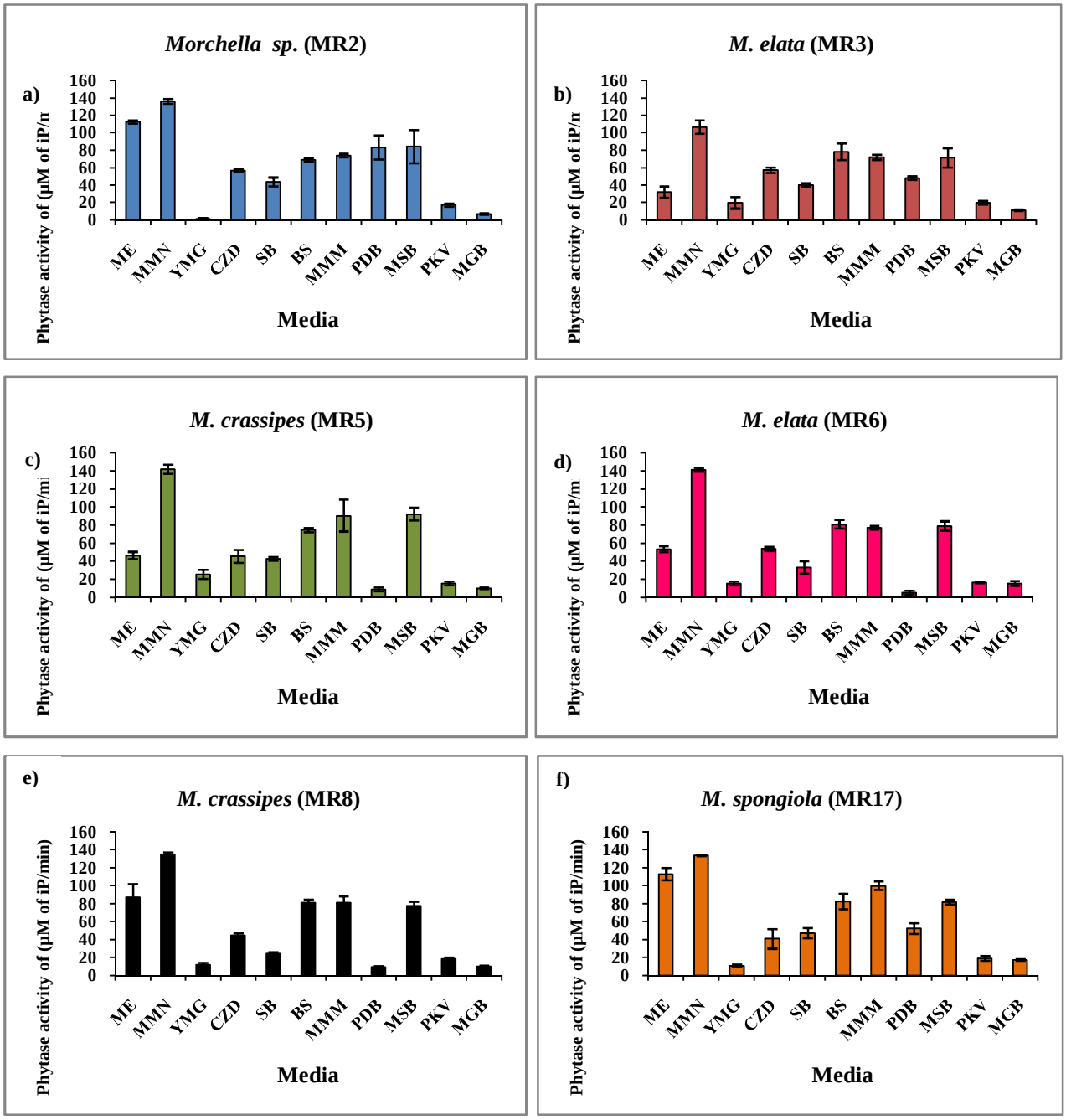


Fig 5.6: Effect of different media on phytase activity of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). ME, Malt Extract; MMN, Modified Melin Norkrans; CZD, CzapekDox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media.Values are Mean ± SEM (n=3).

5.1.6. Laccase activity

Laccase activity was found to be highly influenced by different *Morchella* species in different media. It was found that laccase activity was dependent on the media components as no activity was detected in ME, YMG, SB, BS, MMM, PDB, PKV and MGB media. All the *Morchella* spp. were able to produce high titers of laccase in MSB than other media (Tab 5.7). Laccase activity was also detected in MMN and CZD, but the amount produced was found to be significantly less as compared to that produced in MSB media. Maximum laccase activity was observed in *M. crassipes* (MR8) in MSB media. The activity produced was almost 20-fold higher in MSB than that produced in MMN or CZD media. Similar pattern of high titers of laccase activity were detected in *M. elata* (MR3 and MR6) and *M. spongiola* MR17. Not much significant differences were observed in laccase production in MSB by all the spp. (Table 5.7, Fig 5.7).

Table 5.7: Effect of different media on laccase activity (U/ml) of different *Morchella* spp.

Media	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR5)	*M. elata* (MR6)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
ME	ND	ND	ND	ND	ND	ND
MMN	0.054 ± 0.01b B	0.10 ± 0.02a B	0.14 ± 0.03a B	0.045 ± 0.01b B	0.11 ± 0.03a B	0.054 ± 0.02b C
CZD	0.076 ± 0.03b B	0.076 ± 0.01b C	0.11 ± 0.01a B	0.054 ± 0.01b B	0.098 ± 0.02a B	0.11 ± 0.02a B
YMG	ND	ND	ND	ND	ND	ND
SB	ND	ND	ND	ND	ND	ND
BS	ND	ND	ND	ND	ND	ND
MMM	ND	ND	ND	ND	ND	ND
PDB	ND	ND	ND	ND	ND	ND
MSB	**1.0 ± 0.14a A**	**0.75 ± 0.09b A**	**1.65 ± 0.3a A**	**1.30 ± 0.34a A**	**1.91 ± 0.12a A**	**1.46 ± 0.2a A**
PKV	ND	ND	ND	ND	ND	ND
MGB	ND	ND	ND	ND	ND	ND

ME, Malt Extract; MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; YMG, Yeast Maltose Glucose; SB, Sabourd's media; BS, Basal media; MMM, Minimal Mineral media; PDB, Potato Dextrose broth; MSB, Mineral Salts broth; MGB, morel growth broth; PKV, pikovskaya's media. Values sharing a common lower case letter within the row and uppercase letter within the column are not significant at P<0.05. Values are Mean ± SEM (n=3)**.** Values in bold indicate significant ones. ND=Not detected

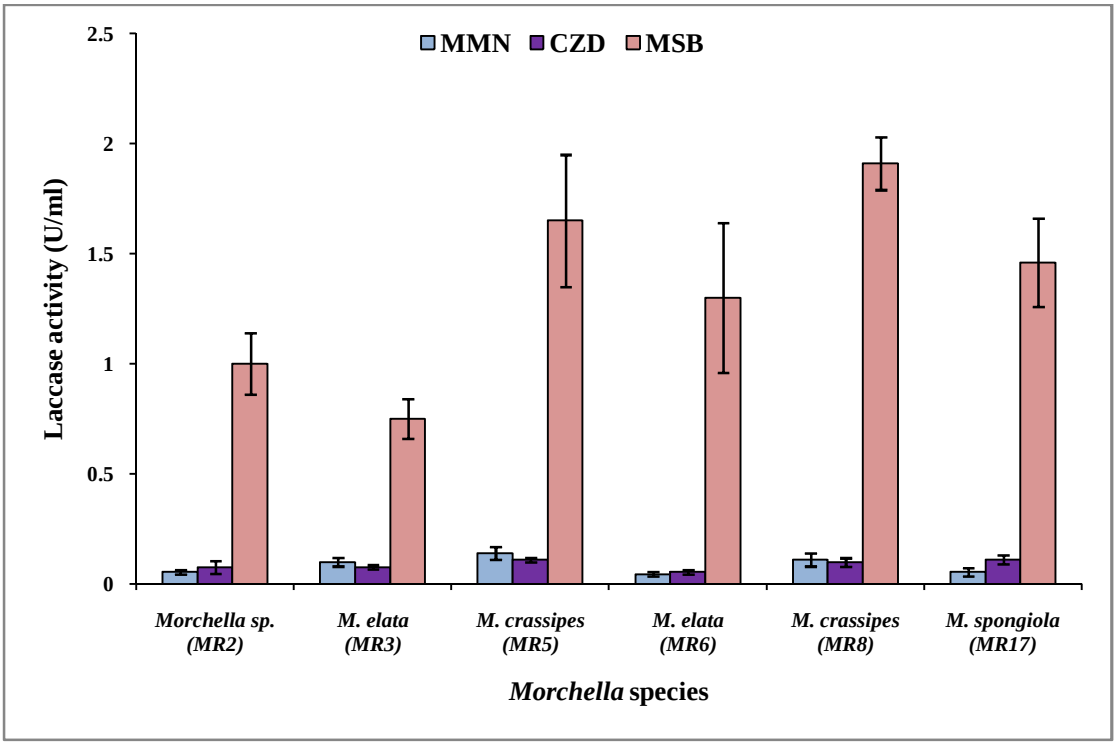


Fig 5.7: Effect of different media on laccase activity of different *Morchella* spp. MMN, Modified Melin Norkrans Media; CZD, Czapek Dox; MSB, Mineral Salts broth. Values are Mean ± SEM (n=3)**.**

5.1.7 Lignin Peroxidase and Manganese peroxidase activity

None of the *Morchella* spp. showed lignin peroxidase or manganese peroxidase activity in the culture filtrate of different media.

The results showed that the malt extract, potato dextrose broth and mineral salts broth were observed to be the best for high biomass yield and protein content in all *Morchella* spp. Acid phosphatase and phytase activity were found to vary with culture and media used in the study. Mineral salts broth was found to be the best for high biomass yield along with high laccase activity by all *Morchella* spp.

5.2 Growth studies with different pH

Mineral salts broth was used to study the effect of pH on the growth studies of *Morchella* spp. Mineral salts broth with different pH of media (2.5, 3.5, 4.5, 5.0, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5) was inoculated with two mycelial discs (each 5 mm in diameter) of different *Morchella* spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17)). Mycelia were harvested after 7 days and dry biomass, pH and protein content of culture filtrates were determined.

5.2.1 Biomass

Morchella spp. were able to grow well from pH 5 to pH 10.5 (Tab 5.8). Unlike most of fungi, *Morchella* spp. preferred slightly alkaline pH for growth and were able to grow well even at pH 10.5.

Maximum growth was observed at pH 10.5 in *M. spongiola* (MR17), *Morchella sp.* (MR2) and *M. elata* (MR3); while in *M. elata* (MR6) and *M. crassipes* (MR8), maximum growth was observed at pH 9.5. In *M. crassipes* (MR5), pH 8.5 was found to be the best for maximum biomass production. It was observed that in *Morchella* spp., pH of 8.5 to 10.5 was found to be highly favorable for growth.

Least biomass was detected at pH 2.5. Significant differences in biomass yield were observed at pH of 9.5 and 10.5 by all *Morchella* spp. Growth was reduced to significant levels at pH of 2.5, 3.5 and 4.5 by all the *Morchella* spp. A significant increase in biomass yield along with an increase in pH was observed from pH 4.5 to pH 5.5 (Tab 5.8, Fig 5.8).

Table 5.8: Effect of different pH on the biomass (mg/50ml) of different *Morchella* spp.

pH	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR5)	*M. elata* (MR6)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
2.5	3.98 ± 0.5d	5.00 ± 0.5f	5.52 ± 0.2c	4.42 ± 0.2d	4.72 ± 0.1c	6.00 ± 0.2e
3.5	5.95 ± 0.3d	4.39 ± 0.7f	6.51 ± 0.2c	5.90 ± 0.2d	5.97 ± 0.9c	6.00 ± 0.7e
4.5	6.66 ± 0.1d	6.08 ± 0.3f	7.85 ± 0.5c	7.14 ± 0.1d	10.00 ± 0.8c	6.89 ± 0.7e
5.0	14.62 ± 1.2d	15.97 ± 2.7e	7.24 ± 0.1c	7.43 ± 0.5d	11.78 ± 5.2bc	23.00 ± 3.9de
5.5	53.83 ± 7.0c	56.73 ± 1.4d	56.99 ± 1.0b	47.20 ± 6.2bc	49.06 ± 10.5ab	40.30 ± 6.0cd
6.5	66.65 ± 1.2bc	62.70 ± 2.5cd	63.80 ± 3.3ab	39.20 ± 7.9c	62.56 ± 4.0a	50.60 ± 4.9c
7.5	56.43 ± 1.7c	65.16 ± 1.7bc	58.63 ± 1.9b	50.33 ± 7.7abc	54.61 ± 6.3a	55.46 ± 11.0bc
8.5	67.20 ± 1.5bc	72.35 ± 1.2ab	**78.18 ± 1.6a**	65.03 ± 1.4ab	63.15 ± 3.4a	77.50 ± 2.7ab
9.5	75.97 ± 4.8ab	72.30 ± 1.2ab	**73.45 ± 1.4a**	**70.07 ± 0.3a**	**76.16 ± 5.0a**	**85.94 ± 1.8a**
10.5	**85.20 ± 2.3a**	**73.90 ± 2.5a**	66.31 ± 1.2ab	63.77 ± 2.3ab	61.54 ± 7.8a	77.23 ± 0.5ab

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

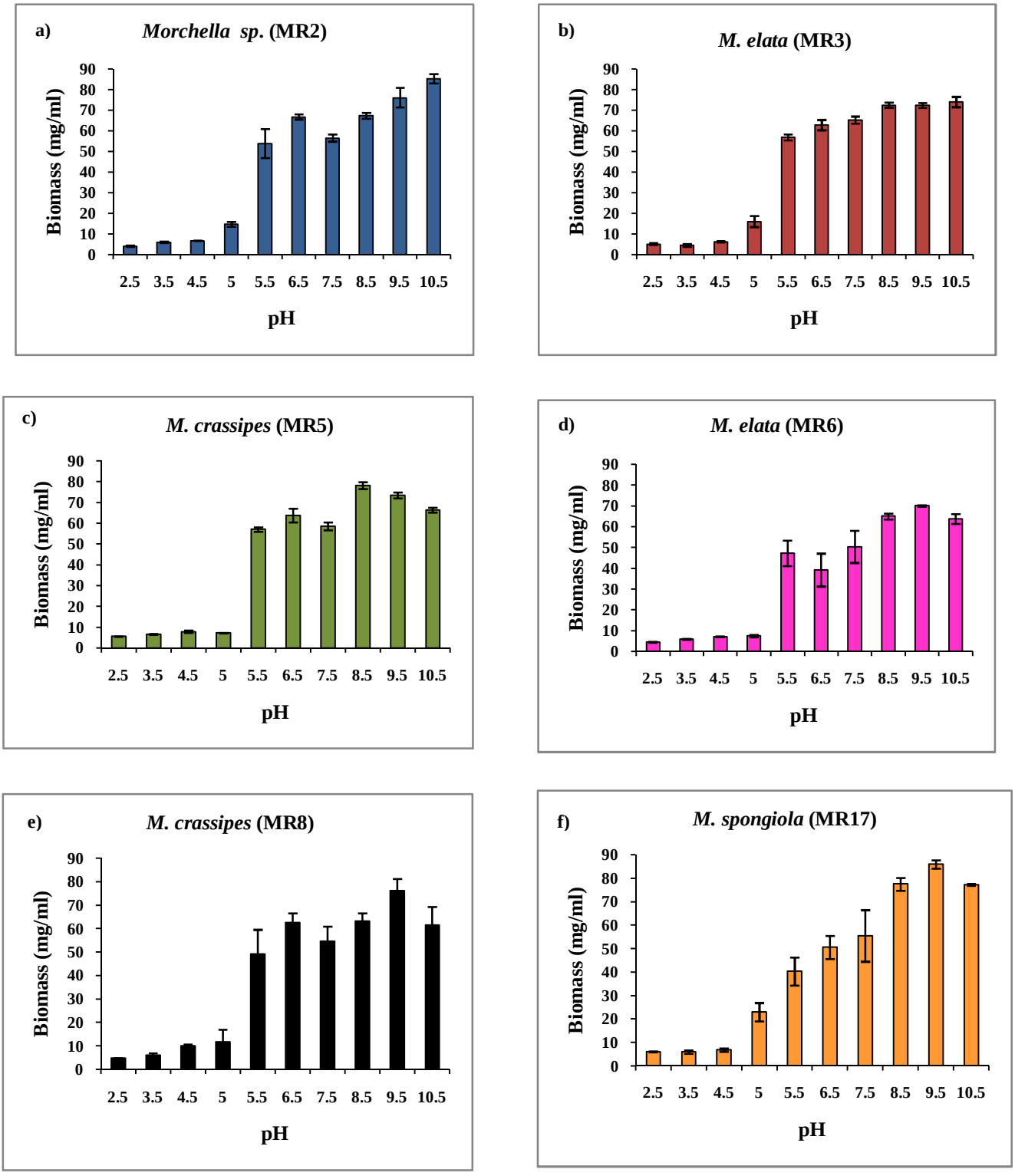


Fig 5.8: Effect of pH on biomass (mg/50ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). Values are Mean ± SEM (n=3)

5.2.2. pH

An increase in pH (compared to pH of respective controls) was observed in all the *Morchella* spp. A significant increase in pH was observed from pH 5.5 till pH of 8.5 in *Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), while it was observed till pH of 7.5 in *M. crassipes* (MR8) and pH of 5.5 in *M. spongiola* (MR17) (Tab 5.9). The pH decreased to significant levels from initial pH of 9.5 and 10.5 to 8.5 (approx.) in all the *Morchella* spp., where maximum biomass was observed as described previously. Significant differences were observed in the increase in pH as compared to respective controls pH (Table 5.9, Fig 5.9).

Table 5.9: Effect of different pH on different *Morchella* spp.

pH	***Morchella sp.*** **(MR2)**	***M. elata*** **(MR3)**	***M. crassipes*** **(MR5)**	***M. elata*** **(MR6)**	***M. crassipes*** **(MR8)**	***M. spongiola*** **(MR17)**
2.5	2.52 ± 0.02f	2.50 ± 0.01g	2.50 ± 0.00f	2.57 ± 0.05f	2.50 ± 0.01h	2.51 ± 0.04f
3.5	3.67 ± 0.11e	3.48 ± 0.14f	3.50 ± 0.09e	3.60 ± 0.11e	3.52 ± 0.02g	3.40 ± 0.23f
4.5	4.59 ± 0.01d	4.54 ± 0.02e	4.60 ± 0.21d	5.47 ± 0.19cd	4.60 ± 0.05f	4.46 ± 0.28e
5.0	5.81 ± 0.05c	5.57 ± 0.05d	5.54 ± 0.26c	5.16 ± 0.17d	5.79 ± 0.10e	5.71 ± 0.05d
5.5	6.19 ± 0.15c	6.16 ± 0.16d	6.30 ± 0.09c	6.09 ± 0.20c	6.93 ± 0.05d	5.58 ± 0.24d
6.5	7.83 ± 0.17b	7.36 ± 0.15c	7.21 ± 0.04b	7.30 ± 0.15b	8.16 ± 0.06c	5.91 ± 0.22cd
7.5	8.30 ± 0.16ab	8.30 ± 0.18b	8.37 ± 0.13a	8.20 ± 0.03a	8.29 ± 0.07bc	6.73 ± 0.15bc
8.5	8.60 ± 0.07a	8.73 ± 0.14ab	8.47 ± 0.11a	8.61 ± 0.20a	8.46 ± 0.07bc	7.39 ± 0.15b
9.5	8.80 ± 0.12a	8.90 ± 0.14ab	8.92 ± 0.13a	8.62 ± 0.20a	9.01 ± 0.13ab	8.59 ± 0.19a
10.5	8.67 ± 0.28a	8.93 ± 0.06a	9.08 ± 0.28a	8.87 ± 0.18a	8.93 ± 0.08a	8.59 ± 0.08a

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

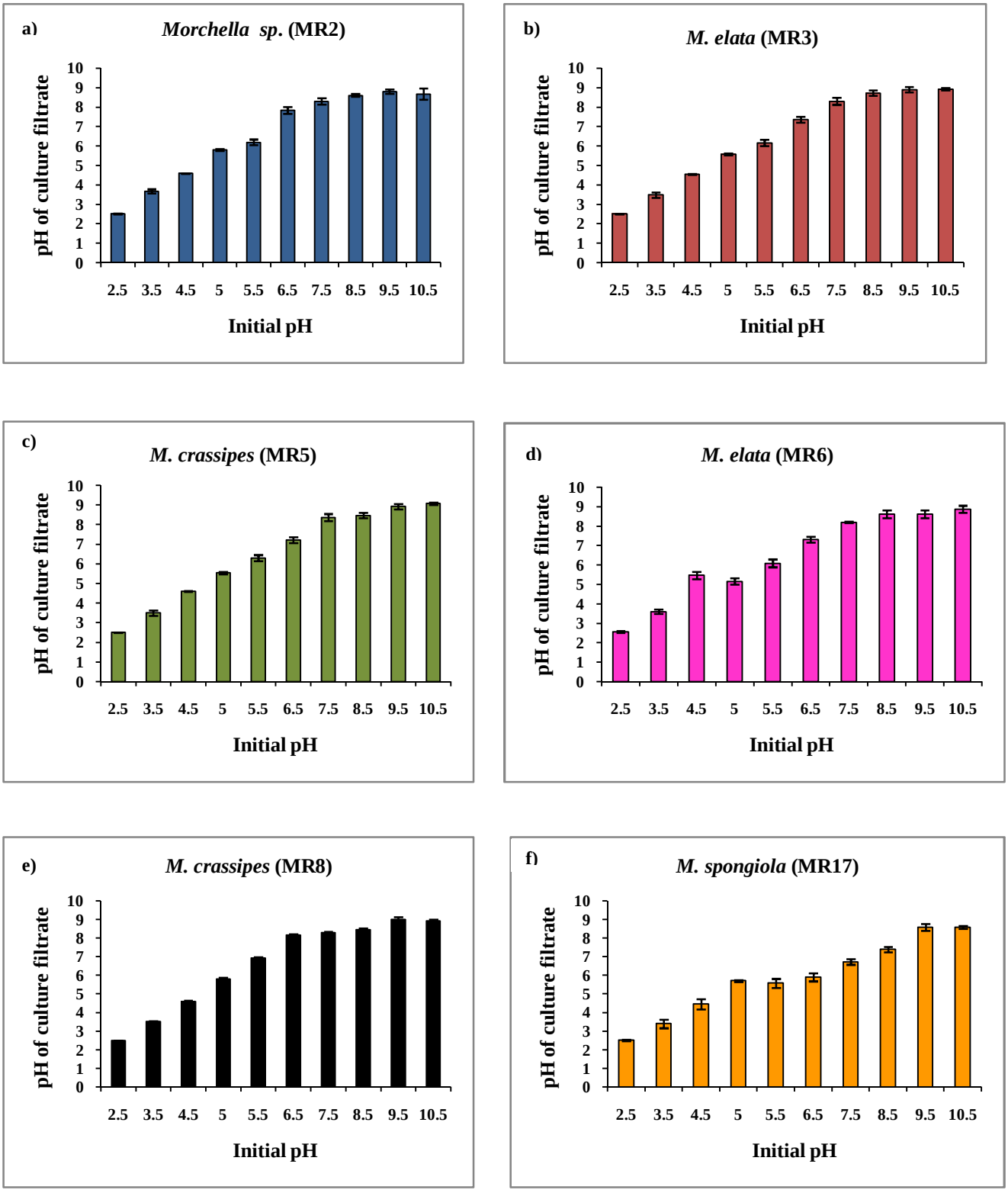


Fig 5.9: Effect of pH on the pH of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). Values are Mean ± SEM (n=3)

5.2.3 Protein content

Significant differences were observed in protein content at highly acidic (pH of 2.5) and alkaline (pH of 10.5) conditions as compared to others in all *Morchella* spp (Tab 5.10). Protein content was observed to be decreased with an increase in pH from 2.5 till 5.5, and after that an increase in protein content along with an increase in pH was observed. Protein content was found to be high under stress conditions of highly acidic and alkaline conditions. Maximum protein content was observed at pH 10.5 in *M. elata* (MR3) and *M. crassipes* (MR5). Significant differences in protein content at pH 2.5 and 10.5 as compared to others were found in *Morchella sp.* (MR2), *M. elata* (MR3), *M. elata* (MR6) and *M. spongiola* (MR17) (Table 5.10, Fig 5.10).

Table 5.10: Effect of different pH on protein content (mg/ml) of different *Morchella* spp.

pH	***Morchella sp.*** **(MR2)**	***M. elata*** **(MR3)**	***M. crassipes*** **(MR5)**	***M. elata*** **(MR6)**	***M. crassipes*** **(MR8)**	***M. spongiola*** **(MR17)**
2.5	**2.64 ± 0.2a**	**2.11 ± 0.1ab**	**2.05 ± 0.1bc**	**2.43 ± 0.2a**	**1.98 ± 0.1abc**	**2.68 ± 0.3a**
3.5	1.50 ± 0.2bc	1.71 ± 0.1bc	1.81 ± 0.1cd	1.65 ± 0.2abc	1.51 ± 0.2bcde	1.56 ± 0.9bc
4.5	0.97 ± 0.1bcd	1.16 ± 0.0bcd	1.12 ± 0.0cde	1.27 ± 0.0bcd	0.81 ± 0.1fg	0.89 ± 0.3cd
5.0	0.57 ± 0.2cd	0.61 ± 0.2cd	0.75 ± 0.1de	1.02 ± 0.1cd	1.16 ± 0.2def	1.13 ± 0.0bc
5.5	0.48 ± 0.2d	0.31 ± 0.1d	0.53 ± 0.1e	0.97 ± 0.1cd	0.43 ± 0.1g	0.17 ± 0.0d
6.5	0.95 ± 0.2bcd	1.09 ± 0.1bcd	0.93 ± 0.0cde	1.16 ± 0.1bcd	0.85 ± 0.1efg	0.71 ± 0.2cd
7.5	1.33 ± 0.1bcd	1.28 ± 0.3bcd	1.20 ± 0.1cde	0.77 ± 0.1d	1.68 ± 0.1abcd	1.02 ± 0.1bcd
8.5	1.64 ± 0.0b	1.68 ± 0.2bc	1.56 ± 0.1cde	1.26 ± 0.2bcd	2.19 ± 0.2ab	0.72 ± 0.1cd
9.5	1.86 ± 0.1ab	1.30 ± 0.7bcd	3.15 ± 0.5ab	1.45 ± 0.4bcd	1.44 ± 0.1cdef	1.95 ± 0.2ab
10.5	**2.75 ± 0.5a**	**3.41 ± 0.2a**	**3.41 ± 0.5a**	**1.94 ± 0.1ab**	**2.24 ± 0.1a**	**2.68 ± 0.2a**

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

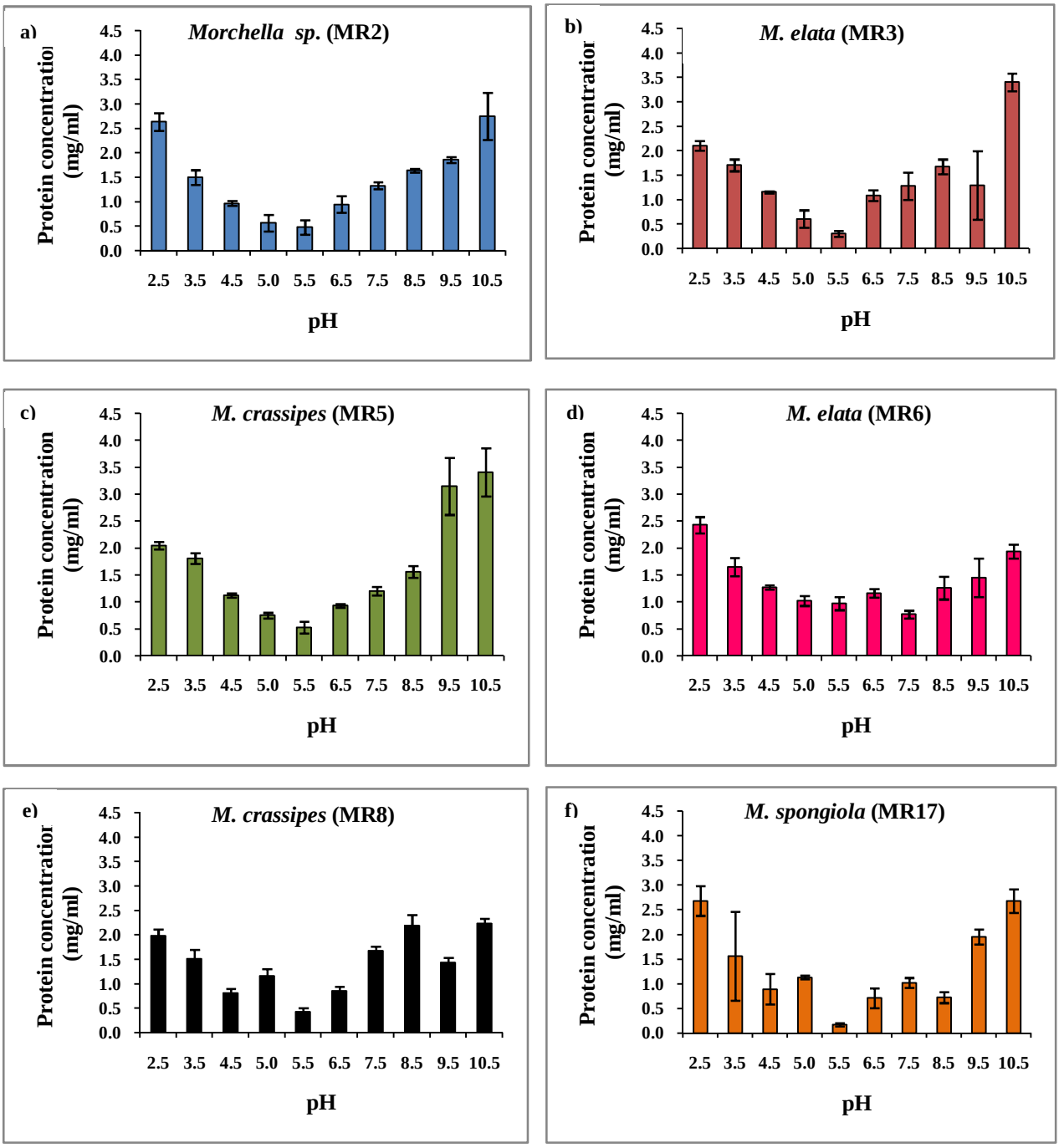


Fig 5.10: Effect of pH on the protein content (mg/ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). Values are Mean ± SEM (n=3)

5.3 Growth studies with temperature variations

In order to study the effect of temperature, mineral salts broth media (pH 5.6) was inoculated with two mycelial discs (each 5 mm in diameter) of *Morchella* spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17)) and kept in dark under different temperature conditions of 4, 15, 25, 30, 35, 40, and 50 °C. the results showed that temperature of 25 °C was observed to bethe best for growth studies in all *Morchella* spp. Maximum biomass was observed at 25 °C compared to other temperatures. Mycelial growth had initiated at 4 °C, but the growth was very slow and least biomass was recorded at 4 °C. Mycelia were able to grow well at temperature of 30 °C but in other temperature stress of 35, 40 and 50 °C, no growth was observed. *Morchella crassipes* (MR5) and (MR8), *M. spongiola* (MR17) and *Morchella sp.* (MR2) grew well at temperature 25 °C. Significant differences were observed in the growth of *Morchella* spp. at 25 °C as compared to other temperatures (Table 5.11, Fig 5.11).

Table 5.11: Effect of different temperature on different *Morchella* spp**.**

Temp °C	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
4	4.3 ± 0.7b	5 ± 0.9b	6.8 ± 1.3c	4.2 ± 1.2b	7.9 ± 1.0b	2.0 ± 0.5c
15	15.2 ± 1.5ab	7.3 ± 1.2ab	16.4 ± 2.8b	8.5 ± 0.9ab	15.3 ± 1.2ab	4.7 ± 0.7c
25	33.9 ± 2.0a	15.1 ± 1.5a	32.7 ± 2.7a	14.7 ± 1.5a	35.5 ± 3.8a	35.8 ± 2.6a
30	28.5 ± 1.7a	10.8 ± 2.0a	29.5 ± 1.6a	11.9 ± 2.5a	30.5 ± 2.6a	20.6 ± 1.7b
35	-	-	-	-	-	-
40	-	-	-	-	-	-
50	-	-	-	-	-	-

Values sharing a common letter within the column are not significant at P<0.05. Values are Mean ± SEM (n=3). - denotes no growth

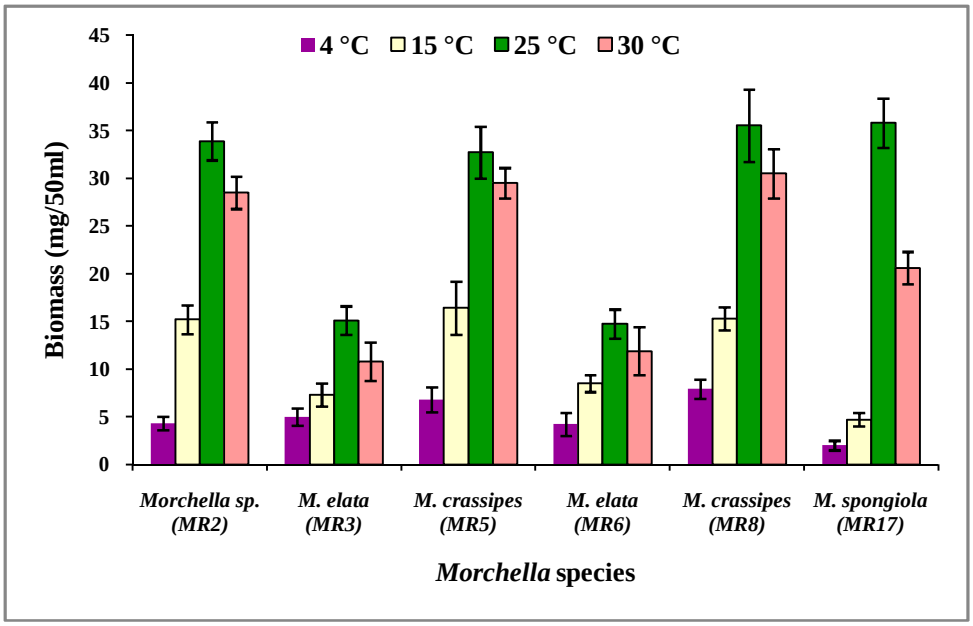


Fig 5.11: Effect of different temperatures on the growth of different *Morchella* spp. Values are Mean ± SEM (n=3).

The results showed that alkaline pH of 8.5 to 10.5 and temperature of 25 ℃ was favourable for the growth of all *Morchella* spp.

5.4 Growth and enzymatic studies with different carbon sources

Different species of *Morchella* (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17)) were tested for their ability to utilize different carbon sources. The fungi were grown in mineral salts broth by replacing glucose with fructose or sucrose or mannose or cellobiose or ribose or rhamnose or galactose or arabinose or xylose or sorbose or mannitol. After incubation for 7 days, biomass and ligninolytic enzyme activities such as laccase, lignin peroxidase and manganese peroxidase were determined.

5.4.1 Biomass

All *Morchella* species showed considerable variations in utilization of carbon sources. Maximum biomass was observed in cellobiose, followed by fructose in *Morchella sp.* (MR2) and *M. spongiola* (MR17). Glucose was utilized most effectively by *M. crassipes* ((MR5) and (MR8)) (Table 5.12, Fig 5.12). *Morchella sp.* (MR2) preferred cellobiose the most than other carbon sources. Fructose, sucrose, mannitol and mannose were the other carbon sources that were utilized well by *Morchella sp*. (MR2). Poorly utilized carbon source included ribose, where least biomass was obtained. In general, the utilization of carbon sources in *Morchella sp.* (MR2) followed the pattern as: cellobiose>fructose>sucrose>mannitol>mannose> sorbose>glucose>galactose>rhamnose>xylose>arabinose>ribose

Most preferred carbon source for growth in *M. elata* (MR3) was found to be mannitol, followed by xylose. Other carbon sources that acted as promising carbon sources for growth were fructose, galactose, cellobiose, rhamnose and ribose. Sorbose was poorly utilized by *M. elata* (MR3). The pattern of carbon utilization in *M. elata* (MR3) was: mannitol>xylose>fructose>galactose>cellobiose>rhamnose>ribose>sucrose>glucose>manno se >arabinose>sorbose

In *M. crassipes* (MR5), glucose was utilized the most than other carbon sources. Fructose, mannose, galactose and mannitol were found to be optimum souces of carbon for *M. crassipes* (MR5) growth. Poorly utilized carbon source was found to be rhamnose. The pattern of carbon utilization in *M. crassipes* (MR5) was: glucose>fructose>mannose >galactose>mannitol>sorbose>sucrose>ribose>xylose>arabinose>cellobiose>rhamnose;
while in *M. crassipes* (MR8), the pattern follows as: glucose>fructose>mannitol >galactose>mannose>arabinose>rhamnose>sorbose>cellobiose> sucrose>ribose>xylose

Most preferred carbon source for *M. elata* (MR6) included sucrose and ribose, while the least utilized carbon source included arabinose. In general, the preference of carbon

source utilization decreases as: sucrose>ribose>xylose>cellobiose>mannitol>glucose> galactose>sorbose>rhamnose>mannose>fructose>arabinose

In *M. spongiola* (MR17), cellobiose was found to be the most preferred carbon source for growth. Mannitol and xylose were the other carbon sources that were utilized well by *M. spongiola* (MR17). Poorly utilized carbon source included ribose. The trend of carbon utilization decreases as: cellobiose>mannitol> xylose> fructose> mannose> glucose > galactose> arabinose>sucrose> rhamnose> sorbose> ribose

Table 5.12: Effect of different carbon sources on biomass yield (mg/50ml) of different *Morchella* spp.

Carbon sources	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
Glucose	33.9 ± 2.0c	15.1 ± 1.5ab	**32.7 ± 2.7a**	14.8 ± 1.5ab	**35.5 ± 3.8a**	35.3 ± 2.6b
Fructose	63.9 ± 5.8ab	18.6 ± 1.6a	15.0 ± 2.5b	12.5 ± 0.8ab	17.0 ± 2.5b	39.1 ± 2.0b
Sucrose	56.3 ± 1.0b	15.2 ± 2.1ab	12.4 ± 1.4b	**17.4 ± 0.6a**	9.7 ± 3.8d	33.3 ± 1.0b
Mannose	52.8 ± 1.2b	14.7 ± 1.5ab	14.9 ± 1.4b	13.1 ± 1.9ab	12.0 ± 1.7c	39.5 ± 2.5b
Cellobiose	**70.9 ± 2.8a**	18.4 ± 0.6a	5.5 ± 0.5d	15.0 ± 0.4ab	10.0 ± 4.0d	**54.4 ± 1.7a**
Ribose	13.3 ± 1.0e	15.3 ± 2.8ab	12.1 ± 1.6b	**17.3 ± 1.4a**	10.0 ± 2.0d	23.7 ± 2.2c
Rhamnose	25.8 ± 1.9d	16.5 ± 1.6ab	10.6 ± 1.6c	13.4 ± 1.0ab	11.0 ± 2.2c	30.4 ± 2.3b
Galactose	27.8 ± 1.6d	18.6 ± 3.0a	13.9 ± 0.8b	13.9 ± 0.8ab	13.0 ± 2.4c	37.2 ± 3.6b
Arabinose	22.3 ± 1.9d	12.8 ± 0.8b	7.4 ± 1.7d	11.8 ± 2.0b	12.4 ± 2.1c	33.9 ± 1.9b
Xylose	25.6 ± 2.6d	20.0 ± 1.2a	11.9 ± 1.9b	16.6 ± 0.7a	8.2 ± 1.7e	41.9 ± 1.4ab
Sorbose	37.6 ± 2.0c	7.7 ± 1.3c	12.6 ± 1.4b	13.5 ± 0.6ab	10.0 ± 2.2d	25.8 ± 2.7c
Mannitol	56.1 ± 2.4b	**24.9 ± 1.2a**	13.1 ± 1.7b	14.8 ± 0.5ab	15.7 ± 1.9c	43.2 ± 2.0ab

Values sharing a common letter in the column are not significant at P<0.05. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

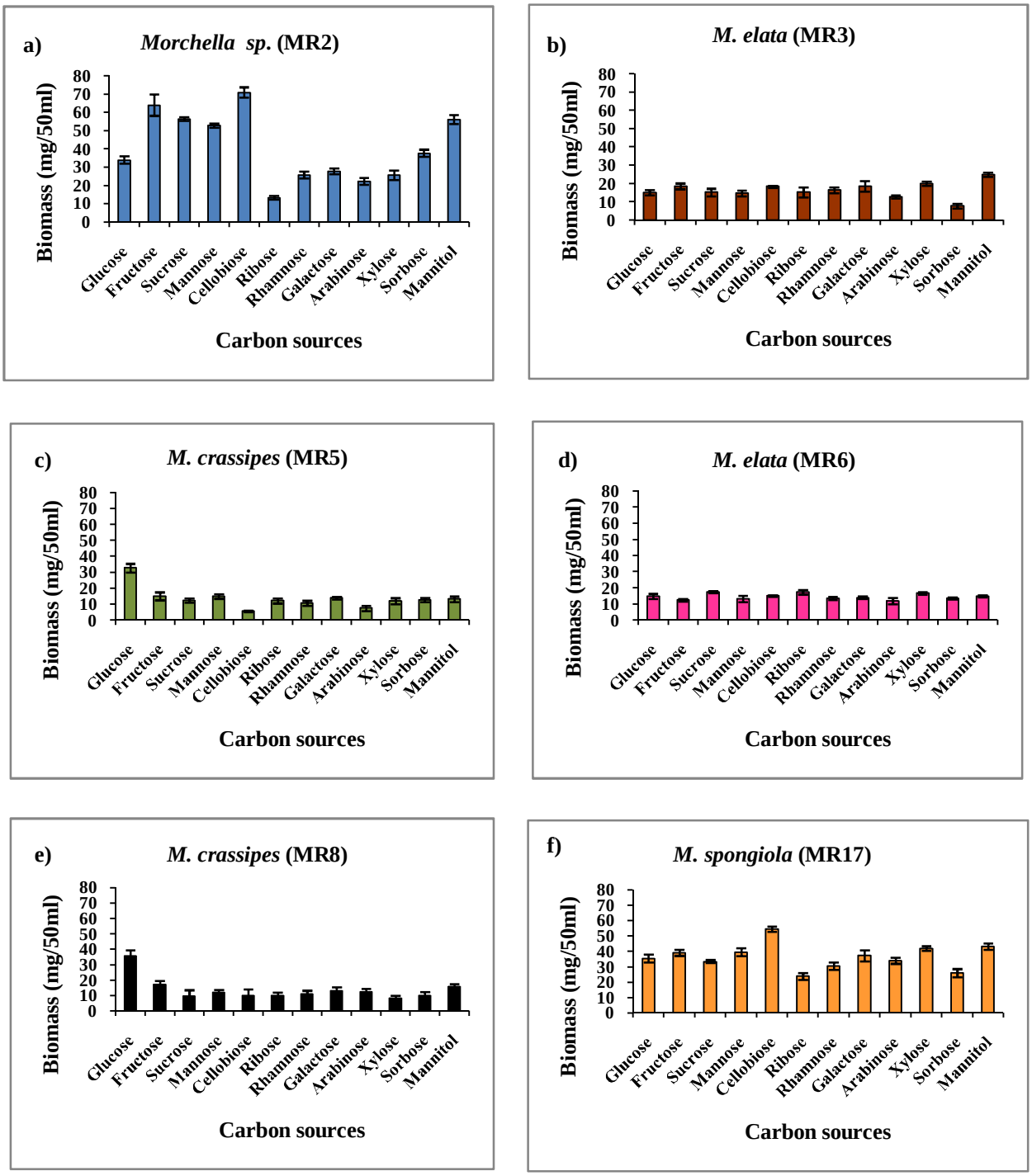


Fig 5.12: Effect of different carbon sources on biomass (mg/50ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). Values are Mean ± SEM (n=3)

5.4.2 Laccase activity

Laccase activity was highly influenced by different carbon sources. Mannose was found to be the most preferred source for laccase activity in all the *Morchella* spp (Table 5.13). Maximum laccase was produced by *M. crassipes* (MR8), followed by *M. crassipes* (MR5), using mannose as the carbon source. Optimum amounts of laccase activity were observed in *Morchella sp.* (MR2), *M. elata* (MR3) and *M. elata* (MR6). *Morchella spongiola* (MR17) showed the least laccase activity that was highly significant compared to that produced in other carbon sources (Table 5.13, Fig 5.13).

