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Ectomycorrhiza and Fungal Diversity in the Mycorrhizosphere of *Pinus gerardiana*

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ABSTRACT

The four types (white, dark radish brown, dark brown and light brown to pale brown types) of ectomycorrhizae were collected from natural habitat of P. gerardiana. Anatomically ectomycorrhizal roots showed presence of fungal mantle and well developed Hartig net. Fungal diversity in the mycorrhizosphere of P. gerardiana was studied and revealed the presence of 32 fungal species and it was found that maximum fungi were present during rainy season (30 fungi) followed by spring (24 fungi), autumn (18 fungi) and winter (14 fungi). It was also concluded from the study that the three members of Zygomycotina (Cunninghamella echinulata, Mucor elegans and Rhizopus nigricans), one member of Ascomycotina (Penicillium citrinum) and five members of Deuteromycotina (Alternaria alternata, Cephalosporium acremonium, Fusarium equiseti Gliocladium penicillioides and Trichoderma viride) were isolated during all the seasons (i.e. winter, spring, rainy and autumn season) from the mycorrhizosphere of Pinus gerardiana.

Key words: *Pinus gerardiana, Ectomycorrhiza, Mycorrhizosphere, Fungal Diversity.*

INTRODUCTION

The term mycorrhiza (Greek: *mykes* = Fungus; *rhiza* = root), a mutualistic association formed between specialized soil fungi (Basidiomycetes, Ascomycetes and Zygomycetes) and plant roots (most vascular plants), was first described late in the 19th century by a forest pathologist called Frank[1]. Soil contains a large number of diverse microbial communities. The influence of plant assimilates on microbial communities has been defined in relation to the rhizosphere, the narrow zone of soil surrounding living roots[2]. The rhizosphere is characterized by increased microbial activity stimulated by the leakage and exudation of organic substances from the root[3]. However, since plant roots in natural and semi-natural ecosystems are commonly mycorrhizal, the rhizosphere concept has been widened to include the fungal component of the symbiosis, resulting in the term "mycorrhizosphere"[4].

The photosynthates flowing into soil through roots and mycorrhizae support a diverse community of soil microorganisms, many of which influence plant growth[4,5]. Fluctuations in soil fungal biomass occur seasonally[6].

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The mycelial growth pattern in soil was proposed[7] and later shown[8] to be dynamic, involving competition between different types of fungi and different fungal species. Fungi contribute significant biomass to soil where they have important functions in nutrient cycling[9] and microaggregate formation[10]. The parasitic nature of these associations does not imply that all root-inhabiting fungi are pathogens. In fact, many fungi growing with roots are beneficial, as exemplified by mycorrhizal symbionts[11] and nonpathogenic parasites associated with roots[12].

There are considerable variations in the structure and function of ectomycorrhiza formed by one host associating with different fungi[13]. The degree of short root branching and the structure of the mantle and Hartig net vary because of the presence of different ECM fungi[13,14]. Plant-fungus specificity varies considerably in ECM associations, from narrow host range fungi that associate with a single host species to broad host range fungi that associate with different families of host plants[15].

Mycorrhizosphere interactions may be quite specific and have important effects on mycorrhizal formation. Microbial activity in soil is controlled by several environmental factors, such as availability of C, mineral nutrient and growth factors, availability of water, favourable temperature and pH, composition of soil microflora and ecological interactions between microorganisms[16]. Microorganisms play an important role in soil fertility because they oxidize organic matter and promote the biogeochemical cycles of C, N, P, and S[17]. Soil microorganisms are important components in the natural soil ecosystem because not only can they contribute to nutrient availability in the soil, but also bind soil particles into stable aggregates, which improve soil structure and reduce erosion potential[18].

P. gerardiana is economically important high altitude conifer and its seed are well known in dry fruit trade and are highly prized and valuable as an edible nut. The export of seeds contributes appreciably to the annual income of most of the families living in the area of its distribution. So it is important, therefore, to identify the best strains of beneficial microbes for the planting situation and employ this combination inoculum in afforestation practices of this conifer. Tailoring the microbes to fit the growth situation will increase the chances of successful exploitation of the mycorrhizosphere phenomenon.

