Isolation and characterization of epiphytic Orchidaceous Mycorrhizas associated with the populations of *Rhynchostylis retusa* from Kangra valley, Himachal Pradesh

A dissertation

submitted in the partial fulfilment of the requirement

for the award of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **"Isolation and characterization of epiphytic Orchidaceous Mycorrhizas associated with the populations of *Rhynchostylis retusa* from Kangra valley, Himachal Pradesh"** in partial fulfilment of the requirement for the award of Master of Science, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of six months from January 2014 to July 2014, under the supervision of Dr. **M Sudhakara Reddy**, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Place: Patiala

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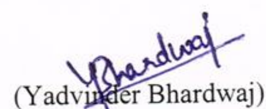

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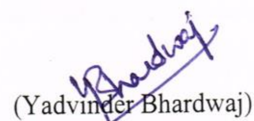

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Abstract

Orchids are most extravagant group of flowering plants and are getting depleted and threatened due to habitat destruction, macroclimatic changes, shifting cultivation, overexploitation and developmental activities. Orchids produce the smallest seeds (nearly microscopic in size) of any flowering plant. These dust-like seeds are produced in great numbers, often over a million seeds per plant and lack an endosperm, resulting in a small embryo covered only by a thin protective wall. This lack of storage tissues for food reserves and protection makes the seeds extremely vulnerable to their environment, resulting in a high mortality rate unless optimum conditions are found for germination.

The rate of seed germination in nature is very poor, that is, 2-5%. The symbiotic association between epiphytic orchids and mycorrhizal fungi is vital for essential nutrient supply during germination and seed establishment. Most of the mycorrhizal fungi of orchids fall into a non- sporing group known as *Rhizoctonia, Rhizoctonia solani* is a common symbiont of the members of Orchidaceae. *Rhynchostylis retusa*(L.) *Bl.* is a epiphytic orchid species found in Kangra, Himachal Pradesh, India. The ITS region is highly variable among fungal species, allowing to discrimination between even closely related species-based mycological studies at the sub-generic level and used for fungal species identification. In India till date a liitle work has been done on the mycorrhizal species related to orchids. This study focuses on the *Tulasnella* specific mycorrhizal interaction with *Rhynchostylis retusa*, which is an orchid specific mycorrhizal association.

The isolated and pure seven fungal strains were amplified with *Tulasnella* specific primers set: ITSI/ITS4-Tul, out of which five showed positive results. The five fungal isolates were morphologically and microscopically characterized. Media optimazation on MMN media by OFAT method and various enzyme activity tests like acid phosphatase

activity, phytase enzyme activity, biomass and pH reduction, cellulose enzyme reduction and phosphate solublization activity were performed and the results were analysed statistically on Graph Pad Prism 5.0 software and 2 Way-ANOVA also was performed to find the possible interactions and the significant statistical values. All of the fungal isolates showed varying significant results positive results. The molecular characterization of the fungal isolates was performed by sequencing the *Tulasnella* specific amplified PCR products. Out of five fungal species two sequences were able to be sequenced and the other three are under process. The two sequences were analysed and using BLAST search tool to homology of unknown sequences with those present in databases. The results showed that the sequences T1 and T2 were homologous to *Fusarium sp. 208f* and *Leptosphaerulina chartarum* respectively. The further studies are to be continued.

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1. Introduction

1.1 Orchids

Orchids are most extravagant group of flowering plants distributed throughout the globe (White and Sharma, 2000), but they are very sensitive to habitat change. Estimates indicate the presence of 800 genera and 25,000-35,000 species of orchids worldwide, according to the World Conservation Monitoring Centre 1992. Orchid populations get depleted and threatened due to habitat destruction, macroclimatic changes, shifting cultivation, overexploitation and developmental activities (Joshi et al., 2009).They are regarded as the ecological indicators (Rao, 2005) and also important aesthetically and medicinally. They have been used in medicinal purpose at first in china, and in India also as one of the ingredients in ancient Indian system of medicine called the "Ayurveda" which has been used worldwide. They have been used in pharmacological treatments as anti-inflammatory, anti-viral, anti-carcinogenic, anti-convulsive, diuretic, neuro-protective, anti-aging, wound healing, anti-tumor and anti-rheumatic (Bijaya, 2013).

Orchids are considered monocots, distinguished from other angiosperms by having only one cotyledon, or the leaf that first emerges from the seed. Orchids are species having storage organs and with high activity of polyphenoloxidase, they can function as bio-filters and air cleaners in hermetic capacity (Cherevchenko et al., 2001). Considering both endangering, the natural beauty of epiphytic orchids, and their important in canopy ecosystems, comprehensive efforts for their conservation are needed.

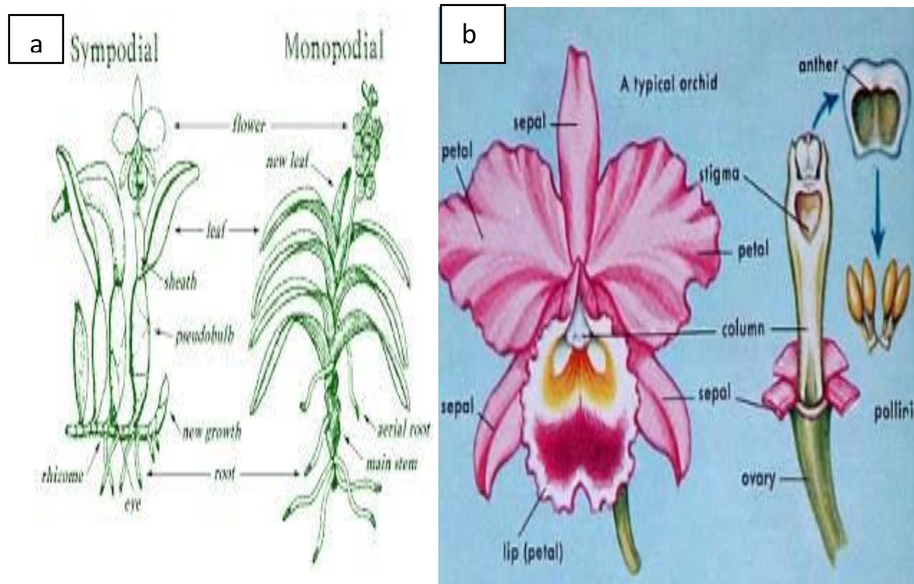


Fig 1.1) a) Difference between parts of sympodial and monopodial orchids.

b) Floral parts of a typical orchid.

1.2 Epiphytic Orchids

Epiphytes are canopy elements, independent from ground habitats. Epiphytes generally grow on trees or shrubs without harming them (Adhikari, 2013). They are divided into two groups; holo-epiphytes (complete life cycle on host trees) and hemi-epiphytes (only part of life cycle on host trees). Moisture rich and mossy habitats are especially suitable for luxuriant growth of epiphytic orchids. Epiphytic orchids are both of the sympodial (stems of determinate growth; e.g. Dendrobium) and the monopodial (indeterminate terminal growth; e.g. Vanda) type. Epiphytic orchids are able to absorb and store atmospheric moisture. The lack of forest floor contact combined with the diversity of nutrient and water sources in the canopy life has led to specific adaptation mechanisms in epiphytes (Cardelus and Mack, 2010).

The main nutrient and water sources of epiphytic orchids are precipitation, canopy through fall, and cloud water from which they shape their functional morphologies. For

example, thick leathery leaves, water loaded pseudo bulbs; pendant orchids with velamen-coated aerial root uptake serve the double purpose of anchorage and absorption (Benzing, 1990). The symbiotic association between epiphytic orchids and mycorrhizal fungi is vital for essential nutrient supply during germination and seed establishment (Weston et al., 2005). Epiphytic orchids have modified aerial roots that can sometimes be a few meters long. In the older parts of the roots, a modified spongy epidermis, called Velamen, has the function to absorb humidity. It is made of dead cells and can have a silvery-grey, white or brown appearance. Nutrients mainly come from animal droppings and other organic detritus on their supporting surfaces.

1.3 Host trees and abiotic variables:

The diversity of epiphytic orchids and their abundance are mainly influenced by the available host trees and their micro-site conditions. Abiotic variables are host characteristics as micro-site conditions (e.g. host bark pH, bark rugosity, and water holding capacity of barks). Different abiotic substrates of the host bark play a vital role for aggregation and abundance of epiphyte species. Therefore, vascular epiphytes are largely found in tropics and subtropics (Nieder and Michaloud, 2001).

The combination of host tree traits like host tree size, the host tree as substrate, the chemical characteristics of host bark, the bark structure, and rugosity (Adhikari et al, 2012), appears to be important in determining the epiphytes presence and diversity. The knowledge of host tree species, their traits and chemical characteristics including micro-site conditions as well as knowledge of their ecological formation is important for conservation of epiphytic communities in the future.

1.4 Micro-site conditions

Host bark pH, host bark rugosity, host sun light intensity, and host exposure to wind in different human impact categories are the different micro-site conditions that could be analysed with respect to orchids (Adhikari et al., 2012). The best suitable native tree species which offer the best micro-site conditions (e.g. *Ficus religiosa, Mangifera indica, Schima wallichii, Alnus nepalensis*, and *Rhododendron arboreum*) not only as single trees but in groups, developing some forest habitats for epiphytic orchids as “future host trees ” for the epiphytic plant *Rhyncostylis retusa.*. Old trees are better hosts for epiphytic orchids than younger ones because the larger the surface area of the host, the larger the area for colonization and higher the possibilities for epiphyte seed to contact a host. Furthermore, the older the host the more time it has been exposed, thus increasing the chances of epiphytic seed contact (Migenis and Ackerman, 1993).

1.5) Mycorrhiza

Mycorrhizal fungi are species of fungi that intimately associate with plant roots forming a symbiotic relationship, with the plant providing sugars for the fungi and the fungi providing nutrients such as phosphorus and carbon to the plants. Mycorrhizal fungi can absorb, accumulate and transport large quantities of phosphate within their hyphae and release to plant cells in root tissue. The ability of plant species to survive in many environments depends on mycorrhizae and they do play a major role in nutrient recycling in soils .

There are three major groups of mycorrhiza: Ectomycorrhiza, Ectendomycorrhiza and Endomycorrhiza. Ectomycorrhiza and endomycorrhiza are important in agriculture and forestry. Plants inoculated with endomycorrhiza have been shown to be more resistant to some root diseases. The most common type of endomycorrhizae is the Arbuscular

mycorrhizas (AM), which occur in both herbaceous and woody species. AM are most abundant in systems where phosphorus is limited and in warmer or drier climates.

1.5. a) Taxonomy of AM fungi

AM fungi show the peculiar characteristics in morphology and physiology. Spores of AM fungi are generally formed in soil and their sizes (50-500 μm in diameter) are much larger than those of other fungi. There is no septum in their hyphae. No sexual growth-phase has been observed i.e anamorphic in nature. Spores germinate when they are under favourable conditions, extend their hyphae and colonized plant roots. The fungi penetrate the hyphae into cortex layer of roots and form the hyphal organs, “vesicles” and “arbuscules” which are characteristics to AM fungi (Fig 1.2) (Bergero et al., 2003). Colonization on plant roots is essential for proliferation of AM fungi. AM fungi are thus recognized as obligate symbiotic fungi (Driver et al., 2005). The interaction between AM fungi and plants is generally mutualism based upon nutrient exchange. A new phylum Glomeromycota has been proposed for AM fungi.

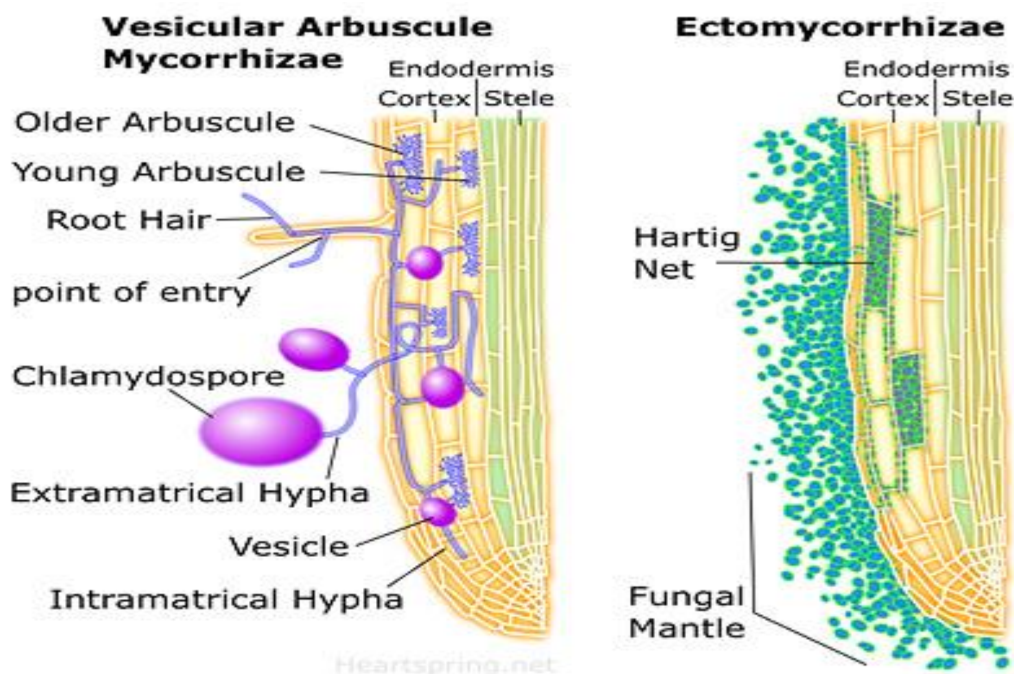


Fig 1.2) Difference between Vesicular Arbuscule Mycorrhizae (AM) and Ectomycorrhiza.

1.6) Orchid Mycorrhizae and their function

Orchid mycorrizae are symbiotic relationships between roots of plants of the family Orchidaceae and a variety of fungi. Orchid mycorrhizae are critically important during orchid germination, as orchid seed has virtually no energy reserve and obtains its carbon from the fungal symbiont. The fungi that form orchid mycorrhiza are typically basidiomycetes. These fungi come from a range of taxa including *Ceratobasidium (Rhizoctonia), Sebacina, Tulasnella* and *Russula* species.

1.6.1) Functions

a) Nutrient exchange

Orchid mycorrhizas are different from other types of mycorrhizas in the nature of the nutrient exchange. After establishment of a mycorrhiza, organic carbon and other nutrients are passed from the fungus to the seed. Because asymbiotic germination requires sugars, amino acids and vitamins, it is assumed that these are also obtained from the fungus. The fungus continues to supply the protocorm with all its organic energy until such times as the plant starts to photosynthesis (Adhikari, 2013). The source of carbon is assumed to be organic materials surrounding the plant.

Mycorrhizal fungi have the potential to solubilise carbohydrates, including cellulose. The fungi translocate cellulose in the hyphae and the sugar is made available at the interface. Thus the fungus acquires and translocates organic energy to the plant. This is significant even in adult plants, especially epiphytes that exist in shadows of the canopy.

b) Seed germination (a symbiotic effect)

Orchids produce the smallest seeds (nearly microscopic in size) of any flowering plant. These dust-like seeds are produced in great numbers, often over a million seeds per plant. Unlike other plant species, these minute seeds lack an endosperm, or food source, resulting in a small

embryo covered only by a thin protective wall. This lack of storage tissues for food reserves and protection makes the seeds extremely vulnerable to their environment, resulting in a high mortality rate unless optimum conditions are found for germination. In nature, association with a specific fungal partner, the orchid mycorrhiza is a pre-requisite for the germination of orchid seeds (Mitra, 1971). The rate of seed germination in nature is very poor, that is, 2-5% (Rao, 1977). Symbiotic associations between orchids and mycorrhizal fungi are a competitive struggle. The fungi always try to invade the cytoplasm of orchid cells to obtain nutritional compounds. On the other hand, the orchid cells restrict the growth of the infecting hyphae and obtain nutrition by digesting them. It is assumed that antifungal compounds are involved in the restriction of fungal growth inside the orchid (Shimura et al, 2007)

Germination depends on colonisation by a specific mycorrhizal fungus. Germination follows a similar pattern in most cases. The seed imbibes water. The fungus penetrates the testa of the seed and enters either through epidermal hairs or the suspensor of the undifferentiated embryo. The fungus forms a tight coil or peleton following invagination of the plasma-membrane and extension into the cell. The peleton remains active for some time, but then collapses. The fungus colonises further cells, and the mycorrhiza spreads. Initial contact between fungus and imbibed seed can have one of three results: the fungus and plant form a functional mycorrhizae, the fungus can parasitise the seeds, or the fungus remains outside the seeds. In any one batch of seed, all three processes seem to take place. That is, some seed are potentially germinable, some are parasitised and die, and others remain ungerminated, because of a lack of recognition between symbionts. The process of differentiation follows initiation of colonisation. The imbibed seed starts to expand and cells multiply (Adhikari, 2013). The fungus colonises further cells. Cells apart from the peletons start to differentiate to eventually become the shoot tissue. The tip becomes slightly pointed. From this time, the plant is called a protocorm. The uncolonized tip then continues to grow

and becomes the shoot. The basal region remains the absorbing region, eventually differentiating into structures that resemble roots.

Orchid seeds cannot utilize their own reserve or do so very slowly, they also cannot hydrolyse large molecules like starch or cellulose (Fig 1.3). As a result, asymbiotic germination in the absence of sugar proceeds only to the early protocorm stage, after which they wait for external supply of simple sugars through the help of mycorrhizal fungus. The fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid which is called symbiotic germination (Arditti J, 1992). Most of the mycorrhizal fungi of orchids fall into a non- sporing group known as *Rhizoctonia*, the major species being *Rhizoctonia repens, Rhizoctonia mucoroides* and *Rhizoctonia languinosa*. *Rhizoctonia solani* is a common symbiont of the members of Orchidaceae. It is an endophytic fungus occurring in the roots of orchids in the form of pelotons, which is the distinctive characteristic of orchid mycorrhiza (Kaushik and Pal, 2011) and in the case of saprophytic terrestrial orchids and form dead bark in case of epiphytic orchid .

Figure 1.3) Longitudinal Section of orchid capsule showing immature seeds.

1.7 ***Rhynchostylis retusa*** **(foxtail orchid)**

Rhynchostylis retusa(L.) *Bl.* is a monopodial, epiphytic orchid species with beautiful flowers arranged in racemose inflorescence, which rank the species among the important Indian ornamental orchids (Parab and Krishnan, 2008). Its leaves have been used for the treatment of rheumatism and its root juice can be applied to cuts and wounds. It is closely linked with the people of Assam and known as *Kopou Phul* in Assamese language and regarded as the symbol of love. In India, the plant is most common in North-East, Orissa and Andhra Pradesh. In Andhra Pradesh, the plant is called by Telugu name *Chintaranamu*. Due to bio-piracy, the plant is in the verge of extinction in India. *Rhychostylis retusa* is recognized as the state flower of Arunachal Pradesh and Assam. Likewise, in Uttarkhand it is called *Banda* or *Rasna. Rhynchostylis* is a genus of three species, commonly known as 'fox-tail orchid' because of its brush-like spikes of colourful flowers (Bose et al. 1999).

Scientific classification	
Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Monocots
Order:	Asparagales
Family:	Orchidaceae
Subfamily:	Epidendroideae
Tribe:	Vandeae
Subtribe:	Aeridinae
Genus:	*Rhynchostylis*

Fig 1.4) Scientific classification of *Rhynchostylis retusa*

The plant is found in semi-deciduous and deciduous dry lowland forests woodlands at elevations of sea level up to 1,200 m (3,900 ft), and can be found in Bangladesh, Benin, Burma, Burma, China, India, Indonesia, Laos, Malaysia, Nepal, Philippines, Singapore, Sri Lanka, Thailand and Vietnam. The orchid has a bunch consisting of more than 100 pink-spotted white flowers (Fig 1.5 a). They have stout, repent, short stem carrying up to 12, curved, fleshy, deeply channelled, keeled, retuse apically leaves and blooms on an axillary pendant to 60 cm (24 inch) long, racemose, densely flowered, cylindrical inflorescence that occurs in the winter and early spring (Fig 1.5 b).