Maximum laccase activity was detected in mannose as the carbon source in *Morchella sp*. (MR2). High titers of laccase activity were also detected in sorbose, mannitol, galactose and ribose as the carbon sources. Laccase activity was found to decrease in the order as: mannose> sorbose> mannitol> galactose> ribose> rhamnose> arabinose> glucose> xylose> cellobiose> sucrose> fructose

In *M. elata* (MR3), mannose and rhamnose served as best sources of carbon for laccase production; whereas fructose served as poor carbon source for laccase activity. The trend for laccase activity decreases as: mannose> rhamnose >sorbose> ribose> galactose> mannitol> glucose> arabinose> xylose>cellobiose> sucrose> fructose; whereas in *M. elata* (MR6), this pattern follows as: rhamnose> sorbose> ribose> mannitol> galactose> mannose> glucose> arabinose> xylose>cellobiose> fructose> sucrose

In *M. crassipes* (MR5), mannose followed by rhamnose produced maximum laccase activity. Minimum laccase activity was produced in sorbose and fructose as carbon sources. The pattern of laccase production in *M. crassipes* (MR5) decreases as: mannose> rhamnose> mannitol> galactose> ribose> sorbose> arabinose> glucose> xylose>cellobiose> sucrose> fructose;

while in *M. crassipes* (MR8), the pattern follows as: mannose> rhamnose> galactose> ribose> sorbose> mannitol> glucose> arabinose> xylose>sucrose> fructose> cellobiose

In *M. spongiola* (MR17), maximum laccase activity was observed in mannose and rhamnose as the carbon sources whereas minimum activity was detected in cellobiose and fructose as the carbon sources. Laccase activity decreases in the order as: mannose> rhamnose> galactose> ribose> sorbose> mannitol> arabinose> glucose> xylose>sucrose> cellobiose> fructose

Table 5.13: Effect of different carbon sources on laccase activity (U/ml) of different *Morchella* species**.**

Carbon sources	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
Glucose	1.62 ± 0.07bc	1.84 ± 0.20bc	1.86 ± 0.20cd	1.88 ± 0.10abc	1.91 ± 0.11bc	1.42 ± 0.10abcd
Fructose	0.69 ± 0.11d	0.76 ± 0.11d	0.67 ± 0.09e	0.81 ± 0.07cd	1.08 ± 0.40cd	0.52 ± 0.08d
Sucrose	0.80 ± 0.04d	0.83 ± 0.11d	0.70 ± 0.09e	0.64 ± 0.07d	1.21 ± 0.24cd	0.64 ± 0.10d
Mannose	**2.46 ± 0.18a**	**2.90 ± 0.29a**	**3.09 ± 0.18a**	**2.31 ± 0.70ab**	**3.48 ± 0.20a**	**2.31 ± 0.15a**
Cellobiose	0.82 ± 0.05d	0.86 ± 0.19d	0.87 ± 0.04e	0.92 ± 0.10cd	0.78 ± 0.07d	0.65 ± 0.11cd
Ribose	2.28 ± 0.06ab	2.34 ± 0.12ab	2.59 ± 0.10abc	2.42 ± 0.06ab	2.67 ± 0.17ab	2.26 ± 0.13ab
Rhamnose	2.10 ± 0.20ab	2.90 ± 0.09a	3.08 ± 0.10a	2.70 ± 0.22a	3.10 ± 0.20a	2.34 ± 0.45a
Galactose	2.27 ± 0.16ab	2.26 ± 0.22ab	2.60 ± 0.30abc	2.30 ± 0.16ab	2.77 ± 0.18ab	2.34 ± 0.22a
Arabinose	1.94 ± 0.10ab	1.90 ± 0.15bc	2.00 ± 0.14bcd	1.60 ± 0.18abcd	1.89 ± 0.18bc	1.62 ± 0.14abc
Xylose	1.07 ± 0.06cd	1.24 ± 0.05cd	1.35 ± 0.08de	1.26 ± 0.12bcd	1.40 ± 0.14cd	1.31 ± 0.24bcd
Sorbose	2.38 ± 0.29a	2.35 ± 0.09ab	2.50 ± 0.19abc	2.52 ± 0.10a	2.62 ± 0.08ab	2.18 ± 0.10ab
Mannitol	2.38 ± 0.10a	2.24 ± 0.10ab	2.68 ± 0.15ab	2.47 ± 0.10ab	2.49 ± 0.15ab	2.16 ± 0.14ab

Values sharing a common letter in the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

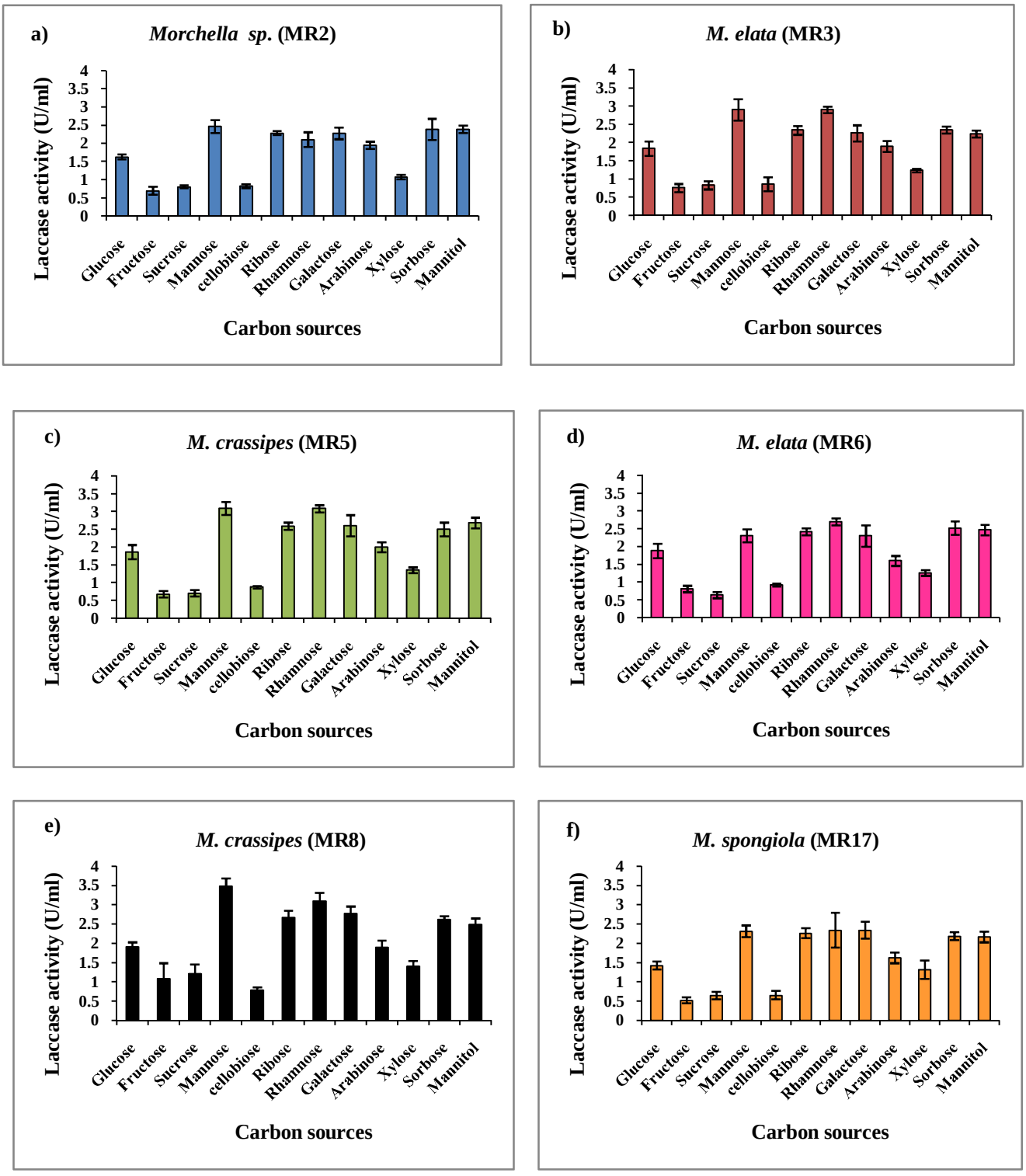


Fig 5.13: Effect of different carbon sources on laccase activity (U/ml) of different *Morchella* species a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). Values are Mean ± SEM (n=3)

5.4.3 LiP and MnP activities

Lignin peroxidase and manganese peroxidase activity were not detected with different carbon sources as no inducer was added in the media.

5.5 Growth and enzymatic studies with different nitrogen sources

Growth and enzyme production was studied in mineral salts broth having different nitrogen sources such as, ammonium nitrate (NH_4NO_3), sodium nitrite ($NaNO_2$), sodium nitrate ($NaNO_3$), peptone, yeast extract, casein and tryptone. *Morchella* spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17)) were inoculated into minera salts broth with glucose as the carbon source and different nitrogen sources. The flasks were incubated at 25 °C in dark for 7 days. After incubation and harvesting, biomass and ligninolytic enzyme activities were studied.

5.5.1 Biomass

All *Morchella* spp. were tested for their ability to utilize both organic and inorganic nitrogen sources such as ammonium nitrate (NH_4NO_3), sodium nitrite ($NaNO_2$), sodium nitrate ($NaNO_3$), peptone, yeast extract, casein and tryptone. All nitrogen sources were readily utilized by the *Morchella* spp (Table 5.14). *Morchella sp.* (MR2) and *M. spongiola* (MR17) produced significantly more biomass in yeast extract as the nitrogen source. Tryptone, peptone, $NaNO_3$ and casein were the other nitrogen sources that were utilized significantly well by *Morchella sp.* (MR2).

Morchella elata (MR3 and MR6) utilized tryptone, peptone and $NaNO_2$ more readily as compared to others. Significant differences in the biomass yield were observed in these nitrogen sources as compared to others. Yeast extract and NH_4NO_3 were found to act as

optimum sources of nitrogen for biomass production. Casein was found to act as poor source of nitrogen in *M. elata* (MR3 and MR6).

Morchella crassipes (MR5 and MR8) produced significantly more biomass in peptone and $NaNO_3$ as compared to other nitrogen sources. NH_4NO_3, $NaNO_2$, yeast extract and casein were the other nitrogen sources that were moderately utilized by *M. crassipes* (MR5 and MR8). Tryptone was observed to be the poorest source of nitrogen for growth studies.

Maximum biomass in *M. spongiola* (MR17) was observed in yeast extract. Casein, $NaNO_3$ and tryptone were found to be optimum sources of nitrogen for *M. spongiola* (MR17). Poorly utilized sources of nitrogen included $NaNO_2$ in *Morchella sp.* (MR2) and *M. spongiola* (MR17), Casein in *M. elata* (MR3), NH_4NO_3 in *M. elata* (MR6) and tryptone in *M. crassipes* (MR5 and MR8) (Table 5.14, Fig 5.14).

Table 5.14: Effect of different nitrogen sources on the mycelial growth (mg/50ml) of different *Morchella* spp.

Nitrogen sources	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
NH_4NO_3	18.3 ± 1.5b	22.0 ± 0.8a	24.5 ± 1.3ab	15.1 ± 1.3b	23.5 ± 4.2ab	21.0 ± 0.6b
$NaNO_2$	15.7 ± 2.6b	25.1 ± 1.1a	25.9 ± 2.0ab	**23.7 ± 1.7a**	24.4 ± 1.4ab	14.8 ± 1.7c
$NaNO_3$	34.0 ± 2.0a	15.1 ± 1.5b	**32.7 ± 2.7a**	14.7 ± 1.5b	**35.5 ± 3.9a**	35.3 ± 2.6ab
Peptone	35.1 ± 2.5a	24.0 ± 1.9a	**35.4 ± 6.0a**	15.0 ± 1.6b	**33.4 ± 11.0a**	28.0 ± 2.6b
Yeast extract	**54.1 ± 3.5a**	22.1 ± 0.9a	16.1 ± 0.4b	14.7 ± 0.7b	25.9 ± 3.0ab	**55.1 ± 2.0a**
Casein	29.8 ± 4.0ab	13.1 ± 0.9b	23.6 ± 1.9ab	18.0 ± 6.0b	22.6 ± 2.0ab	43.0 ± 1.0ab
Tryptone	41.8 ± 3.4a	**25.5 ± 1.5a**	15.5 ± 1.7b	21.0 ± 3.0a	15.3 ± 3.0b	29.5 ± 1.3b

Values sharing a common letter in the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

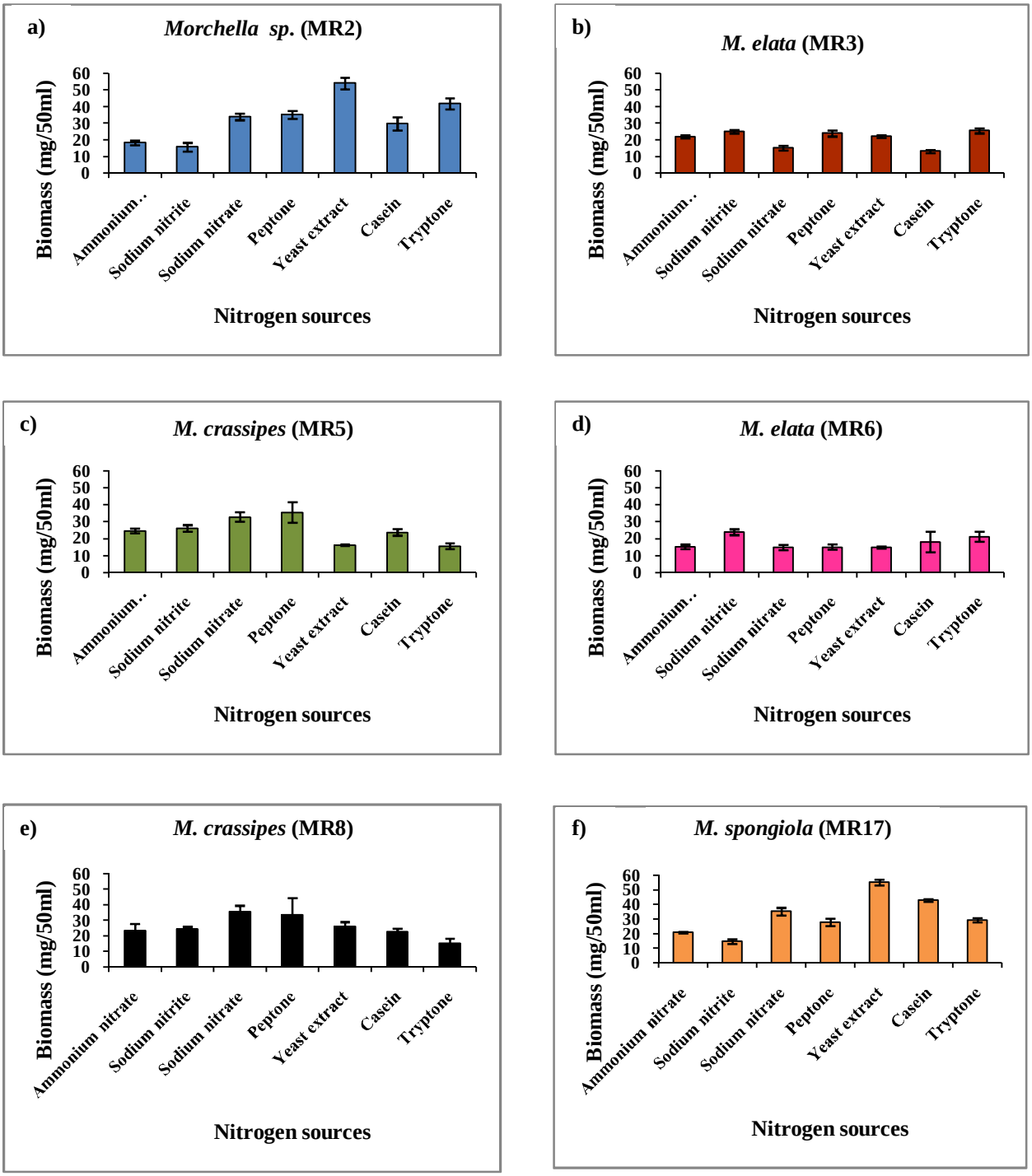


Fig 5.14: Effect of different nitrogen sources on the mycelial growth (mg/50ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). Values are Mean ± SEM (n=3)

5.5.2 Laccase

Among the different nitrogen sources, organic sources increased the laccase production as compared to the inorganic ones. Among these, peptone and casein exhibited significant influence on laccase production by all *Morchella* spp. (Table 5.15).

Maximum laccase activity was observed in peptone followed by casein in *M. crassipes* (MR8). In *Morchella sp.* (MR2), maximum laccase activity was observed in significant amounts in casein as the nitrogen source. However, other nitrogen sources such as peptone and yeast extract and NH_4NO_3, too, produced laccase to considerable amounts. In *M. elata* (MR3 and MR6), casein was observed to be the best nitrogen source which significantly produced high titers of laccase as compared to other nitrogen sources. Laccase production in organic nitrogen sources was almost twofold of that produced in inorganic nitrogen sources (Table 5.15, Fig 5.15).

Table 5.15: Effect of different nitrogen sources on laccase activity (U/ml) of different *Morchella* spp.

Nitrogen sources	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
NH_4NO_3	1.48 ± 0.2b	1.29 ± 0.1b	1.29 ± 0.3b	1.38 ± 0.1c	1.54 ± 0.3b	1.19 ± 0.2b
$NaNO_2$	1.39 ± 0.2b	1.17 ± 0.2b	1.00 ± 0.1c	1.19 ± 0.2c	1.47 ± 0.4b	1.15 ± 0.1b
$NaNO_3$	1.28 ± 0.3b	1.29 ± 0.1b	1.00 ± 0.1c	1.20 ± 0.2c	1.90 ± 0.2b	1.60 ± 0.3a
Peptone	**1.86 ± 0.2a**	1.29 ± 0.1b	**1.94 ± 0.1a**	1.77 ± 0.2b	**2.58 ± 0.3a**	1.58 ± 0.1a
Yeast extract	1.50 ± 0.2b	1.68 ± 0.1ab	1.37 ± 0.1b	1.57 ± 0.3b	1.35 ± 0.2b	1.66 ± 0.1a
Casein	2.23 ± 0.2a	**1.99 ± 0.1a**	1.86 ± 0.1a	**2.14 ± 0.1a**	2.43 ± 0.2a	**1.74 ± 0.2a**
Tryptone	1.38 ± 0.2b	1.50 ± 0.2ab	1.76 ± 0.1a	1.64 ± 0.1b	1.90 ± 0.2b	1.68 ± 0.2a

Values sharing a common letter in the column are not significant at P<0.05. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

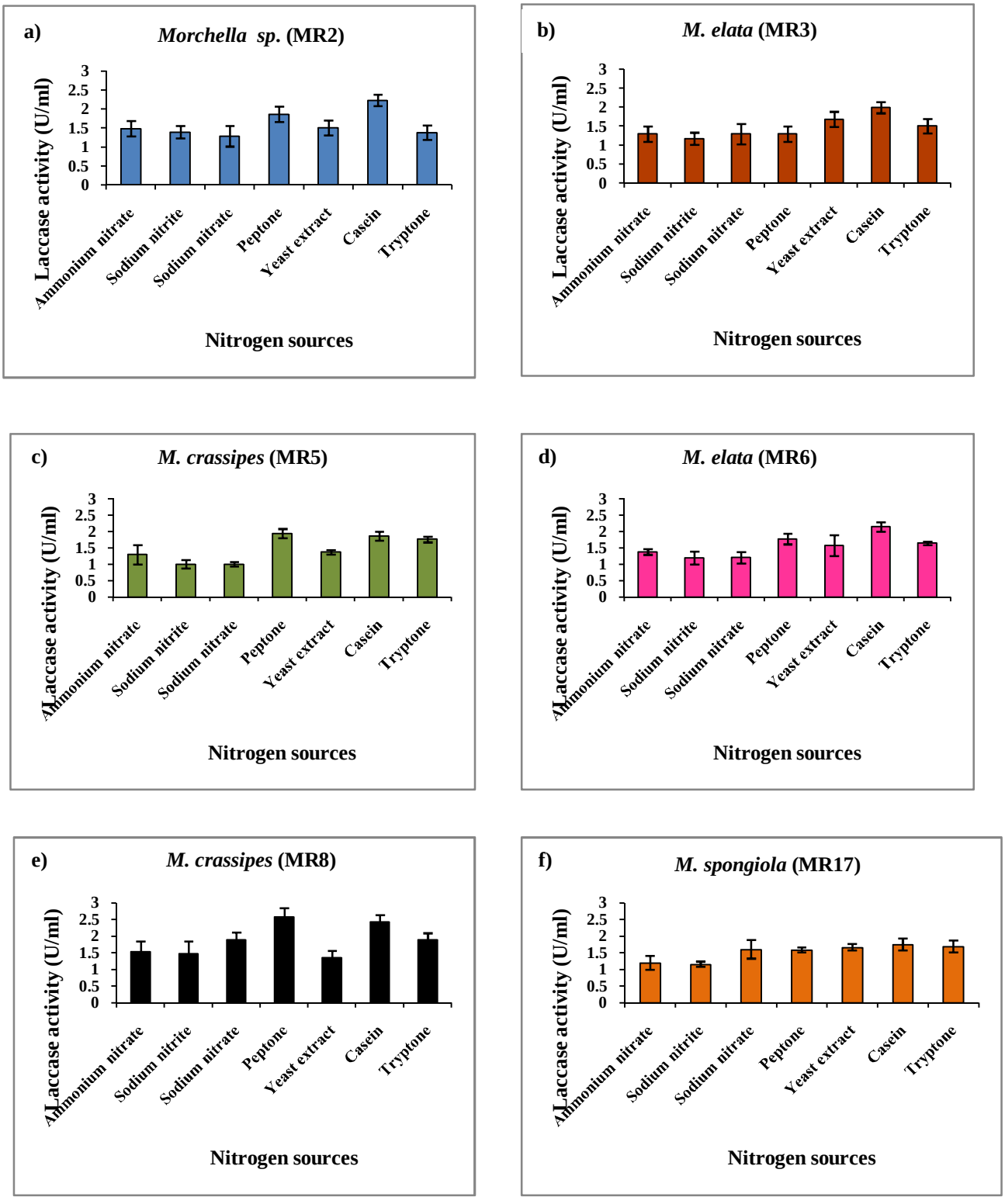


Fig 5.15: Effect of different nitrogen sources on laccase activity (U/ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). Values are Mean ± SEM (n=3)

The results of the effect of different carbon and nitrogen sources on the biomass and laccase showed that glucose served as the best carbon and peptone served as the best nitrogen source for high biomass production. Carbon sources as mannose and rhamnose and nitrogen sources as peptone and casein were found to be the best for maximum laccase production in *M. crassipes* (MR8).

5.6 Growth and enzyme activities with different ligninolytic inducers

Different ligninolytic inducers (chemical inducers such as DNBA (DiNitrobenzoic acid), DOPA (3,4-Dihydroxyphenylalanine), pyrogallol (PG), 8-Hydroxyquinoline (8-OHQ), p-Nitroaniline (p-NO2), ferulic acid (FA), veratryl alcohol (VA), phenol red (PR), resorcinol (RC) and 2,2 -azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) at a concentration of 1 mM as well as natural substrates such as finely crushed eucalyptus leaves (EL), leaf litter (LL), saw dust (SD), pine needles (PN), groundnut shell (GS), wheat straw (WS) and rice straw (RS) at a concentration of 1% were added to mineral salts broth (MSB) media containing glucose as carbon and sodium nitrate as nitrogen source for studying their effect on growth and ligninolytic enzymes. MSB media with culture and without any inducer served as the control. The jars were incubated at 25 °C for 7 days and after incubation the cultures were harvested and their biomass and ligninolytic enzyme activities were studied.

5.6.1 Biomass

Addition of various inducers to MSB containing *Morchella* cultures had significant effects on the growth of the mycelia (Table 5.16). Growth was reduced in most of the chemical inducers than natural substrates compared to controls. Maximum biomass was observed in *M. crassipes* (MR5) in veratrylalcohol as the substrate. *Morchella sp*. (MR2) and *M. spongiola*

(MR17) were found to be effected much by the inducers than other *Morchella* spp. used in the study.

Morchella sp. (MR2) produced maximum biomass in veratrylalcohol as compared to controls. Inducers such as ABTS, leaf litter, wheat straw, rice straw and groundnuts shell did not have any effect on biomass as compared to control group. Poorly utilized substrates were found to be resorcinol, pyrogallol, 8-OHQuinoline and p-nitroaniline where growth was retarded significantly as compared to control.

In *M. elata* (MR3 and MR6), maximum biomass was observed in veratrylalcohol, Phenol red, ABTS and DNBA followed by natural inducers as rice straw, wheat straw, groundnuts shell, leaf litter and pine needles. Significantly less biomass was observed in the chemical inducers such as 8-OHQuinoline, resorcinol, eucalyptus leaves and p-nitroaniline.

In *M. crassipes* (MR5 and MR8), maximum biomass was observed in veratrylalcohol and ABTS followed by wheat straw and rice straw. These inducers were found to have a positive effect on biomass yield. Inducers such as, DNBA, DOPA, phenol red, leaf litter and saw dust had a negative effect on biomass as growth in these substrates were found to be reduced to an extent. Less biomass was observed in resorcinol and eucalyptus leaves, where significantly least biomass was observed as compared to control.

In *M. spongiola* (MR17), all the inducers had a negative effect on growth. Among the inducers, maximum biomass was observed in veratrylalcohol, ABTS, phenol red, wheat straw and rice straw. Resorcinol and eucalyptus leaves served as the poor substrates for growth studies.

Natural inducers had a positive effect on biomass yield as compared to chemical inducers. Significant difference was observed among the biomass in chemical inducers as compared to natural inducers (Table 5.16, Fig 5.16).

Table 5.16: Effect of different inducers on the mycelia growth (mg/50ml) of different *Morchella* spp.

Inducers	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR5)	*M. elata* (MR6)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Control	**33.9 ± 2.0a**	15.1 ± 1.5cd	32.7 ± 2.7a	14.8 ± 1.5c	35.5 ± 3.8ab	**35.3 ± 2.6a**
DNBA	13.6 ± 1.3c	26.5 ± 2.0b	21.4 ± 2.0b	25.9 ± 2.0b	22.2 ± 2.0b	14.8 ± 1.0b
DOPA	22.1 ± 1.0ab	19.5 ± 3.0c	19.5 ± 3.0b	22.3 ± 1.2b	19.5 ± 4.0bc	14.2 ± 2.0b
Pyrogallol	4.8 ± 0.6d	12.0 ± 1.3d	12.9 ± 1.3bc	7.4 ± 0.5d	13.3 ± 6.0c	13.3 ± 1.8b
8-OHQuinoline	4.6 ± 1.3d	5.9 ± 1.0f	13.5 ± 2.0bc	4.3 ± 1.0e	13.4 ± 5.3c	8.4 ± 0.8cd
p-NO2-aniline	3.9 ± 0.6d	4.9 ± 1.0f	12.5 ± 1.2bc	3.2 ± 0.3e	13.2 ± 3.4c	12.2 ± 1.0b
Ferulic acid	18.8 ± 2.0b	12.9 ± 1.6d	18.2 ± 2.0b	8.4 ± 0.6d	15.8 ± 4.3bc	10.1 ± 1.0c
Veratrylalcohol	26.2 ± 2.0ab	**35.6 ± 2.3a**	35.7 ± 4.0a	33.8 ± 2.0a	34.4 ± 5.7ab	28.2 ± 1.3ab
Phenol red	17.0 ± 1.0b	27.4 ± 1.8b	19.4 ± 2.0b	22.9 ± 1.3b	24.7 ± 3.0b	24.6 ± 1.0ab
Resorcinol	3.1 ± 0.4d	5.8 ± 1.2f	9.5 ± 1.0c	5.7 ± 0.6de	19.0 ± 1.0bc	4.5 ± 1.0d
ABTS	26.2 ± 2.0ab	29.6 ± 2.6b	28.4 ± 3.0b	32.5 ± 1.0a	29.9 ± 4.9ab	25.2 ± 2.7ab
Eucalyptusleaves	11.3 ± 2.0c	7.1 ± 1.5e	8.4 ± 1.5c	6.0 ± 1.5de	12.2 ± 3.0c	6.6 ± 0.7d
Leaf litter	27.0 ± 2.0ab	28.8 ± 3.0b	20.4 ± 1.0b	25.8 ± 1.9b	25.1 ± 4.6b	14.7 ± 1.2b
Saw dust	12.2 ± 1.5c	17.0 ± 1.0cd	13.7 ± 1.5bc	15.0 ± 1.3c	23.0 ± 3.5b	10.5 ± 2.0c
Pine needles	16.8 ± 3.0b	23.4 ± 1.5bc	35.6 ± 6.0a	22.7 ± 1.0b	37.6 ± 4.5ab	13.2 ± 1.5b
Groundnutshell	21.3 ± 3.0ab	29.6 ± 1.7b	24.2 ± 3.0b	24.5 ± 1.0b	42.8 ± 7.2a	19.4 ± 3.0b
Wheat straw	24.6 ± 2.5ab	29.0 ± 0.8b	**39.8 ± 3.0a**	**31.0 ± 3.4a**	45.0 ± 6.0a	23.4 ± 1.8ab
Rice straw	21.7 ± 1.0ab	29.6 ± 1.8	34.6 ± 2.4a	30.0 ± 1.8a	**44.3 ± 9.2a**	21.0 ± 2.0ab

Values sharing a common letter in the column are not significant at P<0.05. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

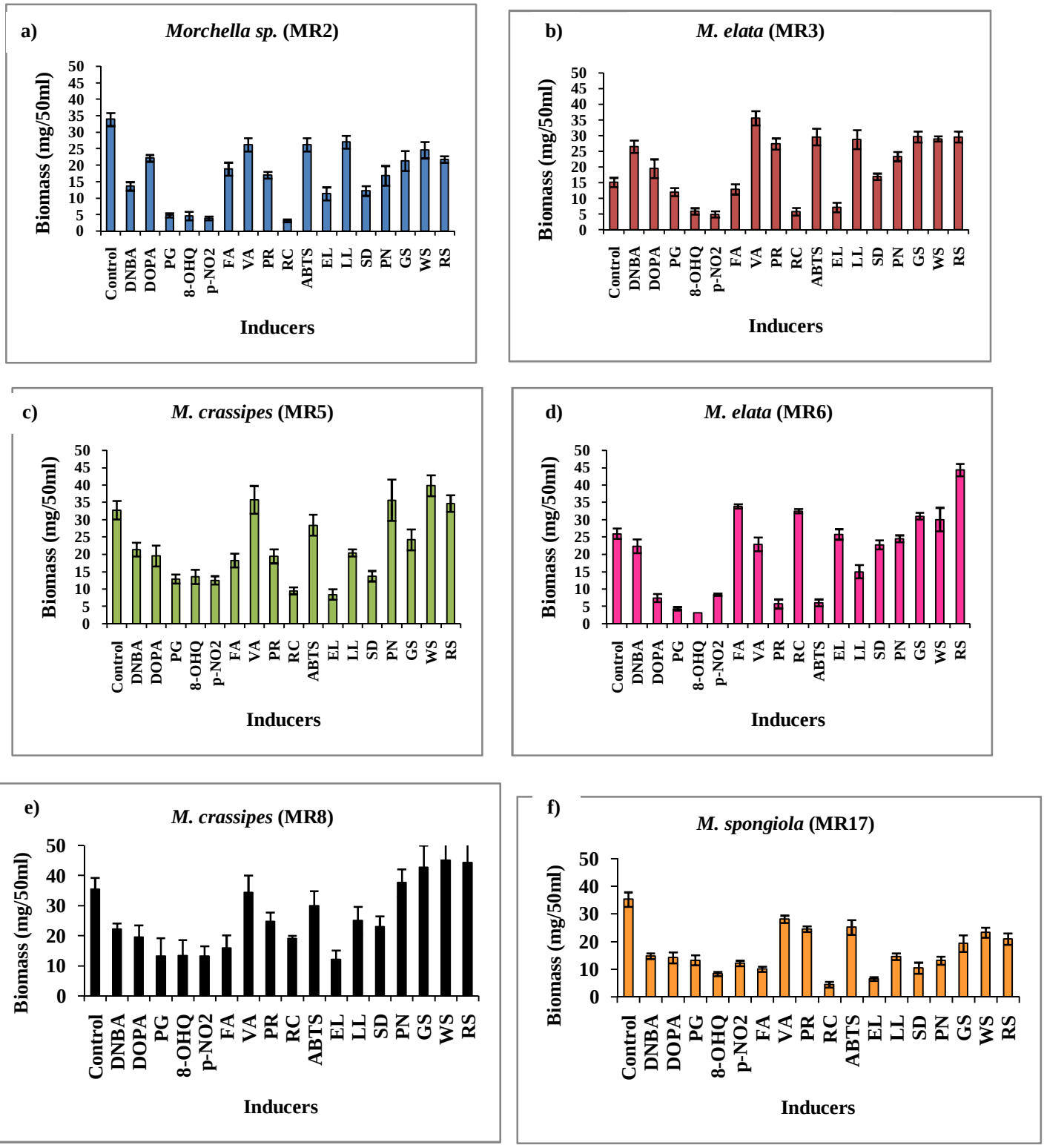


Fig 5.16: Effect of inducers on biomass (mg/50ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). DNBA, DiNitrobenzoic acid; DOPA, 3,4-Dihydroxyphenylalanine; PG, pyrogallol; 8-OHQ, 8-Hydroxyquinoline; p-NO2, p-Nitroaniline; FA, ferulic acid; VA, veratryl alcohol; PR, phenol red; RC, resorcinol; ABTS, 2,2 -azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid); EL, eucalyptus leaves; LL, leaf litter; SD, sawdust; PN, pine needles, GS, groundnut shell; WS, wheat straw; RS, rice straw. Values are Mean ± SEM (n=3).

5.6.2 Laccase activity

Different chemical and natural inducers were capable of enhancing the laccase activity compared to controls without inducers. Among the natural inducers, rice straw, groundnut shell, wheat straw and pine needles appeared to be the best sources for laccase production Table (5.17). Among the chemical inducers, ABTS was the most efficient inducer for laccase production followed by veratrylalcohol by all *Morchella* spp. Other inducers such as DNBA, DOPA, 8-OHQuinoline, p-nitroaniline, ferulic acid, resorcinol, and eucalyptus leaves failed to induce the laccase activity.

Maximum laccase activity was observed in *M. crassipes* (MR8 and MR5) in rice straw and ABTS as substrates. Significantly high laccase activity was observed in these inducers as compared to controls. Wheat straw, groundnuts shell, pine needles and veratrylalcohol, too served as good inducers for laccase production in *M. crassipes* (MR5 and MR8).

In *Morchella sp.* (MR2), maximum laccase activity was detected in ABTS and veratrylalcohol. Optimum inducers for laccase activity included wheat straw, pine needles, and groundnuts shell. *Morchella spongiola* (MR17) showed least laccase actiivty as compared to other cultures. Maximum laccase activity was observed in ABTS, and veratrylalcohol, but the activity produced was significantly less as compared to *M. elata* (MR3 and MR6) and *M. crassipes* (MR5 and MR8).

In *M. elata* (MR3 and MR6), maximum laccase production was observed in rice straw and ABTS as the inducers. High titers of laccase production was observed in wheat straw, veratrylalcohol, pine needles and leaf litter. Significantly less amounts of laccase activity was detected in resorcinol and 8-OHQuinoline as the inducers. Significant differences were observed among the laccase production in these inducers as compared to controls without inducers (Table 5.17 and Fig 5.17).

Table 5.17: Effect of inducers on laccase activity of different *Morchella* spp.

Inducers	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR5)	*M. elata* (MR6)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Control	1.62 ± 0.1b	1.84 ± 0.2b	1.86 ± 0.2cd	1.88 ± 0.1c	1.91 ± 0.1d	1.42 ± 0.1ab
DNBA	ND	ND	ND	ND	ND	ND
DOPA	ND	ND	0.44 ± 0.0d	ND	0.93 ± 0.0e	0.15 ± 0.1bc
Pyrogallol	0.44 ± 0.2d	0.81 ± 0.1c	3.65 ± 0.4bc	0.89 ± 0.2d	5.36 ± 0.9c	0.64 ± 0.1b
8-OHQuinoline	ND	0.39 ± 0.1c	0.20 ± 0.1d	0.14 ± 0.1de	0.27 ± 0.1e	0.07 ± 0.0c
p-NO2-aniline	ND	0.10 ± 0.1c	2.35 ± 0.2c	0.03 ± 0.0e	2.15 ± 0.4d	ND
Ferulic acid	ND	ND	0.16 ± 0.1d	ND	0.27 ± 0.1e	ND
Veratrylalcohol	1.74 ± 0.2b	4.30 ± 0.6ab	6.16 ± 0.5b	4.70 ± 2.0b	6.57 ± 0.8c	**2.60 ± 0.2a**
Phenol red	1.13 ± 0.1b	1.60 ± 0.4b	2.53 ± 0.2c	1.90 ± 0.4c	1.80 ± 0.3d	0.14 ± 0.0bc
Resorcinol	ND	0.24 ± 0.1c	1.63 ± 0.2cd	0.13 ± 0.1de	1.90 ± 0.4d	ND
ABTS	**2.29 ± 0.1a**	9.80 ± 0.4a	11.30 ± 0.6a	9.00 ± 0.2a	11.62 ± 0.9a	3.33 ± 0.2a
Eucalyptusleaves	ND	ND	ND	ND	ND	ND
Leaf litter	0.15 ± 0.0d	2.35 ± 0.3b	1.69 ± 0.2cd	1.68 ± 0.1c	2.15 ±0.2d	1.25 ± 0.3ab
Saw dust	0.09 ± 0.0e	ND	2.14 ± 0.7c	ND	3.12 ± 0.5d	ND
Pine needles	0.89 ± 0.1c	2.66 ± 0.2b	4.80 ± 0.7bc	2.60 ± 0.3bc	5.41 ± 1.4c	0.67 ± 0.1b
Groundnutshell	0.76 ± 0.1c	5.00 ± 0.6ab	9.30 ± 0.3ab	5.40 ± 1.0b	8.52 ± 1.0b	1.00 ± 0.1ab
Wheat straw	0.86 ± 0.0c	4.00 ± 0.7ab	5.80 ± 0.9b	3.28 ± 0.3bc	6.52 ± 0.7c	1.24 ± 0.3ab
Rice straw	1.00 ± 0.1b	**9.60 ± 0.2a**	**12.20 ± 0.8a**	**10.36 ± 0.6a**	**12.64 ± 0.3a**	1.00 ± 0.1ab

Values sharing a common letter in the column are not significant at P<0.05. Values are Mean ± SEM (n=3) * ND = Not Detected. Values in bold indicate significant ones.

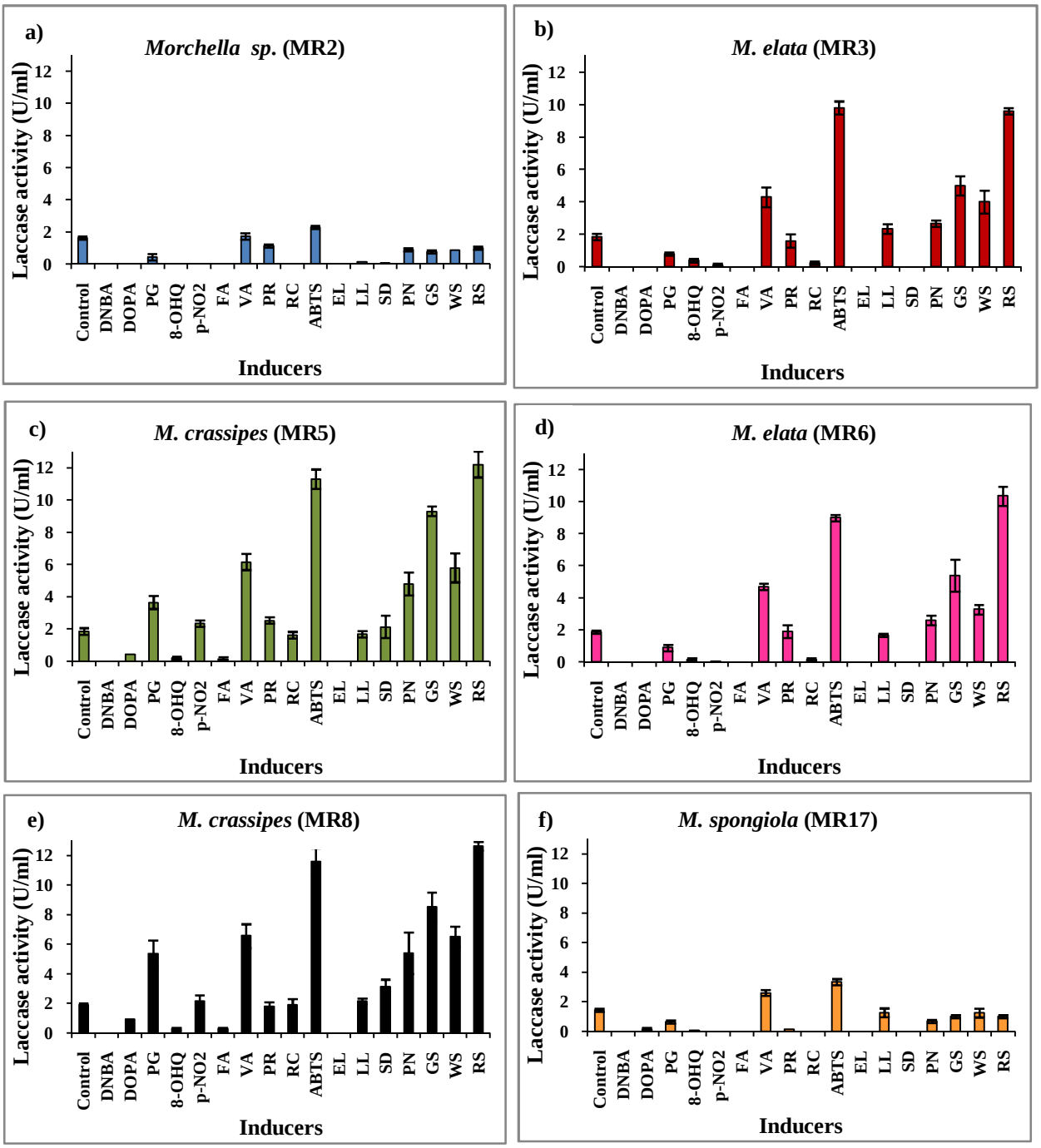


Fig 5.17: Effect of inducers on laccase activity of different *Morchella* spp. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). DNBA, DiNitrobenzoic acid; DOPA, 3,4-Dihydroxyphenylalanine; PG, pyrogallol; 8-OHQ, 8-Hydroxyquinoline; p-NO2, p-Nitroaniline; FA, ferulic acid; VA, veratryl alcohol; PR, phenol red; RC, resorcinol; ABTS, 2,2 -azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid); EL, eucalyptus leaves; LL, leaf litter; SD, sawdust; PN, pine needles, GS, groundnut shell; WS, wheat straw; RS, rice straw. Values are Mean ± SEM (n=3).

5.6.3 Manganese peroxidase activity

Manganese peroxidase activity was produced in traces in response to some of the inducers used in the study. Inducers such as ferulic acid, veratrylalcohol and rice straw were found to be positive inducers for manganese peroxidase activity in different *Morchella* spp., whereas no manganese peroxidase activity was observed in DNBA, DOPA, pyrogallol, 8-OHQuinoline, p-nitroaniline, resorcinol, eucalyptus leaves, leaf litter, and saw dust (Table 5.18).

Maximum manganese peroxidase activity was produced in presence of phenol red followed by rice straw in *M. elata* (MR6). Maximum activity was observed in ABTS as the inducer in *Morchella sp.* (MR2), followed by phenol red. No significant differences were found in manganese peroxidase activity among the inducers as ferulic acid, veratrylalcohol, groundnuts shell, wheat straw and rice straw in *Morchella sp.* (MR2).

Significantly high manganese peroxidase activity was observed in phenol red and rice straw as inducers in *M. elata* (MR3 and MR6) and *M. crassipes* (MR5 and MR8). Inducers such as, wheat straw, pine needles, groundnuts shell and ABTS acted as good manganese peroxidase inducers in *M. crassipes* (MR5 and MR8). Moderate amounts of manganese peroxidase activity was observed in ABTS, ferulic acid, veratrylalcohol, wheat straw and groundnuts shell as the inducers in *M. elata* (MR3 and MR6).

In *M. spongiola* (MR17), maximum manganese peroxidase activity was observed in wheat straw as the inducer. Rice straw, phenol red and pine needles produced moderate amounts of manganese peroxidase activity in *M. spongiola* (MR17). Inducers such as pyrogallol, 8-OHQuinoline, p-Nitroaniline, resorcinol, eucalyptus leaves, leaf litter and saw dust failed to induce manganese peroxidase activity. Manganese peroxidase activity was found to vary with the different inducers along with different *Morchella* spp. (Tab 5.18, Fig 5.18).

Table 5.18: Effect of different inducers on manganese peroxidase activity (U/L) of different *Morchella* spp.

Inducers	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR5)**	***M. elata* (MR6)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
DNBA	ND	ND	ND	ND	ND	ND
DOPA	ND	ND	ND	ND	ND	ND
Pyrogallol	ND	ND	ND	ND	ND	ND
8-OHQuinoline	ND	ND	ND	ND	ND	ND
p-NO2-aniline	ND	ND	ND	ND	ND	ND
Ferulic acid	3.8 ± 0.6b	6.2 ± 0.9c	7.0 ± 0.5c	4.0 ± 1.3b	8.5 ± 1.0d	1.3 ± 0.3b
Veratrylalcohol	3.9 ± 0.6b	5.3 ± 0.3cd	4.7 ± 0.3d	5.4 ± 0.6b	5.3 ± 1.1e	2.1 ± 0.2b
Phenol red	8.9 ± 1.0a	12.7 ± 0.5b	13.8 ± 0.9a	12.9 ± 1.2a	15.0 ± 1.1a	5.2 ± 0.8b
Resorcinol	ND	ND	ND	ND	ND	ND
ABTS	**9.9 ± 0.6a**	7.9 ± 0.7c	9.2 ± 0.6b	6.7 ± 0.9b	11.0 ± 1.1bc	1.5 ± 0.8b
Eucalyptusleaves	ND	ND	ND	ND	ND	ND
Leaf litter	ND	ND	ND	ND	ND	ND
Saw dust	ND	ND	ND	ND	ND	ND
Pine needles	1.9 ± 0.5c	3.6 ± 0.6d	10.1 ± 0.7b	6.0 ± 0.8b	10.8 ± 1.7bc	3.8 ± 0.4b
Groundnutshell	3.5 ± 0.1b	5.9 ± 0.3c	10.0 ± 0.3b	5.2 ± 0.9b	10.1 ± 0.5c	2.5 ± 0.1b
Wheat straw	4.2 ± 0.4b	4.7 ± 0.9cd	11.6 ± 0.3ab	5.6 ± 1.0b	12.2 ± 0.9b	**10.7 ± 0.7a**
Rice straw	3.6 ± 0.2b	**16.0 ± 1.0a**	**14.8 ± 0.6a**	**16.5 ± 1.6a**	**14.3 ±1.9a**	5.2 ± 0.2b

Values sharing a common letter in the column are not significant at P<0.05. Values are Mean ± SEM (n=3) * ND = Not Detected. Values in bold indicate significant ones.

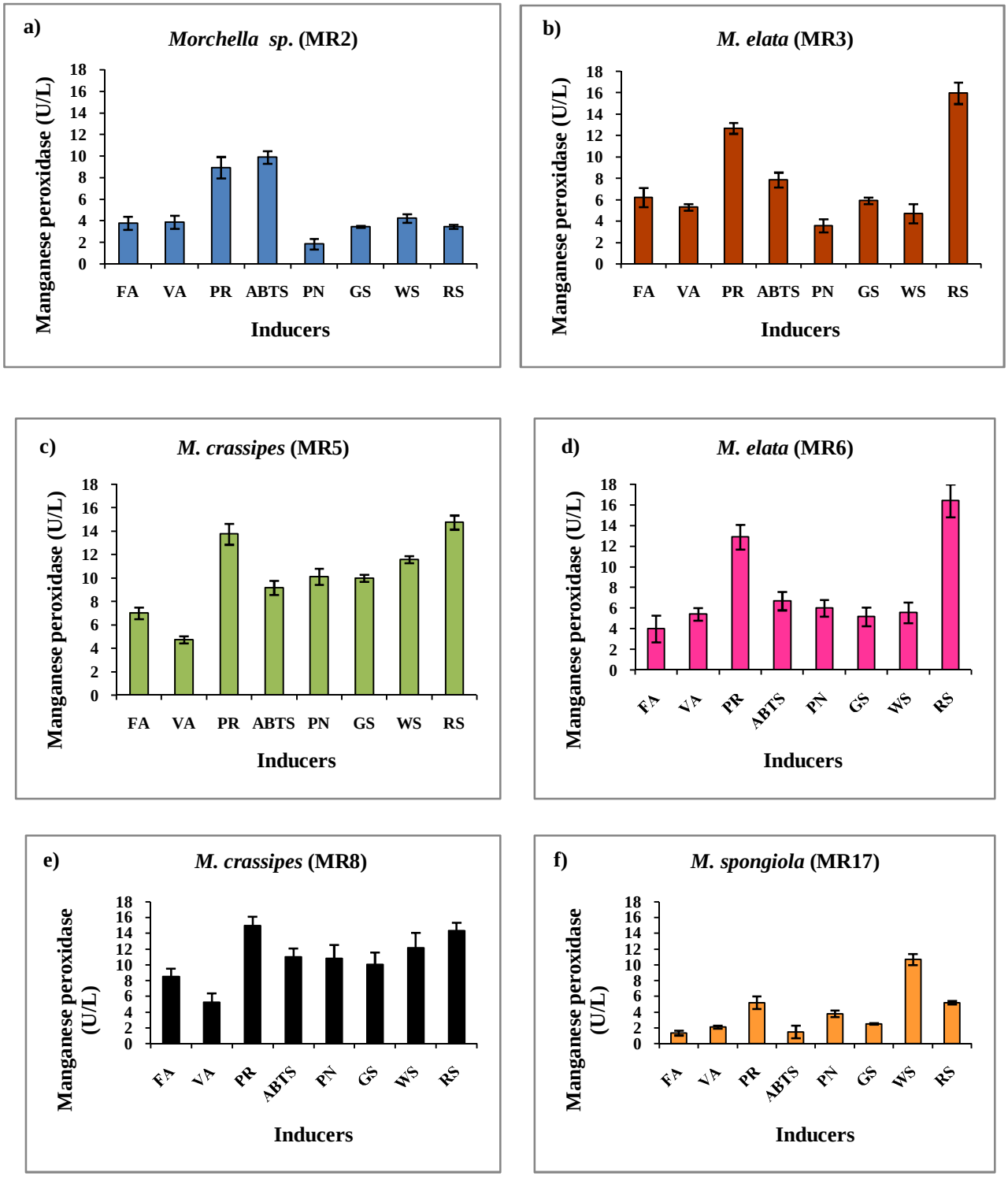


Fig 5.18: Effect of different inducers on Manganese peroxidase activity of different M*orchella* spp. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). DNBA, DiNitrobenzoic acid; DOPA, 3,4-Dihydroxyphenylalanine; PG, pyrogallol; 8-OHQ, 8-Hydroxyquinoline; p-NO2, p-Nitroaniline; FA, ferulic acid; VA, veratryl alcohol; PR, phenol red; RC, resorcinol; ABTS, 2,2 -azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid); EL, eucalyptus leaves; LL, leaf litter; SD, sawdust; PN, pine needles, GS, groundnut shell; WS, wheat straw; RS, rice straw.Values are Mean ± SEM (n=3).