MATERIALS AND METHODS

Morpho-anatomical Studies on Mycorrhiza:

Morpho-anatomical details of natural mycorrhizal roots of *P. gerardiana* were worked out following Zak[19], whereas anatomical details were carried out from the wet material. Mycorrhizal roots were fixed in F.A.A. (Formalin-acetic acid-alcohol) for 24 hours, and then preserved in 70% alcohol for further investigations. Sectioning was done following Johansen[20].

Isolation of Mycorrhizosphere Fungi:

Soil samples were collected from mycorrhizosphere (soil in the direct influence of mycorrhizae) of *P. gerardiana* and sampling was done during four seasons (i.e. winter, spring, rainy and autumn) of the year 2008. For the isolation of mycorrhizosphere fungi, dilution plate method of Wakesman[21] and Warcup[22] was followed. This method includes shaking of a known amount of soil (1g) taken from region surrounding fresh and active root in 1000ml of distilled water. Then 1ml of this suspension is taken and is added to 100ml of distilled water. From this, 1ml material is taken and various suspensions are made and are dispersed over medium under aseptic conditions.

The media used for culturing mycorrhizosphere fungi were Czapek's Dox[23] and Potato Dextrose Agar[24]. The sterilized medium was poured into the Petri plates under aseptic conditions (laminar flow hood) and allowed to solidify. Samples of each type were taken with sterilized graduated dropper and poured on Petri plates containing medium. The samples were uniformly spread on the medium by tilting the Petri plates. The inoculated Petri

plates were placed in incubator and incubated at 22±1ºC for seven days and checked regularly for fungal colony growth.

For obtaining pure culture, slants of media were made in test tubes and fungi separated from soil were subcultured in them. For identification, temporary mounts of fungi were made in 0.1% cotton blue and Lactophenol. The mycorrhizosphere fungi were identified following Barnett and Hunter[25] and Gillman[26].

RESULTS AND DISCUSSION

Mycorrhizae of *Pinus gerardiana* in its Natural Habitat:

Studies on mycorrhiza of *Pinus gerardiana* in its natural habitat showed that ectomycorrhiza arises as a lateral outgrowth from the mother root. On the basis of the colour, general shape, texture and thickness of mantle and degree of spreading of hyphae into the cortex the mycorrhizal roots can be distinguished into following four types (**Fig.1**)

a) White Type: Mycorrhizal roots were white in colour, reticulate, coralloid and profusely branched. These roots were 0.3-0.5 cm long. Mantle was 10-20 µm in thickness. Root hairs were absent (**Fig.1** A, B).

b) Dark Reddish Brown Type: Mycorrhizal roots were dark reddish brown in colour, dichotomously branched and reticulate. Mycorrhizal roots were 0.5-0.7 cm long. Mantle is 15-20 µm in thickness. (**Fig.1** C, D).

c) Dark Brown Type: Colour of mycorrhizal roots was dark brown. Roots were monopodial in general shape, 0.3-0.5 cm long. Mantle was 35-35 µm in thickness. (**Fig.1** E, F).

d) Light Brown to pale brown Type: Colour of mycorrhizal roots was light brown to pale brown. Roots were monopodial pinnate in shape, 0.5-0.7 cm long. Mantle is 25-30 µm in thickness. Root hairs were absent (**Fig.1**G, H).

In the similar studies Bakshi *et al*.,[27] described ectomycorrhizae to be creamish, pinkish, brown or black in colour in blue pine, spruce and deodar. They also observed dichotomous and coralloid type of branching in mycorrhizal roots. Rudawska *et al*., [28] characterized nine types of ectomycorrhizae from the roots of 1 year old and 2 year old *Pinus sylvestris* seedlings from nurseries situated in the North-west part of Poland. In recent studies[29] on the beech ectomycorrhizae formed by *Pachyphloeus* spp. ectomycorrhizal roots were whitish-yellow or Ochre to light brown in colour and was monopodial-pyramidal, densely ramified with short stout ends whereas in the root system of *Pinus pinaster* 15 different types of ectomycorrhizae were observed[30].

