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ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM SPIKES OF PINUS ROUXBURGHII GROWING IN HIMALAYAN REGION

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ABSTRACT

Endophytes constitute a remarkably multifarious group of microorganisms ubiquitous in plants and maintain an imperceptible association with their hosts for at least a part of their life cycle. Their enormous biological diversity coupled with their capability to biosynthesize bioactive secondary metabolites has provided the impetus for a number of investigations on endophytes. Intention of present report is to provide studies on endophytic fungi, particularly on diversity of fungal endophytes from *Pinus rouxburghii* of Pauri, Garhwal forest. Different fungal taxawas isolated from spikes of *Pinus rouxburghii*. These are majorly *Penicillium frequenta*, *Alternaria alternate*, *Geotrichium albida*, *Thielaviopsis basicola* and two unidentified groups identified by using morphotypic methods of characterization. The colonization rate of fungal endophyte produced from different species is 0.121%. Endophytic fungi showed as *Penicillium frequentaus showed maximum* colonoization frequency (41.1%) followed by *Thielaviopsis basicola* (29.40%), *Geotrichium albida* (11.76%), *Alternaria alternate* (5.88%),) and two unknown fungi show colonization frequency as unidentified-1&2(5.88%).

KEY WORDS: Endophytic fungi, morphotypic characterization, diversity, *Pinus rouxburghii*.

INTRODUCTION

The need for new and safe bioactive compounds to provide aid and comfort in all aspects of mankind life is ever increasing. Emergence of new diseases, development of drug resistant

pathogenic microorganisms, appearance of life threatening viruses, management of post-operative complication in patient with organ transplantation, are some of the challenges in front of scientists. This situation has forced scientists to explore different natural sources for the safe and potent agents to meet the challenges of the twenty first century.

Fungi have been widely investigated as a source of bioactive compounds. The first milestone in this field was the discovery of penicillin from *Penicillium notatum* by Alexander Fleming in 1928. Soon thereafter, there was an explosion of new isolation of antimicrobial compounds from fungi. For example, griseofulvin, an effective antifungal agent for the systemic treatment of fungal infections of skin, hair, and nails, was first isolated from the *Penicillium griseofulvum*[1]. Similarly, cyclosporine, an effective immunosuppressive drug used routinely in clinical was isolated from fungi *Tolypocladium niveum* (initially classified as *Tricoderm polysporum*) and *Cylindrocarpon lucidum* [2]. Furthermore, taxol, a diterpene, is one of the most promising anticancer agents obtained from *Taxomyces andereanae* [3] and mevastatin which is a hypocholesterolemic agent was isolated from a number of fungi, including *T. Koningii* and *T. Longibra* [4]. These filamentous fungi are continuously being used for the search of potential pharmaceutical products. As a result, the list of potent drugs from fungal sources is increasing. The additions of potent clinical useful drugs of fungal origin have created interest in the discovery of new secondary metabolites from fungi.

Microorganisms like endophytic fungi generally reside asymptomatically in the tissues of higher plants. The host endophyte relationship is supposed to be complex and different from plant to microbe. Endophytic organisms have received considerable attention after they were found to protect their host against insect, pest, pathogens and even domestic herbivorous [5]. Almost all the plant species (400,000) harbour one or more endophytic organisms [6]. To date, only a few plants are investigated for their endophyte biodiversity and their potential to produce bioactive secondary metabolites. Studies have been conducted at different parts of the world about the endophytic biodiversity, taxonomy, reproduction, host ecology and their effect on host [7]. Currently, endophytes are viewed as outstanding sources of bioactive natural products, because many of them are occupying literally millions of unique biological niches growing in so many unusual environments. Endophytic fungi that live inside the tissues of living plants are under explored group of microorganisms.