5.6.4 Lignin peroxidase activity

Lignin peroxidase was induced to small amounts in response to various inducers used in this study. Lignin peroxidase activity was produced in considerable amounts in presence of veratrylalcohol, pyrogallol, ABTS, 8-OHQuinoline, rice straw, groundnuts shell, and wheat straw (Table 5.19). No lignin peroxidase activity was detected in DNBA, DOPA, p-nitroaniline, ferulic acid, phenol red, resorcinol, eucalyptus leaves, and leaf litter. Maximum lignin peroxidase activity was observed in rice straw in *M. crassipes* (MR8).

Significantly high lignin peroxidase activity was also detected in rice straw, veratrylalcohol and wheat straw as the inducers in *M. crassipes* (MR8 and MR5). Other inducers such as, pyrogallol, 8-OHQuinoline, ABTS, pine needles and groundnuts shell, too produced considerable levels of Lignin peroxidase activity. Saw dust, an inducer that is widely used for inducing high lignin peroxidase activity produced only small amounts of lignin peroxidase. In *Morchella sp.* (MR2), maximum lignin peroxidase activity was observed in wheat straw, rice straw, groundnuts shell, ABTS and veratrylalcohol. Rice straw served as efficient MnP inducer for *M. elata* (MR3) and veratrylalcohol was found to be the best inducer for *M. elata* (MR6). Optimum inducers for MnP production in *M. elata* (MR3) included veratrylalcohol, ABTS, and wheat straw. In *M. spongiola* (MR17), maximum lignin peroxidase activity was observed in wheat straw and rice straw, followed by veratrylalcohol and groundnuts shell. *Morchella crassipes* (MR5 and MR8) were observed to produce more lignin peroxidase activity than the other cultures. *Morchella spongiola* (MR17) and *Morchella sp.* (MR2) were found to produce significantly least lignin peroxidase activity (Table 5.19, Fig 5.19).

The results showed that inducers such as rice straw, wheat straw, veratrylalcohol and ABTS had a positive effect on biomass yield, laccase, lignin peroxidase, and manganese peroxidase activities in all the *Morchella* spp. *Morchella crassipes* (MR5 and MR8) was

found to be the best for high biomass yield, laccase, lignin peroxidase, and manganese peroxidase activities with rice straw and wheat starw as the best inducer.

Table 5.19: Effect of different inducers on lignin peroxidase activity of different *Morchella* spp.

Inducers	***Morchella sp.*** **(MR2)**	***M. elata*** **(MR3)**	***M. crassipes*** **(MR5)**	***M. elata*** **(MR6)**	***M. crassipes*** **(MR8)**	***M. spongiola*** **(MR17)**
DNBA	ND	ND	ND	ND	ND	ND
DOPA	ND	ND	ND	ND	ND	ND
Pyrogallol	3.2 ± 0.5c	1.5 ± 0.5c	11.0 ± 1.6b	5.7 ± 0.8c	9.9 ± 2.0b	3.2 ± 0.6d
8-OHQuinoline	ND	8.9 ± 1.4b	8.5 ± 0.5c	10.0 ± 1.0ab	8.7 ± 3.2b	8.0 ± 0.3bc
p-NO2-aniline	ND	ND	ND	ND	ND	ND
Ferulic acid	ND	ND	ND	ND	ND	ND
Veratrylalcohol	6.2 ± 0.5b	9.8 ± 0.9b	**18.2 ± 3.2a**	**14.2 ± 0.8a**	**20.6 ± 8.0a**	11.3 ± 1.2a
Phenol red	ND	ND	ND	ND	ND	ND
Resorcinol	ND	ND	ND	ND	ND	ND
ABTS	7.0 ± 1.0b	9.6 ± 0.9b	7.3 ± 0.6c	8.3 ± 1.2b	8.2 ± 2.5bc	6.4 ± 1.5c
Eucalyptusleaves	ND	ND	ND	ND	ND	ND
Leaf litter	ND	ND	ND	ND	ND	ND
Saw dust	ND	0.8 ± 0.5d	1.4 ± 0.4d	ND	5.3 ± 1.7bc	ND
Pine needles	3.9 ± 1.5c	7.6 ± 0.7b	8.6 ± 1.0c	2.7 ± 0.9d	6.8 ± 1.8bc	1.4 ± 1.0D
Groundnutshell	8.0 ± 0.9b	6.8 ± 0.9b	6.4 ± 1.4c	11.0 ± 1.3ab	8.5 ± 1.0bc	10.0 ± 0.6a
Wheat straw	**10.6 ± 0.7a**	7.0 ± 0.7b	9.8 ± 0.7c	11.2 ± 0.8ab	16.9 ± 2.6a	**14.3 ± 0.8a**
Rice straw	**9.7 ± 2.5a**	**16.5 ± 1.5a**	**19.4 ± 2.0a**	**13.7 ± 1.2a**	**22.0 ± 5.3a**	**13.9 ± 2.7a**

Values sharing a common letter in the column are not significant at P<0.05. Values are Mean ± SEM**.** ND denotes Not Detected. Values in bold indicate significant ones.

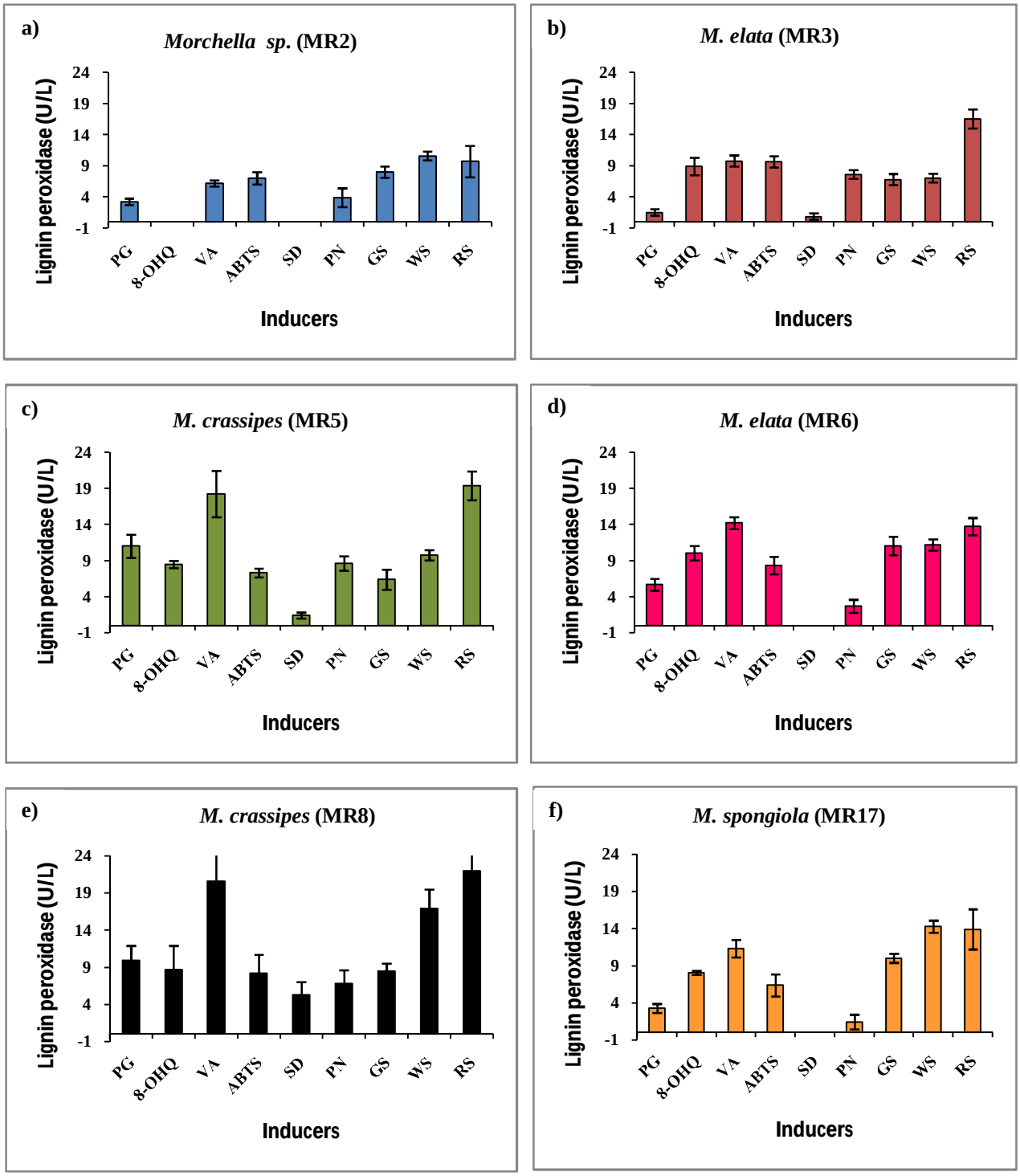


Fig 5.19: Effect of different inducers on lignin peroxidase activity of different *Morchella* spp. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR5) d) *M. elata* (MR6) e) *M. crassipes* (MR8) f) *M. spongiola* (MR17). DNBA, DiNitrobenzoic acid; DOPA, 3,4-Dihydroxyphenylalanine; PG, pyrogallol; 8-OHQ, 8-Hydroxyquinoline; p-NO2, p-Nitroaniline; FA, ferulic acid; VA, veratryl alcohol; PR, phenol red; RC, resorcinol; ABTS, 2,2 -azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid); EL, eucalyptus leaves; LL, leaf litter; SD, sawdust; PN, pine needles, GS, groundnut shell; WS, wheat straw; RS, rice straw. Values are Mean ± SEM (n=3).

Chapter VI

Sclerotial studies of Morchella spp.

6.0 Sclerotial Studies in different media and natural substrates

Sclerotium is a hard surfaced resting body of fungal cells that acts as a nutrient reservoir during unfavorable environmental conditions. *Morchella* species (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR8) and *M. spongiola* (MR17)) were grown in different synthetic media and natural substrates so as to study their effect on sclerotia formation.

6.1 Sclerotia formation in different media

Morchella spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR8) and *M. spongiola* (MR17)) were inoculated in Malt Extract (ME), Modified Melin Norkrans Medium (MMN), Czapek Dox (CZD), Yeast Maltose Glucose (YMG), Sabourd's medium (SB), Basal medium (BS), Minimal Mineral medium (MMM), Potato Dextrose broth (PDB), Mineral Salts broth (MSB), morel growth broth (MGB) and pikovskaya's (PKV) medium. Cultures were incubated at 25 °C in dark for 15 days and after 15 days of mycelial growth, petriplates with cultures were placed at 4 °C in dark. None of the media tested supported sclerotia formation, except malt extract, where small, rounded sclerotia were observed in petriplates in *M. elata* (MR3) (Fig 6.1 a and c) and *M. crassipes* (MR8) (Fig 6.1 b and d) after 15 days. Sclerotia were formed only in *M. crassipes* (MR8) and *M. elata* (MR3). Sclerotia were not observed in *M. spongiola* (MR17) and *Morchella sp*. (MR2).

6.2 Sclerotia formation in split plates

Morchella spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR8) and *M. spongiola* (MR17)) were inoculated on split plates containing both nutrient rich and poor media (Buscot medium A and B; and nutrient rich medium-A and nutrient poor medium-B) and incubated at 25 °C in dark. The mycelium was inoculated in the centre of smaller petriplate containing

medium A and afer the growth of mycelium in medium A, the mycelium grew towards the other medium B. the plates were incubated at 25 °C in dark and observed daily for sclerotia formation.

Light brown colored dense mycelium was observed in split plates of *Morchella sp.* (MR2), containing Buscot A medium and Buscot B medium; and Nutrient rich A and nutrient poor B media. Mycelium was more concentrated towards Buscot B medium as compared to Buscot A medium. Mycelial growth was retarded as the growing mycelia in Buscot B reached Buscot A layer (Fig 6.2a). The same result was observed in nutrient rich A and nutrient poor B media, where, growth of mycelium stopped on reaching nutrient poor medium B (Fig 6.2b). No sclerotia were seen in any of the media tested

In *M. elata* (MR3), brown colored mycelium was observed in both Buscot A and Buscot B media. Growth was found to be even in both the media. Profusely branched aerial hyphae were present in both the medium. Small rounded sclerotial initials were observed in Buscot B (Fig 6.2c). In nutrient media, cream colored mycelium was observed in Nutrient rich A, whereas no growth was observed in Nutrient B (Fig 6.2d). No sclerotial initials were observed in nutrient rich or poor medium.

In *M. crassipes* (MR8), light brown colored mycelium was observed in both Buscot A and Buscot B media (Fig 6.2e); whereas brown colored mycelium was observed in Nutrient A medium (Fig 6.2f). No growth was observed in Nutrient B medium. Profusely, highly branched hyphae in both media were observed. No sclerotial initials were observed in any of the medium tested.

In *M. spongiola* (MR17), more mycelial growth was observed in Buscot B as compared to Buscot A medium. Mycelium, initially creamish in color, changed to brownish in color (Fig 6.2g). Creamish mycelium was observed in Nutrient A and Nutrient B medium (Fig 6.2h). None of the media tested supported sclerotia formation.

Sclerotia were formed in Buscot A, Buscot B and nutrient A media. Sclerotia formed were small, and white colored, in appearance. On maturity, white color of sclerotia was changed to brown color.

Microscopic examination of the main body of sclerotia revealed cells to be swollen and reduced in length when compared to hyphae. Those hyphae at the perimeter of the sclerotial structure were swollen and densely packed together. The "fluffy" appearance of sclerotia appeared to be caused by longer hyphae extending from the sclerotium surface Sclerotia formed were connected with the other sclerotia through the network of fungal hyphae. (Fig 6.1 c and d).

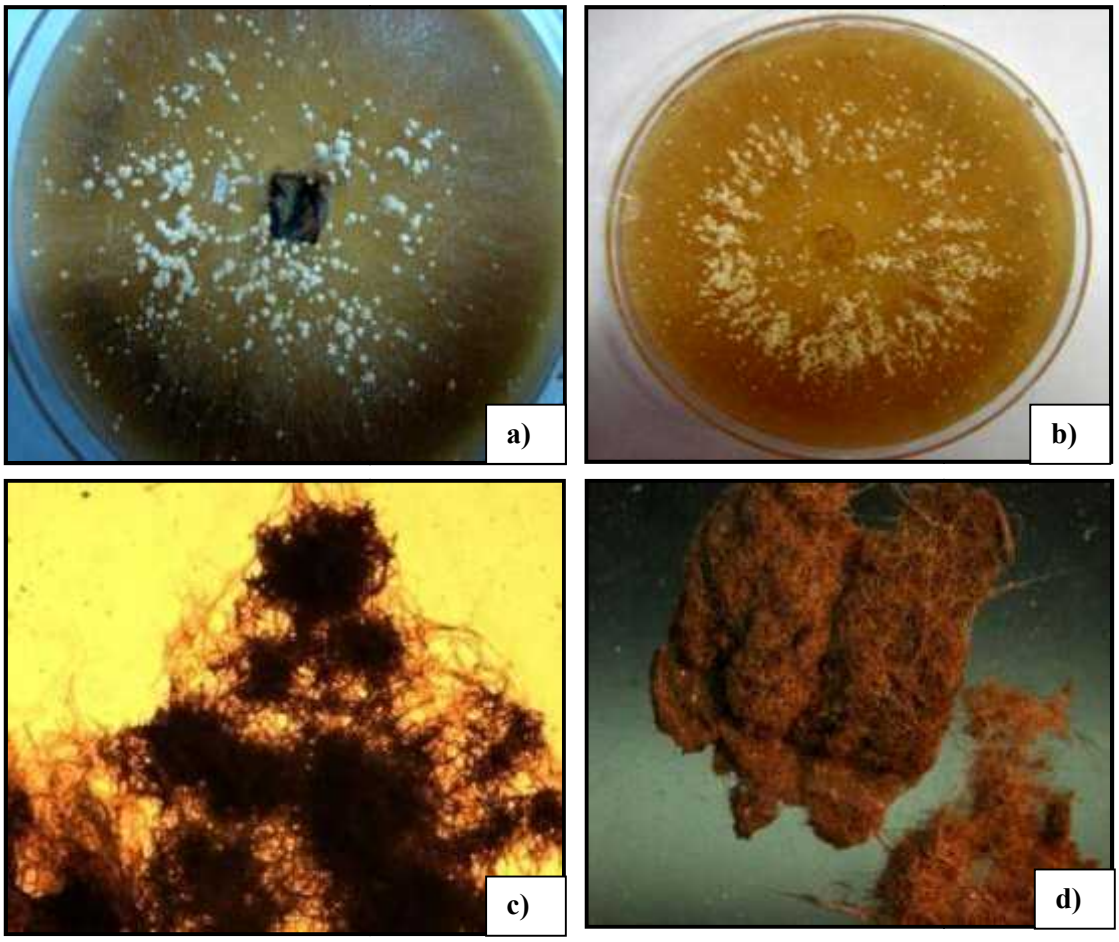


Fig 6.1a-d: Sclerotia of *Morchella* spp. in ME media a) *M. elata* (MR3) in petriplate b) *M. crassipes* (MR8) in petriplate c) *M. elata* (MR3) in petriplate at 4 °C (4X) d) Microscopic slides of *M. elata* (MR3) and d) Microscopic slides of *M. crassipes* (MR8) (4X)

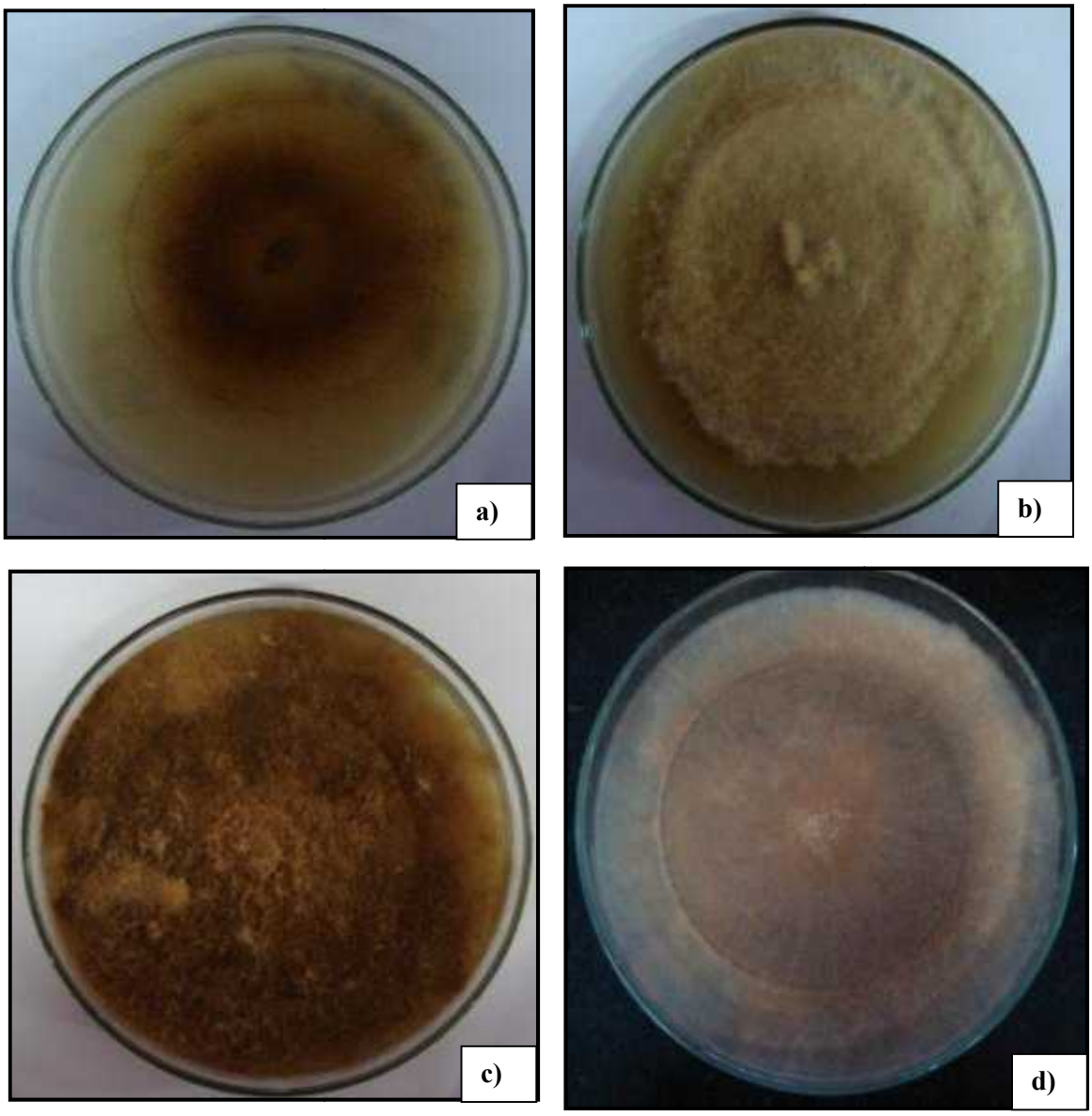


Fig 6.2a-d: *Morchella* cultures grown on split plates with different media a) *Morchella sp.* (MR2) on Buscot A and Buscot B media b) *Morchella sp.* (MR2) on Nutrient A and Nutrient B c) *M. elata* (MR3) on Buscot A and Buscot B media and d) *M. elata* (MR3) on Nutrient A and Nutrient B media

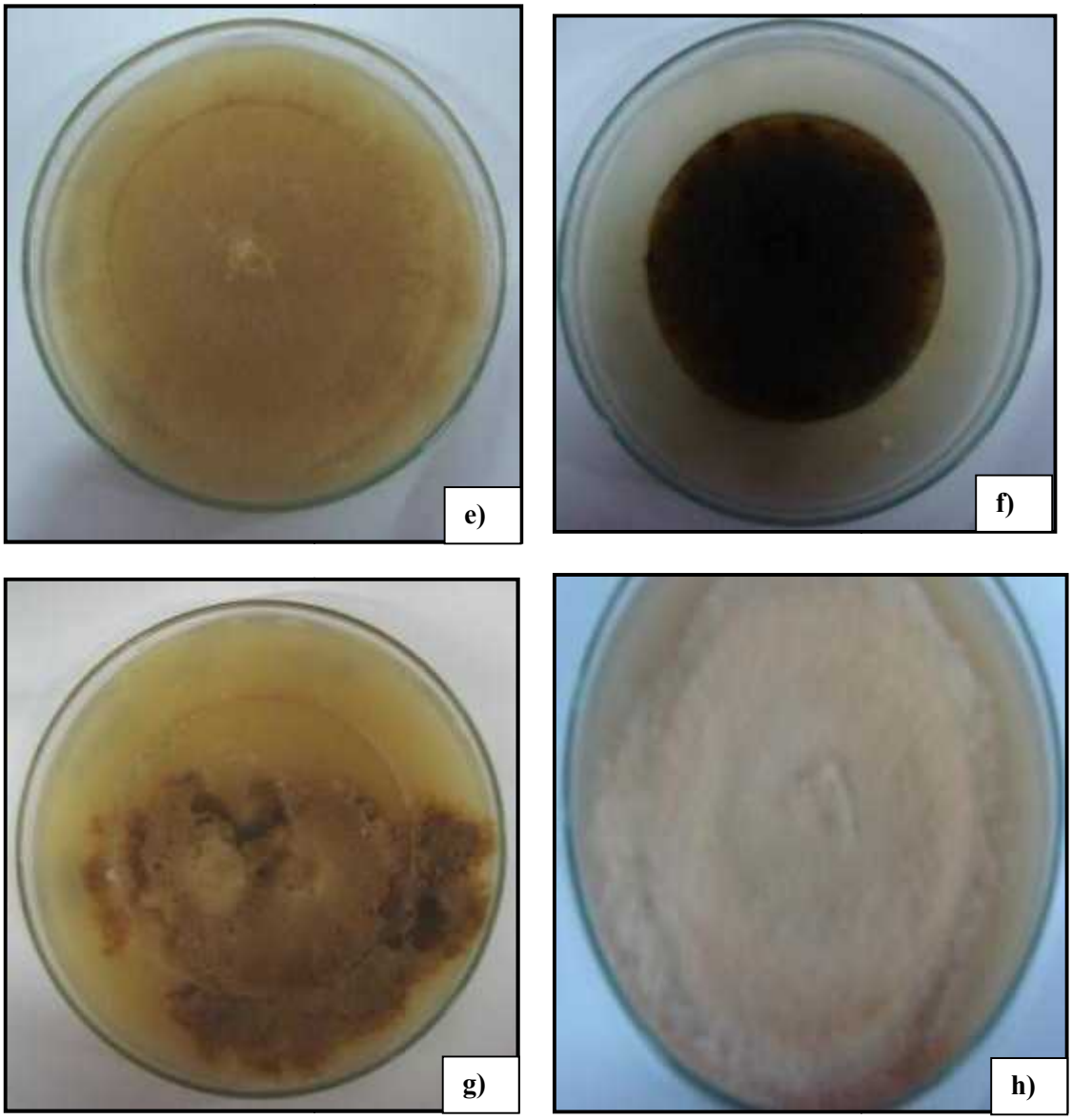


Fig 6.2e-h: *Morchella* cultures grown on split plates with different media e) *M. crassipes* (MR8) on Buscot A and Buscot B media f) *M. crassipes* (MR8) on Nutrient A and Nutrient B g) *M. spongiola* (MR17) on Buscot A and Buscot B and h) *M. spongiola* (MR17) on Nutrient A and Nutrient B media

6.3 Dual culture studies for sclerotia formation

Morchella spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR8) and *M. spongiola* (MR17)), in pairs, were inoculated on the perimeter of petriplates containg malt extract medium and incubated at 25 °C in dark. In case of *Morchella sp.* (MR2) and *M. elata* (MR3) combination, cream colored mycelia were observed in both the cultures (Fig 6.3a). *Morchella elata* (MR3) was observed to be fast growing as compared to *Morchella sp.* (MR2). Mycelial meld (characterized by a ridge of hyphae forming at the juncture where growth from the two colonies meet) was formed at the central line of interference. Brown colored sclerotia (9 in number with an average size of 0.24 cm) were formed in *M. elata* (MR3).

In case of *Morchella sp.* (MR2) and *M. crassipes* (MR8) combination; brown colored mycelia was observed in *M. crassipes* (MR8), whereas cream colored mycelia was observed in *Morchella sp.* (MR2) (Fig 6.3b). Color of mycelium in *Morchella sp.* (MR2) did not change on maturation, whereas brown colored mycelium in *M. crassipes* (MR8) changed to dark brown on maturity. Small sclerotia were formed in *M. crassipes* (MR8) near the central line of interference. Sclerotia were 34 in number, and creamish in color initially on formation but on maturation, their color changed to brown.

In *Morchella sp*. (MR2) and *M. spongiola* (MR17) combination, cream colored, profusely branched, highly dense hyphae were formed by both the cultures (Fig 6.3c). *Morchella sp.* (MR2) was fast growing fungus as compared to *M. spongiola* (MR17) as it had covered more than half of the petriplate. Neither mycelial meld nor sclerotia was observed in any of the culture.

In *M. elata* (MR3) and *M. crassipes* (MR8) combination, cream colored mycelia were observed in both the cultures (Fig 6.3d). *Morchella elata* (MR3) was found to be slow grower as compared to *M. crassipes* (MR8). Mycelia meld was observed at the point of contact of both the cultures. Sclerotia were observed in both the cultures. In *M. elata* (MR3), 16 small

sized sclerotia were observed towards the mycelia meld layer whereas in *M. crassipes* (MR8), only 8 sclerotia were formed away from mycelial meld layer i.e. towards the perimeter of the petriplate. Sclerotia formed in *M. crassipes* (MR8) were larger as compared to sclerotia formed by *M. elata* (MR3).

In *M. elata* (MR3) and *M. spongiola* (MR17); and *M. crassipes* (MR8) and *M. spongiola* (MR17) combination, cream colored mycelium was observed in *M. elata* (MR3) (Fig 6.3e) whereas brown colored mycelium was observed in *M. crassipes* (MR8) and *M. spongiola* (MR17) (Fig 6.3f). Growth was even in all the cultures. Sclerotia were not observed in *M. spongiola* (MR17).

The results showed that *M. crassipes* (MR8) and *M. elata* (MR3) produced large number of sclerotia in Buscot B medium as compared to the other cultures in the study.

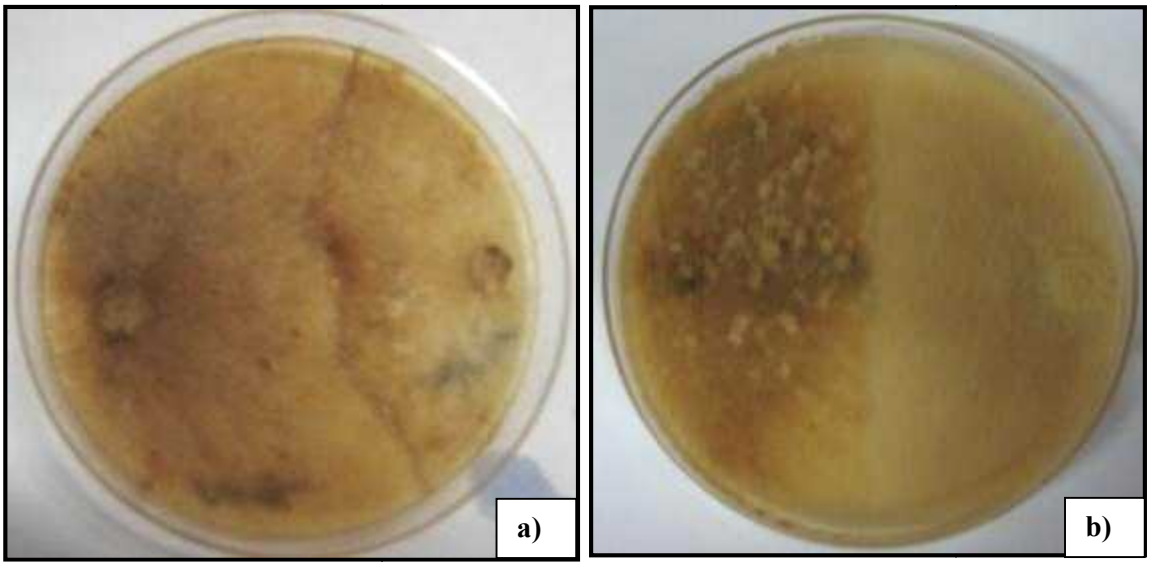


Fig 6.3a-b: Dual culture studies of *Morchella* cultures a) *M. elata* (MR3) (Left) and *Morchella sp.* (MR2) (Right) and b) *M. crassipes* (MR8) (Left) and *Morchella sp*. (MR2) (Right)

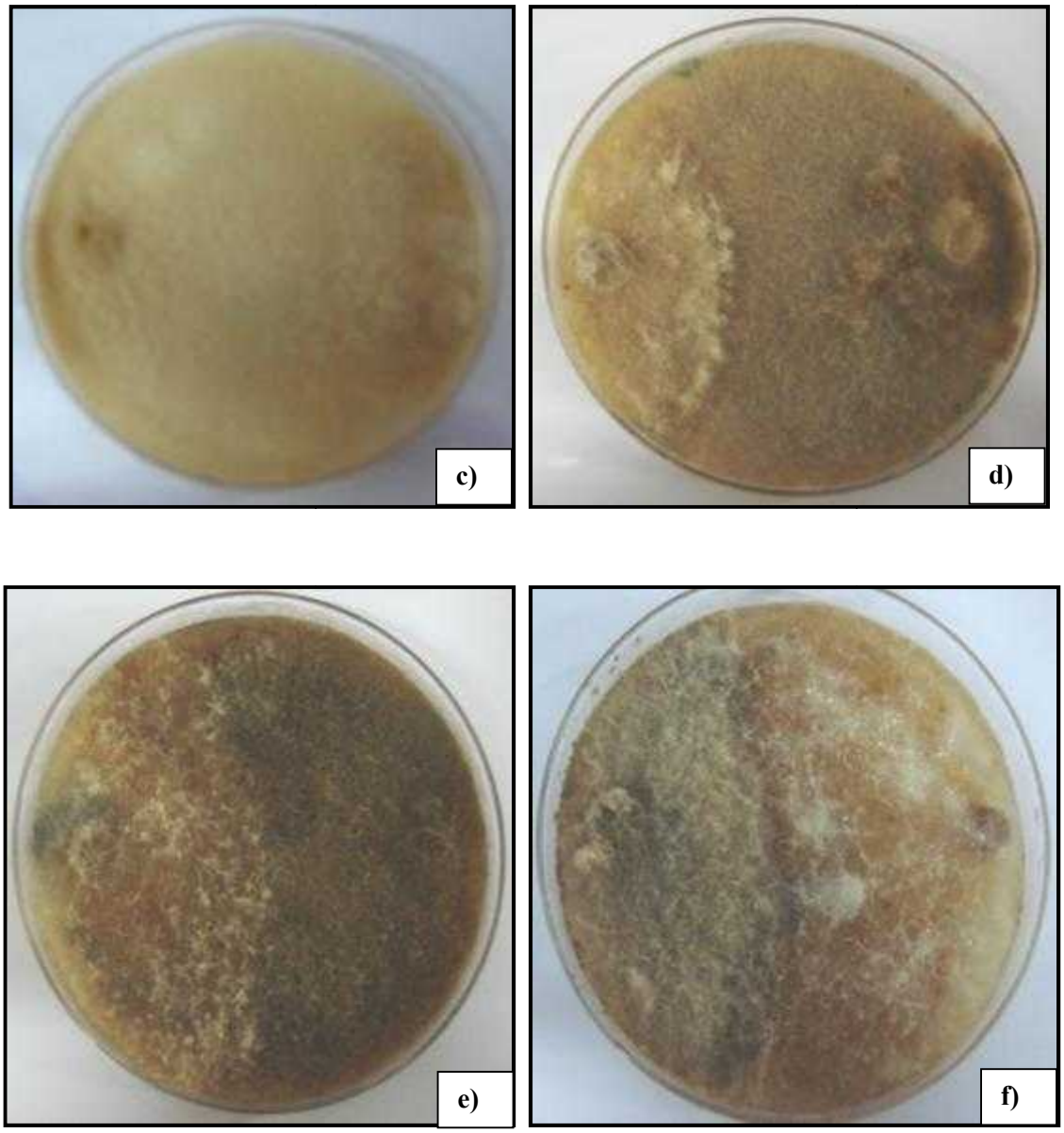


Fig 6.3c-f: Dual culture studies of *Morchella* cultures c) *Morchella sp.* (MR2) (Left) and *M. spongiola* (MR17) (Right) d) *M. elata* (MR3) (Left) and *M. crassipes* (MR8) (Right) e) *M. elata* (MR3) (Left) and *M. spongiola* (MR17) (Right) and f) *M. crassipes* (MR8) (Left) and *M. spongiola* (MR17) (Right)

6.4 Effect of carbon and nitrogen sources on sclerotia formation in jars.

Sclerotial formation was evaluated on Buscot agar media containing different carbon sources (ribose, arabinose, xylose, mannose, galactose, fructose, sorbose, rhamnose, sucrose, cellobiose, starch soluble, mannitol at a concentration of 10g/L) in place of dextrose and different nitrogen sources (casein, peptone, yeast extract, tryptone, sodium nitrite ($NaNO_2$), ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) in place of sodium nitrate ($NaNO_3$) (250mg/L). Small sclerotia were observed in *M. elata* (MR3) and *M. crassipes* (MR8) whereas cultures of *Morchella sp.* (MR2) and *M. spongiola* (MR17) did not form any sclerotia. The mycelial color was creamish brown in both these cultures. For *M. crassipes* (MR8), dense and circular growth of brown colored mycelium was observed. Ribose, cellobiose, galactose, glucose, xylose, sucrose and mannitol served the best carbon sources for sclerotia formation (Table 6.1, Fig 6.4). Rhamnose, mannose, fructose, soluble starch and sorbose did not promote the sclerotia formation. Large-sized sclerotia were observed in presence of mannitol. Younger sclerotia were light yellow in color and turned to brown with maturity. Sclerotia formed in xylose (average size - 0.2 cm) and sucrose (average size - 0.16 cm) were much smaller in size compared to mannitol (average size - 0.43 cm) and formed on the agar surface rather than the sides of glass jars. Sclerotial formation was observed only with sodium nitrate, ammonium nitrate, peptone and yeast extract, where small-sized, less numerous sclerotia were observed (Table 6.1).

In *M. elata* (MR3), dense profusely branched, light brown mycelium was observed in all of the carbon sources tested. Sclerotia were smaller in comparison to the sclerotia formed by *M. crassipes* (MR8) (Table 6.1). Ribose, galactose, glucose, sorbose and mannitol promoted the formation of numerous small sized sclerotia (Fig 6.4) The sclerotia were brown in color when young and turned to dark brown on maturity. No sclerotia were formed in media containing rhamnose, cellobiose, mannose, xylose, fructose, sucrose and soluble starch,

although media containing these substances supported the vegetative growth of the mycelium. Among the different nitrogen sources, sclerotia formed only in $NaNO_3$ and yeast extract (Table 6.1). For the latter, small-sized, brown-pigmented, irregular sclerotia formed on the perimeter of agar medium.

Table 6.1: Details of *Morchella crassipes* and *Morchella elata* along with the average number of sclerotia (n=3) formed under different carbon and nitrogen sources after 18 days.

	M. crassipes **(MR8)**		***M. elata*** **(MR3)**	
Nutritional sources	**Number of sclerotia/jar**	**Average size of sclerotia (cm)**	**Number of sclerotia/jar**	**Average size of sclerotia (cm)**
		Carbon sources		
Ribose	125 ± 14.0a	0.33 ± 0.1b	105 ± 10.0a	0.28 ± 0.1a
Rhamnose	–	–	–	–
Cellobiose	73 ± 9.4b	0.30 ± 0.1b	–	–
Galactose	18 ± 4.1d	0.21 ± 0.1c	82 ± 13.8b	0.20 ± 0.1c
Mannose	–	–	–	–
Xylose	47 ± 5.8c	0.20 ± 0.1c	–	–
Fructose	–	–	–	–
Sucrose	19 ± 4.3d	0.16 ± 0.1d	–	–
Soluble starch	–	–	–	–
Glucose	23 ± 5.7d	0.24 ± 0.1c	97 ± 7.3a	0.23 ± 0.1b
Sorbose	–	–	20 ± 3.8c	0.16 ± 0.1d
Mannitol	74 ± 7.2b	0.43 ± 0.1a	65 ± 14.9a	0.23 ± 0.1b
		Nitrogen sources		
Casein	–	–	–	–
Peptone	14 ± 2.8b	0.14 ± 0.0c	–	–
Yeast extract	24 ± 3.3a	0.26 ± 0.1a	5 ± 2.0b	0.11 ± 0.0b
Tryptone	–	–	–	–
Sodium nitrate	12 ± 2.0b	0.27 ± 0.1a	9 ± 2.9a	0.12 ± 0.0a
Sodium nitrite	–	–	–	–
Ammonium chloride	–	–	–	–
Ammonium nitrate	6 ± 1.7c	0.18 ± 0.1b	–	–

Values sharing a common letter within the column within the C & N sources are not significant (P<0.05).). Values are Mean ± SEM (n=3). – denotes no sclerotia

Studies of *Morchella* spp. with different carbon and nitrogen sources showed that ribose as carbon source and peptone as nitrogen source was found to be the best for large size and number of sclerotia formation in *M. crassipes* (MR8) and *M. elata* (MR3).

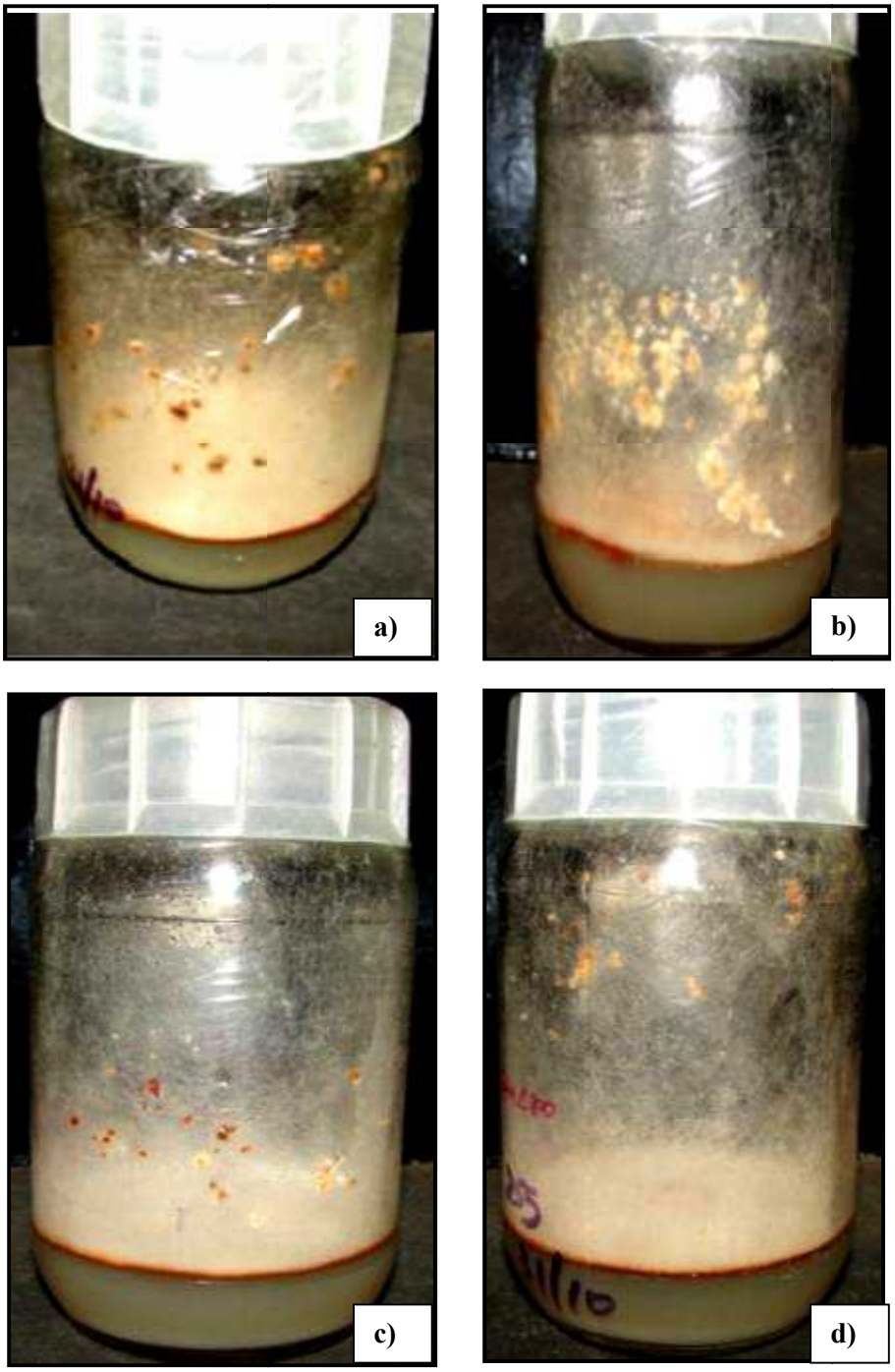


Fig. 6.4: Sclerotia formation by *M. crassipes* with a) mannitol b) cellobiose c) xylose and d) sucrose as carbon source.

