CHAPTER- I

1. INTRODUCTION

The need for new and useful compound to provide assistance and relief in all aspects of human condition is ever growing. There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact. So, in recent years a lot of attention has been deducted to novel molecules derived from natural sources that exhibit a range of clinical and pharmacological activities. Endophytes, an example of novel microorganisms that reside in the tissues of living plants, are relatively unstudied, unexplored component of biodiversity. They are the potential sources of novel natural products for exploitation in medicine, agriculture, and industry. Of the nearly 300,000 plant species that exist on earth, each individual plant is host to one or more endophytes, only a few these plants have ever been completely studied relative to their endophytic biology (Strobel and Daisy, 2003).

Modern usage of the term endophyte in mycology refers to those fungi which live almost within the lives and stems of apparently healthy host plants, doing so asymptomatically causing no visible signs of infection. The term endophyte was originally defined by De Barry (1887) to distinguish those species, which reside within the host tissues or cells from epiphytes, those fungi living on the outer surfaces of host plants (Isaac, 1996; Petrini, 1986). Endophytic fungi have been isolated from leaves, stems and roots of woody plants in the temperate regions and tropics (Rodrigues *et al*., 2000).

Currently, endophytic fungi are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of biological niches (higher plants) growing in many unusual environment. It seems quite obvious that they are rich and reliable source of genetic diversity and may represent previously

undescribed species and to add more, novel microbes often have associated with them novel natural products.

The history of endophytes is not too long. It has just been two decades but in these past decades a great deal of information on the role of endophytic microorganisms in nature has been collected. These endophytes constitute a rich bio-resource for exploration to discover new natural products. Endophytes holds a promising potential in the field of modern medicine, industry and agriculture. The capability of colonizing internal host tissues has made endophyte valuable for agriculture as a tool to improve crop performance. New species are continually being described from cultural and molecular studies and endophyte biology is a burgeoning field in Mycology. These studies indicate that the breadth of endophyte diversity and ecology is just beginning to be discovered (Arnold *et al.,* 2000).

An individual plant may be host to a range of endophytic fungal symbionts simultaneously (Isaac, 1996). Endophytic fungi live in almost all higher plants including conifers and have symbiotic relationship with the host plant (Shrestha *et al.*, 2001). Fungal endophytes of conifers are common although many belong to little known species; probably these fungi are inconspicuous and are rarely collected. Some species live almost entirely within a host plant cell. The hyphae are often swollen with constrictions at the septa and may fill the whole lumen (Isaac, 1996).

Natural products are naturally derived metabolites and/or byproducts from microorganisms, plants or animals. These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine (Strobel and Daisy, 2003). What is so interesting about endophytes is that they can increase the plant's resistance to water shortage, accelerate the plant's growth and protect them from pathogens, and the bioactive substances called the secondary metabolites synthesized by the endophytes is responsible for these amazing outcomes. Endophytes produce plethora of bioactive secondary metabolites to protect themselves as well as the host plant from their enemies. They also protect the host plant from damp

and adverse environmental conditions. Therefore, they have many applications in the production of drugs to cure the deadly diseases like cancer (Shrestha *et al.*, 2001) and as biocontrol agents of plant pathogens (Isaac, 1996).

The nature and biological role of endophytic fungi with their plant host is variable. Endophytic fungi are known to have mutualistic relation to their hosts, often protecting plants against herbivory, insect attack or tissue invading pathogens (Sigel *et al.*, 1985) and in some instances the endophyte may survive as a latent pathogen, causing or quiescent infections for a long period and symptoms only when physiological or ecological conditions favors virulence (Bettucci and Saravay, 1993; Carroll, 1986).

Plant pathology originated from the convergence of microbiology, botany and agronomy; its ultimate goal is the control of plant disease. Microbiologists have been attracted to this field of research because of the need for identification of agent's infectious diseases in economically important crops and its ultimate treatment. Plants, like humans and other animals, also get sick, exhibit disease symptoms and die. Nearly 70% of the cause of the disease happens to be fungi, the best examples being potato wart, cabbage club root, potato blight, downy mildews, rusts and smuts, red rot of sugar cane (Singh, 1998).

Finally, a call from the public for the better and more environmentally sound ways to grow the world's food indicates that new methods of controlling pests and pathogens are needed. In the past, the major source of pesticidal agent was organic synthesis. Recently, there has been an increased interest in using more environmentally friendly methods in agricultural production (Overton,1996) It now appears that a relatively untapped source of microbial diversity for use in agriculture as well as in medicine is the microbial endophytes, which live in the interstitial spaces of living plant tissue (Bacon and White, 2000).

Human and plant infections caused by pathogenic microorganisms are continuing and posing a serious problem. Thus, discovery and characterization of novel and effective products for its treatment is extremely important. As these novel natural products and

organisms that make them offer opportunities for innovation in drug and agrochemical discovery, this fulfills the alternative ways to control farm pests and pathogens. Endophytes offer as a promising candidate as an alternative to control animal and plant diseases (Strobel and Daisy, 2003).

In addition to the economical aspects, the study of endophytic microorganisms has strong academic interests, concerning the discovery of new microbial species. The presence of specific endophytes, the lack of signs of disease and the long lived association indicate that further study of the association may yield interesting information of the precise benefits to plants associated with these colonists. Hence, this dissertation work is mainly focused to study the endophytic fungal diversity associated with some Himalayan conifers of our country, Nepal collected from different places viz: ‘Kavre’ and ‘Kirtipur’; and the antifungal activity of the isolated and identified endophytic fungi against different fungal plant pathogens.

CHAPTER –II

2. OBJECTIVES

2.1 General objective

To study the biodiversity and bioactivity of endophytic fungi of some Himalayan conifers of Nepal.

2.2 Specific Objectives

- To isolate and identify the endophytic fungi from the conifers used in the study.
- To perform the screening of the identified isolates against fungal plant pathogens based on the antifungal activities.
- To perform the fermentation of the priority isolates and extract the secondary metabolites from the culture medium.
- To evaluate the antifungal activity of the extracts against the fungal plant pathogens.
- To analyze the fungal extracts for the presence of taxol by TLC.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Endophytes

Endophytes are the microbial entities that live within living tissues of plants without causing any immediate overt negative effects (Bacon and White, 2000). They are found virtually in every plant on earth. An endophyte is a bacterial (including actinomyctes) or fungal microorganisms which spends the whole or a part of its lifecycle colonizing inter and/or intracellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease (Tan and Zou, 2001). Endophytes are important component of microbial biodiversity; accordingly they are presumably ubiquitous in the plant kingdom with the population being dependent on host species and location.

3.1.1 History and Discovery

The discovery of endophyte dates back to mid 1900s, when plants in the pastures, where the animal gains were lowest, contained a fungal endophyte and found that it produced (or caused the plant to produce) many toxic compounds (Morris, 2001). Microscopical evidence for the presence of endophytic fungi in aerial plant tissues was demonstrated as early as 1887 by De Barry and more recently by Bernstein and Carrol (1997). Subsequently, the presence of endophytes in more than 200 plants species belonging to different families and from different climatic regions of the world was reviewed by Petrini (1986).

In the 1970s endophyte were considered as neutral, not causing benefits nor showing detrimental effects to plants, but later on it was found that they had an important role in host protection against predators and pathogens. Although they were described in the past century, endophytic microorganisms only received considerable attention in the last two decades, when their capacity to protect their host against insects, pests, pathogens and even domestic herbivores was recognized. In the early 1980s, the specialized literature published in the first reports showing that the endophytic

microorganisms play an important role inside plants. It was demonstrated that the presence of these microorganisms in their respective host could result in the reduction of insect attacks. Landmark report on the subject started in 1981, less than two decades ago, from 1981-1985, which may be considered as a historical period to the field (Claydon *et al.,* 1985; Webber, 1981).

After the discovery of endophytes, it has been defined in different ways by different investigators. The term endophyte was originally defined by De Barry to distinguish these species, which invade and reside within host tissues or cells from epiphytes, those that live in the outer surfaces of the host plants (Issac, 1996). Most of the frequently isolated endophytes are fungi (Strobel and Daisy, 2003), this may be the reason that most of the investigators have defined endophytes as a fungus that inhabit the healthy plant tissues without causing disease (Arnold *et al.,* 2000).

The discovery of endophytes led researchers to look more closely at the benefits that endophytes might provide the plant. Since then there had been a number of researches on the endophytes of grasses, conifers, angiosperms, etc but still, the endophytes are still considered as one of the untapped resources and there are many facets left unexplored and unturned regarding the properties of endophytes and their possible application in agriculture, industry and medicine.

3.1.2 Distribution and Biodiversity

Endophytes are microorganisms that colonize and cause asymptomatic symbiotic associations in healthy plant tissues (Wilson, 1997). They have been found in every plant species examined to date and appear to be important, but largely unquantified components of microbial biodiversity (Arnold *et al.,* 2000). Both fungi and bacteria (including actinomycetes) are the common microbes existing as endophytes. It seems that other microbial forms, like mycoplasmas and archaeobacteria certainly do exist in plant as endophytic association, but no evidence for them has yet been presented. Saikkonem *et al.,* (1998) reviewed multiple studies showing that individual plants in the temperate zone may harbour dozens of endophytic species.

Endophytes occupy unique ecological niches and can influence the distribution, ecology, physiology and biochemistry of plants (Sridhar and Raviraja, 1999). Endophytes are important component of microbial biodiversity; they are presumably ubiquitous in plant kingdom with the population being dependent on host species and location (Tan and zou, 2001). The plant- associated habitat is a dynamic environment in which many factors affect the structure and species composition of the microbial communities that colonize roots, stems, branches and leaves. It has previously been shown that endophytic communities vary spatially in the plant or may be dependent on interaction with other endophytic or pathogenic microorganisms (Fisher *et al.*, 1991)

Fungal endophytes are considered at least as ubiquitous as mycorrhizal association among temperate zone plants (Carrol, 1988). The most frequently isolated endophytes are the fungi (Strobel and Daisy, 2003). There might be at least one million species of endophytic fungi alone (Dreyfuss and Chapela, 1994). Endophytic fungi are found in all division of fungi, so have presumably evolved the association independently on many occasions. The most common endophytes are the anamorphic member of the Ascomycotina and they are closely related to the fungi known to cause disease, either in healthy tissues or as secondary invaders of damaged tissues. This suggests that the endophyte may have evolved from pathogen or vice-versa. The mechanism of host recognition and development of colonization may also be common (Redlin and Carris, 1985).

According to Carrol, 1986 and Petrini, 1986 most work has centered on fungal endophytes of grasses and conifers, suggesting that the majority of living plants are host to endophytic fungi. The majority of the identified endophytic fungal species belong to Ascomycetes and Deuteromycetes with a few Basidiomycetes and a very small number of Oomycetes (Issac, 1996).

The environmental conditions under which the host also effects the endophytic population and endophytic profile may be more diversified in tropical areas (Tan and Zou, 2001). Tropical rainforests are remarkable in the context of its environment. The

competition is great, resources are limited and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structure and biologically active compounds (Redell and Gorden, 2000). The host plant also influences the general metabolism of endophytic microbes (Bills *et al.,* 2002). The tropical endophytes provide more active secondary metabolites than the temperate endophytes, not only this, significantly high number of tropical endophytes provide more active secondary metabolites than the fungi from other tropical substrata (Bills *et al.,* 2002). Commonly several to hundreds of endophyte species can be isolated from a single host plant, at least one species showing the host specificity (Tan and Zou, 2001). An individual plant may be host to a range of endophytic fungal symbionts simultaneously (Issac, 1996).

Dispersal of the endophytic fungi remains puzzling, apart from *Neotyphoidium* and related species, endophytes are transmitted horizontally i.e. each plant is colonized by fungal propagules that arrive from the environment. Aerial dispersal either in the wind or on vector is probably the most common mechanism for fungal dispersal. Endophytic fungi colonize various parts of plants. Many of the fungi sporulate in the culture indicating the potential to release spores in the air. Indeed, sporulation is seen after the senescence of plant tissues. However, few cases of dispersal have been documented in the wild and the various mechanisms remain unexplored (Redlin and Carris, 1985).

A number of endophytic fungi have been isolated from leaves, stems and roots of woody plants in temperate regions and the tropics (Mahesh *et al.,* 2005). Besides this a number of endophytes have been isolated from crown, stem and leaves of grasses (Morris, 2001) and from the seeds of the maize as well (Mc Inroy *et al.,* 1991).Fungal endophytes reside in healthy tissues of all terrestrial plant taxa studied to date are diverse and abundant. This group of fungi represents an interesting, diverse microbiota associated with healthy tissues of terrestrial plants and also representing a large reservoir of genetic diversity (Tejesvi *et al*., 2005). The endophytes are a rich and reliable source of genetic diversity and are novel microbes associated with novel natural products. It turns out that the vast majority of plants have been studied for their

endophytes heading for enormous opportunities that exist for the recovery of novel fungal forms, taxa and biotypes. Different genotypes and morphtypes are even isolated from the same plant (Strobel and Daisy, 2003).

3.1.2.1 Distribution and biodiversity of endophytes of conifers

Geographical position in the middle of the Himalayas provided Nepal, a complex assemblage of physiographic and climatic diversities that created a surprisingly rich biodiversity against a small area which covers 0.03% of world's land. Among the higher plants in Himalayas, gymnosperms dominate in the forest flora of Nepal. Nepalese gymnosperms consist of 10 families (Hara *et al.,* 1982) and are distributed in the Himalayan regions where extensive areas are covered with conifer trees. In Nepal, twenty species of conifers were reported from tropical to alpine zones of the country (Shrestha, 1985). Some of the important conifers of our country are *Abies spectabilis, Cedrus deodara, Podocarpus nerifolius, Taxus wallichiana, Tsuga dumosa* and *Juniperus indica*.

The geographical distribution of existing species of conifers is of great interest, some genera are wide spread, many have a markedly restricted distribution and a few are endemic. All are temperate or subtropical plants, those found in topical latitudes being confined to elevations where climatic conditions are temperate to subtropical (Dallimore and Jackson, 1996). Conifers are the most primitive of seed plants whose seeds are not enclosed in the seed case. They are among the economically most important plant species, especially because of their timber and other forest products. However, compared to other group of plants, very little basic research has been done on them. It is only recently that foresters have begun to treat conifer as a "crop plant" instead of simply obtaining their supplies from wild population (Bhatnagar and Moitra, 1996). In Nepal, a number of Himalayan conifers dominate the forest flora in the temperate region. Some of the conifers are of great interest and importance due to their use in Ayurvedic medicines as well as their phytochemical and biological significance.

This research work is mainly focused on revealing and studying the biodiversity of the endophytic fungi of some selected indigenous Himalayan conifers from different locations, and their bioactivity as well. The description regarding the conifers used in this study has been portrayed in Appendix-III. The main objective of selecting the indigenous Himalayan conifers is that these conifers inhabit in the high altitude in the adverse environmental conditions. In order to combat the adverse environmental effect, they produce an array of spectacular secondary metabolites, and it is a well known fact that endophytes produce the same or the similar compounds, which are present in the host plants having similar biological activities. For e.g. subglutinols, an immunosuppressive compound produced by the endophyte of a well known Chinese medicinal plant possessing immunosuppressive properties (Lee *et al.,* 1995). Several reasonable hypothesis govern plant selection strategy, like, plants from unique environmental settings, especially those with unusual biology, and possessing novel strategies for survival, plants having ethnobotanical history, plants that are endemic that have an unusual longevity, plants growing in areas of great biodiversity have the prospect of housing endophytes with great biodiversity, and also plants generating bioactive natural products such as *Taxus* spp. that may have associated endophytes that produce the same natural products are seriously considered for study (Strobel and Daisy, 2003).

Wilson (1997) isolated endophytic fungi from four tree species: red pine (*Pinus resinosa Ait.*), white pine (*Pinus strobes L.*), white spruce (*Picea glauca* (Monench Voss)) and balsam fir (*Abies balsamea (L.Mill*)) from three widely separated locations in New Brunswick. From 2400 needles collected from 240 trees, 2697 fungal isolates were obtained. Twenty one taxa were identified on the basis of morphology and an additional nine groups were identified among which *Leptosporoma* spp. was the largest group accounting for 67% of the total number of isolates obtained from all tree species.

Infections of plants by endophytic fungi are common and their presence is not revealed by external symptoms. Camacho *et al.,* (1997) found the spruce needle DNA being contaminated by endophytic fungal DNA. Isolation of endophytic fungi from *Picea*

foliage collected from the same location as the original sample was done, and was examined to identify the source of contaminating DNA. The ITS region of the isolates were screened by southern blotting using an oligonucleotide probe homologous to a unique portion of the reported "Spruce" sequences. This report identified a DNA sequence originally attributed to *Picea engelmannii* (Engelmann spruce) as *Hormonema dematioides*, a ubiquitous foliar endophyte of conifers.

Caruso *et al.*, (2000) investigated the fungal and actinomycetes population of internal tissues of woody branches, shoots and leaves of different plant belonging to genus *Taxus.* Twenty two plants of *Taxus baccata* and one of *Taxus brevifoila* were sampled in different habitats located in central-northern Italy. 150 fungal and 71 actinomycetes strains were isolated. Endophytes were more frequent in woody tissues than in herbaceous ones. The composition of endophytic fungal population of *Taxus* spp. is varied: 105 out of 150 strains belonging to 25 different genera, one was not determined and 44 didn't produce any reproductive structure in solid cultures. The most frequently isolated genera were *Alternaria, Fusarium* and *Mucor*. In particular *Alternaria* was isolated from all the analysed plant material and can be considered as resident genus of *Taxus* spp. *Aspergillus, Drechslera, Pestalotia, Phoma, Phomopsis, Penicillium, Geotrichum, Trichoderma* and *Rhizopus* were also among the the genera that were identified. In this research, 15 fungal strains produced taxane. The amount of taxane produced was rather low: the analysis detected the amount of taxane from 10-100 ng/L in the liquid media.

Muller and Hallaksela (2000) described the fungal diversity of the above ground parts of a 61 years old Norway spruce tree lacking visible signs of damage or disease. The problem involved with the identification of the fungi to named species was circumvented by classifying them into operational chemotaxic units (OCTUS) by using their combined fatty acids and sterol profiles (FAST-profiles). 99 OCTUS were identified from 666 fungal isolates obtained. Bacteria were found occasionally from the inner bark samples (3 isolates) and from needles (15 isolates). Their result suggested

that an undamaged, apparently healthy Norway spruce, harbors in its above ground parts nearly 200 fungal species.

Shrestha (2002) isolated a number of endophytes from the inner bark of *Taxus wallichiana* (Himalayan Yew), in this study, altogether 36 endophytes were isolated from from *Taxus wallichiana* collected from different places of Nepal between the altitudes of 2200-3572m. Most of the endophytic fungi were Deuteromycetes and some were Ascomycetes; 4 isolates were identifies as the *Pestalotiopsis microspora*. Rests were *Aspergillus* spp., *Sporormia minima*, *Phomopsis* spp.