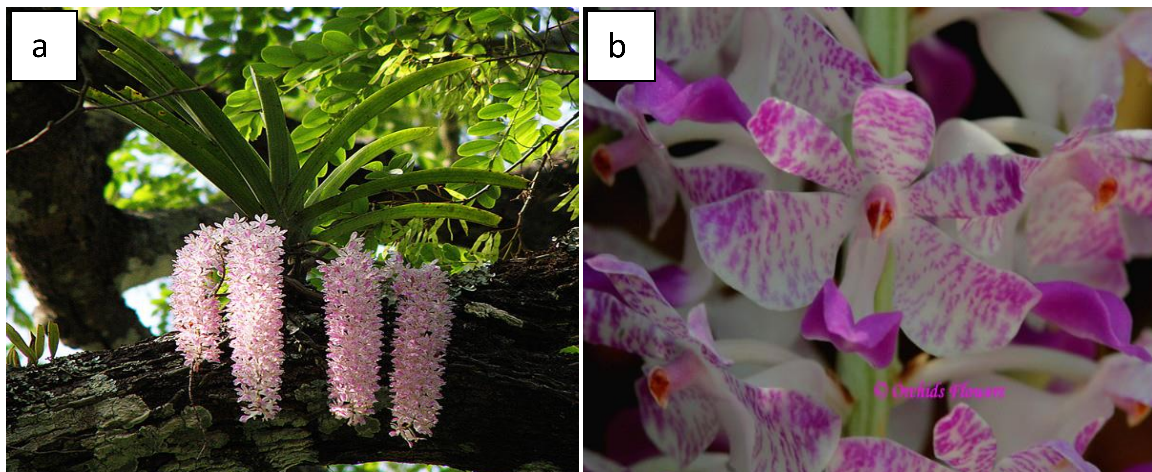


Fig 1.5 (a) Epiphytic plant *Rhynchostylis retusa* on its host *Mangifera indica* .

(b) pink-white spotted flowers of the epiphyte.

It can tolerate a wide range of temperature from 3°C to 31°C. Preferred light intensity is 40-80% of full sunlight, rough bark with pH around 6.5 and bark with a wide range of water holding capacity. A high number of *Rhynchostylis* individuals are found on *Mangifera indica*, *Ficus religiosa* and *Alnus nepalensis* having moderate water holding capacity of 74% and 70%, respectively. *Ficus religiosa* is the most common host species. The ranking of the host bark rugosity in facilitating the occurrence of *Rhynchostylis* was rough>medium>smooth bark.

1.8) Media optimization for increased production of fungal isolates

To enhance the seed germination rates of epiphytic orchids (*Rhynchostylis retusa*) there is a great need to find way out for increased production of endophytic fungal biomass symbiotically linked with the orchid species. So for, large scale production in defined media often needs to be optimized for conditions which provide maximum biomass levels. It is prerequisite to find all the factors and conditions involved in an optimization process and optimize the parameters so that their response reaches the optimum level. In order to achieve this, there are several conventional methods for optimization of a media based on 'one factor at a time' (OFAT) approach. The **one-factor-at-a-time method** (OFAT) is a method of designing experiments involving the testing of factors, or causes, one at a time instead of all simultaneously with a primary goal is to attain improvements in the system, and experimental error is not large compared to factor effects.

1.9) Enzymatic assays for the biochemical properties of the fungal isolates

Fungi are microorganisms well known for the range of novel enzymes they produce. Extracellular enzymes or exoenzymes produced by fungi are synthesized inside the cell and then secreted outside the cell, where their function is to break down complex macromolecules into smaller units to be taken up by the cell for growth and assimilation. These enzymes degrade complex organic matter such as cellulose and hemi-cellulose into simple sugars that enzyme-producing organisms use as a source of carbon, energy, and nutrients. Extracellular enzymes target macromolecules such as carbohydrates (cellulases), lignin (oxidases), organic phosphates (phosphatases), amino sugar polymers (chitinases) and proteins (proteases) and break them down into soluble sugars that are subsequently transported into cells to support heterotrophic metabolism. Fungi play an important role in

carbon cycling as they use secreted enzymes to breakdown lingo-cellulose and polymers then transporting the resulting products into the cells as their foods.

The secreted proteins in plant associated fungi play important roles in plant and fungi symbioses. Fungal enzymes have found a wide range of application in food, feed, pulp and paper, bio-ethanol and textile industries. Fungal enzyme play a critical role in everything from brewing beer and baking bread to make wine and converting plant matter into biofuel. Enzyme assays are laboratory methods for measuring enzymatic activity. They are vital for the study of enzyme kinetics and enzyme inhibition. Fungal mycelia mass and pigment is major obstacle to investigating the secretion of bioactive substances such as fungal activities using a plate assay. All enzyme assays measure either the consumption of substrate or production of product over time. A large number of different methods of measuring the concentrations of substrates and products exist and many enzymes can be assayed in several different ways.

1.10) Molecular characterization (ITS-region)

The most popular locus for DNA-based mycological studies at the sub-generic level for fungal species identification is the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit (Horton and Bruns, 2001; Bridge et al., 2005). The ITS region is preferred because it is relatively short (600-800 base pairs long), and there are hundreds of copies of rDNA, making it easier to amplify with PCR. Most importantly, the ITS region is highly variable among fungal species, allowing for discrimination between even closely related species (Gardes et al., 1993). In eukaryotes the nucleotide sequence of Ribosomal DNA (rDNA) changes very slowly and it is arranged in tandemly repeated units containing the coding regions for highly conserved regions and variable regions such as internal

transcribed spacer (ITS) regions and 18S, 5.8S, and 28S ribosomal RNA separated by spacers (Fig. 1.6).

Fungal rRNA operons contain two ITS regions out of which one is located between the 18S and 5.8S rRNA genes (ITS1) and the other exists between the 5.8S and 28S rRNA genes (ITS2). The sequence of the two ITS regions accumulate mutations at a faster rate than the 5.8S, 18S, and 28S rRNA genes because the two ITS sequences are excised and not required for any functional purpose after the transcription of rRNA operon. Hence the analysis of ITS regions (variation in the spacers) has proven useful for distinguishing among a wide diversity of fungal taxa. The ITS region is now perhaps the most widely sequenced DNA region in fungi. (Gardes and Bruns, 1993) designed two taxon selective primers, ITS1F and ITS4-R, intended to be specific for fungi and basidiomycetes, respectively.

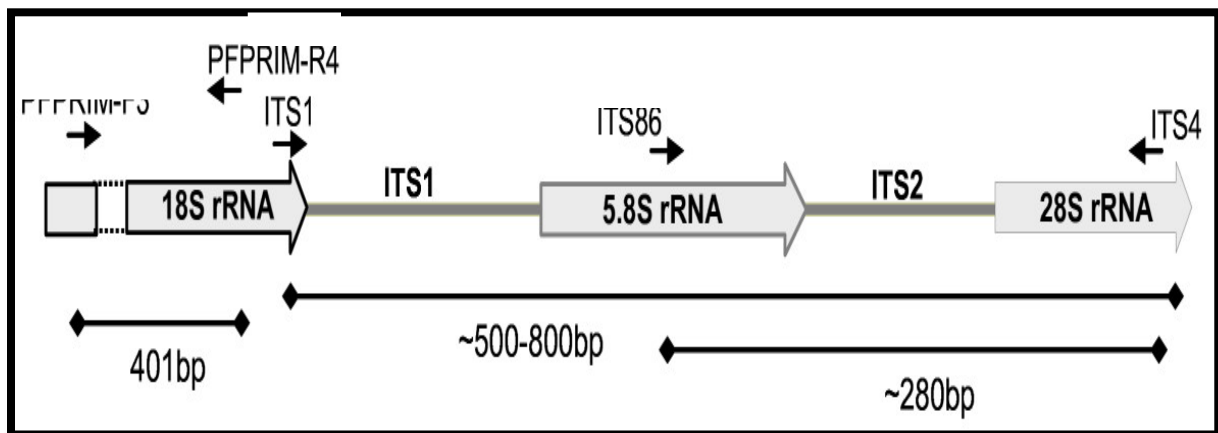


Fig1.6) Schematic representation of the fungal ribosomal 18S rRNA gene and ITS regions with primer binding locations.

1.11) Objectives of study

a) Isolation and identification of endophytic orchidaceous mycorrhizal fungi, *Tulasnella* species associated with the epiphytic orchid plant (*Rhynchostylis retusae*).

b) Physiological and biochemical characterization of *Tulasnella* species.

c) Molecular analysis of *Tulasnella* species associated with *Rhynchostylis retusae.*

Review of literature

2.1) Introduction

Orchids are herbaceous plants that are classified on their name in the family, the Orchidaceae. This family is estimated to have between 20,000-35,000 species (Dressler, 1993) with new species often being described. Habitat destruction due to logging is viewed as the most obvious cause of orchid diversity loss; however, habitat modification due to road construction, fire, urbanization, drainage, and other anthropogenic influences can also directly affect orchid habitats (Hágsater and Dumont, 1996; Cribb et al., 2003).

India is very rich in orchid flora. Orchids are found distributed in different parts of the country having different altitude, rainfall and temperature. They are located at an altitude up to 5000 meters from the sea level and in areas with rainfall ranging from 60 to 1100 cm per year. Epiphytic orchids are distributed up to 1800 meters and their frequency decreases with the increase in altitude. Most of the terrestrial orchids are limited to the temperate zone. In India, five major orchid zones such as plains, tropical, sub-tropical, temperate and sub-temperate, alpine and sub-alpine zone are classified based on their occurrence in different vegetations (Bose et al., 1999). Majority of orchids in India are distributed in the Eastern Himalayas, the Western and South Indian hills.

2.2) Economic importance and uses

In the Khasi hills, juice prepared from the leaves of *Cymbidium giganteum* is used to prevent blood flow in wound, while powdered flower of *Vanda spathulata* is effective against hysteria. *Dendrobium fimbriatum* is used against liver upsets and nervous debility (Bose et

al., 1999). Many of the Indian orchids such as *Cymbidium, Dendrobium, Paphiopedilum, Renanthera* and *Vanda* are currently in the International market. It was reported that about 70% of orchid plants are illegally imported by USA from India and Thailand (Chadha, 1992).

2.3) Epiphytic orchids

Epiphytic orchids are entirely depending on trees for structural and nutritional support. They are not parasitic and do not feed on the host plants (Dematte and Dematte, 1996); (Jonathan and Raju, 2005). Orchids obtain nutrients from rains and debris which are accumulated on the barks of host plant. They have adapted to have aerial roots and do not have the advantage of absorption of water from the soil.

Usually in orchids, water is absorbed by a spongy structure called "velamen tissue" which formed by dead cells layers in the roots. Orchids withstand severe drought, however, they die if there is inadequate drainage (Bomba, 1975; Batchelor, 1981).

2.4) Orchids seed germination

Orchid seeds are very small and dust-like usually ranging from 0.05–6.0 mm long and 0.01–0.9 mm wide and weighing from 0.31–24 µg (Arditti & Ghani, 1999). Orchid seeds are highly buoyant, with the embryo occupying only a minor proportion of the volume inside the testa of most species that have been examined, the rest of the seed being filled with air.

The seeds of most orchids can thus be modelled as miniature balloons and, indeed the seeds of some species resemble tiny balloons in form (Arditti & Ghani, 1999).In addition to their hydrophobic nature, the seed coats of some orchids contain inhibitory compounds, such as abscisic acid (Kinderen, 1987).

These compounds must be leached out of the seed coats with water before the seeds can germinate and it has been speculated that this may be a major factor in long dormancy

periods experienced by many orchid species in situ (Rasmussen, 1995). Despite the production of large number of seeds in orchids, only 0.2-0.3% of seeds germinates in nature (Singh, 1992; Murthy and Pyati, 2001). Most orchid seeds cannot germinate naturally in the absence of mycorrhiza. Noel Bernard discovered the role of mycorrhiza in seed germination in the late 1800s and Hans Buergeff worked on orchid mycorrhiza in the early part of last century (Arditti, 1992).

Their work showed that orchid mycorrhizal interactions were fairly specific and orchid seeds would not germinate without a fungal symbiont. It is now known that though some species do have specific interactions with certain species of fungi, others have a general relationship with many species of fungi (Arditti et al., 1980). (Masuhare and Katsuga, 1994) showed that seeds germinated in an turf grassland were infected by a single species of *Rhizoctonia* even though field isolates of other *Rhizoctonia* species were able to induce germination in vitro.

Mycorrhizal interactions also allow temperate orchids to persist for many years in a dormant state as the plant feeds on its fungal symbiont (Arditti, 1992). Endophytic fungi in the roots of mature pants of epiphytic orchid *Cattleya skinneri* to test their potential in seed germination of the plant and 108 strains were isolated to 11 genera out of which 8 strains were manageable in laboratory and their mycorrhizal potential was observed (Isidro et al., 2005).

2.5) Mycorrhizal interactions

The relationship of orchids with fungi is relatively unique in the plant kingdom. Of the greater than 80 percent of plant species that form mycorrhizal relationships, orchids along with members of the order Ericales do not form endomycorrhizal relationships with genera of the Zygomycota. The main group of fungi inhabiting orchid roots is Basidiomycetes, though Ascomycetes have been found (Currah et al., 1997).

Some of the Basidiomycetes with which orchids form a relationship are pathogenic on other crops, e.g., *Rhizoctonia solani* (Hadley, 1982). Even within the Orchidaceae, symbiotic fungi of one orchid species may be pathogenic on another. However, most orchids are able to control the infection and growth of endomycorrhizal fungi.

All the complexity of the mycorrhizae, its isolation and inoculation methods as fertilizer and all its benefits had been explained in Mycorrhiza: Complex fungi, a chapter abstract in (2004). Orchid mycorrhizal fungi are found intracellularly in cells of the cortex and they are confined to roots (Hadley, 1982).Within the cells, the mycorrhizae form dense coils of mycelium called pelotons which are thought to be adaptations to the host cell (Hadley, 1982).

Within the cell, the pelotons are surrounded by a membrane and interfacial matrix material (Peterson et al., 1997). The orientation of microtubules and cell wall microfibrils is altered during infection and may be necessary to alteration in the cytoplasm and synthesis of the membrane surrounding the pelotons (Peterson et al., 1997). (Knudson, 1951) successfully germinated *Cattelya* seeds without any symbiotic association of fungus on nutrient medium.

Research into mycorrhizal fungi in orchids has revealed that a relatively small number of fungal genera form associations with orchids. Identification of these fungi has mostly been achieved through the in vitro isolation of the fungi from parts of mature orchid plants (roots, tubers and protocorms) and subsequent morphological and genetic comparisons with known teleomorphic test cultures (Roberts 1999, Sneh et al., 1991).

Fungal endophytes are either binucleate or multinucleate and are members of the form genus *Rhizoctonia*, and species of the teleomorphic genera *Ceratobasidium, Tulasnella, Sebacina, Thanatephorus, Oleveonia and Serendipita* (Roberts, 1999, Sneh et al. 1991, Warcup 1971, Warcup & Talbot 1967). The growth of *Rhizoctonia solani* in different carbon and nitrogen sources were studied. Growth rate of fungus was measured by taking the dry mycelial weight

produced in the medium at particular time intervals. (Pal and Kaushik , 2012). Orchidaceous mycorrhizas are characterized by the fungus growing into the plant cell, invaginating the cell membrane and forming hyphal coils within the cell. The coils are active for only a few days, after which they lose turgor and degenerate and the nutrient contents are absorbed by the developing orchid (Sylvia, 2006).

2.6) *Rhynchostylis retusa*

Culturing of immature seeds of *Rhynchostylis retusa* was performed on MS media and specific growth regulators which helped out in the high frequency plantlet regeneration and multiple shoot induction and resulted with 40 out of 45 plantlet regenerated survived in the soil and the standardized protocol followed enabled rapid propagation and conservation of this precious orchid (Thomas and Michael, 2007) .

Traditional knowledge of the orchids growing in Uttarakhand, Western Himlaya, was elaborated and valuable products for herbal drug industry were discussed (Jeewan et al., 2008). Medhi et al. (2009) described the information of the traditional knowledge of the people of north-eastern region to conserve the valuable wild orchid germplasm and it comprises of the 876 orchid species of 151 genera with their ornamental and medicinal values.

Distribution pattern of the epiphytic orchid *Rhynchostylis retusa* (L.) Blume with respect to (i) site characteristics and host conditions, and (ii) the type and intensity of land use in Kathmandu Valley, central Nepal The correlation between *R. retusa* occurrence and microclimate condition was weak. *R. retusa*, to a certain degree, preferred light intensity of 40-80% of full sun light, rough bark with pH around 6.5 and bark with a wide range of water holding capacity. The distribution pattern of *R. retusa* was influenced by certain types of land

use. The probability to find *R. retusa* was highest in forest patches and parks and lowest in agricultural and dense populated area (Adhikari and Fischer, 2011).

2.7) Phosphate solublizing activity

With the help of phosphate solubilizers, phosphate availability in soil and the utilization of phosphate by plants can be increased (Gaur and Sacher, 1980). Venkateswaralu et al.(1984) isolated phosphate-solubilizing actinomycetes from orchid soils using modified Pikovskaya medium. (Darmwal et al., 1989) isolated phosphate solubilizing fungal cultures from different samples of rhizosphere of wheat, gram, garden soils and composed materials.

Arora and Gaur (1979) inoculated the bacterial and fungal culture into Pikovskaya broth and after incubation the water-soluble phosphate in the supernatant was estimated by the King's B method improved by Sherman with Klett-Sommerson colorimeter using red filter. Acid phosphatase and phytases secreted by microorganisms also have an important role in phosphate solubilization (Richardson et al., 2000). The Mineral Phosphate Solubilization (MPS) was studied in ten *Aspergillus niger* strains. MPS activity was measured in solid (Pikovskaya's medium) as well as liquid media using different phosphate sources, carbon sources and nitrogen sources All the strains showed a zone of clearance of Tricalcium Phosphate in Pikovskaya's medium in plates and solubilized Dicalcium and Tricalcium Phosphates in broth efficiently. Among the carbon sources *Aspergillus niger* preferred mannitol for higher phosphate solubilization. Nitrogen in the form of nitrate was very effective in solubilizing inorganic phosphates. Xylose and urea were the poorest sources of carbon and nitrogen for all the strains of *Aspergillus*. Phosphate release was associated with reduction in pH (Seshadri et al., 2004).

Eupenicillium parvum, a phosphate-solubilizing microorganism was isolated from the tea rhizosphere. The fungus developed a phosphate solubilization zone on modified Pikovskaya agar, supplemented with tri calcium phosphate. Quantitative estimation of phosphate solubilization in Pikovskaya broth showed high solubilization of Tri Calcium Phosphate and Aluminium phosphate. The fungus also solubilized North Carolina Rock Phosphate and Mussoorie Rock Phosphate, and exhibited high levels of tolerance against desiccation, acidity, salinity, Aluminium, and Iron. Solubilization of inorganic phosphates by the fungus was also observed under high stress levels of Aluminium, Iron, and desiccation, though the significant decline in phosphate solubilization was marked in the presence of Aluminium than Iron (Vyas et al. 2007).

Srividya et al.(2009) isolated and characterized the fungal strains from agriculture soil, having potential to solubilize insoluble inorganic phosphates on Pikovskya's medium with Tricalcium Phosphate. Aspergillus niger and Penicillium sp. showed high phosphate solubilisation efficiency on Pikovskya's medium with Tricalcium Phosphate in liquid broth in 5 days of growth. A. niger, showed maximum phosphate solubilization efficiency on Pikovskya's agar solid and liquid medium in 5 days of growth. Aspergillus sp showed diverse levels of phosphate solubilization activity in both solid and liquid broth culture in presence of various carbon and nitrogen sources and different media. Phosphate Solubilizing Microorganisms convert insoluble phosphates into soluble forms generally through the process of acidification, chelation and exchange reactions.

2.8) Molecular Studies in Orchidaceous mycorrhiza (*Tulasnella sp.)*

Gardes and Bruns, (1993) designed two taxon-selective primers for the internal transcribed spacer (ITS) region in the nuclear ribosomal repeat unit. The designed primers, ITS1-F and

ITS4-B, were intended to be specific to fungi and basidomycetes, respectively which was concluded by testing the specificity against a number of ascomycetes, basidomycetes and plants species.