Transverse sections of these roots revealed the presence of a thick fungal mantle as the outer most layer which gives protection against root pathogens. Between the cortical cells of these roots was observed a well developed Hartig net. Profusely branched root morphology, presence of fungal mantle and Hartig net confirmed that the natural mycorrhizal roots of *P. gerardiana* were of ectomycorrhizal type. Similar observations were reported on the anatomical details of Scots pine (*Pinus sylvestris*) and beech (*Fagus sylvatica*) ectomycorrhizae[31].

Fungal Diversity in the Mycorrhizosphere of *Pinus gerardiana*:

Thirty two species of fungi belonging to Zygomycotina, Ascomycotina, Basidiomycotina and Deuteromycotina have been isolated from the mycorrhizosphere of *Pinus gerardiana* during four ((i.e. winter, spring, rainy and autumn) seasons (Table 1).

Mycoflora of Winter Season

During winter season 12 species of fungi isolated from the mycorrhizosphere soil samples of *Pinus gerardiana*. The fungal genera isolated were *Alternaria*, *Cephalosporium*, *Cladosporium*, *Cunninghamella*, *Fusarium*, *Gliocladium Mucor*, *Penicillium*, *Rhizopus*, *Trichoderma* and two unidentified basidiomycetous mycelia. All these twelve genera were represented by single species i.e. *Alternaria alternata*, *Cephalosporium acremonium*, *Cladosporium sphaerospermum*, *Cunninghamella echinulata*, *Fusarium equiseti*, *Gliocladium penicillioides*, *Mucor elegans*, *Penicillium citrinum*, *Rhizopus nigricans*, *Trichoderma viride* and two unidentified basidiomycetous mycelia.

Out of these fungi, three genera belong to Zygomycotina (*Cunninghamella*, *Mucor* and *Rhizopus*), one to Ascomycotina (*Penicillium*), six to Deuteromycotina (*Alternaria*, *Cephalosporium*, *Cladosporium*, *Fusarium*, *Gliocladium* and *Trichoderma*). Basidiomycotina is represented by two white sterile mycelia (Table 1).

Mycoflora of Spring Season

During spring season 24 species of fungi belonging to 17 genera were isolated from the mycorrhizosphere of *Pinus gerardiana*. The fungal genera were *Absidia*, *Alternaria*, *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Cunninghamella*, *Fusarium*, *Gliocladium*, *Mucor*, *Penicillium*, *Rhizoctonia*, *Rhizopus* and *Trichoderma*. Basidiomycotina is represented by four white sterile mycelia.

Fusarium was represented by four species (*F. equiseti*, *F. moniliforme*, *F. oxysporum* and *F. solani*) followed by *Aspergillus* (*A. flavus* and *A. wentii*), *Cladosporium* (*C. cladosporioides* and *C. herbarum*), *Mucor* (*M. elegans* and *M. hiemalis*) and *Trichoderma* (*T. pseudokoningii* and *T. viride*). Seven fungal genera were represented by single species i.e. *Absidia ramosa*, *Alternaria alternata*, *Cephalosporium acremonium*, *Cunninghamella echinulata*, *Gliocladium penicillioides*, *Penicillium citrinum*, *Rhizoctonia solani* and *Rhizopus nigricans*. Basidiomycotina is also represented by four sterile mycelia. Out of these fungi, four genera belong to Zygomycotina (*Absidia*, *Cunninghamella*, *Mucor* and *Rhizopus*), two to Ascomycotina (*Aspergillus* and *Penicillium*) and seven to Deuteromycotina (*Alternaria*, *Cephalosporium*, *Cladosporium*, *Fusarium*, *Gliocladium*, *Rhizoctonia* and *Trichoderma*). Basidiomycotina is represented by four white sterile mycelia (Table 1).