Most endophytes live almost entirely within the host plant tissues, often without causing any visible signs of infection. Fungal hyphae penetrate between plant cells or may also grow intracellularly and must obtain nutrient materials through this intimate contact with host but the occurrence of specialized feeding structure has not been reported in these fungi. Hyphae are sometimes wide in diameter (10-15m) in association with plant cells and may be distorted, irregular or bulbous in form. In some instances, considerable amounts of fungal biomass are supported in host plant tissues (Issac, 1996). Beside this, endophytes colonizing inside plant tissues also get protection from host plant (Tan and Zou, 2001).

Shrestha (2002) isolated more than 60 endophytes from inner bark of a Himalayan conifer, Hemlock spruce collected from the Khumbu region of east Nepal including the most representative genera *Trichoderma, Fusarium, Phoma, Phomopsis, Sclerotium, Alternaria, Nigrospora, Nodulosporium* and different yeasts, whereas few were identified as endomycorrhizae. All the endophytic fungi were forwarded for screening for antifungal/ antibacterial activities by dual culture method on PDA in which all the endophytic fungi showed antifungal/ antibacterial activities against different plant pathogenic fungi such *Pythium, Geotrichum, Phytopthora, Rhizoctonia* and the bacterium, *Bacillus subtilis* (ATCC-6635).

Upadhyayay (2005) carried out a study based on the biodiversity and bioactivity of endophytic fungi of *Tsuga dumosa* D. Don, a Himalayan conifer collected from

Tangboche at an altitude of 4000m. Altogether thirty isolates of endophytic fungi were isolated from the inner bark of the several twig samples, out of which only thirteen isolates were identified up to the genus level belonging to the genera: *Pestalotiopsis, Phacidiella, Peacilomyces, Aspergillus* and *Endobotryella*.

The endophytic fungi of woody plants may be diverse as often claimed, and likewise, they may be functionally novel as demonstrated in a few studies. However, the endophyte taxa that are most frequently reported tend to belong to fungal groups composed of morphologically similar endophytes and parasites.

According to the report of Ganley *et al.,* (2004), seven distinct parasites of *Pinus monticola* do not occur as endophytes. The majority of endophytes of *P. monticola* (90% of 2,019 cultures) belonged to one fungal family, the Rhytismataceae. However, not a single rhytismataceous endophyte was found to be most closely related by sequence homology to the three known rhytismataceous parasites of *P. monticola*. Similarly, neither endophytic *Mycosphaerella* nor *Rhizosphaera* isolates were most closely related to known parasites of *P. monticola*. Morphologically, the endophytes of *P. monticola* can be confounded with the parasites of the same host. However, they are actually most closely related to, but distinct from, parasites of other species of *Pinus*. If endophytes are generally unknown species, then estimates of 1 million endophytes (i.e., approximately 1 in 14 of all species of life) seem reasonable.

Most endophytes of *Pinus* tend to belong to the aforementioned Rhytismataceae Within this family, those fungi that are proven parasites tend to be specialized, at least to a single subgenus of *Pinus*. For example, the only rhytismataceous parasites of *P. monticola* are *Bifusella linearis, Lophodermella arcuata*, and *Meloderma desmazieresii* (Funk, 1985; Hansen and Lewis, 1997). The first is known to parasitize *Pinus strobus, P. monticola, Pinus flexilis*, and *Pinus albicaulis*, all white pines in subgenus Strobus. The host range of *Lophodermella arcuata* is also restricted to subgenus Strobus. Only *Meloderma desmazieresii* parasitizes both subgenus Strobus and subgenus Pinus. However, there are rhytismataceous fungi that parasitize subgenus Pinus only (e.g.,

Cyclaneusma minus, Elytroderma deformans, Davisomycella medusa, Lophodermium baculiferum, Lophodermium seditiosum, and others) (Hoff, 1988; Hansen and Lewis, 1997).

Specialized parasites are not alone in infecting plants. Endophytic fungi also infect plants, although as nonpathogenic colonists. At least in some plants, endophytic fungi perform novel ecological functions e.g., thermotolerance of plants growing in geothermal soils. Endophytes can influence community biodiversity or even directly enhance plant growth (Ernst *et al.*, 2003). In woody plants, endophytes also may function in specific defense roles (Wilkinson *et al.*, 2000) or more generally function to limit pathogen damage. Together with mycorrhizal fungi, endophytes form an intregal part of the extended phenotype or symbiotic community of a plant. The full range of ecological functions of endophytes of woody plants is poorly understood, but it is likely to be correlated with their species diversity (Purvis and Hector, 2000), and many of these so-called endophytes are actually cryptic or latent, but known, parasites. Thus, these fungi remain inherently ambiguous (Arnold *et al.*, 2003).

Wang and Guo (2005) investigated the endophytic fungi associated with Chinese oil pine (*Pinus tabulaeformis*) from two distinct climatic sites, i.e. Fenghuangshan and Linguyan in northeast China, there were higher overall colonization and isolation rates of endophytic fungi from Fenghuangshan than Linguyan . In the Fenghuangshan site, the colonization and isolation rates significantly increased with ageing of xylem, but not with that of bark. In the Lingyuan site, the colonization and isolation rates significantly increased with ageing of xylem, however, there were no significant differences between two and three year old needles and bark, except that they both had less endophytes in one year old as compared with two and three year old needles and bark. *Alternaria alternate*, *Phoma* spp. and *Phomopsis archeri*, *Leptostroma* spp. were the dominant taxa in bark and needles respectively. In this research it was concluded that some fungi show certain degree of tissue recurrence or specificity and composition of endophytic assemblages is not influenced by the geographical or climatic factors.

Huang *et al.,* (2001) screened the endophytic fungi having antitumor or antifungal activity, which were isolated from the inner barks of three different pharmaceutical plants viz: *Taxus mairei, Cephalotaxus fortunei* and *Torreya grandis* collected from Fujan Province China. The endophytic fungi isolated showed both antifungal and antitumor activitiy, displayed growth inhibition on at least one pathogenic fungi, such as *Neurospora* spp., *Trichoderma* spp. and *Fusarium* spp. Among all the endophytic fungi isolated *Paecilomyces* spp. had the highest positive rate of antitumor and antifungal activity.

The distinctions between endophytic and pathogenic relationship are often not clear. Additionally, the growth requirements of endophytic species indicate the utilization of a limited range of materials as substrates, a characteristic that is often associated with pathogenic species. Two strategies of endophytic mutualisms have been described (Carroll, 1986, 1988). In grasses fungal species have been identified which do not leave host plants at reproduction and do not produce any external fruiting bodies, spread of the fungus is achieved by vegetative growth of hyphae into the ovules of the host so that dispersed seed is already infected by these fungi. This has been termed as constitutive mutualism (Carroll, 1986). Such species do not appear to harm the host plant but often produce toxins, which may have deterrent effects on grazing herbivores and may therefore provide protection to the plant, and the presence of these endophytes within ovule tissues results the host plant sterilization resulting more vigorous vegetative growth enhancing competitive ability in ecological situations. An alternative strategy, adopted by some endophytic species, is an inducible mutualism (Carroll, 1986, 1988) in which the distribution of endophyte in plant tissues is patchy and normally inhabit senescent host tissues and only penetrate metabolically active regions when the plant is stressed.

In physiological terms, relatively little is known about the endophytic interactions between host and fungus and it is not easy to see how a host plant may benefit from such a relationship. Endophytic associations do not lead to the development of disease symptoms but do result in some morphological and physiological changes in host

tissues, which increase in the survival and vigor of the plants concerned. Such physiological enhancements would be likely to increase the capacity of the plant to resist disease. The benefit to the host plant by such endophytic mutualism include growth promotion of the host plant which is at least in part due to the endophytes production of phytohormones such as indole-3-acetic acid (IAA), cytokines and other plant growth promoting substances and/ or partly owing to the fact that endophytes could have enhanced the host uptake of nutritional elements such as nitrogen and phosphorus (Tan and Zou, 2001), toxic effects on the insects making plant tissues unacceptable and unpalatable to insects, toxicosis in domestic herbivores owing to the presence of endophytes in grasses, increase stress tolerance (Belesky *et al.,* 1987) and drought tolerance (West *et al.*,1988) and most importantly plant resistance to microbial pathogens (Carroll, 1988). It has also been suggested that endophytic species may be antagonistic towards other fungal species.

3.1.2.2 Bioactivity of Endophytes

Endophytes (fungal and bacterial) have attracted great interest over past few years because their presence benefits the host plant (development and defense) and they are a good source of novel secondary metabolites of potential interest and thus play an important role in regulation of plant community and their herbivores. There are an increasing number of reports on the isolation, identification of endophytes and their production of secondary metabolites (Gimenez *et al.,* 2007).

The secondary metabolites are also referred to as “Natural Products”. It is believed that more than 100,000 different structures of secondary metabolites may be synthesized by organisms, to a tune of 109 tons per year. Out of these more than 80% are found in plants (Harborne, 1993). Secondary metabolites are believed to detoxify substances accumulated in the primary metabolism and to provide chemical signals to coordinate metabolism of multicellular organisms. They are believed to coordinate activities of different individuals of the same species. Although secondary metabolism was first recognized in 1873, its function was elucidated only in 1888 (Stahl, 1888), but up to the 1950s, secondary metabolites were regarded as end products of deluxe metabolism and

relegated to the rank of ‘waste products’. It was only during the 1960s, that their eco-relations were discovered (Kurz and Constabel, 1988).

Fungi represent one of the most understudied and diverse group of organisms. They produce some thousands of secondary metabolites (Turner and Aldridge, 1983), including plant growth hormones, mycotoxins etc. Antibiotic is also the secondary metabolite, which is capable of inhibiting the growth of another microorganisms or even destroying them (Waksman, 1945). Plant associated microbes or endophytes produce a large number of known classes of phytotoxins, trichothecins (Jarvis *et al.*, 1988), diterpenes (Strobel *et al.*, 1996a) and antibiotics (Strobel *et al.*, 1996b).

Commonly, endophytes make association with higher life forms and may proceed to biochemically mimic the host organisms. It has well documented that endophytes have the ability to produce the same type of secondary metabolites, which the host plant produces, via co-evolution or genetic transfer from their hosts. An excellent e.g. is the anticancer drug taxol, which had been previously supposed to occur only in the plant genus Taxus (Strobel *et al.*, 1996).

The host adopted pathogens can affect a number of taxonomically related plants. Sometimes they are family adapted genus adapted or species specific which may be due to similar types of defense chemicals within that taxon. Preformed phycotoxins act as general resistance mechanism. The host adopted microorganisms are able to detoxify these phycotoxins by producing a specific enzyme as whereas other pathogens cannot detoxify it and cannot invade the plant tissues. Host adaptation is explained by gene-for-gene relationship with the host. A single phycotoxin- detoxifying gene can change the host range of a fungus (Van Ettan *et al.*, 1995).

Plants are commonly hosts to multitude of microbes including parasites, symbionts, endophytes, epiphytes and mycorrhizal fungi. These organisms may influence the production of secondary metabolites such as phytoalexins, whose presence can be triggered by elicitors from microbes. Such microbes may also be capable of production of secondary metabolites similar to those produced by plants (Puri *et al.*, 2006). Like

other microorganisms invading plant tissues, endophytes produce extracellular hydrolases as a resistance mechanism to overcome attack by the host against pathogenic invasion, and/or to get nutrition from the host. Such enzymes including pectinases, esterases, cellulases and lipases, proteinase, α-1, 4-glucan lyase and phospatases have been documented with different endophytes. Enzymatic activities closely related to the host-specificity of the endophytes were demonstrated. The action of these enzymes gives rise to the possibility that the 'genetic recombination' of the endophyte with the host may occur in evolutionary time. This could be the reason why some endophytes can produce some phytochemicals originally characteristics of the host (Tan and Zou, 2001).

Wang *et al.,* (2000) isolated an endophytic fungus (strain TF5) which was identified as *Tubercularia* spp. according to the morphology of fungal culture, the mechanism of spore production and characteristics of spores. This particular fungal isolate was shown to produce taxol when grown in liquid potato dextrose medium, analyzed by TLC, HPLC, UV and mass spectrometry. The fungal taxol showed strong cytotoxic activity as well.

Puri *et al.,* (2006) isolated a novel endophytic fungal strain having accession number MTCC5124, from the inner bark of tree *Nothapodytes foetida*. This novel fungal strain produced camptothecin and camptothecinoides. It is also a well known fact that various microorganisms exhibit biological activities so as to be useful to control different plant diseases. *Muscodor albus* is a newly described endophytic fungus isolated from small limbs of *Cinnamomum zeylanicum* (cinnamon tree), that produces a mixture of volatile antibiotics with activity on specific plant pathogens, bacteria, nematodes and insects (Worapong *et al.,* 2001).

Inorder to meet the needs for new antifungal drug, Anke *et al.,* (2003) performed screening of 510 endophytic fungi isolated from plants collected from southern Europe and South America. A total of 64 strains isolated, exhibited antifungal activities. Antifungal activity was assayed with Candida albicans, *Candida glabrata*, *Candida*

krusei, *Cryptococcus neoformans*, *Aspergillus flavus*, Aspergillus fumigatus, *Rhizopus orizae*, *Trichophyton rubrum* and *Microsporum canis*. Six compounds responsible for the antifungal activity were isolated and characterized. Structural elucidation yielded spaheropsidin A, aurindifungin, 5-(1,3-butadien-1-yl)-3-(pronen-1-yl)-2-(5H)-furanone, cerulenin, ascosteroside and a new derivative of ascosteroside. The new compound exhibited significant in vitro antifungal activities against a wide spectrum of clinically relevant fungi.

Taxol (Palitaxel) was first isolated in 1971 by Monroe Wall and Mansukh Wani as a cytotoxic agent from the inner bark of the Pacific yew, *Taxus brevifolia* (Wani *et al.*, 1971), which showed antitumour activity against advanced ovarian cancer, breast cancer, head and neck, bronchial carcinoma in human (Shrestha, 2002). The presence of taxol in yew species has prompted in the study of their endophytes. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of efforts, a novel palitaxel producing endophytic fungus, *Taxomyces andreanae* was discovered from *Taxus brevifolia* after which a search for a taxol producing microorganism among the endophytic fungi continued (Stierle *et al.,* 1993). Another relative of yew that does not synthesize taxol such as *Taxodium disticum* and *Torryea grandfolia* were also found to be the sources of taxol producing fungi, *Pestalotiopsis microspora* and *Periconia* respectively (Li *et al.,* 1996, 1998). In addition, two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora,* an endophyte isolated from combretaceous plant, *Terminalia morobensis*, growing in Sepik river drainage of Papua New Guinea that displayed antimicrobial as well as antioxidant activity (Harper *et al.,* 2001; Strobel *et al.,* 2002).

The concept that was proposed as a mechanism to explain why T. andreanae may be producing palitaxel is that some endophytes produce certain phytochemicals, originally characteristic of the host, might be related to the genetic recombination of the endophyte with the host that occurred in evolutionary time (Stierle *et al.,* 1993; Tan and Zou, 2001). Thus, if endophytes can produce the same rare and important bioactive

compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants but also help to preserve the world's ever-diminishing biodiversity. Further more it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price. Quite commonly, endophytes do produce secondary metabolites when placed in suitable culture medium. However, the temperature, the composition of the medium, and degree of aeration will affect the amount and kinds of compounds that are produced by an endophytic fungus. Sometimes endophytic fungi produce antibiotics. Natural products from fungi have been observed to inhibit or kill a wide variety of harmful microorganisms including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals (Strobel and Daisy, 2003).

Increasing interest has recently focused on the biology and chemistry of endophytic microorganisms. Since these organisms live in very intimate association with plant tissue and may also be present in the cell level, they can produce compounds similar or even identical to those produced by the host plant. An excellent example is palitaxel, an anticancer diterpene formerly obtained from Pacific yew tree.Other palitaxel-like diterpenes have also been produced by endophytic fungi (Bashyal *et al.,* 1999; Lee *et al.,* 1996; Strobel *et al.,* 1996). The production of gibberelic acid derivatives by fungus and trichothecene macrocyclic lactones by the female plant *Baccharis megapotaneau* are further examples of results of this intimate association between plant and fungi. These ecological-biochemical phenomena have been tentatively explained by a possible plasmid exchange between the associated organisms. The expression of these genetic exchanges would result in the codification of specific enzymes responsible for the transformation of substrates and the production of interesting secondary metabolites. In general these compounds show insecticidal and antimicrobial activities (Huang *et al.,* 1998).

Tan and Zou (2001) recently reviewed the diversity of metabolites that have been isolated from endophytic fungi emphasizing their potential ecological role. These secondary metabolites of endophytes are synthesized via various metabolic pathways, e.g. polyketide, isoprenoid, or aminoacid derivation, and belong to diverse structural groups, i.e. steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoides, depsipeptides and cytochalasines and benzopyranone. Endophytes may contribute to their host plant by producing this plethora of substances to provide protection and ultimately survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine, agriculture, and industry.

Cryptocandin A, an antifungal lipopeptide, was isolated and characterized from the endophytic fungus *Cryptosporiopsis quercina*. This compound contained a number of unusual hydroxylated amino acids and a novel amino acid, 3-hydroxy-4hydroxymethylproline. Cryptocandin A is active against some important human fungal pathogens including *Candida albicans* and *Trichophyton* spp. and also against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Cryptocin, a tetrameric acid antifungal compound was also obtained from *C. quercina*, this unusual compound possesses potent activity against *Pyricularia orizae*, the causal organism of one of the worst plant diseases in the world as well as a number of plant pathogenic fungi (Strobel *et al.,* 1999).

Another fascinating product from an endophytic fungus is the antidiabetic agent. A nonpeptidal fungal metabolite was isolated from an endophytic fungus (*Pseudomassaria* spp.) collected from an African rainforest near Kingshasha in the Democratic republic of Congo. This compound acts as insulin mimetic and unlike insulin is not destroyed in the digestive tract and may be given orally, oral administration of which in the mouse models of diabetes resulted in significant lowering of blood glucose levels (Zhang *et al.,* 1999). Besides this, antiviral compounds like, two novel human cytomegalovirus protease inhibitors, cytotonic acids A and B,

have isolated from the solid state fermentation of the endophytic fungus Cytonaema sp. (Guo *et al.,* 2000).

A total of 582 pure isolates of endophytic fungi were obtained from 81 Thai medicinal plants, collected from forests in four geographical regions of Thailand. Out of 582 pure isolates, 360 morphologically distinct fungi were selected for cultivation on malt czapek broth and yeast extract sucrose broth, from which extracts were tested for biological activity. The result showed that medicinal plants could provide a wide variety of endophytes that might be a potential source of bioactive compounds, as the extracts efficiently displayed antimicrobial, anticancer and antimalarial activities (Wiyakrutta *et al.*, 2004).