There are enormous scopes exist for the recovery of novel fungal species, genera and biotypes from this ecological niche. To some estimate approximately 1.5 million fungal

species exist in the world [8], while only 100,000 have been estimated that there may be at least one million species of endophytic fungi alone[9]. *Pinus rouxburghii* is a tree having different medicinal value and is used as an ornamental plant. This tree is a native of the Himalayan region and may be found in Bhutan, Nepal, Kashmir ,Sikkim ,Tibet and other parts of north India. This plant belongs to family Pinaceae commonly known as Chir Pine [10]. The wood of *Pinus rouxburghii* is aromatic deodorants, haemostatic stimulant and anthelmintic. It's also has the properties of being digestive, acting as a liver tonic, diaphoretic and diuretic. The plant has ethno botanical importance in terms of its use in eye, ear, and pharynx diseases. It is also used across cultures for the treatment of foul ulcers, haemorrhages, warm infections, liver diseases, bronchitis, inflammations, etc. Skin disease, pruritus, and giddiness are also said to be cured [11]. Hence, endohytes from this plant attract the attention of researches all over the world.

This study was conducted to characterize and study the biodiversity of endophytic fungi of *Pinus rouxburghii* collected from specific location in the Pauri District region of Uttrakhand, which falls in Gangetic plains and a major part in the Himalayan North encompasses an area of and situated between (29^0 45' to $30^0$15'W Latitude and 78^0 24' to 79^0 23' E Longitude) and explore these endophytic fungi for their bioactive potential.

MATERIAL AND METHODS

Plant Collection and Isolation of the Endophytic Fungi

Healthy (showing no visual disease symptom) and mature plants of *Pinus rouxburgii* were carefully chosen for sampling. Spikes of *Pinus rouxburgii* from different sites of Pauri, Gharwal region of Uttrakhand and randomly collected for study. The material was brought to the laboratory in sterile bags and processed within a few hours of sampling. Fresh plant materials were used for isolation work to reduce chance of contamination.

Processing and Disinfecting Samples for Isolation of Endophytic Fungi

The plant material was rinsed gently in running water to remove to dust and derbies. After proper washing spike cut into small pieces, and spike were selected for further processing under aseptic condition. Highly sterile condition maintained for isolation of endophytic fungi. All the work is performed in laminar air hood. Sterile glass ware and mechanical things such as scissor, forceps, scalpel, and blades were used in all experiment. The spikes of cut into 0.5-1cm in length. The isolation of endophytic fungi according to the method described by[12]. It is further treated with 1% sodium hypochlorite for 1 to 2 minutes depending upon the type of

surface and then washed with autoclaved water. It is then further sterilised by treating it with 70% ethanol for 1 minute and washing with autoclaved water for 2 to 3 times. The sterilized explants are then inoculated in PDA plates. The control plates of PDA medium are also run by placing the sample spikes on the medium without any surface sterilization [13]. In each the hyphal tips growing out of the inoculated sample are transferred on the plates containing potato dextrose agar (PDA). PDA is used as a maintenance medium. The Petri plates were supplemented with antibiotics streptomycin 100µg/ml to suppress bacterial growth. PDA plates were incubated at 28°C for 2-3 weeks. Most of the fungal growth was initiated within two weeks of inoculation. The incubation period for each fungus was recorded. It was almost similar for the same species. The day of first visual growth was observed from the plating date was considered as an incubation period for growth. Isolation from the master plates was done by the transfer of hyphal tips to fresh potato dextrose agar (PDA) plates without addition of antibiotics to obtain pure culture of endophytic fungi.

Diversity Analysis of Different Fungal Species

To analyze the diversity of fungal entophytes, the isolates were grouped according to their macro and micro morphological characteristics. They were identified by their macroscopic vegetative characteristics, which were color, texture, topography, diffuse pigmentation, color, and topography of the back of the colony, and well as by their microscopic reproductive structures, using the microculture technique and comparing the obtained results with taxonomic keys [14]. The colonization Rate (CR) of the fungi isolated were achieved by the ratio between the total number of isolates and the number of fragments within the sample, and the relative frequency (RF) was based on the ratio of the total number of isolates of a group and the total number of isolates [15].

Identification of Endophytic Fungi

The identification procedure of endophytic fungi was based on morphology. All seventeen isolated species were described according to their macroscopic features such as colour, shape and growth of cultured colonies on culture media, as well as microscopic characteristics like structure of hyphae, conidia and conidiophores by staining techniques. Obtained data then compared with the descriptions of endophytic fungi species present in the literature. When the morphological investigation fails to reveal the identity of the isolated fungus, the species is marked as ‘unknown’. Identification of endophytc fungi were further confirmed by Pathology division, FRI, Dehradun.