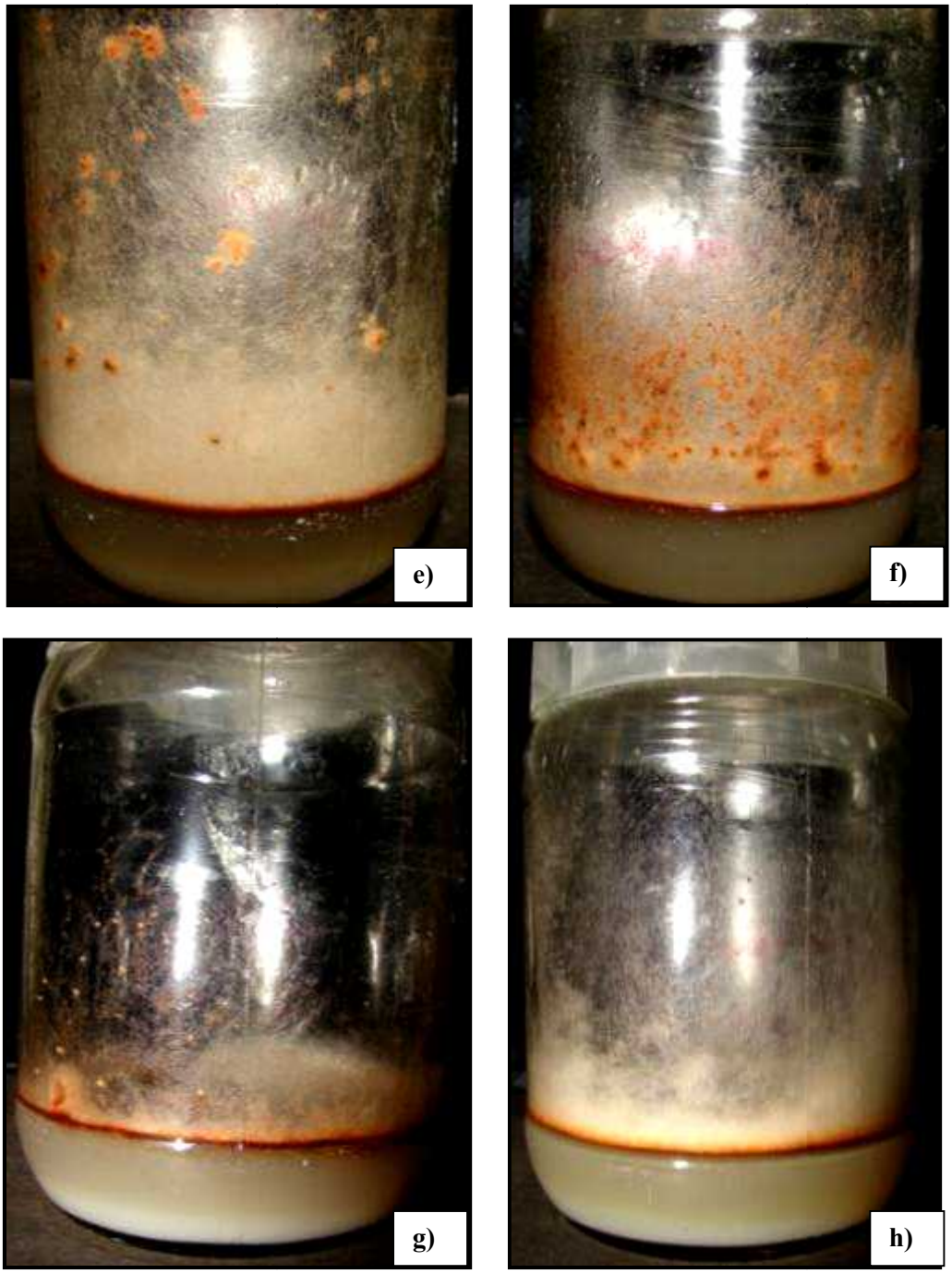


Fig 6.4e-h: Sclerotia formation by *M. elata* with e) galactose f) ribose g) sorbose and h) sucrose as carbon source.

6.5 Effect of substrates on enzyme activity

Different *Morchella* spp. *(Morchella sp*. (MR2), *M. elata* (MR3), *M. crassipes* (MR8) and *M. spongiola* (MR17)) were grown on finely chopped and steam sterilized substrates namely, wheat straw (WS), rice straw (RS), saw dust (SD), apple leaves (AL), groundnuts shell (GS), eucalyptus leaves (EL), bamboo leaves (BL), orange peels (OP), pine needles (PN), wheat bran (WB), wheat grains (WG), and carrot peels (CP) (Fig 6.5). A control group without any culture was also incubated alongwith treatments. pH and enzyme activities such as acid phosphatase, phytase, cellulase, laccase, lignin peroxidase and manganese peroxidase were determined.

6.5.1 pH of the spent culture filtrate

pH in most of the substrates increased to small amounts as compared to their respective controls. Maximum increase in pH was observed in orange peels by *M. elata* (MR3), followed by groundnuts shell in *M. crassipes* (MR8) and carrot peels by *Morchella sp.* (MR2).

pH in most of the substrates increased towards neutral pH even from highly acidic conditions as in case of orange peels in all *Morchella* species. No or little growth was found in eucalyptus and sawdust so not much significant variation in pH was observed by all the *Morchella* spp. in this case. Similarly, not much significant difference in pH variations were observed in rice straw, wheat straw, apple leaves and pine needles; even though *Morchella* mycelium was found to grow well on these substrates. The observation of increased pH was of much significance as the reports from most of the lignolytic enzymes from fungi generally decrease the pH.(Tab 6.2, Fig 6.6).

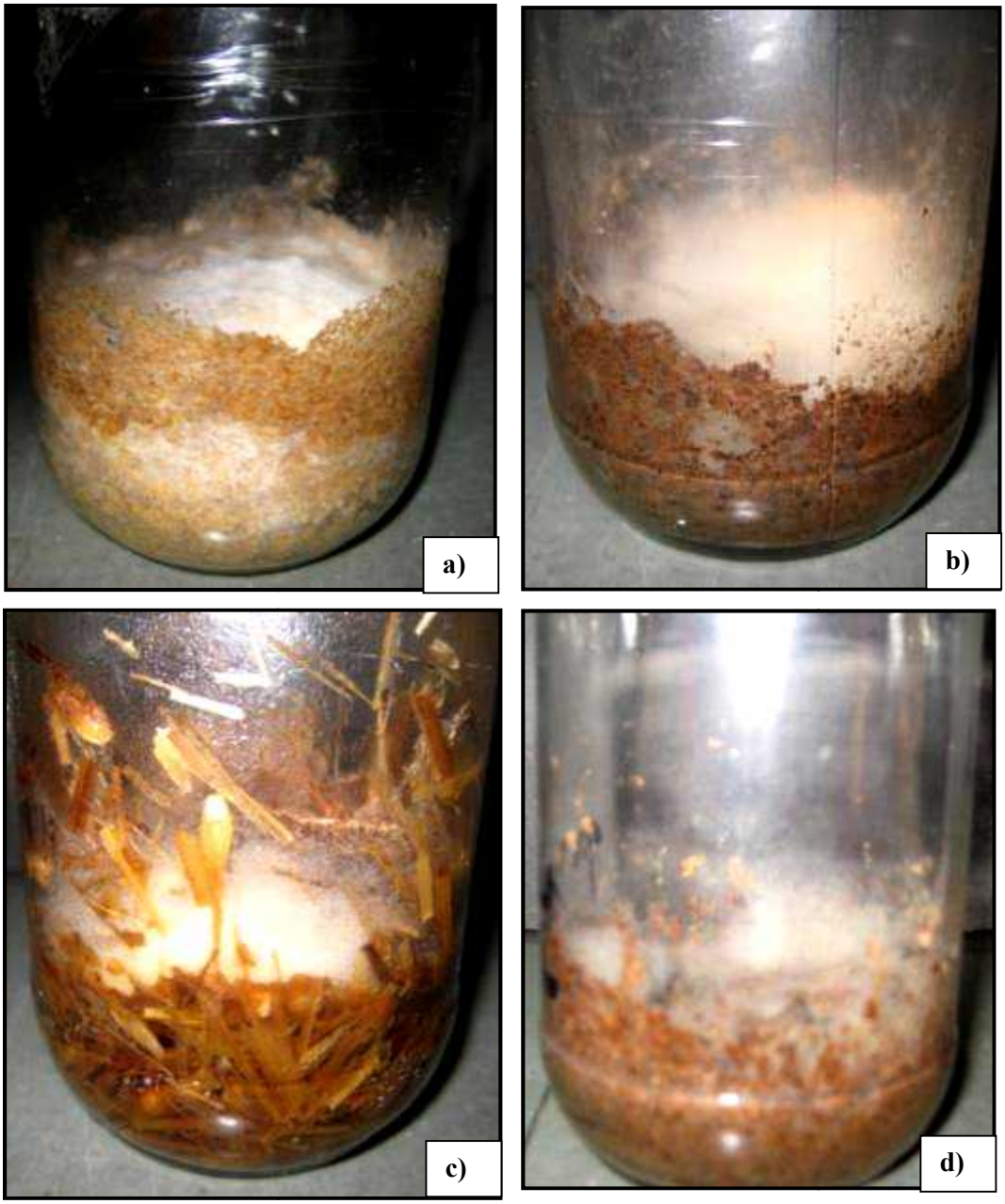


Fig 6.5a-d: Growth of *Morchella* spp. on different substrates a) *M. crassipes* (MR8) on wheat bran b) *M. crassipes* (MR8) on groundnuts shell c) *M. crassipes* (MR8) on wheat straw, and d) *M. elata* (MR3) on groundnuts shell

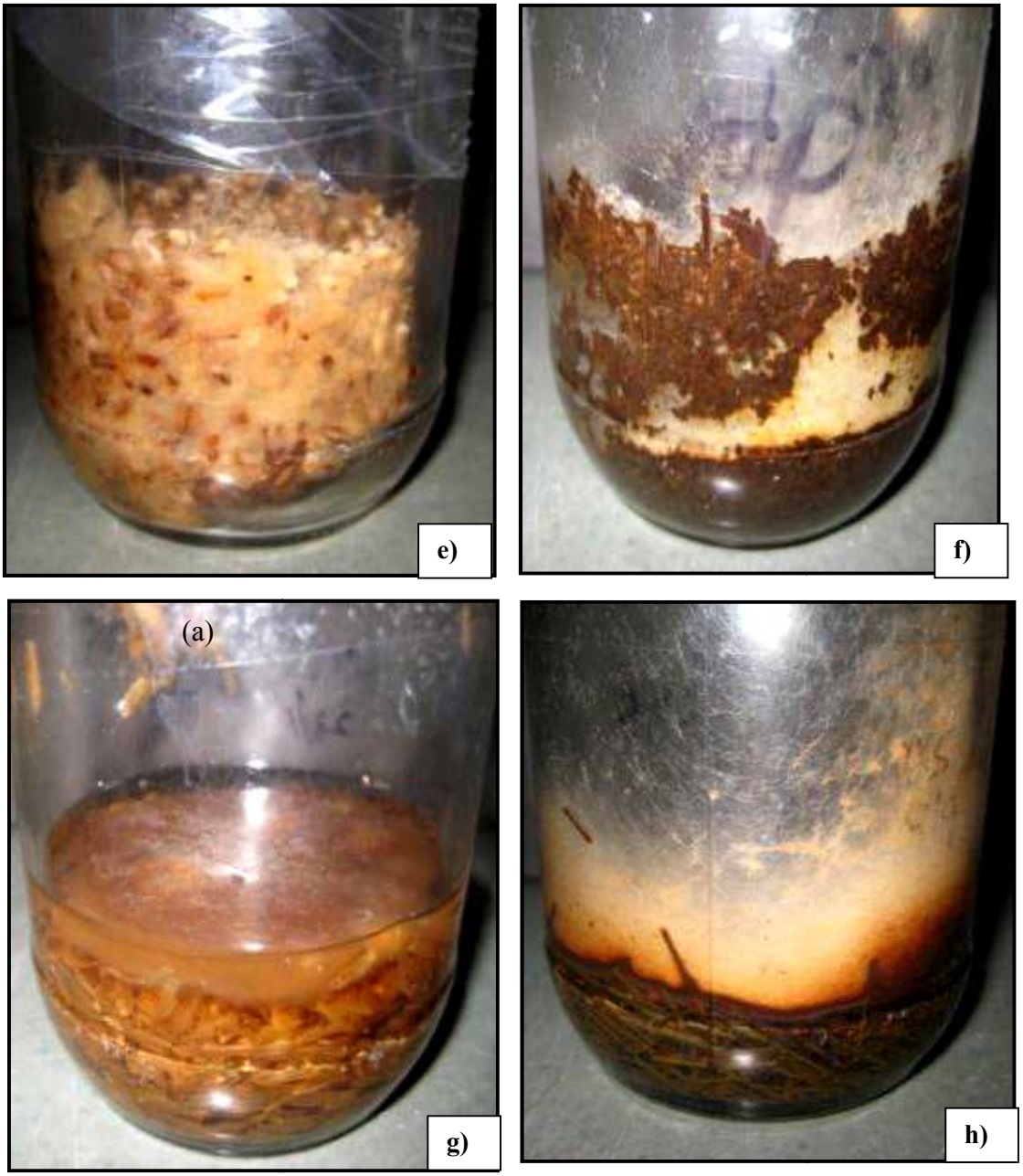


Fig 6.5e-h: Growth of *Morchella* spp. on different substrates e) *M. crassipes* (MR8) on wheat grains f) *M. elata* (MR3) on apple leaves g) *Morchella sp.* (MR2) on rice straw, and h) *M. elata* (MR3) on pine needles

Table 6.2: pH of spent culture filtrate of different *Morchella* spp. inoculated on different substrates.

SUBSTRATES	Control pH	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Groundnuts shell	6.30	7.59 ± 0.1a	7.54 ± 0.1a	7.70 ± 0.2a	7.59 ± 0.1a
Sawdust	6.89	7.27 ± 0.2ab	7.13 ± 0.1ab	7.22 ± 0.1ab	6.63 ± 0.3b
Rice straw	6.83	7.44 ± 0.3ab	6.51 ± 0.1	7.25 ± 0.1ab	7.23 ± 0.1ab
Wheat straw	6.77	7.01 ± 0.1ab	7.05 ± 0.1ab	7.00 ± 0.0ab	7.04 ± 0.1ab
Wheat grains	6.43	6.33 ± 0.1b	6.33 ± 0.0 b	6.30 ± 0.1b	6.49 ± 0.2b
Wheat bran	6.50	6.58 ± 0.2b	6.73 ± 0.4b	6.40 ± 0.1b	6.47 ± 0.1b
Apple leaves	6.24	6.34 ± 0.2b	6.41 ± 0.1	6.76 ± 0.1b	6.49 ± 0.1b
Carrot peels	6.59	7.71 ± 0.1a	7.44 ± 0.2a	7.72 ± 0.2a	7.43 ± 0.2a
Orange peels	4.57	7.12 ± 0.1ab	7.21 ± 0.1ab	7.13 ± 0.1ab	7.08 ± 0.2ab
Bamboo leaves	6.28	7.49 ± 0.1ab	7.29 ± 0.2ab	7.27 ± 0.1ab	7.34 ± 0.1ab
Pine needles	6.00	6.58 ± 0.1b	6.64 ± 0.2b	6.57 ± 0.2b	6.62 ± 0.2b
Eucalyptus leaves	4.59	4.56 ± 0.1c	4.67 ± 0.1c	4.68 ± 0.1c	4.66 ± 0.2c

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3)

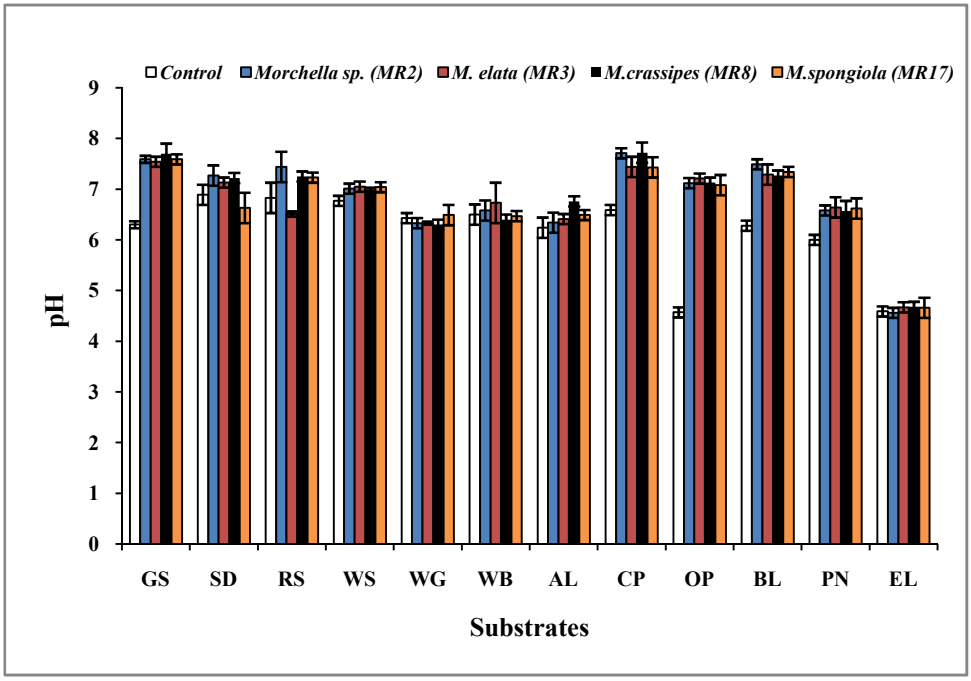


Fig 6.6: pH of spent culture filtrate of different *Morchella* species inoculated on different substrates. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3)

6.5.2 Protein content of the spent culture filtrate

Protein content in different *Morchella* spp. showed varying concentrations according to different substrates. Highest protein concentration was observed in groundnuts shell, wheat straw, rice straw, wheat grains, wheat bran, carrot peels, orange peels and pine needles. Maximum protein content was observed in groundnuts shell followed by pine needles by *M. crassipes* (MR8). Significant differences were observed in protein contents of *M. crassipes* (MR8) in groundnuts shell and pine needles as compared to others. In *Morchella sp*. (MR2), maximum protein content was observed in wheat grains, followed by groundnuts shell, wheat straw, pine needles, orange peels, wheat bran and carrot peels. In *M. elata* (MR3), maximum amount of proteins were observed in wheat grains followed by pine needles, wheat bran and

groundnuts shell. Optimum sources of substrates for protein concentration include wheat straw, apple leaves, carrot peels and orange peels. In *M. spongiola* (MR17), maximum amount of protein content was observed in groundnuts shell, pine needles, wheat grains and wheat straw. Optimum sources for protein content included orange peels, wheat bran, apple leaves, carrot peels. Significant differences in amount of proteins were found on sawdust and eucalyptus in all the *Morchella* spp. (Tab 6.3, Fig 6.7).

Table 6.3: Protein content (mg/ml) of different *Morchella* spp. inoculated on different substrates.

SUBSTRATES	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Groundnuts shell	16.95 ± 1.6ab	14.87 ± 1.2b	31.13 ± 2.6a	17.87 ± 1.2a
Sawdust	3.34 ± 1.4e	2.17 ± 0.1e	0.94 ± 0.5f	1.12 ± 0.1d
Rice straw	5.80 ± 0.1d	6.82 ± 0.5d	4.15 ± 2.0ef	3.09 ± 0.5c
Wheat straw	15.97 ± 0.4ab	10.53 ± 0.6c	9.67 ± 0.2d	15.04 ± 0.5ab
Wheat grains	17.95 ± 4.0a	20.20 ± 1.1a	17.27 ± 1.4c	17.38 ± 0.6a
Wheat bran	13.20 ± 1.6ab	14.93 ± 0.2b	7.84 ± 0.4de	11.2 ± 0.8b
Apple leaves	7.07 ± 0.4c	10.34 ± 0.5c	6.55 ± 2.0e	11.28 ± 0.8b
Carrot peels	12.11 ± 2.0b	10.67 ± 1.6c	6.42 ± 0.4e	11.33 ± 0.7b
Orange peels	13.00 ± 1.6ab	8.11 ± 0.5cd	5.63 ± 1.6e	12.32 ± 1.5b
Bamboo leaves	5.28 ± 1.0d	1.79 ± 0.9ef	0.68 ± 0.2f	2.60 ± 0.4c
Pine needles	15.16 ± 1.4ab	15.48 ± 0.3b	25.33 ± 1.4b	17.62 ± 1.5a
Eucalyptus leaves	ND	0.39 ± 0.2f	ND	ND

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). *ND denotes not detected

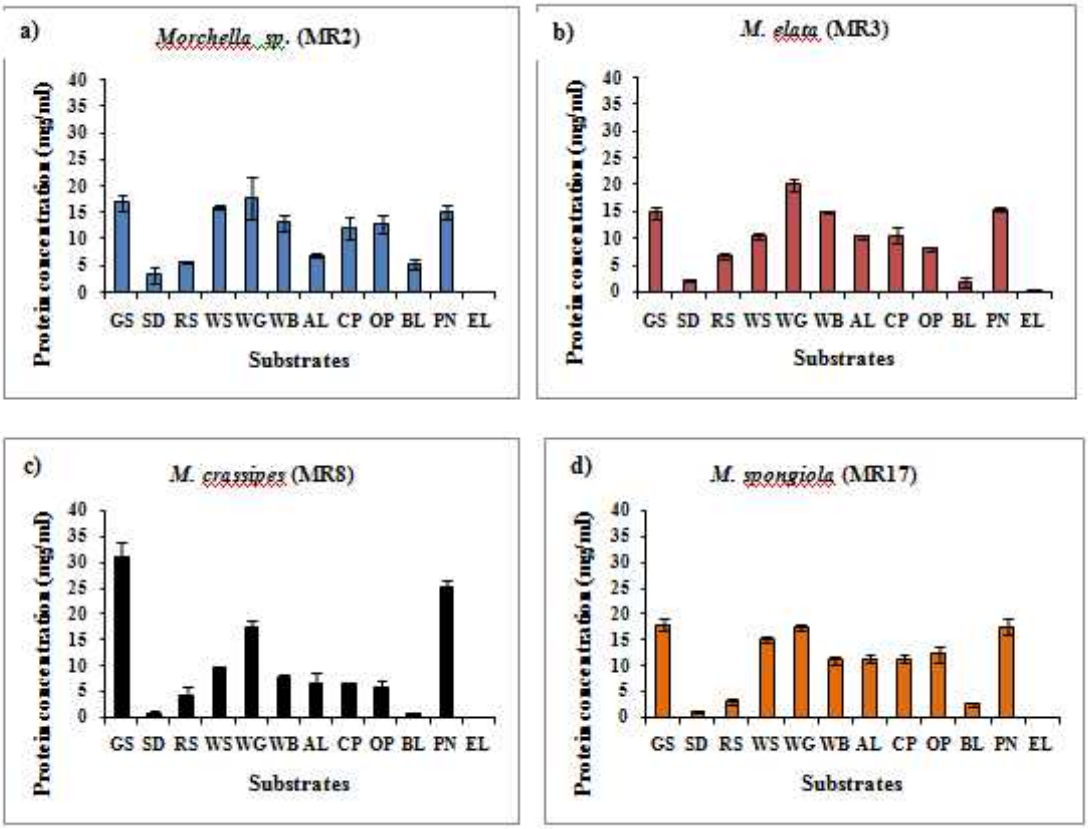


Fig 6.7: Protein content (mg/ml) of different *Morchella* spp. inoculated on different substrates. a) *Morchella sp*. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR8) and d) *M. spongiola* (MR17), inoculated on different substrates. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3)

6.5.3 Acid phosphatase

Acid phosphatase was found to vary with different substrates by all *Morchella* spp. Maximum acid phosphatase was observed in orange peels by *M. spongiola* (MR17), followed by *M. crassipes* (MR8) in carrot peels (Tab 6.4, Fig 6.8). In *Morchella sp.* (MR2), maximum acid phosphatase was observed in bamboo leaves, followed by carrot peels, orange peels and wheat straw. In *M. elata* (MR3), maximum acid phosphatase was observed in pine needles, carrot peels, bamboo leaves and wheat straw. In *M. crassipes* (MR8), maximum acid phosphatase was observed in carrot peels and wheat straw, followed by bamboo leaves. Maximum acid phosphatase in *M. spongiola* (MR17) was observed in pine needles and wheat straw as the substrates. Least acid phosphatase and no acid phosphatase was detected in case of eucalyptus and saw dust, respectively (Tab 6.4, Fig 6.8).

Table 6.4: Extracellular acid phosphatase (µM/hr/ml**)** of different *Morchella* spp. inoculated on different substrates.

SUBSTRATES	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Groundnuts shell	56.0 ± 2.0b	52.3 ± 3.0bc	68.3 ± 4.0c	49.2 ± 6.0cd
Sawdust	ND	ND	ND	ND
Rice straw	43.0 ± 2.5c	39.8 ± 1.0c	38.5 ± 7.0d	42.3 ± 7.0cd
Wheat straw	85.6 ± 2.0ab	85.8 ± 3.0ab	112.5 ± 8.0a	108.1 ± 3.0ab
Wheat grains	68.6 ± 4.0b	67.3 ± 6.0b	65.4 ± 4.0c	83.9 ± 6.0b
Wheat bran	59.2 ± 2.0b	45.2 ± 2.0c	52.9 ± 3.0c	54.2 ± 3.0c
Apple leaves	60.6 ± 2.0b	63.3 ± 4.0b	69.8 ± 5.0c	56.2 ± 2.0c
Carrot peels	92.1 ± 7.0ab	96.4 ± 5.0a	118.0 ± 4.0a	71.4 ± 13.0bc
Orange peels	84.6 ± 18.0ab	66.2 ± 9.0b	65.8 ± 7.4c	125.0 ± 23.0a
Bamboo leaves	102.1 ± 4.0a	92.7 ± 9.0a	98.7 ± 5.0b	42.7 ± 7.0cd
Pine needles	49.8 ± 2.0c	99.8 ± 11.0a	56.8 ± 2.0c	109.0 ± 3.0ab
Eucalyptus leaves	31.0 ± 2.0d	23.9 ± 4.0d	37.3 ± 3.0d	32.5 ± 2.0d

Values sharing a common letter within the column are not significant at P<0.05. Values are Mean ± SEM (n=3). *ND denotes not detected

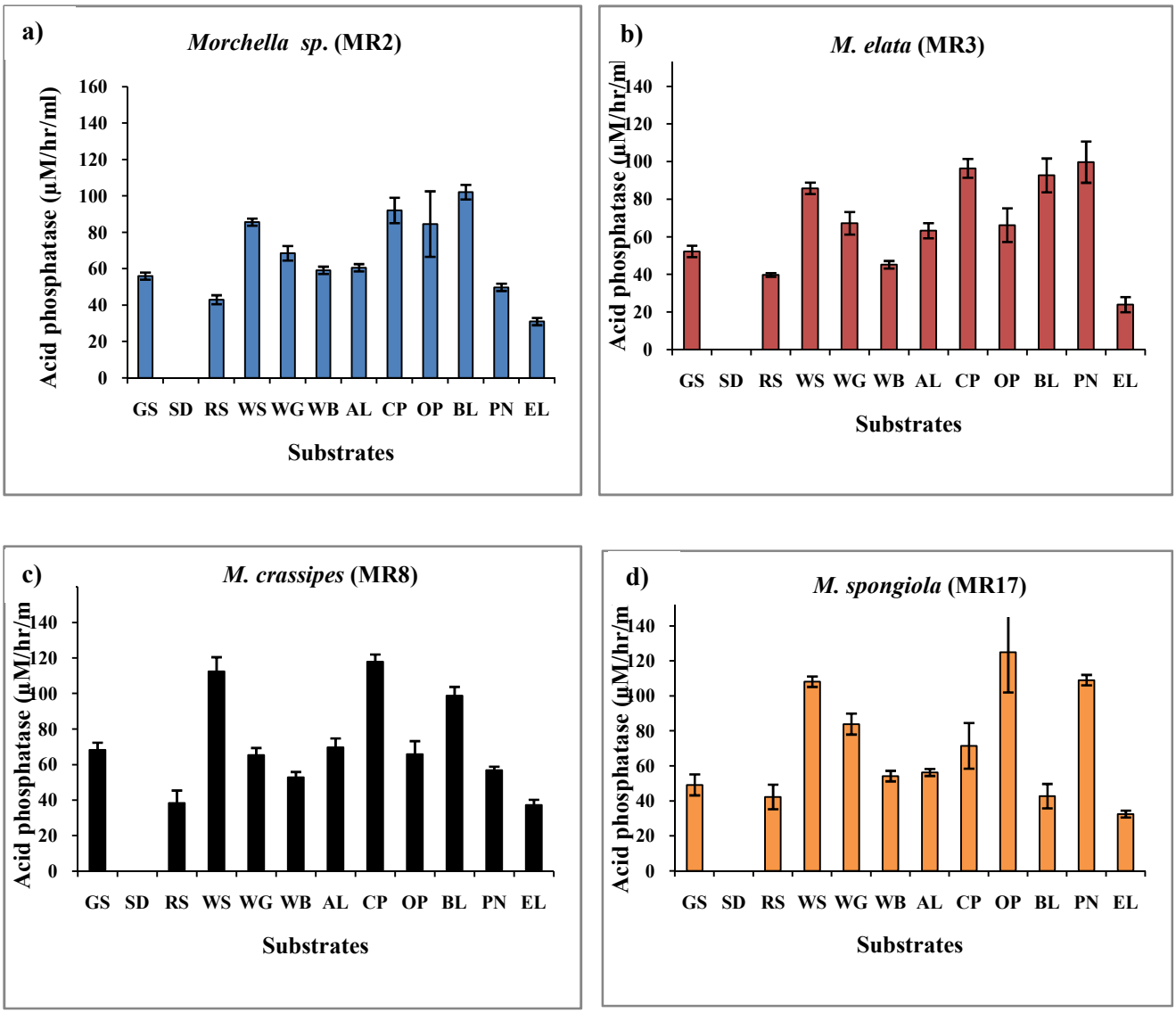


Fig 6.8: Extracellular acid phosphatase (µM/hr/ml**)** of different *Morchella* spp. inoculated on different substrates. a) *Morchella sp*. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR8) and d) *M. spongiola* (MR17), inoculated on different substrates. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3)

6.5.4 Phytase activity of the culture filtrate

None of the *Morchella* spp. showed phytase activity with different substrates used in the present study.

6.5.5 Laccase

Laccase activity was highly influenced by different substrates. Substrates such as wheat grains, wheat bran and wheat straw served as the most promising substrates for laccase production. Maximum laccase activity was observed in wheat grains by *M. crassipes* (MR8) followed by *M. elata* (MR3). In *Morchella sp*. (MR2), maximum laccase production was observed in wheat grains and wheat bran followed by pine needles. Significant difference in laccase activity was observed in between the different substrates. Optimum sources of substrates for laccase production included rice straw, wheat straw, apple leaves, orange peels, carrot peels and groundnuts shell. Least or no activity was detected in bamboo leaves, eucalyptus and sawdust. In *M. elata* (MR3), maximum laccase activity was observed in wheat grains, followed by wheat bran. Substrates such as, pine needles, wheat straw and rice straw, too produced laccase to significant amounts.

In *M. crassipes* (MR8), maximum laccase activity was found in wheat grains, followed by wheat bran. Moderate level of activity was found in wheat straw, pine needles, groundnuts shell, rice straw, apple leaves, orange peels and carrot peels. Least activity was found in sawdust, where least or no growth was found. In *M. spongiola* (MR17), wheat grains produced maximum laccase activity, followed by wheat bran. Laccase activity was found to decrease in the order of substrates as, wheat straw, pine needles, apple leaves, rice straw, carrot peels, orange peels, and groundnuts shell. Least activity was detected in bamboo leaves, eucalyptus and sawdust. Significant levels of difference were observed in laccase activity by different *Morchella* spp. in different substrates (Tab 6.5, Fig 6.9).

Table 6.5: Laccase activity (U/ml) of different *Morchella* spp. inoculated on different substrates.

SUBSTRATES	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Groundnuts shell	13.5 ± 1.5de	25.3 ± 3.0c	38.7 ± 1.3de	9.1 ± 1.0e
Sawdust	ND	2.0 ± 1.0f	9.6 ± 0.4f	ND
Rice straw	22.0 ± 3.0c	34.7 ± 3.0bc	44.3 ± 1.3d	15.2 ± 1.0d
Wheat straw	28.0 ± 5.0c	44.8 ± 2.0b	53.3 ± 3.0c	24.4 ± 1.0c
Wheat grains	**79.0 ± 3.0a**	**93.2 ± 2.0a**	**106.0 ± 12.0a**	**63.2 ± 1.5a**
Wheat bran	**70.0 ± 2.0a**	**82.6 ± 1.0ab**	**83.1 ± 5.0b**	**47.0 ± 8.0b**
Apple leaves	23.0 ± 1.4c	23.1 ± 1.0c	33.2 ± 1.4de	16.0 ± 2.0d
Carrot peels	14.0 ± 1.7de	11.6 ± 2.0d	9.1 ± 1.0g	12.8 ± 2.0d
Orange peels	17.3 ± 1.0d	23.1 ± 1.0c	16.5 ± 2.0e	12.0 ± 1.0d
Bamboo leaves	6.0 ± 2.0e	7.8 ± 2.0de	2.4 ± 0.5h	4.4 ± 1.0f
Pine needles	42.0 ± 1.3b	47.3 ± 2.0b	52.4 ± 2.0c	24.0 ± 2.0c
Eucalyptus leaves	ND	4.9 ± 0.3e	5.3 ± 0.4gh	3.0 ± 0.6f

Values sharing a common letter within the column are not significant at P<0.05. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

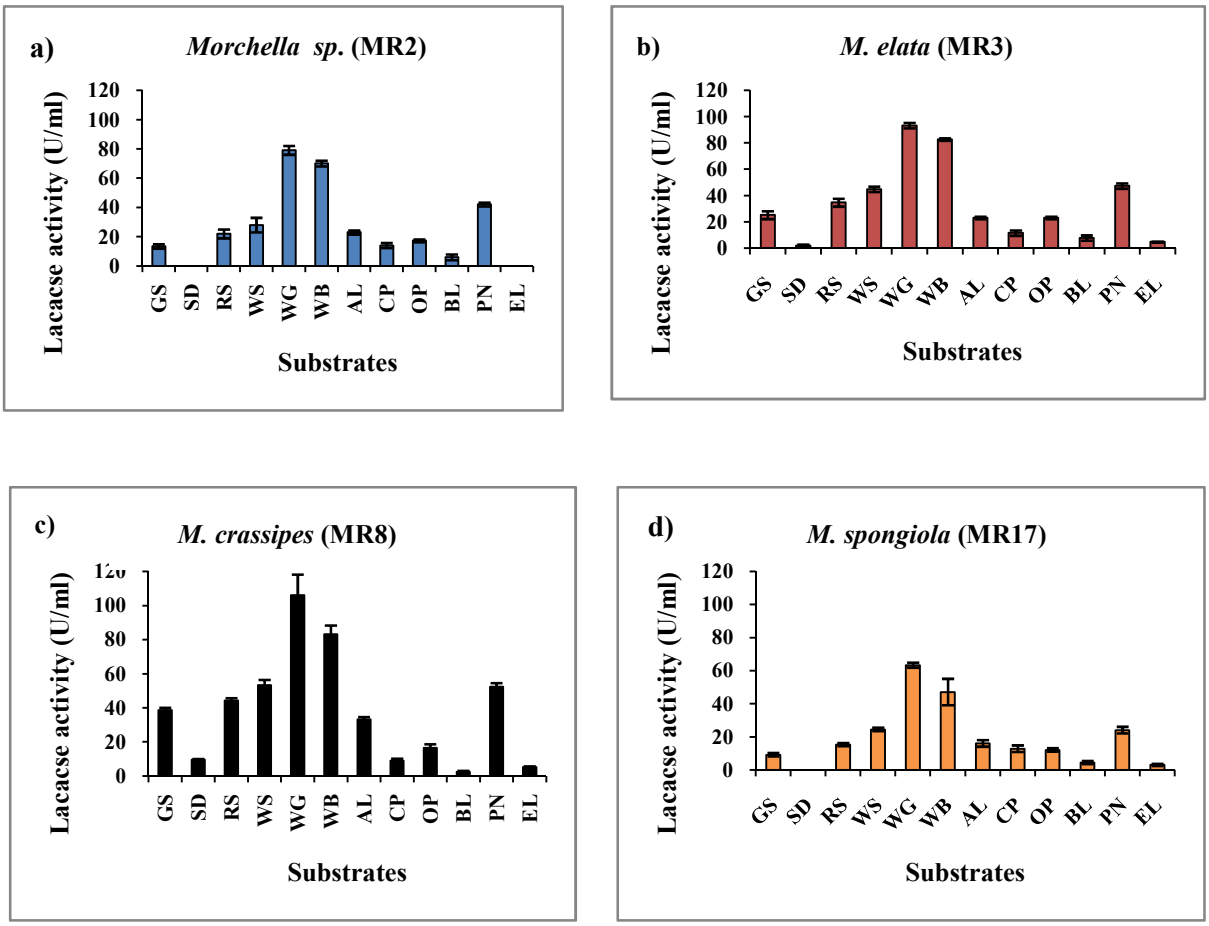


Fig 6.9: Laccase activity (U/ml) of different *Morchella* spp. a) *Morchella sp.* (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR8) and d) *M. spongiola* (MR17), inoculated on different substrates. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3)

6.5.6 Lignin peroxidase

Lignin peroxidase activity was found to be highly influencd in different substrates by different *Morchella* spp., even though the amount produced was less than other fungi (basidiomycetes).

Maximum lignin peroxidase activity was observed in rice straw followed by apple leaves and wheat straw in *M. crassipes* (MR8) (Tab 6.6, Fig 6.10). The enzyme activity was significantly higher in these substrates. Optimum levels in enzyme activity were found in pine needles, groundnuts shell, and wheat grains. Significantly less lignin peroxidase was detected in wheat bran, bamboo leaves, orange peels, carrot peels, eucalyptus, and sawdust.

In *Morchella sp.* (MR2), maximum lignin peroxidase activity was observed in pine needles followed by apple leaves. Rice straw and wheat straw also produced significantly high lignin peroxidase. Least or no lignin peroxidase activity was observed in bamboo leaves, wheat bran, carrot peels, groundnuts shell, wheat grains, bamboo leaves and eucalyptus.

Maximum lignin peroxidase activity was observed by *M. elata* (MR3) in rice straw, followed by wheat straw and apple leaves. Moderate amounts of lignin peroxidase activity was observed in pine needles and groundnuts shell. Significantly least lignin peroxidase activity was detected in wheat bran, bamboo leaves, wheat grains, eucalyptus, carrot peels, orange peels and sawdust.

In *M. spongiola* (MR17), maximum lignin peroxidase acticity was observed in rice straw, followed by wheat straw, pine needles and apple leaves. Other substrates such as groundnuts shell, wheat bran, wheat grains, carrot peels, bamboo leaves, eucalyptus and orange peels served as poor sources for lignin peroxidase production as least lignin peroxidase was detected in them (Tab 6.6, Fig 6.10).

Table 6.6: Lignin peroxidase (U/L) of different *Morchella* spp. inoculated on different substrates.

SUBSTRATES	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Groundnuts shell	1.8 ± 0.8d	9.9 ± 1.0c	15.9 ± 1.0c	4.5 ± 1.0c
Sawdust	ND	0.7 ± 0.2f	1.6 ± 0.5f	0.2 ± 0.1f
Rice straw	7.9 ± 1.5b	28.8 ± 1.0a	**41.4 ± 1.0a**	**12.0 ± 0.5a**
Wheat straw	5.9 ± 1.4c	23.8 ± 3.0a	28.9 ± 1.0b	9.9 ± 1.6ab
Wheat grains	1.4 ± 0.7d	4.6 ± 1.0e	14.8 ± 0.5c	2.5 ± 0.6d
Wheat bran	2.2 ± 0.5d	6.7 ± 2.0d	4.4 ± 1.7de	2.7 ± 0.8d
Apple leaves	**9.1 ± 4.0a**	**23.6 ± 3.0a**	29.2 ± 5.0b	8.5 ± 1.5b
Carrot peels	2.1 ± 0.4d	3.5 ± 0.4e	3.7 ± 1.4e	1.9 ± 0.5de
Orange peels	1.0 ± 0.6e	2.2 ± 0.5ef	5.9 ± 0.8d	0.7 ± 0.4e
Bamboo leaves	2.6 ± 0.8d	5.3 ± 0.5de	5.4 ± 0.8d	1.8 ± 0.8de
Pine needles	9.4 ± 1.2a	14.0 ± 1.5b	21.9 ± 1.0bc	9.2 ± 2.0ab
Eucalyptus leaves	0.7 ± 0.3e	3.1 ± 1.0e	4.8 ± 1.0de	0.9 ± 0.5e

Values sharing a common letter within the column are not significant at P<0.05. Values are Mean ± SEM (n=3). *ND denotes Not detected. Values in bold indicate significant ones.

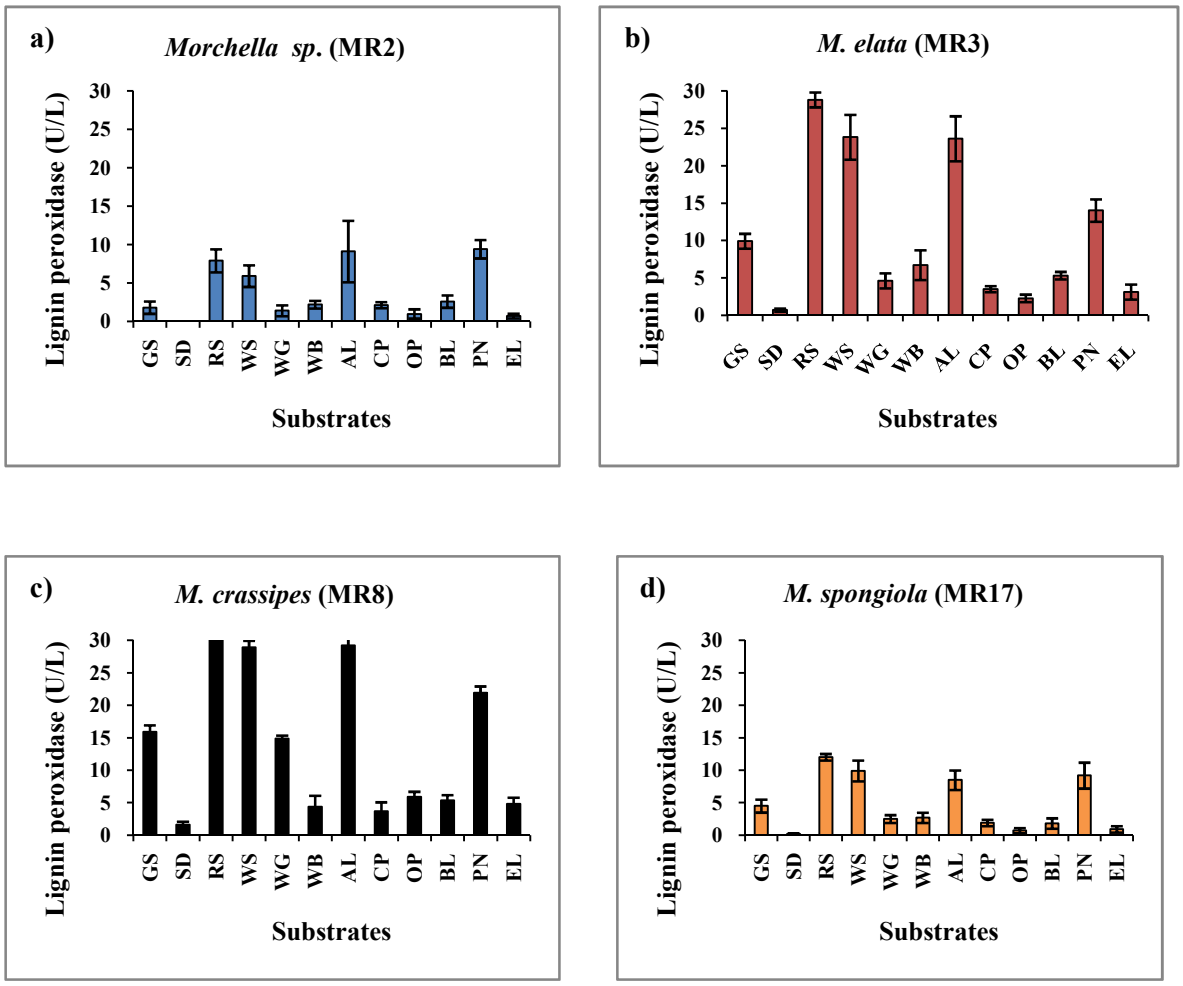


Fig 6.10: Lignin peroxidase (U/L) of different *Morchella* spp. a)*Morchella sp*. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR8) and d) *M. spongiola* (MR17), inoculated on different substrates. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3).

6.5.7 Manganese Peroxidase activity

Manganese peroxidase activity was found to be influenced by different substrates used in the study. Maximum manganese peroxidase acticity was observed in wheat straw and rice straw by *M. crassipes* (MR8) (Tab 6.7, Fig 6.11). Moderate manganese peroxidase acticity was observed in wheat grains, wheat bran, groundnuts shell and pine needles. Least activity was

detected in bamboo leaves and eucalyptus and no activity was detected in sawdust and apple leaves. In *Morchella sp*. (MR2), maximum manganese peroxidase activity was detected in rice straw and wheat straw as the substrates. Pine needles, wheat grains, wheat bran and groundnuts shell too produced manganese peroxidase activity to significant levels. Least activity was observed in bamboo leaves and eucalyptus. Rice straw and wheat straw served as the promising substrates for manganese peroxidase actiivty by *M. elata* (MR3) and *M. spongiola* (MR17). Moderate levels of Lignin peroxidase activity was observed in groundnuts shell, wheat grains and wheat straw by *M. elata* (MR3) and *M. spongiola* (MR17). Least or no activity was observed in sawdust, eucalyptus and apple leaves.

Table 6.7: Manganese peroxidase (U/L) of different *Morchella* spp. inoculated on different substrates.

SUBSTRATES	***Morchella sp.* (MR2)**	***M. elata* (MR3)**	***M. crassipes* (MR8)**	***M. spongiola* (MR17)**
Groundnuts shell	18.0 ± 5.0bc	32.2 ± 5.0b	43.5 ± 5.0c	13.0 ± 2.5c
Sawdust	ND	ND	ND	ND
Rice straw	**44.0 ± 5.0a**	**62.2 ± 1.5a**	**81.9 ± 1.5a**	**28.8 ± 2.6b**
Wheat straw	**41.0 ± 6.0a**	**53.1 ± 7.0ab**	**84.2 ± 7.0a**	**36.2 ± 6.0a**
Wheat grains	24.9 ± 5.0b	27.1 ± 3.0c	71.2 ± 3.0ab	7.3 ± 1.5d
Wheat bran	20.9 ± 3.0bc	20.9 ± 6.0d	67.8 ± 6.0b	10.7 ± 2.5cd
Apple leaves	6.2 ± 2.0d	7.3 ± 0.4f	ND	1.1 ± 1.0f
Carrot peels	10.1 ± 2.0c	7.9 ± 1.4f	37.8 ± 8.0cd	5.6 ± 1.5d
Orange peels	10.1 ± 3.0c	11.3 ± 4.0e	26.0 ± 5.0d	6.9 ± 2.6d
Bamboo leaves	2.3 ± 1.0e	2.3 ± 2.0g	7.9 ± 2.0e	2.8 ± 1.5e
Pine needles	25.4 ± 2.0b	35.6 ± 7.0b	45.2 ± 7.0c	28.2 ± 2.5b
Eucalyptus leaves	0.6 ± 0.2f	ND	1.6 ± 0.9f	ND

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

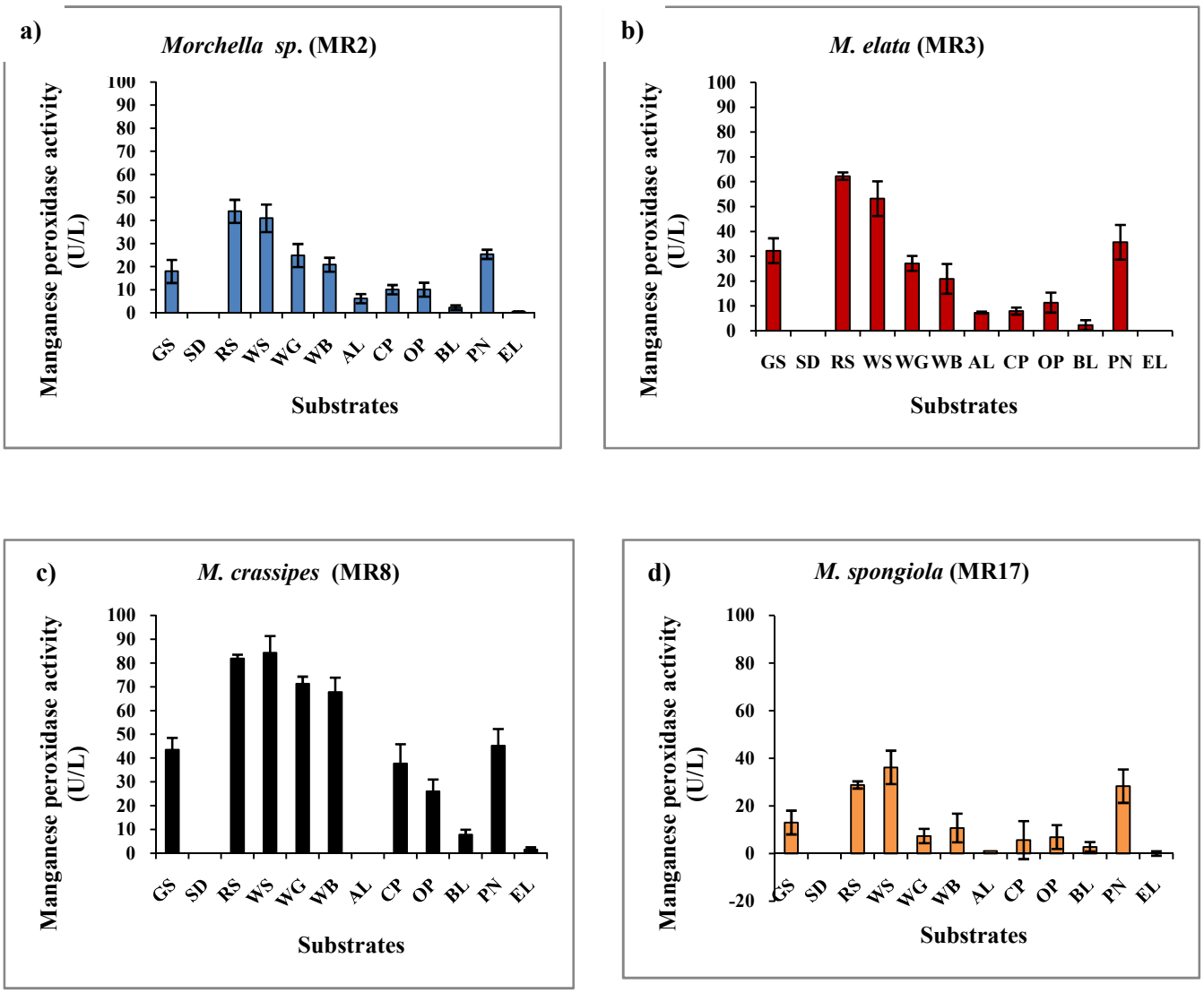


Fig 6.11: Manganese peroxidase (U/L) of different *Morchella* spp. a) *Morchella sp*. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR8) and d) *M. spongiola* (MR17), inoculated on different substrates. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3)

6.5.8 Cellulase activity of culture filtrate

Cellulase activity was found to be highly influenced by different *Morchella* spp. Maximum cellulase activity was observed in wheat grains, wheat bran, and wheat straw as the substrates by *M. spongiola* (MR17), followed by *Morchella sp*. (MR2). Significant differences in enzyme productions were observed among these substrates with the other ones.