Mycoflora of Rainy Season

Thirty species of fungi belonging to 22 genera were isolated from the mycorrhizosphere of *Pinus gerardiana* during rainy season. The fungal genera were *Absidia*, *Alternaria*, *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Cunninghamella*, *Epicoccum*, *Fusarium*, *Gliocladium*, *Mortierella*, *Mucor*, *Paecilomyces*, *Penicillium*, *Rhizoctonia*, *Rhizopus*, *Stachybotrys*, *Trichoderma* and five basidiomycetous fungi.

The genus *Cladosporium* and *Fusarium* was represented by three species each (i.e. *C. cladosporioides*, *C. herbarum*, *C. sphaerospermum*, *F. equiseti*, *F. moniliforme* and *F. oxysporum*) followed by *Aspergillus*, *Mucor*, *Penicillium* and *Trichoderma* which are represented by two species each (i.e. *A. niger*, *A. wentii*, *M. elegans*, *M. hiemalis*, *P. citrinum*, *P. funiculosum*, *T. pseudokoningii* and *T. viride*). Other eleven genera (*Absidia*, *Alternaria*, *Cephalosporium*, *Cunninghamella*, *Epicoccum*, *Gliocladium*, *Mortierella*, *Paecilomyces*, *Rhizoctonia*, *Rhizopus* and *Stachybotrys*) were represented by one species each (i.e. *Absidia ramosa*, *Alternaria alternata*, *Cephalosporium acremonium*, *Cunninghamella echinulata*, *Epicoccum nigrum*, *Gliocladium penicillioides*, *Mortierella minutissima*, *Paecilomyces lilacinus*, *Rhizoctonia solani*, *Rhizopus nigricans* and *Stachybotrys atra*). Out of these fungi, five genera belong to Zygomycotina (*Absidia*, *Cunninghamella*, *Mortierella*, *Mucor* and *Rhizopus*), two belong to Ascomycotina (*Aspergillus* and *Penicillium*) and ten to Deuteromycotina (*Alternaria*, *Cephalosporium*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Gliocladium*, *Paecilomyces*, *Rhizoctonia*, *Stachybotrys* and *Trichoderma*). Basidiomycotina is represented by five white sterile mycelia (Table 1).

Mycoflora of Autumn Season

17 species of fungi belonging to 15 genera were isolated from the mycorrhizosphere of *Pinus gerardiana* during autumn season. The isolated fungal genera were *Alternaria*, *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Cunninghamella*, *Fusarium*, *Gliocladium*, *Mucor*, *Penicillium*, *Rhizoctonia*, *Rhizopus*, *Stachybotrys*, *Trichoderma* and two basidiomycetous white sterile mycelia.

Fusarium and *Mucor* were represented by two species each (*F. equiseti*, *F. moniliforme*, *M. elegans* and *M. hiemalis*) while all other fungal genera were represented by single species i.e. *Alternaria alternata*,

Aspergillus flavus, Cephalosporium acremonium, Cladosporium sphaerospermum, Cunninghamella echinulata, Gliocladium penicillioides, Penicillium citrinum, Rhizoctonia solani, Rhizopus nigricans, Stachybotrys atra, Trichoderma viride and two basidiomycetous mycelia. Out of these fungi, three genera belong to Zygomycotina (*Cunninghamella, Mucor* and *Rhizopus*), two to Ascomycotina (*Aspergillus* and *Penicillium*), eight to Deuteromycotina (*Alternaria, Cephalosporium, Cladosporium, Fusarium, Gliocladium, Rhizoctonia, Stachybotrys* and *Trichoderma*). Basidiomycotina is represented by two white sterile mycelia (Table 1).

It is concluded from the above study that three members of Zygomycotina (*Cunninghamella echinulata, Mucor elegans* and *Rhizopus nigricans*), one member of Ascomycotina (*Penicillium citrinum*) and five members of Deuteromycotina (*Alternaria alternata, Cephalosporium acremonium, Fusarium equiseti Gliocladium penicillioides* and *Trichoderma viride*) were isolated during four seasons (i.e. winter, spring, rainy and autumn season) from the mycorrhizosphere soil samples of *Pinus gerardiana*.