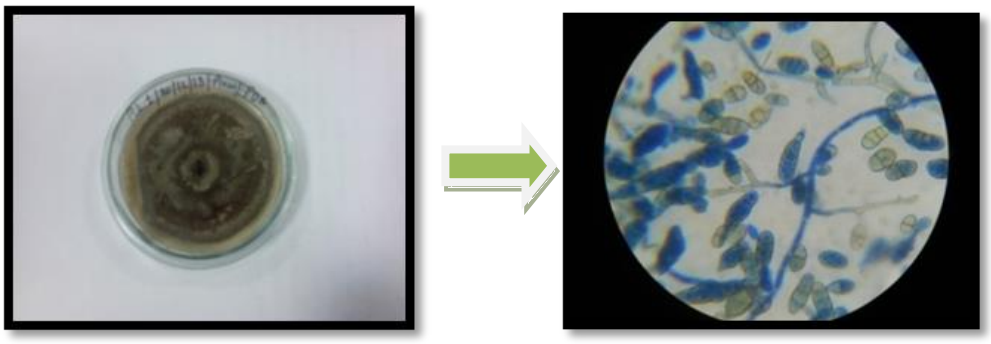
RESULT AND DISCUSSION

Sample Collection and Isolation of Endophytic Fungi

A systematic study about the endophytic fungi associated with spikes of forest plant, *Pinus rouxburghii* were located Pauri Gharwal region of Uttrakhand to carry out to evaluate their biodiversity. A total of 17 endophytic fungi were isolated from spike of *Pinus rouxburghii* by using Potato Dextrose Agar medium. Thesse endophytic fungi were characterzed morphotipically (Table-1). Out of 17, a total 6 distinct morphotpes were characterized and identified. Further confirm identification have been done by by Forest Reaserch Institute (FRI), Deharadun and endophytic fungi characterized as *Alternaria alternata***,** *Thielaviopsis basicola, Geotrichum albida, Penicillium frequentaus* and two species remain unknown(Fig1).

Morphytic Charecterization of Endophytic Fungi

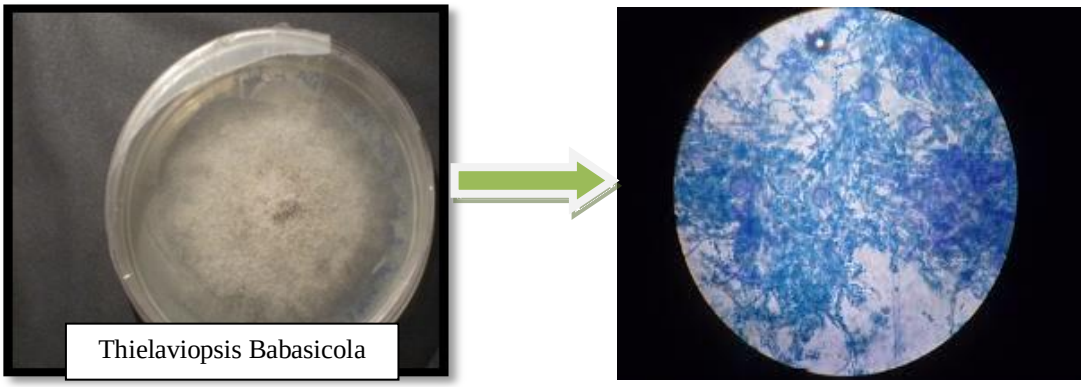
a) ***Alternaria alternate*****:** *Alternaria alternate* produced olivaceous black colour colonies on potato dextrose agar (PDA). Reverse side of the colonies were dark brown. Hyphae were colourless. Conidiophores arose singly or in small groups, often branched, straight and flexuous. The conidiophores were smooth in appearances, 50µ long, and 3-6 µ thick in size and processed one or more conidial scars. The conidia were formed in long chain and mostly obclavate, obphyrium, and ovoid in shape. Several transverse and longitudinal or oblique septa were present on conodia. Beaks of condia were short cylindrical, and smooth. The overall length of conidia was 20-63 (37) µ, and thickness was 9-18(13) µ in the broadest part and 2-5 µ in the beak pale.