The study conducted by Huang *et al.,* (2006) showed that the endophytic fungi isolated from *Nerium oleander*, a medicinal plant, can be a potential antioxidant source. A total of 42 endophytic fungal strains were isolated from the host plant, and total antioxidant capacity, xanthine oxidase inhibitory activity, antimicrobial activity and total phenolic content were evaluated for 16 representative fungal cultures. Wide range of antimicrobial activity was exhibited which were not very strong, but much better than those of the host plant. *Chaetomium* spp. showed the strongest antioxidant capacity, contained the highest level of phenolics, and to some extent inhibited xanthine oxidase activity. The major bioactive constituents of the fungal cultures detected were phenolics (e.g. phenolic acids and their derivatives, flavonoids) and the volatile and aliphatic compounds.

According to Regina *et al.,* (2003), over 30 different fungi were isolated from the leaves, stems and roots of *Melia azedarachta* after surface sterilization and cultivated on sterilized rice. This particular plant is a good producer of limonoides. One of the fungus identified as *Penicillium* spp. was found to produce limonoides, similar to the host plant, which are important insecticidal agents.

In addition, several endophytes are reported to possess anti-insect properties. For example an endophytic fungus, *Muscodor vitigenus* isolated from liana (*Paullinia paulliniodes*) showed a promising preliminary results as an insect deterrent, possess naphthalene and has exhibited a potent repellency against the adult stage of wheat stem sawfly (*Cephus cinctus*) (Daisy *et al.*, 2002).

Rodrigues *et al.*, (2000) performed a search for the bioactive compounds produced by fungal endophytes from *Spondias mombin* (Anacardiaceae). The endophytes obtained were *Guignardia* spp., *Phomopsis* spp. and *Phomopsis guepinii*. The crude extract of these endophytes were tested against 14 organisms including actinomycetes, gram positive and gram negative bacteria, yeast and filamentous fungi. All fungal extracts inhibited actinomycetes growth; *Guignardia* spp. was also active against *E.coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Geotrichum* spp. and *Penicillium* spp.

Despite the undergoing researches in the field of endophytes, all aspects of biology and interrelatedness of endophytes with their respective host is a vastly under investigated exciting field. Currently, none is quite certain of the role of endophytes in nature and what appears to be their relationship to various host plant species. While some endophytic fungi appears to be ubiquitous (for e.g. *Fusarium* spp., *Pestalotiopsis* spp., *Xylaria* spp.), one cannot definitely state that endophytes are truly host specific or even systemic within plants. Anymore than one can assume that their associations in plants are chance encounters. Frequently, many endophytes (biotypes) of the same species are isolated from the same plant and only one of the endophytes will produce a highly biologically active compound in culture (Li *et al.*, 2000). A great deal of uncertainty also exists between what an endophyte produces in culture and what it may produce in nature. It does seem apparent that the production of certain bioactive compounds by the endophyte in situ may facilitate the domination of the biological niche within the plant or even provide protection to the plant from harmful pathogens. This may be especially true if the bioactive product may be of great importance in the biocontrol of plant pathogens. For example, colletric acid, a bioactive metabolite of *Collectrotrichum*

gloeosporiodes, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria as well as against a fungus *Helminthosporium sativum* (Zou *et al.*, 2000).

3.2 Biological control of plant pathogens

Biological control is defined as "any condition under which or practice where by survival or activity of pathogen is reduced through the agency of any other living organisms except man himself with the result that there is reduction in the incidence of diseases caused by the pathogen" (Dubey, 1998). Biological control is principally achieved through antagonism, which means a relation between organisms in which one organism, the antagonist, creates adverse circumstances for other. Biological control systems are preferred to the use of chemicals and in the recent years, a great deal of research activity has been directed towards the development of efficient and reliable systems. In environmental terms, the effects and particularly longer consequences of biological control are less damaging than the routine use of pesticides or fungicides. The practical use of any antagonistic fungi or any other microorganisms, in disease control in the form of biological control provides a great challenge for plant pathologists and phytomicrobiologists (Mukerji, 1989).

Plant disease is caused by abiotic and biotic factors, plants like humans and other animals also get sick exhibit disease symptoms and die. Plant diseases are caused by environmental stress, genetic or physiological disorders and infectious agents including viroids, viruses, bacteria and fungi. Infections by fungal pathogens cause reduction in the growth rate and development of plants, either rapidly or in the long term, which may be so severe as to be fatal. In general, reductions in biomass occur and reduction in yields which have economic importance (Issac, 1996). A pathogenic fungus, when attacks its host, more or less rapidly kills the tissues. The killing is known as necrosis and the diseased part of the plant where such necrosis has taken place is known as a lesion (Mundkur, 1991). Plant disease due to fungus may arise from very specific damage to structural components or to uptake systems, perhaps are the result of toxic

activity by the pathogen, or the fungus may act as a sink for nutrient materials, essentially starving the plant of resources.

Interactions between biocontrol fungi and fungal plant pathogens continue to be a focus among the researches. There is an extra dimension in the quality of the interactions between the fungi and biocontrol fungi, as the fungi has greater potential than bacteria to grow and spread through soil and in the rhizosphere through possession of hyphal growth (Whipps, 2001). Since, most of the biotic cause of the plant disease is fungi, the interactions among fungi are exclusively considered. The interactions between fungi can be manipulated to reduce the damage caused by one fungus on the plant. Essentially these interactions include: mycoparasitism, antibiotic interactions and competition for resources.

Mycoparasitism is an act where one fungus parasitizes another (Upadhayay and Rai, 1989). In the case of mycoparasitism one fungus derives its nutrition from another without any benefit in return. The interaction can be where the parasite is biotrophic or necrotrophic. Biotrophs tend to have highly specialised relations with specific hosts. The parasite is extremely difficult to manipulate, and as a consequence few have ever been used in biocontrol. Necrotrophs tend to kill the cell or tissue from which they feed. They tend to have a broad host range, and their activity commonly includes excretion of antibiotics. For instance, the necrotroph *Trichoderma* has the capacity to parasitise a great number of fungi, including pathogens *Rhizoctonia*, Sclerotinia, *Fusarium* and *Verticillium*, and the *Straminopiles Pythium* and *Phytophthora*. *Trichoderma* grows towards hyphae of other fungi where it binds following branching. *Trichoderma* releases hydrolytic enzymes that digest the walls of the host prior to penetration. *Trichoderma* is also thought to release a range of toxins that reduce any response from the host to invasion (Lewis *et al.,* 1990; Whipps *et al.,* 1988). The mycoparasitism is of common occurrence and example can be found among all groups of fungi from chytrids to the higher basidiomycetes.

The most important and highly studied mechanism of fungal biological control is antibiosis. Antibiosis can be defined as the inhibition of one organism by metabolites of another. Metabolites that kill another organism tend to be called toxins. Thus, dilute solutions of toxins may inhibit and high concentrations of antibiotics may kill. Most fungi produce inhibitory metabolites. For instance, *Gliocladium* produces a diketopiperazine that kills *Pythium* probably because of coagulation of proteins in the cytoplasm. Use of the fungus on seed appears to reduce seedling damping-off as much as a common seed treatment. Volatile pyrones produced by *Trichoderma* appear to reduce damping off caused by *Rhizoctonia*. The potential to exploit these metabolites in biocontrol appears feasible. The role of antibiotics in biocontrol is still unclear. Some spectacular experiments, when repeated in the field, have resulted in enormously variable outcomes indicating that other mechanisms may be important. Antibiosis not only involves the inhibition of the growth of microorganisms by toxic metabolites from the antagonist (e.g. antibiotics) but also possibly lysis that is dissolutions of the cellular structure (Mukerji, 1989).

The competitive abilities in some fungi render them highly antagonistic and ideal as potential combative organisms. Competition is the active requirement for resources in excess of those immediately available to two or more organisms (Issac, 1996). The resource most commonly considered to be important for fungi is organic energy. Thus much competition exists for access to energy for growth and maintenance. However, competition between fungi is extremely difficult to quantify. The mycelium is constantly changing. Resources within the hyphae are constantly being shifted between different parts, with the consequence that over very short periods of time the nature of what is being assessed changes. However, competition for nutrients can take place between survival propagules, germinating units and mycelium in any arrangement. The result of competition is stasis of the less successful competitor. Inoculum density does not decline and the potential to initiate disease remains. Thus the application of competition by itself to biocontrol appears limited. Among microorganisms, competition exists for nutrients, including oxygen, temperature or pH. Competition for carbon, nitrogen and iron has been shown to be mechanism associated with biocontrol

or suppression of *Fusarium* wilt in several systems by non-pathogenic *Fusarium* and *Trichoderma* species and competition for thiamine as significant process in the rhizosphere of wheat (Whipps, 2000).

3.2.1 Biocontrol of plant pathogens by use of endophytes

Biological control is defined as the control of pathogens or insects by the use of a secondary organism. It is well known that various microorganisms exhibit biological activity so as they are useful to control plant diseases. Although progress has been made in the field of identifying and developing biological pesticides for controlling various plant diseases of agronomic and horticulture importance, most of the pesticides in use are still synthetic compounds, many of which are classified as carcinogens and are toxic to wildlife and other non-target species. So there is a great need to find safer replacement for this and other synthetic pesticides.

The natural and biological control of pathogens and pests affecting cultivated plants has gained much attention in the past decades as a way of reducing the dependence and use of chemical products in agriculture. The control of pests and pathogens by means of biological process i.e. use of entomopathogenic microorganisms or those that inhibit or antagonize other microorganisms pathogenic to plants is a better alternative that may contribute to reduce or eliminate the use of dependence of chemical products in agriculture.

In the past two decades, a great deal of information regarding the role of endophytic microorganisms in nature has been collected. The capability of colonizing internal host tissues has made endophyte valuable for agriculture as a tool to improve crop performance. The products, such as insecticides and fungicides aim to control pests and phytopathogenic fungi. However, they are responsible for eliminating important species of insects that control other pests and microorganisms that are performing a crucial role in the environment, inhibiting the growth and multiplication of other microorganisms. One group of the microorganisms that is affected by the anthropogenic modifications is the ‘Endophytes’. In the 1970s they were initially considered neutral, not causing

benefits nor showing any detriments to plants, but later it was discovered that they have an important role in host protection against predators and pathogens of plants.

Plant diseases are caused by environmental stress, genetic or physiological disorders and infectious agents including viriods, viruses, bacteria and fungi. Many fungal pathogens cause the production of characteristics symptoms in host plants. A fungus may kill a plant in pre-emergence or seedling stage. Others may affect the roots, stems, leaves, inflorescence, flowers or fruits. Sometimes the same fungus may be responsible both for the seedling phase of the disease and the disease in the adult plant. Specific forms have been applied to the physical manifestations of the host-pathogen interactions, which result in order to accurately describe the symptoms that are visualized. Many of these have arisen as the result of historical usage. Such terminology has been listed (FBPP, 1973), in attempts to clarify, standardized and clearly define the terms, which are used. Some of the important symptoms of fungal plant disease are damping off, leaf spots, cankers, scabs, anthracnose, mildew(powdery mildew and downy mildew), blights, foot rot, root rot, necrosis, rust diseases, leaf curl, witches broom, epinasty, club root disease, galls and warts, wilts, Dutch Elm disease, ergot of cereals and grasses and smuts. The classification and characteristics of fungal plant pathogens used in this study for the screening purpose of antifungal activity of fungal endophytes have been illustrated in Appendix-IV.

Muscodor vitigenus, a recently described endophytic fungus of *Paullinia paullinioides*, produces volatile antimicrobials (especially naphthalene) exclusively, which can be used as a repellant for the adult stage of wheat stem sawfly (Daisy *et al.*, 2002). Another endophytic fungus *Muscodor albus* can also be applied as a microbial control agent of Potato tuber moth. Potato tuber moth (PTM) is a serious pest of stored potatoes in most countries. This novel fungus produces a mixture of antimicrobial volatile organic chemicals, which can be used effectively in the biocontrol of this pest (Lacey and Neven, 2005).

Radopholus similes is recognized as the most important plant-parasitic nematodes infecting bananas worldwide (Gowen *et al.,* 2005). Nematode control basically relies on the use of granular organophosphate and carbamate nematicides (Marin, 2005). Some biocontrol products including bacteria and fungus such as *Paecilomyces lilacinus* are available for the nematode management. In addition, to improve the activity and thereby increase the options for the biological management of *R. similis* in banana, novel biological control agents, such as endophytic fungi from promising locations are isolated and applied in controlling nematodes in banana plants. Clay (1989) first suggested the potential of endophytes from endosphere as biocontrol agents of insect's pests. More recently, fungal endophytes from endorhiza have been identified as biocontrol agents in bananas (Pocasangre *et al.*, 2002). In the study carried out by Felde *et al.,* (2006), fungal endophytes, *Fusarium oxysporum* and *Trichoderma atroviridae* were used for nematode control. The results revealed the total number and the density of nematodes were significantly lower in plants inoculated with endophytes than in the control plants. Also *Fusarium* isolates tended to suppress nematodes better than the *Trichoderma* isolates.

Endophytic fungi were isolated from the healthy stems and pods of *Theobroma cacao* trees. These endophytic fungi can be used to control the serious fungal diseases of cacao known as frosty pod rot and witches broom that threaten the production of the cacao crops. These fungi may compete with the pathogenic fungi to live inside the plant and there by keeping them out of the healthy plants, serve as the biological control agents. In addition these endophytic fungi may produce chemicals that keep away the disease causing organisms (Crozier *et al.,* 2006).

Endophytic fungi isolated from oak tissues were evaluated for its ability to control *Diplodia corticola,* a fungus that is the causal agent of cankers, vascular necrosis and dieback of various oak species. The isolates that were used in the biocontrol of the pathogen were *Trichoderma viride*, *Epicoccum nigrum*, *Fusarium tricintum*, *Alternaria alternata*, *Sclerotinia sclerotiorum* and *Cytospora*. Among the isolates tested *T.viride*

and *F.tricinctum* showed maximum effect in controlling the fungal pathogen (*D.corticola*) (Campanile *et al.,* 2007).

Kim *et al.,* (2007), isolated endophytic fungi from vegetable plants and examined their in vivo anti-oomycete activity against *Phytopthora infestans* in tomato plants. A total of 152 isolates were obtained from healthy tissue samples of cucumber, red pepper, tomato, pumpkin and Chinese cabbage. The fermentation broths of 23 isolates showed potent in vivo anti-oomycete activity against tomato late blight with control value over 90%. The *Fusarium oxysporum* strain (EF 119), that was isolated from roots of red pepper showed the most effective disease control efficacy against tomato late blight. In dual culture tests it inhibited the growth of Pythium ultimum, Phytopthora infestans and *Phytopthora capsici*.

According to Dingle and Mcgee (2003), endophytic fungi can be used to reduce the density of pustules of *Puccinia recondita* that causes leaf rust in wheat plants. The interactions between the endophytes and this *Puccinia* are most probably mediated by defense mechanisms induced in the host plant. Kelemu *et al.,* (2001) isolated 11 species of endophytes from species of *Brachiaria*, which is tropical forage grass. Inoculation method to introduce endophytes in various genotypes of *Brachiaria* is also developed. It is determined that these endophytes play a role in the protection of the *Brachiaria* from some fungal disease e.g. leaf spot caused by *Drechslera* spp. and some insects.

On this abundance of fungi used with the prospect of potential biocontrol against different plant pathogens are ‘Endophytic fungi’, which under normal conditions do not show symptoms of disease and perfectly live as a symbiont (Strobel *et al.,* 1996). When the host is stressed, however, some endophytes cause disease in host plant that indicates there is an equilibrium between chemical factors of the host and the endophytes i.e. equilibrium between phycotoxins and mycotoxins. Many pathogens grow as endophytes yet other endophytes are important as biocontrol agents of plant pathogens.

Shrestha (2002) studied the antifungal activity of endophytic fungi isolated from the inner bark of Hemlock spruce collected from Khumbu region in which all of the

representative genera of the endophytic fungi showed the bioactivity against the test fungal plant pathogens- *Pythium* spp., *Geotrichum* spp., *Phytopthora* spp. and *Rhizoctonia* spp. indicated by the formation of inhibition zone between the colonies indicating antibiosis. Similarly, Bashyal *et al.,* (2002) also studied the antifungal activity of the endophytic fungi isolated from the sisoo trees suffering from dieback disease, against the fungal plant pathogens- *Pythium* spp. and *Geotrichum* spp. in which *Fusarium* spp. and *Phoma* spp. showed the highest bioactivity against *Pythium* spp. and *Trichothecium* spp. showed the highest activity against *Geotrichum* spp.

In the similar manner Upadhyayay (2005) studied the antifungal activity of the endophytic fungi isolated from the inner bark of Hemlock spruce collected from Tangboche from Solukhumbu district. The observations showed that the endophytic fungal isolates possessed the maximum inhibitory activities against *Gloeosporium sporioides* followed by that against *Fusarium* spp. and the lowest inhibitory activities against *Pythium* spp. The genera *Verticillium* and *Pestalotiopsis* showed broad spectrum of antifungal activities against the test fungal plant pathogens used in the study.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Materials

All the materials that are used to accomplish this study are listed in Appendix-I.

4.2 Methods

The methods followed in this study are given below:

4.2.1 Collection of plant material

Several twig samples of the following Himalayan conifers were collected randomly, viz; *Abies spectabilis*; *Taxus wallichiana; Podocarpus nerifolius; Juniperus indica*. The plant samples were collected from different places like 'Kavre' district (*Abies spectabilis, Taxus wallichiana*) Kathmandu district (*Podocarpus nerifolius, Juniperus indica*). The twigs of 1-2 cm. in diameter were cut into 20cm. long and both ends were sealed with parafilm to prevent drying out during transport and kept in sealed plastic bags. The samples were transported to Natural Product Research Laboratory at NAST, 'Khumaltar' and preserved for few days at 4 ºC (Stierle *et al.,* 1993).

4.2.2 Isolation of endophytic fungi

After the collection of the samples, transportation and storage at the laboratory at 4 ºC, the endophytic fungi were isolated within a week. The stem fragments were first of all surface sterilized with 70% (v/v) ethanol for 2 minutes to remove the epiphytic microbes. The outer bark was removed aseptically with sharp sterilized blade carefully. The small pieces of inner bark were placed carefully on water agar plates at the rate of 3-4 pieces per plate. It was then incubated at 28 ºC for 2 weeks. The plates were monitored regularly for the growth of endophytic mycelia. Endophytic mycelia growing on surface sterilized inner bark segments on water agar plates were then transferred to PDA plates by the hyphal tip method and incubated at 28 ºC for at least

10 days. Each plate was eventually examined for the purity of culture (Stierle *et al.*, 1993).

4.2.3 Maintenance of fungal culture

Each pure culture of isolate was noted with a code and stored in sterile distilled water and PDA slants at 4 ºC (Aneja, 1996).