The different fungal genera exhibited considerable fluctuations in their occurrence in different soil samples collected during various seasons. Such variations have also been recorded by earlier workers, working with different plants. Large number of bacterial and fungal species other than those forming the mycorrhiza was observed in the mycorrhizosphere of *Pinus radiata*[32]. Two unidentified basidiomycetous mycelia and eleven fungi were isolated from the mycorrhizosphere of *Picea smithiana*[33] and in a similar study, 22 fungi were isolated from the mycorrhizosphere of *Abies spectabilies*, 14 fungi from *Pinus roxburghii* and 27 fungi from the mycorrhizosphere of *Taxus baccata* [34].

Mycorrhizosphere effect is a dynamic process initiated by root exudation, other release of organic nutrients and is influenced by host factors such as species, age and stage of development, soil factors such as fertility and moisture level; environmental conditions such as light, temperature and soil microbial interactions[35]. Micro-organisms associated with developing and already established mycorrhizae were also studied by earlier worker[36] and an increased development of mycorrhizae were observed if certain associative bacteria and fungi, such as *Azotobacter* and *Trichoderma* were also present at the time of inoculation.

Table 1. Morpho-anatomical characteristics of *Pinus gerardiana* ectomycorrhizae collected from its natural habitat

Sr. No.	Characters	ECTOMYCORRHIZA			
		White Type	**Dark Reddish Brown Type**	**Dark Brown Type**	**Light Brown to pale brown Type**
	Macroscopic				
1	Colour	White	Dark Reddish Brown	Dark Brown	Light brown to pale brown
2	Shape of mycorrhiza	Reticulate, coralloid and profusely branched	Dichotomously branched and reticulate	Roots were monopodial in general shape	Roots were monopodial pinnate in shape
3	Texture	Smooth to shiny	Smooth to shiny	Smooth	Smooth
5	Emanating Hyphae	Many long irregularly radiating outwards	Absent	Rare	Infrequent
6	Root Hairs	Absent	Absent	Absent	Absent
	Microscopic				
7	Thickness of Mantle	Mantle was 10-20 µm in thickness	Mantle is 15-20 µm in thickness	Mantle was 35-35 µm in thickness	Mantle is 25-30 µm in thickness
8	Hartig net	Present	Present	Present	Present