1 a) Alternaria alternate **colony performance on the media PDA and by staning technique.**

b) ***Thielaviopsis basicola*****:** Black root rot is caused by the fungus *Thielaviopsis basicola* and can affect a wide range of greenhouse crops. Affected roots become black and rotted, hence it's common name, black root rot. Above ground symptoms include stunting, chlorosis or

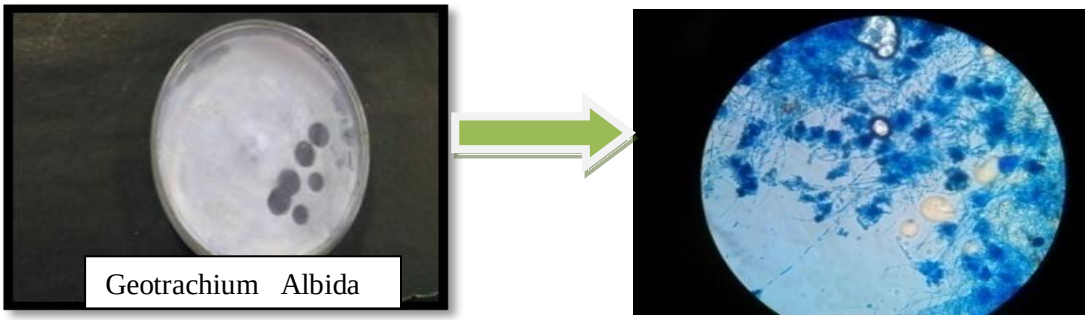
yellowing and plant dieback. Roots become black and rotted, but are not as water soaked as may occur with Pythium root rot infections. *Thielaviopsis* can also produce toxins that adversely affect plant growth, so root systems may not be that stunted for infected plants to look unhealthy.



***1 b) Thielaviopsis basicola* colony performance on the media PDA and by staining techniques.**

c) *Geotrichum albida*

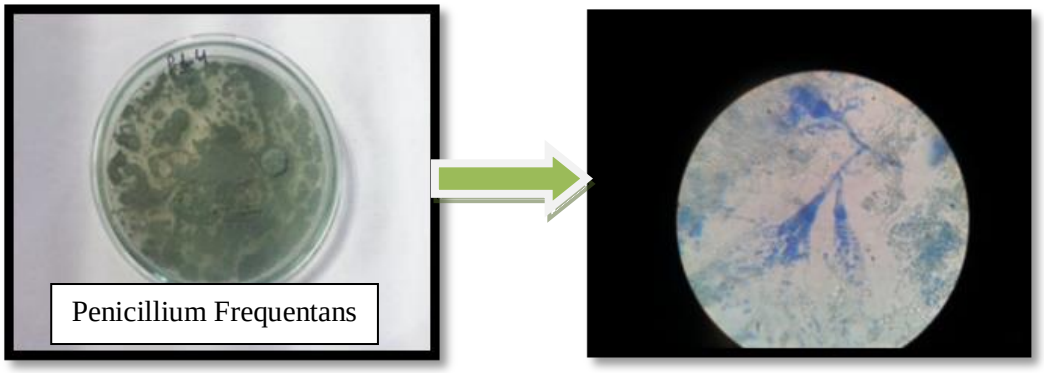
Species of the genus *Geotrichum* produce chains of hyaline, smooth, one-celled, subglobose to cylindrical, slimy arthroconidia (ameroconidia) by the holoarthric fragmentation of undifferentiated hyphae. The arthroconidia, which are quite variable in size, may germinate at one end giving the appearance of a bud. However, the latter develops into a septate mycelium. True blastoconidia production is not found in the genus. This characteristic distinguishes the genus *Geotrichum* from *Trichosporon*, which usually does produce blastoconidia. On Potato dextrose agar, colonies are fast growing, flat, white to cream, dry and finely suede-like with no reverse pigment. Hyphae are hyaline, septate, branched and break up into chains of hyaline, smooth, one-celled, subglobose to cylindrical arthroconidia. They are 6-12 x 3-6 um in size and are released by the separation of a double septum.