Bougoure et al. (2005) used ITS-RFLP and sequence analysis to determine the identities of the fungal endophytes of six terrestrial orchid species from South-eastern Queensland, a region previously unexplored in this context. Pure cultures of orchid – colonising fungi were obtained and fungal identities were assessed by means of ITS-PCR, RFLP analysis, sequence comparison, and protocorm colonisation tests. ITS-PCR and RFLP analysis resulted in five main groupings. Sequencing and GenBank comparison of these five groups showed that the fungal endophytes isolated from the three *Pterostylis* species were probably *Thanatephorus* species. Analysis of the ITS and LSU sequences of the fungus isolated from *Caladenia carnea* showed high identities with a sequence from a *Sebacina vermifera* originally isolated from *Caladenia dilatata*. These results show that there is specificity for fungal partners within the orchid genera *Acianthus, Caladenia* and *Pterostylis.*

Suarez et al. (2006) identified diverse *Tulasnelloid* fungi forming mycorrhizas with epiphytic orchids in an Andean cloud forest. Root samples of adult individuals of the epiphytic orchids *Stelis hallii, S. superbiens, S. concinna* and *Pleurothallis lilijae* were collected in a tropical mountain rainforest of southern Ecuador. Ultrastructural evidence of symbiotic interaction was combined with molecular sequencing of fungi directly from the mycorrhizas and isolation of mycobionts. Ultrastructural analyses displayed vital orchid mycorrhizas formed by fungi with an imperforate parenthesome and cell wall slime bodies typical for the genus *Tulasnella*.

Three different *Tulasnella* isolates were obtained in pure culture. Phylogenetic analysis of nuclear rDNA sequences from coding regions of the ribosomal large subunit (nucLSU) and the 5.8S subunit, including parts of the internal transcribed spacers, obtained

directly from the roots and from the fungal isolates, yielded seven distinct *Tulasnella* clades. All *Tulasnella* sequences were new to science and distinct from known sequences of mycobionts of terrestrial orchids. The results indicate that *Tulasnella* fungi, adapted to the conditions on tree stems, might be important for orchid growth and maintenance in the Andean cloud forest.

Alfaro and Bayman, (2007) studied diversity, specificity and function of mycorrhizal fungi in Vanilla, a pantropical genus that is both terrestrial and epiphytic. Cultured and uncultured mycorrhizal fungi were identified by sequencing the internal transcribed spacer region of nuclear rDNA (nrITS) and part of the mitochondrial ribosomal large subunit (mtLSU) Vanilla spp. were associated with a wide range of mycorrhizal fungi: *Ceratobasidium, Thanatephorus* and *Tulasnella*. *Tulasnella* was more common in roots on tree bark. *Ceratobasidium and Tulasnella* were tested for effects on seed germination of Vanilla and effects on growth of Vanilla plants. Results confirmed that a single orchid species can be associated with several mycorrhizal fungi with different functional consequences for the plant.

The findings of Roche et al.(2010) indicated that plant groups with highly specific mycorrhizal partners can have a widespread distribution. The study showed that narrow group of monophyletic *Tulasnella* symbiont lineages are associated with multiple species of *Chiloglottis* (Orchidaceae). Mycorrhizal diversity was examined in the terrestrial, photosynthetic orchid genus *Chiloglottis* to test the hypothesis of mycorrhizal-mediated diversification in the genus *Chiloglottis.* Phylogenetic analyses revealed that six representative *Chiloglottis* species spanning a broad survey of the genus were all associated with a narrow group of monophyletic *Tulasnella* fungal lineages. It concluded that the specific pollination system of *Chiloglottis*, rather than specific orchid fungal interactions has been the key driving force in the diversification of the genus.

3. Materials and methods

3.1 Sample collection areas and geographical features

Kangra valley, selected as the site for root samples collection, is a sub-Himalayan region sheltered by the Dhauladhar range in Himachal Pradesh, India. It is situated at average elevation of 730-750 metres and its coordinates are 32.1° N 76.27° E.(Fig 3.1). About 45 square kilometres of area was covered for sampling and seven sites chosen for sampling were: Takipur, Kangra khad, Kangra city, Kangra airport road, Kohli, Matour and Palampur road. The samples were collected in the month of February during which the climatic conditions were semi-humid and temperature variations was between 15° C - 20° C. All selected plants were epiphytic on trunks or branches of standing trees 150 cm to 250 cm above the forest floor. Roots of one flowering individual orchid per tree stem were collected. One to four roots per plant individual were packed in aluminium foil to prevent desiccation and carefully transported to the laboratory the same day. The samples were then stored in cold storage, for further experimental work.

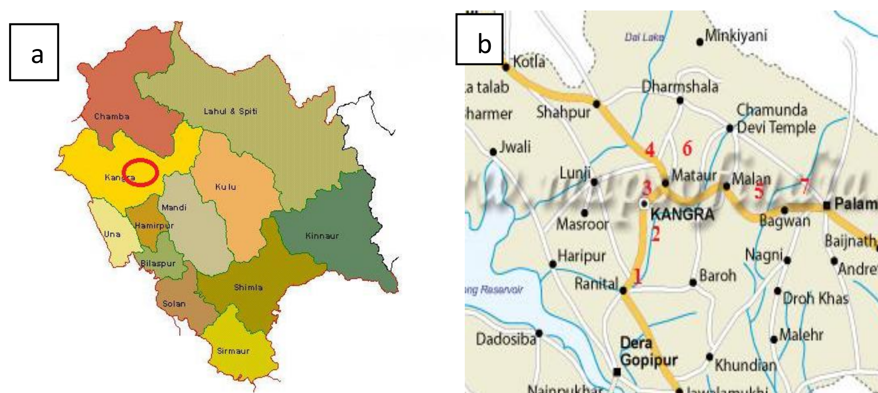


Fig 3.1) a) marked circular region shows the collection sites in Kangra valley. **(b)** seven sites marked on the map as 1) Takipur, 2) Kangra khad, 3) Kangra city, 4) Kangra airport road, 5) Kohli, 6) Matour and 7) Palampur road.

3.2 Surface sterilization

As pre-investigations have shown that mycorrhizal fungi colonize only with roots in contact of the stems of host tree, best when also covered by mosses or a minute humus layer. While collecting the samples, only such roots were selected. Root pieces were surface sterilized by washing under running tap water and then rinsed in distilled water with some drops of liquid soap. For surface sterilization, the roots were immersed in ethanol (70%) for 1 minute and then in 10% sodium hypochlorite (NaOCl) for 7 min and finally rinsed in sterile distilled water inside laminar air flow (LAF). The velamen (a protective cell layer of orchids root), was then removed using dissection microscope with a sterile surgical blade and forceps. About 3 to 4 transverse root sections of 1-3 mm thickness were cut from the middle of the roots.

3.3 Fungal isolation and sub-culturing

3 transverse root sections per root were inoculated onto freshly prepared Potato Dextrose Agar (PDA) (Appendix) plates supplemented with 1 mg/ml amplicillin. The plates were incubated in dark at temperature 25° C until mycelium started growing from the root sections onto the medium. The actively growing mycelium of the fungal colonies were transferred to fresh PDA plates and incubated for 7 days. The fungal colonies were sub-cultured 2-3 times to get the purified cultures. The plates were periodically checked for the growth of endophytic fungal colonies and culture purity. All the fungal isolates were coded and stock cultures were maintained by sub-culturing at monthly intervals.

3.4 Morphological characterization

3.4. a) Macroscopic characterisation of fungal isolates

For studying the cultural and morphological characters, endophytic isolates were sub-cultured onto fresh media (PDA) and incubated at 25° C for 7 days. Cultural characters such as colour, appearance, nature of the growth, pigmentation and special characteristics of the colony were determined by visual observation.

3.4. b) Microscopic characterization of fungal isolates

Morphological characteristics of the fungus like mycelia, conidiophores and conidia were microscopically (Nikon Eclipse 50i) examined at 10X and 100X magnification.(Barnett et al.1998; Wei 1979).

3.5) Molecular screening of *Tulasnella*-specific fungi

3.5. a) Fungal sub-culturing in broth

About 5 mm discs of fungal mycelium grown on PDA plates were cut and transferred to 250 ml screw-cap conical flasks containing 50 ml of potato dextrose broth (PDB) and incubated in shaker at 25° C with 130 revolutions per minute (rpm) till 14 days for DNA extraction.

3.5. b) Isolation of genomic DNA

1. Took 5 gm of mycelium in an autoclaved mortar, grinded the mycelium in liquid nitrogen to fine powder with pestle. Immediately transferred the powder (prevented thawing) to 50 ml oak ridge tube and added preheat CTAB buffer (Appendix) to make slurry along with 200 µL β-mercaptoethanol. Incubated at 60ºC for 1hr in water bath and mixed at regular intervals.

2. Added equal volume of chloroform:iso-amyl alcohol (24:1), mixed for about 3 min. Centrifuged for 10 min at 5000 rpm.
3. Removed aqueous phase with wide-bore pipette (tip was cut-off from mouth) to clean oak ridge tube. Repeated chloroform extraction if extract was still colored.
4. Precipitated DNA with 0.66 volumes of cold isopropanol and incubated for 1 hr at -20ºC.
5. Centrifuged at 10000×g for 15 min.
6. Discarded supernatant and dissolved pellet in 1 mL TE buffer (Appendix) and took solution in microfuge tube.
7. Added 2 µL RNase solution (10 mg/mL stock) and incubated at 37ºC for 1hr. It is important to pre-heat RNase stock solution for 5 min at 60ºC.
8. Added equal volume of phenol:chloroform (1:1 v/v) and shaked slowly. Centrifuged at (1000 x g, 10 min) and retained aqueous phase.
9. Added 0.3 volume of 3M sodium acetate and 0.6 volume of chilled iso-propanol. Incubated for 1 hr at -20ºC.
10. Centrifuged (10000 x g, 8 min) and retained pellet. Washed pellet with 30 µL of 70% EtOH(ethanol) and air-dried pellet.
11. Dissolved pellet in TE buffer (Appendix) and stored at -20ºC.

3.5. c) Visualization of DNA by gel-electrophoresis

Separation of nucleic acids on gel is usually carried out on a suitable gel in which DNA molecules differing in length by a single base or base pair could readily be separated at pH 7.0-8.5. Nucleic acids are negatively charged and move towards anode. Gel retards larger molecules disproportionately so that smaller molecules move faster. In general, agarose gel electrophoresis is used for viewing of larger double stranded molecules under U.V. trans-illuminator.

Following steps were done:

- Made 0.7 % (w/v) gel in TBE buffer (Appendix) by boiling in microwave. Added ethidium bromide (0.5µL/1mL) after cooling down to stain and poured the gel in gel tray with comb. Allowed the gel to solidify.
- Placed gel tray in electrophoresis unit, filled the unit with 0.5X TBE buffer and removed the comb carefully.
- Prepared samples by adding 6X loading dye (Appendix) to DNA samples and mixed.
- After loading the samples in wells, electrophoresis was carried out at 60 V for 60-75 min and visualized the gel on a U.V. transilluminator.

3.5. d) DNA quantification

Concentration of isolated fungal DNA samples was quantified using Nanodrop spectrophotometer which measures 1 µl samples with high accuracy and reproducibility. This full spectrum (220nm-750nm) 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).

3.6) *Tulasnella*-specific DNA amplification by polymerase chain reaction(PCR)

The polymerase chain reaction (PCR) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences in genomic DNA resulting in the exponential increase of target DNA copies. As the work focused on *Tulasnella*-specific fungus, the isolated genomic DNA of all fungi was given a specific

amplification by using Inter transcribed spacer (ITS) region and 5.8S region as a selectable marker for the desired results.

3.6. a) Amplification of isolated DNA using PCR

First amplification for the ITS and 5.8S region of Tulasnella- specific fungi was carried out using two sets of universal fungal primers: Set 1- ITS1F/TW14 and Set 2- ITS1/TW14 (Table 3.1) which are specific to the higher fungi (basidiomycetes).

Table 3.1) The two universal fungal primer sets with their primer sequences and melting temperatures used for first PCR amplification.

Set No.	Primers	Sequence (5'-3')	Melting temperature(T_m)
1	ITS1F (Forward)	CTTGGTCATTTAGAGGAAGTAA	T_m - 49 ºC
	TW14 (Reverse)	GCTATCCTGAGGGAAACTTC	T_m - 52 ºC
2	ITS1 (Forward)	TCCGTAGGTGAACCTGCGG	T_m - 57 ºC
	TW14 (Reverse)	GCTATCCTGAGGGAAACTTC	T_m - 52 ºC

Reaction mixture for the PCR contained 2.5 µl of 10X PCR buffer (Aligent Technologies, USA) with final concentration of 2 mM $MgCl_2$, 1.5µl of 100 mM dNTP mix (25 mM each dNTP), 1 µl of each primer at a concentration of 0.1 µM, 0.5 µl (2.5 units) of Paq 5000 DNA polymerase (Aligent Technologies, USA) and 1 µl of 50 ng DNA with the final volume made up to 25 µl with distilled H_2O. The amplification of ITS and 5.8S region from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA). The PCR programme consisted of the following primer extension conditions for both the primer sets:

Cycle No.	Reaction step	Time	Temperature
1	Denaturation of template DNA	3 min	94ºC
	Denaturation	30 sec	94ºC
35	Annealing of primers Combination	45 sec	**53ºC**
	Elongation	1 min	72ºC
1	Final elongation	7 min	72ºC

Controls containing no DNA template were included for verifying that there is no contamination of reagents and reaction buffer. Successful amplifications were confirmed by agarose gel (0.7% w/v) electrophoresis and ethidium bromide staining.

3.6. b) Nested PCR for *Tulasnella*-specific amplification

First amplified product was then used for the *Tulasnella*-specific nested PCR. *Tulasnella* specific primers set was used to amplify the first amplified PCR product and the primers used were: ITS1/ITS4-Tul.(Table 3.2)

Table 3.2 The two tulasnella-specific fungal primers with their primer sequences and melting temperatures used for nested PCR amplification.

primers	Sequence (5'-3')	Melting temperature(T_m)
ITS1(forward)	TCCGTAGGTGAACCTGCGG	T_m - 57°C
ITS4-Tul (reverse)	CCGCCAGATTCACACATTGA	T_m - 54°C

Reaction mixture for the PCR contained 2.5 µl of 10X PCR buffer (Fermentas, USA) with final concentration of 2 mM $MgCl_2$, 1.5 µl of 100 mM dNTP mix (25 mM each dNTP), 1 µl of each *Tulasnella*-specific primer at a concentration of 0.1 µM, 0.5 µl (2.5 units) of Paq 5000 DNA polymerase (Aligent Technologies, USA) and 1.0 µl of 50 ng first amplification DNA product with the final volume made up to 25 µl with deionised dH_2O.

The amplification of ITS and 5.8S region from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA). The PCR programme consisted of the following primer extension conditions:

Cycle No.	**Reaction step**	**Time**	**Temperature**
1	Denaturation of template DNA	3 min	94ºC
	Denaturation	30 sec	94ºC
35	Annealing of primers	45 sec	**54ºC**
	Elongation	1 min	72ºC
1	Final elongation	7 min	72ºC

Controls containing no DNA template were included for verifying that there is no contamination of reagents and reaction buffer. Successful amplifications were confirmed by agarose gel (1.3% w/v) electrophoresis and ethidium bromide staining.

3.7) Purification of PCR products

Amplified *Tulasnella*-specific ITS products were purified by HiPurA^{TM} PCR Product purification spin kit (HIMEDIA). All the steps in the kit were followed carefully with

precision and the purified product was eluted with 50μl of Elution buffer. Purified PCR products were stored in -20̊ C for direct use in sequencing.

3.8) Media optimization for fungal isolates by One factor at a time approach (OFAT)

3.8 a) Fungal cultivation on defined media

For the media, optimization the isolated fungal strains were grown in a defined Melin's modified Norkans medium (MMN) (Appendix) with varying concentration of carbon and nitrogen sources and pH set at 5.5 (acidic). All the fungal isolates which were *Tulasnella* positive were grown in 250 ml screw-cap flasks with 50 ml of MMN media in them. 5 mm discs of fungal mycelium on PDA plates were cut and transferred to 50 ml MMN media in screw-cap conical flasks. The flasks were sealed tight and incubated in an incubator at temperature 25° C for 14 days. The flasks were shaken at regular interval of 3 days for uniform availability of nutrients in medium and their consumption.

3.8. b) Carbon source (Factor 1)

The carbon source in MMN media i.e Glucose was taken under consideration as factor 1 for media optimization, which was varied with four different carbon sources and glucose kept as control source for media optimisation. The pH of medium was fixed at 5.5. The four other carbon sources used were: Fructose, Lactose, Sucrose and Mannitol. The component concentration in the media (10 g/l) was not disturbed and was kept same for all the four test sources as in control source. The media with different carbon sources were made and transferred 50 ml of each altered form in 250 ml screw-cap conical flasks. The pH of medium was set at 5.5 (acidic) and flasks were autoclaved at 121° C at 15 psi for 15 min. Then the flasks were inoculated with 5 mm discs of fungal mycelium on PDA plates and were labelled

properly with specific annotations to distinguish the flasks with different fungal strains and varied carbon sources. The inoculation was done in triplicates per carbon source per *Tulasnella*-specific fungus strain.

3.8. c) Nitrogen source (Factor 2)

The nitrogen source in MMN media i.e Di-ammonium hydrogen phosphate was considered to be the Factor 2 for media optimization and was varied with four different nitrogen sources and itself kept as control source for media optimisation. The four other nitrogen sources used were: Peptone, Sodium nitrate, Yeast extract and Urea. The component concentration in the media (250 mg/l) was not disturbed and was kept same for all the four test sources as in control source. The media with different nitrogen sources were made and transferred 50 ml of each altered form in 250 ml screw-cap conical flasks. The pH of medium was fixed at 5.5 (acidic) and the flasks were autoclaved at 121° c at 15psi for 15 min. Then the flasks were inoculated with 5 mm discs of fungal mycelium on PDA plates and were labelled properly with specific annotations to distinguish the flasks with different fungal strains and varied nitrogen components. The inoculation was done in triplicates per nitrogen source per *Tulasnella*-specific fungus strain.

3.9) Parameters for characterization

3.9. a) Biomass determination

For biomass determination was done after 14 days of incubation by filtering the contents of the flasks with muslin cloth. The fungal mycelium was dried at 70ºC for 24 hours and then the dry weight of fungal mycelium was recorded by weighing. The biomass determination for both carbon and nitrogen sources with varied forms were done in triplicates per fungus.

3.9. b) pH determination

As the pH of the MMN media was fixed at 5.5 (acidic), prior to incubation. The pH determination of culture filtrate in inoculated flasks, after 14 days of incubation was done by pH meter. The pH determination for both carbon and nitrogen sources with varied forms were done in triplicates per fungus.

3.10) Biochemical screening of fungal isolates

3.10. a) Acid phosphatase activity (Tabatabai and Bremner 1969)

Isolated *Tulasnella*-specific cultures were grown with varying five carbon and nitrogen sources respectively in 50 ml of Melin's Modified Norkans(MMN) medium at 25̊ C in incubator for 14 days. After incubation the culture filtrate was used for Acid phosphatase estimation and was performed in triplicates.

Procedure of determination using culture filtrate:

1. 0.5 ml of culture filtrate was taken in a test tube.

2. Added 4.0 ml of the diluted MUB (Appendix) (pH 5.5 for acid phosphatase).

3. One ml of filter sterilized 0.115 M disodium p-nitro phenyl phosphate solution (Appendix) was added (of the same pH as of MUB).

4. The content was incubated at 37 ºC for 1 hour in dark, after 1 hour of incubation added 5 ml of 0.5 N NaOH (Appendix) solution to stop the reaction.

5. The content was then checked for phosphatase activity by measuring the yellow color intensity with UV-Vis spectrophotometer at 410 nm.

6. Acid phosphatase enzyme activity was indicated as the amount of p-nitro phenol released in the filtrate from the p-nitro phenyl phosphate substrate as per ml of supernatant.

The p-nitro phenol (Appendix) content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 10-100 µg ml^{-1} of p-nitro phenol.