xxxx

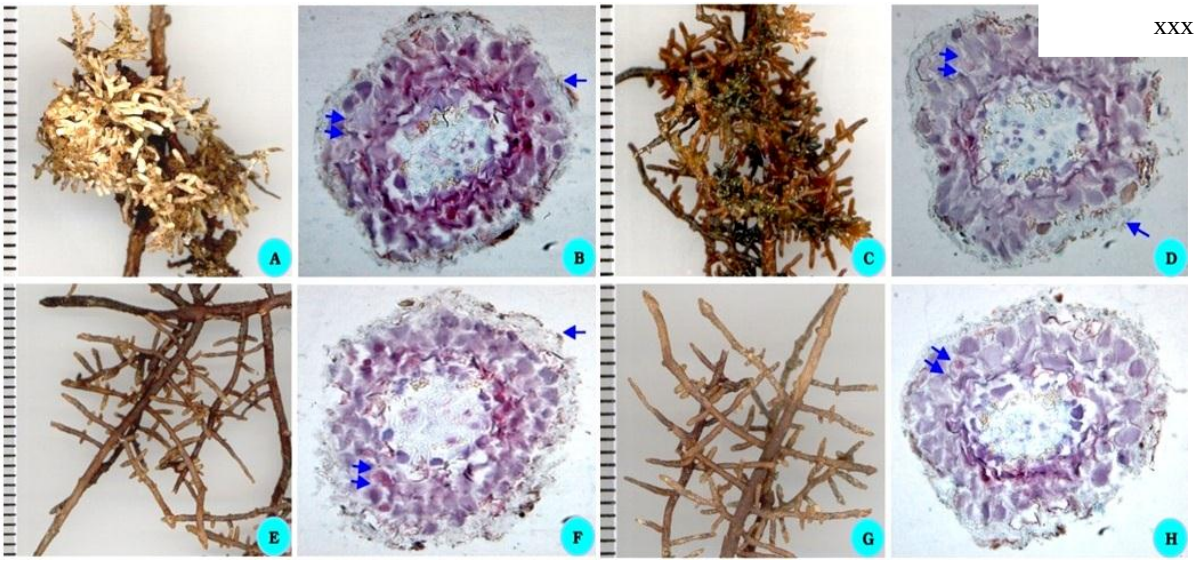

Fig. 1: Four Types of Ectomycorrhizae: (a) White type (b) T. S. of White type (c) Dark Reddish Brown type (d) T. S. of Dark Reddish Brown type (e) Dark Brown type (f) T. S. of Dark Brown type (g) Light Brown type (h) T. S. of Light Brown type. Fungal mantle (→) and Hartig net (⇉)

Table 2. Seasonal Distribution of the Fungi Isolated from Mycorrhizosphere of *Pinus gerardiana*

Sub Division	Name of the Fungus	Winter	Spring	Rainy	Autumn
Zygomycotina	*Absidia ramosa*	-	+	+	-
	Cunninghamella echinulata	+	+	+	+
	Mortierella minutissima	-	-	+	-
	Mucor elegans	+	+	+	+
	Mucor hiemalis	-	+	+	+
	Rhizopus nigricans	+	+	+	+
Ascomycotina	*Aspergillus flavus*	-	+	-	+
	Aspergillus niger	-	-	+	-
	Aspergillus wentii	-	+	+	-
	Penicillium citrinum	+	+	+	+
	Penicillium funiculosum	-	-	+	-
Deuteromycotina	*Alternaria alternata*	+	+	+	+
	Cephalosporium acremonium	+	+	+	+
	Cladosporium cladosporioides	-	+	+	-
	Cladosporium herbarum	-	+	+	-
	Cladosporium sphaerospermum	+	-	+	+
	Epicoccum nigrum	-	-	+	-
	Fusarium equiseti	+	+	+	+
	Fusarium moniliforme	-	+	+	+
	Fusarium oxysporum	-	+	+	-
	Fusarium solani	-	+	-	-
	Gliocladium penicillioides	+	+	+	+
	Paecilomyces lilacinus	-	-	+	-
	Rhizoctonia solani	-	+	+	+
	Stachybotrys atra	-	-	+	+
	Trichoderma pseudokoningii	-	+	+	-
	Trichoderma viride	+	+	+	+
Basidiomycotina	Basidiomycetous mycelium	+	+	+	+