4.2.3.1 Storage in sterile distilled water

For storage, 3ml of distilled water was pipetted out and dispensed in a number of capped autoclavable plastic vials and made sterile by autoclaving at 15lbs per sq.inch pressure at 121 ºC for 15 minutes. The water in the vial, after sterilization, was allowed to cool. The surface of mold colony on PDA was scrapped with sterile inoculating loop and transferred to the sterile distilled water in vial aseptically so as to prepare a suspension of broken mycelium and spores in water. The vial was capped tightly and sealed with parafilm. The vials were labeled with the isolate code and date and stored at 4º C from which cultures could be revived later by pipetting 1-2ml of suspension on PDA and incubating at 28 ºC for 2-3 weeks as per the requirement.

4.2.3.2 Storage in PDA slants

Fungal culture from a typical sporulating colony was selected and transferred aseptically with a sterile inoculating wire onto the PDA slant (hyphal tip method) (Strobel and Daisy, 2003) and incubated at 28 ºC for 2-3 weeks. The slant was observed for good colony growth with typical spore production and checked if it is pure i.e., free from contaminants. Finally, the screw cap was sealed with parafilm; the tubes were labeled with isolate code and date and stored at 4 ºC.

4.2.4 Identification of the fungal isolates

The endophytic fungi were identified on the basis of color, morphology and spores they produced on PDA, if not produced, then it was subjected to water agar (WA) with sterilized carnation leaf. Identification procedure of endophytic fungi is quite

complicated because some of them do not sporulate on PDA and are to be encouraged to sporulate on specific plant material (Strobel and Daisy, 2003) and are eventually identified via standard morphological study following Ainsworth *et al.,* 1973; Barnet, 1965; Benson, 1979; Booth, 1971 and Sutton, 1973.

4.2.4.1 Colony morphology on PDA

Each of the isolate was cultured by hyphal tip method on PDA at 28 ºC for at least 10 days. Colour, colony morphplogy (waxy, leathery, velvety, fluffy, cottony etc), presence of moisture above the colony, presence of concentric rings and any peculiar underside colour were also noted. Further, the cultures were examined for the change in colour and colony morpohlogy daily and any change was noted down.

4.2.4.2 Lactophenol cotton blue mounting of the fungal specimen

A drop of lactophenol cotton blue was placed on a clean slide and a small tuft of the each of the sample fungus from PDA was transferred onto the drop of lactopenol cotton blue, using a flamed cooled needle. The material was gently teased by the help of cooled flamed needle. The stain was gently mixed with the mold structure. A cover glass slip was placed over the preparation taking care that no air bubbles trapped in the stain. The characteristic type and arrangement of the spore was noted down.

4.2.4.3 Cover slip culture

In this process, the sterilized cover slip was carefully inserted at an angle of 45º into the solidified PDA in petridish until about half of the cover slip was buried in the medium. The isolates were inoculated along the line where the medium meets the surface of the cover slip. After incubation at 28 ºC for 7-10 days, the cover slip was carefully removed and placed over a drop of lactophenol cotton blue which was placed on a clean glass slide, and was observed under light microscope (Kawato and Shinobu, 1959). This method thus aided in observing the arrangement of spores in its natural state of growth as the fungus formed a thin film of growth on the surface of coverslip.

4.2.4.4 Culture on water agar (WA) with sterilized carnation leaf

Those endophytic fungal isolates that did not sporulate easily on PDA were forwarded for the sporulation on water agar (WA) with gamma irradiated carnation leaf. In this method, sterilized carnation leaf was placed on the surface of solidified water agar plate with the help of a sterile forcep and pure culture of the isolate was transferred by the hyphal tip method near the leaf and incubated at 28 ºC for two to four weeks (Nelson *et al.,* 1983). Fruiting bodies and spores on the carnation leaf were examined by stereomicroscope and light microscope for identification. For better visualization, spore was carefully placed on a drop of lactophenol cotton blue on a clean and grease free slide aseptically with a sterile wire, placed a cover slip and observed under light microscope.

4.2.5 Screening of identified endophytic fungal isolates for antifungal activities

Screening was done on PDA medium according to the dual culture method used by Carruthers *et al.,* 1994 (Shrestha, 2002). Petridish containing solidified sterile PDA was inoculated 2cm. from its edge with a fungal agar disc (5mm diameter) cut out from actively growing plant pathogenic fungus (7 days old culture) grown on PDA.

Similarly, 5mm fungal agar disc of actively growing fungal endophyte was also inoculated at the center of the plate. The plates were then incubated at 28 ºC for 2-3 weeks and observed for the antagonistic effect of the endophytic fungi on the plant pathogenic fungal colony. The isolates were noted as highly inhibitory (+++), moderately inhibitory (++), low inhibitory (+), and not inhibitory (-) on the basis of extent of inhibition shown on the plant pathogenic fungal colony. The fresh cultures of *Fusarium oxysporum, Fusarium moniliforme, Helminthosporium* spp., *Sclerotinia* spp., *Gloeosporium sporioides, Alternaria* spp. *and Geotrichum* spp. were used for studying antagonistic or inhibitory properties.

4.2.6 Extraction of secondary metabolites of a priority isolate of endophytic fungi

The priority isolate for fermentation was selected on the basis of the result obtained during screening of the identified isolates for antifungal activities and the review of literature. The fermentation of the priority isolate was carried out in S-7 medium, extracted with chloroform, extract checked for its antifungal activity and finally analyzed by TLC.

Four to five serial hyphal tip transfers of the organism were carried out before culturing in liquid media to assure that the bioactive secondary metabolite is not carried from the source tree.

4.2.6.1 Fungal culture in liquid S-7 media

The amount of each of the constituents of S-7 media (listed in Appendix-II) was calculated for 1000 ml the of media. Each of the constituents was weighed and dissolved in 1000 ml of distilled water. The pH of the medium was adjusted at 5.5 by adding drops of 0.1 M HCL (w/v). The media was distributed in two 1L conical flasks (500 ml of media in each flask) and closed with the tight cotton plugs. The media was then autoclaved at 15lbs per sq. inch pressure at 121ºC for 15 minutes. Altogether four sets (8 conical flasks each containing 500 ml of S-7 media) of media was prepared.

4.2.6.2 Fermentation

The selected isolate of endophytic fungi was grown on PDA plates. The actively growing 7-days culture free of any contamination was used for inoculation. The fungal colony was cut into small pieces with sterile sharp blade on PDA and transferred aseptically to sterilized liquid S-7 media. Few colonies of the fungal isolate along with the media (PDA) were inoculated to the one of the two S-7 media, while second media flask was left uninoculated and maintained as control. The mouth of the flasks were cotton plugged and wrapped with aluminium foil properly, labeled and thus fermentation was carried out in 500 ml of S-7 media supplemented with 1gm Bacto

Soytone at 28 ºC in the incubator for 3 weeks (21 days). The control was also maintained under similar condition (Shrestha *et al.,* 2001).

4.2.6.3 Extraction of secondary metabolite

After 3 weeks, the culture media was filtered through four folds of cheesecloth to remove solids. The filtrate was mixed with equal volume of chloroform and extracted two times in a 2l separating funnel. The chloroform fraction was combined, dried over anhydrous sodium sulphate and evaporated in a rotary vacuum evaporator under reduced pressure (Shrestha, 2002). The process was carried out separately for all different flasks of culture media. The control was also processed in the similar manner.

4.2.6.4 Determination of dry weight of chloroform extract

After evaporation of the extract in rotary evaporator, the extract was dissolved in small volume of chloroform and transferred to a small glass vial (whose weight was already taken before) taking care that no extract was left over. The vial was left overnight in order to allow the chloroform to evaporate. After the complete drying of the crude extract, the weight of vial along with the extract was taken and the dry weight of the extract obtained from each 500ml of S-7 media was calculated as:

Dry weight of extract = Total weight of the vial and extract –Weight of empty vial

The dry weight of extract of control media, if obtained any, is found out in the similar manner.

4.2.7 Study of antifungal activity of the endophytic fungal extract extracted with chloroform

To the extract thus obtained, 500µl of DMSO was added and dissolved well. The crude extract thus obtained was subjected for screening for its antifungal activity against previously mentioned seven different plant pathogenic fungi i.e., determination of zone of inhibition of the extract against the tested organism by agar well diffusion method. The test plant pathogenic fungus was inoculated on the PDA plates.Three wells of 6mm

diameter were made towards the edge in solidified PDA plates by removing agar disc from the media using a sterile cork borer. Then, 50µl of the fungal extract was dispensed carefully with the help of a micropipette. Into the second of the three wells, 50µl of extract of control media was dispensed carefully and similarly into the third well 50µl of the solvent (DMSO) was added to the test if the solvent itself exhibited the antifungal activity. The plates were then left for half an hour with the lid closed so that the extract diffused into the media. After diffusion, the inoculated plates were incubated at 28 ºC for 4 days. Growth inhibition was observed after 4 days. The zones of inhibition were measured using a scale and recorded.

4.2.8 TLC analysis of the fungal extract

The chloroform fraction of the fungal extract was developed on a percolated silica plate (20x5cm, 0.25mm thickness) along with authentic taxol in chloroform: methanol (7:1, v/v) as the solvent system.

The TLC tank was well equilibrated with the lower layer of solvent at the bottom and the filter paper was equilibrated for at least 16 hours prior to the development. 5µl of the authentic taxol, fungal extract and extract of control media each of which dissolved in 40µl of methanol was loaded on to TLC plate, making at least 1.5cm from the edge, 2cm from the bottom and 1cm between each spot with the help of a 5µl capillary. Blow drier was used to prevent the unwanted diffusion. Then, the plate was kept in a tank with the solvent system and developed until the solvent front reached $2/3^{rd}$ of the length of the plate. The solvent front was marked as soon as the plate was taken out of the solvent system. The plate was then dried and the spots were first visualized under UV light with the wavelength of 254nm. Then, the plate was sprayed with vanillin/sulphuric acid (1%w/v) reagent followed by heating (Cardellina, 1991). The plate was also observed 24 hours after the reagent was sprayed. R_f values were calculated for each spot obtained and compared with that of authentic taxol.

The movement of the analyte was expressed by its retardation factor, R_f:

R_f = Distance moved by analyte from origin/Distance moved by solvent from origin

CHAPTER-V

5. RESULTS

5.1 Isolation and identification of endophytic fungi from the selected conifers

The endophytic fungi isolated from different selected Himalayan conifers (authentically identified) were identified following the standard methodology.

5.1.1 Isolation of endophytic fungi from the inner bark of selected conifers

A total of 47 endophytic fungal isolates were isolated from the inner bark of the selected conifers. Seventeen endophytic fungal isolates were isolated from *Abies spectabilis*, 12 from *Taxus wallichiana*, 10 from *Juniperus indica* and 8 from *Podocarpus nerifolius*. The fungal isolates were obtained by culturing on water agar (WA) plates, followed by their purification on PDA plates by hyphal tip method 4-5 times in order to get pure cultures. Each of the endophytic fungal isolates were noted with proper code and forwarded for the identification process.

5.1.2 Identification of the fungal isolates

Identification of the endophytic fungi isolated from the selected conifers was done on the basis of color, morphology, fruiting structure and/or spore they produced on PDA or water agar (WA) incorporated with sterilized carnation leaves.

Table 1: Identification of endophytic fungal isolates of *Abies spectabilis*

Isolate code	Colony characteristics on PDA	Fruiting structure and/or spore	Remark
NA-902	Initially white, later turned to grass green, powdery, irregularly shaped with sulfur yellow margin. Ventrally cream colored	Single celled spores (conidia) in chains developing at the end of the sterigma arising from the terminal bulb of the conidiophore, the vesicle; long conidiophore arise from a septate mycelium.	*Aspergillus* spp.
NA-906	Dirty pink colored, powdery and raised dorsally, irregularly shaped.	Well developed conidiophores, basal portion of phialide nearly cylindrical, tapering gradually or abruptly to a long slender tube. Conidia produced successively and held together in chains.	*Paecilomyces* spp.
NA-907b	Cream colored and raised dorsally, small convex colonies.	Elliptical conidia produced arranged in long chains borne on phialides on conidiophore	*Paecilomyces* spp.
NA-907a	White colored colony, bit wrinkled , green colored spores appeared as the culture matured	Conidiophores arising from foot cell, basocatenate conidium arrangement in phialides on vesicle	*Aspergillus* spp.
NA-903	Lemon green, irregularly shaped, gave fluorescence. Ventrally dark colored, produced spikes as colonies matured. Color of the media changed to dark color as well.	Elliptical spores arranged singly	Unidentified
NA-904	Lemon green, irregularly shaped, gave fluorescence. Ventrally dark colored, produced spikes as colonies matured. Color of the media changed to dark as well.	Elliptical spores arranged singly	Unidentified
NA-901	Initially white cottony, rapid growth with green patches of conidia, covering whole plate. Concentric rings of alternate green and white color.	Conidiophores hyaline, upright, much branched, not verticillate, phialides single or in groups, conidia hyaline 1-celled, ovoid, borne in small terminal clusters.	*Trichoderma* spp.
NA-909	Light purple dorsally, attached to media. Yellow and black ventrally.	No spores were observed	Unidentified

NA-913	Dirty pink colored, powdery and raised dorsally, irregularly shaped	Elliptical conidia in produced in short chains borne on phialides on conidiophore.	*Paecilomyces* spp.
NA-905	Lemon green, irregularly shaped, gave fluorescence. Ventrally dark colored. Produced spikes as the colonies got matured. Color of the media changed to dark as well.	Elliptical spores arranged singly	Unidentified
NA-910	Dirty brown, raised from the media dorsally, black ventrally	Spherical spores were observed	*Glomus* spp.
NA-911	Initially brown turned dirty black on maturing, irregularly shaped, leathery. Ventrally black.	Conidiophores short or long, septate, irregular or bent bearing conidia on new growing tips at the side. Conidia elliptical	*Drechslera* spp.
NA-913	Initially white cottony, rapid growth with green patches of conidia, covering entire plate. Concentric rings of alternate green and white color.	Conidiophores hyaline, upright, much branched, not verticillate, phialides single or in groups, conidia hyaline 1-cellled, ovoid, borne in small terminal clustures.	*Trichoderma* spp.
NA-915	Grass green, powdery, irregularly shaped producing sulphur yellow margin later on.	Conidiophore arising from foot cell basocatenate conidium arrangement in phialides on vesicle	*Aspergillus* spp.
NA-908	Lemon green, irregularly shaped, gave fluorescence. Ventrally dark colored. Produced spikes as the colonies got matured. Color of the media changed to dark as well.	Elliptical spores arranged singly	Unidentified
NA-912	Dirty brown, cottony, raised from the media, dark mycelium in culture, black ventrally	Conidiophores short or long, septate, irregular or bent, bearing conidia successively on a new growing tip, conidia dark containing more than three cells, cylindrical slightly curved	*Helminthospo-rium* spp.
NA-916	Grass green, powdery, irregularly shaped producing sulphur yellow margin later on.	Conidia in chains developing at the end of sterigma arising from vesicle, long conidiophore arise from a septate mycelium.	*Aspergillus* spp.

Table 2: Identification of endophytic fungal isolates of *Taxus wallichiana*

Isolate code	Colony characteristics on PDA	Fruiting structure and/ or spore	Remarks
NT-803	Grayish green with grey edges, rapidly swarming over entire plate, aerial mycelium not very dense	Multicellular spores(conidia) are pear shaped and attached to single conidiophore arising from a septate mycelium	*Alternaria* spp.
NT-802	White cottony, presence of moisture, ventrally cream colored initially but turned to yellowish black as the colony matured.	Conidiophores hyaline, short or elongate, conidial filaments thin tightly coiled.	*Helicomyces* spp.
NT-801a	Light pinkish white, felted, dorsally with irregular margin and blackish brown ventrally	Black colored fruiting body produced on WA with sterilized carnation leaves, fusiform transversely septate conidia with 3 median cells pigmented and two end cells hyaline with 3 apical and 1 basal cellular and simple appendages.	*Pestalotiopsis* spp.
NT-806	White cottony mycelium, spreaded on the media, having slow growth, ventrally light brown.	No spores were observed	Unidentified
NT-807	Dorsally white, slightly cottony towards the periphery, light brown ventrally	No spores were observed	Unidentified
NT-805	Initially white but later turned to grey and finally to black as the colony matured. Ventrally dark.	Conidiophores short, cells somewhat inflated, dark mostly simple. Conidia black 1 celled, globose to somewhat flattened, situated on a flattened, hyaline vesicle at the end of the conidiophore.	*Nigrospora* spp.
NT-807	Dirty green, mycelium immersed and also partly superficial	Multicellular spores (conidia) are pear shaped and attached to single conidiophores arising from a septate mycelium	*Alternaria* spp.

NT-801b	White cottony, presence of moisture, ventrally cream colored initially but turned to yellowish black as the colony matured.	Conidiophores hyaline, short or elongate, conidial filaments thin, tightly coiled.	*Helicomyces* spp.
NT-810	Dorsally light pink, somewhat superficial mycelium, irregularly shaped, ventrally black along with light orange	Black colored fruiting body produced on WA with sterilized carnation leaves, fusiform transversely septate conidia with 3 median cells pigmented and two end cells hyaline with 3 apical and 1 basal cellular and simple appendages.	*Pestalotiopsis* spp.
NT-804	Initially white but later turned to grey and finally to black as the colony matured. Ventrally dark.	Conidiophores short, cells somewhat inflated, dark mostly simple. Conidia black 1 celled, globose to somewhat flattened, situated on a flattened, hyaline vesicle at the end of the conidiophore.	*Nigrospora* spp.
NT-808	White, slightly cottony and raised in the middle dorsally, cream colored ventrally	No spores were observed	Unidentified
NT-809	Light pink dorsally, attached to the media, blackish to orange ventrally	No spores were observed	Unidentified

Table 3: Identification of the fungal isolates of *Podocarpus nerifolius*

Isolate code	Colony characteristics on PDA	Fruiting structure and/ or spore	Remarks
NP-603	Initially white cottony, rapid growth with green patches of conidia, covering entire plate. Concentric rings of alternate green and white color.	Conidiophores hyaline, upright, much branched, not verticillate, phialides single or in groups, conidia hyaline 1-cellled, ovoid, borne in small terminal clustures.	*Trichoderma* spp.
NP-601	Pinkish white, raised from the media, irregularly shaped. Ventrally cream colored with black at the center	Black colored fruiting body produced on WA with sterilized carnation leaves, fusiform transversely septate conidia with 3 median cells pigmented and two end cells hyaline with 3 apical and 1 basal cellular and simple appendages.	*Pestalotiopsis* spp.
NP-602	Initially white cottony, rapid growth with green patches of conidia, covering entire plate. Concentric rings of alternate green and white color.	Conidiophores hyaline, upright, much branched, not verticillate, phialides single or in groups, conidia hyaline 1-cellled, ovoid, borne in small terminal clustures.	*Trichoderma* spp.
NP-604	Round, dirty pink, raised from the media, quite leathery. Ventrally dirty pink with black colored mixed	No spores were observed	Unidentified
NP-605	Round, dirty pink, raised from the media, quite leathery. Ventrally dirty pink with black colored mixed	No spores were observed	Unidentified
NP-606	Brown colored dorsally, cottony aerial mycelium, rapid growth. Presence of moisture. Ventrally dark.	No spores were observed	Unidentified
NP-607	Brown colored dorsally, cottony aerial mycelium, rapid growth. Presence of moisture. Ventrally dark.	No spores were observed	Unidentified
NP-608	Brown colored dorsally, cottony aerial mycelium, rapid growth. Presence of moisture. Ventrally dark.	No spores were observed	Unidentified

Table 4: Identification of endophytic fungal isolates of *Juniperus indica*

Isolate code	Colony characteristics on PDA	Fruiting structure and/ or spore	Remarks
NJ-701	White and cottony towards the periphery, a bit raised from the media, irregular margin. Black and orange ventrally.	Black colored fruiting body produced on WA with sterilized carnation leaves, fusiform transversely septate conidia with 3 median cells pigmented and two end cells hyaline with 3 apical and 1 basal cellular and simple appendages.	*Pestalotiopsis* spp.
NJ-702	White and cottony towards the periphery, a bit raised from the media, irregular margin. Black and orange ventrally.	Black colored fruiting body produced on WA with sterilized carnation leaves, fusiform transversely septate conidia with 3 median cells pigmented and two end cells hyaline with 3 apical and 1 basal cellular and simple appendages.	*Pestalotiopsis* spp.
NJ-703	White, small round raised colonies, wrinkled margin, ventrally cream colored	Conidia in chains developing at the end of sterigma arising from vesicle, long conidiophore arise from a septate mycelium.	*Aspergillus* spp.
NJ-707	White cottony, spreading to the whole media plate. Yellow ring at the center. Ventrally cream colored with black center	No spores were observed	Unidentified
NJ-710	Brown woolly, presence of moisture. ventrally grayish black	No spores were observed	Unidentified
NJ-708	White and cottony towards the periphery, a bit raised from the media, irregular margin. Black and orange ventrally.	Black colored fruiting body produced on WA with sterilized carnation leaves, fusiform transversely septate conidia with 3 median cells pigmented and two end cells hyaline with 3 apical and 1 basal cellular and simple appendages.	*Pestalotiopsis* spp.