***1 c) Geotrichum albida* colony performance on the media PDA and staining techniques**

d) *Penicillium frequentans*

Species of penicillium are recognised by their dense brush like spore bearing structure called penicillin (sing: penicillus). Conidiophores are simple or branched and are terminated by clusters of flask shaped phialides. The spores (conidia) are produced in dry chains from the tips of the phialides, with the youngest spore at the base of the chain and are nearly always green .Branching is the important features for identifying penicillium species. Some such as *Penicillium glabrum* at left, branched and simply bear a cluster of phialides at the top of the stipe. The images have a cluster of branches, each bearing a cluster of phialides. *Penicillium atramentosum* , represent a type haing branches that bear a second oder of branches third bearing in turn a cluster of phialides. These three types of spore bearing system (penicilli) are called monoverticillate, biverticillate and terverticillate respectively. Penicillium is a large and difficult genus encountered almost everywhere and usually the most abundant genus of fungi in soils.



***1 d) Penicillium frequentans* colony performance on the media PDA and by staining techniques**.

Table1: Endophytic fungi were isolated from the needles of *the P.rouxburghii* the details of the same are given below.

S. No	Source of Endophytic Fungi	Colony charecterstics on PDA media	Colony Charecterstics By cotton blue lactophenol staining techniques	Probable endophytic fungus	Class
1	Spike	Appears dark and olive spoting	Individual conidiophores consisting of 4–8 large catenate conidia chains, Pale brown to light brown Obclavate to obpyriform orellipsoid, short conical beak at the tip, or beakless, Several vertical and transverse septa	*Alternaria alternata*	*Dothideomycetes*

2	Spike	Appears green in colour	Conidiophores are simple or branched and are terminated by clusters of flask shaped phialides. Conidia produced in dry chains from the tips of the phialides *Phialides with branched metulae*	*Penicillium frequentans*	*Deuteromycetes*
3	Spike	Appears blakish in colour	Unstained endoconidia could be seen within the hyphal tubes, Hyphal tubes from which the endoconidia had been liberated appeared as empty cylinders	*Thielaviopsis basicola*	*Sordariomycetes*
4	Spike	White dry powder to cottony colonies	Hyphae are hyaline, septate, branched and break up into chains of hyaline, smooth, one-celled, subglobose to cylindrical arthroconidia	*Geotrichium albida*	*Leotiomycetes*
5	Spike	Appears in orange colour	Chain of conidia	Unidentified-1	-
6	Spike	Appears in olive gray	*Chain of conidia with phialides*	*Unidentified 2*	-

Endophytic Fungi Diversity

Colonization rate of all endophytic fungi were observed as 0.121. All isolated fungi were assembled in six morpho groups, four of known and two of unknown genera. Their relative frequencies were maximum in *Penicilium frequentaus* (41.1%) followed by *Thielalaviopsis basicola* (29.4.0%), *Geotrochium albida* (11.76%),*Alternaria alternata* (5.88%), unidentified-1 (5.88%), and unidentified-2 (5.88%) (Table-2).

Table-2: Morphological Group and Relative Frequency of Endophytic Fungi Isolated From Spikeso of *Pinus Rouxburghii.*

Sources of isolates	**Spikes**	**Spikes**	**Spikes**	**Total no of isolates**	**Relative frequency**
Morphotypes	**Specimen-1**	**Specimen2**	**Specimen -3**		
Alternaria alternata	1	--	-	1	5.88
Thielaviopsis basicola	2	2	1	5	29.4
Geotrichium albida	1	1	-	2	11.7
Penicilliumfrequentas	3	2	2	7	41.1
Unidentified -1	-	1	-	1	5.88
Unidentified -2	-	1	-	1	5.88