In *M. spongiola* (MR17), substrates such as groundnuts shell, rice straw, apple leaves, carrot peels, orange peels and pine needles produced cellulase activity to significant levels. Least activity was detected in bamboo leaves, saw dust and eucalyptus.

In *Morchella sp.* (MR2), maximum cellulase activity was determined in wheat grains and wheat straw. Optimum sources of cellulase activity were observed to be groundnuts shell, apple leaves, wheat bran, rice straw, carrot peels, orange peels and pine needles.

In *M. elata* (MR3), maximum cellulase production was estimated in wheat grains, wheat straw and rice straw. Other substrates such as groundnuts shell, pine needles, wheat bran and apple leaves, too produced cellulase activity to significant levels. Least activity was detected in case of bamboo leaves, eucalyptus and sawdust as the substrates.

Maximum cellulase activity was observed in wheat straw, groundnuts shell and wheat grains followed by wheat bran, rice straw, carrot peels, orange peels and pine needles by *M. crassipes* (MR8). Least activity was detected in case of apple leaves, eucalyptus, bamboo leaves and sawdust (Tab 6.8, Fig 6.12).

Table 6.8: Cellulase activity (U/L) of different *Morchella* spp. inoculated on different substrates.

SUBSTRATES	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Groundnuts shell	2.65 ± 0.2b	2.38 ± 0.5b	3.33 ± 0.1a	3.75 ± 0.6b
Sawdust	0.32 ± 0.2d	0.48 ± 0.2d	0.19 ± 0.5d	0.40 ± 0.2d
Rice straw	1.66 ± 0.3b	3.34 ± 0.2a	2.65 ± 0.7b	3.76 ± 0.3b
Wheat straw	**4.00 ± 0.4a**	**3.38 ± 0.7a**	**3.33 ± 0.6a**	**5.25 ± 0.3ab**
Wheat grains	**4.16 ± 0.2a**	**3.72 ± 0.3a**	**3.14 ± 0.2a**	**6.75 ± 0.2a**
Wheat bran	1.97 ± 0.1b	2.20 ± 0.2b	2.53 ± 0.2b	6.50 ± 0.3a
Apple leaves	2.58 ± 0.1b	2.29 ± 0.3b	0.90 ± 0.1c	2.19 ± 0.2c
Carrot peels	1.59 ± 0.2b	1.66 ± 0.2c	2.31 ± 0.2b	2.84 ± 0.3c
Orange peels	1.17 ± 0.1c	0.72 ± 0.1d	2.44 ± 0.2b	2.42 ± 0.2c
Bamboo leaves	0.27 ± 0.2d	0.81 ± 0.3d	0.69 ± 0.3c	0.71 ± 0.3d
Pine needles	1.13 ± 0.1c	2.35 ± 0.1b	2.10 ± 0.5b	2.35 ± 0.3c
Eucalyptus leaves	0.45 ± 0.1d	0.44 ± 0.3d	0.71 ± 0.3c	0.29 ± 0.2d

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3). Values in bold indicate significant ones.

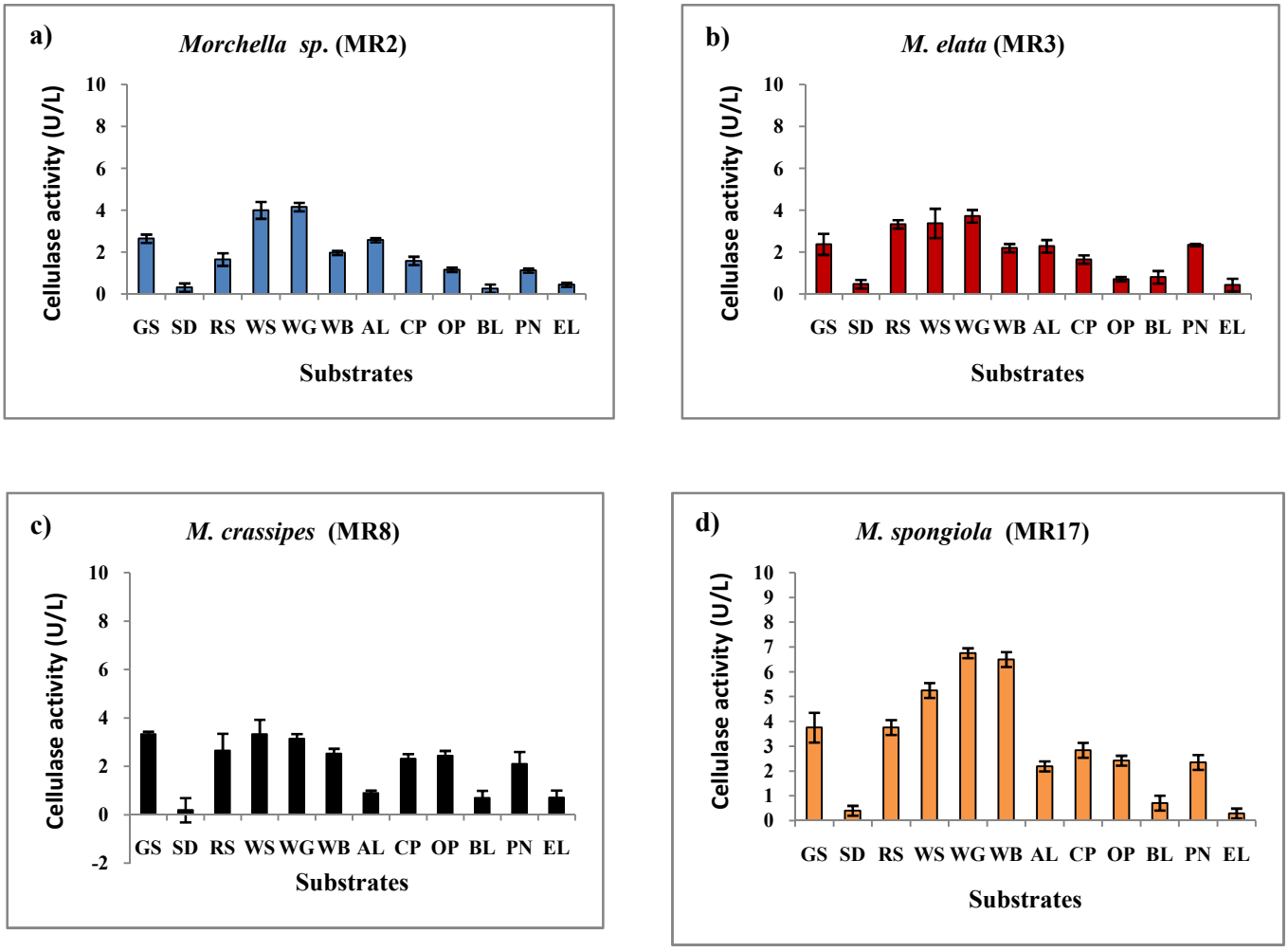


Fig 6.12: Cellulase activity (U/L) of different *Morchella* spp. a) *Morchella sp*. (MR2) b) *M. elata* (MR3) c) *M. crassipes* (MR8) and d) *M. spongiola* (MR17), inoculated on different substrates. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3)

6.5.9 Characterisation of substrates

The substrates were characterized for the ash, cellulose and lignin content in them. Table 6.9 shows the % ash, cellulose and lignin content of the substrates used in the study.

Ash content was found to vary with the substrates used in the study. Maximum ash content was observed in case of apple leaves, bamboo leaves and pine needles. Cellulose content was observed to be the highest in agricultural residues rather than other substrates. Maximum cellulose content was observed in case of wheat bran and wheat grains, followed by wheat straw and rice straw. Lignin content was observed to vary with the substrate used in the study. Maximum lignin content was observed in sawdust and pine needles, followed by groundnuts shell, wheat straw, rice straw, and bamboo leaves.

Table 6.9: Chemical characterisation (%) of different substrates used for inoculation of different *Morchella* spp.

SUBSTRATES	Ash	Cellulose	Lignin
Groundnuts shell	23.0 ± 2.9ab	8.4 ± 1.8b	33.5 ± 1.8b
Sawdust	7.5 ± 1.1bc	8.5 ± 0.5b	85.3 ± 3.0a
Rice straw	12.5 ± 0.7b	18.0 ± 1.7ab	34.5 ± 4.2b
Wheat straw	5.0 ± 1.1c	19.7 ± 0.6ab	29.5 ± 1.2b
Wheat grains	11.5 ± 1.1b	27.7 ± 1.4a	19.4 ± 1.7c
Wheat bran	21.5 ± 1.4ab	28.8 ± 2.1a	17.3 ± 1.0c
Apple leaves	27.0 ± 2.4a	11.7 ± 0.3b	24.6 ± 1.7bc
Carrot peels	13.5 ± 0.8b	10.7 ± 1.5b	11.6 ± 1.2c
Orange peels	15.5 ± 1.2b	14.2 ± 0.5ab	12.8 ± 1.4c
Bamboo leaves	33.0 ± 0.1a	10.0 ± 1.2b	31.7 ± 1.4b
Pine needles	28.5 ± 0.5a	8.2 ± 2.5b	64.3 ± 2.1ab
Eucalyptus leaves	12.0 ± 1.6b	16.2 ± 0.7ab	12.0 ± 6.3c

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3)

6.5.10 Lignin degradation

The lignin degradation (%) varied with the substrates and *Morchella* spp. used. Highest lignin degradation was observed by *M. crassipes* (MR8) and *M. elata* (MR3) in wheat straw and rice straw as the substrates. Maximum lignin degradation was observed in (Tab 6.10, Fig 6.13); and *Morchella sp.* (MR2) and *M. spongiola* (MR17) were found to be poor degraders (Tab 6.10, Fig 6.13). In *Morchella sp.* (MR2), maximum lignin degradation was observed in rice straw and pine needles as the substrate, followed by wheat straw, orange peels and wheat straw. Least or no lignin degradation was observed in saw dust and eucalyptus.

Morchella elata (MR3) was found to degrade wheat straw the maximum, followed by rice straw degradation. Other substrates that were degraded included wheat bran, apple leaves, groundnuts shell and apple leaves. Least degradation was observed in sawdust and eucalyptus leaves. In *M. crassipes* (MR8), maximum lignin degradation was observed in wheat straw followed by rice straw. Lignin degradation was also observed in groundnuts shell, orange peels, wheat bran, pine needles, carrot peels and wheat grains as the substrates.

Morchella spongiola (MR17) was found to be degraded the least. Maximum degradation was observed in wheat straw, followed by wheat bran. Other substrates that were degraded include pine needles, rice straw, and wheat grains. Significantly less degradation was observed in *Morchella sp.* (MR2) and *M. spongiola* (MR17) as compared to *M. elata* (MR3) and *M. crassipes* (MR8) (Table 6.10, Fig 6.13).

In the present study, lignin degradation was observed to be carried out by a combined effect of all these three ligninolytic enzymes (laccase, lignin peroxidase and manganese peroxidase); though the amount of laccase production was higher in most of the substrates used compared to the amount of manganese peroxidase and lignin peroxidase. The enzyme activities were comparatively higher in agricultural substrates such as, wheat grains, wheat straw and rice straw, than other substrates.

The results of ligninolytic activities on different substrates showed that wheat straw, rice straw and wheat grains were observed to be the best for ligninolytic enzyme production in *Morchella* spp. *Morchella crassipes* (MR8) was found to be the best for high ligninoltic enzyme activity along with more lignin degradation on agricultural substrates; while *M. elata* (MR3), *M. spongiola* (MR17) and *Morchella sp.* (MR2) were found to be poor producers of ligninolytic activity and poor degraders of lignin.

Table 6.10: Lignin degradation (%) of different substrates used for inoculation of different *Morchella* spp.

SUBSTRATES	*Morchella sp.* (MR2)	*M. elata* (MR3)	*M. crassipes* (MR8)	*M. spongiola* (MR17)
Groundnuts shell	1.88 ± 0.15d	5.37 ± 0.55c	11.70 ± 2.00abc	1.07 ± 0.14b
Sawdust	0.20 ± 0.16e	ND	1.00 ± 0.40e	ND
Rice straw	4.48 ± 0.45b	9.70 ± 0.67ab	13.80 ± 2.10ab	1.49 ± 0.46b
Wheat straw	2.89 ± 0.37c	12.70 ± 0.77a	16.20 ± 2.70a	5.20 ± 0.30a
Wheat grains	5.45 ± 0.25ab	4.00 ± 0.06c	7.60 ± 1.10cd	1.81 ± 0.68c
Wheat bran	4.08 ± 0.75b	7.06 ± 0.13b	9.30 ± 1.10bc	3.34 ± 0.34ab
Apple leaves	1.48 ± 0.34d	6.40 ± 0.84b	2.90 ± 1.30de	1.47 ± 0.27b
Carrot peels	8.69 ± 0.40a	3.19 ± 0.41c	8.20 ± 1.00bcd	0.45 ± 0.15c
Orange peels	2.26 ± 0.24c	1.35 ± 0.06d	11.30 ± 1.30abc	0.45 ± 0.08 c
Bamboo leaves	1.19 ± 0.14d	1.79 ± 0.03d	1.79 ± 0.69e	ND
Pine needles	4.78 ± 0.51b	4.40 ± 0.50c	8.60 ± 1.30bcd	2.20 ± 0.20b
Eucalyptus leaves	ND	0.62 ± 0.03e	3.77 ± 0.93e	ND

Values sharing a common letter within the column are not significant at $P<0.05$. Values are Mean ± SEM (n=3)

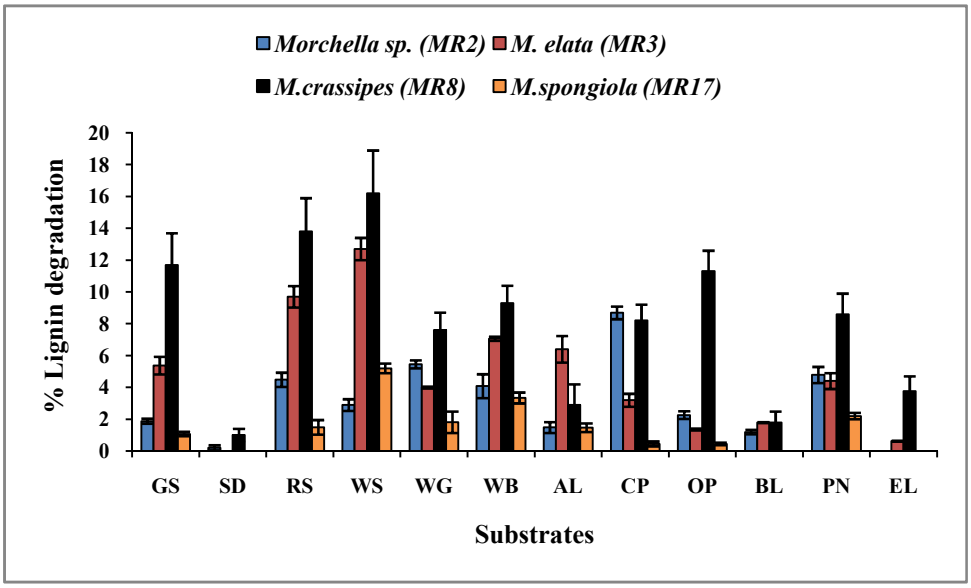


Fig 6.13: Lignin degradation (%) of different substrates used for inoculation of different *Morchella* spp. WS, wheat straw; RS, rice straw; SD, sawdust; AL, apple leaves; GS, groundnuts shell; EL, eucalyptus leaves; BL, bamboo leaves; OP, orange peels; PN, pine needles; WB, wheat bran; WG, wheat grains; CP, carrot peels. Values are Mean ± SEM (n=3)

6.6 Sclerotia formation on substrates in *M. crassipes* (MR8)

The method of Ower et al. (1986) was used to produce sclerotia. The hyphae grew through the garden soil and the perforated aluminium layer; and reached the substrates layer. Both wheat grains and wheat straw produced a large number of sclerotia (Table 6.11, Fig 6.14, 6.15, 6.16). White colored, circular to oval, small-sized sclerotia were formed within 15 days from the day of inoculation in *M. crassipes* (MR8). Sclerotia were formed mostly on the garden soil layer but some of the sclerotia were observed in substrates layer and on the walls

of the jar, too (Fig 6.14). Sclerotia of *M. crassipes* (MR8) turned to creamish in color on maturity. Enzyme activities were found to be highly influenced by the combination of soil and substrates, number of sclerotia formed with an increase in the number of incubation days.

Laccase activity was the only enzyme activity that was observed. No MnP or lignin peroxidase activity could be detected in the spent culture filtrate. High titers of laccase during period of sclerotia formation was observed in soil and wheat grain combination as compared to that in soil and wheat straw combination (Table 6.11 and 6.12). Maximum laccase activity was observed on day 24 of sclerotia maturation, when maximum number of sclerotia were formed in case of soil and wheat grains as the substrate; wherein soil and wheat straw combination, maximum laccase activity was observed on day 21 of sclerotia formation. In case of soil and wheat grains as the substrate; with an increase in the incubation period of sclerotia, an increase in laccase activity was observed. However, this trend was not observed in soil and wheat straw combination, where laccase activity increased till day 21 of sclerotia maturation.

The combination of soil and wheat grains yielded more cellulase activity as compared to soil and wheat straw combination (Table 6.11 and 6.12). But the detected level of cellulase activity was found to be constant whereas laccase activity (Ligninolytic activity) was found to vary during the phase of sclerotia formation and maturation.

Table 6.11: Enzyme activities (U/gm) and number of sclerotia formation by *M. crassipes* on soil and substrates (wheat grains).

Days	Soil and wheat grains			
	Lac (U/gm)	**Cel (U/gm)**	**Sclerotia no.**	**Size (cm)**
15	78.80 ± 10.64d	0.38 ± 0.03a	18.33 ± 7.09c	0.28 ± 0.01b
18	130.83 ± 8.80b	0.43 ± 0.02a	95.67 ± 10.26b	1.30 ± 0.12ab
21	141.00 ± 12.53a	0.41 ± 0.05a	99.33 ± 14.57b	1.92 ± 0.52ab
24	144.61 ± 16.92a	0.42 ± 0.09a	116.00 ± 9.54a	2.63 ± 0.84a
27	113.67 ± 6.26c	0.41 ± 0.02a	121.00 ± 19.85a	2.71 ± 0.76a

Values sharing a common letter within the column are not significant at P<0.05. Values are Mean ± SEM (n=3)

Table 6.12: Enzyme activities (U/gm) and number of sclerotia formation by *M. crassipes* on soil and substrates (wheat straw).

Days	Soil and wheat straw			
	Lac (U/gm)	**Cel (U/gm)**	**Sclerotia no.**	**Size** (cm)
15	7.34 ± 1.76c	0.066 ± 0.006a	21.33 ± 6.70d	0.16 ± 0.01b
18	19.05 ± 2.75b	0.07 ± 0.01a	67.33 ± 18.17c	0.58 ± 0.02b
21	25.31 ± 5.02a	0.068 ± 0.002a	74.00 ± 8.71b	1.14 ± 0.09ab
24	18.65 ± 2.21b	0.057 ± 0.004b	83.66 ± 8.74a	2.06 ± 0.12a
27	15.09 ± 3.12bc	0.056 ± 0.002b	84.33 ± 12.22a	2.31 ± 0.27a

Values sharing a common letter within the column are not significant at P<0.05. Values are Mean ± SEM (n=3)

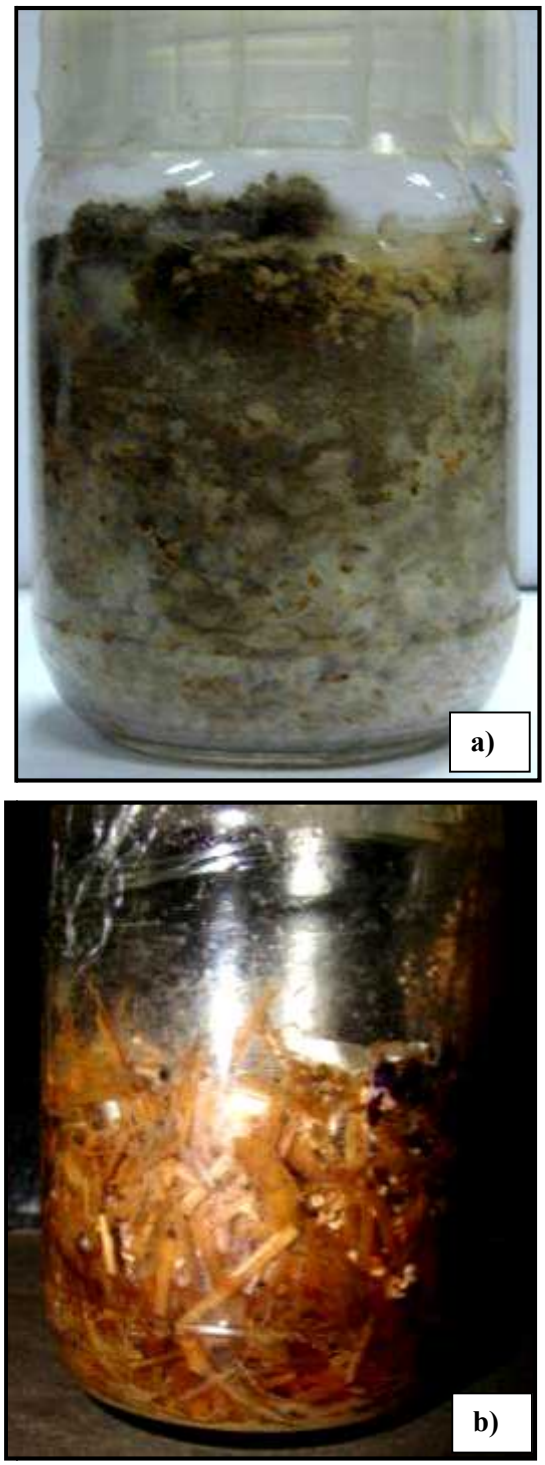


Fig 6.14: Sclerotia on different substrates a) *M. crassipes* (MR8) on soil and wheat grains combination and b) *M. crassipes* (MR8) on soil and wheat straw combination

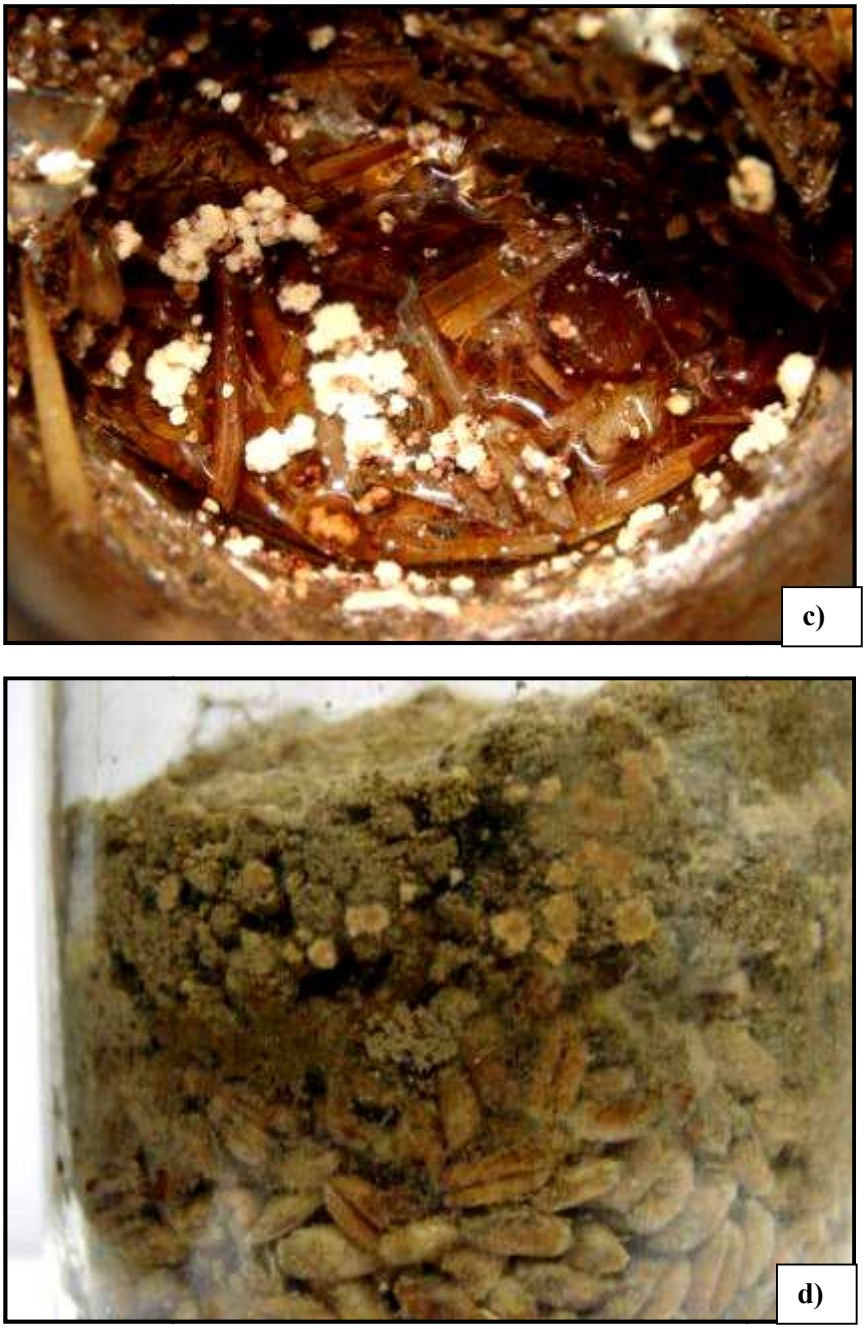


Fig 6.14: A closer view of sclerotia on different substrates c) *M. crassipes* (MR8) on soil and wheat grains combination and d) *M. crassipes* (MR8) on soil and wheat straw combination

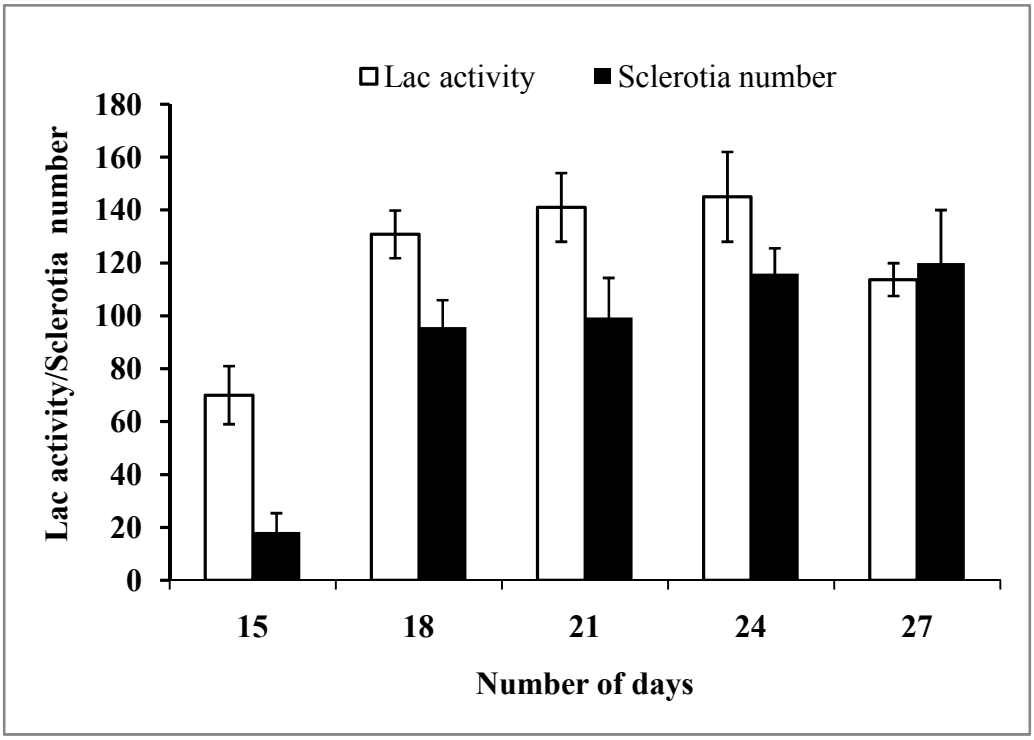


Fig 6.15: Laccase activity (U/gm) and number of sclerotia formation by *M. crassipes* (MR8) on soil and wheat grains. Values are Mean ± SEM (n=3)

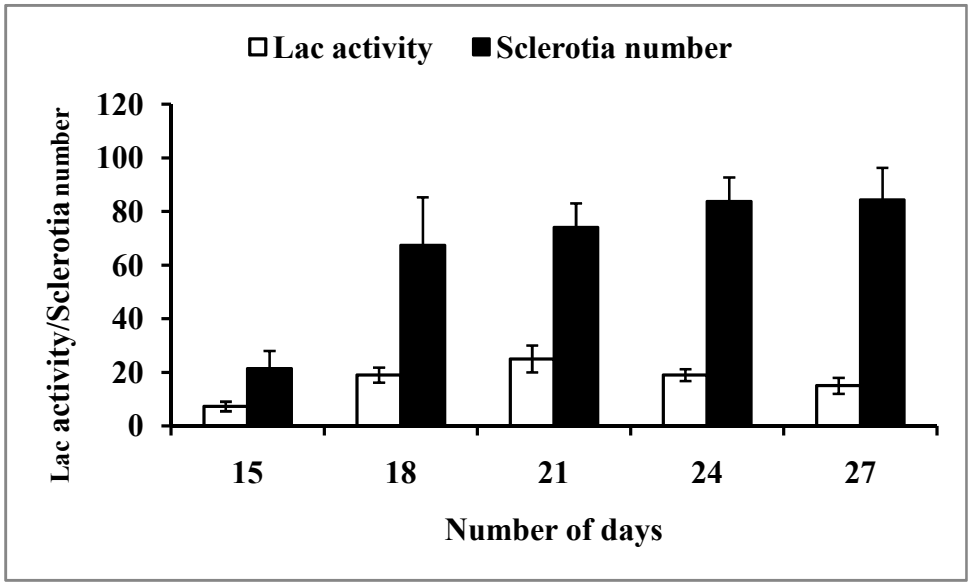


Fig 6.16: Laccase activity (U/gm) and number of sclerotia formation by *M. crassipes* (MR8) on soil and wheat straw. Values are Mean ± SEM (n=3)

Chapter VII

DISCUSSION

DISCUSSION

Diversity of *Morchella species*

Morchella, the true morels, (known as **"Guchhi"** in India), is a genus of edible mushrooms closely related to anatomically simpler cup fungi. Ascocarps of *Morchella* are highly prized for their culinary and medicinal values. The fruiting bodies of the *Morchella* are highly polymorphic in appearance, exhibiting variations in shape, color and size; that has contributed to uncertainties regarding taxonomy (Masaphy et al., 2010). Discriminating between the various species is complicated by uncertainty regarding which species are truly biologically distinct. Some authors suggest that the genus only contains as few as 3 to 6 species (Weber, 1988), while others place up to 50 species in the genus (Kimbrough, 1970).

There is no clear distinction in the classification of morels. Classical fungal taxonomy in morels, is dependent on the use of external morphological features. On the basis of color, morels are broadly classified as yellow morel and black morel. But species differentiation in morels cannot be solved merely on the basis of classical taxonomy. Nowadays, molecular data through phylogenetic analyses has given new insights in solving the species discrimination in morels (Stefani et al., 2010; O'Donnell et al., 2011).

The diversity of thirty-two different *Morchella* cultures/fruiting bodies, collected from the Western Himalayan region of India was studied in this study. The results showed that in the Western Himalayan region of India, both yellow (*M. crassipes*, *M. spongiola*) and black morels (*M. elata, M. angusticeps* and *M. gigas*) are present. Classical taxonomy involves the study of morphological descriptions such as type of fruiting body, type of ascus (a microscopic, unicellular, frequently globose, saccate, or cylindrical structure which often contains eight ascospores produced after meiosis, and is usually developed in the cavity of

fruiting bodies), shape, color and the opening of ascomata (Ainsworth, 1973). These macroscopic and microscopic features of fruiting bodies were analysed and compared with the classical features reported by Waraitch (1976). Based on these features, the fruiting bodies could be grouped together into two groups - one consisting of yellow morels (containing MR1, MR8, MR21, MR25, MR26, MR27, MR28, MR29, MR11, MR13, MR14, and MR20) with morphological features such as yellow or dirty white colored fruiting body, size of apothecia ranging from 15-18 cm, size of pileus (9-12 × 3-6), size of stipe (9-13 × 1-3), ascus (245-350 × 15-29), spore (21-28 × 11-16), color of pits (yellowish) and the other as black morels (containing MR3, MR6, MR23, MR24, FK, and F1) with morphological features such as grey or black colored fruiting body, size of apothecia (5-12 cm, size of pileus (6-10 × 4-6), size of stipe (6-9 × 2-4), ascus (230-390 × 18-26), spore (20-28 × 10-20), color of pits (brownish). In India, six species of *Morchella*: *M. deliciosa* Fr. (delicious morels), *M. esculenta* (L) Pers. ex Fr. (Common morel), *M. conica* Pers. (conical morels), *M. crassipes* (thick stemmed morel), *M. angusticeps* Peck. (Black morel) and *M. hybrida* (hybrid morel) have been reported by Waraitch (1976). The basis of differentiation of these species were the morphological characters i.e. base of the pileus free or adnate from stipe, color of the ribs and pits, size and shape of pileus and stipe.

The morphological details of the fungal specimens studied were not found to vary much so as to classify them up to species level. Among the yellow morels, species differentiation between *M. crassipes* and *M. esculenta* is very difficult, as there are minute variations in their morphology. Morphologically, these two are similar in color, but the size of apothecia (15-18.5 cm), stipe (10-12.5 × 1.8-2.2 cm), pileus (10-12 × 4.5-5.5 cm), ascus (255-325 × 18-25 µm), ascospores (21-26 × 12-15.5 µm) of *M. crassipes* differs from *M. esculenta* (the size of apothecia 15-19 cm, stipe 8.5-10 × 3.2-5.4 cm, pileus 7-8.6 × 5-6.2 cm,

ascus 215-355 × 16.5-25 µm, ascospores 17-25 × 11-13.5 µm) (Waraitch, 1976; Weber, 1988). Different developmental stages might lead to the variations in the morphological characters (Yoon et al., 1990; Lakhanpal et al., 2010).

Genetic studies could solve the problem of species differentiation. The development of molecular biology techniques for the genetic differentiation of species has resulted in substantial advances in taxonomy due to their sensitivity and specificity. For studying the molecular diversity of any fungi, the ideal region of choice for PCR amplification should be a) present in all fungi of interest, b) easy to amplify, c) preferentially amplified from fungi, when plant and fungal DNAs are mixed and d) variable enough to enable probes to be designed for several taxonomic hierarchies (species, genera, families) (Bruns and Gardes, 1993; Nilsson et al., 2005; Ryber et al., 2008). Genes coding for rRNA satisfy many of these criteria and thus, they have been extensively analyzed. Repeated copies of rDNA genes are present in the genome of both prokaryotes and eukaryotes. Molecular techniques based on variations in ITS region of nuclear rDNA amongst/within ectomycorrhizal fungal species are good candidates for distinguishing fungal taxa (Egger, 1995; Gardes and Bruns, 1996). The amplification of internal transcribed spacer (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) by the polymerase chain reaction (PCR) (Mullis and Faloona, 1987, Lanfranco et al., 1998), combined with sequencing of the amplicon and analysis of similarity between the sequences obtained and those already deposited in the GenBank, has been frequently employed for identification of fungal species.

In our study, PCR amplification of ITS-rRNA region of morels using ITS1 and ITS4 primers resulted in different size products ranging from 700 bp to 1,200 bp. Among the *Morchella* spp., sixteen isolates showed ITS size of ~1.2 kbp (MR1, MR5, MR8, MR9, MR11, MR12, MR13, MR14, MR18, MR20, MR21, MR25, MR26, MR27, MR28, and

MR29), eleven showed a size of ~750 bp (MR3, MR4, MR6, MR10, MR15, MR16, MR19, MR23, MR24, F1, and FK), and other species showed sizes of ~1000 bp (MR17), ~950 bp (MR2), and ~800 bp (MR7). The results are in accordance to Wipf et al. (1996, 1999), who observed ITS/5.8S rRNA gene region lengths of 740–760 bp (black morels) and 1150–1220 bp (yellow morels) by carrying out genetic studies of the whole of ITS/5.8S rRNA gene region in morels and clearly separated morels into black and yellow morels. In the present study, RFLP analyses of all 16 isolates using the 1.2 kbp ITS region with different restriction enzymes showed five different restriction patterns, resulting into five groups. Based on RFLP pattern of ITS region, Buscot et al. (1996) and Mcfarlane et al. (2005) classified morels into five major types.

Further, to classify the *Morchella* spp. up to species level, cloning and sequencing and sequence analyses of ITS/5.8S rRNA gene region was done in the present study. Sequence analysis using BLASTN revealed seven fruiting bodies (MR1, MR5, MR8, MR12, MR18, MR20 and MR21) having the ITS size of 1.2 kbp to be *M. crassipes*; eight (MR3, MR4, MR6, MR10, MR23, F1 and FK) having an ITS size of 750 bp to be *M. elata*; one of them (MR17) having ITS size of 1.2kbp as *M. spongiola*; and the other (MR2) having ITS of 955 bp to be a new species of *Morchella* as it did not correspond to any similar sequence, and was designated as *Morchella sp.* (MR2). Sequence data from BLAST results showed that out of thirty-two morel species studied, thirty belonged to *Morchella* species and the two were *Verpa* species (MR22 and FK).

The differences between M. *crassipes* and *M. esculenta* at the molecular level were even less evident from the reported NCBI databases. Kellner et al. (2005) differentiated *M. crassipes, M. spongiola* and *M. esculenta* based on the RFLP pattern of ITS fragments with different enzymes, such as *Alu*I, *Taq*I, *Mbo*I, *BsuR*I and *Msp*I. But the restriction patterns of

M. crassipes and *M. spongiola* in the present study were different for the same species reported by Kellner et al. (2005). In our study, among the cultures having ITS size of 1.2 kbp, (showing 99% homology to *M. crassipes)*, no *Alu*I site was reported except in one of the isolates (MR21). These results suggest that in yellow morels, species differentiation using restriction enzyme analysis in the ITS region does not consistently perform for morels at the species level. These results are contrary to those reported by Kellner et al. (2005). BLAST search using the 1.2 kbp ITS sequence from our collections had shown close similarity with *M. crassipes,* but not with *M. esculenta.* There are a few reports of the occurrence of *M. esculenta* from North India (Waraitch, 1976; Jandaik and Sharma, 1995), but the identification is entirely based on the external morphological features and not supported with molecular taxonomy. Hence the identification of *M. esculenta* (which might be *M. crassipes*) might be incorrect, as morphologically there are very small differences that exist between *M. crassipes* and *M. esculenta*. According to the available literature, six species of *Morchella* have been reported in India: *M. deliciosa, M. esculenta, M. conica, M. crassipes, M. angusticeps* and *M. hybrida* (Waraitch, 1976). Morphological variability of ascomata development is influenced by climatic and biogeographical conditions. Nevertheless, our study revealed six different (putative) species of *Morchella* and two different species of *Verpa* in the Western Himalayan region.

The phylogenetic trees separated the yellow (*M. crassipes* and *M. spongiola*) and black morels (*M. elata, M. angusticeps, M. conica* and *M. gigas*) into distinct clades. This data supports the findings of some of the researchers (Royse and May, 1990; Bunyard et al., 1994, 1995; Wipf et al., 1999; Stefani et al., 2010). Many authors have provided evidence for conspecificity of *M. esculenta, M. crassipes* and *M. deliciosa* (Gessner et al., 1987; Volk and Leonard, 1989; Yoon et al., 1990; Jung et al., 1993).

The phylogenetic tree did not resolve the relationships of some of the *Morchella* species of the present study with other morel clades. Within the crassipes groups, little intraspecific variation was found. Slight variation was found in *M. crassipes* (MR21) versus the other collections corresponding to *M. crassipes*. The variation could be due to geographical isolation of different areas. Bunyard et al., (1994, 1995), too reported variations in the ITS regions of *Morchella* isolates collected from two different areas due to geographical isolation. Misidentification of the species on the basis of morphological variations can be the other reason for variations in the ITS regions. More variation may occur between geographically isolated populations of the same species of *Morchella* than between two putatively distinct species (Yoon et al., 1990; Jung et al., 1993).

In the present study, black morels were grouped together into a single clade containing *M. conica*, *Morchella* sp. (MR2), *M. elata*, *M. gigas* and *M. angusticeps.* Phylogenetic studies of Bunyard et al., (1994, 1995); Wipf et al., (1996) and Singh et al., (2004), too, had shown, black morels as a separate clade with higher intraspecific variability. In the present study, *M. crassipes* and *M. elata* were found to be cryptic species. Similar results were observed by Taskin et al., (2010) where they discovered 15 putatively phylogenetically distinct but morphologically cryptic *Morchella* species using multigene phylogenetic approach from fruiting bodies collected in Turkey.

Similar studies on phylogenetic analyses of *Morchella* spp. in North America, too, had shown three discrete clades consisting of *Morchella rufobrunnea*, the yellow morels (*M. esculenta* and others), and the black morels (*M. elata* and others) (Kuo, 2009). Stefani et al. (2010) reported a new clade of morels i.e. tomentosa clade through phylogenetic analyses based on ITS-rRNA regions and nLSU gene regions by Bayesian and maximum parsimony methods. Strong genetic divergence was observed between *M. tomentosa* and the other

morels that suggested very different biological behaviour of tomentosa group. Phylogenetic analyses of morels based on coding genes are required to better resolve the relationships of the different morel clades and to increase understanding of their ecology (Stefani et al., 2010).

Within the yellow and black clades, there are dozens of individual species, most endemic to individual continents or regions (O'Donnell et al., 2011). Phylogenetic analyses in the study of O'Donnell et al. (2011) identified three lineages within *Morchella*: a basal monotypic lineage represented by *Morchella rufobrunnea*, and two sister clades comprising the black morels (Elata Clade, 26 species) and yellow morels (Esculenta Clade, 16 species). Molecular work of Masaphy et al. (2009) had identified a new species of *Morchella* named as *Morchella rufobrunnea* (MS5-IL1) by sequencing of its nuclear ribosomal internal transcribed spacer region and phylogenetic analyses. Similarly, Pilz et al. (2004) carried out morphological, genetic, and ecological data to identify and characterize five putative species found at their study sites.

The phylogenetic reconstructions clustered *Verpa* sequences into two clades comprising of bohemica and conica groups. Based on the phylogenetic analysis, MR22 and F2 were designated as *Verpa bohemica*. Phylogenetic studies by Bunyard et al. (1995) on the large subunit (28S) of the ribosomal DNA have separated *Morchella*, *Verpa*, *and Disciotis* from each other.

Physiological studies of a few *Morchella* species

The developmental form of an organism may be viewed as a result of the interaction of the physiology of an organism with environmental and nutritional factors (Nickerson and Edwards, 1946). These physiological studies dealing with nutritional and environmental

studies of fungi helps in the formulation of substrate for achieving the ultimate target of domestication under controlled conditions. So it is necessary to find out the differences in physiology of different species of *Morchella*.

Six different *Morchella* species (*M. elata* (MR3 and MR6), *M. spongiola* (MR17), *M. crassipes* (MR5 and MR8) were selected (on the basis of availability of pure cultures) for studying the physiological behaviour in different environmental and nutritional parameters. Morphological variations of different species of *Morchella* were carried out by studying the growth pattern in different media. Among the different media tested, *Morchella* spp. (*M. elata* (MR3 and MR6), *M. spongiola* (MR17), *M. crassipes* (MR5 and MR8)) were able to grow well in all the media tested with maximum biomass being produced in malt extract, mineral salts broth and potato dextrose broth. Potato dextrose agar and malt extract agar have been reported to be the most suitable media for mycelial growth of *Morchella* spp. In the studies of Kalm and Kalyoncu (2008) and Lakhanpal et al. (2010) on different media. Similarly, Guler et al. (1999) and Guler and Okazaya (2009) did extensive studies on the growth and morphology of *M. esculenta* and *M. conica* mycelia on potato dextrose agar, malt extract agar plates, wheat agar and complete medium yeast agar and found remarkable differences in colony morphology with different parameters. Mineral salts broth having major and minor elements too supported good growth of *Morchella* spp. in our study. Metals such as Boron (10 ppm) and calcium (5 and 10 ppm) and vitamins such as folic acid, ascorbic acid, nicotinic acid and biotin have been observed to be utilized well by *Morchella esculenta* (Devi and Sharma, 1999). Studies by Winder (2006) on the effect of different nutritional parameters on cultural characteristics of *Morchella elata* showed that maltose and potato dextrose media provided stress to the fungus, triggering slower growth and plumose culture margins or dark pigmentation.