NJ-704	Bright green with yellow margin, presence of concentric rings. Dark ventrally	Conidiophore dark, simple, elongate, typically bearing a single or branched chain of conidia; conidia dark typically with cross and longitudinal septa, conidia borne acropetally in long chains.	*Alternaria* spp.
NJ-706	White cottony, spreading to the whole media plate. Yellow ring at the center. Ventrally cream colored with black center	No spores were observed	Unidentified
NJ-705	Bright green with yellow margin, presence of concentric rings. Dark ventrally	Conidiophore dark, simple, elongate, typically bearing a single or branched chain of conidia; conidia dark typically with cross and longitudinal septa, conidia borne acropetally in long chains.	*Alternaria* spp.
NJ-709	White cottony, spreading to the whole media plate. Yellow ring at the center. Ventrally cream colored with black center	No spores were observed	Unidentified

Only 29 isolates could be identified out of total 47 isolates (characteristics described in table 1). Remaining 18 isolates didn't produce any fruiting body and/ or spores neither on PDA nor on WA with sterilized carnation leaves. Out of 29 fungal isolates 19 isolates easily produced spores on PDA when incubated at 28 °C for at least a week to ten days. The isolates that produced spore on PDA were: NA-902, NA-906, NA-907b, NA-914, NA-912, NA-901, NA-915, NA-913, NA-916, NA-907a, NT-803, NT-807, NT-805, NT-804, NP-603, NP-602, NJ-703, NJ-704 and NJ-705. Those that didn't produce spore on PDA but on WA incorporated with sterilized carnation leaves were: NA-911, NT-802, NT-801b, NT-801a, NT-810, NP-601, NJ-708 and NJ-701, which were observed under the light microscope. The results are shown in Table 1, 2, 3 and 4.

5.1.3 Class wise and Genera wise distribution of the identified isolates of endophytic fungi

Table 5: Class wise and Genera wise distribution of the identified isolates of endophytic fungi from *Abies spectabilis*

No.	Isolate code No.	Identified genera	Sub division	Class
1.	NA-902	*Aspergillus* spp.	Deuteromycotina	Hyphomycetes
2.	NA-906	*Paecilomyces* spp.	Deuteromycotina	Hyphomycetes
3.	NA-907b	*Paecilomyces* spp.	Deuteromycotina	Hyphomycetes
4.	NA-914	*Paecilomyces* spp.	Deuteromycotina	Hyphomycetes
5.	NA-910	*Glomus* spp.	Zygomycotina	Zygomycetes
6.	NA-911	*Drechslera* spp.	Deuteromycotina	Hyphomycetes
7.	NA-912	*Helminthosporium* spp.	Deuteromycotina	Hyphomycetes
8.	NA-901	*Trichoderma* spp.	Deuteromycotina	Hyphomycetes
9.	NA-915	*Aspergillus* spp.	Deuteromycotina	Hyphomycetes
10.	NA-913	*Trichoderma* spp.	Deuteromycotina	Hyphomycetes
11.	NA-916	*Aspergillus* spp.	Deuteromycotina	Hyphomycetes
12.	NA-907a	*Aspergillus* spp.	Deuteromycotina	Hyphomycetes

In case of *Abies spectabilis*, 12 fungal isolates were obtained. Most of the identified fungal isolates belonged to class Hyphomycetes of sub division Deuteromycotina while one isolate belonged to class Zygomycetes of sub division Zygomycotina. The results are given in Table 5. From 12 isolates, 6 different genera were identified and their distribution is shown in Figure 5.

Table 6: Class wise and Genera wise distribution of identified isolates of endophytic fungi from *Taxus wallichiana*

No.	Isolate code No.	Identified genera	Sub division	Class
1.	NT-803	*Alternaria* spp.	Deuteromycotina	Hyphomycetes
2.	NT-807	*Alternaria* spp.	Deuteromycotina	Hyphomycetes
3.	NT-802	*Helicomyces* spp.	Deuteromycotina	Hyphomycetes
4.	NT-801b	*Helicomyces* spp.	Deuteromycotina	Hyphomycetes
5.	NT-801a	*Pestalotiopsis* spp.	Deuteromycotina	Coelomycetes
6.	NT- 810	*Pestalotiopsis* spp.	Deuteromycotina	Coelomycetes
7.	NT-805	*Nigrospora* spp.	Deuteromycotina	Hyphomycetes
8.	NT-804	*Nigrospora* spp.	Deuteromycotina	Hyphomycetes

In case of *Taxus wallichiana*, altogether 8 fungal isolates were identified. All the identified isolates belonged to 2 different classes of sub division Deuteromycotina. Out of 8, 6 isolates belonged to class Hyphomycetes and 2 belonged to class Coelomycetes. The results are shown in Table 6. From 8 isolates, 4 different genera were identified and their distribution is shown in Figure 6.

Table 7: Class wise and Genera wise distribution of the identified isolates of endophytic fungi isolated from *Podocarpus nerifolius*

No.	Isolate code No.	Identified genera	Sub division	Class
1.	NP-603	*Trichoderma* spp.	Deuteromycotina	Hyphomycetes
2.	NP-602	*Trichoderma* spp.	Deuteromycotina	Hyphomycetes
3.	NP-601	*Pestalotiopsis* spp.	Deuteromycotina	Coelomycetes

Table 7 shows that only 3 fungal isolates were identified from *Podocarpus nerifolius* which belonged to subdivision Deuteromycotina. Two belonged to class Hyphomycetes and one isolate belonged to class Coelomycetes. From 3 isolates, 2 different genera were identified and their distribution is shown in Figure7.

Table 8: Class wise and Genera wise distribution of the identified isolates of endophytic fungi isolated from *Juniperus indica*

No.	Isolate code No.	Identified genera	Sub division	Class
1.	NJ-702	*Pestalotiopsis* spp.	Deuteromycotina	Coelomycetes
2.	NJ-708	*Pestalotiopsis* spp.	Deuteromycotina	Coelomycetes
3.	NJ-703	*Aspergillus* spp.	Deuteromycotina	Hyphomycetes
4.	NJ-704	*Alternaria* spp.	Deuteromycotina	Hyphomycetes
5.	NJ-705	*Alternaria* spp.	Deuteromycotina	Hyphomycetes
6.	NJ-701	*Pestalotiopsis* spp.	Deuteromycotina	Coelomycetes

As shown in Table 8, altogether 6 fungal isolates were identified from *Juniperus indica* all belonging to subdivision Deuteromycotina. 3 isolates belonged to class Hyphomycetes and 3 belonged to class Coelomycetes. From 6 isolates, 3 different genera were identified and their distribution is shown in Figure 8.

5.2 Screening of the identified fungal isolates for their antifungal activities

Screening of the identified endophytic fungal isolates (29 isolates) for their antifungal activities was accomplished by using the fresh cultures of the plant pathogenic fungi viz. *Fusarium oxysporum*, *Fusarium moniliforme*, *Sclerotinia* spp., *Helminthosporium* spp., *Alternaria* spp., *Geotrichum* spp. and *Gloeosporium sporioides*. The antagonistic or inhibitory properties of the endophytic fungal isolates against the plant pathogenic fungi were studied according to the dual culture method performed on PDA. The endophytic fungal isolates were noted as highly inhibitory (+++), moderately inhibitory (++), low inhibitory (+) and not inhibitory (-) to the plant pathogenic fungi.

Table 9: Screening of the identified isolates of endophytic fungi for their inhibitory activities against plant pathogenic fungi

Conifer	Isolate code	Identified genera	Test plant pathogenic fungi						
			F. oxysporum	***F. moniliforme***	***Sclerotinia* spp.**	***Helmithosporium* spp.**	***Alternaria* spp.**	***Geotrichum* spp.**	***Gloeosporium sporioides***
Abies. spectabilis	NA-902	*Aspergillus* spp.	-	++	++	+	+	-	+
	NA-906	*Paecilomyces* spp.	-	++	+++	+++	-	-	+
	NA-907b	*Paecilomyces* spp.	-	++	+++	+++	++	+	+++
	NA-914	*Paecilomyces* spp.	-	+	+	+++	++	+	++
	NA-910	*Glomus* spp.	-	-	++	+	-	++	+
	NA-911	*Drechslera* spp.	-	-	+	+	-	-	+
	NA-912	*Helminthosporium* spp.	+++	+++	+++	+++	+++	+	+++
	NA-901	*Trichoderma* spp.	+++	+++	+++	+++	+++	+++	+++
	NA-915	*Aspergillus* spp.	-	-	-	+	-	-	-
	NA-913	*Aspergillus* spp.	+	+	+	-	-	-	+
	NA-916	*Aspergillus* spp.	+	++	++	+	++	++	++
	NA-907a	*Aspergillus* spp.	-	+	+	+	+	-	-

Table 9 (contd..)

Conifer	Isolate code	Identified genera	Test plant pathogenic fungi						
			F. oxysporum	***F. moniliforme***	***Sclerotonia* spp.**	***Helminthosporium spp.***	***Alternaria spp***	***Geotrichum spp.***	***Gloeosporium sporioides***
Taxus wallichiana	NT-803	*Alternaria* spp.	+++	++	+++	+++	+++	+++	++
	NT-807	*Alternaria* spp.	+++	++	++	+++	+++	++	++
	NT-802	*Helicomyces* spp.	+	+	+	+	+	+	+
	NT-801b	*Helicomyces* spp.	+	+	+	+	+	+	++
	NT-801a	*Pestalotiopsis* spp.	+++	+++	++	+++	++	++	++
	NT-810	*Pestalotiopsis* spp.	++	+++	+++	+++	++	++	++
	NT-805	*Nigrospora* spp.	-	+	+	++	-	++	++
	NT-804	*Nigrospora* spp.	-	+	+	++	-	+++	++
Podocarpus nerifolius	NP-603	*Trichoderma* spp.	+++	+++	+++	+++	+++	+++	+++
	NP-602	*Trichoderma* spp.	+++	+++	+++	+++	+++	+++	+++
	NP-601	*Pestalotiopsis* spp.	++	++	++	++	+	++	+++

Table 9 (contd..)

Conifer	Isolate code	Identified genera	Test plant pathogenic fungi						
			F. oxysporum	***F. moniliforme***	***Sclerotinia* spp.**	***Helminthosporium* spp.**	***Alternaria spp.***	***Geotrichum spp.***	***Gloeosporium sporioides***
Juniperus indica	NJ-702	*Pestalotiopsis* spp.	+++	+++	+++	+++	+++	+++	+++
	NJ-708	*Pestalotiopsis* spp.	++	++	++	+++	+++	++	+++
	NJ-703	*Aspergillus* spp.	+	+	+	-	+	+	+
	NJ-705	*Alternaria* spp.	+++	+++	++	+	+++	++	+++
	NJ-701	*Pestalotiopsis* spp.	++	++	+++	+++	+++	+++	+++
	NJ-704	*Alternaria* spp.	+	++	+++	++	++	+++	++

Where +++ = Highly inhibitory
++ = Moderately inhibitory
+ = Low inhibitory
- = Not inhibitory

In case of fungal isolates obtained from *Abies spectabilis*, *Trichoderma* spp. (NA-901) was found to be highly inhibitory against the fungal plant pathogens used in the study while *Aspergillus* spp. (NA-915) were found to be not inhibitory against most of the pathogen except *Helminthosporium* spp. Similarly, in case of fungal isolates obtained from *Taxus wallichiana, Alternaria* spp (NT-803, NT-807) and *Pestalotiopsis* spp. (NT-801a, NT-810) showed high inhibitory effect against most of the fungal plant pathogens used in the study, where as *Nigrospora* spp. (NT-805) showed less inhibitory effect in

comparision to other isolates obtained. In case of *Podocarpus nerifolius, Trichoderma* spp. (NP-603, NP-602) showed high inhibitory effect towards all the fungal plant pathogens. *Pestalotiopsis* spp. (NP-601) showed high inhibitory effect towards only one one fungal plant pathogen (*Gloeosporium sporioides).* In case of *Juniperus indica,* high inhibitory effect was shown by *Pestalotiopsis* spp. (NJ-702) against all the fungal plant pathogens used and *Aspergillus* spp. (NJ-703) gave low inhibitory effect towards most of the fungal plant pathogens. The results are given Table 9.

5.3 Extraction of secondary metabolite of a priority isolates of endophytic fungi from different conifers

Based upon the results of screening process and the review of the literature, the priority isolates that were chosen for fermentation for the purpose of extraction of bioactive secondary metabolites were NT-801a (from *Taxus wallichiana*), NP-601 (from *Podocarpus nerifolius*) and NJ-702 (from *Juniperus indica*), all belonging to the genera *Pestalotiopsis*. Fermentation was carried out in each flask containing 500 ml of S-7 medium, incubated at 28° C for 3 weeks followed by its extraction, by using equal volume of chloroform twice. Control flask was as well processed in the similar manner. The flasks were processed separately and dried crude extracts was obtained after drying in rotary vacuum evaporator and its weight was recorded. The dry weight of the extract of control flask was found to be 10 mg/500ml.

Table 10: Extraction of secondary metabolite of isolated endophytic fungi isolated from different conifers

Conifer	Isolate code	Identified genus	Dry weight of extract (mg/500ml)
Taxus wallichiana	NT-801a	*Pestalotiopsis* spp.	33
Podocarpus nerifolius	NP-601	*Pestalotiopsis* spp.	17
Juniperus indica	NJ-702	*Pestalotiopsis* spp.	32

Pestalotiopsis spp. (NT-801a) isolated from *Taxus wallichiana* gave highest yield of fungal extract (33 mg/500ml) while *Pestalotiopsis* spp. (NP-601) isolated from *Podocarpus nerifolius* gave lowest yield of the fungal extract (17 mg/500ml). The results are given in Table 10.

5.4 Study of antifungal activity of the endophytic fungal extract extracted with chloroform

The fungal extracts obtained were dissolved in 500 µl of DMSO in separate glass vials, so that the final concentration of the extracts would be 33 mg/500µl (for NT-801a), 17 mg/500µl (for NP-601) and 32 mg/500µl (for NJ-702). Similarly 10 mg of the control extract was dissolved in 1.2 ml of DMSO. Study of the antifungal activity was carried out by the agar well diffusion method, in which 50 µl of the crude fungal extract was loaded into one of the three wells made on PDA. Similarly, 50 µl each of the control extract and the solvent i.e DMSO were loaded separately into the wells made on same PDA plate, initially inoculated with test plant pathogenic fungal organism for assaying the bioactivity. The results confirmed that the inhibitory effect was solely due to the presence of the bioactive secondary metabolites that was present in the crude extract, as the control media extract and the solvent themselves didn’t give any kind of inhibitory effect.

Table 11: Potency of the extract for the antifungal activities

Endophytic fungi	Code	Source of extract	Dry weight of extract used (mg)	Vol. of solvent in dissolving extract	Vol.loaded into the well	Diameter of zone of inhibition (mm) (ZOI)						
						Fusarium oxysporum	***Fusarium moniliforme***	***Sclerotinia* spp.**	***Helmithosporium* spp.**	***Alternaria* spp.**	***Geotrichum* spp.**	***Gloeosporium sporioides***
NT-801 a	E	Culture broth	33	500 µl of DMSO	50µl	10	20	12	25	10	13	16
	C	Control media	10	500 µl of DMSO	50µl	-	-	-	-	-	-	-
	S (solvent)				50µl	-	-	-	-	-	-	-
NP-601	E	Culture broth	17	500 µl of DMSO	50µl	-	15	16	10	-	15	25
	C	Control media	10	500 µl of DMSO	50µl	-	-	-	-	-	-	-
	S (solvent)				50µl	-	-	-	-	-	-	-
NJ-702	E	Culture broth	32	500 µl of DMSO	50µl	10	16	30	25	20	30	20
	C	Control media	10	500 µl of DMSO	50µl	-	-	-	-	-	-	-
	S (solvent)				50µl	-	-	-	-	-	-	-

Where - = No zone of inhibition
Diameter of the well = 6 mm
Height of the well = 4 mm

Among three fungal extract, highest ZOI was given by NJ-702 against *Geotrichum* spp. i.e. 30mm while fungal extract NP-601 didn't give any ZOI towards *Fusarium oxysporum* and *Alternaria* spp.The result is shown in Table 11.