7. For controls, followed the procedure described for the assay but made the addition of 1ml p-nitro phenyl phosphate after the addition of 0.5N NaOH.

8. Phosphatase activity was calculated in the unit of µM PNP/ g mycelium/hour.

Calculations:

$$\text{Phosphatase activity } (\mu\text{M PNP/g mycelium/hour}) = \frac{\text{Conc. of PNP } (\mu\text{M})}{2 \times \text{weight of mycelium}}$$

3.10. b) Phytase activity (Heinonen and Lahti 1981)

Isolated cultures grown with varying five carbon and nitrogen sources respectively in 50 ml of Melin's Modified Norkans (MMN) medium incubated at 25̊ C in incubator. After incubation the culture filtrate was used for phytase enzyme estimation and was performed in triplicates.

Procedure of determination using culture filtrate:

1. 0.5 ml of culture filtrate was taken in a test tube.

2. Added 0.5 ml substrate solution (Appendix) and incubated for 10 min at 37 ℃.

3. The reaction was stopped by addition of 2 ml ice cold color stop solution (Appendix).

4. Added 100 µl 1 M citric acid (Appendix) and O.D. was taken at 380 nm.

5. Un-inoculated medium of experiment was taken as control.

6. Calculated the phytase activity per ml of sample with reference to a calibration graph plotted from the results obtained by standards containing 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg of potassium di-hydrogen phosphate ($KH_2 PO_4$)

7. Enzyme activity was expressed as micromoles of inorganic P released per hour per millilitre (µM PNP/g mycelium/hour) 1 of culture filtrate from sodium phytate at 37 °C.

Calculation:

$$\text{Phosphatase activity } (\mu M \text{ PNP/g mycelium/hour}) = \frac{\text{Conc. of PNP } (\mu M)}{2 \times \text{weight of mycelium}}$$

3.10. c) Cellulase enzyme production activity

Evidence for the microbial utilization of cellulose and production of cellulose enzyme by the *Tulasnella*-specific strains was detected qualitatively by inoculating the agar plates of Modified Czapek-mineral salt medium (Appendix) containg carboxymethyl cellulose (CMC) with the fungal discs and then using hexadecyltrimethyl ammonium bromide (CTAB) (Appendix) to detect the activity. This reagent precipitated intact carboxymethyl cellulose (CMC) in the medium and thus clear zones around a colony in an otherwise opaque medium indicated degradation of CMC.

Procedure

1. Dissolved agar in 400 ml of hot distilled water by added in small amounts and stirring with a glass rod.

2. Magnesium sulphate, potassium chloride, peptone, sodium nitrate were dissolved in 200 ml of water.

3. Dissolved potassium phosphate in 100 ml of water and CMC in 200 ml of water with heat and mix.

4. Mixed all the solutions and made up to 1000 ml volume.

5. The pH of the medium was adjusted to 6.5 with the addition of acid or alkali and autoclaved at 15 lb/in^2 pressure (121 ºC) for 15 minutes.

6. Poured the autoclaved medium cooled to 45-50 ºC into sterile Petri plates and allow the media to solidify.

7. The appropriately labelled plates were inoculated with the respective fungal isolates.

8. Incubated the plates at 35 ºC for 2-5 days.

9. The plates were flooded with 1 % aqueous solution of hexadeceyltrimethyl ammonium bromide (CTAB) and the plates were observed for the formation of a zone around the growth.

The formation of zone depicts the cellulose degradation by cellulose enzyme produced by the fungal cultures.

3.11) Phosphate solubilizing activity

3.11. a) Qualitative screening of phosphate-solubilising fungi (Pikovskaya, 1948)

For qualitative screening 5 mm diameter disc, cut from the periphery of the actively growing colonies of tulasnella-specific fungal isolates, and were inoculated on Pikovskaya's agar medium (PKV) (Appendix) agar plates. The halo zone of phosphate solubilization around

growth was recorded (in mm) after 9 days of incubation incubation. The colonies forming more than 5.0 mm zone of solubilization were selected as efficient strains.

3.11. b) Quantitative Assay for P-solubilization in liquid medium

The tulasnella-specific fungal isolates were further tested for solubilization of tri-calcium phosphate in quantitative terms. About 5 mm diameter disc, cut from the periphery of the actively growing colonies of fungal isolates were inoculated in 100 ml PKV broth in 250 ml conical flask. The flasks were incubated at 25 °C in a rotary shaker at 130 rpm for 9 days.

Procedure for estimation of soluble P in culture filtrate (Jackson 1973)

1) On the 9th day 5 ml of culture suspension was centrifuged at 10,000 rpm for 10 min from each culture grown in PKV broth.

2) Then 500 µl aliquot of each supernatant was transferred to 50.0 ml volumetric flask.

3) This was followed by addition of 10.0 ml chloromolybdic acid (Appendix) along the sides of the flask. 4) The contents of the flasks were diluted to 40.0 ml with distilled water.

5) Then 1 ml of chlorostannous acid (Appendix) was added and mixed well.

6) After mixing, the volume was made up to 50.0 ml with distilled water.

7) The blue color intensity of the solution was measured against the blank (without culture filtrate) in a spectrophotometer (Hitachi U-2900) at O.D. 600 nm.

8) The soluble 'P' was estimated from standard curve of KH_2PO_4 (100 ppm) (Appendix) drawn against O.D. 600 nm with UV-VIS spectrophotometer.

3.11. c) Biomass and pH estimation

The biomass estimation of the contents of the flasks was done by filtering the fungal mycelium grown in PKV broth and was dried at 70º C for 48 hours. Dry weight of the mycelium represented growth. pH of the culture filtrates was also recorded.

3.12) Statistical analysis of data generated

The result was expressed as mean values and standard deviation (SD) and the data were analyzed by 2-way analysis of variance (2 way-ANOVA) and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.0 software.

3.13) Phylogenetic analysis of sequence data

The ITS nrDNA sequences of all the isolates were compared with those available in GenBank databases using BLAST search program (Altschul et al., 1997) to find the possible homologous sequences of the newly sequenced taxa for each fungus. The sequences of closely related strains and uncultured fungi retrieved from GenBank were aligned to minimize the number of inferred gaps. The sequences were edited with BioEdit 5.0.6 (Hall, 1999) and aligned using multiple alignments ClustalW software (Larkin et al., 1997). The phylogenetic analysis was carried out by the maximum parsimony method and the Kimura two-parameter distance calculation by MEGA5 software. The bootstrap was 1,000 replications to assess the reliable level for the nodes of the tree (Tamura et al., 2011). All sequences were annotated using Sequin software and submitted to the NCBI GenBank database.

Chapter 4

Results and discussions

4.1 Sample collection

The samples of the roots of *Rhynchostylis retusae* were collected in the month of February from Kangra valley. This epiphytic orchid had two varying host species in 45 square kilometres of sampling area. The areal roots were avoided from sampling and roots intact with the bark of the host tree, differentially having mosses and humus layer on them were only collected (Fig 4.1). One to two root samples were orchid individual per tree per site were collected and labelled properly before being transported to the laboratory.

(a) **(b)**

Fig 4.1) *Rhynchostylis retusa* on its two host trees **a)** Mango tree (*Mangifera indica* L.) and **b)** Peepal tree *(Ficus religiosa)*

Table 4.1 Different sampling sites for *Rhynchostylis retusae* and number of root samples collected per orchid plant from varying host trees.

Name of site	Host Tree	No of plant individual	No of roots per Individual
Takipur	(*Mangifera indica* L.) Mango	2	3
Kangra khad	(*Mangifera indica* L.) Mango	5	1
Kangra city	(*Mangifera indica* L.) Mango	2	3
Kangra airport road	*(Ficus religiosa)* Peepal	4	2
Kohli	(*Mangifera indica* L.) Mango	1	3
Matour	(*Mangifera indica* L.) Mango	1	3
Palampur road	(*Mangifera indica* L.) Mango	1	3

4.2) Fungal isolation and sub-culturing

The surface sterilized transverse sections of roots, inoculated onto Potato dextrose Agar (PDA) in triplicates and incubated in dark at temperature 25° C showed fungal mycelium growth on the 3rd day of the incubation with mixed mycelia growth. The actively growing mycelium with variation in morphology growing from the root sections were immediately transferred to the freshly PDA plates with one type of mycelium on one plate each and were again incubated for 7 days at 25° C. There were seven morphologically different fungal isolates which could be observed after incubation. These fungal isolates were further sub-cultured 2-3 times to achieve the pure fungal isolates. The pure fungal isolates were coded with annotations varying from T1 – T7 and were used further.

4.3) Fungi morphological characterization

a) Macroscopic characterization of fungal isolates

The isolated seven pure fungal cultures had several different characters and growth patterns. The characteristics which could be observed were like: colony colour, appearance, growth, elevation and some special characteristics of particular fungus (Table 4.2).

Table 4.2) Colony morphology of seven fungal isolates.

Sl. No.	**Fungus code**	**Color**		**Appearance**		**Growth (on PDA at 25°C)**	**Growth (on PDA at 25°C)**	**Elevation**
		Front		**Centre**		**Edges**	**Back**	
1	**T1**	Orangish red	Orangish red	White	Pinkish	Round, wooly	5 cm/7 days; fast, compact but dense with sharp edges	Raised
2	**T2**	White	White	White	White	Flat and cottony	7 cm/7days; moderate, non dense, circular	Complete flat spreading
3	**T3**	Whitish	White	Rich white	Fade white	Bulby , round	5.4cm/7 days; moderate, dense, compact, margins regular and smooth	Flat but slightly raised
4	**T4**	Green	Dark green	Green	Yellowish green	Cottony, round with rings	3cm/7 days; moderate, circular	Raised
5	**T5**	White	White and bulb	Pale white	Black with light brownish edges	Cottony, round	2cm/7 days; dense, circular and sharp edges	Raised
6	**T6**	White	White and flat	White and raised	Dark green with a centric ring	Cottony and round	6cm/7days;dense circular and broken edges	Raised
7	**T7**	White	White	White	Pale yellow a	Cottony	6cm/7days; Circular	Raised

4.4) Molecular screening of *Tulasnella*-specific fungi

4.4 a) Fungal sub-culturing in PDB

The seven fungal isolates were grown in potato dextrose broth (PDB) at 25º C till 14 days in a shaker which resulted in maximal fungal biomass after completion of the incubation and the mycelia was further used for DNA extraction.

4.4 b) Isolation of genomic DNA

The fungal biomass grown in PDB was filtered from the media after the incubation and was subjected to genomic DNA isolation by CTAB method. The DNA pellet at the end of the extraction was mixed in the 30 ml TE buffer, were coded with same annotations respectively as the fungal culture was coded and was stored at -20º C for the further molecular studies.

4.4 c) DNA quantification using Nanodrop spectrophotometer

The isolated fungal DNA was quantified using Nanodrop 1000 spectrophotometer which gave us the value of unknown concentrations of isolated DNA samples with high accuracy and reproducibility (Table 4.3).

Table 4.3) Quantified concentration of isolated DNA samples using Nanodrop spectrophotometer..

Sample no.	Sample code	Concentration (ng/µl)	Absorbance ratio (260/280)
1	T1	1604.3	1.61
2	T2	1351.8	1.63
3	T3	772.1	1.52
4	T4	1062.3	1.57
5	T5	1464.2	1.68
6	T6	1036.9	1.59
7	T7	1839.8	1.65

4.5) *Tulasnella*-specific DNA amplification by polymerase chain reaction(PCR) and its amplification

All the isolated fungal DNA samples were subjected to *Tulasnella* specific DNA amplification with the ITS and 5.8S region as the selective markers. The amplification was carried out using the universal fungal primers sets: Set 1- ITS1F/TW14 and Set 2- ITS1/TW14 which are specific to the higher fungi. Successful amplifications were confirmed by agarose gel (0.7% w/v) electrophoresis and ethidium bromide staining. The samples were run parallel with a specific DNA ladder (1 kb) to identify the size of the amplified product. Out of the both primer sets, the Set-1 gave the best fungal amplification for all seven fungal DNA. The band size of amplified ITS and 5.8S region product was about 1.5 kb and above for all fungal DNA samples (Fig 4.2).

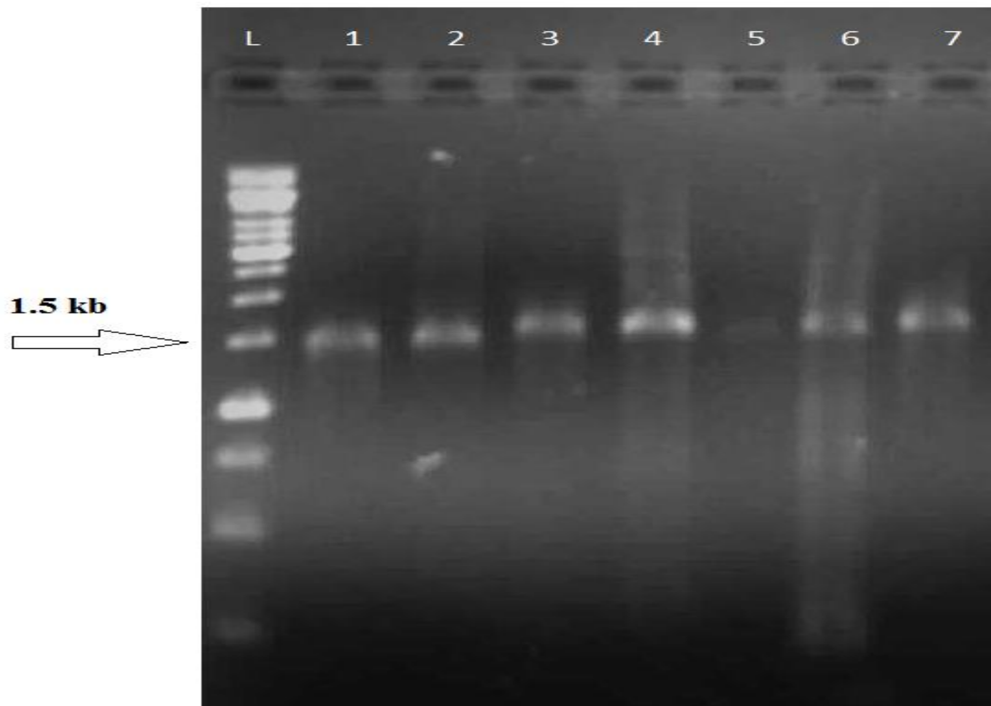


Fig 4.2) Gel electrophoresis showing the amplified DNA product with a size of 1.5 kb and the annotations given on the gel are: L-1kb ladder; 1 to 7- DNA samples.

4.6) Nested PCR for *Tulasnella*-specific amplification

After first amplification, the DNA products were subjected to *Tulasnella* specific nested PCR DNA amplification which only amplified the ITS and 5.8S region of *Tulasnella* positive DNA products. The amplification was carried out using the *Tulasnella*-specific fungal primers set: ITS1/ITS4-Tul. Successful amplifications were confirmed by agarose gel (0.7% w/v) electrophoresis and ethidium bromide staining. The samples were run parallel with a specific DNA ladder (1 kb) to identify the size of the amplified product. Out of all seven DNA amplified products only five samples: T1, T2, T4, T5 and T7 showed the positive results and two samples: T3 and T6 did not produce *Tulasnella*-specific amplification (Fig 4.3). The band size of amplified *Tulasnella*-specific ITS and 5.8S region product was also about 1.5 kb and above, for all five DNA amplified products (Fig 4.4 and 4.5).

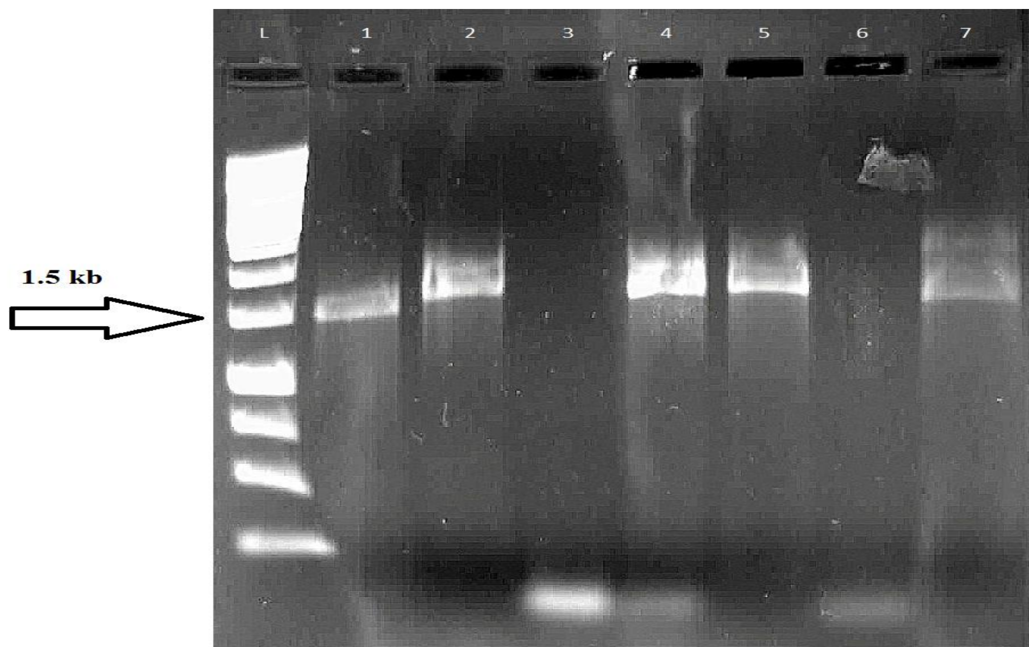


Fig 4.3) Gel electrophoresis of the amplified nested PCR with DNA products TI, T2, T4, T5 and T7showing positive amplification with size of 1.5 kb and the products T3 and T6 showing negative results with no amplification.

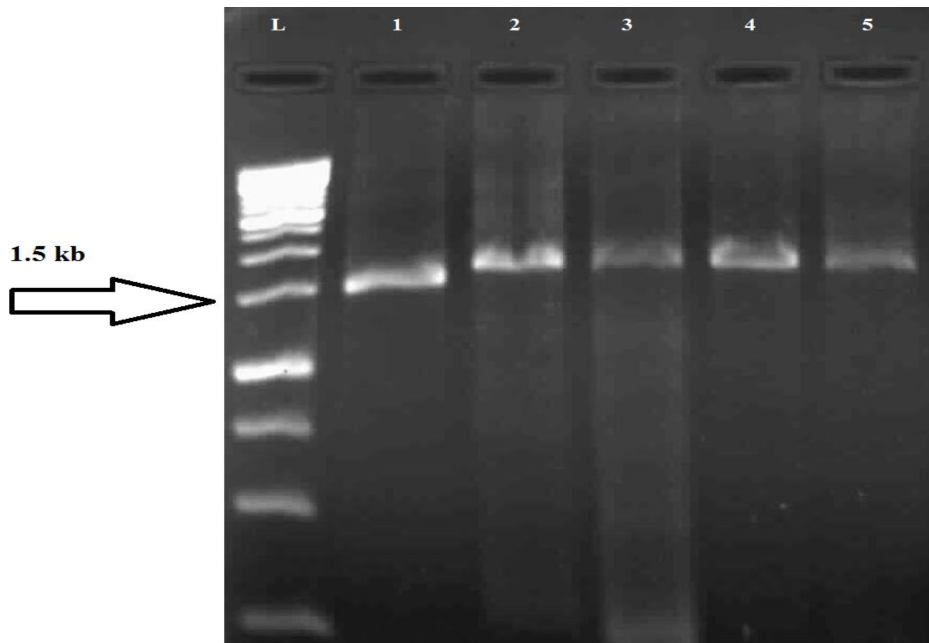


Fig 4.4) Conformation of the size of the *Tulasnella*-specific nested PCR products which was around 1.5 kb and above. The annotations given on the gel are: L-1kb ladder; 1 to 5- nested PCR products.