Morchella spp. exhibited different growth patterns according to the different carbon and nitrogen sources used in the experiment. Maximum growth was recorded in case of glucose, fructose, mannose and cellobiose, whereas arabinose, sorbose limited the growth and caused changes in colony morphology. It was observed that each species exhibited different pattern of carbon utilization. Mannose and sucrose have been proved to be good carbon sources for *Morchella* growth (Kaul, 1977; Winder, 2006). Among the nitrogen sources studied, organic nitrogen sources (casein, tryptone, peptone and yeast extract) were found to be good for the growth rather than inorganic nitrogen sources. The physiology of *Morchella* vegetative mycelia has been relatively well studied compared with other aspects of the morel life cycle (Fron, 1905; Hawker, 1956). Brock (1951) and Kaul (1977) did an extensive study on the nutrition of *M. esculenta* in which they compared various carbon and nitrogen sources for their ability to support vegetative growth in liquid culture. They suggested that the physiology of different morel strains, *M. esculenta* and *M. crassipes* was quite similar.

The optimum range of temperature for vegetative growth of different *Morchella* spp. was found to be from 20 - 30 °C, with maximum growth observed at 25 °C in our study. *Morchella* mycelium was able to grow well even at 4 °C. Optimum pH range was found to be between 8.5 to 9.5. Similar results were obtained in the studies of Winder (2006) and Lakhanpal et al. (2010) in which optimum temperature of 16-24 °C and pH of 7 had supported the maximum growth in *Morchella spp*. In different studies by Devi and Sharma (1999) and Sud and Sharma (1999) on the growth studies of *Morchella sp.* with different nutritional and environmental parameters had concluded temperature optima of 20 °C and pH of 6.0 to be the best.

Enzymatic profiles

Morchella spp. are an important fungi due to its rich nutritional and medicinal properties. Carbon and nitrogen sources are important to study the physiology and metabolism of enzyme production by fungi. A correlation has been found between productivity parameters and some enzymatic profiles (Sainos et al., 2006). *Morchella esculenta* has been found to be efficient degrader of agrowastes and in turn a good producer of ligninolytic enzymes. Fungal ligninolytic enzymes are a diverse group of enzymes consisting of lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and a copper containing phenoloxidase, laccase (EC 1.10.3.2). The carbon and nitrogen sources having enhanced ability of producing the ligninolytic enzymes can be utilized further for producing morel fruiting bodies on commercial scale. To elucidate which of the media, carbon/nitrogen source influences the best for ligninolytic enzyme production, we studied the effect of these factors on ligninolytic enzyme production.

In the present study ligninolytic enzyme activity has been found to be highly influenced by different media, carbon and nitrogen sources and inducers. It was observed that mineral salts broth was observed to be the best media for laccase production. Arora and Gill (2005) had used mineral salts broth as a suitable media for laccase production in fungi. Among the different carbon sources tested, mannose served as the best source for laccase production in *M. crassipes* (MR8). In most of the fungi, glucose, mannitol, sodium gluconate and cellobiose are the efficient and rapidly utilized carbon sources for laccase production (Elisashvili et al., 2001, 2002). It is reported that apple (*Malus domestica*), a plant often associated with morels (Weber, 1988), produces root exudates that contain a major proportion of mannose and/or mannitol, depending on stage of growth of apple trees (Wittenmayer and Szabo, 2000).

Manganese peroxidase and lignin peroxidase activities were found to be absent in the media tested with different carbon and nitrogen sources. This may be due to the reason that manganese peroxidase and lignin peroxidase are inducible enzymes that require inducers to be expressed. Papinutti and Lechner (2008) reported that laccase was the only enzyme from the group of ligninolytic enzymes that was produced by *Morchella esculenta* and no manganese peroxidase or lignin peroxidase activity was detected at all in their study. Studies have shown that both the nature and concentration of nitrogen sources are powerful nutrition factors in ligninolytic enzymes (Elisashvili et al. 2001; Galhaup et al. 2002).

In this study, organic nitrogen sources peptone, yeast extract and casein were found to be the best sources for higher biomass production. Organic nitrogen sources were also found to be the best for higher laccase production compared to inorganic ones. These results were in line with other studies dealing with ligninolytic enzyme production (Arora and Gill, 2000, 2005; Mikiashvili et al., 2006).

Aromatic and phenolic compounds have been widely used to elicit enhanced ligninolytic enzyme production by different organisms and the nature of compound that induces these enzyme activities differs greatly with the species (Leonowicz et al., 2001; De Souza et al., 2004). In the present study, these inducers were able to enhance as well as induce the ligninolytic enzyme activities. The chemical inducers such as, ABTS, veratrylalcohol and pyrogallol increased the laccase activity to significant levels. The positive inductive effect of these inducers on laccase has earlier been reported in many organisms including fungi (Ulmer et al., 1984; Ardon et al., 1998; Niladevi and Prema, 2008). There are only a few reports on the effect of inducers on lignin peroxidase and manganese peroxidase are available. Inducers such as, phenol red and rice straw produced the maximum manganese peroxidase activity; even though the amount produced was less in comparison to that in

basidiomycetes (Arora and Gill, 2005). Rice straw and veratrylalcohol were the potential inducers for lignin peroxidase activity. These agrowastes and lignin related compounds have been used as inducers for lignin peroxidises (Spiker et al., 1992).

Acid phosphatase activity and phytase activity differ among species of fungi (Bartlett and Lewis, 1973; Williamson and Alexander, 1975; Ho and Zak, 1979). Release and utilization of organophosphorus by trees consumes both energy and nitrogen. Spreading of mycelia through a large volume of soil and coming in contact with available, mineralized nutrients could be advantageous in low organic soils. In the present study, less acid phosphatase activity and phytase activity were detected in the culture filtrates whereas more acid phosphatase activity was detected by the mycelia of different *Morchella* species. In high organic soils, fungi with higher phosphatase and decompositional capacity might have a competitive advantage in obtaining nutrients (Ho, 1989).

Sclerotial studies

Fungal sclerotia are hard subterranean structures that are believed to act as a resting stage, resistant to unfavourable environmental or physiological conditions. They remain dormant until favourable conditions occur and they form new mycelium, or an ascocarp, and continue the life cycle (Alexopoulos et al., 1996; Nelson, 1996). Sclerotia in morels, remain dormant for long periods of time and resume growth on the return of favorable environmental conditions (Volk and Leonard, 1990). Two types of sclerotia- EES- early encrusting sclerotia (smaller in size) and LIS- Late isolated sclerotia (bigger in size) have been found in morels (Buscot, 1993a). LIS have similar temperature requirements, cold resistance and biochemical properties (quality and type of mycosporins) to the subterranean mycelial structure connected with ascocarp in nature. In the present work, sclerotial studies were carried out with different media and natural substrates. Sclerotia were observed in *M. elata* (MR3) and *M. crassipes*

(MR8), whereas the rest of the cultures did not produce any sclerotia. Malt extract medium, buscot medium and nutrient rich medium were found to be the best for sclerotia formation in our study. Development of sclerotia in morels had been reported by Robbins and Hervey (1959) and Kaul (1981) who recorded sclerotia formation on 2% malt extract media. Similarly, Mehta and Sharma (1992) and Buscot (1993b), too reported a large number of sclerotia on potato dextrose agar medium and Buscot medium, respectively.

Different cultures grown on the perimeter of the same potato dextrose agar and malt extract agar containing petriplates resulted in the mycelial meld in the centre of the plate. Mycelia meld is a ridge of hyphae forming at the juncture where growth from the two colonies meets. Hervey et al. (1978) and Volk and Leonard (1989, 1990) reported these 'mycelial melds', and interpreted them as a secondary heterokaryotic mycelial phase in morels.

Cultures grown on Buscot media A, Buscot media B, nutrient rich media A, and nutrient poor B media resulted in the formation of numerous creamish to brown colored sclerotia. Growth of mycelia in split plates (Amir et al., 1993) having media as buscot media (buscot media A and buscot media B) and nutrient media (nutrient rich media A and nutrient poor B) produced less number of sclerotia. Many researchers have suggested that both nutrient rich and nutrient poor media were necessary for sclerotia formation in morels and sclerotia were formed only after the exhaustation of nutrients in the nutrient poor layer (Volk and Leonard, 1989; Amir et al., 1992; Faris et al., 1996). Amir et al. (1995) proposed a model for translocation during sclerotial development and showed turgor pressure gradients in fungi between source and sink by direct measurement. Experimental work by Singh and Verma (2000) had contradicted the fact that both nutrient rich and nutrient poor substrates are necessary for sclerotia formation in morels.

The substrate selected for the production of sclerotia should have an optimum carbon and nitrogen source that can yield large number of sclerotia in less time span (Kues and Liu, 2000). In this study, dense vegetative growth was found in all the carbon sources by both the cultures. Carbon sources such as starch, maltose, fructose, glucose, glycogen and sucrose have been reported to be good carbon sources for all the *Morchella* spp. whereas ribose, sorbose, rhamnose, tartaric acid and malic acid as poor sources for mycelia growth (Kaul, 1978, 1997, 1981; Winder, 2006). Both *M. elata* (MR3) and *M. crassipes* (MR8) showed different patterns of sclerotia formation with different carbon sources. Large sized and more sclerotia were formed by *M. crassipes* as compared to *M. elata* in many carbon sources. Sugars have been found to produce many sclerotia due to turgor pressure created by them (Amir et al., 1992, 1995). Previous reports indicated that carbon sources such as glucose, sucrose, maltose and starch are better sources for sclerotia formation in *Morchella* spp. (Volk and Leonard, 1989; Guler and Ozkaya, 2008). In the present study, mannitol produced large sized sclerotia in both the cultures. Many sclerotia forming fungi excrete water and soluble carbohydrates during the early stages of sclerotium development. This leads to a decrease in endogenous mannitol, causing a change in internal physiological mechanism, thus allowing development of large sized sclerotia (Cooke, 1969). Different substrates and soil amendments, too, have been found as the important factors affecting the sclerotia formation in *Morchella esculenta* (Pal et al., 1999; Sharma and Devi, 2000).

Among the different nitrogen sources, sodium nitrate and yeast extract served good source for sclerotia formation in both the cultures. Casamino acids and yeast extract in combination with rye have been reported to enhance sclerotia formation in *M. crassipes* (Volk and Leonard, 1989). The addition of potassium nitrate and starch to boiled wheat grain (in the ratio of 1:4:400) respectively, were found to produce high yields of *M. esculenta*

sclerotia within 35 to 40 days when grown at a temperature of 25 ºC without light (Sharma et al., 1997).

Sclerotia formation on lignocellulosic substrates and enzyme activities

Due to the structure, porosity and nutritional status of substrates, fungal mycelium was able to grow well on orange peels, carrot peels, groundnuts shell and wheat. But all the substrates failed to produce sclerotia during incubation at 25 °C. Sclerotia were not observed even after cold stress given to the mycelia as described by Ower et al. (1986). Morels have been found to grow well on citrus wastes (Labaneiah et al., 1979), wheat bran, and wheat bran plus corn starch (Papinutti and Lechner, 2008). Substrates such as wheat and vegetable wastes had been successfully used for cultivating morels (Ower et al., 1986). Recent report by Miller (2005) had emphasized on the cultivation of morel along with apple trees in mycorrhizal association with each other.

Fungi have the capability to produce ligninolytic enzymes in the utilization of lignocelluloses (Kachlishvili et al., 2006; Isikhuemhen et al., 2009; Sun et al., 2004). Many enzymes are involved in the oxidative degradation of lignin, most being laccases, manganese peroxidase and lignin peroxidase. Lignin peroxidase and manganese peroxidase are heme containing glycoproteins, which require H_2O_2 as an oxidant.

The present study revealed that the ligninolytic enzyme activities exhibited remarkable differences against different substrates. The substrates affected strongly the production of laccase. Laccase activity was detected in all the substrates; although enzyme activity varied to a large extent with substrates. Maximum laccase activity was found in wheat grains, wheat bran, wheat straw, rice straw, pine needles as the substrates. Studies on the growth of *Morchella* mycelium on wheat, wheat straw, and wheat bran have shown them to be good inducers for laccase activity (Papinutti and Lechner, 2008; Elisashvili et al., 2008,

2009). Fruit residues have been shown as appropriate growth substrates for the laccase production by many researchers (Songulashvili et al. 2006; Rosales et al. 2007; Elisashvili et al. 2008).

The lignified materials, wheat straw and rice straw appeared to be the most appropriate for the manganese peroxidase secretion in the present study. But the detected levels of manganese peroxidase activity were less as compared to other basidiomycetes, where these lignified materials, namely, wheat and rice straw, fruiting peelings and plant parts have been reported as efficient manganese peroxidase inducers (Kapich et al., 2004; Elisashvili et al., 2009).

Like manganese peroxidase, lignin peroxidase activity too was produced in less amounts as compared to other ligninolytic fungi. Lignin peroxidase activity was found to be the maximum in lignin related substrates such as, rice straw, wheat straw, wheat grains, wheat bran, pine needles, apple leaves and groundnuts shell. These natural substrates and lignin related compounds have been reported to be good inducers for lignin peroxidases (Spiker et al., 1992).

Variations among the cellulase enzyme activity were observed with different substrates used in the present study. Maximum cellulase activity was observed in wheat straw, groundnuts shell and wheat grains followed by wheat bran, rice straw, carrot peels, orange peels and pine needles. The cellulase activity was produced in less titers compared to ligninolytic enzyme activities studied in this case. This shows that *M. crassipes* can have low cellulolytic activity and high ligninolytic activity. Similar results were observed by Liew et al. (2011) where they observed high ligninolytic activity and low cellulase activity of white rot fungi for lignin degradation of *Acacia mangium* wood chips.

The lignin content and lignin degradation of substrates varied with the substrates used. Lignin degradation was found to be the maximum in wheat straw and rice straw. Extracellular ligninolytic enzymes are involved in the lignin degradation and the role of these has been extensively studied in fungi (Lopez et al., 2007; Isikhuemhen et al., 2009). In some of the fungi, laccase acts as a sole source for lignin degradation (Eggert et al., 1996; Pointing et al., 2000; Papinutti and Lechner, 2008), whereas in others, all of these enzymes are responsible for the lignin degradation. A combined effect of all these three enzymes was observed to influence lignin degradation; even though the amount of laccase production was higher in most of the substrates used in the study as compared to the amount of manganese peroxidase and lignin peroxidase. The enzyme activities were comparatively higher in agricultural substrates. In some of the fungi, laccase acts as a sole source for lignin degradation (Eggert et al., 1996; Pointing et al., 2000; Papinutti and Lechner, 2008) whereas in others, all of these three enzymes (Lac, MnP and LiP) are responsible for the lignin degradation. These agricultural substrates are being used on commercial scale for mushroom cultivation. The use of these sources for enhanced ligninolytic enzyme production plays an important role in improving the cultivation aspects in morels.

Ligninolytic enzyme production during sclerotia formation and maturation

To study the relationship of sclerotia and ligninolytic enzymes produced during the formation and maturation of sclerotia, the mycelia of *Morchella crassipes* (MR8), was grown in the soil and substrate combination by the method described by Ower et al. (1986). The culture that produced the highest ligninolytic activity (*M. crassipes* (MR8)) was selected for carrying out this study. Sclerotia were produced in considerable numbers on both the substrates (wheat grains/wheat straw) and soil layer. Wheat grains and wheat straw supported sclerotia formation with the method of Ower et al. (1986) used in the present study. The mycelium was

inoculated on soil layer, where it flourished with the period of time; and colonised the lower substrate layer. Nutrient deficiency (soil layer) might have triggered the formation of sclerotia. White colored, circular to oval, small-sized sclerotia were formed within 15 days after the inoculation of *M. crassipes.* Sclerotia were formed mostly on the soil layer but some of the sclerotia were observed in substrate layer and on the walls of the jar, also. Sclerotia of *M. crassipes* turned to creamish in color on maturity. The number of sclerotia increased with an increase in incubation period. Sclerotia formation was observed only on soil and substrates combination; but not with substrates alone. This shows that the combination of nutrient poor and nutrient rich substrate is an essential condition for sclerotia formation in morels. Both the structure and composition of substrate are the main factors for sclerotia formation in morels (Volk and Leonard, 1989; Amir et al., 1995). Singh et al. (1999) reported large number of sclerotia on different agricultural residues and sand layers. Sharma (2001) carried out extensive experimental studies on the soil collected from the morel bearing sites; no direct relationship between the soil pH and morel growth could be traced in their experiment, even though positive relation was observed between the growth of *M. esculenta* and nutrient supply (Sharma et al., 2001). Recently, Masaphy (2010) reported the successful fruiting body formation of *Morchella rufobrunnea* from sclerotia on soil and wheat grain combination. In his study, the fungal mycelium was inoculated into a sterile layer of potting soil placed above the layer of nutritionally rich medium based on wheat grains.

Sclerotia formed on substrates and soils combination were larger than the sclerotia formed in the synthetic media. Sclerotia formed on agricultural residues have been reported to be produced in large numbers and sizes as compared to media (Volk and Leonard, 1989; Singh et al. 1999; Sharma and Devi, 2006).

For commercial mushroom production, fruiting body development is thus often induced by vegetative mycelium by nutrient poor layer (Scarse and Elliot, 1998). Moreover, water shock and depletion of nutrients are the main factors that are required for the the induction of sclerotia to form fruiting body (Ower et al., 1986). Further, for the induction of sclerotia to form fruiting body, it is important to keep a balance between carbon and nitrogen sources (Mandelin, 1956). Since the carbon sources utilized by fungi are usually of a lignocellulosic character, the fungi during vegetative growth produce a wide range of enzymes to degrade the lignocellulosic substrates. The enzymes are peroxidises and laccases for lignin degradation and various types of glucanases, cellulases and xylanases for cellulose and hemicellulose degradation (De Groot et al., 1998). Studies have proved that low concentration of nitrogen in substrates enhances the sclerotia formation (due to depletion of nutrients) and consequently, low concentration favours laccase activity (De Groot et al., 1998).

Laccase activity was the only enzyme activity that was observed along the course of sclerotia formation and maturation and MnP or LiP activity could not be detected. Maximum laccase activity was observed on day 24 where maximum numbers of sclerotia were also observed in case of soil and wheat grains as the substrate. On soil and wheat straw combination, maximum laccase activity was observed on day 21 of sclerotia formation. In case of soil and substrate combination; an increase in laccase activity along with an increase in sclerotia number was observed. There was a positive correlation between sclerotia number and laccase activity (for soil and wheat grain combination r=0.85; for soil and wheat straw combination r=0.73). Cellulase activity (Non-ligninolytic activity) was determined in the present study in order to study the role of this enzyme during sclerotia formation and maturation. The combination of soil and wheat grains yielded more cellulase activity as compared to soil and wheat straw combination. But the detected level of cellulase activity

was found to be significantly less and constant whereas laccase activity (Ligninolytic activity) was found to be more and vary during the sclerotia formation. An increase in laccase activity but constant cellulase activity along with an increase in sclerotia numbers suggests that in *M. crassipes,* laccase activity plays an important role in sclerotia formation and maturation. Maximum activity of laccase in presence of wheat grains than wheat straw was observed in the present study. Researchers have shown that starch based substrates have a positive influence on laccase activity (Sainos et al., 2006; Papinutti and Lechner, 2008). Ligninolytic enzyme activity, particularly, manganese peroxidase activity, was found to be the main enzyme rather than cellulase enzyme that was responsible for growth and lignin degradation by *Trametes versicolor* and *Phellinus sp.* (Liew et al., 2011).

In the present study, high titers of laccase activity were detected during sclerotia formation as compared to the growth of mycelia on substrates alone. Sclerotial metamorphosis in filamentous fungi has been found to be induced in response to various oxidative and nitrogen stresses (Volk and Leonard, 1989; Amir et al., 1995; Georgiou et al., 2006). Laccase was found to be induced in response to nitrogen stress (Buswell, 1992). Many fungi are known to produce laccase during sclerotia formation (Chet and Huttermann, 1982; Ryan et al., 2003) and so laccase play an important role during fruiting body formation (Das et al., 1997; Chen et al., 2004). In *Aspergillus bisporus* and *Lentinula edodes*, enzyme activities have been found to vary with mycelium and fruiting bodies. Laccase activities were the highest just before fruiting body initiation and decline rapidly with aggregate formation. Similarly, Eriksson et al. (1990) showed the positive influence of ligninolytic enzymes along with mushroom production. Recently, Papinutti and Lechner (2008) and Zhang et al. (2010) studied the ligninolytic enzyme activities in *Morchella esculenta* on different agricultural substrates. High laccase activity was observed with wheat bran as the substrate. Sclerotia

were formed on the substrates but was not analysed quantitatively and also relationship between enzyme production and sclerotia number was not observed in their study.

In the present study, high titers of laccase activity was detected during sclerotia formation as compared to mycelia on substrates alone. As sclerotia is an intermediate stage during fruiting body formation in morels and as laccase has been observed to be produced in high amounts during the formation of sclerotia, these laccase have a positive impact on sclerotia formation in morels.

Many reports are available on the diversity of *Morchella* spp. from western countries. However in the present study, the diversity, physiology and cultivation attempts of *Morchella* spp. collected from western Himalayan region of India, have been studied. As indicated previously, *Morchella* spp. have been classified up to species level only on the basis of morphological descriptions, so there is a lot of confusion regarding the species differentiation in them. No study has been carried out for the genetic diversity and identity of morels occurring in the western Himalayan region of India. In the present study, diversity of *Morchella* spp. has been carried out by both classical methods along with molecular methods by studying the phylogenetic relationships between them. Many reports on the ligninolytic nature of basidiomycetes are available (Elisashvili et al., 2009; Liew et al., 2011) but reports on the ligninolytic nature of ascomycetes are scarce. The secretion of ligninolytic enzymes is regulated to a large extent by carbon, nitrogen sources and various chemical and natural inducers. The present study focuses on the selection of selected carbon, nitrogen sources along with the inducers for higher growth and enhanced ligninolytic enzyme production by *Morchella* species.

In the study of Papinutti and Lechner (2008), ligninolytic enzyme activities of *Morchella esculenta* were assayed but the relationship of ligninolytic enzyme activities with

lignin degradation was not studied. The present study points out a decrease in lignin content of the substrates along with an increase in laccase, manganese peroxidase and lignin peroxidase enzyme activities, indicating the ligninonlytic nature of *Morchella spp.*

Reports on the sclerotia formaion on lignocellulosic substares are available, but there is no information about the relationship of ligninolytic enzymes and sclerotia formed on substrates. In the present study, increase in laccase activity alongwith increase in number of sclerotia were observed in *M. crassipes.* As sclerotia is an intermediate stage during fruiting body formation in morels and as laccase has been observed to be produced in high amounts during the formation of sclerotia, these laccase have a positive impact on sclerotia formation in morels.

From these studies, it could be concluded that in the Western Himalayan region of India, both yellow (*M. crassipes, M. spongiola*) and black morels (*M. elata, M. angusticeps, and M. gigas*) are present. *Morchella* species has the ability to degrade lignin by producing ligninolytic enzymes during its growth on various substrates. Laccase activity was found to produce in high titers during sclerotia formation. To the best of our knowledge, there is no data available on laccase being produced during sclerotia formation and maturation in ascomycetous fungi and that too, by morel mushroom, *M. crassipes.* Moreover, laccase has been observed to play an important role during sclerotia formation and maturation in morels. This important role of laccase production during sclerotia formation can be exploited further for morel cultivation on substrates.

The study helps in understanding the diversity of morels and the mystery behind its cultivation using sclerotia. Cultivation of morels not only improves the socioeconomic conditions of the locals of the higher Himalayan region, but also helps in conserving Himalayan Biodiversity.

Salient features

- The diversity of *Morchella* species from the Western Himalayan region of India was studied.
- Amplification of ITS/5.8S rRNA region of morels resulted in different size products ranging from 700 bp to 1200 bp. Out of total thirty two different morphotypes, sixteen isolates showed ITS size of ~1.2 kbp (MR1, MR5, MR8, MR9, MR11, MR12, MR13, MR14, MR18, MR20, MR21, MR25, MR26, MR27, MR28 and MR29), eleven showed a size of ~750 bp (MR3, MR4, MR6, MR10, MR15, MR16, MR19, MR23, MR24, F1 and FK), two of 650 bp (MR22 and FK) and others showed sizes of ~1000 bp (MR17), ~950 bp (MR2), and ~800 bp (MR7).
- Phylogenetic analysis clustered all *Morchella* sequences into two clades differentiating them into yellow and black morles. These include *M. elata*, *M. gigas*, *Morchella* sp., *M. spongiola*, and *M. crassipes*.
- *Morchella* spp. exhibited different growth patterns to different nutritional and environmental conditions. Malt extract, mineral salts broth and potato dextrose broth served as the best media for the growth of *Morchella* spp.
- Carbon sources such as glucose, fructose, mannose and cellobiose were utilized well by all the *Morchella* spp.
- Organic nitrogen sources (casein, tryptone, peptone and yeast extract) were found to be good sources for growth rather than inorganic nitrogen sources.
- Optimum range of temperature for the growth of *Morchella* spp. was found to be from 20 - 30 °C and pH range was between 8.5 and 9.5.
- Laccase activity increased in the presence of mannose, rhamnose, galactose, ribose, sorbose and mannitol. *Morchella crassipes* (MR8 and MR5) produced highest laccase activity using mannose as the carbon source.

- Organic sources increased the laccase production as compared to the inorganic ones with peptone and casein being the best. Maximum laccase activity was observed in peptone followed by casein in *M. crassipes* (MR8).
- Rice straw, groundnut shell, wheat straw, pine needles, ABTS and veratrylalcohol appeared to be the best for laccase production. Maximum laccase activity was observed in *M. crassipes* (MR8 and MR5) in rice straw and ABTS as the substrates.
- Split plate technique showed that both *M. elata* (MR3) and *M. crassipes* (MR8) were able to form small sized, cream colored sclerotia in nutrient rich and Buscot media.
- Carbon sources, such as ribose, cellobiose, galactose, glucose, xylose, sucrose and mannitol served the best carbon sources for sclerotia formation. Yeast extract and $NaNO_3$ promoted the formation of small coloured sclerotia.
- Different substrates such as wheat straw, rice straw, apple leaves, groundnuts shell, eucalyptus leaves, bamboo leaves, orange peels, pine needles, wheat bran, wheat grains, and carrot peels supported the growth of *Morchella* spp.
- *Morchella* spp. were found to produce ligninolytic enzymes during their growth on different substrates. Substrates such as wheat grains, wheat bran and wheat straw served the best substrates for ligninolytic enzyme production.
- Large number of sclerotia was formed on combination of soil and substrate (wheat starw and wheat grains) along with the ligninolytic enzyme activities during the course of sclerotia formation and maturation in *M. crassipes* (MR8).
- Laccase activity plays an important role in sclerotia formation and maturation in *Morchella crassipes* (MR8).

SUMMARY

Summary

The Himalayan region in India is known for its great biodiversity. The Himalayan morel (*Morchella*) popularly known as "Guchhi" is an economically important, edible and the most desirable wild mushroom, which belongs to ascomycetes. Morels are highly prized mushrooms known for their medicinal and nutritional properties. In India, morels grow abundantly in Jammu and Kashmir, Himachal Pradesh and Uttaranchal during March to May and during August to September. Black fleshed fruiting bodies are produced by *M. angusticeps*, *M. elata* and *M. conica* while *M. esculenta*, *M. crassipes* and *M. deliciosa* produce yellow/white fruiting bodies. Morels have been important to mankind since a long time but yet humans could not get hold of them till date. Even though, it has been an exciting field for research, still the taxonomy of morels is poorly known. Till date, classification of morels has been defined according to classical taxonomy. Due to similar morphological features among *Morchella* spp., there is a lot of confusion regarding their species identification. But, nowadays, the development and application of molecular techniques based on the analysis of Internal Transcribed Spacer (ITS), D1 & D2 regions of Large Subunit (LSU) of 28S rRNA, protein coding genes such as RNA Polymerase II subunit (*RPB1, RPB2)* and Elongation factor (*EF-1α)* amplified from extracted DNA, have been extensively used to study fungal diversity in natural environments.

Morels cannot be cultivated artificially due to the inability of sclerotium (intermediate stage) to fructify. The formation of a fruiting body from sclerotium requires very specific environmental conditions of nutrition, humidity and temperature. *Morchella* will usually generate new mycelium because the environmental requirements for the formation of fruiting body are less specific. The carbon sources utilized by fruiting body producing fungi are mostly of lignocellulosic character; so the fungi during vegetative growth produce a wide

range of extracellular enzymes to degrade the lignocellulosic substrates for lignin degradation. Extensive collection of morels each year and failed attempt of its domestication have originated the need to document the diversity of *Morchella* spp. so as to conserve it.

In the present study, the diversity of morels had been studied both by classical and molecular approach. The physiology of different *Morchella* spp. too had been studied in different nutritional and environmental conditions. Enzymatic parameters such as laccase, lignin peroxidase, manganese peroxidase, phytase and acid phosphatase were also studied. Optimal substrates for the formation, growth and maturation of sclerotia that can act as spawn cultures for fructification, have also been studied. Moreover, the study evaluates the ligninolytic enzyme activities and lignin degradation during growth of mycelium and sclerotia on *Morchella crassipes* on different substrates. Further, the role of these ligninolytic enzymes during the formation and maturation of sclerotia on combination of soil and substrates was studied.

Diversity of *Morchella* spp.

Different isolates of *Morchella* (MR1 to (MR2)9, F1, F2 and FK) were isolated/procured from the Western Himalayan region of India. The fruiting bodies were analysed through classical and molecular taxonomy so as to identify them up to species level. Macroscopic morphological details of specimens such as size, shape, color of apothecia, stipe, pileus, and spores were observed. The spore measurements were recorded on twenty-five randomly selected matured spores using Nikon *50i* eclipse microscope. Considerable variations in the morphological characteristics were found in the different *Morchella* species in this present study. Based on these features, the fruiting bodies could be grouped together into two groups - one consisting of yellow morels (containing MR1, (MR8), (MR2)1,

(MR2)5, (MR2)6, (MR2)7, (MR2)8, (MR2)9, MR11, MR13, MR14, (MR2)0) with morphological features such as yellow or dirty white colored fruiting body, size of apothecia ranging from 15-18 cm, size of pileus (9-12 × 3-6), size of stipe (9-13 × 1-3), ascus (245-350 × 15-29), spore (21-28 × 11-16), color of pits (yellowish) and the other as black morels (containing (MR3), (MR6), (MR2)3, (MR2)4, FK, F1) with morphological features such as grey or black colored fruiting body, size of apothecia (5-12 cm, size of pileus (6-10 × 4-6), size of stipe (6-9 × 2-4), ascus (230-390 × 18-26), spore (20-28 × 10-20), color of pits (brownish). The Scatter plot of spores of different fruiting bodies with the spore sizes clearly revealed that there are variations in the spores of different fruiting bodies, which may be due to different developmental stages or geographical isolation. Due to the similarity found in all the microscopic features in different *Morchella* spp., it was difficult to classify them up to species level.

To study the genetic variations among different *Morchella* spp. (MR1 to MR29, F1, F2 and FK) collected from different parts of western Himalayan region, ITS region of rDNA was used. Different size products, ranging from 700 bp to 1200 bp, were observed in the amplification reaction. Out of total thirty two isolates, ITS size of ~1.2 kbp was shown by MR1, MR5, MR8, MR9, MR11, MR12, MR13, MR14, MR18, MR20, MR21, MR25, MR26, MR27, MR28 and MR29; ITS size of ~ 750 bp was observed in MR3, MR4, MR6, MR10, MR15, MR16, MR19, MR23, MR24, F1 and FK; ITS size of 650 bp was shown in MR22 and FK, and ITS sizes of ~1000 bp, ~ 950 bp and ~ 800 bp were observed in MR17, MR2, and MR7, respectively. Restriction Fragment Length Polymorphism analysis of all 16 isolates having 1.2 kbp ITS region with restriction enzymes *TaqI, HinfI, AluI and MboI* showed 5 different restriction patterns comprising Group I with MR1, MR9, MR11, MR12, MR13 and MR14; group II with MR5, MR25, MR26, MR27, MR28 and MR29; group III with MR8;

group IV with MR18, MR20 and group V with MR21. Restriction digestion of 11 isolates having ITS size of 750 bp showed similar banding patterns. The ITS products of the representative fruiting bodies from each group of 1.2 kbp MR1, MR5, MR8, MR12, MR18, MR20 and MR21 and 750 bp MR3, MR4, MR6, MR10, MR23, MR24, F1 and FK as well as different-sized ITS products of the fruiting bodies MR2, MR7 and MR17 were cloned into *E. coli* DH5*α* cells and sequenced.

Sequence analysis using BLASTN revealed that sequences of seven fruiting bodies (MR1, (MR5), (MR8), MR12, MR18, (MR2)0 and (MR2)1) showed close similarity (99%) to *M. crassipes;* (MR17) to *M. spongiola* (similarity 99%); *Morchella* isolates (MR3), MR4, (MR6), MR10, (MR2)3, F1 and FK to *M. elata* (similarity 99%)*;* (MR2)4 to *M. angusticeps* (similarity 97%)*;* and MR7 to *M. gigas* (similarity 99%). (MR2) (ITS of 955 bp) did not correspond to any similar sequence in the NCBI databases indicating that this might be a new species of *Morchella*.

Phylogenetic analysis was performed by maximum likelihood method and Bayesian method. In case of *Morchella* diversity, phylogenetic reconstructions clustered all *Morchella* sequences into two clades differentiating yellow and black morels. Phylogenetic analysis clearly divided yellow morels into different groups; crassipes group, spongiola group, rufobrunnea group and esculenta group, whereas the black morels were divided into elata, conica, gigas, tomentosa, costata and angusticeps groups. *Morchella spongiola, M. esculenta, M. rufobrunnea* and *M. crassipes* belonged to yellow morels as they were grouped into a cluster that was supported by high bootstrap and PP values. Fruiting bodies/cultures of MR1, MR5, MR8, MR12, MR18, MR20, and MR21, belonged to the crassipes group, while MR17 belonged to the spongiola group. Phylogenetic analysis clearly clustered *M. elata, M. angusticeps, M. costata, M. tomentosa* and *M. gigas* into the clade of black morels.

Sequences such as F1, FK, MR4, MR3, MR 10, MR6 and MR23 were grouped with *M. elata*, MR24 was clustered with angusticeps group, and MR7 clustered to the gigas group. The sequence of (MR2) branched separately from the black morel cluster, (supported by high PP and BP values), and clustered with *M. tomentosa*, indicating a new species from India.

Similar results were observed in the phylogenetic tree of *Verpa*, where two clades- one consisting of bohemica and the other consisting of conica were observed. *Verpa* spp. (MR22 and F2) clustered with bohemica group, indicating them to be *V. bohemica*. The branch of (MR22 and F2) was supported by high BP and PP values.

Physiological studies of *Morchella* spp.

In the present study, morphological variations of different species of *Morchella* were carried out by studying growth and enzyme activities in different nutritional parameters such as media, carbon and nitrogen sources and environmental parameters such as pH and temperature. *Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR5), *M. elata* (MR6), *M. crassipes* (MR8) and *M. spongiola* (MR17) were able to grow well in all the media tested with best results being obtained in malt extract, mineral salts broth and potato dextrose broth. Pigmentation showed a variety of colors ranging from cream to light yellow to yellow brown and brown. In the present study, *Morchella* spp. exhibited different growth patterns according to the different carbon and nitrogen sources used in the experiment. Maximum growth was recorded in case of glucose, fructose, mannose and cellobiose, whereas arabinose, sorbose limited the growth. Maximum dry biomass was observed in cellobiose, followed by fructose in *Morchella sp.* (MR2) and *M. spongiola* (MR17). Among the nitrogen sources studied, organic nitrogen sources (casein, tryptone, peptone and yeast extract) were found to be good sources for growth rather than inorganic nitrogen sources. *Morchella sp.* (MR2) and *M.*

spongiola (MR17) produced significantly more biomass in yeast extract as the nitrogen source.

The optimum range of temperature for vegetative growth of different *Morchella* spp. was found to be from 20 - 30 °C, with maximum growth observed at 25 °C in our study. Optimum pH range was found to be between 8.5 to 9.5, with maximum vegetative growth recorded at pH 8.5 by *M. crassipes* (MR5).

Enzymatic profiles

Carbon and nitrogen sources are important to study the physiology and metabolism of enzyme production by fungi. In the present study, ligninolytic enzyme activity has been found to be highly influenced by carbon, nitrogen sources and inducers. All the *Morchella* spp. were able to produce high titers of laccase in mineral salts broth than other media. Mannose served as the best carbon source for laccase activity. Rhamnose, galactose, ribose, sorbose and mannitol, also produced high titers of laccase activity; whereas, laccase activity was very less in xylose, sucrose, glucose, fructose and cellobiose. *Morchella crassipes* (MR8 and MR5) were observed to be the best culture for laccase activity whereas *M. spongiola* (MR17) showed the poor laccase activity. Optimum amounts of laccase activity were observed in *Morchella sp.* (MR2), *M. elata* (MR3 and MR6). Among the different nitrogen sources studied, organic sources increased the laccase production as compared to inorganic ones with peptone and casein being the best. Among the natural inducers, rice straw, groundnuts shell, wheat straw and pine needles appeared to be the best for laccase production. Among the chemical inducers, 2,2-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid (ABTS) was the most efficient inducer for laccase production followed by veratrylalcohol by all *Morchella* spp. Other inducers such as DiNitrobenzoic acid (DNBA),

3,4-Dihydroxyphenylalanine (DOPA), 8-OHQuinoline, p-nitroaniline, ferulic acid, resorcinol, and eucalyptus leaves failed to induce the laccase activity as reported with other organisms. Maximum laccase activity was observed in *M. crassipes* (MR8 and MR5) in rice straw and ABTS as the substrates. Manganese peroxidase and lignin peroxidase activities were found to be absent in the media tested with different carbon and nitrogen sources, but were induced to small extent with chemical and natural inducers. Inducers such as ferulic acid, veratrylalcohol and rice straw were found to be positive inducers for manganese peroxidase activity in different *Morchella* spp. Maximum manganese peroxidase activity was produced in presence of phenol red followed by rice straw in *M. elata* (MR6). Lignin peroxidase activity was produced in considerable amounts in the presence of veratryl alcohol, rice straw, groundnuts shell and wheat straw. Maximum lignin peroxidase activity was observed in rice straw in *M. crassipes* (MR8). Rice straw and veratrylalcohol were the potential inducers for lignin peroxidase activity.

Sclerotial studies

Sclerotia are usually compact bodies of aggregated fungal hyphae. These sclerotia act as intermediate stages during fruiting body formation. To study the effect of different nutritional and environmental parameters on sclerotia formation, different *Morchella* spp. (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR8) and *M. spongiola* (MR17), were inoculated in different media in petriplates, test tubes and jars. Dual culture tests and split plate techniques were also used for sclerotia formation for these cultures. *Morchella elata* (MR3) and *M. crassipes* (MR8) were able to form small sized, cream colored sclerotia in nutrient rich and Buscot media.

To study the effect of different carbon and nitrogen sources, sclerotial formation was evaluated on Buscot agar media containing different carbon sources (10g/L) in place of dextrose and different nitrogen sources in place of sodium nitrate ($NaNO_3$) (250mg/L). In *M. crassipes* (MR8), ribose, cellobiose, galactose, glucose, xylose, sucrose and mannitol served the best carbon sources for sclerotia formation; whereas rhamnose, mannose, fructose, soluble starch and sorbose did not promote the sclerotia formation. In *M. crassipes* (MR8), sclerotia formed in xylose (average size - 0.2 cm) and sucrose (average size - 0.16 cm) were much smaller in size compared to mannitol (average size - 0.43 cm). In *M. elata* (MR3); sclerotia were smaller in comparison to the sclerotia formed by *M. crassipes*, and galactose, glucose, sorbose and mannitol promoted the formation of numerous small sized sclerotia. Rhamnose, cellobiose, mannose, xylose, fructose, sucrose and soluble starch didn't promote any sclerotia formation in *M. elata* (MR3). Sodium nitrate and yeast extract promoted the formation of small colored sclerotia in *M. elata* (MR3) and *M. crassipes* (MR8).

To study the effect of different substrates on sclerotia formation and enzyme activities, all the *Morchella* cultures (*Morchella sp.* (MR2), *M. elata* (MR3), *M. crassipes* (MR8) and *M. spongiola* (MR17)), were inoculated on finely chopped and steam sterilized substrates for sclerotial and enzymatic studies. Sclerotia were found to be absent on all the substrates tested. pH in most of the substrates increased to small amounts as compared to their respective controls. Maximum increase in pH was observed in orange peels by *M. elata* (MR3), followed by groundnuts shell in *M. crassipes* (MR8) and carrot peels by *Morchella sp*. (MR2) and *M. crassipes* (MR8). Highest protein concentration was observed in groundnuts shell, wheat straw, rice straw, wheat grains, wheat bran, carrot peels, orange peels and pine needles. Only small amounts of acid phosphatase were observed in the filtrates. Maximum acid phosphatase was observed in orange peels by *M. spongiola* (MR17), followed

by carrot peels by *M. crassipes* (MR8). Ligninolytic enzyme activity was highly influenced by different substrates. Substrates such as wheat grains, wheat bran and wheat straw served as the most promising substrates for laccase production. Maximum laccase activity was observed in wheat grains by *M. crassipes* (MR8) followed by *M. elata* (MR3). *Morchella sp.* (MR2) and *M. spongiola* (MR17) were found to be poor producers of laccase activity. Least or no activity was detected in bamboo leaves, eucalyptus and sawdust, respectively. Lignin peroxidase activity was found to be highly influenced in different substrates by different *Morchella* spp., even though the amount produced was less as compared to other basidiomycetous fungi. Maximum lignin peroxidase activity was observed in rice straw followed by apple leaves and wheat straw in *M. crassipes* (MR8). Maximum manganese peroxidase acticity was observed in wheat straw and rice straw by *M. crassipes* (MR8). Maximum cellulase activity was observed in wheat grains wheat bran and wheat straw by *M. crassipes* (MR17) followed by *Morchella sp.* (MR2). Along with ligninolytic enzyme activity, maximum lignin degradation was observed in wheat straw and rice straw as the substrates. Maximum lignin degradation was observed in *M. crassipes* (MR8) and *M. elata* (MR3). *Morchella sp*. (MR2) and *M. spongiola* (MR17) were found to be poor degraders.

The method of Ower et al. (1986) was used to produce sclerotia on combination of soil and substrate and the ligninolytic enzyme activities were observed during the course of sclerotia formation and maturation. Both wheat grains and wheat straw produced a large number of sclerotia. Laccase enzyme was produced in high titers during sclerotia formation and maturation of *M. crassipes* (MR8) on the combination of soil and wheat grains/wheat straw. The combination of soil and wheat grains was observed to be the best for large number of sclerotia (116 in number) production along with high amounts of laccase production (145 U/gm) on day 24^{th} of sclerotia maturation.

From the present study, it is concluded that in the Western Himalayan region of India, two types of morels occur- yellow morels comprising *M. crassipes* and *M. spongiola*; and black morels comprising *M. elata, M. angusticeps, Morchella sp.* (MR2), and *M. gigas*. Sequence analysis showed that out of thirty-two morphotypes of morels studied, thirty belonged to *Morchella* species and two were false morels i.e. *Verpa* species (MR22 and FK). Physiologically, a lot of variations were observed among these *Morchella* spp. in respect of different media, carbon and nitrogen sources, pH, temperature variations and enzyme activities such as laccase, lignin peroxidase, manganese peroxidase, acid phosphatase and phytase activity. These physiological variations might be due to different environmental or developmental stages among those *Morchella* spp. as all of them have been isolated from different parts of western Himalayan region of India. Sclerotial studies of these *Morchella* spp. with different media, carbon and nitrogen sources also concluded the variations among these species. The present work also deals with involvement of laccase enzyme by *M. crassipes* mycelium during sclerotia formation on agricultural substrates. So this type of study can be helpful in exploring the fungal biodiversity of India and for selecting the suitable isolates for cultivation purposes. The use of these sources for enhanced ligninolytic enzyme production plays an important role in improving the cultivation aspects in morels.