5.5 TLC analysis of the endophytic fungal extracts

Table 12: R_f values of the spots obtained on TLC analysis

Analyte	Solvent front (cm.)	Distance moved by the spot (cm.)	Rf value
Authentic taxol	17	13.2	0.77
10-Deacetyl Baccatin (10-DAB)	17	8.2	0.48
Fungal extract (NT-801a)	17	13.3	0.78
Fungal extract (NP-601)	17	13.5	0.79
Fungal extract (NJ-702)	17	13.4	0.78
Control extract	17	-	-

Where R_f = distance moved by analyte from origin/ distance moved by solvent from origin

- = No spot obtained on TLC

TLC analysis of the endophytic fungal extracts was carried out on a precoated silica plate using the solvent system of chloroform: methanol (7:1). The fungal extracts of the NT-801a, NP-601, NJ-702 and control extract was made to run on precoated silica plate along with the authentic taxol and its precursor compound (10-Deacetyl Baccatin III) as reference compounds. The fungal analytes, control extract and the reference compounds as well were dissolved in 50 µl of methanol. About 5 µl of each of them were loaded and was allowed to run on the precoated silica plate till it reached a marked solvent front which was 17 cm. The plate was dried, visualized under UV-light with the wave length of 254 nm. Here, the control extract gave no spot, while the fungal analytes and the reference compounds showed the spots/spot. The authentic taxol and its precursor compound (10-Deacetyl Baccatin III) gave a single distinct spot whose R_f values were 0.77 and 0.48 respectively. The fungal analytes gave a hazy, not very well separated band of spots, which could be clearly visualized under UV-light. The spots gave

fluorescence of blue color. Only the R_f values of those spots were taken which fell near about the spot exhibited by the authentic taxol. The R_f calculated for the fungal extract analytes for NT-801a, NP-601 and NJ-702 are 0.78, 0.79 and 0.78 respectively. Then, the plate was sprayed with vanillin/sulphuric acid (1% w/v) reagent followed by heating. The spots were seen as bluish coloration which turned to brown after 24 hours.

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

The study was conducted in Natural Product Research Laboratory of NAST from September 2005 to September 2006 for the purpose of studying the endophytic fungi isolated from different Himalayan conifers viz. *Abies spectabilis, Taxus wallichiana, Podocarpus nerifolius* and *Juniperus indica* from different locations.

Fungi are a group of eukaryotic organisms that are of great practical and scientific interest. The systematic study of fungi is only 250 years old, but the manifestations of this group of organisms have been known for thousands of years. Fungi play such an important role in the slow but constant changes taking place around us because of their ubiquity (Cooke, 1975).Fungi are both destructive and beneficial to agriculture. Specifically, fungi are the agents responsible for much of the disintegration of organic matter and as much affect directly by destroying food, fabrics, leather and other consumer goods manufactured from raw materials are subject to fungal attack. On the other hand they are responsible for millions of dollars worth of damage to crops by causing plant diseases, yet in their role as saprobes, fungi together with bacteria have been responsible for recycling of many important chemical elements that without their activity would remain forever locked up in dead plant and animal bodies(Alexopoulos and Mims, 1979).

Biological control using microorganisms to initiate disease in pest population has the potential of reducing agricultural reliance on chemical pesticides. The negative interactions (ammensalism, predation and parasitism) among microbial population and between microbes and higher organism have always formed a natural basis for the biological control of pest and pathogens, and biological control methods take advantage of those relationship (Atlas and Bartha, 2005). *Metarrhizium anisopliae, Verticillium*

lecanii and *Trichoderma* spp. and potential of many others as fungicides, insecticides and herbicides is currently being developed (Ramachandran, 1991).

Fungi are present in most plant parts, especially the leaves where the tissue is apparently healthy, the fungi may be either endophytes, epiphytes or latent pathogen (Redlin and Carris, 1985). It was reported that only 5% of the world's fungi are currently known (Hawksworth, 1991). Though Nepal covers less than 0.03% of the world's land, due to the wide range of altitudinal variation, Nepal constitutes 1882 fungi (2.60%) out of 70,000 fungi of the world. Out of 552 genera with 1,822 species, 150 species are endemic to Nepal (WCMC, 1992). Endophytic fungi are important, beneficial, extremely significant yet largely untapped resources that are likely to hold several economically important applications. Endophytes are contained within the plant without diseases; plant tissues remain entire and functional. Such resources 'endophytic fungi' are currently being chosen as the subject of research globally, as these resources are recently considered to be a wellspring of secondary metabolites that offers a huge potential for medical, agriculture, and/or industrial exploitation (Strobel and Daisy, 2003) and are equally important as biological agents for controlling plant pathogens.

Endophytic fungi colonize plant tissue and remain within the tissue except that fruiting structures may emerge through the surface. Indeed, leaves may be fully colonized by a variety of fungi within a few weeks of emergence. The colonies remain asymptomatic and some in perennial parts may have a very long life (Redlin and Carris, 1985). Endophytic fungi are found in all division of fungi so have presumably evolved the association independently on many occasions. The most common endophytes are anamorphic members of the Ascomycotina, and they are often closely related to fungi known to cause disease, either in healthy tissue or as secondary in healthy tissue or as secondary invaders of damaged tissue. The *Discula* spp. and *Alternaria* spp. both include pathogenic strains, they do occur as endophytes in diverse taxa (Arnold *et al.,* 2007; Kirk *et al.,* 2001).This suggests that the endophytes may have evolved from pathogens or vice-versa. The mechanisms of host recognition and development of colonization may also be common. The endophytic microorganisms have peculiar

characteristics which favor their growth inside plant tissues. Some particular metabolic functions permit the microbe to overcome the chemical barriers, natural or induced present in the host tissues. From this point of view the taxane production by the population of yews could be considered as an adaptation (Caruso *et al.,* 2000).

Endophytic fungi are often divided into two groups: the 'clavicipitaceous system' (ascomyctes family clavicipitaceae) which contains endophytes of grass that offer resistance to herbivory and pathogens, and endophytes representing all other fungal groups on diverse herbaceous and woody hosts (Petrini, 1991), and fungal endophytes have been found in all woody plants studied to date (Saikkonem *et al.,* 1998).A wide range of plants have now been examined for endophytes, and endophytes have been found in nearly all of them. An enormous number of different fungi can be isolated from plants growing in their native habitat. Most of the fungi are uncommon and narrowly distributed taxonomically and geographically. However, a few fungi are widely distributed with the host, suggesting a long standing, close and mutually beneficial interaction. Endophytes occupy unique ecological niches and can influence the distribution, ecology, physiology and biochemistry of plants (Sridhar and Raviraja, 1995).

The study was carried out basically on four Himalayan conifers, viz.*Abies spectabilis*, *Taxus wallichiana*, *Podocarpus nerifolius* and *Juniperus indica* from different places ('Kavre' district, 'Kavre' district, 'Kirtipur' district and 'Kirtipur' district respectively), with the objective of studying the biodiversity of endophytic fungi and the bioactivity of the secondary metabolites from the most potent one.

Several twig samples of the coniferous plants viz. *Abies spectabilis* (from 'Kavre' , 2,930m); *Taxus wallichiana* (from 'Kavre', 2,930m); *Podocarpus nerifolius* (from 'Kirtipur', 1,372m) and *Juniperus indica* (from 'Kirtipur', 1,372m) were collected and cut in the size of 1-2 cm. diameter and 20 cm. long. Both the ends were sealed with properly with parafilm inorder to prevent drying out during transport and kept in sealed plastic bags. The samples were transported to NPRL at NAST, 'Khumaltar' and

preserved for few days in refrigeration at 4 °C (Stierle *et al.,* 1993). During the collection of plant material, care was taken that the twigs were from the healthy source and not from the diseased one. This is because it is of utmost importance to understand the methods and rationale used to provide the best opportunities to isolate novel endophytic microorganisms as well as the ones making novel bioactive products. The rationale that should be considered while selecting the plants include ,those from unique environmental settings, plants having longevity, plants growing in areas of great biodiversity also have the prospect of housing endophytes with great biodiversity (Strobel and Daisy, 2003).

The plant materials used were already authentically identified. These plant materials were further subjected for isolation of the endophytic fungi housing in the tissue of the plant material. To avoid contamination and to isolate the endophytic fungi only, from the inner tissues, the branches were peeled after surface disinfection. Surface disinfection was achieved by treating the sample i.e. stem fragments with 70% ethanol; air drying under laminar flow hood in order to eliminate surface contaminating microbes. After peeling of the outer bark the inner bark was excised and careful placement was done on the water agar plates. The similar procedure was applied by Shrestha, 2002; Striele *et al.,* 1993; Strobel *et al.,* 1996 and Upadhyayay, 2005 for the isolation of endophytic fungi from plant materials. Fungi can grow very well on water agar. Since our goal was to isolate the endophytic fungi, water agar was the choice of media that could avoid the growth of bacteria. Antibiotics can be as well incorporated in the media for the isolation of fungal communities. Antibiotics like streptomycin, penicillin or tetracycline (250 mg/L) have been incorporated in the plates containing Malt extract agar in order to avoid the growth of unwanted microorganisms like bacteria (Fisher *et al.,* 1991; Sridhar and Barlocher, 1992)

The water agar plates were incubated at 28 °C, regular monitoring was done for the growth of endophytic fungi. After 4-5 days the growth of hyphal tips was observed upon the surface sterilized inner barks. These hyphal tips were transferred on to the PDA plates 4-5 times so as to get the pure culture (Stierle *et al.,* 1993) and were

incubated for at least 10 days. This proves the point that endophytic fungi are relatively having slow growth on laboratory culture media and also are taxonomically difficult group (Deacon, 1997). These, endophytic fungi took more than 10 days to some even a month to grow on laboratory culture medium.

Pure culture was obtained by transfer of hyphal tip method. Identification was performed by studying the macroscopic and microscopic appearance (Aneja, 1996). Altogether 47 isolates of endophytic fungi were isolated; from *Abies spectabilis* (17 isolates), *Taxus wallichiana* (12 isolates), *Podocarpus nerifolius* (8 isolates), and *Juniperus indica* (10 isolates) out of which only 29 fungal isolates could be identified. Some of the fungi didn't produce spores on PDA so for inducing sporulation gamma irradiated carnation leaves were used on water agar plates (Nelson *et al.*, 1983). The fungi were identified on the basis of spores they produced. The unidentified fungi didn't sporulate even upon incubation in water agar with sterilized carnation leaves. Cover slip culture was also performed in order to reveal the exact morphology of the fungus. The fungi that produced characteristic spore on PDA were identified NT-803, NT-807, NJ-704 and NJ-705 to be *Alternaria* spp., NA-907b, NA-914 and NA-906 were identified as *Paecilomyces* spp., NA-901, NA-913, NP-603 and NP-602 were identified to be belonging to genus *Trichoderma*. The isolates NA-902, NA-915, NA-916, NA-907a and NJ-703 were identified as *Aspergillus* spp., NT-805 and NT-804 was found to be *Nigrospora* spp., and NA-912 was identified as *Helminthosporium* spp.

Those that didn't produce spore on PDA were further subjected to sporulation process, by incubating on water agar incorporated with sterilized carnation leaves. Later those isolates were identified on the basis of the spores they produced and were identified NT-801a, NT-810, NP-601, NJ-702, NJ-708 and NJ-701 to be *Pestalotiopsis* spp. and NA-911 was identified as *Drechslera* sp. Adverse condition was artificially created in laboratory, devising such medium i.e. WA medium, sterilized plant material was incorporated, which encourages the sporulation of the endophyte (Strobel and Daisy, 2003). Many species sporulate better on the included plant material than agar itself only. Using sterilized natural substrate such as wheat straw, macerated leaves into basic

agar has proved to be quite advantageous (Sutton, 1973). Fruiting body and the spore on the carnation leaves were observed under light microscope for identification. Similar procedure was applied previously by Shrestha, 2002 and Upadhyayay, 2005 for the identification of endophytic fungi of *Tsuga dumosa* from Khumbu region and 'Tangboche', 'Solukhumbu' district respectively.

In our study identification of the isolated fungal strains posed a problem, as a consequence only 29 fungal isolates could be identified out of 47 isolates. The reason is that the endophytic fungi grow extremely slowly in the laboratory culture media (Deacon, 1997) and also requires a considerable incubation time (Issac, 1996). Incubation period of at least 10 days to 1 month was provided in this study which might not have been sufficient for the sporulation of fungi as these fungi requires incubation time sometimes many months. Besides this, some of them might have lost this ability altogether because they always grow within the plant and are transferred from generation to generation by growing as endophytes. However; the environment also has a profound influence on growth and sporulation of fungi. Almost all environmental conditions including nutrient supply, aeration, temperature, pH, humidity, light and injury have been shown to influence fungal sporulation (Turian, 1974). Fruiting structure of fungi was influenced by nutrient concentration (Smith, 1978). Sucrose concentration influenced sporulation in *Plectosporium tabacinum* (Zhang *et al.*, 2001) and carbohydrate starvation was favorable for sporulation in *Penicillium* (Jicinska, 1968).

The endophytic fungi were identified on the basis of their spores and fruiting bodies, which they produced on water agar containing sterilized carnation leaves. Some species produced spore quite easily on PDA, while some species do not produce spores easily. Mycelia that do not differentiate reproductive structure can also be easily found. This particular aspect agrees with the result obtained on different plant species both conifers and deciduous trees. The reason for failure in the identification could be connected to their taxonomic position, Basidiomycotina or heterothallic species of Ascomycotina. Particularly these genera cause the biodegradation of the woody organs and that are the

only structure on which they can produce the characteristic reproductive structure (Caruso *et al.,* 2000). This may be the plausible reason regarding the identification of fungal isolates. Most of the identified fungal isolates belonged to deuteromycetes. Deuteromycetes have septate mycelium and reproduce by means of conidia. Since, these fungi apparently lack a sexual phase, these are commonly known as Fungi Imperfecti. Deuteromycotina (fungi imperfecti) are an assemblage of fungi typically reproducing by spores, which are formed without nuclear fusion followed by meiosis. This artificial subdivision embraces not only the imperfect, asexual or conidial states of the Ascomycotina and Basidiomycotina but also those asexual fungi with which no perfect stages have been yet been correlated if indeed any are in existence. The Deuteromycotina are actually comprised of three orders, i. The Sphaeropsidales (eg. *Phomopsis*) in which the conidia are borne hymenium enclosed in a round, flask shaped or flattened sporophore (pycnidium) and the conidia are set free by a narrow pore or silt.

ii. The Melancoliales in which conidia are borne on crowded conidiophores arising from an immersed stromatic base in a cavity of which the outer wall is formed by the tissues of the host (acervulus) the best example being Pestalotiopsis sp. (one of the identified fungal isolates of endophytic fungi in the study) which formed black colored fructification (conidiomata) which was acervular on WA with sterilized carnation leaves. The conidia are borne in an acervulus of the tips of the conidiophore that were packed closed together on the cushion of hyphae. The conidiophores are hyaline branched and septate at the base and above. The conidia are fusiform, straight or slightly curved and transversely septate with three pigmented median cells and two ends hyaline. The apical cells had three unbranched appendages and the basal cell had one simple unbranched appendage observed under light microscope (Kendrick and Carmichael, 1973; Sutton, 1973).

iii. The Moniliales with superficial separate or aggregated conidiophores that bear conidia, to which eight of the identified genera of endophytic fungi belonged are *Aspergillus*, *Paecilomyces*, *Alternaria*, *Helminthosporium*, *Helicomyces*, *Trichoderma*,

Drechslera and *Nigrospora* (Barnett, 1965;Kendrick and Carmichael, 1973).Moniliales includes all those hyphomycetous fungi that produce conidia. Moniliales is a very large group consisting of probably over 7000 species. Many of the fungi are of immense importance to us as either plant pathogens, human pathogens or industrial fungi (Alexopoulos and Mims, 1979).

The term Coelomycetes was introduced to accommodate in single category the extremes of fructification type shown by the pycnidial and acervular genera. Later two classes were recognized in the Deuteromycotina: the Hphomycetes characterized by conidia borne on the exterior of the substrate in which the fungus grows and the Coelomycetes for those fungi with conidia that are formed within a cavity of the substrate in which the fungus grows. This class now includes all immersed to superficial pycnidial and acervular fungi in which conidia are initiated within a country lined by either the fungal or host tissue, or the combination of both. In our study all the isolated endophytic fungi from different conifers, belonged to above mentioned two classes i.e. Hyphomycetes and Coelomycetes. The identification was based on fructifications and /or spores they produced. The term conidioma (conidiomata) was introduced by Kanaskis II as a comprehensive noun that can be applied to the fruit-bodies in or on which conidiogenous cells and conidia develop in the Deuteromycotina, which includes all the five types of fructification, recognized by Von Hohnel: pycnidia, acervuli, stromata and cupulate and pycnothyriod pycnidia (Sutton, 1973).

Shrestha (2002) isolated more than 60 isolates from the inner bark of a Himalayan conifer, Hemlock spruce collected from 'Khumbu' region of east of Nepal. The representing fungal genera belonged to *Trichoderma*, *Fusarium, Phoma, Phomopsis*, *Sclerotium, Alternaria, Nigrospora*, *Nodulosporum* and different yeasts. Whereas few were identified as endomycorrhizae. When forwarded for the screening for antifungal/antibacterial activities against different pathogenic fungi such as *Pythium*, *Geotrichum*, *Phytopthora*, *Rhizoctonia* and bacterium *Bacillus subtilis* (ATTC 6635) due to the production of novel secondary metabolites produced by these endophytic fungal community.

Upadhyayay (2005) isolated 30 isolates of endophytic fungi from the inner bark of a Himalayan conifer, Hemlock spruce collected from 'Tangboche' from altitude of 4000-4500m, 'Solukhumbu' district. The identified representative fungal genera included *Aspergillus, Paecilomyces, Verticillium, Pestalotiopsis*, *Fusarium, and Endobotryella*. These endophytes were subjected to screening regarding its bioactivity against plant pathogens, the result revealed that some endophytic fungus could be used in biocontrol of plant pathogens.

Shrestha (2002) also did isolated 20 isolates of endophytic fungi from *Taxus wallichiana* collected from 'Shivapuri' (2200 m). The prominent genera belonged to *Pestalotiopsis microspora*, *Trichoderma* spp., *Aspergillus flavipis*, *Culvularia* spp., *Sporormia minima*, were also among the identified genera. Rest one was identified as dimorphic fungus. In the similar manner, Shrestha (2002) isolated 5 fungal isolates from the inner bark of *Taxus wallichiana* collected from 'Kavre' (2930 m). The identified isolates were identified to be *Pestalotiopsis microspora* (2 isolates), *Phomopsis* (2 isolates) while one remained unidentified. Similarly, Shrestha (2002) isolated 12 endophytic fungal isolates from *Podocarpus nerifolius* collected from 'Kirtipur' (1372 m) among which 10 of them were identified to be *Pestalotiopsis microspora*, one was identified to be *Aspergillus sulphurius* and one was left unidentified. 8 isolates from *Abies spectabilis* from 'Kavre' (2930 m) in which 6 were identified to be *Pestalotiopsis microspora*, one *Phomopsis* spp., and other one was Sclerotia forming fungus. *Pestalotiopsis microspora* and *Fusarium* spp. was isolated from very old *Cedrus deodara* collected from 'Lalitpur' (1350 m).