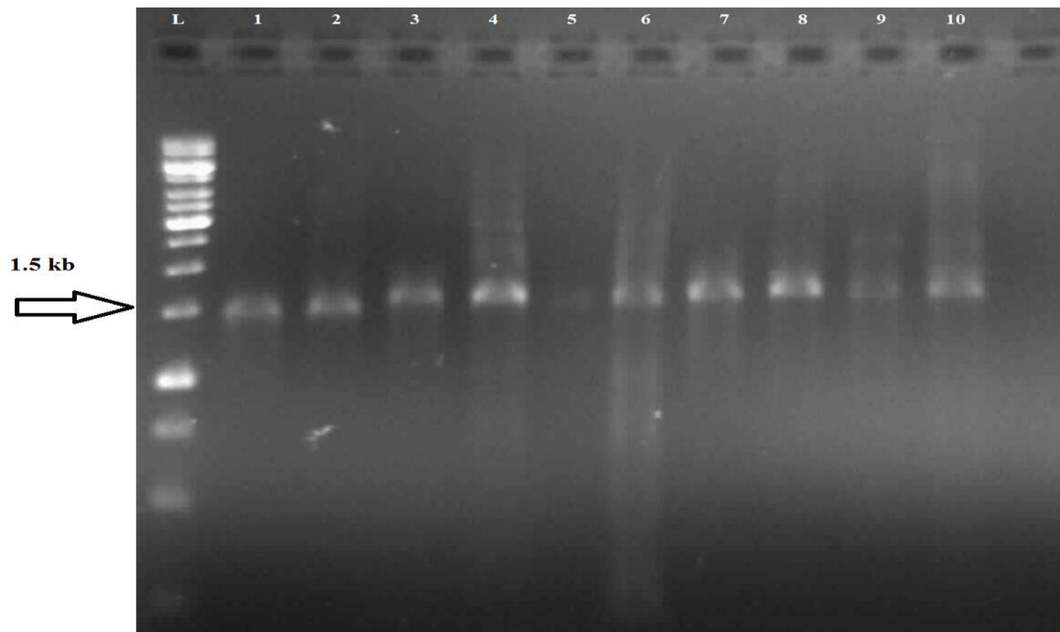


Fig 4.5) Comparison of the size of the amplified PCR product and *Tulasnella*-specific nested PCR products in which both the products had same size around 1.5 kb and above. The annotations given on the gel are: L-1kb ladder; 1, 3, 5, 7 and 9- universal primer amplification PCR products and 2, 4, 6, 8 and10- nested PCR products.

4.7) Macroscopic and microscopic characterization of fungal isolates.

4.7 a) Macroscopic and microscopic characterization of fungus T1

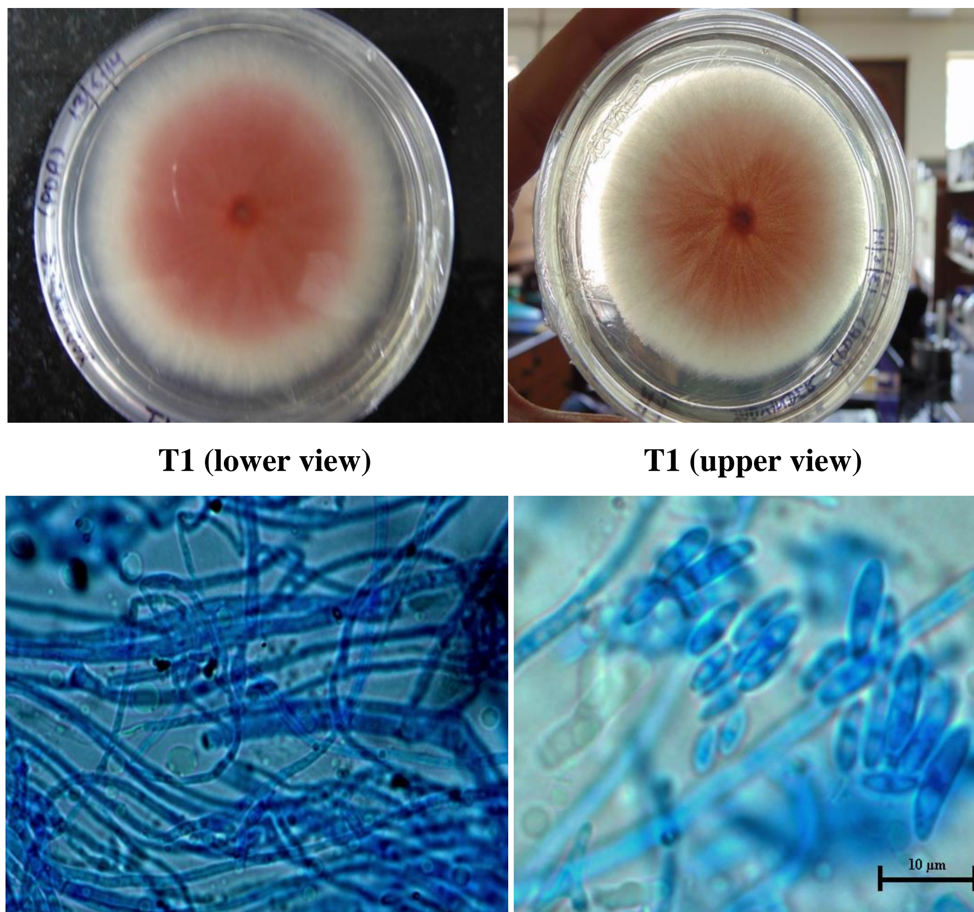


Fig. 4.6) Macroscopic and microscopic characteristics of ***Fusarium sp.*** **208f** (T1). **A-B**: colony features on PDA plates lower and upper surface respectively; **C**: conidiomatal formation; **D**; conidia

On PDA media at 25° C under dark conditions, T1 aerial mycelia produced red pinkish pigments. The fungus grew with a radial growth rate of 5 cm per week, appearing cottony with round spreading (Fig 4.6)

Under microscope both macro and micro conidia were observed (Fig. 4.6). Conidiophores are short, single, lateral monophialides in the aerial mycelium, later arranged in densely branched clusters. Macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, 23-54 x 3-4.5 µm. Microconidia are abundant,

never in chains, mostly non-septate, ellipsoidal to cylindrical, straight or often curved, 5-12 x 2.3 - 3.5 µm. Chlamydospores are terminal or intercalary, hyaline, smooth or rough-walled, 5-13 µm.

4.7 b) Macroscopic and microscopic characterization of fungus T2

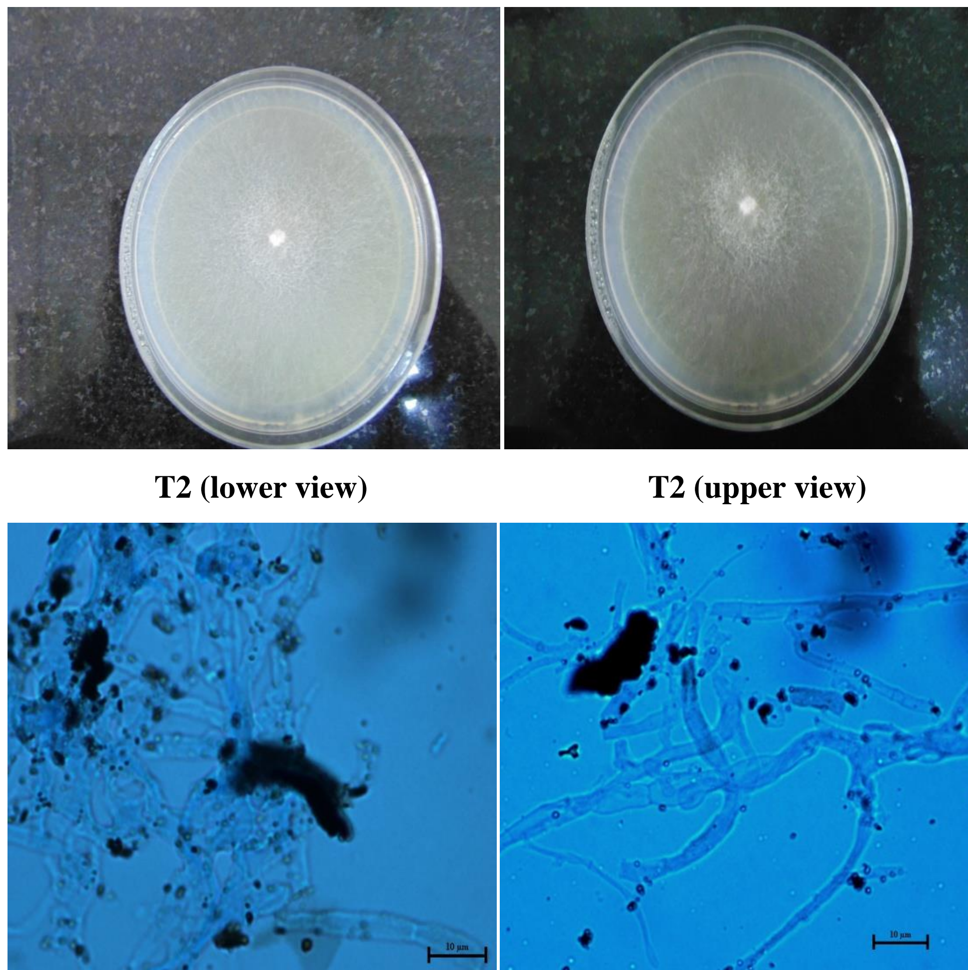
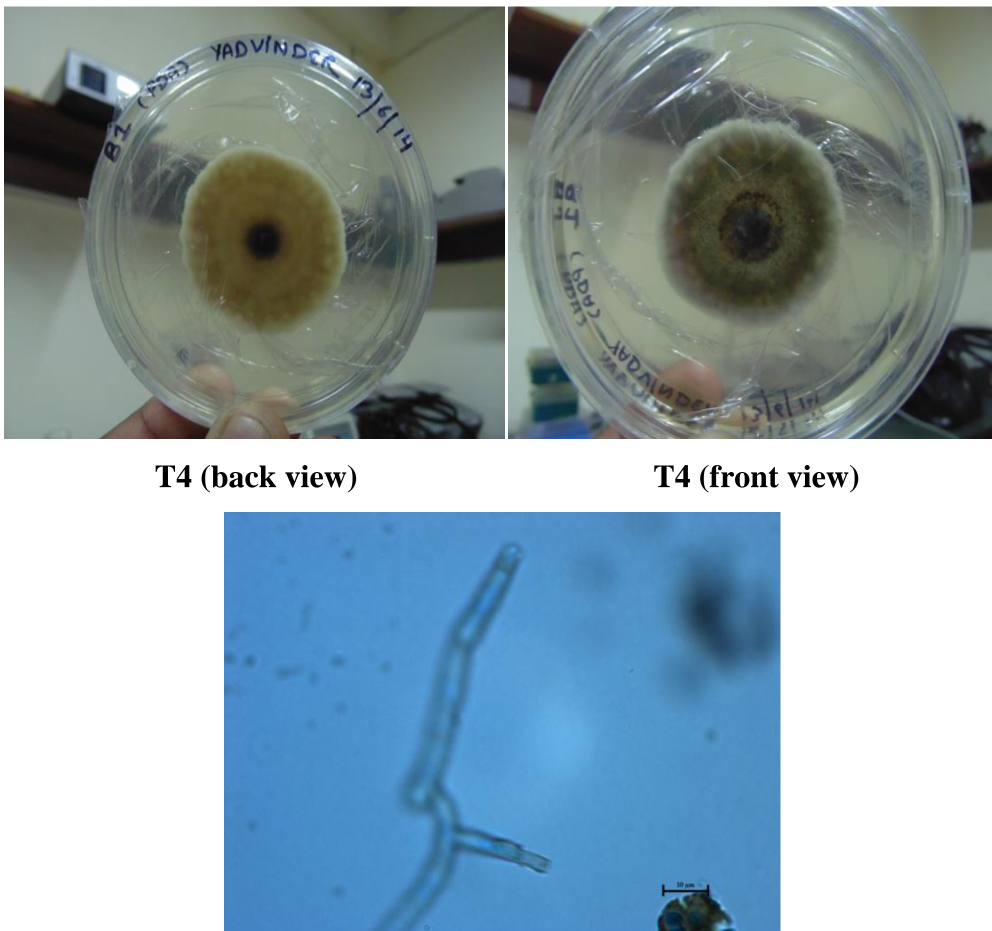


Fig. 4.7) Macroscopic and microscopic characteristics of (T2). **A-B**: colony features on PDA plates lower and upper surface respectively; **C**: conidiomatal formation; **D**; conidia

On PDA media at 25°C under dark conditions, T2 aerial mycelia produced were flat and cottony. The fungus grew with a radial growth rate of 7 cm per week, appearing non dense with round spreading. Under microscope both macro and micro conidia were observed (Fig. 4.7).

Filamentous ascomycetes that produce, dark colored pseudothecia. showed muriform (brick wall pattern) conidia on conidiophores; conidiophore was indistinguishable from hyphae (Figure 1). Conidia were broadly ellipsoidal, and echinulate (spikes) or verrucose (wart-like projections). An annular frill at the base of the conidium was visible after detachment.

4.7 c) Macroscopic and microscopic characterization of fungus T4



T4 (back view) **T4 (front view)**

Fig 4.8) Macroscopic and microscopic characteristics of (T4). **A-B**: colony features on PDA plates lower and upper surface respectively; **C**: conidiomatal formation; **D**; conidia

On PDA media at 25°C under dark conditions, T4 aerial mycelia produced were cottony, round and with rings. The fungus grew with a radial growth rate of 3 cm per week, appearing

dense with round spreading. Under microscope both macro and micro conidia were observed (Fig. 4.8). the hyphae were septate , branched and without spores.

4.7 d) Macroscopic and microscopic characterization of fungus T5

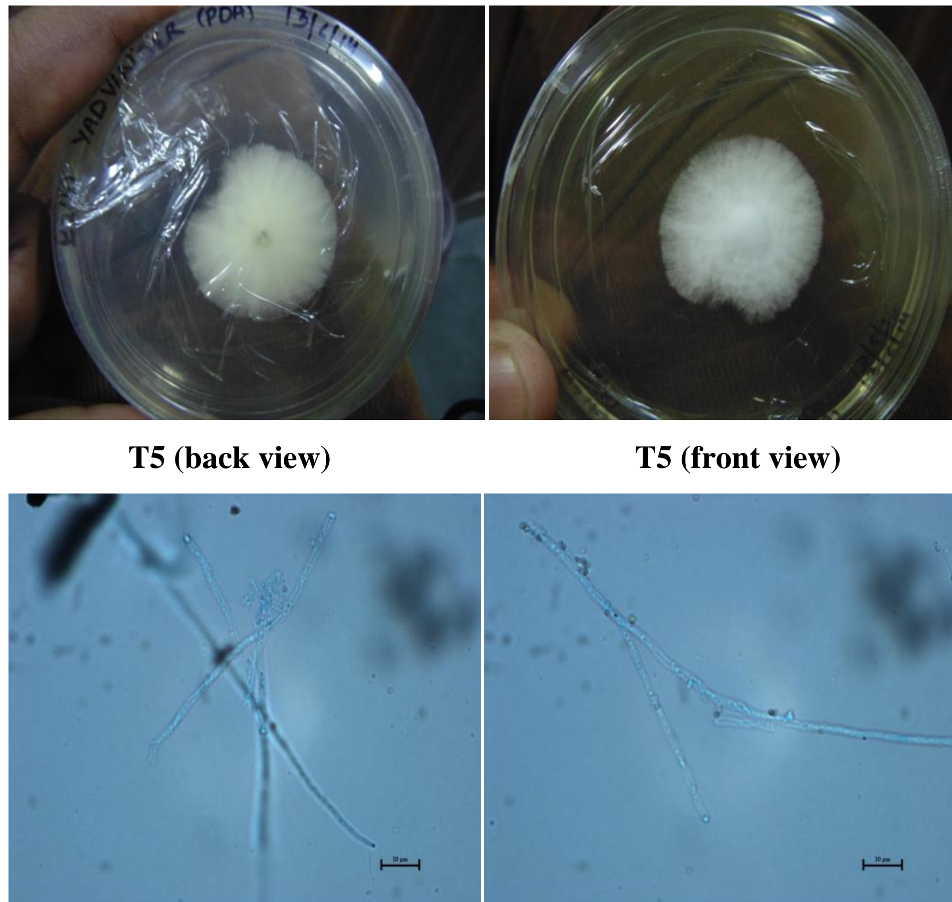


Fig. 4.9 Macroscopic and microscopic characteristics of (T5). **A-B**: colony features on PDA plates lower and upper surface respectively; **C**: conidiomatal formation; **D**; conidia

On PDA media at 25°C under dark conditions, T5 aerial mycelia produced were flat and cottony. The fungus grew with a radial growth rate of 2 cm per week, appearing dense, cottony with round spreading and broken edges.

Under microscope both macro and micro conidia were observed (Fig. 4.9). The hyphae were branched,and long and have small sized conidiospores.

4.7 e) Macroscopic and microscopic characterization of fungus T7

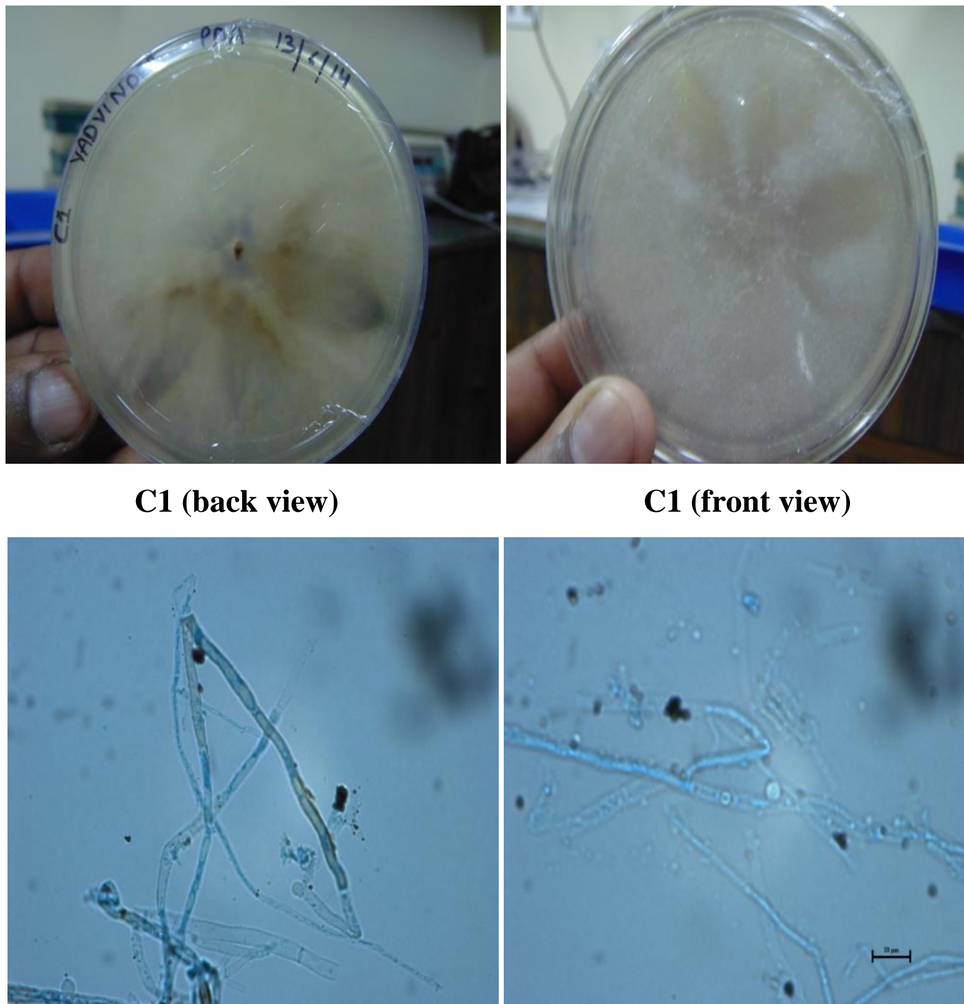


Fig. 4.10) Macroscopic and microscopic characteristics of (T7). **A-B**: colony features on PDA plates lower and upper surface respectively; **C**: conidiomatal formation; **D**; conidia

On PDA media at 25°C under dark conditions, T7 aerial mycelia produced were flat and cottony. The fungus grew with a radial growth rate of 7 cm per week, appearing non dense with round spreading.