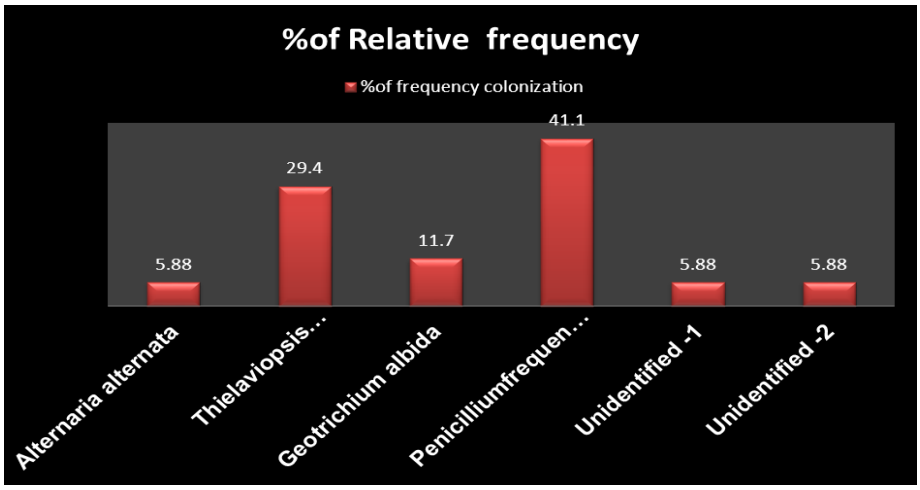


Fig 2: Relative Frequency of Endophytic fungi isolated from *spikes of Pinus rouxburghii*

DISCUSSION

Fungi are known to colonize, multiply and survive in diverse habitats besides parasitizing plants as obligate parasites and biotrophs. There is substantial evidence of fungal diversity associated with a particular host plant. For example, around 40–60 fungal species are associated as endophytes with grasses [16]. While the study of tropical trees for sustenance of fungal diversity has not been carried out seriously, a single leaf, simultaneously supporting 6– 10 fungal species as biotrophs and endophytes, has been recorded in *Eucalyptus* **[17].** Several fungal endophytes have been isolated from a variety of plant species which have proved as a rich source of secondary metabolites **[18].** In this present work a total of 17 fungal endophytes were isolated from *spikes of Pinus rouxburghii*. Similarly, endophytic fungi were successfully isolated from *Pinus roxburghi* and identified as penicilium species[19]. Endophyte *also have been reported within apparently healthy pine cones*[20]. Diversity and saline resistance of endophytic fungi were observed in spikes of *Pinus thunbergii*[21].

In the present study 6 different species of endophytic fungi such as *Penicilium frequentaus* (41.1%) followed by *Thielalaviopsis basicola* (29.4.0%), *Geotrochium albida* (11.76%), *Alternaria alternata* (5.88%), unidentified-1 (5.88%), and unidentified-2 (5.88%) were isolated from *Pinus rouxburghii*. In Korea, total 59 isolates and 19 species of endophytic fungi were isolated from the leaves of *Juniperus rigida, Larix kaempferi and Pinus densiflora* and identified using morphological and molecular characteristics [22]. Similarly, endophytic fungi were also isolate from *Calotropis Procera* and *Withania somnifera*[23].

Morphological investigations, using both macroscopic and microscopic features, have resulted in the identification of four fungal species: *Penicilium frequentaus, Thielalaviopsis*

basicola, *Geotrochium albida*, *Alternaria alternata*. Two fungal species, although subjected to the same morphological investigations, remains unidentified. Similarly, endophytic fungi *were also* isolated from Indian medicinal plant Calotropis procer and identified as *Alternaria alternata* based on morphological characters [24].

CONCLUSION

Fungal endophytes from forest plants are under attention as these plants tend to produce natural products beneficial for us. A number of forests have been screened in different regions of world for endophytes. Still knowledge on comprehensive diversity of endophytic fungi from forest plant is scanty. The present investigations have been carried on spikes of *Pinus rouxburghii* plants which provides evidence that isolated endophytes are capable to survive inside plants. Results of present study indicate that selected plants and their parts are highly colonized by microbial endophytes and endophytes are not host specific. This is preliminary study for isolation and identification of endophytic fungi from spikes of *Pinus rouxburghii* from Pauri, garhwal region in Himalaya. In my knowledge, this is first preliminary report on isolation and characterization of novel *Geotrichum albida* in spikes of *Pinus rouxburghii*.

REFERENCES

1. Oxford A E, Raistrick H, and Simonart P. The biochemistry of microorganism LX. *Griseopfulvin*, C17H17O6CL, a metabolic product of *Penicillium grisofulvum* Dierckx. *Biochem. j*. 1939; 33:240-248.
2. Weber G, Sehorgendorfer K, Schneider-Scherzer E, and Leitner E. The peptide catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by againt 45.8kilobase open reading frame current genetics. 1994; 26:120-125.
3. Sterile A, Strobel G A, Sterile D. Taxol and Taxane Production by *Taxomyces andereanae* an endophytic fungus of Pacific yew. Science. 1993; 260 (5105):214-6.
4. Ghisalberti EL. Antiinfective agent produced by hypomycetes genra *Trichoderma and Glicoladium*. Current medicinal chemistry anti infective agent. 2002; 1:343-347.
5. Webber J. A natural control of dutch elm disease. Nature, London. 1981; (292): 449-451.
6. Tan RX, Zou W X. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep*, 2001; 18: 448–459.
7. Bandra WMM, Seneviratne G, Kulasooroya SA. Infection among Endophytic Bacteria And Fungi: Effects And Potential. *J. Of Biosci.* 2001; 3(1): 645-650.