References

Ainsworth GC, Sparrow FK, Sussman AS (1973) The Fungi: An Advanced Treatise. Vol IVa and IVb, Academic Press, New York

Alexopoulos CJ, Mims CW, Blackwell M (1996) Introductory Mycology. 4th edn, Wiley & Sons, New York

Altschul SF, Gish W, Miller W, Myers FW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology **215:** 403-410

Amir R, Levanon D, Hadar Y, Chet I (1992) Formation of sclerotia by *Morchella esculenta*: relationship between media composition and turgor potential in the mycelium. Mycological Research **96:** 943-948

Amir R, Levanon D, Hadar Y, Chet I (1993) Morphology and physiology of *Morchella esculenta* during sclerotia formation. Mycological Research **97:** 683-689

Amir R, Levanon D, Hadar Y, Chet I (1995) Factors affecting translocation and sclerotial formation in *Morchella esculenta*. Experimental Mycology **19:** 61-70

Amir R, Steudle E, Levanon D, Hadar Y, Chet I (1995) Turgor changes in *Morchella esculenta* during translocation and sclerotial formation. Experimental Mycology **19:** 129-136

Aptroot A, Diederich P, Sérusiaux E, Sipman HJM (1997) Lichens and lichenicolous fungi from New Guinea. Bibliotheca Lichenologica **64:** 1-239

Ardon O, Kerem Z, Hadar Y (1998) Enhancement of lignin degradation and laccase activity in *Pleurotus ostreatus* by cotton stalk extract. Canadian Journal of Microbiology **44:** 676-680

Arkan O (1992) Hyphal fusions and heterokaryon formation betwen monoascosorous strains of *Morchella conica*. Turkish Journal of Biology **16:** 203-216

Arora DS, Gill PK (2000) Laccase production by some white rot fungi under different nutritional conditions. Bioresource Technology **73:** 283-285

Arora DS, Gill PK (2005) Production of ligninolytic enzymes by *Phlebia floridensis*. World Journal of Microbiology and Biotechnology **21:** 1021-1028

Baker BF, Matkin OA (1959) An unusual occurrence of morels in cultivated beds of *Cymbidiums*. Plant Disease Reporter **43:** 1032-1034

Bartelli I (1990) May is morel month in Michigan. Extension Bulletin E-614, Michigan State University, East Lansing, pp 22

Bartlett EM, Lewis DH (1973) Surface phosphatase activity of mycorrhizal roots of beech. Soil Biology and Biochemistry **5:** 249-252

Beilharz V, Cunnington J (2003) Two new closely related species of *Pseudocercospora* on unrelated host families from south-eastern Australia. Mycological Research **107:** 445-451

Berthet P (1964) Essai biotaxonomique sur les discomyce`tes. PhD Thesis, Faculte´ des Sciences de l'Universite´ de Lyon, Lyon, France

Bhattacharya B, Baruah HK (1953) Fungi of Assam. Journal of University of Gauhati **4:** 287-312

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72:** 248-254

Brock TD (1951) Studies on the nutrition of *Morchella esculenta* Fries. Mycologia **43:** 402-422

Bruns TD, Gardes M (1993) Molecular tools for the identification of ectomycorrhizal fungi: taxon-specific oligonucleotide probes for suilloid fungi. Molecular Ecology **2:** 233-242

Bruns TD, Szaro TM, Gardes M, Cullings KW, Pan JJ, Taylor DL, Horton TR, Kretzer A, Garbelotto M, Li Y (1998) A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. Molecular Ecology **7:** 257-272

Bruns TD, White TJ, Taylor JW (1991) Fungal molecular systematics. Annual Review of Ecology, Evolution, and Systematics **22:** 525-564

Bunyard BA, Nicholson MS, Royse DJ (1994) A systematic assessment of *Morchella* using RFLP analysis of the 28S ribosomal RNA gene. Mycologia **86:** 762-772

Bunyard BA, Nicholson MS, Royse DJ (1995) Phylogenetic resolution of *Morchella*, *Verpa* and *Disciotis* [Pezizales: *Morchellaceae*] based on restriction enzyme analysis of the 28S ribosomal gene. Experimental Mycology **19:** 223-233

Buscot F (1993a) Synthesis of two types of association between *Morchella esculenta* and *Picea abies* under controlled culture conditions. Journal of Plant Physiology **141:** 12-17

Buscot F (1993b) Mycelial differentiation of *Morchella esculenta* in pure culture. Mycological Research **97:** 136-140

Buscot F, Bernillon J (1991) Mycosporins and related compounds in field and cultured mycelia structures of "*Morchella esculenta*". Mycological Research **95:** 752-754

Buscot F, Kottke I (1990) The association of *Morchella rotunda* (Pers.) Boudier with roots of *Picea abies* (L.) Karst. New Phytologist **116:** 425-430

Buscot F, Munch JC, Charcosset JY, Gardes M, Nehls U, Hampp R (2000) Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbiosis in ecosystems. FEMS Microbiology Reviews **24:** 601-614

Buscot F, Roux J (1987) Association between living roots and ascocarps of *Morchella rotunda* (Pers.) Boudier. Transactions of British Mycological Society **89:** 249-252

Buscot FD, Wipf C, De Battista JC, Munch B, Martin F (1996) DNA polymorphism in morels. I. PCR/RFLP analysis of the ribosomal DNA spacers and microsatellite primed PCR. Mycological Research **100:** 63-71

Buswell JA (1992) Fungal degradation of lignin. In: Arora DK, Rai B, Mukerji KG, Kundsen G (eds) Marcel Dekker Handbook of Applied Mycology, Soil and Plants, Vol. 1, New York, pp 425-480

Butler EJ, Bisby GR (1931) The fungi of India. Indian Council of Agricultural Research India, Scientific Monograph 1. Calcutta, India

Carlile MJ, Watkinson SC (1994) The fungi. Academic Press, Ltd., London, UK

Carpenter SE, Trappe JM, Hunt GA (1981) Observations on fungal succession on recent volcanic deposits of Mount St. Helens. In: Evett JF (ed) Procceedings, 39th Meeting of the Oregon Academy of Sciences, pp 36-44

Chang ST (1991) Applied Mycology. In: Arora, DK, Mukerji KG, Marth EH (eds) Marcel Dekker Hand Book of Applied Mycology. New York, pp 221-240

Chapela IH (1991) Spore size revisited: analysis of spore populations using automated particle size. Sydowia **43:** 1-14

Chaverri P, Samuels GJ (2003) *Hypocrea/Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): Species with green ascospores. Studies in Mycology **48:** 116-118

Chen S, Ge W, Buswell JA (2004) Molecular cloning of a new laccase from the edible straw mushroom *Volvariella volvacea:* possible involvement in fruit body development. FEMS Microbiology Letters **230:** 171-176

Chet I, Huttermann A (1982) De novo synthesis of polyphenol oxidase (Laccase) during formation of sclerotia in *Sclerotium rolfsii.* FEMS Microbiology Letters **14:** 211-215

Chung H, Park M, Madhaiyan M, Seshadri S, Song J, Cho H, Sa T (2005) Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biology and Biochemistry **37:** 1970-1974

Cooke MC (1969) Kashmir Morels. Transactions of the Botanical Society Edinburgh **10:** 439-443

Criquet S, Farnet AM, Tagger S, Le Petit J (2000) Annual variations of phenoloxidase activities in an evergreen oak litter: influence of certain biotic and abiotic factors. Soil Biology and Biochemistry **32:** 1505-1513

Crowe JD, Olsson S (2001) Induction of Laccase Activity in *Rhizoctonia solani* by Antagonistic *Pseudomonas fluorescens* Strains and a Range of Chemical Treatments. Applied and Environmental Microbiology **67:** 2088-2094

Cullings KW (1994) Molecular phylogeny of the Monotropoideae with a note on the placement of the Pyroloideae. Journal of Evolutionary Biology **7:** 501-516

Dahlstrom JL, Smith J, Weber NS (2000) Mycorrhiza-like interaction by "*Morchella*" with species of the *Pinaceae* in pure culture synthesis. Mycorrhiza **9:** 279-285

Dalgleish HJ, Jacobson KM (2005) A first assessment of genetic variation among *Morchella esculenta* (Morel) populations. Journal of Hereditary **96:** 396-403

Das N, Sengupta S, Mukherjee M (1997) Importance of laccase in vegetative growth of *Pleurotus florida*. Applied and Environmental Microbiology **10:** 4120-4122

De Groot PWJ, Visser J, Van Griensven LJLD, Schaap PJ (1998) Biochemical and molecular aspects of growth and fruiting of the edible mushroom *Agaricus bisporus.* Mycological Research **102:** 1297-1308

De Souza, CGM, Tychanowicz GK, De Souza DF, Peralta RM (2004) Production of laccase isoforms by *Pleurotus pulmonaris* in response to presence of phenolic and aromatic compounds. Journal of Basic Microbiology **44:** 129-136

Devi P, Sharma BM (1999) Effect of vitamins on the mycelial growth and sclerotial production of *Morchella esculenta.* Indian Phytopathology **53:** 365

Devi P, Sharma BM (1999) Role of minerals on the mycelial growth and sclerotial production of *Morchella esculenta.* Indian Phytopathology **53:** 365

Dorn G, Rivera W (1966) Kinetics of Fungal Growth and Phosphatase Formation in *Aspergillus nidulans.* Journal of Bacteriology **92:** 1618-1622

Dunham SM, O'Dell TE, Molina R (2003) Analysis of nrDNA sequences and microsatellite allele frequencies reveals a cryptic chanterelle species *Cantharellus cascadensis* sp. nov. from the American Pacific Northwest. Mycological Research **107:** 1163-1177

Egger KN (1995) Molecular analysis of ectomycorrhizal fungal communities. Canadian Journal of Botany **73:** 1415-1422

Eggert C, Temp U, Eriksson KE (1996) The liginolytic system of the white rot fungus *Pycnoporous cinnabarinus*: purification and characterization of the laccase. Applied and Environmental Microbiology **602:** 1151-1158

Elisashvili V, Kachlishvili E, Penninckx M (2009) Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes. Journal of Industrial Microbiology and Biotechnology **35:** 1531-1538

Elisashvili V, Kachlishvili E, Tsiklauri N, Bakradze M (2002) Physiological regulation of edible and medicinal higher Basidiomycetes lignocellulolytic enzymes activity. International Journal of Medicinal Mushrooms **4:** 159-166

Elisashvili V, Parlar H, Kachlishvili E. Chichua D, Kvesitadze G (2001) Lignocellulolytic activity of Basidiomycetes grown under submerged and solid-state fermentation on plant raw material. Advances in Food Sciences **23:** 117-123

Elisashvili V, Penninckx M, Kachlishvili E, Tsiklauri N, Metreveli E, Khardziani T et al (2008) *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. Bioresource Technology **99:** 457-462

Eriksson KE, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer Series Wood Science, pp 407

FAO (2004) Wild edible fungi: A global overview of their use and importance to people, by Eric Boa, FAO NWFP Series No. 17, Rome, pp 147

Faris H, Broderick A, Nair NG (1996) Occurrence and Initial Observations of "*Morchella*" in Australia. In: Royse DJ (ed) Mushroom Biology and Mushroom Products - Proceedings of the Second International Conference. Pennsylvania State University, Pennsylvania, pp 393-399

Franz G (1989) Polysaccharides in pharmacy. Current applications and future concepts. Planta Medica **55:** 493-497

Fron G (1905) Sur les conditions de development du mycelium de la morille. Comptes Rendus Academy of Sciences, Paris **140:** 1187-1189

Gaitán-Hernández R, Salmones D (2008) Obtaining and characterizing *Pleurotus ostreatus* strains for commercial cultivation under warm environmental conditions, Scientia Horticulturae **118:** 106-110

Galhaup C, Wagner H, Hinterstoisser B, Haltrich D (2002) Increased production of laccase by the wood degrading basidiomycete *Trametes pubescens*. Enzyme Microbial Technology **30:** 529-536

Gardes M, Bruns TD (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Canadian Journal of Botany **74:** 1572-1583

Gargas A, JW Taylor (1995) Phylogeny of discomycetes and early radiation of the apothecial ascomycetes inferred from SSU rDNA sequence data. Experimental Mycology **19:** 7-15

Georgiou DC (1997) Lipid peroxidation in *Sclerotium rolfsii*: A new look into the mechanism of sclerotial biogenesis in fungi. Mycological Research **101:** 460-464

Georgiou CD, Patsoukis N, Papapostolou I, Zervoudakis G (2006) Sclerotial metamorphosis in filamentous fungi is induced by oxidative stress. Integrative Comparative Biology **46:** 691-712

Gessner RV, Romano MA, Schultz RW (1987) Allelic variation and segregation in *Morchella deliciosa* and *M. esculenta*. Mycologia **79:** 683-687

Ghosh TK (1987) Measurement of cellulase activitites. Pure and Applied Chemistry **59:** 257-268

Ghosh A, Frankland JC, Thurston CF, Robinson CH (2003) Enzyme production by *Mycena galopus* mycelium in artificial media and in *Picea sitchensis* F-1 horizon needle litter. Mycological Research **107:** 996-1008

Ghosh RN, Pathak NC (1962) Fungi of India-1 *Morchella, Verpa* and *Helvella*. Bulletin of National Botanical Gardens **71:** 1-19

Ghurde VR, Wakode DD (1981) A new report of *Morchella* from central India. Indian Journal of Mycology and Plant Pathology **11:** 314-315

Golenberg EM, Giannasi DE, Clegg MT, Smiley CJ, Durbin M, Henderson D, Zurawski G (1990) Chloroplast DNA sequence from a miocene *Magnolia* species. Nature **344:** 656-658

Guarro J, Gene J, Stchigel AM (1999) Developments in fungal taxonomy. Clinical Microbiology Reviews **12:** 454-500

Guler P, Arkan O (1999) Cultural Characteristics of *Morchella esculenta* Mycelium on Some Nutrients. Turkish Journal of Biology **24:** 783-794

Guler T, Erta ON, Ciftci M, Dalkılıc B (2005) The effect of coriander seed (*Coriandrum sativum* L.) as diet ingredient on the performance of Japanese quail. South African Journal of Animal Sciences **35:** 261-267

Guler P, Ozkaya EG (2008) Morphological development of *Morchella conica* mycelium on different agar media. Journal of Environmental Biology **30:** 601-604

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series **41:** 95-98

Hammel KE (1997) Fungal degradation of lignin - Plant litter quality and decomposition CAB-International, UK

Hansen K, LoBuglio KF, Pfister DH (2005) Evolutionary relationships of the cupfungus genus Peziza and Pezizaceae inferred from multiple nuclear genes: RPB2, β-tubulin, and LSU rDNA. Molecular Phylogenetics and Evolution **36:** 1-23

Hawker LE (1956) The Physiology of Reproduction in Fungi. Cambridge University Press, Cambridge, USA

Hawksworth DL (1991) The fungal dimension of biodiversity: magnitude, significance and conservation. Mycological Research **95:** 641-655

Hawksworth DL (2004) Fungal diversity and its implications for genetic resource collections. Studies in Mycology **50:** 9-18

Hawksworth DL, Mueller GM (2005) Fungal communities: their diversity and distribution. In: Dighton J, Oudemans P, White J (eds) The fungal community: its organization and role in the ecosystem, 3rd edn. Marcel Dekker, New York, pp 27–37

Hawksworth DL, Rossman AY *(*1997) Where are all the undescribed fungi? Phytopathology **87:** 888-891

Henkel TW, Terborgh J, Viglalys R (2002) Ectomycorrhizal fungi and their leguminous hosts in the Pakaraima Mountains of Guyana. Mycological Research **106:** 515-531

Hennings P (1901) Aliquot Fungi Africae borealis a cl. Dr. G. Schweinfurth collecti. Hedwigia **40:** 98-101

Hervey A, Bistis G, Leong I (1978) Cultural studies of a single ascospore isolates of "*Morchella esculenta*". Mycologia **70:** 1269-1274

Ho I, Zak B *(*1979) Acid phosphatase activity of six ectomycorrhizal fungi. Canadian Journal of Botany **56:** 2813-2817

Ho I (1989) Acid phosphatase, alkaline phosphatase and nitrate reductase activity of selected ectomycorrhizal fungi. Canadian Journal of Botany **67:** 750-753

Hofrichter M (2000) Formation and degradation of a synthetic humic acid derived from 3-fluorocatechol. Applied Microbiology and Biotechnology **53:** 441-446

Hong SG, Jung HS (2004) Phylogenetic analysis of *Ganoderma* based on nearly complete mitochondrial small-subunit ribosomal DNA sequences. Mycologia **96**: 742-755

Hyde KD, Ho WH, Mckenzie EHC, Dalisay T (2001) Saprobic fungi on bamboo culms. Fungal Diversity **7:** 35-48

Isikhuemhen OS, Mikiashvili NA, Kelkar V (2009) Application of solid waste from anaerobic digestion of poultry litter in *Agrocybe aegerita* cultivation: mushroom production, lignocellulolytic enzymes activity and substrate utilization. Biodegradation **20:** 351-361

Jacquetant E (1984) Les morilles. Plantanida, Lausanne, Switzerland

Jandaik CL, Sharma SR (1995) Present status of *Morchella* in India. In: Chadha KL, Sharma SR (eds.) Advances in Horticulture, India, pp 171-194

Jorgenson RD, Cluster PD (1988) Modes and tempos in the evolution of nuclear ribosomal DNA: a new characters for evolutionary studies and new markers for genetic and population studies. Annals of Missouri Botanical Gardens **75:** 1238-1247

Jung SW, Gessner RV, Keudell KC, Romano MA (1993) Systematics of *Morchella esculenta* complex using enzyme-linked immunosorbent assay. Mycologia **85:** 677-684

Kachlishvili E, Penninckx MJ, Tsiklauri N, Elisashvili V (2006) Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. World Journal of Microbiology and Biotechnology 22:391-397

Kalm E, Kalyoncu F (2008) Mycelial growth rate of some morels (*Morchella* spp.) in four different microbiological media. American-Eurasian Journal of Agricultural and Environmental Sciences **3:** 861-864

Kapich AN, Prior BA, Botha A, Galkin S, Lundell T, Hatakka A (2004) Effect of lignocellulose-containing substrate on production of ligninolytic peroxidises in submerged cultures of *Phanerochaete chrysosporium* ME-446. Enzyme Microbial Technology **34:** 187-195

Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinfomatics **9:** 286-298

Kaul TN (1976) Studies on genus "*Morchella*" in Jammu and Kashmir I. Soil composition in relation to carpophore development. Bulletin of Botanical Society of Bengal **29:** 127-134

Kaul TN (1977) Physiological studies on *Morchella species*. II. Nitrogen utilization. Mushroom Journal **58:** 328-332

Kaul TN (1978) Physiological studies on *Morchella species*. I. Carbon utilization. Bulletin of Botanical Society of Bengal **31:** 35-42

Kaul TN (1997) Recent Developments in Morel Biology. In: Dhar R, and Verma BL (eds), Advances in Mushroom Biology and Production. Mycological Society of India, Solan, India, pp 67-68

Kaul TN (1981) Common edible mushrooms of Jammu and Kashmir. Indian Mushroom Science **11:** 79-82

Kellner H, Renker C, Buscot F (2005) Species diversity within the *Morchella esculenta* group (Ascomycota: Morchellaceae) in Germany and France. Organisms, diversity and Evolution **5:** 101-107

Kim TW, Lei XG (2005) An improved method for a rapid determination of phytase activity in animal feed. Journal of Animal Sciences **83:** 1062-1067

Kimbrough JW (1970) Current trends in the classification of Discomycetes. Botanical Reviews **36:** 91-161

Koroleva OV, Gavrilova VP, Stepanocva EV, Lebedeva VI, Sverdlova NI, Landesman EO, et al. (2002) Production of lignin modifying enzymes by co-cultivated white rot fungi *Cerrena maxima* and *Coriolus hirsutus* and characterization of laccase from *Cerrena maxima*. Enzyme Microbial Technology **30:** 573-580

Kroken S, Taylor JW (2001) Outcrossing and recombination in the lichenized fungus *Letharia*. Fungal Geneics and Biology **34:** 83-92

Kues U, Liu Y (2000) Fruiting body production in basidiomycetes. Applied Microbiology and Biotechnology **54:** 141-152

Kuo M (2009) *Morchella tomentosa*, a new species from western North America, and notes on *M. rufobrunnea*. Mycotaxon **105:** 441-446

Labaneiah MEO, Abou-Donia S A, Mohamed M S, El-Zarki EM (1979) Utilization of citrus wastes for the production of fungal protein. Journal of Food Technology **14:** 95-100

Lakhanpal TN, Shad OS (1986) Studies on wild edible mushrooms of Himachal Pradesh (N.W.Himalayas). II. Ecological relationships of *Morchella* species. Indian Journal of Mushrooms **12:** 15-20

Lakhanpal TN, Shad OS, Sagar A (1991) Mycorrhiza: a possible deterrent in artificial cultivation of morels. Current Science **60:** 375-377

Lakhanpal TN, Shad OS, Rana M (2010) Biology of Indian Morels. IK international Publishing house, New Delhi, India

Lanfranco L, Perotto S, Bonfante P (1998) Applications of PCR for studying the biodiversity of mycorrhizal fungi. In: Bridge PD, Arora DK, Reddy CA, Elander RP (eds) Applications of PCR in Mycology. CAB International, UK, pp 107-124

Langfelder K, Jahn B, Gehringer H, Schmidt A, Wanner G, Brakhage AA (1998) Identification of a polyketide synthase gene (*pksP*) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. Medical Microbiology and Immunology **187:** 79-89

Leonowicz A, Cho NS, Luterek J, Wilkolazka A, Wotjas-Wasilewska M, Matuszewska A, Hofrichter M, Wesenberg D, Rogalski J (2001) Fungal laccase: properties and activity on lignin. Journal of Basic Microbiology **41:** 185-227

Liew CY, Husaini A, Hussain H, Muid S, Liew KC, Roslan HA (2011) Lignin biodegradation and ligninolytic enzyme studies during biopulping of *Acacia mangium* wood chips by tropical white rot fungi. World Journal of Microbiology and Biotechnology **27:** 1457-1468

Lopez MJ, Vargas-Garcia MDC, Suarez-Estrella F, Nicholas NN, Dien BS, Moreno J (2007) Lignocellulose-degrading enzymes produced by the ascomycete *Coniochaeta ligniaria* and related species: Application for a lignocellulosic substrate treatment. Enzyme Microbial Technology **40:** 794-800

Maidak BL, Olson GJ, Overbook R, McCaughey MJ, Woese CR (1997) the RDP (Ribosomal Database Project) Nucleic Acids Research **25:** 109-110

Mandelin MF (1956) Studies on the nutrition of Coprinus lagopus Fr, especially as affecting fruiting. Annals of Botany **20:** 467-480

Manoharachary C, Sridhar K, Singh R, Adholeya, Suryanarayanan TS, Rawat S and Johri BN (2005) Fungal Biodiversity: Distribution, Conservation and Prospecting of Fungi from India. Current Science **89:** 58-71

Masaphy S (2005) External ultrastructure of fruit body initiation of *Morchella* spp. Mycological Research **109:** 508-512

Masaphy S (2010) Biotechnology of morel mushrooms: successful fruiting body formation and development in a soilless system. Biotechnology letters **32:** 1523-1527

Masaphy S, Zabari L, Goldberg D, Jander-Shagug G (2010) The Complexity of *Morchella* Systematics: A Case of the Yellow Morel from Israel. Fungi **3:** 14-18

Masaphy S, Zabari L, Goldberg D (2009) New long season ecotype of *Morchella rufobrunnea* from Northern Israel. Micología Aplicada International **21:** 45-55

Mayr R (1982) Untersuchungen zum Wachstum und zur Entwicklung einiger Arten der Mykorrhiza bildenden Gattung "*Morchella*". Universitat Gieben, Germany.

McFarlane EM, Pilz D, Weber NS (2005) High-elevation gray morels and other *Morchella* species harvested as non-timber forest products in Idaho and Montana. Mycologist **19:** 62-68

Mehmann B, Egli S, Braves GH, Brunner I (1995) Coincidence between molecularly or morphologically classified ectomycorrhizal morphotypes and fruit bodies in a Spruce forest. In: Stocchi VP, Bonfante P, Nuti M (eds) Biotechnology of ectomycorrhizae: Molecular approaches. Plenum Press, London, pp 41-52

Mehta KB, Sharma SR (1992) Screening of substrates for mycelia growth and sclerotial formation in *Morchella* species. Indian Journal of Mycology and Plant Pathology **22:** 109-110

Mihail JD, Bruhn JN, Bonella P (2007) Spatial and temporal patterns of morel fruiting. Mycological Research **3:** 339-346

Mikiashvili N, Wasser SP, Nevo E, Elisashvili V (2006) Effects of carbon and nitrogen sources on *Pleurotus ostreatus* ligninolytic enzyme activity. World Journal of Microbiology and Biotechnology **22:** 999-1002

Miller SC (2005) Cultivation of *Morchella* (Morels). *Patent Number 6,907,691*.

Mitchell JI, Roberts PJ, Moss ST (1995) Sequence or Structure? A short review on the application of nucleic acid sequence information to fungal taxonomy. Mycologist **9:** 67-76

Molina MC, Crespo A, Blanco O, Lumbsch HT, Hawksworth DL (2004) Phylogenetic relationships and species concepts in *Parmelia* s.str. (*Parmeliaceae*) inferred from nuclear ITS rDNA and β-tubulin sequences. Lichenologist **36:** 37-54

Molliard M (1905) Forme conidienne et sclérotes de "*Morchella esculenta*". *Revue générale de Botanique*, **14:** 209-218

Moore D (1998a) Fungal Morphogenesis. Cambridge University Press, New York

Mueller GM, Bills GF, Foster MS (2004) Biodiversity of Fungi: Inventory and Monitoring Methods. Elsevier Academic Press, San Diego, Califnornia pp 777

Mullis KB, Faloona FA (1987) Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. Methods in Enzymology **155:** 335-350

Negi CS (2006) Morels (*Morchella* spp.) in Kumaun Himalaya. Natural Products Radiance **5:** 306-310

Nelson MA (1996) Mating systems in ascomycetes: a romp in the sac. Trends in Genetics **12:** 69-70

Nickerson WJ, Edwards GA (1946) Studies on the physiological bassis of morphogenesis in fungi. Journal of General Physiology **33:** 41-55

Niladevi KN, Prema P (2008) Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its applications in dye colourization. Bioresource Technology **99:** 4583-4589

Nilsson RH, **Kristiansson E Ryberg M, Larsson KH (2005)** Approaching the taxonomic affiliation of unidentified sequences in public databases – an example from the mycorrhizal fungi. BMC Bioinformatics **6:** 178-180

Nitha B, Meera CR, Janardhanan KK (2007) Anti-inflammatory and antitumour activities of cultured mycelium of morel mushroom, *Morchella esculenta*. Current Science **92:** 235-239

Nelson MA (1996) Mating systems in ascomycetes: a romp in sac. Trends in Genetics **12:** 69-70

O'Donnell K, Cigelnik E, Weber NS, Trappe JM (1997) Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia **89:** 48-65

O'Donnell K, Rooney AP, Mills GL, Kuo M, Nancy Weber SN, Rehner SA (2011) Phylogeny and historical biogeography of true morels (*Morchella*) reveals an early Cretaceous origin and high continental endemism and provincialism in the Holarctic. Fungal Genetics and Biology **48:** 252-265

Ohga S (1992) Comparison of extracellular enzyme activities among different strains of *Lentinus edodes* grown on sawdust-based cultures in relationship to their fruiting abilities. Journal of Japan Wood Research Society **38:** 310-316

Ohga S, Smith M, Thurston C, Wood DA (1999) Transcriptional regulkation of laccase and cellulose genes in the mycelium of *Agaricus bisporus* during fruit body development on a solid substrate. Mycological Research **103:** 1557-1560

Onions AHS, Eggins HOW, Allsop D (1981) Smiths introduction to Industrial Mycology. John Wiley & Sons, Inc, New York

Ower RD (1982) Notes on the development of the morel ascocarp; *Morchella esculenta*. Mycologia **74:** 142-144

Ower RD, Mills GL, Malachowski JA (1986) Cultivation of *Morchella*. U.S. *Patent No: 4,594,809*

Ower RD, Millls GL, Malachowski JA (1989) Cultivation of *Morchella. U.S. Patent No. 4,866,878*

Pal J, Singh BM, Sharma BM (1999) Evaluation of different substrates combinations for multiplication of sclerotia of *Morchella sp.* Indian Phytopathology **53:** 364

Pandey PK, Reddy MS, Suryanarayanan TS (2003) ITS-RFLP and ITS sequence analysis of a foliar endophytic *Phyllosticta* from different tropical trees. Mycological Research **107:** 439-444

Papinutti L, Lechner B (2008) Influence of the carbon source on the growth and lignocellulolytic enzyme production by *Morchella esculenta* strains. Journal of Industrial Microbiology and Biotechnology **35:** 1715–1721

Pegler DN (2001) Taxonomy, nomenclature and description of *Armillaria*. In: RTV Fox, Intercept, Andover (ed) *Armillaria* root rot: biology and control of honey fungus. pp 81-93

Pegler DN (2003) Useful fungi of the world: morels and truffles. Mycologist **17:** 174-175

Pilz D, Molina R (2002) Commercial harvest of edible mushrooms from the forests of the Pacific Northwest United States: issues, management and monitoring for sustainability. Forest Ecology and Management **155:** 3-16

Pilz D, Weber N, Carter M, Parks C, Molina R (2004) Productivity and diversity of morel mushrooms in healthy, burned and insect-damaged forests of northeastern Oregon. Forest Ecology and Management **198:** 367-386

Pointing SB, Jones EB, Vrijmoed LLP (2000) Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. Mycologia **92:** 139-144

Prasad P, Chauhan K, Kandari LS, Maikhuri RK, Purohit A, Bhatt RP, Rao KS (2002) *Morchella esculenta* (Guchhi): Need for scientific intervention for its cultivation in Central Himalaya. Current Science **82:** 1098-1100

Purkayastha RP, Chandra A (1985) Manual of Indian Edible Mushrooms. Today and tomorrows Printers and publishers, New delhi

Ramsbottom J (1953) Mushrooms and toadstools: A study of activities of fungi, Collins London

Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia **97:** 84-98

Riva S (2006) Laccases: Blue enzymes for green chemistry. Trends in Biotechnology **5:** 219-225

Ronquist F, Huelsenbeck JP (2003) MRBAYES3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572-1574

Rosales E, Rodriguez Couto S, Sanroman MA (2005) Increased laccase production by *Trametes hirsuta* grown on ground orange peelings. Enzyme Microbial Technology **40:** 1286-1290

Rowe RF (1997) The Commercial harvesting of Wild Edible Mushrooms in the Pacific Northwest Region of the United States. Mycologist **11:** 10-15

Royse DJ, May B (1990) Interspecific allozyme variation among *Morchella* spp. and its interferences for systematics within the genus. Biochemical Systematics and Ecology **18:** 475-479

Roze ME (1883) La parasitisme du Morchella esculenta Pers. Sur I' *Helianthus tuberous* L. Bulletin of Society of Botany of France **30:** 139-143

Ryan S, Schnitzhofer W, Tzanov T, Cavaco-Paulo A, Gubitz GM (2003) An acid-stable laccase from *Sclerotium rolfsii* with potential for wool dye decolourization. Enzyme Microbial Technology **33:** 766-774

Ryber M, Nilsson RH, Kristiansson E, Topel M, Jacobsson S, Larsson E (2008) Mining metadata from unidentified ITS sequences in GenBank: A case study in *Inocybe* (Basidiomycota). BMC Evolution and Biology **8:** 50-54

Sainos E, Dıaz-Godınez G, Loera O, Montiel Gonzalez AM, Sanchez C (2006) Growth of *Pleurotus ostreatus* on wheat straw and wheat-grain-based media: biochemical aspects and preparation of mushroom inoculum. Applied Microbiology and Biotechnology **72:** 812-815

Sanchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnological Advances **27:** 185-194

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proceedings of National Academy of Sciences, USA **74:** 5463-5467

Sarbhoy AK, Vashney JL, Agarwal DK (1996) Fungi of India (1982-92). CBS Publisher & Distributer, New Delhi

Scarse RJ, Elliot TJ (1998) Biology and technology of mushroom culture. In: Wood RJB (ed) Microbiology of fermented food Vol 2, 2nd edn. Blackie, London pp 543-584

Schmidt E (1983) Spore germination of and carbohydrate colonization by *Morchella esculenta* at different soil temperatures. Mycologia **75:** 870–875

Scott K, Mohammed C (2004) Specialty Mushroom Production Systems: Maitake and Morels. Australian Government Rural Industries Research and Development Corporation RIRDC Publication No 04/024

Seifert KA, Wingfield BD, Wingfield MJ (1995) A critique of DNA sequence analysis in the taxonomy of filamentous Ascomycetes and ascomycetous anamorphs. Canadian Journal of Botany **73:** 760-767

Sharma BM (2001) Studies on soil microflora of morel bearing sites. Mushroom Research **10:** 51-53

Sharma BM, Deepika S, Singh BM (1997) Natural occurrence of two strains of *Morchella esculenta* in Himachal Pradesh. Mushroom Research **6:** 63-64

Sharma BM, Deepika S, Singh BM (1997) Production of sclerotial spawn for the cultivation of *Morchella esculenta*. Proceedings of Indian National Science Academy, India **63:** 647-654

Sharma BM, Devi P (2000) Effect of soil amendments on sclerotial production of *Morchella esculenta*. Indian Phytopathology **53:** 365

Sharma BM, Devi P (2006) Nutritional requirements for production of sclerotia for cultivation of *Morchella esculenta.* Mushroom Research **15:** 83-86

Sharma BM, Pal J, Sharma PK (2001) Studies on the quality of soils supporting growth of *Morchella esculenta*. Mushroom Research **10:** 47-50

Sharma KK, Kuhad RC (2008) Laccase: enzyme revisited and function redefined. Indian Journal of Microbiology **48:** 309-316

Sharma OP (1989) Textbook of fungi. Tata McGraw-Hill Publishing Company Limited, New delhi pp 134-136

Sharma S, Madan M, Vasudevan P (1997) Biology and applications of mycorrhizal fungi. Microbiology **13:** 427-436

Singer R, Harris B (1987) Mushrooms and Truffles: Botany, Cultivation, and Utilization. Koeltz Scientific Books, Koenigstein, Germany

Singh SK, Dhar BL, Verma RN (1999) Mass production of carpogenic sclerotial spawn in *Morchella esculenta*- An attempt to its domestication. *3rd ICMBMP*

Singh SK, Shwet K, Mugdha T, Yadav MC, Upadhaya RC (2004) Arbitrary primer based RAPD-PCR a useful genetic marker for species identification in morels. Journal of Plant Biochemistry **13:** 7-12

Singh SK, Verma RN (2000) Effect of nutrients on mycelial growth and sclerotia formation. In Griensven, LJLD (ed), Science and Cultivation of Edible Fungi. Balkema, Rotterdam, pp 531-534

Sohi HS, Kumar S, Seth PK (1965) Some interesting fleshy fungi from Himachal Pradesh. Journal of Indian Botanical Society **54:** 69-73

Songulashvili G, Elisashvili V, Wasser S, Nevo E, Hadar Y (2006) Laccase and manganese peroxidases activities of *Phellinus robustus* and *Ganoderma adspersum* grown on food industry wastes in submerged fermentation. Biotechnology Letters **28:** 1425-1429

Spiker JK, Crawford DL, Thiel EC (1992) Oxidation of phenolic and non-phenolic substrate by the lignin peroxidase of *Streptomyces viridosporus* T7A. Applied Microbiology and Biotechnology **37:** 518-523

Spooner B, Işiloğlu M, Alli H, Solak H (2010) *Morchella anatolica* (Ascomycota), a new species from southwestern Anatolia, Turkey. Mycologia **102:** 455-458

Stamatakis A (2006a) Phylogenetic models of rate heterogeneity: a high performance computing perspective. In: Proceedings of IPDPS 2006. Rhodos p. 8

Stamatakis A (2006b) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22:** 2688-2690

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML Web Servers. Systematics of Biology **57:** 758-771

Stamets P (1993b) Growth parameters. In Growing Gourmet and Medicinal Mushroom. Ten Speed Press and Mycomedia, Olympia, USA, pp 370-379

Stefani OPF, Sokolski S, wurtz T, Piche Y, Hamelin R, Fortin A, Berube JA (2010) *Morchella tomentosa*: a unique belowground structure and a new clade of morels. Mycologia **102:** 1082-1088

Sud D, Sharma BM (1999) Physiological studies on a strain of *Morchella esculenta*. Indian Phytopathology **53:** 365

Sun X, Zhang R, Zhang Y (2004) Production of lignocellulolytic enzymes by *Trametes gallica* and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels. Journal of Basic Microbiology **44:** 220-231

Tabatabai MA, Bremner JM (1969) Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry 1:301-307

TAPPI-T222 om-88 (1992–1993) Acid-insoluble lignin in wood and pulp. Numerical index of TAPPI standards and provisional methods. Technical Association of Pulp and Paper Industry, Atlanta, Georgia, USA

Taskin H, Büyükalaca, Dogan HH, Rehner SA, O'Donnell K (2010) A multigene molecular phylogenetic assessment of true morels (*Morchella*) in Turkey. Fungal Genetics and Biology **47:** 672-682

Ten Have R, Teunissen PJM (2001) Oxidative mechanisms involved in lignin degradation by white-rot fungi. Chemical Reviews **11:** 3397-3414

Tien M, Kirk TK (1984) Lignin degrading enzyme from *Phanerochaete chrysosporium*: purification, characterisation and catalytic properties of a unique-H_2O_2 requiring oxygenase. Proceedings of the National Academy of Sciences USA, **81:** 2280-2284

Tilak KVBR, Ranganayaki N, Pal KK, De R, Saxena AK, Nautiyal CS,Mittal S, Tripathi AK, Johri BN (2005) Diversity of plant growth and soil health supporting bacteria. Current Science **89:** 136-150

Ulmer DC, Leisola MSA, Fiechter A (1984) Possible induction of the lignolytic system of *Phanerochaete chrysosporium*. Journal of Biotechnology **1:** 13-24

Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Analytical Biochemistry **32:** 420-424

Vaiterbo A, Yagen B, Mayer AM (1993) Cucurbitacins, attack enzymes and laccase in *Botrytis cinerea*. Phytochemistry **32:** 61-65

Valmaseda M, Martinez MJ, Martinez A (1991) Kinetics of wheat straw solid-state fermentation with *Trametes versicolor* and *Pleurotus ostreatus*—lignin and polysaccharide alteration and production of related enzymatic activities. Applied Microbiology and Biotechnology **35:** 817-823

Vankan JAL, Ackerveken V, De Wit (1991) Cloning and characterization of the cDNA of avirulence avr9 pathogen *Cladosporium fulvum*, the causative agent of tomato leaf mild. Molecular Plant Microbe Interactions **4:** 52-59

Viaud M, Pasquier A, Brygoo Y (2000) Diversity of soil fungi studied by PCR-RFLP of ITS. Mycological Research **104:** 1027-1032

Volk TJ, Leonard TJ (1989) Physiology and environment studies of sclerotium formation and maturation in isolates of *Morchella crassipes.* Applied and Environmental Microbiology **55:** 3095-3100

Volk TJ, Leonard TJ (1990) Cytology of the life-cycle of *Morchella*. Mycological Research **94:** 399-406

Volk TJ, Leonard TJ (1992) Production of new edible mushrooms in North America: shiitake and morels. In: Leatham GF (ed). Frontiers in Industrial Mycology. Chapman Hall, pp 1-23

Wakode DD (1983) Distribuition of *Morchella* in time and space with special reference to Melghat forests. In: Silver Jubilee symposium on Science and cultivation of edible fungi. Regional research laboratory, Sanatnagar, Srinagar, pp 87

Waraitch KS (1976) The genus *Morchella* in India. Kavaka **4:** 69-76

Warishi H, Valli K, Gold MH (1992) Manganese (II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Journal of Biology and Chemistry **267:** 23688- 23695

Weber NS (1988) A morel hunters companion. Two peninsula Press, Lansing, Michigan

Weber NS (1995) A morel hunter's companion. Thunderbay Press, Holt, Michigan

Weber NS (1997) A Morel Hunter's Companion: a Guide to True and False Morels. Lansing, MI: Thunder Bay Press, Holt, Michigan

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a Guide to Methods and Applications. Academic Press, San Diego pp 315-322

Williamson B, Alexander IJ (1975) Acid phosphatase localized in the sheath of beech mycorrhizae. Soil Biology and Biochemistry **7:** 195-198

Winder RS (2006) Cultural studies of *Morchella elata*. Mycological Research **110:** 612-623

Wipf D, Fribourg A, Munch, JC, Botton B, Buscot F (1999) Diversity of the internal transcribed spacer of rDNA in morels. Canadian Journal of Microbiology **45:** 769-778

Wipf D, Munch JC, Botton B, Buscot F (1996) DNA polymorphism in morels: complete sequences of the internal transcribed spacer of genes coding for rRNA in *Morchella esculenta* (yellow morel) and *Morchella conica* (black morel). Applied and Environmental Microbiology **62:** 3541-3543

Wittenmayer L, Szabo K (2000) The role of root exudates in specific apple (*Malus domestica* Borkh) replant disease (SARD). Journal of Plant Nutrition and Soil Science **163:** 399-404

Wong AL, Willetts HJ (1973) Polyacrylamide gel electrophoresis of enzymes during morphogenesis of sclerotia of *Sclerotinia sclerotiorum*. Journal of General Microbiology **81:** 101-109

Wong AL, Willetts HJ (1980) The biology of *Sclerotinia sclerotiorum, S. trifoliorum*, and *S. minor* with emphasis on specific nomenclature. Botanical Reviews **46:** 101-165.

www.flickr.com/photos/52345239@N05/5226817921/in/photostream

www.mushroomthejournal.com

www.ncbi.nlm.nih.gov

www.taxonomy.zoology.gla.ac.uk/rod/rod.html

www.thefullwiki.org/Ascomycete

www.weatherpulse.info/2011/04/mushroomweather

Yoon CS, Gessner RV, Romano MA (1990) Population genetics and systematics of the *Morchella esculenta* complex. Mycologia **82:** 227-235

Zhang GP, Zhang F, Ru WM, Han JR (2009) Solid state fermentation of cornmeal with the ascomycete *Morchella esculenta* for degrading starch and upgrading nutritional value. World Journal of Microbiology and Biotechnology **26:** 15-20

Zhou DP, Sun JQ, Yu HY, Ping WX, Zheng XQ (2001) *Nodulisporium*, a genus new to China. Mycosystema **2:** 277-278

Appendix I

Genomic DNA Extraction buffer

Sodium acetate	100 mM
Na2EDTA	50 mM
NaCl	500 mM
SDS	1%

TBE Buffer (10x)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.90 M
EDTA	0.02 M (pH 8)

LB broth

Bacto-tryptone	10 g/L
Bacto-yeast extract	5 g/L
NaCl	5 g/L

Adjust pH to 7.0 with 5 N NaOH and autoclave 20 minutes at 121°C.

LB/amp+ agar plates

Prepare LB broth as above. Add agar (18 g/L), autoclave, and cool to 50°C. Add ampicillin to 50 μg/ml. Pour plates and store at 4°C.

IPTG stock solution (0.1 M)

Take 1.2 g IPTG and add water to 50 ml final volume. Filter sterilize and store at 4°C.

X-Gal (2 ml)

Take 100 mg 5-bromo-4-chloro-3-indolyl-Dgalactoside and dissolve in 2 ml N,N´-dimethylformamide. Cover with aluminum foil and store at 20°C.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

Plasmid extraction solution I (10X)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na2EDTA	10 mM

Plasmid extraction solution II

NaOH	5 M
SDS	10%

Plasmid extraction solution III

5.0 M K-acetate (pH 4.5)

Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.00%

AppendixII

Malt Extract (ME)

Malt extract	20 g/L

Autoclave 15 minutes at 121℃ for 15 minutes.

Melin's modified Norkans medium(MMN)

$(NH_4)HPO_4$	250.0 mg/L
KH_2PO_4	500.0 mg/L
$MgSO_4.7H_2O$	150.0 mg/L
$CaCl_2.H_2O$	50.0 mg/L
NaCl	25.0 mg/L
1 % (w\v) $FeCl_3$	1.2 ml/L
Thiamine HCl	40.0 mg/L
Biotine	0.4 mg/L
Glucose	2.5 g/L

Heller's micronutrient (100x) 10 ml/L (Heller, 1953)

$FeCl_3.6H_20$	100 mg/L
$ZnSO_4.7H_20$	100 mg/L
H_3BO_3	100 mg/L
$MnSO_4.4H_20$	10 mg/L
$CuSO_4$ $.5H_20$	3 mg/L
$AlCl_3$	3 mg/L
$NiCl_3.6H20$	3 mg/L
KI	1 mg/L

Autoclave 15 minutes at 121℃ for 15 minutes.

Czapek Dox (CZD)

Glucose	30.00 g/L
$NaNO_3$	3.00 g/L
K_2HPO_4	1.00 g/L
MgSO4.7H2O	0.50 g/L
KCl	0.50 g/L
$FeSO_4.7H2O$	0.01 g/L

Autoclave 15 minutes at 121℃ for 15 minutes.

Yeast Maltose Glucose (YMG)

Yeast extract	4 g/L
Malt extract	10 g/L
Glucose	4 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Sabourd's media (SB)

Peptone	10 g/L
Maltose	30 g/L
Yeastextract	500 mg/L

Autoclave 15 minutes at 121°C for 15 minutes.

Basal media (BS)

K_2HPO_4	0.62 g/L
KH_2PO_4	2.00 g/L
$(NH_4)_2SO_4$	1.00 g/L
$MgSO_4.7H_2O$	1.10 g/L
Yeast extract	0.60 g/L
Glucose	10.00 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Minimal Mineral media (MMM)

Yeast extract	14.3 g/L
$(NH_4)_2SO_4$	2.1 g/L
$MgSO_4.7H_2O$	3.0 g/L
$CaCl_2.H_2O$	300.0 mg/L
$FeSO_4 \cdot 7H_2O$	500.0 mg/L
KH_2PO_4	10.0 g/L
Glucose	10.0 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Potato Dextrose broth (PDB)

Potato infusion	200 g/L
Dextrose	20 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Mineral Salts broth (MSB)

Sodium acetate	20.0 mM
Glucose	10.0 g/L
KH_2PO_4	2.0 g/L
$MgSO_4.7H_2O$	0.5 g/L
$CaCl_2.H_2O$	0.1 g/L
Thiamine HCl	1.0 mg/L
$NaNO_3$	0.2 g/L
Trace element solution	10.0 ml/L

Trace element solution (100X)

Nitrilotriacetic acid	1.5 g/L
$MnSO_4 \cdot 7H_2O$	0.5 g/L
NaCl	1.0 g/L
$FeSO_4 \cdot 7H_2O$	100.0 mg/L
$CoSO_4 \cdot 7H_2O$	100.0 mg/L
$ZnSO_4 \cdot 7H_2O$	100.0 mg/L
$CuSO_4$.$5H_20$	10.0 mg/L
$AlK(SO_4)_2.2H_2O$	10.0 mg/L
H_3BO_3	10.0 mg/L
$Na_2MoO_4.2H_2O$	10.0 mg/L

Autoclave 15 minutes at 121℃ for 15 minutes.

Morel growth broth (MGB)

Sucrose	6.5 g/L
Mannose	3.4 g/L
Yeast extract	140.0 mg/L

Autoclave 15 minutes at 121℃ for 15 minutes.

Buscot media A (Bu-A)

Malt extract	5 g/L
Glucose	10 g/L

Autoclave 15 minutes at 121℃ for 15 minutes.

Buscot media B (Bu-B)

Yeast extract	4 g/L
Starch	15 g/L
Glucose	10 g/L
KH_2PO_4	1 g/L
MgSO4.7H2O	500 mg/L
Agar	15 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Nutrient rich media A (Nut A)

Malt extract	20 g/L
Agar	15 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Nutrient poor B (Nut B)

Agar	15 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Glucose Noble agar (GNA)

Glucose	90 g/L
$NaNO_3$	250 mg/L
Agar	15 g/L

Autoclave 15 minutes at 121°C for 15 minutes.