Under microscope both macro and micro conidia were observed (Fig. 4.10).the hyphae were septate and knotted to each other. There were no reports of spores in this during observations.

4.8) Media optimization for fungal isolates by One factor at a time approach (OFAT)

4.8 a) Fungal cultivation on defined media

For the media optimization the isolated fungal strains were grown in a defined Melin's modified Norkans medium (MMN) (Appendix) in which the concentration of all media components were known. The concentration of carbon and nitrogen sources was varied in the media and pH was maitained at 5.5 (acidic). 5 mm discs of fungal mycelium on PDA plates were cut and inolculated to 50 ml MMN media in screw-cap conical flasks. The flasks were incubated in an incubator at temperature 25° C for 14 days. A remarkable growth was noticed in all the flasks with the varied component concentration of media.

4.8 b) Carbon source (FACTOR 1)

The carbon source in MMN media i.e Glucose was taken under consideration as factor 1 for media optimization, which was varied with four different carbon sources and glucose kept as control source for media optimisation. The pH of medium was fixed at 5.5. The four other carbon sources used were: Fructose, Lactose, Sucrose and Mannitol. The component concentration in the media (10 g/l) was not disturbed and was kept same for all the four test sources as in control source. After 14 days of fungal cultivation the filtrate (media) and biomass were used for measurements of different parameters like biomass, pH variation in media, acid phosphatase activity and phytase enzyme activity.

All the measurements and analysis were done in triplicates per carbon source per each fungal strain. The results were expressed as mean values and standard deviation (SD) and the data were analyzed by 2-way analysis of variance (2 way-ANOVA) and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.0 software.

4.9) Biomass estimation of fungal isolates with varying carbon sources

The biomass of the *Tulasnella* positive fungal isolates with varying carbon source concentration in MMN media was estimated. It was observed that isolate T4 showed the maximum biomass production both in glucose (control) and other carbon sources (Table 4.4 a). The best results were shown in the control itself and then other sources. The poorest biomass production among all isolates was observed in isolate T7 in mannitol. The carbon source which could be used for increasing biomass is fructose. These results also gave a conclusion that simple sugars are efficient carbon source for fungul isolates than other as it required less energy for its breakdown and requires less alterations in the biochemical pathways of the fungus (Fig 4.11). Significance variation between carbon source and fungi were observed as revealed by two way ANOVA (Table 4.4 b)

Table 4.4 a) Effect of different carbon sources in biomass yield of five different isolates

FUNGI	**CARBON SOURCES**				
	Glucose	**Fructose**	**Lactose**	**Sucrose**	**Mannitol**
T1	69.70 ± 3.58^{cd}	60.33 ± 2.48^{cd}	32.57 ± 3.35^{cd}	51.03 ± 1.16^{cd}	26.97 ± 1.95^{cd}
T2	55.83 ± 3.82^{bc}	63.63 ± 2.03^{bc}	40.67 ± 1.84^{bc}	40.47 ± 1.78^{bc}	16.70 ± 1.30^{bc}
T4	120.97 ± 3.07^{a}	118.20 ± 4.39^{a}	87.70 ± 1.57^{a}	64.17 ± 3.91^{a}	27.40 ± 2.17^{a}
T5	82.10 ± 2.69^{b}	70.10 ± 5.87^{b}	21.90 ± 1.71^{b}	31.27 ± 1.80^{b}	10.07 ± 1.74^{b}
T7	93.30 ± 4.73^{d}	54.73 ± 2.87^{d}	31.93 ± 2.17^{d}	33.57 ± 0.90^{d}	4.90 ± 0.36^{d}

Values sharing a common letter within the column are not significant at (P<0.05). All values are mean ± SD (n = 3).

Table 4.4 b) Two-way ANOVA results for biomass estimation

Source of variation	**BIOMASS**				
	df	**SS**	**MSS**	**F**	**P-value**
Carbon source	4	42906	10727	1337	***
Fungus	4	18596	4649	579.7	***
Interaction	16	8127	507.9	63.33	***
Residual error	50	401.0	8.020		

df: degree of freedom, SS: sum-of-squares, MSS: mean square, ns: not significant; **P<0.01; ***P<0.0001

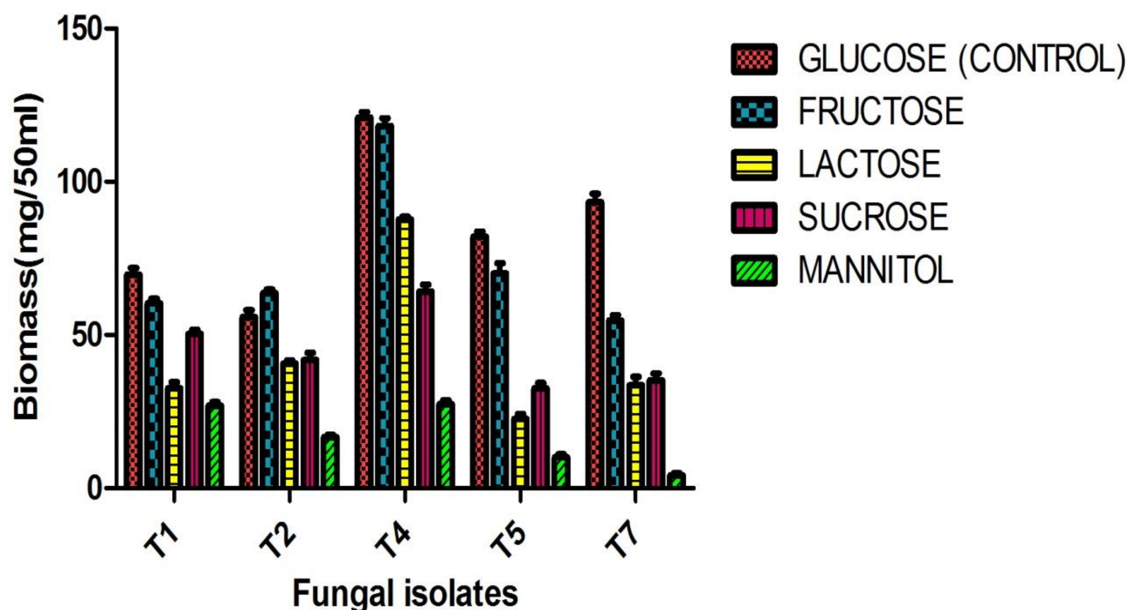


Fig 4.11) Amount of biomass (mg/50ml) estimated for different fungal isolates with varying carbon sources

4.10) pH reduction in different fungal isolates with varying carbon sources

The other parameter for the biochemical characterization of *Tulasnella* fungal species was measured pH estimation of the filtrate left after 14 days when the mycelia had been filtered. The initial pH of medium was adjusted to 5.5 for all the varying carbon sources in MMN media. It was observed that the maximum pH reduction was in T4 fungal sample with sucrose as the carbon source and the least pH reduction was seen in the T5 and T7 isolates with mannitol and lactose as carbon sources respectively. Other best pH reduction was seen in T2 with sucrose as carbon source (Table 4.5a and Fig 4.12). Moreover, all the fungal isolates had significant reduction in the pH (Table 4.5 b). The reduction in pH was an indicator of good metabolic activity of the fungal isolates and increased enzyme activity and secondary metabolite production. Table 4.5b showed that there was a significant variation between carbon and fungal sources in reduction of pH.

Table 4.5 a) Estimation of pH reduction in five isolates with varying carbon sources.

FUNGI	CARBON SOURCES				
	Glucose	**Fructose**	**Lactose**	**Sucrose**	**Mannitol**
T1	2.71 ± 0.01 [a]	2.84 ± 0.03 [a]	2.62 ± 0.01 [a]	2.89 ± 0.02 [a]	2.84 ± 0.02 [a]
T2	2.63 ± 0.03 [c]	2.70 ± 0.01 [c]	2.77 ± 0.11 [c]	2.99 ± 0.02 [c]	2.66 ± 0.04 [c]
T4	2.70 ± 0.02 [ab]	2.80 ± 0.03 [ab]	2.74 ± 0.01 [ab]	3.08 ± 0.27 [ab]	2.69 ± 0.04 [ab]
T5	2.77 ± 0.01 [b]	2.79 ± 0.01 [b]	2.74 ± 0.02 [b]	2.87 ± 0.02 [b]	2.03 ± 0.04 [b]
T7	2.75 ± 0.01 [d]	2.56 ± 0.05 [d]	2.05 ± 0.04 [d]	2.86 ± 0.04 [d]	2.13 ± 0.09 [d]

Values sharing a common letter within the column are not significant at ($P<0.05$). All values are mean ± SD (n = 3).

Table 4.5 b) Two-way ANOVA results for pH reduction estimation

Source of variation	pH reduction				
	df	**SS**	**MSS**	**F**	**P-value**
Carbon source	4	1.863	0.4656	110.6	***
Fungus	4	1.145	0.2862	67.94	***
Interaction	16	1.862	0.1164	27.63	***
Residual error	50	0.2106	0.004212		

df: degree of freedom, SS: sum-of-squares, MSS: mean square, ns: not significant; **$P<0.01$; ***$P<0.0001$

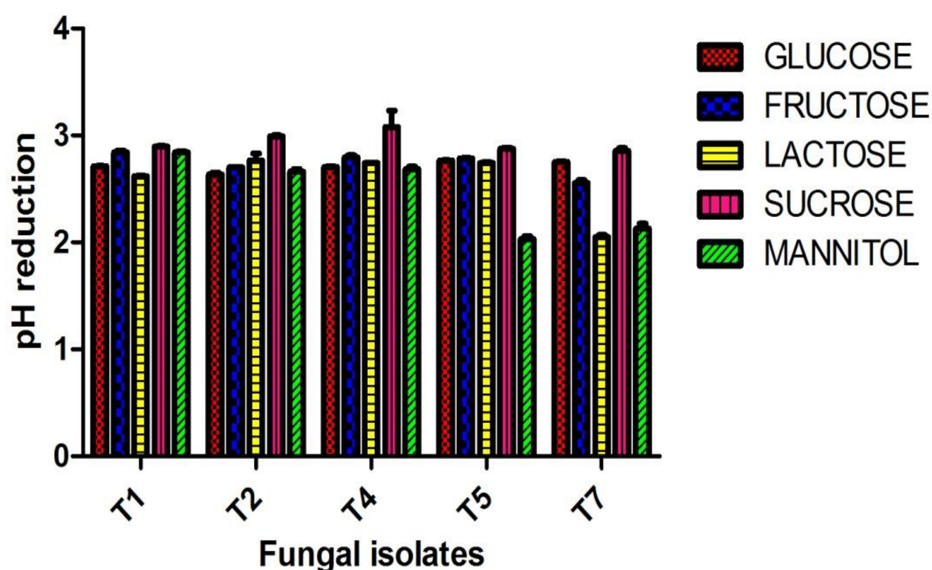


Fig 4.12) Amount of pH reduction estimated for different fungal isolates and varying carbon sources.

4.11) Acid phosphatase activity in different fungal isolates with varying carbon sources

Maximum acid phosphatase activity was seen in the strain T7 in mannitol as carbon source in MMN media at the end of incubation (Table 4.6 a and (Fig 4.13). the lowest acid phosphatase activity was reported in T1,T2and T5 strain with glucose as carbon source. The two way ANOVA revealed the significal variation between carbon and fungal sources in response to acid phosphatase activity (Table 4.6 b)

Table 4.6 a) Acid phosphatase activity in different fungal isolates with varying carbon sources

FUNGI	CARBON SOURCES				
	glucose	**fructose**	**lactose**	**sucrose**	**mannitol**
T1	8.86 ± 0.80^{b}	10.99 ± 3.61^{b}	14.65 ± 3.13^{b}	24.25 ± 8.81^{b}	37.53 ± 6.46^{b}
T2	6.44 ± 0.77^{c}	6.77 ± 2.27^{c}	23.10 ± 7.26^{c}	16.63 ± 2.24^{c}	47.63 ± 2.67^{c}
T4	10.70 ± 1.89^{b}	12.78 ± 0.86^{b}	13.24 ± 0.36^{b}	15.45 ± 3.63^{b}	21.46 ± 2.97^{b}
T5	7.59 ± 1.63^{bc}	10.59 ± 0.72^{bc}	21.91 ± 4.92^{bc}	21.60 ± 1.80^{bc}	76.01 ± 4.66^{bc}
T7	11.59 ± 8.72^{a}	21.79 ± 3.50^{a}	35.66 ± 7.16^{a}	38.05 ± 3.67^{a}	198.69 ± 6.45^{a}

Values sharing a common letter within the column are not significant at ($P<0.05$). All values are mean ± SD (n = 3).

Table 4.6 b) Two-way ANOVA results for Acid phosphatase activity

Source of variation	Acid phosphatase activity				
	Df	**SS**	**MSS**	**F**	**P-value**
Carbon source	4	44829	11207	575.5	***
Fungus	4	21193	5298	272.1	***
Interaction	16	42112	2632	135.2	***
Residual error	50	973.7	19.47		

df: degree of freedom, SS: sum-of-squares, MSS: mean square, ns: not significant; **$P<0.01$; ***$P<0.0001$

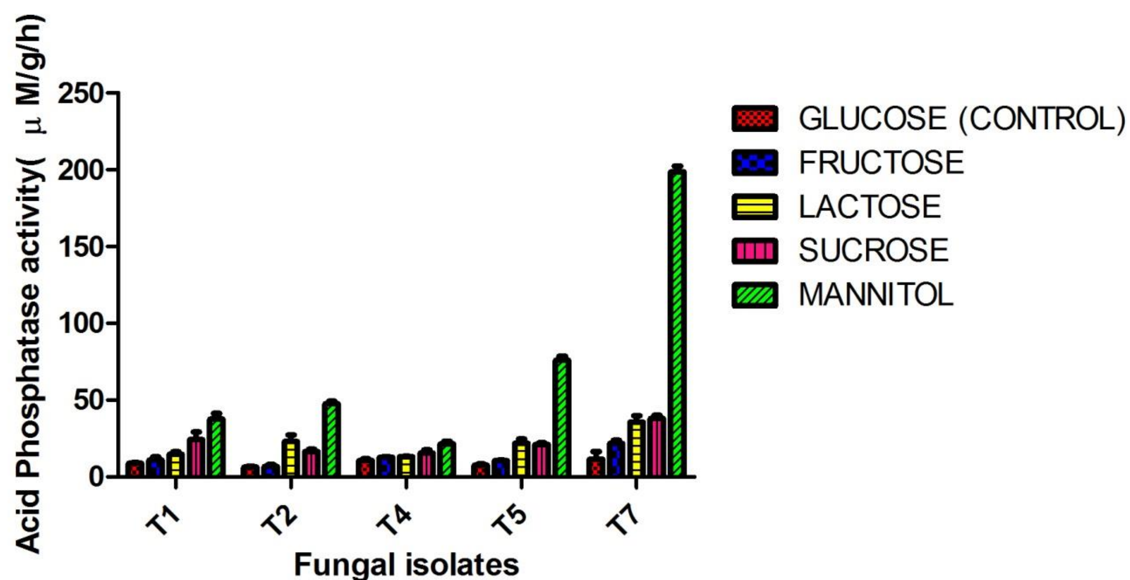


Fig 4.13) Estimation of acid phosphatase activity (µM/g/h) for different fungal isolates and varying carbon sources

4.12) Phytase activity of fungal isolates with varying carbon sources

Since the assimilation of phytate is likely dependent on the phytate dephosphorylating activites, phytate activity in culture filtrate was determined. The isolate T7 showed the maximum phytase activity in mannitol and the least activity was observed in T2 and T5 isolates as seen in (Table 4.7 a) and (fig 4.14). The two way ANOVA revealed the significal variation between carbon and fungal sources in response to acid phosphatase activity (Table 4.7 b)

Table 4.7 a) Estimation of phytase activity in different carbon sources with five different isolates

FUNGI	CARBON SOURCE				
	glucose	**fructose**	**lactose**	**sucrose**	**mannitol**
T1	16.04 ± 0.63 [a]	17.25 ± 1.95 [a]	22.94 ± 1.72 [a]	15.07 ± 0.77 [a]	45.86 ± 2.59 [a]
T2	18.17 ± 1.02 [cd]	7.77 ± 0.53 [cd]	19.91 ± 1.86 [cd]	12.35 ± 1.80 [cd]	61.78 ± 7.96 [cd]
T4	18.06 ± 1.15 [c]	9.50 ± 1.37 [c]	14.51 ± 0.33 [c]	18.47 ± 1.81 [c]	17.19 ± 2.54 [c]
T5	9.12 ± 1.19 [d]	7.08 ± 0.60 [d]	52.33 ± 4.52 [d]	40.10 ± 4.60 [d]	75.74 ± 6.64 [d]
T7	41.67 ± 1.38 [b]	13.10 ± 0.71 [b]	32.56 ± 3.23 [b]	86.65 ± 5.29 [b]	196.13 ± 9.90 [b]

Values sharing a common letter within the column are not significant at (P<0.05). All values are mean ± SD (n = 3)

Table 4.7 b) Two-way ANOVA results for phytase activity estimation

Source of variation	Phytase activity				
	df	SS	MSS	F	P-value
Carbon source	4	41926	10481	805.5	***
Fungus	4	32394	8098	195.8	***
Interaction	16	40771	2548	622.3	***
Residual error	50	650.6	13.01		

df: degree of freedom, SS: sum-of-squares, MSS: mean square, ns: not significant; **P<0.01; ***P<0.0001

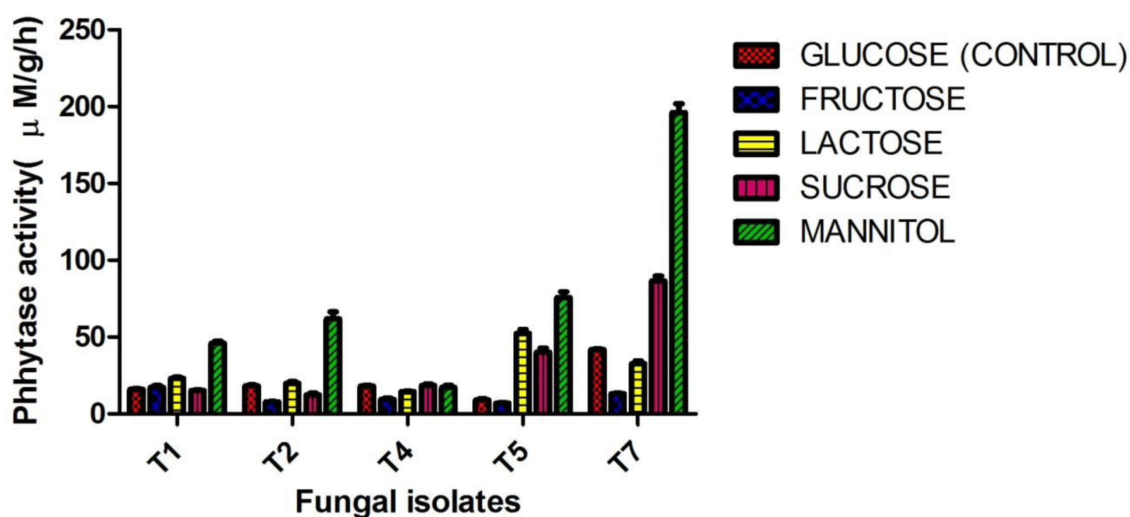


Fig 4.14) Amount of biomass (mg/50ml) estimated for different fungal isolates and varying carbon sources

From the above parametric analysis it could be concluded that there is direct relationship between solublization and activity of these enzymes. The production of acid phosphates and phytase activity is favoured at low pH.

4.13) Nitrogen (Factor 2)

The nitrogen source in MMN media i.e Di-ammonium hydrogen phosphate was considered to be the Factor 2 for media optimization and was varied with four different nitrogen sources and itself kept as control source for media optimisation. The four other nitrogen sources used were: Peptone, Sodium nitrate, Yeast extract and Urea. The pH of medium was fixed at 5.5. After 14 days of fungal cultivation the filtrate (media) and biomass were used for measurements of different parameters like biomass, ph variation in media, acid phosphatase activity and phytase enzyme activity. All the measurements and analysis were done in triplicates per carbon source per *Tulasnella*-specific fungus strain. The results were expressed as mean values and standard deviation (SD) and the data were analyzed by 2-way analysis of variance (2 way-ANOVA) and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.0 software.