The present study did show that majority of endophytic fungal genera belonged to the sub-division Deuteromycotina that is very much comparable with the former studies in which most of the identified fungi belonged to the sub division Deuteromycotina, while one belonged to the sub-division Zygomycotina. Majority of the endophytic fungal isolated belonged to the class hyphomyctes, which may provide products useful in biotechnology and agriculture (Bills and Polishok, 1992; Dreyfuss and Chapela, 1994; Petrini, 1991).

Shrestha (2002) isolated endophytic fungi and found that the dominant genus of the conifers under study was *Pestalotiopsis*. In our present study, total of 47 isolates of endophytic fungi was isolated from the conifers (*Taxus wallichiana, Abies spectabilis, Podocarpus nerifolius and Juniperus indica*), out of which only 29 isolates were identified up to genus level. Among the identified endophytic fungal isolates, majority of the isolates were found to be *Pestalotiopsis* spp. (6 isolates), *Aspergillus* spp. (5 isolates), *Trichoderma* spp. (4 isolates), Alternaria spp. (4 isolates), *Paecilomyces* spp. (3 isolates), Nigrospora spp. (2 isolates), *Helicomyces* spp. (2 isolates) *Glomus* spp. (1 isolate), *Helminthosporium* spp. (1 isolate) and *Drechslera* spp. (1 isolate). In this study, 18 out of 47 isolates were left unidentified. This may be probably because the majority of microbes living in forest trees are still unnamed and our knowledge of their species richness is still vague and also endophytic fungi of conifers are inconspicuous and are rarely collected and studied. Similar results were obtained in the study carried out by Shrestha (2001) in which some of the endophytic isolates of *Taxus wallichiana* from 'Shivpuri', *Tsuga dumosa* from 'Mustang' and *Podocarpus nerifolius* from 'Kirtipur' remained unidentified. Similarly, Upadhyayay (2005) also couldn't identify the fungal isolates from the inner bark of *Tsuga dumosa* collected from 'Tangboche', 'Solukhumbu' district.Identification part posed problem, and most of them couldn't be identified as they didn't produce fruiting bodies on PDA as well as the spores on sterilized carnation leaves.

According to Shrestha (2002), first report of endomycorrhizae living endophytically was disclosed to be *Glomus* spp. i.e. Zygomycetes, from *Tsuga dumosa*. In our current study, endomycorrhizae *Glomus* spp. was isolated from *Abies spectabilis* collected from 'Kavre' district (2930 m).

The isolated endophytic fungal strains were further subjected for screening purpose regarding its bioactivity. Dual culture method on PDA was used for this purpose. Dual culture method has always been a rapid and easy method for the screening or in vitro selection of antagonist for the purpose of biocontrol of plant pathogenic fungi. Result was noted, considering the colony interaction between the test fungal plant pathogen

and the fungal endophyte giving zone of inhibition as the indication of their antifungal activity. The study revealed most of endophytic fungal isolates showed varying degree of inhibitory effect against plant pathogenic fungi used. The study showed the wide range of activity shown by *Pestalotiopsis* spp. (NJ-702, NT-801a) towards the test plant pathogens. Similar result was reported during the study conducted by Upadhyayay (2005), in the fungi isolated from conifer *Tsuga dumosa*.

According to Bashyal *et al.*, (2002), 71 endophytic fungi were isolated from the die-back sisso (*Dalbergia sisso*) that was collected from various parts of different regions of Nepal. Dual culture method was used in order to study the bioactivity of isolated endophytic fungi against the two common plant pathogenic fungi, *Pythium* spp. and *Geotrichum* spp. The report showed *Fusarium* spp. and *Phoma* spp. were the ones among showing highest bioactivity against *Pythium* spp. and endophytic *Trichoderma* spp. showed the highest bioactivity against *Geotrichum* spp. Our result revealed that *Pestalotiopsis* spp., *Alternaria* spp., *Trichoderma* spp. and *Helminthosporium* spp. were the ones among showing highest bioactivity against all the fungal plant pathogens used in the study.

Wang *et al.*, (2005) isolated endophytic fungi associated with Chinese oil pine, *Pinus tabulaeformis* from two distinct climatic sites i.e. Fenghuangstan and Lingynan in northest China. The identified endophytic fungi were *Alternaria alternate*, *Phoma* spp., *Phomopsis archeri* and *Leptostroma* spp., these were found to be dominant taxa in the bark and needles.

Pestalotiopsis spp. was one of the dominant taxa in our study, which was isolated from the inner bark of *Taxus wallichiana* (from 'Kavre'), *Podocarpus nerifolius* (from 'Kirtipur'), and *Juniperus indica* (from 'Kirtipur'). Upon screening these isolates showed maximum inhibitory effect against most of the plant pathogenic fungi used in this study. Among the *Pestalotiopsis* spp. that were isolated, the most effective result was exhibited by the *Pestalotiopsis* spp. (NJ-702) isolated from the conifer *Juniperus indica*.

Pestalotiopsis microspora (isolate NE-32) is an endophyte isolated from the Himalayan Yew (*Taxus wallichiana*), that produces taxol an important chemotherapeutic drug used in the treatment of breast and ovarian cancers (Metz *et al.*, 2000). *Pestalotiopsis microspora* appears to be a ubiquitous organism in global rainforest system. It has been isolated as an endophyte from the stems, leaves, flower and fruits of virtually all plants. This prevalence may suggest an important role for this fungus in ecosystem of the forest. The organism has been dubbed as the *E .coli* of the temperate and tropical rainforest system. Its relationship seems to be either as a weak pathogen invading aged leaves or as an endophytic symbiont functioning with the higher plant in as-yet mysterious ways. Besides its possible importance in nature, *P. microspora* has a great potential in the pharmaceutical industry as a taxol producer in laboratory and as a model biological system for the study of telomers. Despite of its prevalence in forest and its potential utility in laboratory, this microbe is poorly understood (Metz *et al*., 2000).

The endophytic fungi produces bioactive secondary metabolites, the bioactivity expressed by the endophytic fungi against plant pathogenic fungi in dual culture method is simply believed to be due to the production of novel bioactive secondary metabolites (Shrestha, 2002). *Pestalotiopsis* spp. isolated from different conifers was chosen for extraction of its bioactive secondary metabolites, as this species had shown a broad spectrum of antifungal activity during the dual culture method. The *Pestalotiopsis* spp., an isolate NS-835 showed broad spectrum of antifungal activity against different plant pathogens used (Upadhyayay, 2005). A number of endophytic species of *Pestalotiopsis* has been known for its production of antifungal compounds. One such secondary metabolite is ambuic acid, an antifungal agent which has been recently described from several isolates of *Pestalotiopsis microspora* found as representative isolates of the world's rainforest (Li *et al.*, 2001). Several compounds, having antifungal activity including pestaloside and two pyrones: pestalopyrone and hydroxypestalopyrone have been found being produced by a strain of *P. microspora* isolated from endangered tree *Torreya taxifolia* (Lee *et al.*, 1995). A newly described species of *Pestalotiopsis*, namely *P .jesteri* from the Sepik river area of Papua New Guinea, produced jesterone

and hydroxyjesterone that exhibited antifungal activity against a variety of plant pathogenic fungi (Li and Strobel, 2001).

According to Schulz *et al.*, (2002), in optimizing the search for new bioactive secondary metabolites, it is relevant to consider that secondary metabolite the fungus synthesizes may correspond with its respective ecological niche, e.g. the mycotoxins of plant pathogens and that metabolic interaction may enhance the synthesis of secondary metabolites. Thus the fungi screened should originate from biotypes which have metabolic interactions with their environment. This is one of the intelligent screening strategies for exploiting the untapped resource. Endophytic fungi are one good source for intelligent screening that fulfills the above mentioned criteria. Secondary metabolites particularly from endophytic fungi have yielded a large number of different compounds with significant biological activities. Keeping this fact in mind, the endophytic fungal isolates NT-801a (from *Taxus wallichiana*), NP-601 (from *Podocarpus nerifolius*) and NJ-702 (from *Juniperus indica*) were chosen for the extraction of its bioactive secondary metabolites, based upon the results obtained in dual culture method , as these isolates exhibited broad spectrum of antifungal activity against the plant pathogenic fungi used, and also on the basis of review of literature this particular species has shown to produce taxol, the novel anticancer drug. Hence, these particular species were chosen as the priority isolates. The fermentation of the priority isolates were carried out in 500 ml of S-7 medium for three weeks at 28°C. After proper incubation, the solids were removed and the culture broth was extracted with the equal volume of chloroform twice. The control media was also processed in the similar manner. Finally, the dry weight of the extract was determined after drying the extracts in rotary vacuum evaporator. The dry weight of the extract of NT-801a isolate was 33 mg/500ml of the culture media; for NP-601 isolate it was 17 mg/500ml of culture media and for NJ-702 isolate the dry weight was found to be 32 mg/500ml of the culture media, where as the control extract had dry weight of 10 mg/500ml of the culture media. Similarly, Upadhyayay (2005), also performed fermentation of the priority isolate NS-835 isolated from *Tsuga dumosa* collected from Tangboche, identified as *Pestalotiopsis* spp. using S-7 media as the culture medium.

For the antifungal activity of the extracts, the dried extracts were dissolved in 500µl of DMSO, and its antifungal activity was studied qualitatively by agar well diffusion method. The fungal extract NT-801a gave ZOI of 10mm against *Fusarium oxysporum*; 20mm against *Fusarium moniliforme*; 12mm against *Sclerotonia* spp.; 25mm against *Helminthosporium* spp.; 10mm against *Alternaria* spp.; 13mm against *Geotrichum* spp. and 16mm against *Gloeosporium sporioides*. The fungal extract NP-601 gave ZOI of 15mm against *F. proliferum*; 16mm against *Sclerotonia* spp.; 10mm against *Helminthosporium* spp.; 15mm against *Geotrichum* spp. and 25mm against *Gloeosporium sporioides*, while it gave negligible ZOI against *F. oxysporum* and *Alternaria* spp. Finally, the fungal extract NJ-702 gave ZOI of 16mm against *F. proliferum*; 30mm against *Sclerotonia* spp.; 25mm against *Helminthosporium* spp.; 20mm against *Alternaria* spp.; 30mm against *Geotrichum* spp. and 20mm against *Gloeosporium sporioides* but gave negligible ZOI against *F. oxysporum.* The zone of inhibition exhibited was definitely due to the bioactive secondary metabolites present in the fungal extract, as this was confirmed by the absence of ZOI due to the control media extract and the solvent used i.e. DMSO against the test plant pathogenic fungal organism. This suggests that neither solvent nor the constituent of S-7 medium extractable by chloroform possessed the antifungal activity. Upadhyayay (2005) also studied the antifungal activity of the extract qualitatively by agar well diffusion method.

Shrestha (2002) isolated endophytic fungi from different conifers which produced secondary metabolites with potent antibacterial compounds. *Pestalotiopsis microspora* (NP-105), although didn't produce taxol, but produced secondary metabolites with antibiotic activities against *B .subtilis* (ATCC 6635). The compounds were isolated from six liters of MID liquid culture media by extracting with chloroform and purifying over preparative silica gel TLC plates using chloroform/methanol (7:1) as the solvent system, where the compounds NPE-1 to NPE-5 had the retention times of 0.72, 0.66, 0.59, 0.46, and 0.39 respectively. The three major compounds with R_f 0.72, 0.46 and 0.66 were identified as Pestalotin, Hdroxypestalotin and 5, 6-Dehydropestalotin based on spectral data. Another strain of *Pestalotiopsis microspora* (NP-104), isolated from *Podocarpus nerifolius* produced taxol in the concentration of 31.6 ng per liter of culture

media, where the dry weight of the chloroform extract was 33.8 mg/ liter. Similarly, in the same study, *Pestalotiopsis microspora* (NC-202) isolated from *Cedrus deodara* produced taxol in the concentration of 26.4 ng per liter of the culture media, where as the dry weight of the chloroform extract was 15.2 mg/liter. In the same study, *Pestalotiopsis microspora* (NA-502) didn't produce any taxol, whose dry weight of the chloroform extract was found to be 21.4 mg/liter. The taxol was measured qualitatively by Competitive Inhibition Enzyme Immunoassay (CIEIA) method, using a specific anti-taxol monoclonal antibody.

The dry weight of extracts in the present study was found to be of *Pestalotiopsis* spp. (NT-801a) isolated from *Taxus wallichiana* 33 mg/500ml of the culture media; *Pestalotiopsis* spp. (NP-601) isolated from *Podocarpus nerifolius* 17 mg/500ml of the culture media and that of *Pestalotiopsis* spp. (NJ-702) isolated from *Juniperus indica* was 32 mg/500ml of culture media. Based on the literature review and study conducted by Shrestha (2001), these isolated endophytic fungi from different locations also might be the source of taxol. A number of endophytic fungi and their secondary metabolites are reported from *Taxus wallichiana* of Nepal (Strobel *et al.*, 1996). Since, *Abies spectabilis*, *Podocarpus nerifolius* and *Juniperus indica* grows in the same environment, where *Taxus* grows, the fungal isolates isolated from these plant sources were suspected for the presence of taxol. Hence, the crude extract was made to run on a percolated silica plate along with the control extract, authentic taxol and its precursor compound (10-Deacetyl Baccatin III), using chloroform: methanol (7:1) as the solvent system, in which the fungal extract gave fuzzy bands that was more or less in separated form. Within the band bright spots could be easily visualized when observed under UV-light. The dried extract was dissolved in 50 µl of methanol and 5 µl of the analyte was loaded. Since, our concern was to find out the presence of taxol in the fungal extracts so only those spots were analyzed for R_f values that were observed near the spot visualized by the reference compound i.e. taxol. The R_f values of the compounds of Fungal extract of NT-801a, NP-601 and NJ-702 isolates were found to be 0.78, 0.79 and 0.78 respectively. The authentic taxol had the R_f value of 0.77 and the precursor compound (10-Deacetyl Baccatin III) had R_f value of 0.48. This suggests that in our study, since

the retention value of the fungal analytes was found to be similar to the authentic taxol, there might be possibility that the fungal extract of *Pestalotiopsis* spp., NT-801a (from *Taxus wallichiana*); NP-601 (from *Podocarpus nerifolius*) and NJ-702 (from *Juniperus indica*) might possess the taxol. The fungal extracts were also tested for the presence of the precursor compound of taxol as well, but all three fungal analyte moved quite away from the spot of precursor compound. The separation into several spots suggests that the fungi produced a secondary metabolite; in fact a wide variety of secondary products is produced by a single strain of endophytic fungi. The variation in the amount and kinds of product found in the fungus i.e. *Pestalotiopsis* spp. depends on both the cultural condition of the organism as well as the original plant source from which it is isolated.

According study conducted by Shrestha, *Pestalotiopsis* isolated from *Abies spectabilis* (NA-501) and *Cedrus deodara* (NC-202) produced taxol in the concentration of 15.0 and 26.4 ng/L respectively. The amounts of taxol produced by these isolates were lower than that was produced by *Pestalotiopsis microspora* isolated from *Taxus wallichiana*. This indicates that *P. microspora* strains were genetically variable, while appearing morphologically identical; endophytes didn't behave biochemically in the same way (Lee *et al.,* 1996; Turner and Aldridge, 1983). Though in our study we couldn't determine the concentration of taxol content, which was not our main concern, it is possible that there could be variation in terms of the genetics in taxol content and in terms of genetics of *Pestalotiopsis* spp. isolated from different conifers. The presence of such endophytic fungi in different conifers indicates that they can tolerate different kinds of cytotoxic compounds like taxol. These fungi can survive in extreme environmental condition between altitude 1350-3572 m with very damp and adverse condition by living in the inner bark of the conifers. Their survival may be related to the similar type of defense chemicals within that taxon or it could be due to the power of detoxification of phycotoxins and phytoalexins present in the plant tissues. On the other hand, it could be due to the adaptation towards tolerance of toxins acquiring the ability to use new materials for the growth (Van etten *et al.,* 1995).

Two decades of the history of endophytes and still going on researches suggests that endophyte research has become a global enterprise. However, disparities in methodology as well as phylogenetic differences among the host taxa, obscure comarisions across studies such that large scale patterns of fungal diversity are difficult to discern. Although much research is needed to determine the true number of fungal species, it can be concluded that endophytes are an important component of diversity estimates and contribute substantially to fungal diversity.

6.2 Conclusion

The present study indicates the biodiversity of endophytic fungi inhabiting the conifer plants in its habitat. Most of the endophytic fungal isolates belong to Hyphomycetes (47%), followed by coelomycetes (38%) and zygomycetes (2%). The fungal isolates exhibited bioactivity, as majority of endophytic fungal isolates were found to be antagonistic against different fungal plant pathogens used in the study. The selected fungal isolate *Pestalotiopsis* spp., a common rain forest endophyte, revealed a broad spectrum of antagonistic effect against all of the fungal plant pathogens included in this study, whose fermentation yielded fungal extract that, as well caused inhibitory effect towards the fungal plant pathogens. This clearly reveals the fact that these endophytic fungi have great potential in the biocontrol of plant pathogens. The TLC result showed all the *Pestalotiopsis* spp. from different plant sources had high probability of the presence of taxol, which is a novel anticancer drug.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

1. Four authentically identified Himalayan conifers viz. *Abies spectabilis, Taxus wallichiana, Podocarpus nerifolius* and *Juniperus indica* were used for the present study. The endophytic fungi were isolated from twig samples on water agar (WA) plates after incubation of two weeks at 28 °C that were subcultured on PDA to obtain pure culture. Altogether 47 isolates of endophytic fungi were obtained, out of which only 29 isolates were identified up to genus level. Identification was done on the basis of fruiting bodies, and the spores they produced.

2. Of the fungal isolates, six were found to be *Pestalotiopsis* spp., five *Aspergillus* spp., four *Alternaria* spp. and *Trichoderma* spp., three *Paecilomyces* spp., two *Helicomyces* spp. and *Nigrospora* spp., followed by one isolates each belonging to genera *Helminthosporium, Drechslera*, and an endomycorrhizal fungi identified as *Glomus* spp.

3. The inhibitory effect of the endophytic fungal isolates was studied by using different plant pathogenic fungi, by using dual culture method performed on PDA. The different plant pathogenic fungi used were *Fusarium oxysporum*, *Fusarium moniliforme, Sclerotinia* spp., *Alternaria* spp., *Helminthosporium* spp., *Geotrichum* spp. and *Gloeosporium sporioides*, against which, *Pestalotiopsis* spp., *Alternaria* spp., *Trichoderma* spp. and *Helminthosporium* spp. showed highest bioactivity.

4. Owing to the broad spectrum of antifungal activity, three endophytic fungal isolates of *Pestalotiopsis* spp.isolated from different plant source were selected for fermentation. *Pestalotiopsis* spp. (NT-801a) isolated from *Taxus wallichiana* gave the highest yield of fungal extract (33 mg/500ml) while

Pestalotiopsis spp. (NP-601) isolated from *Podocarpus nerifolius* gave the lowest yield of fungal extract (17 mg/500ml).