Fresh colouring reagent

Three volumes of 1 M sulfuric acid

One volume of 2.5% ammonium molybdate,

One volume of 10% ascorbic acid (wt/vol)

Appendix III

Sequence of Internal transcribed spacer (ITS) region of *Morchella crassipes* isolate MR1

```
LOCUS       GQ228461               1208 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella crassipes isolate MR 1 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.
ACCESSION   GQ228461
VERSION     GQ228461.1  GI:294653280
KEYWORDS    .
SOURCE      Morchella crassipes
  ORGANISM  Morchella crassipes
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1208)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1208)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1208
                     /organism="Morchella crassipes"
                     /mol_type="genomic DNA"
                     /isolate="MR 1"
                     /db_xref="taxon:62754"
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                     /product="18S ribosomal RNA"
     misc_RNA        34..613
                     /product="internal transcribed spacer 1"
     rRNA            614..776
                     /product="5.8S ribosomal RNA"
     misc_RNA        777..1150
                     /product="internal transcribed spacer 2"
     rRNA            1151..>1208
                     /product="28S ribosomal RNA"
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       61 agaggcgacc ataacggtgg aagacgccgt catataggcg ggttccataa ccggcacaag
      121 atgttggaag ggcccagggc gggccggccg ggtcaacctc atccgcgtaa tcctgctgcc
      181 cactgcgctc cctccccacg ctttagggca gcaacccccc gcattggggg gtggagccgg
      241 atctaagcaa tcaatgcttc gcatccatcc acaacacagt tgggtgccgg tgcggagtag
      301 actattgcgg ctaaaggagg ttcacccgga tggaggctga ctgcgcatgc aggaccatca
      361 tggatggatt ccaaccatgc gttccttccc ctggggcctg aggggtgtaa aaacccctca
      421 gcgacctgct ccgacggcgt gaagaggacc cccaccgttt ggcaccctct cgccattgtc
      481 ccaaccaaaa ccctctgtgt acccttccct gttgcttccc ccgggcaact ggctccggcc
      541 agccgggggg agaaaccaag caaaaaccct tttcgcaaaa cagacgtctg atgtaaaaaa
      601 acaaaaaaca aaagttaaaa ctttcaacaa cggatctctt ggttcccaca tcgatgaaga
      661 acgcagcgaa atgcgataag taatgtgaat tgcagaattc agtgaatcat cgaatctttg
      721 aacgcacatt gcgccctctg gtattccgga gggcatgcct gttcgagcgt cataaatacc
      781 gctccccctc ggattgcttg cggtccctgg ggggttctgg caatgtggat tccccccgtg
      841 ctttgagggc atgcgaacgg gctcccagtg ctgaaagaca taatgttccc agccgaaacc
      901 ggtgatttat ttcatcggca ggattcgtgg caggcagact gagggcgtca accgcggagt
      961 catgaggata gaaacctccc cctttgcaag taacattgct ctggcagtta gatctgcagg
     1021 cccgccggtc tggggatgga ccctcccact cgtaggcgtc acggccacga tagcgggcgt
     1081 taaatggaat ccgatccgcc cctccccggg tggttgaaga tccttgtggg ctagcaaccc
     1141 ctaaacacat tttgacctcg gatcaggtag ggatacccgc tgaacttaag catatcaata
     1201 agcggagg
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Sequence of Internal transcribed spacer (ITS) region of *Morchella crassipes* isolate MR5

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LOCUS       GQ228462               1206 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella crassipes isolate MR 5 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.
ACCESSION   GQ228462
VERSION     GQ228462.1  GI:294653281
KEYWORDS    .
SOURCE      Morchella crassipes
  ORGANISM  Morchella crassipes
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1206)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1206)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1206
                     /organism="Morchella crassipes"
                     /mol_type="genomic DNA"
                     /isolate="MR 5"
                     /db_xref="taxon:62754"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..613
                     /product="internal transcribed spacer 1"
     rRNA            614..776
                     /product="5.8S ribosomal RNA"
     misc_RNA        777..1147
                     /product="internal transcribed spacer 2"
     rRNA            1148..>1206
                     /product="28S ribosomal RNA"
ORIGIN
        1 tccgtaggtg aacctgcgga aggatcatta ccagattcca aaccaaaagt cagtggagag
       61 agaggcggtc ataacggtgg aagacgtcgt cacattggcg ggttccataa ccggcacaag
      121 atgttggaag ggcccagggc gggccggccg ggtcaacctc atccgtgtaa tcctgctgcc
      181 cactgcgctc cctccctacg ctttagggca gcaacccccc gcattggggg gtggaggccg
      241 gatctatgca atcaatgctt cgcatccatc cacacagttg ggtgccggtg cggagtagac
      301 tattgcggct aaaggaggtt cacccggatg gtggctgact gcgcatgcag gaccatcatg
      361 gatggatcca accatgcgtt ccttcccctg gggcctgagg ggtgtaaaaa cccctcagcg
      421 acctgctccg acggcgtgaa gaggacccca accgtttggc accctctcgc cattgtccca
      481 accaaaaccc tctgtgtacc cttccctgtt gcttcccccg ggcaactggc tccggccagc
      541 cgggggggaga aacctagcaa aaacccttct cgcaaaacag acgtctgatg tagaaaacaa
      601 caaaaaaaaa taagttaaaa ctttcaacaa cggatctctt ggttcccaca tcgatgaaga
      661 acgcagcgaa atgcgataag taatgtgaat tgcagaattc agtgaatcat cgaatctttg
      721 aacgcacatt gcgccctctg gtattccgga gggcatgcct gttcgagcgt cataaatacc
      781 gctccccctc ggattgcttg cggtccctgg ggggttctgg caatgtggat tccccgtgct
      841 ttgagggcat gcgaacgggc tcccagtgct gaaagacata atgttcccag ccgaaaccgg
      901 cgattaattt catcggcagg attcgtggca ggcagactga gggcgtcaac cgtggagtca
      961 tgaggataga aacctccccc tttgcaagta acattgctct ggcagttaga tctgcaggcc
     1021 tgccggtctg gggatgggcc tcccactcgc aggcgtcacg gccacgatag cgggcgttaa
     1081 acggaatccg atccgccccc ccctgggtgg ttgaagatcc ttgtgggcta gcaaccccta
     1141 aacacatttt gacctcggat caggtaggga tacccgctga acttaagcat atcaataagc
     1201 ggagga
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Sequence of Internal transcribed spacer (ITS) region of *Morchella crassipes* isolate MR12

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LOCUS       GQ228463               1210 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella crassipes isolate MR 12 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.
ACCESSION   GQ228463
VERSION     GQ228463.1  GI:294653282
KEYWORDS    .
SOURCE      Morchella crassipes
  ORGANISM  Morchella crassipes
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1210)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1210)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1210
                     /organism="Morchella crassipes"
                     /mol_type="genomic DNA"
                     /isolate="MR 12"
                     /db_xref="taxon:62754"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..614
                     /product="internal transcribed spacer 1"
     rRNA            615..777
                     /product="5.8S ribosomal RNA"
     misc_RNA        778..1151
                     /product="internal transcribed spacer 2"
     rRNA            1152..>1210
                     /product="28S ribosomal RNA"
ORIGIN
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       61 agaggcgacc ataacggtgg aagacgccgt catataggcg ggttccataa ccggcacaag
      121 atgttggaag ggcccagggc gggccggccg ggtcaacctc atccgcgtaa tcctgctgcc
      181 cactgcgctc cctccccacg ctttagggca gcaacccccc gcattggggg gtggagccgg
      241 atctaagcaa tcaatgcttc gcatccatcc acaacacagt cgggtgccgg tgcggagtag
      301 actattgcgg ctaaaggagg ttcacccgga tggaggctga ctgcgcatgc aggaccatca
      361 tggatggatt ccaaccatgc gttccttccc ctggggcctg aggggtgtaa aaacccctca
      421 gcgacctgct ccgacggcgt gaagaggacc cccaccgttt ggcaccctct cgccattgtc
      481 ccaaccaaaa ccctctgtgt acccttccct gttgcttccc ccgggcaact ggctccggcc
      541 agccgggggg agaaaccaag caaaaaccct tttcgcaaaa cagacgtctg aatgtaaaaa
      601 aacaaaaaac aaaagttaaa actttcaaca acggatctct tggttcccac atcgatgaag
      661 aacgcagcga aatgcgataa gtaatgtgaa ttgcagaatt cagtgaatca tcgaatcttt
      721 gaacgcacat tgcgccctct ggtattccgg agggcatgcc tgttcgagcg tcataaatac
      781 cgctcccccc cggattgctt gcggtccctg gggggttctg gcaatgtgga ttccccccgt
      841 gctttgaggg catgcgaacg ggctcccagt gctgaaagac ataatgttcc cagccgaaac
      901 cggtgattta tttcatcggc aggattcgtg gcaggcagac tgagggcgtc aaccgtggag
      961 tcatgaggat agaaacctcc ccctttgcaa gtaacattgc tctggcagtt agatctgcag
     1021 gcccgccggt ctggggatgg accctcccac tcgtaggcgt cacggccacg atagcgggcg
     1081 ttaaatggaa tccgatccgc ccctccccgg gtggttgaag atccttgtgg gctagcaacc
     1141 cctaaacaca ttttgacctc ggatcaggta gggatacccg ctgaacttaa gcatatcaat
     1201 aagcggagga
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Sequence of Internal transcribed spacer (ITS) region of *Morchella crassipes* isolate MR18

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LOCUS       GQ228464               1207 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella crassipes isolate MR 18 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.
ACCESSION   GQ228464
VERSION     GQ228464.1  GI:294653283
KEYWORDS    .
SOURCE      Morchella crassipes
  ORGANISM  Morchella crassipes
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1207)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1207)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1207
                     /organism="Morchella crassipes"
                     /mol_type="genomic DNA"
                     /isolate="MR 18"
                     /db_xref="taxon:62754"
     rRNA            <1..32
                     /product="18S ribosomal RNA"
     misc_RNA        33..614
                     /product="internal transcribed spacer 1"
     rRNA            615..777
                     /product="5.8S ribosomal RNA"
     misc_RNA        778..1148
                     /product="internal transcribed spacer 2"
     rRNA            1149..>1207
                     /product="28S ribosomal RNA"
ORIGIN
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       61 gaggcggtca taacggtgga agacgtcgtc acattggcgg gttccataac cggcacaaga
      121 tgttggaagg gcccagggcg ggccggccgg gtcaacctca tccgtgtaat cctgctgccc
      181 actgcgcttc ctccctacgc tttagggcag caacccccg cattgggggg tggaggccgg
      241 atctatgcaa tcaatgcttc gcatccatcc acacagttgg gtgccggtgc ggagtagact
      301 attgcggcta aaggaggttc acccggatgg tggctgactg cgcatgcagg accatcatgg
      361 atggatccaa ccatgcgttc cttcccctgg ggcctgaggg gtgtaaaaac ccctcagcga
      421 cctgctccga cggcgtgaag aggaccccca ccgtttggca ccctctcgcc attgtcccaa
      481 ccaaaaccct ctgtgtaccc ttccctgttg cttcccccgg gcaactggct ccggccagcc
      541 ggggggagaa acctagcaaa aacccttttc gcaaaacaga cgtctgaatg tagaaaacaa
      601 acaaaaaaaa ataagttaaa actttcaaca acggatctct tggttcccac atcgatgaag
      661 aacgcagcga aatgcgataa gtaatgtgaa ttgcagaatt cagtgaatca tcgaatcttt
      721 gaacgcacat tgcgccctct ggtattccgg agggcatgcc tgttcgagcg tcataaatac
      781 cgctcccccc cggattgctt gcggtccctg gggggttctg gcaatgtgga ttccccgtgc
      841 tttgagggca tgcgaacggg ctcccagtgc tgaaagacat aatgttccca gccgaaaccg
      901 gcgattaatt tcatcggcag gattcgtggc aggcagactg agggcgtcaa ccgtggagtc
      961 atgaggatag aaacctcccc ctttgcaagt aacattgctc tggcagttag atctgcaggc
     1021 ctgccggtct ggggatgggc ctcccactcg caggcgtcac ggccacgata gcgggcgtta
     1081 aacggaatcc gatccgcccc tccctgggtg gttgaagatc cttgtgggct agcaacccct
     1141 aaacacattt tgacctcgga tcaggtaggg atacccgctg aacttaagca tatcaataag
     1201 cggagga
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Sequence of Internal transcribed spacer (ITS) region of *Morchella crassipes* isolate MR21

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LOCUS       GQ228465               1215 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella crassipes isolate MR 21 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.
ACCESSION   GQ228465
VERSION     GQ228465.1  GI:294653284
KEYWORDS    .
SOURCE      Morchella crassipes
  ORGANISM  Morchella crassipes
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1215)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1215)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1215
                     /organism="Morchella crassipes"
                     /mol_type="genomic DNA"
                     /isolate="MR 21"
                     /db_xref="taxon:62754"
     rRNA            <1..30
                     /product="18S ribosomal RNA"
     misc_RNA        31..623
                     /product="internal transcribed spacer 1"
     rRNA            624..786
                     /product="5.8S ribosomal RNA"
     misc_RNA        787..1155
                     /product="internal transcribed spacer 2"
     rRNA            1156..>1215
                     /product="28S ribosomal RNA"
ORIGIN
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       61 agagagccaa ctgcggtggg ggacgccgtc acttcggcgg gttccgtgta ccactgtgtt
      121 ggtccagggc gggccggcca gggagacctc atccgtgtaa tcaaatcctg ctgaccctaa
      181 tgcgctccct ccccacgcct taggcagcaa ccccccgcat tgggggggtgg agccggatct
      241 tagcaatcaa tgctccgcat ccatccacac agttgggtgc cggtgcggag tagactattg
      301 cagctaaagg aggattccct ggcaggtggc ggacaacaca tgcccggctc cttacgcctt
      361 ccgctcggtt ggatccgtct gcgtagccgc tcattgctgt tgattcccct ggggctggag
      421 gtgtagcgac ctccagcgac ccccatcgac ggcgtggaaa cccatcgttt ggcaccctct
      481 cgccattgcc ccaaccaaaa ccctctgtgt acccttttccc tgttgcttcc cccggcacct
      541 ggctccggcc agtcgggggg agaaaccaag caaaaaccct tttcgcaaaa cagacgtctg
      601 aatgtaaaaa aacaaaacaa aaagttaaaa ctttcaacaa cggatctctt ggttcccaca
      661 tcgatgaaga acgcagcgaa atgcgataag taatgtgagt tgcagaattc agtgaatcat
      721 cgaatctttg aacgcacatt gcgccctctg gtattccgga gggcatgcct gttcgagcgt
      781 cataaatacc gctccccctc ggattgcttg cggtccctgg ggggttctgg caatgtggat
      841 tccccgtgct ttgagggcat gcgaacgggc tcccagtgct gaaagacata atgttcccag
      901 ccgaaaccgg tgattaattt catcggcagg attcgtggca ggcagactga gggcgtcaac
      961 cgtggagtca tgaggataga aacctccccc tttgcaagta acattgctct ggcagttaga
     1021 tctgcaggcc tgccggtctg gggatggacc tcccactcgc aggcgtcgcg gccacgatag
     1081 cgggcgttaa acggagtccg atccgccttc attggtggtt gaagatcctt gtgggctagc
     1141 aacccctaaa cacattttga cctcggatca ggtagggata cccgctgaac ttaagcatat
     1201 caataagcgg aggaa
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Sequence of Internal transcribed spacer (ITS) region of *Morchella crassipes* isolate MR8

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LOCUS       GQ228466               1210 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella crassipes isolate MR 8 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.
ACCESSION   GQ228466
VERSION     GQ228466.1  GI:294653285
KEYWORDS    .
SOURCE      Morchella crassipes
  ORGANISM  Morchella crassipes
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1210)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1210)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1210
                     /organism="Morchella crassipes"
                     /mol_type="genomic DNA"
                     /isolate="MR 8"
                     /db_xref="taxon:62754"
     rRNA            <1..34
                     /product="18S ribosomal RNA"
     misc_RNA        35..617
                     /product="internal transcribed spacer 1"
     rRNA            618..780
                     /product="5.8S ribosomal RNA"
     misc_RNA        781..1151
                     /product="internal transcribed spacer 2"
     rRNA            1152..>1210
                     /product="28S ribosomal RNA"
ORIGIN
        1 ttccgtaggt gaacctgcgg aaggatcatt accagattcc aaaccaaaag tcagtggaga
       61 gagaggcggt cataacggtg gaagacgtcg tcacattggc gggttccata accggcacaa
      121 gatgttggaa gggcccaggg cgggccggcc gggtcaacct catccgtgta atcctgctgc
      181 ccactgcgct ccctccctac gctttagggc agcaaccccc cgcattgggg ggtggaggcc
      241 ggatctatgc aatcaatgct tcgcatccat ccacacagtt gggtgccggt gcggagtaga
      301 ctattgcggc taaaggaggt tcacccggat ggtggctgac tgcgcatgca ggaccatcat
      361 ggatggatcc aaccatgcgt tccttccccc ggggcctgag gggtgtaaaa acccctcagc
      421 gacctgctcc gacggcgtga agaggacccc caccgtttgg caccctctcg ccattgtccc
      481 aaccaaaacc ctctgtgtac ccttccctgt tgcttccccc gggcaactgg ctccggccag
      541 ccgggggggag aaacctagca aaaacccttt tcgcaaaaca gacgtctgaa tgtagaaaac
      601 aaacaaaaaa aaaataagtt aaaactttca acaacggatc tcttggttcc cacatcgatg
      661 aagaacgcag cgaaatgcga taagtaatgt gaattgcaga attcagtgaa tcatcgaatc
      721 tttgaacgca cattgcgccc tctggtattc cggagggcat gcctgttcga gcgtcataaa
      781 taccgctccc cctcggattg cttgcggtcc ctgggggttt ctggcaatgt ggattccccg
      841 tgctttgagg gcatgcgaac gggctcccag tgctgaaaga cataatgttc ccagccgaaa
      901 ccggcgatta atttcatcgg caggattcgt ggcaggcaga ctgagggcgt caaccgtgga
      961 gtcatgagga tagaaacctc ccccttgca agtaacattg ctctggcagt tagatctgca
     1021 ggcctgccgg tctggggatg ggcctcccac tcgcaggcgt cacggccacg atagcgggcg
     1081 ttaaacggaa tccgatccgc ccctccctgg gtggttgaag atccttgtgg gctagcaacc
     1141 cctaaacaca ttttgacctc ggatcaggta gggatacccg ctgaacttaa gcatatcaat
     1201 aagcggagga
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Sequence of Internal transcribed spacer (ITS) region of *Morchella crassipes* isolate MR20

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LOCUS       GQ228467               1210 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella crassipes isolate MR 20 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.
ACCESSION   GQ228467
VERSION     GQ228467.1  GI:294653286
KEYWORDS    .
SOURCE      Morchella crassipes
  ORGANISM  Morchella crassipes
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1210)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1210)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1210
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                     /mol_type="genomic DNA"
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                     /db_xref="taxon:62754"
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     misc_RNA        35..616
                     /product="internal transcribed spacer 1"
     rRNA            617..779
                     /product="5.8S ribosomal RNA"
     misc_RNA        780..1150
                     /product="internal transcribed spacer 2"
     rRNA            1151..>1210
                     /product="28S ribosomal RNA"
ORIGIN
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      121 gatgttggaa gggcccaggg cgggccggcc gggtcaacct catccgtgta atcctgctgc
      181 ccactgcgct ccctccctac gctttagggc agcaaccccc cgcattgggg ggtggaggcc
      241 ggatctatgc aatcaatgct tcgcatccat ccacacagtt gggtgccggt gcggagtaga
      301 ctattgcggc taaaggaggt tcacccggat ggtggctgac tgcgcatgca ggaccatcat
      361 ggatggatcc acccatgcgt tcctttcccc tggggcctga ggggtgtaaa aacccctcag
      421 cgactgctcc gacggcgtga agaggacccc caccgtttgg caccctctcg ccattgtccc
      481 aaccaaaacc ctctgtgtac ccttccctgt tgcttccccc gggcaactgg ctccggccag
      541 ccgggggggag aaacctagca aaaacccttt tcgcaaaaca gacgtctgaa tgtagaaaac
      601 aaacaaaaaa aaataagtta aaactttcaa caacggatct cttggtcccc acatcgatga
      661 agaacgcagc gaaatgcgat aagtaatgtg aattgcagaa ttcagtgaat catcgaatct
      721 ttgaacgcac attgcgccct ctggtattcc ggagggcatg cctgttcgag cgtcataaat
      781 accgctcccc ctcggattgc ttgcggtccc tgggggggttc tggcaatgtg gattccccgt
      841 gctttgaggg catgcgaacg ggctcccagt gctgaaagac ataatgttcc cagccgaaac
      901 cggcgattaa tttcatcggc aggattcgtg gcaggcagac tgagggcgtc aaccgtggag
      961 tcatgaggat agaaacctcc ccctttgcaa gtaacattgc tctggcagtt agatctgcag
     1021 gcctgccggt ctggggatgg gcctcccact cgcaggcgtc acggccacga tagcgggcgt
     1081 taaacggaat ccgatccgcc cctccctggg tggttgaaga tccttgtggg ctagcaaccc
     1141 ctaaacacat tttgacctcg gatcaggtag ggatacccgc tgaacttaag catatcaata
     1201 agcggaggaa
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Sequence of Internal transcribed spacer (ITS) region of *Morchella elata* isolate MR3

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LOCUS       GQ228468                737 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella elata isolate MR 3 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.
ACCESSION   GQ228468
VERSION     GQ228468.1  GI:294653287
KEYWORDS    .
SOURCE      Morchella elata
  ORGANISM  Morchella elata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 737)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 737)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
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                     /db_xref="taxon:39930"
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     misc_RNA        34..240
                     /product="internal transcribed spacer 1"
     rRNA            241..403
                     /product="5.8S ribosomal RNA"
     misc_RNA        404..678
                     /product="internal transcribed spacer 2"
     rRNA            679..>737
                     /product="28S ribosomal RNA"
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      121 cccatctaaa ccctctgcgt acctgtccct tcttgcttcc cccggcatct cgtcgggggg
      181 aggtaacaac caaaactctc tgtgaatcaa acagccgtca gaattataaa acaaacaaaa
      241 gttaaaactt tcaacaacgg atctcttggt tcccacatcg atgaagaacg cagcgaaatg
      301 cgataagtaa tgtgaattgc agaattcagt gaatcatcga atctttgaac gcacactgcg
      361 ccccctggta ttccgggggg catgcctgtt cgagcgtcat aaaaacctcc tccccttcg
      421 ggttttgtta ctatcgttgg ggggttttgg cctaatggga tagcgattgg caattcgttt
      481 cccaatgtcc taaatagacg tagacccgcc tccagatgcg acagcaccga ggccatcaac
      541 cgtggagtta tgggatataa taggcttgca gtaaaatgct cacctctctc cacacgccga
      601 tggcacgaca gttgcagttg cgggcgtaaa ttggagccct tttcaggacc cttgtggcct
      661 agcatccacc atacataatt tgacctcgga tcaggtaggg atacccgctg aacttaagca
      721 tatcaataag cggagga
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Sequence of Internal transcribed spacer (ITS) region of *Morchella elata* isolate MR4

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LOCUS       GQ228469                738 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella elata isolate MR 4 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.
ACCESSION   GQ228469
VERSION     GQ228469.1  GI:294653288
KEYWORDS    .
SOURCE      Morchella elata
  ORGANISM  Morchella elata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 738)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 738)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala, Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..738
                     /organism="Morchella elata"
                     /mol_type="genomic DNA"
                     /isolate="MR 4"
                     /db_xref="taxon:39930"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..241
                     /product="internal transcribed spacer 1"
     rRNA            242..404
                     /product="5.8S ribosomal RNA"
     misc_RNA        405..679
                     /product="internal transcribed spacer 2"
     rRNA            680..>738
                     /product="28S ribosomal RNA"
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      121 cccatctaaa ccctctgcgt acctgtccct tcttgcttcc cccggcatct cgtcgggggg
      181 aggtaacaac ccaaaactct ctgtgaatca aacagccgtc agaattataa aacaaacaaa
      241 agttaaaact ttcaacaacg gatctcttgg ttcccacatc gatgaagaac gcagcgaaat
      301 gcgataagta atgtgaattg cagaattcag tgaatcatcg aatctttgaa cgcacattgc
      361 gccccctggt attccggggg gcatgcctgt tcgagcgtca taaaaacctc ctccccctcc
      421 gggttttgtt actatcgttg gggggttttg gcctaatggg atagcgattg gcaattcgtt
      481 tcccaatgtc ctaaatagac gtagacccgc ctccagatgc gacagcaccg aggccatcaa
      541 ccgtggagtt atgggatata ataggcttgc agtaaaatgc tcacctctct ccacacgccg
      601 atggcacgac agttgcagtt gcgggcgtaa attggagccc ttttcaggac ccttgtggcc
      661 tagcatccac catacataat ttgacctcgg atcaggtagg gatacccgct gaacttaagc
      721 atatcaataa gcggagga
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Sequence of Internal transcribed spacer (ITS) region of *Morchella elata* isolate MR6

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LOCUS       GQ228470                739 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella elata isolate MR 6 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.
ACCESSION   GQ228470
VERSION     GQ228470.1  GI:294653289
KEYWORDS    .
SOURCE      Morchella elata
  ORGANISM  Morchella elata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 739)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 739)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..739
                     /organism="Morchella elata"
                     /mol_type="genomic DNA"
                     /isolate="MR 6"
                     /db_xref="taxon:39930"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..243
                     /product="internal transcribed spacer 1"
     rRNA            244..406
                     /product="5.8S ribosomal RNA"
     misc_RNA        407..681
                     /product="internal transcribed spacer 2"
     rRNA            682..>739
                     /product="28S ribosomal RNA"
ORIGIN
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       61 ggggaccgac agggctagta gcttatacgt tgttgaacgt ccagtatgga cccgaagcct
      121 cccccatcta aaccctctgc gtacccgtcc cttcttgctt ccccccggcat ctcgtcgggg
      181 ggaggtaaca accaaaactc tatgtgaatc aaacagccgt cagaattata aaacaaacaa
      241 aaagttaaaa ctttcaacaa cggatctctt ggttcccaca tcgatgaaga acgcagcgaa
      301 atgcgataag taatgtgaat tgcagaattc agtgaatcat cgaatctttg aacgcacatt
      361 gcgccccctg gtattccggg gggcatgcct gttcgagcgt cataaaaacc tcctccccct
      421 tcgggttttg ttactatcgt tgggggttt tggcctaatg ggatagcgat tggcaattcg
      481 tttcccaatg tcctaaatag acgtagaccc gcctccagat gcgacagcac cgaggccatc
      541 aaccgtggag ttatgggata taataggctt gcagtaaaat gctcacctct ctccacacgc
      601 cgatggcacg acagttgcag ttgcgggcgt aaattggagc ccttttcagg acccttgtgg
      661 cctagcatcc accatacata atttgacctc ggatcaggta gggatacccg ctgaacttaa
      721 gcatatcaat aagcggagg
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Sequence of Internal transcribed spacer (ITS) region of *Morchella elata* isolate MR10

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LOCUS       GQ228471                739 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella elata isolate MR 10 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.
ACCESSION   GQ228471
VERSION     GQ228471.1  GI:294653290
KEYWORDS    .
SOURCE      Morchella elata
  ORGANISM  Morchella elata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 739)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 739)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,Punjab 147 004, India
FEATURES             Location/Qualifiers
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                     /isolate="MR 10"
                     /db_xref="taxon:39930"
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                     /product="18S ribosomal RNA"
     misc_RNA        35..241
                     /product="internal transcribed spacer 1"
     rRNA            242..404
                     /product="5.8S ribosomal RNA"
     misc_RNA        405..679
                     /product="internal transcribed spacer 2"
     rRNA            680..>739
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       61 ggggcctaca gggctagtag cttatacgtt gttgaacgtc ctgcctggac ccggagccgc
      121 ccccatctaa accctctgcg tacctgtccc ttcttgcttc ccccggcatc tcgtcggggg
      181 gaggtaacaa ccaaaactct ctgtgaatca aacagccgtc agaattataa aacaaacaaa
      241 agttaaaact ttcaacaacg gatctcttgg ttcccacatc gatgaagaac gcagcgaaat
      301 gcgataagta atgtgaattg cagaattcag tgaatcatcg aatctttgaa cgcacattgc
      361 gccccctggt attccggggg gcatgcctgt tcgagcgtca taaaaacctc ctccccccttc
      421 gggttttgtt actatcgttg gggggttttg gcctaatggg atagcgattg gcaattcgtt
      481 tcccaatgtc ctaaatagac gtagacccgc ctccagatgc gacagcaccg aggccatcaa
      541 ccgtggagtt atgggatata ataggcttgc agtaaaatgc tcacctctct ccacacgccg
      601 atggcacgac agttgcagtt gcgggcgtaa attggagccc ttttcaggac ccttgtggcc
      661 tagcatccac catacataat ttgacctcgg atcaggtagg gatacccgct gaacttaagc
      721 atatcaataa gcggaggaa
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Sequence of Internal transcribed spacer (ITS) region of *Morchella elata* isolate MR23

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LOCUS       GQ228472                743 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella elata isolate MR 23 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.
ACCESSION   GQ228472
VERSION     GQ228472.1  GI:294653291
KEYWORDS    .
SOURCE      Morchella elata
  ORGANISM  Morchella elata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 743)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 743)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..743
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                     /mol_type="genomic DNA"
                     /isolate="MR 23"
                     /db_xref="taxon:39930"
     rRNA            <1..34
                     /product="18S ribosomal RNA"
     misc_RNA        35..245
                     /product="internal transcribed spacer 1"
     rRNA            246..408
                     /product="5.8S ribosomal RNA"
     misc_RNA        409..683
                     /product="internal transcribed spacer 2"
     rRNA            684..>743
                     /product="28S ribosomal RNA"
ORIGIN
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       61 taggggaccg acagggctag tagcttatac gttgttgaac gtccagtatg gacccgaagc
      121 ctccccatc taaaccctct gcgtacccgt cccttcttgc ttccccggc atctcgtcgg
      181 ggggaggtaa caaccaaaac tctatgtgaa tcaaacagcc gtcagaacta taaaacaaac
      241 aaaaagttaa agctttcaac aacggatctc ttggttccca catcgatgaa gaacgcagcg
      301 aaatgcgata agtaatgtga attgcagaat tcagtgaatc atcgaatctt tgaacgcaca
      361 ttgcgccccc tggtattccg gggggcatgc ctgttcgagc gtcataaaaa cctcctcccc
      421 cttcgggttt tgttactatc gttgggggt tttggcctaa tgggatagcg attggcaatt
      481 cgtttcccaa tgtcctaaat agacgtagac ccgcctccag atgcgacagc accgaggcca
      541 tcaaccgtgg agttatggga tataataggc ttgcagtaaa atgctcacct ctctccacac
      601 gccgatggca cgacagttgc agttgcgggc gtaaattgga gccctttca ggacccttgt
      661 ggcctagcat ccaccataca taatttgacc tcggatcagg tagggatacc cgctgaactt
      721 aagcatatca ataagcggag gaa
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Sequence of Internal transcribed spacer (ITS) region of *Morchella elata* isolate F1

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LOCUS       GQ228473                737 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella elata isolate F1 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.
ACCESSION   GQ228473
VERSION     GQ228473.1  GI:294653292
KEYWORDS    .
SOURCE      Morchella elata
  ORGANISM  Morchella elata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 737)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 737)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..737
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                     /mol_type="genomic DNA"
                     /isolate="F1"
                     /db_xref="taxon:39930"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..240
                     /product="internal transcribed spacer 1"
     rRNA            241..403
                     /product="5.8S ribosomal RNA"
     misc_RNA        404..678
                     /product="internal transcribed spacer 2"
     rRNA            679..>737
                     /product="28S ribosomal RNA"
ORIGIN
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      121 cccatctaaa ccctctgcgt acctgtccct tcttgcttcc cccggcatct cgtcgggggg
      181 aggtaacaac caaaactctc tgtgaatcaa acagccgtca gaattagaaa acaaacaaaa
      241 gttaaaactt tcaacaacgg atctcttggt tcccacatcg atgaagaacg cagcgaaatg
      301 cgataagtaa tgtgaattgc agaattcagt gaatcatcga atctttgaac gcacattgcg
      361 ccccctggta ttccgggggg catgcctgtt cgagcgtcat aaaaacttcc tccccttcg
      421 ggttttgtta ctatcgttgg ggggttttgg cctaatggga tagcgattgg caattcgttt
      481 cccaatgtcc taaatagacg tagacccgcc tccagatgcg acagcaccga ggccatcaac
      541 cgtggagtta tgggatataa taggcttgca gtaaaatgct cacctctctc cacacgccga
      601 tggcacgaca gttgcagttg cgggcgtaaa ttggagccct tttcaggacc cttgtggcct
      661 agcatccacc atacataatt tgacctcgga tcaggtaggg atacccgctg aacttaagca
      721 tatcaataag cggagga
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Sequence of Internal transcribed spacer (ITS) region of *Morchella sp.* isolate MR2

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LOCUS       GQ228474                955 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella sp. MR 2 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.
ACCESSION   GQ228474
VERSION     GQ228474.1  GI:294653293
KEYWORDS    .
SOURCE      Morchella sp. MR 2
  ORGANISM  Morchella sp. MR 2
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 955)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 955)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..955
                     /organism="Morchella sp. MR 2"
                     /mol_type="genomic DNA"
                     /isolate="MR 2"
                     /db_xref="taxon:753977"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..442
                     /product="internal transcribed spacer 1"
     rRNA            443..605
                     /product="5.8S ribosomal RNA"
     misc_RNA        606..896
                     /product="internal transcribed spacer 2"
     rRNA            897..>955
                     /product="28S ribosomal RNA"
ORIGIN
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       61 gcgctcgcgc tgggctcgat agacgcgcta gcacccgtca ggcccggagg gcgaaccagg
      121 ccctgggggc cggcaggtta aacccaaggt acccatccaa ggtggacagt cctcccagag
      181 ttcgcccttc ggcgtctgcg agccatcggg gcatggaacc ttgggggctg tcggtttccc
      241 gctgggcata cgtccgatgc cgcctcggga ggcgaaacgt ggctcgtgca gcgtcgcagc
      301 gttggtcccc cactcactca ccaaaaccct ctgcgtacct tgcccacttg cttcccccgg
      361 ccccccggc cgggggggcga caccaaaaac caaactcttt gcgatgaacc gacgtctgaa
      421 tgccaaaagc aaaacaaaaa cagttaaaac tttcaacaac ggatctcttg gctcccacat
      481 cgatgaagaa cgcagcgaaa tgcgataagt aatgtgaatt gcagaattca gtgaatcatc
      541 gaatctttga acgcacattg cgccccctgg tattccgggg ggcatgcctg tccgagcgtc
      601 ataaaacccc ctccccctcg gattaaatgt tccttggggg gtattggccc aatgggattg
      661 cgagatagca attgttaccc aggcgcccaa atgcatcagt accccgccat ttacagattt
      721 accagcaccg aggccgtcaa ccgtggagtt atgggaatta taggcttgca gtaatatgct
      781 cacctctctc catacgcagg cggcacaccg ggtagagtcg cgggcgtaaa gcggaatccg
      841 aaacggccct cacgggtaga ggaggatcct tgcgggctag tcacccctca tcacaatttg
      901 acctcggatc aggtagggat acccgctgaa cttaagcata tcaataagcg gaga
```

Sequence of Internal transcribed spacer (ITS) region of *Morchella gigas* isolate MR7

```
LOCUS       GQ228475                828 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella gigas isolate MR 7 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.
ACCESSION   GQ228475
VERSION     GQ228475.1  GI:294653294
KEYWORDS    .
SOURCE      Morchella gigas
  ORGANISM  Morchella gigas
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 828)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 828)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..828
                     /organism="Morchella gigas"
                     /mol_type="genomic DNA"
                     /isolate="MR 7"
                     /db_xref="taxon:221242"
     rRNA            <1..32
                     /product="18S ribosomal RNA"
     misc_RNA        33..325
                     /product="internal transcribed spacer 1"
     rRNA            326..488
                     /product="5.8S ribosomal RNA"
     misc_RNA        489..768
                     /product="internal transcribed spacer 2"
     rRNA            769..>828
                     /product="28S ribosomal RNA"
ORIGIN
        1 ccgtaggtga acctgcggaa ggatcattac caaaaaacac gttcggacgg ggggcactag
       61 aggtgggaag ccaaggcgat gctagctgga cgtcgtgtgg cctgtggttg ccgggggtta
      121 aacgttcaca ccccccctcg cgcagcacac atcagtggcc gaacccaggc aaagcaccgg
      181 cccccatcgg ctgccccccgg tcccaaaaca aaaaccctct gcgtaccttc ccctccttgc
      241 ttccccccggc acacgtcggg gggaggaaca caacccaaaa ctctttgcgt gtaaccgccg
      301 tcagaaatac agatacaaaa gaaaagttaa aactttcaac aacggatctc ttggttccca
      361 catcgatgaa gaacgcagcg aaatgcgata agtaatgtga attgcagaat tcagtgaatc
      421 atcgaatctt tgaacgcaca ttgcgccctc tggtactccg gggggcatgc ctgttcgagc
      481 gtcataaaac catctccccc aacgtgttta ttcatttaag cctacgctgg gggggtcttg
      541 gcctgcggga aagcgactgg caatccgctc ccgagagccc aaatttagga gacccgccat
      601 ctgatgcgac agcaccgagg ccctcaaccg tggagtcatg ggacttttgg atttgcagta
      661 atatgctcac ctttcttcac acgccgttgg cacaccagtc gcagttgcgg gcgtaatatg
      721 gagtccccca agggggggacc cttgtggatt agcacccacc tcacaaactt tgacctcgga
      781 tcaggtaggg atacccgctg aacttaagca tatcaataag cggaggac
```

Sequence of Internal transcribed spacer region of *Morchella spongiola* isolate MR17

```
LOCUS       GQ228476               1186 bp    DNA     linear   PLN 28-MAR-2011
DEFINITION  Morchella spongiola isolate MR 17 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.
ACCESSION   GQ228476
VERSION     GQ228476.1  GI:294653295
KEYWORDS    .
SOURCE      Morchella spongiola
  ORGANISM  Morchella spongiola
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 1186)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the Western
            himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 1186)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road, Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..1186
                     /organism="Morchella spongiola"
                     /mol_type="genomic DNA"
                     /isolate="MR 17"
                     /db_xref="taxon:62755"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..595
                     /product="internal transcribed spacer 1"
     rRNA            596..758
                     /product="5.8S ribosomal RNA"
     misc_RNA        759..1127
                     /product="internal transcribed spacer 2"
     rRNA            1128..>1186
                     /product="28S ribosomal RNA"
ORIGIN
        1 tccgtaggtg aacctgcgga aggatcatta ccagattcca taccaaaaaa gacagtgggg
       61 agagagaagg cgcataacca cttgctgaga cctgccgtac cgtgactggc gtgttccggc
      121 gcggcaccta gggtgtccac ggttcagggt gtgtcagcca gggaacctca tccgtgtagt
      181 caaatccacg ctgcttggta gtcaggccag gcaccaggca gtacaaggag ccggatcgag
      241 cattaaacgc tctgcaccca tcccacgcag ttgggtgaag gtgcagagcg gatttctgta
      301 gctaaaggag gtacccctgg caagtggctg acgacgcaag cagggctgaa ataaacccct
      361 gccactgcgg cgattcccct gtggctgtgg gtgtaaagac ccacagcgac tcgcaccgcg
      421 gcaggacacc actcttaggt ttgtcacccc ctcgccactg tcccaaccaa aaccctctgt
      481 gtacccttcc cagttgcttc ccccggggaa ctggctcctg ccagccgggg ggagaaacca
      541 agcaaaaacc ctttttcgc aaaacagacg tccgaattta aaaacaaaac aaaaagttaa
      601 aactttcaac aacggatctc ttggttccca catcgatgaa gaacgcagcg aaatgcgata
      661 agtaatgtga attgcagaat tcagtgaatc atcgaatctt tgaacgcaca ttgcgccctc
      721 tggtattccg gagggcatgc ctgttcgagc gtcataaata ccgctccccc tcggattgat
      781 tgcgatccct ggggggttct ggcaatgtgg tctccccgtg ctttgagggc atgcgaacgg
      841 gctcccagtg ctgaaagaca taacgttccc ggccgaaacc ggtgattaat ttcaccggca
      901 ggattcgtgg caggcagact gagggcgtca accgtggagt catgaggata gaaacctccc
      961 cctttgcaag taacattgct ctggcagtta gatctgcagg cctgccggtc tggggatgga
     1021 cctcccactc gcaggcgtca cggccacgat agcgggcgtt aaacggaatc cgatccgcct
     1081 tcactggtgg ttgaagatcc ttgtgggcta gcaacccta aacatatttt gacctcggat
     1141 caggtaggga tacccgctga acttaagcat atcaataagc ggagga
```

Sequence of Internal transcribed spacer (ITS) region of *Morchella elata* isolate MR24

```
LOCUS       GQ228477                741 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Morchella elata isolate MR 24 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.
ACCESSION   GQ228477
VERSION     GQ228477.1  GI:294653296
KEYWORDS    .
SOURCE      Morchella elata
  ORGANISM  Morchella elata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Morchella.
REFERENCE   1  (bases 1 to 741)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 741)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..741
                     /organism="Morchella elata"
                     /mol_type="genomic DNA"
                     /isolate="MR 24"
                     /db_xref="taxon:39930"
     rRNA            <1..34
                     /product="18S ribosomal RNA"
     misc_RNA        35..243
                     /product="internal transcribed spacer 1"
     rRNA            244..406
                     /product="5.8S ribosomal RNA"
     misc_RNA        407..681
                     /product="internal transcribed spacer 2"
     rRNA            682..>741
                     /product="28S ribosomal RNA"
ORIGIN
        1 ttccgtaggt gaacctgcgg aaggatcatt accaagaacc atacagaaaa gggaggcatt
       61 aggggaccga ccagggctag tagctttacg ttgttgaacg tccagtatgg acccgaagcc
      121 tcccccatct aaaccctctg cgtacctgtc cttcttgctt ccccccggcat ctcgtcgggg
      181 ggaggtaaca accaaaactc tctgtgaatc aaacagccgt cagaattata aaacaaacaa
      241 aaagttaaaa ctttcaacaa cggatctctt ggttcccaca tcgatgaaga acgcagcgaa
      301 atgcgataag taatgtgaat tgcagaattc agtgaatcat cgaatctttg aacgcacatt
      361 gcgccccctg gtattccggg gggcatgcct gttcgagcgt cataaaaacc tcctccccct
      421 tcgggttttg ttactatcgt tgggggttt tggcctaatg ggatagcgat tggcaattcg
      481 tttcccaatg tcctaaatag acgtagaccc gcctccagat gcgacagcac cggggccatc
      541 aaccgtggag ttatgggata taataggctt gcagtaaaat gctcacctct ctccacacgc
      601 cgatggcacg acagttgcag ttgcgggcgt aaattggagc ccttttcagg accctcgtgg
      661 cctagcatcc accatacata atttgacctc ggatcaggta gggatacccg ctgaacttaa
      721 gcatatcaat aagcggagga a
```

Sequence of Internal transcribed spacer (ITS) region of *Verpa sp.* isolate F2

```
LOCUS       GQ281276                866 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Verpa sp. F2 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.
ACCESSION   GQ281276
VERSION     GQ281276.1  GI:254574586
KEYWORDS    .
SOURCE      Verpa sp. F2
  ORGANISM  Verpa sp. F2
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Verpa.
REFERENCE   1  (bases 1 to 866)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 866)
  AUTHORS   Kanwal,H.K., Madhavi,M., Ramesh,G. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..866
                     /organism="Verpa sp. F2"
                     /mol_type="genomic DNA"
                     /isolate="F2"
                     /db_xref="taxon:659681"
     rRNA            <1..34
                     /product="18S ribosomal RNA"
     misc_RNA        35..449
                     /product="internal transcribed spacer 1"
     rRNA            450..612
                     /product="5.8S ribosomal RNA"
     misc_RNA        613..808
                     /product="internal transcribed spacer 2"
     rRNA            809..>866
                     /product="28S ribosomal RNA"
ORIGIN
        1 ttccgtaggt gaacctgcgg aaggatcatt accaatagat attacacccg caagctgagg
       61 ggagaaaaca gttcctcccc atagggaggg ctgttgtatg caaacgctgt gttcatgggg
      121 ggcagattca gttaagttta tcctagtaga atcggtgtga ttccgttgtc cgtcacagag
      181 cattacgtct cgaggatggt taccacgaca tgctggcggg atacctacgt taacgctagt
      241 gaatggacat tgacgccact tgtgtgtagg cggctcgtct ctcggtgctc cggtagacca
      301 tacacctctc cttaagcttg ctcggtatat tactcaaacc ctccgtgtac ctatccatgt
      361 tgctttccca ctgctaaaaa gtggtgggag aggtccaata ccaaatcctt ttgtcaaacc
      421 gctgtcagaa ataaaacaaa aaaaaaacag ttaaaacttt caacaacgga tctcttggtt
      481 cccacatcga tgaagaacgc agcgaaatgc gataagtaat gtgaattgca gaattcagtg
      541 aatcatcgaa tctttgaacg cacattgcgc cctctggtat tccgggggggc atgcctgttc
      601 gagcgtcata aacaactctc ccccaaacat ttgttgtgag gggggtactg gctggtaggc
      661 atttggctcc acaggccttt tgcccggctc aaattattgg tctgtaggcc cgagtgcaaa
      721 cagcggcgta gtggtaacat ttcccgtcgc gattgtttgc ctcaagctcc taggacctta
      781 agcgctttac tgcgccaaaa cccatagatt gacctcggat caggtaggga tacccgctga
      841 acttaagcat atcaataagc ggagga
```

Sequence of Internal transcribed spacer (ITS) region of *Verpa sp.* isolate MR22

```
LOCUS       GQ281277                868 bp    DNA     linear   PLN 28-MAR-
2011
DEFINITION  Verpa sp. MR 22 18S ribosomal RNA gene, partial sequence;
internaltranscribed spacer 1, 5.8S ribosomal RNA gene, and internal
transcribed spacer 2, complete sequence; and 28S ribosomal RNA
gene, partial sequence.
ACCESSION   GQ281277
VERSION     GQ281277.1  GI:254574587
KEYWORDS    .
SOURCE      Verpa sp. MR 22
  ORGANISM  Verpa sp. MR 22
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Pezizomycetes; Pezizales; Morchellaceae; Verpa.
REFERENCE   1  (bases 1 to 868)
  AUTHORS   Kanwal,H.K., Acharya,K., Ramesh,G. and Reddy,M.S.
  TITLE     Molecular characterization of morchella species from the
Western himalayan region of India
  JOURNAL   Curr. Microbiol. 62 (4), 1245-1252 (2011)
   PUBMED   21188589
REFERENCE   2  (bases 1 to 868)
  AUTHORS   Kanwal,H.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JUN-2009) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147 004, India
FEATURES             Location/Qualifiers
     source          1..868
                     /organism="Verpa sp. MR 22"
                     /mol_type="genomic DNA"
                     /isolate="MR 22"
                     /db_xref="taxon:659682"
     rRNA            <1..33
                     /product="18S ribosomal RNA"
     misc_RNA        34..448
                     /product="internal transcribed spacer 1"
     rRNA            449..611
                     /product="5.8S ribosomal RNA"
     misc_RNA        612..807
                     /product="internal transcribed spacer 2"
     rRNA            808..>868
                     /product="28S ribosomal RNA"
ORIGIN
        1 tccgtaggtg aacctgcgga aggatcatta ccaatagata ttacacccgc aagctgaggg
       61 gagaaaacag ttcctcccca tagggagggc tgttgtatgc aaacgctgtg ttcatggggg
      121 gcagattcat ttaagtttat cctagtagaa tcggtgtgat tccgttgtcc gtcacagagc
      181 attacgtctc gaggatggtt accacgacat gctggcggga tacctacgtt aacgctagtg
      241 aatggacatt gacgccactt gtgtgtaggc ggctcgtctc tcggtgctcc ggtagaccat
      301 acacctctcc ttaagcttgc tcggtatatt actcaaaccc tccgtgtacc tatccatgtt
      361 gctttcccac tgctaaaaag tggtgggaga ggtccaatac caaatccttt tgtcaaaccg
      421 ctgtcagaaa taaaacaaaa aaaaaacagt taaaactttc aacaacggat ctcttggttc
      481 ccacatcgat gaagaacgcg gcgaaatgcg ataagtaatg tgaattgcag aattcagtga
      541 atcatcgaat ctttgaacgc acattgcgcc ctctggtatt ccgggggggca tgcctgttcg
      601 agcgtcataa acaactctcc cccaaacact tgttgtgagg ggggtactgg ctggtaggca
      661 tttggctcca caggcctttt gcccggctca aattattggt ctgtaggccc gagtgcaaac
      721 agcggcgtag tggtaacatt tcccgtcgcg attgtttgcc tcaagctcct aggaccttaa
      781 gcgctttact gcgtcaaaac ccatagattg acctcggatc aggtagggat acccgctgaa
      841 cttaagcata tcaataagcg gaggaaat
```