Biomass estimation of fungal isolates with varying nitrogen sources

the biomass of the *Tulasnella* positive fungal isolates with varying nitrogen source concentration in MMN media was estimated. It was observed that isolate T4 showed the maximum biomass production both in sodium nitrate showing the best results were shown in the control itself and then other sources. The poorest biomass production among all isolates was shown by isolate T7 in glucose (Table 4.8a and Fig 4.15). The other best source of nitrogen which could be used for increasing biomass is yeast. The values in the estimation showed highly significant results (Table 4.4 b).

Table 4.8 a) Effect of different nitrogen sources in biomass yield of five different isolates

FUNGI	NITROGEN SOURCE				
	di ammonium hydrgen	**peptone**	**sodium nitrate**	**yeast**	**urea**
T1	22.23 ± 2.06 ^{d}	73.50 ± 3.18 ^{d}	134.13 ± 3.29 ^{d}	55.03 ± 2.12 ^{d}	124.43 ± 11.06 ^{d}
T2	87.70 ± 3.62 ^{c}	102.87 ± 3.14 ^{c}	129.43 ± 6.52 ^{c}	80.13 ± 6.77 ^{c}	81.13 ± 7.50 ^{c}
T4	43.50 ± 5.83 ^{b}	119.93 ± 6.05 ^{b}	268.83 ± 10.39 ^{b}	166.40 ± 3.37 ^{b}	119.43 ± 4.14 ^{b}
T5	57.03 ± 4.80 ^{c}	101.20 ± 6.32 ^{c}	207.83 ± 3.52 ^{c}	99.40 ± 9.00 ^{c}	162.27 ± 3.25 ^{c}
T7	19.93 ± 2.95 ^{a}	166.70 ±4.28 ^{a}	170.83 ±7.82 ^{a}	131.07 ± 7.21 ^{a}	96.63 ± 9.00 ^{a}

Values sharing a common letter within the column are not significant at ($P<0.05$). All values are mean ± SD (n = 3)

Table 4.8 b) Two-way ANOVA results for biomass estimation

Source of variation	Biomass				
	df	**SS**	**MSS**	**F**	**P-value**
Nitrogen source	4	139897	34974	955.8	***
Fungus	4	35414	8853	242.0	***
Interaction	16	62472	3904	106.7	***
Residual error	50	1830	36.59		

df: degree of freedom, SS: sum-of-squares, MSS: mean square, ns: not significant; **$P<0.01$; ***$P<0.0001$

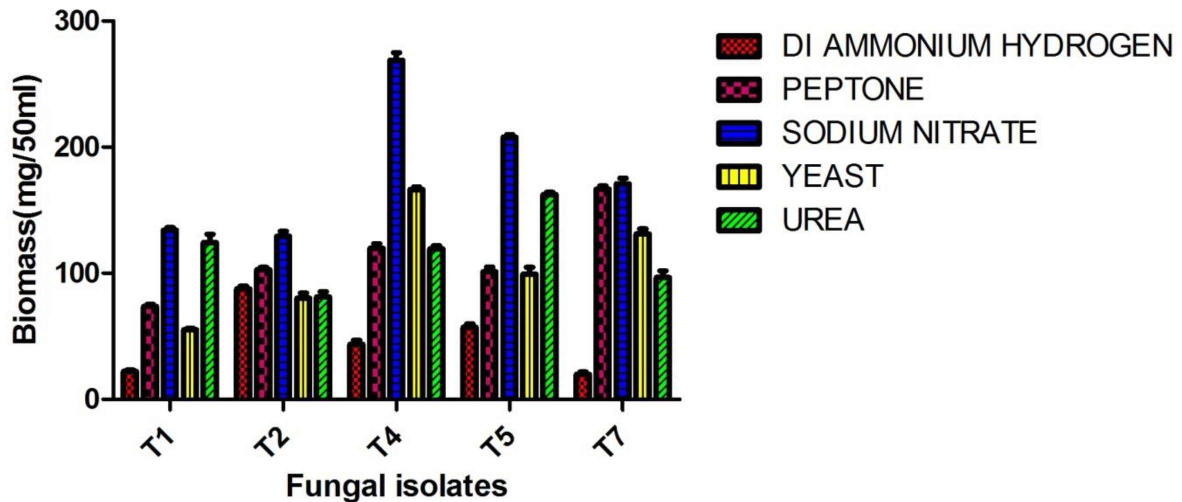


Fig 4. 15) Amount of biomass (mg/50ml) estimated for different fungal isolates and varying nitrogen sources

4.14) Acid phosphatase activity of different fungal isolates with varying nitrogen sources

Maximum acid phosphatase activity was seen in the strain T7 in di-ammonium hydrogen as nitrogen source in MMN media at the end of incubation (Table 4.9 a) and (Fig 4.16). the lowest acid phosphatase activity was reported in sodium nitrate in all the strains. The two way ANOVA revealed the significal variation between carbon and fungal sources in response to acid phosphatase activity (Table 4.9 b)

Table 4.9 a) Acid phosphatase activity of five different isolates with varying nitrogen sources.

FUNGI	NITROGEN SOURCE				
	di ammonium hydrogen	peptone	sodiun nitrate	yeast	urea
T1	30.08 ± 2.52 [b]	9.80 ± 0.99 [b]	2.70 ± 0.06 [b]	16.02 ± 0.64 [b]	6.63 ± 0.84 [b]
T2	6.19 ± 1.53 [c]	3.43 ± 0.12 [c]	2.71 ± 0.19 [c]	9.56 ± 2.61 [c]	7.92 ± 0.96 [c]
T4	17.66 ± 1.82 [c]	4.01 ± 0.17 [c]	1.97 ± 0.15 [c]	5.33 ± 0.15 [c]	6.91 ± 0.30 [c]
T5	12.56 ± 1.21 [c]	3.45 ± 0.24 [c]	3.11 ± 0.15 [c]	8.54 ± 0.98 [c]	23.06 ± 1.38 [c]
T7	121.77 ± 9.82 [a]	29.13 ± 0.59 [a]	3.51 ± 0.22 [a]	4.91 ± 0.36 [a]	8.28 ± 0.59 [a]

Values sharing a common letter within the column are not significant at ($P<0.05$). All values are mean ± SD (n = 3).

Table 4.9 b) Two-way ANOVA results for acid phosphatase activity

Source of variation	Acid phosphatase activity				
	df	SS	MSS	F	P-value
Nitrogen source	4	11089	2772	553.4	***
Fungus	4	7620	1905	380.3	***
Interaction	16	22138	1384	273.2	***
Residual error	50	250.5	5.009		

df: degree of freedom, SS: sum-of-squares, MSS: mean square, ns: not significant; **$P<0.01$; ***$P<0.0001$

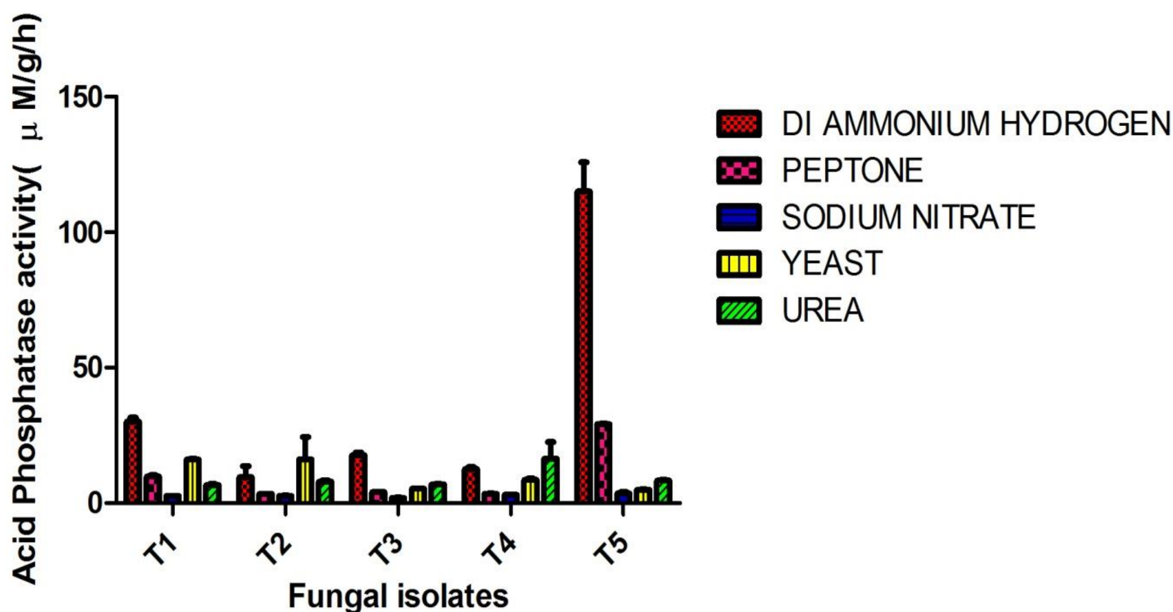


Fig 4.16) Acid phosphatase activity estimated for different fungal isolates and varying nitrogen sources

4.15) Phytase activity of fungal isolates with varying nitrogen sources

Since the assimilation of phytate is likely dependent on the phytate dephosphorylating activites, phytate activity in culture filtrate was determined. The isolate T7 showed the maximum phytase activity in di ammonium hydrogen and the least activity was observed in T5 isolates as seen in T5 in sodium nitrate (Table 4.10a) and (fig 4.17). The two way ANOVA revealed the significal variation between carbon and fungal sources in response to acid phosphatase activity (Table 4.10 b)

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Table 4.10 a) Effect of phytase activity of five different isloates with varying nitrogen sources

FUNGI	NITROGEN SOURCE				
	di ammonium hydrogen	peptone	sodiun nitrate	yeast	urea
T1	37.67 ± 2.03 [a]	12.00 ± 0.55 [a]	6.31 ± 0.52 [a]	24.73 ± 1.70 [a]	8.22 ± 0.84 [a]
T2	8.97 ± 0.65 [b]	10.09 ± 0.20 [b]	7.96 ± 0.34 [b]	16.59 ± 1.92 [b]	16.08 ± 1.25 [b]
T4	22.64 ± 2.61 [b]	9.90 ± 0.48 [b]	8.10 ± 0.61 [b]	8.26 ± 0.39 [b]	24.14 ± 0.71 [b]
T5	13.07 ± 1.48 [c]	8.22 ± 0.72 [c]	5.20 ± 0.36 [c]	8.22 ± 1.26 [c]	8.65 ± 0.35 [c]
T7	72.99 ± 4.53 [d]	5.21 ± 0.51 [d]	12.49 ± 2.56 [d]	8.79 ± 1.44 [d]	26.31 ± 2.31 [d]

Values sharing a common letter within the column are not significant at (P<0.05). All values are mean ± SD (n = 3).

Table 4.10 b) Two-way ANOVA results for phytase activity

Source of variation	Phytase activity				
	df	SS	MSS	F	P-value
Nitrogen sources	4	5185	1296	527.0	***
Fungus	4	2379	594.8	241.8	***
Interaction	16	7340	458.8	186.5	***
Residual error	50	123.0	2.460		

df: degree of freedom, SS: sum-of-squares, MSS: mean square, ns: not significant; **P<0.01; ***P<0.0001

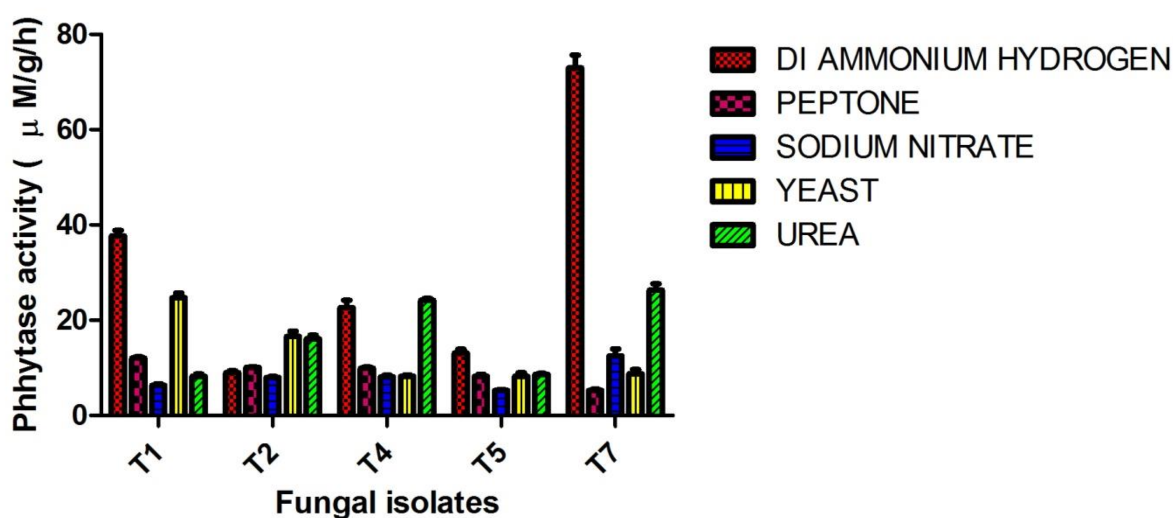
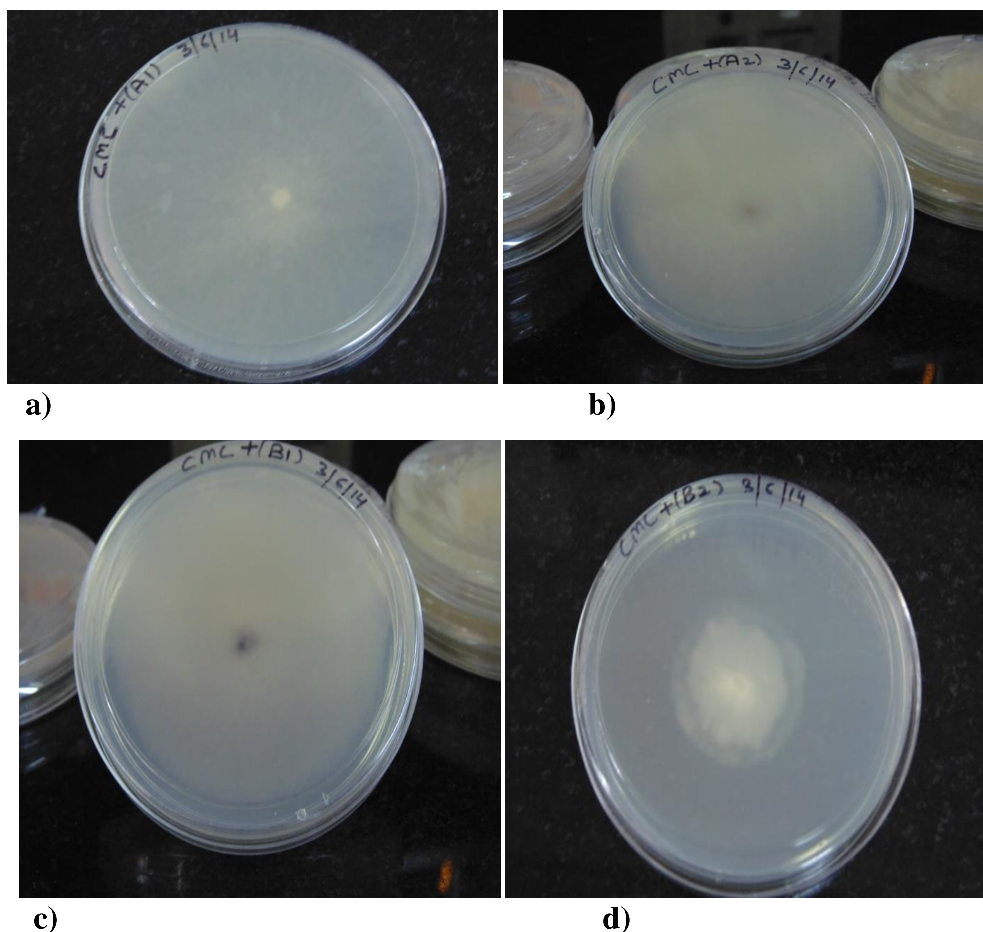


Fig 4.17) Phytase activity estimation for different fungal isolates and varying nitrogen sources

From the above parametric analysis it could be concluded that there is direct relationship between solublization and activity of these enzymes. The production of acid phosphates and phytase activity is favoured at low pH.

4.16) Cellulase enzyme production activity

After the qualitative analysis of the five fungal isolates for the cellulose enzyme production the best cellulose degrading strains observed was T5 and rest of the strains did not show a prominent CMC degrading activity. The positive result could conclude the saprophytic, endophytic and pathogenic nature of the isolated fungal species.



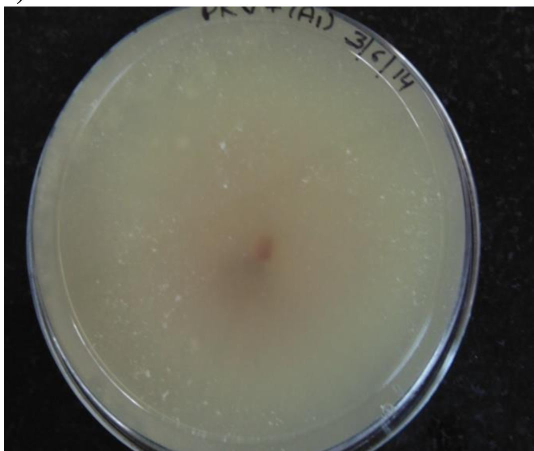
e)

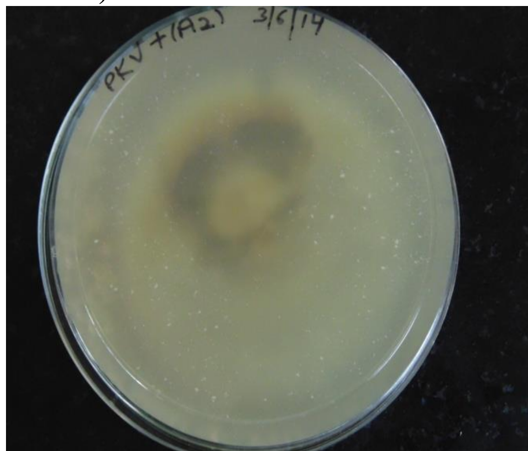
Fig 4.18) Clear zone (Halo) formation was showen by (d) T5 fungal isolate on the CMC agar plates by degradation of cellulose present in the media and giving an opaque appearance to the media in the plates.