8. Hawksworth, D.L. The Magnitude Of Fungal Diversity: The 1.5 Million Species Estimated Revisted. *MycologyReaserch*. 2001; 105:1422-1432.
9. Dreyfuss MM, Chepla IH. Potential of fungi in the dicovery of novel, low molecular weight pharmaceticals. In v.p.gullo (ed), the discovery of natural products with therapeutic potenial. *Butter Worth –Heineemann,London*, 1994; 49-80.
10. Sharad B, Bohra A. Antibacterial Potential Of three naked-seeded (Gymnosperm) plants. *Nat. prod.of radiances.* 2008; 7:420-425.
11. Earle, C.J. *Pinus rouxburghii* Sargent. 1897.
12. Petrini O, Fokkema NJ, Van den Hueval J. Microbiology of the Phyllosphere. Cambridge University Press.plant defence compounds. *Mol. Microbiol*. 1986; 47:257–266.
13. Regina MG, Edson RF, Waldireny CR. Endophytic fungi from Melia azedarach. World Journal of Microbiology & Biotechnology.2003; 19:767-770.
14. Barnett HL, Hunter BB. Illustrated genera of imperfect fungi. Burgess Publishing Company, Minneapolis. 1972.
15. Azevedo JL. Microrganismos endofíticos. In: Melo, I.S. *Ecologia Microbiana*. EMBRAPA- CNPMA, *Jaguariuma, Brazil*. 1999; 117-137.
16. Schulzt B. Endophytic fungi: asources of novel metabolites. In british Mycological Society, International Symposium Proceedings on Bioactive fungal metabolites –Impact and Exploitation at University of wales. 2002.
17. Dingle, J. and McGee, P. A. Some endophytic fungi reduce the density of pustules of *Puccinia recondita* f. sp. *tritici* in wheat. *Mycol. Res.* 2003; 107: 310–316.
18. Bhardwaj A, Agrawal P K. Review fungal endophytes: as a store house of bioactive compound world. Journal of pharmacy and pharmaceutical sciences. 2014; 3(9): 228-237.
19. Mittal RK, Sharma MR.Studies on the mycoflora and its control on the seeds of some forest trees. IV. *Pinus roxburghii*. Indian Journal of Mycological Plant Pathology.1982; 12: 198-205.
20. Smith H, Wingfield MJ, Crous PW, Coutinho TA. Sphaeropsis sapinea and Botryosphaeria dothidea endophytic in Pinus spp. and Eucalyptus spp. in South Africa. S. Afr. J. Bot. 1996; 62: 86-88.
21. Min YJ, Park MS, Fong JJ, Quan Y, Jung S, Lim YW. Diversity and saline resistance of endophytic fungi associated with *Pinus thunbergii* in coastal shelterbelts of Korea. Microbiol Biotechnol. 2014; 28. 24(3):324-33.

22. Chang-Kyun Kim, Ju-Kyeong Eo, Ahn-Heum Eom. Diversity and Seasonal Variation of Endophytic Fungi Isolated from Three Conifers in Mt. Taehwa, Korea. Mycobiology. 2013; 41(2): 82–85.
23. Khan R, Saleem S, M. Iqbal C, Shekeel AK, Aqeel Ahma. Biodiversity of the endophytic fungi isolate from *Calotropis Procera* (AIT) R.BR. Pak J Bot. 2007; 39(6): 2233-2239.
24. Aharwal R P, Kumar S, Sandhu S S. Isolation and antibacterial property of endophytic fungi isolated from indian medicinal plant *Calotropis procera* (linn.) R.BR. World journal of pharmacy and pharmaceutical sciences. 2014; 3(5):678-691.