+ Present; - Absent

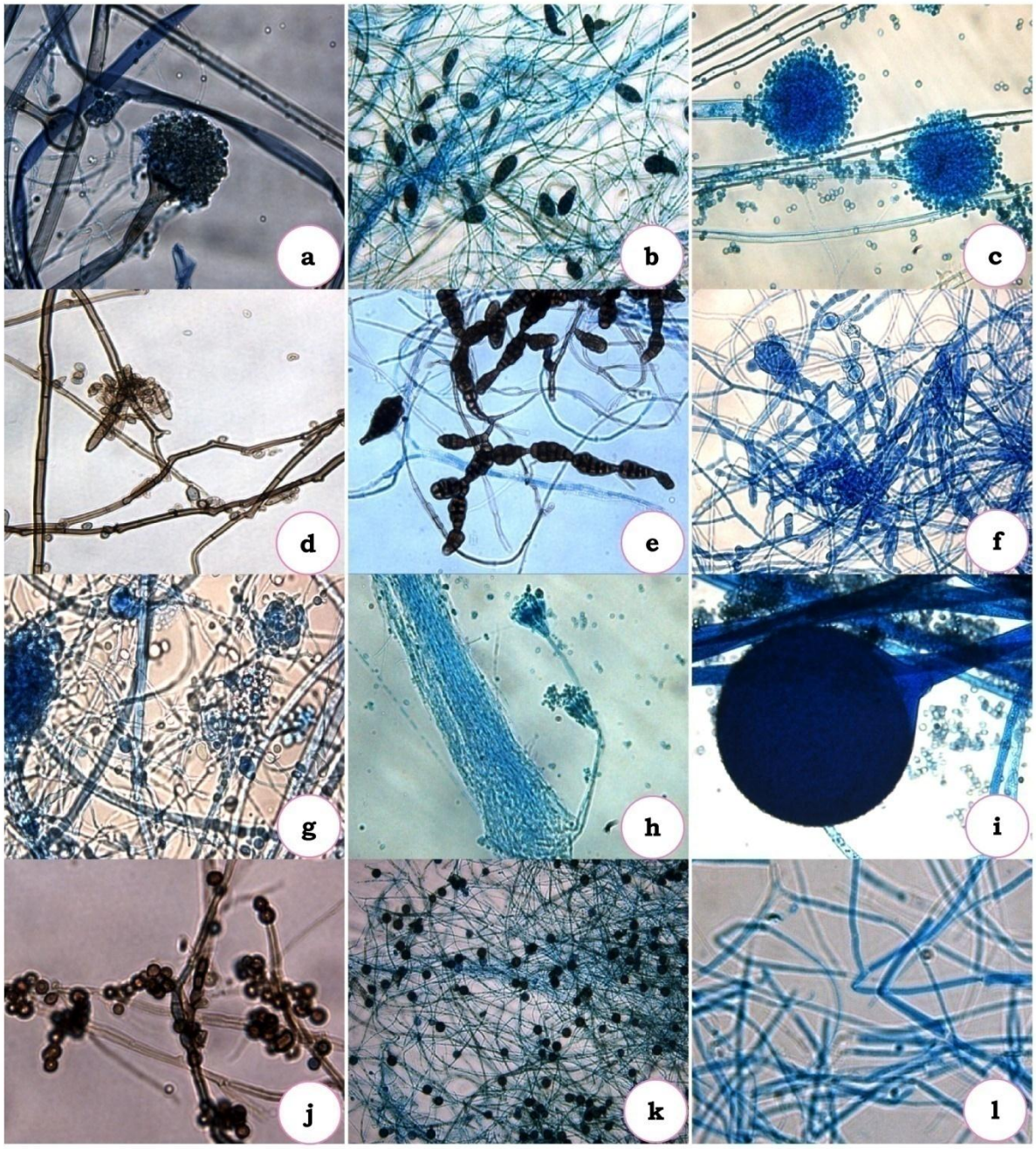

Figure 2. (a) ***Absidia ramose*** **(b)** ***Alternaria alternata*** **(c)** ***Aspergillus niger*** **(d)** ***Cladosporium sphaerospermum*** **(e)** ***Cunninghamella echinulata*** **(f)** ***Fusarium moniliforme*** **(g)** ***Mortierella minutissima*** **(h)** ***Penicillium citrinum*** **(i)** ***Rhizopus nigricans*** **(j)** ***Stachybotrys atra*** **(k)** ***Trichoderma viride*** **(l) Basidiomycetous mycelium**

CONCLUSIONS

The four types (white, dark radish brown, dark brown and light brown to pale brown types) of ectomycorrhizae were collected from natural habitat of *P. gerardiana*. Mycorrhizosphere of *P. gerardiana* revealed the presence of 32 fungi belonging to Zygomycotina, Ascomycotina, Basidiomycotina and Deuteromycotina. The maximum representatives were of Deuteromycotina (16 genera), followed by Zygomycotina (6 genera), Basidiomycotina (5 sterile mycelium) and Ascomycotina (5 genera). Seasonal distribution of Mycoflora in the mycorrhizosphere of *P. gerardiana* was also studied and it was found that maximum fungi were present during rainy season (30 fungi) followed by spring (24 fungi), autumn (18 fungi) and winter (14 fungi). Study can be further extended to see the effect of

mycorrhizosphere fungi on ectomycorrhizae formation and to observe their impact on growth and performance of seedlings of this economically important high altitude conifer.

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