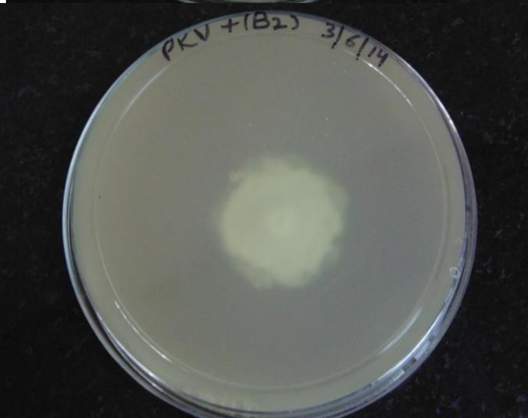
4.17) Soluble P estimation of fungal isolates grown in PKV broth

4.17 a) Qualitative screening of phosphate-solubilising fungi

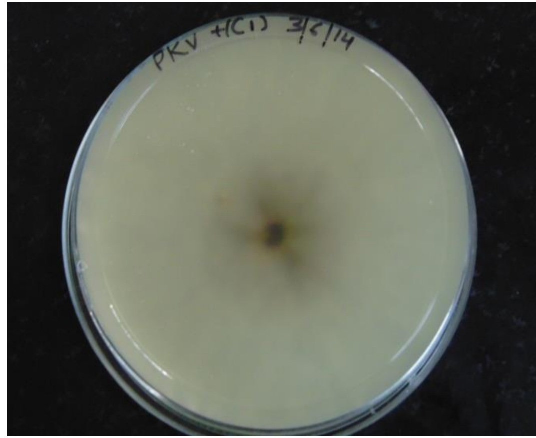
a) **b)**







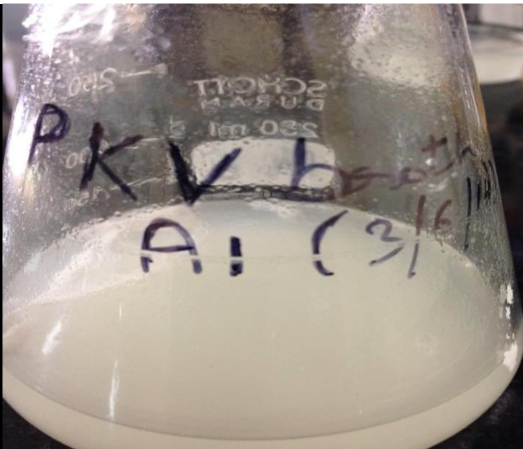
c) **d)**



e)

Fig 4.19) Clear zone formation by the fungal isolates on the PKV agar plates by utilization of the Tri-calcium phosphate in the media

a) b)



c) **d)**

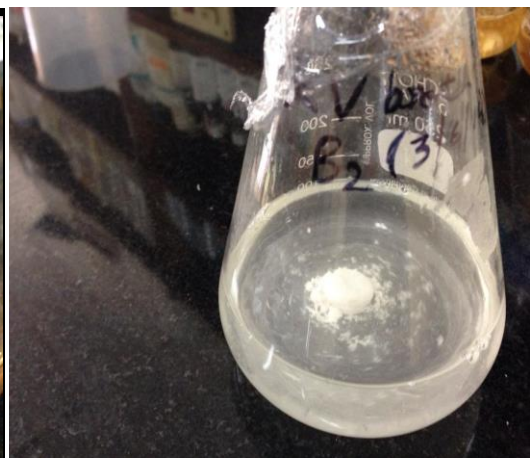


Fig 4.20) Solublization of tri calcium phosphate by, **a)** different fungal isolates, **b)** T1, **c)** T4 and **d)**T5

b) Quantitative Assay for P-solubilization in liquid medium

Table 4.11) Effect of different carbon souces in biomass yield of five different isloates

FUNGUS	SOLUBLE PHOSPHATE (µg/ml)
T1	2.83 ± 0.57 [d]
T2	4.95 ± 0.28 [c]
T4	10.30 ± 0.60 [a]
T5	4.83 ± 0.53 [c]
T7	6.84 ± 0.86 [b]

Values sharing a common letter within the column are not significant at ($P<0.05$). All values are mean ± SD (n = 3).

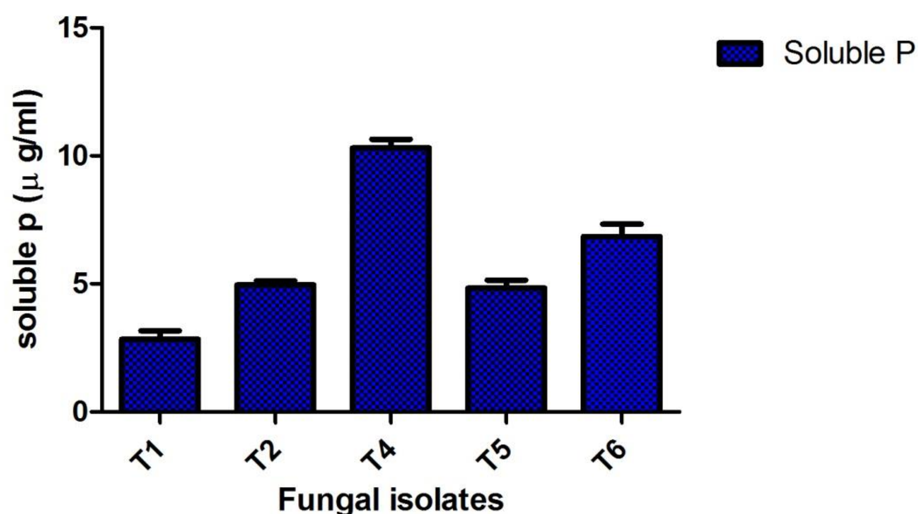


Fig 4.21) Soluble P estimation for different fungal isolates and varying carbon sources

All the five fungal isolates were tested for P solubilization in PKV broth supplemented with tri-calcium phosphate (equivalent to 100 mg P_2O_5 100 ml^{-1}). It was observed that all fungal isolates showed P solubilization activity (Table 4.12). T4 was the fungal isolate which

showed maximum P solublization activity with respect to other isolates. The lowest P solublization activity was of T1. The P soublization of all the fungal isolates was arranged in descending order T4 > T7 > T5 ≥ T2 >T1.

4.18) DNA Sequencing

The purified PCR products of the five fungal strains which showed *Tulasnella* positive amplification in nested PCR out of the seven fungal isolates were sequenced. We were able to to sequence two samples T1 and T2 and the other three sequences are under process. The length of both forward and reverse sequences of the samples was between 700-800 base pairs. The sequences were compared with those available in GenBank databases using BLAST search tool to find the possible homology in the sequences. Both sample sequences showed varied identity with fungal stains present in the database.

Table 4.12) Analysis and putative taxonomic affinities of fungal endophytes isolated from *Rhynchostylis retusa* (foxtail orchid)

Fungal strains	**Length of sequence**	**Molecular identification**	
		BLAST	Identity
T1	760 bp	*Fusarium sp.* 208f	99%
T2	770 bp	*Leptosphaerulina chartarum*	99%
T3			
T4	Under process of sequencing		
T5			

BlastN result of sample T1

Sample T1 sequence showed maximum homology and identity with the *Fusarium sp.* 208f strain., which is an Ascomycetes. It is ubiquitous in nature as it lives as an pathogen and endophyte in *Rhynchostylis retusa*. It acts as an endophyte which is important for the seed

germination and as pathogen for adult orchids. This fungal species have been also reported in numerous other orchids like *Dendrobium* (Siddiquee et al.,2010).

BLAST® Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ Formatting Results - WNC0245G014

Nucleotide Sequence (716 letters)

RID WNC0245G014 (Expires on 07-21 01:50 am)
Query ID lcl|21435
Description None
Molecule type nucleic acid
Query Length 716

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ Citation

Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

Graphic Summary
Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Fusarium sp. 208f internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rit	1291	1291	100%	0.0	99%	JX244011.1
Fusarium sp. 201k internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ri	1291	1291	100%	0.0	99%	JX244004.1
Fusarium sp. 201j internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rit	1291	1291	100%	0.0	99%	JX244003.1
Fusarium sp. BESC883b internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and	1291	1291	100%	0.0	99%	KC007209.1
Fusarium sp. WF157 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, cc	1291	1291	100%	0.0	99%	HQ130713.1
Colletotrichum sp. WF154 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	1291	1291	100%	0.0	99%	HQ130710.1
Fusarium sp. WF153 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, cc	1291	1291	100%	0.0	99%	HQ130709.1
Fusarium sp. WF152 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, cc	1291	1291	100%	0.0	99%	HQ130708.1

BlastN result of sample T2

Sample T2 sequence showed maximum homology and identity with the *Leptosphaerulina chartarum*, which is an Ascomycetes. It lives as a colonized pathogen in *Rhynchostylis retusa*. It acts as pathogen for adult orchids.

BLAST® Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ Formatting Results - WNCCKW0P015

Nucleotide Sequence (751 letters)

RID WNCCKW0P015 (Expires on 07-21 01:57 am)
Query ID lcl|17265
Description None
Molecule type nucleic acid
Query Length 751

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ Citation

Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

Graphic Summary
Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Leptosphaerulina chartarum 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spa	1236	1236	100%	0.0	97%	DQ384571.1
Uncultured fungus clone CMH011 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribe	1101	1101	79%	0.0	99%	KF800102.1
Leptosphaerulina chartarum isolate CY233 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	1101	1101	79%	0.0	99%	HQ608046.1
Leptosphaerulina chartarum isolate ATT044 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal	1101	1101	79%	0.0	99%	HQ607815.1
Leptosphaerulina chartarum isolate PCT20 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	1101	1101	79%	0.0	99%	HQ248190.1
Uncultured fungus clone f3Fc44 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	1101	1101	79%	0.0	99%	GU721572.1
Leptosphaerulina sp. CCG-2012 isolate PanB1b0191SNA2CC46 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, c	1098	1098	87%	0.0	97%	JQ388922.1
Leptosphaerulina sp. CCN4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spa	1096	1096	79%	0.0	99%	DQ092534.1

4.19) Salient findings

a) Seven pure fungal cultures associated with roots of *Rhynchostylis retusae* were isolated.

b) Out of seven, five cultures showed *Tulasnella* positive amplification in the nested PCR.

c) Sporous and non-sporous fungi were characterized in the microscopic study.

d) Three strains showed high enzyme activity, in which one strain had high phosphate solublizing activity.

e) Two-way ANOVA, statistical analysis showed high significance in the results for majority of fungal biochemical screening assays.

f) Sequencing results of two sequences showed homology with *Fusarium sp*. and *Leptosphaerulina chartarum* in BLAST analysis.

Appendix

1) **CTAB**

CTAB (cetyltrimethyl ammonium bromide)	
2% CTAB	20 gm CTAB
20 mM EDTA	40 mL 0.5 M EDTA
100 mM Tris-HCl pH 8.0	100 ml 1M Tris-HCl
1.4M NaCl	280 mL 5 M NaCl
Make up to 1 L with dd H_2O and autoclave	

2) **TE buffer**

TE buffer	
1M Tris HCl pH 8.0	10 ml
0.5M EDTA	2 ml
Bring total volume to 1 L with dd H_2O	

3) **TBE buffer**

TBE Buffer (10x)	
Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

4) **Agarose Gel Loading Dye (6X)**

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

5) Reagents

1. **Chloromolybdic acid:** 15.0 g ammonium molybdate was dissolved in about 400 ml of distilled water. Filtered and then 400 ml of 10 N HCl was added slowly with rapid stirring. Volume was made to 1000 ml with distilled water and stored in amber glass bottle.
2. **Chlorostannous acid:**

   ***Stock Solution*:**

$SnCl_2 .2H_2O$	10.0 g
Conc. HCl	25.0 ml

   $SnCl_2$ crystals were dissolved in conc. HCl and solution was kept in glass under airtight stopper.

   ***Working Solution*:** Fresh working solution was prepared by adding 1.0 ml of the above solution to 132.0 ml of distilled water.
3. **5X modified universal buffer (MUB) (Skujins et al. 1962)**

Tris (hydroxyl methyl) amino methane	12.10 g
Maleic acid	11.60 g
Citric acid	14.00 g
Boric acid	6.28 g
1 N NaOH	488 ml

   Final volume was made up to 1000 ml with distilled water and stored at 4 ºC.

   Before use, the pH of 200 ml of MUB was adjusted to 5.5 with 0.5 N HCl for the assay of acid phosphatase or with 0.5 N NaOH to pH 9.0 for the alkaline phosphatase. The volume of pH modified buffer was made up to 1 liter with distilled water.

d) p-nitrophenyl phosphate solution (0.115 M): Dissolved 4.268 g disodium p-nitrophenyl phosphate hexahydrate in 100 ml of appropriate pH adjusted, diluted MUB (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase. The p-nitrophenyl phosphate substrate was stored at 4 ℃ but no longer than 10 days.

e) NaOH (0.5N): 20 g of NaOH was dissolved in 70 ml distilled water and the volume was made up to 1 litre.

f) **p-nitrophenol standard:** 1 mg per 1 ml p.nitrophenol solution was prepared in modified universal buffer. The solution was stored in dark bottle at 4 ℃ for no longer than 21 days.

g) **Substrate solution:** Dissolved 2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5.

h) **Color stop solution:** (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio)

i) **1 M citric acid**

j) **Standard KH_2PO_4 solution:** mg/ml stock.

6) Composition of Pikovskaya's agar medium (Pikovskaya 1948)

Ingredients	**gms/liter**
Yeast Extract	0.50
Dextrose	10.0
Tri-calcium phosphate	5.00
Ammonium Sulphate	0.50
Potassium Chloride	0.20
Magnesium Sulphate	0.10
Manganese Sulphate	0.001

Ferrous Sulphate	0.001
Agar	15.00
Distilled Water	1000 ml
pH	7.2 ± 0.2

All ingredients except tri-calcium phosphate $Ca_3(PO_4)_2$ were dissolved in 1000 ml of distilled water, pH adjusted to 7.2 and autoclaved at 15 Psi for 15 minutes.

7) Composition of Potato Dextrose Agar

Component	g/l
Potato infusion	200.0g
Dextrose	20.0 g
Agar	15.0 g
Distilled Water	1.0 L

8) Defined Melin's modified Norkans medium

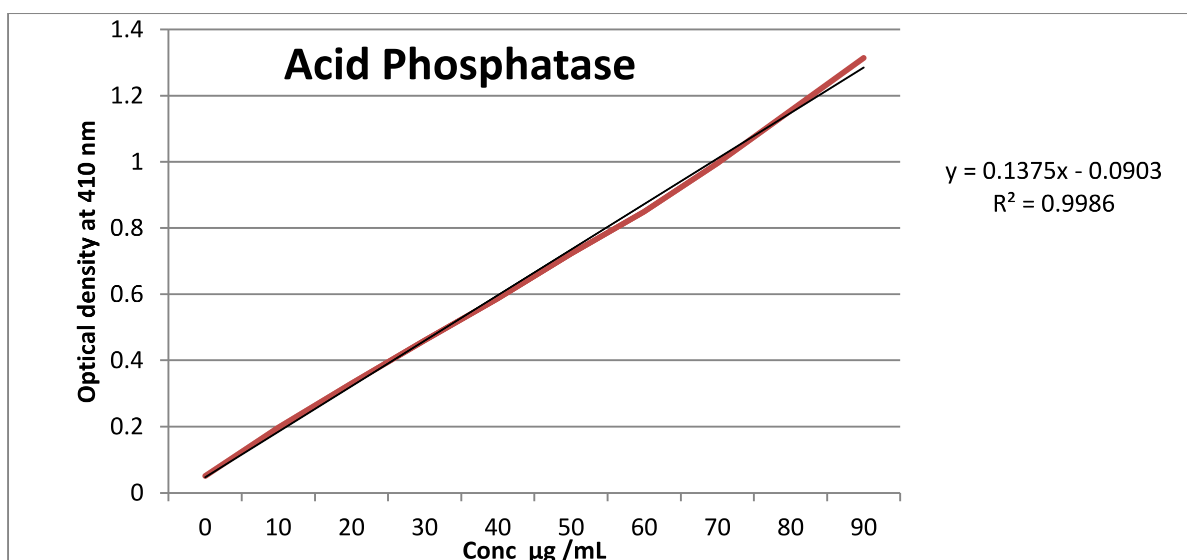
Components	mg/l
Ammonium Phosphate Monobasic	250 mg/l
Potassium Phosphate Monobasic	500 mg/l
Magnesium Sulphate	150 mg/l
Calcium Chloride	50 mg/l
Sodium Chloride	25 mg/l
Thiamine HCl	40 mg/l
1% (w/v) Ferrous Chlorate	1.2 ml/l

Biotine	0.4 mg/l
Glucose	2500 mg/l
Heller's micronutrients (100 X)	10 ml/l
Agar	8000l

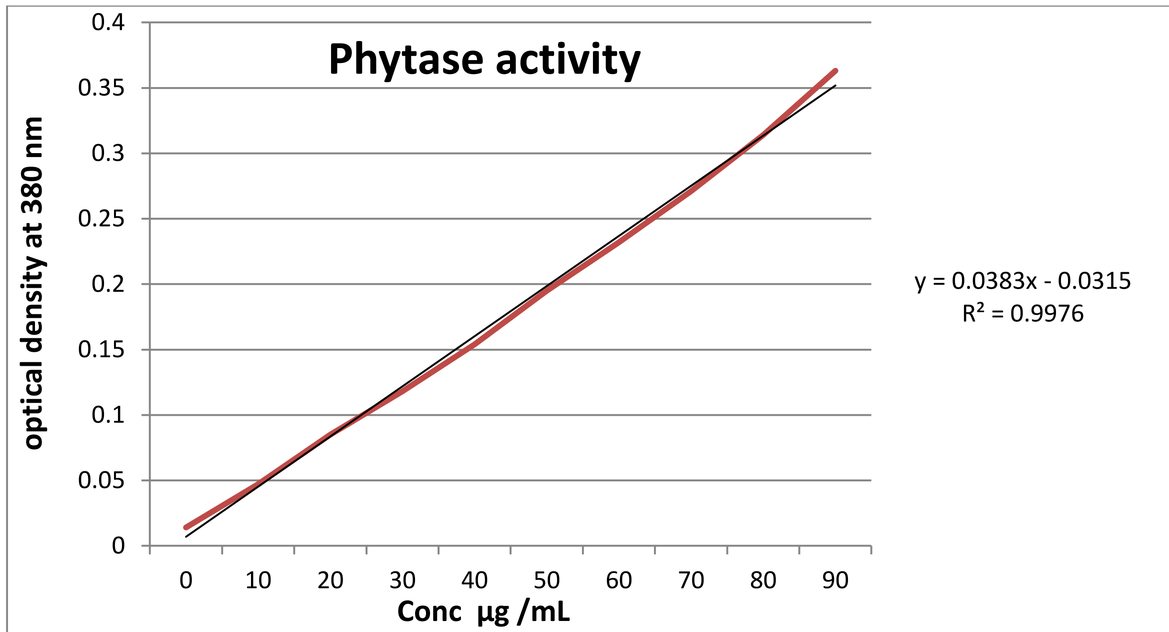
9) Modified Czapek-mineral salt medium

Ingredients	**g/l**
Sodium nitrate	2.0
Potassium phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5
Carboxymethyl cellulose	5.0
Peptone	2.0
Agar	20.0
Distilled water	1000 ml

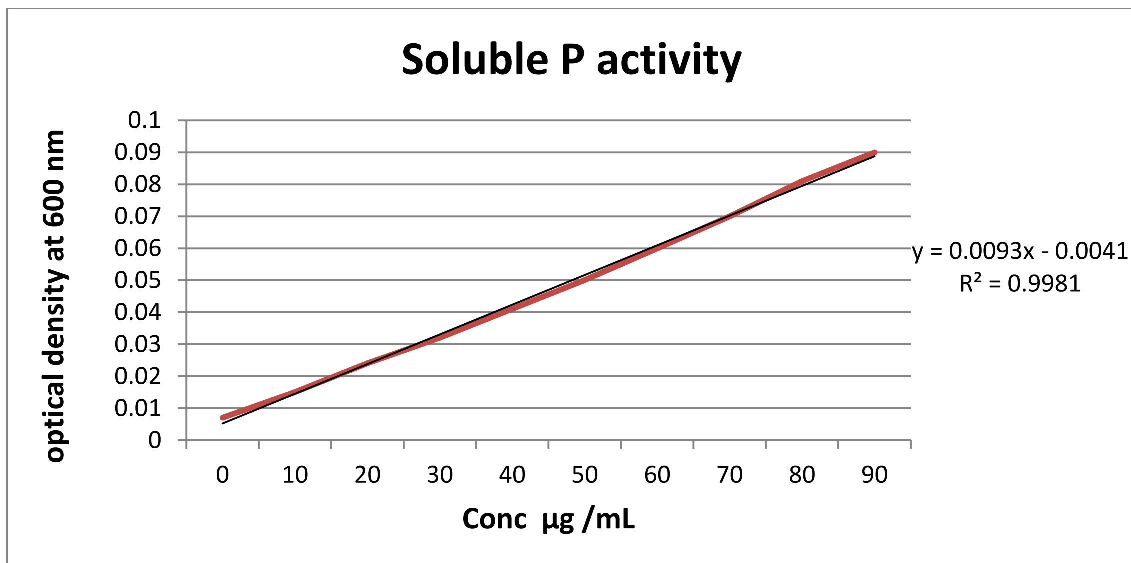
13) Standard curve for Acid Phosphatase Activity (p-nitro phenol)



14) Standard curve for Phytase Activity (potassium di-hydrogen phosphate, KH_2PO_4)



15) Standard curve for Soluble P Activity (potassium di-hydrogen phosphate, KH_2PO_4)



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