5. Potency of the fungal extract for the antifungal activities was studied by the agar well diffusion method against different plant pathogenic fungi viz. *Fusarium oxysporum*, *Fusarium moniliforme*, *Sclerotinia* spp., *Alternaria* spp., *Helminthosporium* spp., *Geotrichum* spp. and *Gloeosporium sporioides*. Among the three fungal extract, highest zone of inhibition (ZOI) was given by NJ-702 against *Geotrichum* spp. i.e. 30 mm, while fungal extract NP-601 didn't give any ZOI towards *Fusarium oxysporum* and *Alternaria* spp.

6. The fungal extracts of NT-801a, NJ-702 and NP-601 were run on TLC plate along with the extract of control media, authentic taxol, and it's precursor i.e 10-Deacetyl Baccatin III (10- DAB). The R_f value of NT-801a, NP-601, NJ-702, authentic taxol and Deacetyl Baccatin III were found to be 0.78, 0.79, 0.78, 0.77 and 0.48 respectively. This indicates the probability of presence of taxol in the extracts obtained from different isolates of *Pestalotiopsis* spp.

7.2 Recommendation

The following Recommendations can be made from the present study:

- In this study, the endophytic fungi were identified only up to genus level. So, effort should be made to identify the fungal isolates up to species level as well.Many of the isolated fungi couldn't be identified, which had huge potential regarding its bioactivity, should also be identified, which may require standard morphological and molecular biological methods. The fermentation and extraction of bioactive secondary metabolite is strongly recommended for other isolates having huge potential.

- Only endophytic fungal isolates were studied in the present research work. So, further study of other endophytic microorganisms like endophytic bacteria, endophytic actinomycetes is highly recommended.
- Indigenous plants from unique environmental settings, plants having medicinal values etc. can be employed for studying the endophytes.
- Besides these plant pathogens used, other significant plant pathogen and human pathogens as well can be exploited for the study of antimicrobial property of the extract.
- In this study, only chloroform was used as the solvent for the extraction. Other active compounds insoluble in chloroform could not be extracted with chloroform. So, other suitable solvents may also be used.
- Determination of action spectrum of bioactive secondary metabolite in the extract by minimum inhibitory concentration (MIC) is recommended.
- For the determination of presence of taxol, the extract is recommended to be checked by CIEIA, which can detect the presence of taxol even in low concentration.
- In our present study, inner barks of the plant source were analysed for the presence of endophytes. So, other plant part like roots, leaves is recommended for studying the endophytes.

APPENDIX-I

1. LISTS OF MATERIALS

1.1 Equipments

1. Autoclave (Life steriware, India)
2. Electric balance (Denver Instruments)
3. Hot air oven (Universal, India)
4. Incubator (Universal, India)
5. Laminar air flow cabinet (Toshiba, India)
6. Microscope (Olympus, Japan)
7. pH meter (TOA pH meter HM-10P)
8. Refrigerator (Samsung)
9. Rotary vacuum evaporator (Aikakai, Japan)
10. Water distillation plant (Ogaweseiki)

1.2 Microbiological Media

1. Potato Dextrose Agar (Hi-media)
2. S-7 medium
3. Water Agar

1.3 Chemicals and Reagents

1. Calcium nitrate-4-Purified (Merck, India)
2. Chloroform (Qualigens)
3. Cotton lactophenol mounting (STABIO, Calcutta)
4. Cupric nitrate (The British Drug House Ltd., England)

5. Dehydrated alcohol (Ethanol) (Bengal chemicals, India)
6. d-Biotin (Hoffmann L-A Roche Inc.)
7. D (-) Fructose (Mercle)
8. D-glucose (anhydrous) (Qualigens)
9. Dimethyl Sulfoxide (Qualigens)
10. Dl-Phenylalanine (Central Drug House (P), India)
11. i-sodium hydrogen phosphate 2-hydrate purified (Merck)
12. Ferric chloride anhydrous (s.d fine chemical. Pvt. Ltd)
13. Magnesium sulphate (heptahydrate) (LOBA CHEMIE)
14. Manganese (II) chloride purified (Merck)
15. Methanol (Qualigens)
16. Pantothenic acid calcium salt (Calcium pantothenate) (Glaxo, India)
17. Potassium Dihydrogen Orthophosphate (Qualigens)
18. Sodium acetate crystals Pure (Merck)
19. Sodium Benzoate (Perkin, India)
20. Soytone (DIFCO)
21. Sucrose Extra Pure (LOBA CHEMIE)
22. Zinc sulphate 7-hydrate Purified (Merck)

1.4 Glasswares

1. Beakers
2. Conical flasks
3. Cover slips
4. Funnels
5. Glass rods
6. Glass vials

7. Graduated Measuring cylinders
8. Microscopic slides
9. Micropippetes
10. Petriplates
11. Plastic vials
12. Round bottom flasks
13. Separating funnel
14. Screw-capped test tubes

1.5 Essential Requirements for TLC

1. Precoated silica plate (20x5 cm)
2. Solvent tank with lid
3. Solvent system- chloroform: Methanol (7:1)
4. Oven
5. Capillary
6. Blow drier
7. Sample spotting template
8. UV chamber
9. Chromogenic reagent:Vanillin/Sulphuric acid (1% w\v) reagent
10. Spray apparatus

1.6 Miscellaneous

1. Aluminium foil
2. Blotting paper
3. Cotton role
4. Cork borer
5. Cotton swabs
6. Dropper
7. Forceps
8. Sterilized carnation leaves
9. Immersion oil
10. Lens paper

11. Inoculating loop
12. Measuring scale
13. Plastic vials
14. Sticker
15. Soap/Detergent
16. Transport Tray

1.7 Test fungal plant pathogens

1. *Alternaria* spp.
2. *Fusarium oxysporum*
3. *Fusarium moniliforme*
4. *Geotrichum* spp.
5. *Gloeosporium sporioides*
6. *Helminthosporium* spp.
7. *Sclerotinia* spp.

APPENDIX-II

2. Composition and Preparation of different types of culture media

2.1 Potato Dextrose Agar

Composition	Grams/ Liter
Potatoes Infusion form	200.00
Dextrose	20.00
Agar	15.00
Final pH (at 25°C)	5.6 +\-0.2

Thirty nine grams of PDA (Hi media) was suspended in 1000ml of cold distilled water in a conical flask and pH was adjusted to 5.6. It was then heated to boiling to dissolve the medium completely. The conical flask was closed with a tight cotton plug. It was sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes. It was then allowed to cool up to 45°C, and then dispensed in sterilized petriplates. The media was finally allowed to solidify before use.

2.2 S-7 Medium

Composition	
Glucose	1 gm
Fructose	3 gm
Sucrose	6 gm
Sodium acetate	1 gm
Soytone	1 gm
Thiamina	1 mg
Biotin	1 mg
Pyridoxal	1mg
Calcium Pantothenate	1mg
Magnesium Sulphate	3.6 mg

Calcium nitrate	6.5 mg
Copper nitrate	1 mg
Zinc sulphate	2.5 mg
Magnesium chloride	5 mg
Ferric chloride	2 mg
Phenylalanine	5 mg
Sodium benzoate	100 mg
1 M KH2PO4 buffer	1 ml

Final pH (at 25°C) 6.8 -per liter of media

2.3 Water Agar

Composition	
Bacto agar	15 gram
Distilled water	1000 ml

Fifteen grams of Bacto Agar was suspended in 1000ml of cold distilled water in a conical flask and heated to boiling to dissolve the medium completely and the flask was closed with a tight cotton plug. It was then sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes. After sterilization, it was cooled to 45°C.

APPENDIX-III

3. Characteristics of the coniferous plants used in this study

3.1 *Taxus wallichiana* Zucc. (Taxaceae)

It is commonly known as “Himalayan yew” and locally known as “Talispatra”. It is a dioecious tree, much branched up to 30 m in height. The leaves are alternate, persistent, linear flattened, spiny tipped, dark glossy- green above and paler beneath. Fruits are red, 8mm long and surrounded the olive green single seed. Flowering and fruiting occurs during March to September. The yew tree has a special place in the history for several reasons. Many ancient European religions regarded this somber looking tree as a symbol of longevity because it is very slow growing and lives for several hundred years.

It is commonly found in temperate forests at 2300 m to 3600 m altitude all along the Himalayan ranges. It is usually found as undergrowth in forests of *Tsuga dumosa* and *Abies spectabilis* on the south of the main Himalayan ranges.

Himalayan yew is the only yew with the history of medicinal use. Its extracts show tranquilizing and sedative activity presumably due to benzodiazepine like activity of biflavones of the amantoflavon type.Current interest in the biological activity of yew constituents is mainly focused on the antitumer activity of taxol. Regarding its conservation status, government has banned *T. wallichiana* for export as raw material without processing under the forest act 1993.

3.2 Abies spectabilis (D.Don) Spach (Pinaceae)

Abies spectabilis is commonly known as “Himalayan silver fir” and locally known as “Gobresalla or Talispatra”. It is monoecious evergreen trees with height up to 40 m. It grows on the slopes of Himalaya, generally occurring in cool, moist situations with deep rich soil. It is a lofty, black and stout tree. Its leaves are crowded all around the branches and arranged spirally and variable in length. The under surface of the leaves is silvery and glaucous on either side of mid-rib.

It is found in the temperate and sub-alpine regions of Nepal. It is distributed uniformly in eastern, central and western parts of Himalayan regions with the altitude of 2100-3900 m.

Leaves are regarded as carminative, expectorant, stomachic, tonic and astringent. It is also used in asthma and bronchitis. Leaves contain essential oil, alkaloid and a biflavonoid abiesin.

Due to its overexploitation and unplanned collection, the plant has now been declared as endangered plant.

3.3 Podocarpus nerifolius D.Don. (Podocarpaceae)

It is a glaborous dioecious, evergreen, medium sized conifer attaining a height of 12-13 m. Its branches are whorled and leaves are linear, leathery and up to 26 cm long. It is commonly known as “Mounteak and Thitmin”and locally known as “Gunsi”.

It is distributed in tropical, sub-tropical forests in central and eastern Nepal at an altitude of 990-1070 m. it has not been found in good abundance like a pure forest as one may expect in Sikkim. Presumably, due to adverse environmental conditions for its growth in Nepal, the plant has not been reported any further west than central Nepal.

3.4 *Juniperus indica* Bertol

Juniperus indica Bertol syn *Juniperus wallichiana* or *Juniperus pseudosabina* is commonly called “Black Juniper”. Its local name is “Dhupi”. It is a wide spread bush of inner Himalaya. *Juniperus indica* is a conifer that belongs to the family Cupressaceae which includes 19 genera and about 140 species of principally evergreen coniferous trees and shrubs. They are of cosmopolitan in distributuion.

Juniperus indica Bertol is a juniper native to high altitudes Himalaya, occurring from the northern Indus valley in Kashmir east to western Yunnan in China. It is of interest as the highest altitude woody plant known, reported growing as high as 5200 m in southern Tibet; the lowest limit being 2600 m. In Nepal they are found in high altitude Himalayan range.

Juniperus indica is dioecious, with male (pollen) and female (seed) cones on separate plants. The leaves are closely appressed and overlapping, arranged in four ranks, and narrowly ovate with points incurved. The leaves are dark grey-green, dimorphic, with adult plants having mostly scale-like leaves 1-3 mm long, while young plants have mostly needle-like leaves 5-8 mm long, but needle-like leaves can also be found on shaded shoots of adult plants.

J. indica is a characteristic of dry upland regions where it forms bushy vegetation of steppe nature. However large trees measuring over 15 m occur in Dhorpatan valley (2700m) which lies on south west of Dhaulagiri Himal. *J.indica* is generally associated with other conifer plants.

APPENDIX-IV

4. Characteristics of Fungal plant pathogens used for the study

4.1 *Fusarium oxysporum*

Class-Deuteromycetes
Order- Hyphomycetales
(Butler and Jones, 1986)

Fusarium oxysporum, apart from being the most economically important member of the genus *Fusarium,* is one of the most liable and variable species. The average growth rate of cultures is 4.5 cm. Cultures are pale, salmon, rosy buff, vinaceous, and violet to pale slate on media of pH 6.7 to 7. Mycelium is delicate, felted to floccose. Microconidia are always present that are unicellular or bicellular, ellipsoidal to allantoid and born on lateral phialides or on phialides produced from short lateral conidiophores. Macroconidia are falcate and are 3-5 septate when mature and are initially formed from branched lateral phialides but later often formed from sporodochia. Chalmydospores are intercalary or terminal on short lateral branches solitary or in chains, hyaline, smooth to rough walled.

The distribution is worldwide and occurs chiefly as a soil saprophyte and appears to survive in winter in mycelial or chlamydospore state.

Numerous strains of this species are wilt pathogens of many crop plants. *Fusarium oxysporum* causes an important wilt disease of sweet potato (*Ipomoea batetas*) in USA but is less important in tropcs. Intervienal yellowing of the leaves is followed by distortation and stunting, and the old leaves fall. There is extensive vascular necrosis that may appear purplish below the soil level; the cortex may rupture. Infected tubers may rot in storage. The fungus infects the roots of many plants without causing any

external symptoms viz. cabbage, cotton, cowpea, maize, okra, soyabean, tobacco, and watermelon (Booth, 1971).

4.2 *Fusarium moniliforme*

Class-Deuteromycetes
Order-Moniliales
Family-Tuberculariaceae
(Alexopoulos and Mims, 1979)

Fusarium moniliforme is wide spread in both humid and sub-humid temperate zones and extending into sub-tropical zones through out the world. Growth is initially rather filmy, colorless and rapid (4.6 cm). Culture from below typically dark violet but occasionally paler, pink, lilac, vinaceous or even cream. Aerial mycelium is generally dense and delicately floccose to felted, often with a powdery appearance due to formation of microconidia. Microconidiophores are simple, lateral, subulate phialides formed on the aerial hyphae; rarely may they be formed on short lateral branches. They are 20-30 µ long by 2-3 µ at the base narrowing to the approximately 1.5µ at the apex.
Fusarium moniliforme is a major parasite of several Gramineae such as rice, sugarcane, maize and sorghum. It also occurs on a very wide range of other hosts. F. moniliforme causes seedling blight, foot rot, stunting and hypertrophy of shoots of rice (bakanae disease), also causes seedling blight, root rot and pink boll of cotton. It also attacks flowers and fruits of banana. It is also associated with storage rots of Freesia corms, pineapple and tomatoes. (Booth, 1971)

4.3 *Sclerotinia* spp.

Class-Ascomycetes
Order-Heliotales
Family-Sclerotiniaceae
(Alexopoulos and Mims,1979)

This family comprises of economically important groups of fungi. Most fungi of this family live parasitically on plants. *Sclerotinia* spp. is an important plant pathogen. These species mostly live saprobically on the soil, on dead wood, on dung or on other organic matter from which they draw nourishment.

Apothecial initials arise from stromata or sclerotia. The apothecia are of medium size, generally brown and are most often borne on long stalks. The ascospores are generally hyaline, one-celled, oval or somewhat elongated (Alexopoulos and Mims, 1979).

Sclerotinia spp. causes a number of diseases in plants. *Sclerotinia sclerotiorum* cause lettuce drop and other vegetable diseases as well. *Sclerotinia frutigena* causes brown rot of fruit apple. *Sclerotinia frutigena*, *Sclerotinia cinerea* and *S. americana* causes brown rot of stone fruits e.g. apricot, plum etc. *Sclerotinia ricini* causes stem rot of castor. *Sclerotinia sclerotiorum* causes sclerotinia rot in wheat and other crops causing leaf spot and blight. It also causes stem blight of mustard (Rangaswami, 1996).

4.4 *Helminthosporium* spp.

Class-Deuteromycetes
Order-Moniliales
(Butler and Jones, 1986)

Helminthosporium is a widespread fungus whose mycelium consists of inter- and intra-cellular prostrate hyphae and more or less erect conidiophores. The mycelium develops as grayish brown to dark brown mat on host parts and on culture. On the host hyphae are short segmented and the conidiophores arise as lateral branches from these hyphae. The conidiophores are characteristically bent and possess knee joints, the points where conidia are attached. The conidia are sub hyaline to yellowish-brown, thin walled, straight, cylindrical to slightly tapering, having rounded ends, 1-7 septate and without constrictions at the septa. This genus causes a number of diseases in plants. *Helminthosporium gramineum* causes stripe disease of barley; Helminthosporium orizae causes brown leaf spot or Helminthosporium disease of rice, an important disease of rice that occurs in almost all rice growing areas of the world, especially under

semi dry conditions; Helminthosporium sativum causes spot blotch of barley and seedling blight and foot rot of wheat and barley; Helminthosporium teres causes net blotch of barley, etc (Singh,1998).

4.5 *Alternaria* spp.

Class-Deuteromycetes
Order-Moniliales
Family-Dematiaceae
(Alexopoulos and Mims, 1979)

Alternaria is a large, universally occurring genus. Several form species are found as sprobes on dead and dying plant parts in soil from which the conidia are picked up by the wind.

Conidiophores dark, simple, rather short or elongate, typically bearing a simple or branched chain of conidia. Conidia dark, typically with both cross and longitudinal septa; variously shaped, obclavate to elliptical or ovoid, frequently borne acropetally in long chains; less often borne singly and having an apical simple or branched appendage.

A number of form species are also parasitic on plants. *Alternaria solani* attacks members of the family solanaceae as well as other hosts. It is particularly troublesome on potato, tomato where it causes the disease known as early blight. Blight is caused in linseed (*A.liniday*), castor (*A.ricini*), mustard (*A.brassicae*).In onion *Alternaria* causes blight, *Alternaria brassicae* and *Alternaria melongenae. solani* cause leaf spots with concentric rings in raddish and egg plant respectively. Leaf spots in lady's finger, chilli, sunflower is shown to be caused by *Alternaria nelianthis*. *Alternaria* also causes fruit rot in tomato (*A.solani*) (Rangaswami, 1996).

4.6 *Gloeosporium sporioides*

Class-Deuteromycetes
Order-Melaconiales
(Butler and Jones, 1986)

Gloeosporium is a widespread fungus whose perfect stage is *Glomerella* found under family Hypocreaceae of order Sphaerials of class Pyrenomycetes under the subdivision Ascomycotina which has small, spherical perithica with dark parenchymatous walls and are formed single or in clusters mostly in host tissues. The ascospores are small, translucent, hyaline, and one-celled. They are mostly parasitic on higher plants causing anthracnose, leaf spots, fruit rots and stalk rot. The conidial stage is found under order Melanconiales of class deuteromycetes where conidia are produced in acervulus. The mycelium consists of sparsely septate hyphae, which at first are hyaline but later take on slightly dark color. The conidia are hyaline but in mass they look pinkish (Singh, 1998).

Besides these, the other test pathogenic fungal organism used in the study was *Geotrichum* spp.

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