CHAPTER 2
LITERATURE REVIEW

All living plants so far investigated have been shown to harbor fungi inside their tissues. Such fungi colonizing the inner part of aerial plant tissues have been referred to as "endophytes" and are now known to be widespread in nature (Rodrigues, 1996). At the most basic level, the term endophyte simply refers to the location of the organism: 'endo' is the Greek word meaning within and 'phyte' is the Greek word for plant, thus, an endophyte is an organism which lives inside a plant. This is in contrast to epiphyte which refers to organisms living on the outer surface of the plant.

The term endophyte was originally defined by De Bary and referred to any organism occuring within plant tissues. Since then it has become deeply embedded in the literature and within the last decade, different authors have proposed a range of similar, but more complex definitions e.g. Carroll 1986, Petrini 1991 (Wilson, 1995). In 1986, Carroll restricted the use of the term endophyte to organisms that cause asymptomatic infections within plant tissues, excluding pathogenic fungi and mutualists such as mycorrhizal fungi (Carroll, 1986 cited in Petrini, 1991). Petrini (1991) proposed an expansion of Carroll's definition and incorporated into it, his concept of latent pathogens known to live symptomlessly inside the host tissues for only part of their life cycle. Wilson (1995) described endophytes as fungi and other microorganisms such as bacteria that invade tissues of plants and cause unapparent and asymptomatic infections for all or part of their life cycle.

Fungal endophytes live within the intercellular spaces of plants but may also grow intracellularly and must obtain nutrient materials through this intimate contact with the host. The occurrence of specialized feeding structures has not been reported in these fungi (Bernstein and Carroll, 1977; Cabral, Stone and Carroll, 1993; Johnson and Whitney, 1989a; Suske and Acker, 1987,1989; Stone, 1987).

Endophytic fungi include species in the family Clavicipitaceae (Clavicipitales; Ascomycota) and other fungal taxa belonging to the Ascomycota Basidiomycota Deuteromycota and Zygomycota, most of which are found associated with a diverse assemblage of plant hosts, including grass, sedge, rushes, shrubs, and trees (Bacon and Hinton,1997). Fungal endophytes of grasses (Poaceae) and sedges (Cyperaceae and Juncaceae) are probably the most extensively studied group (Clay,1988, 1989).

Reports on the presence of endophytes in vascular plants, other than grasses, have focused mainly on ericaceous, dicotyledoneous plants and conifers (Rodrigues, 1996). Symptomless endophytes can be separated into two distinct ecological groups: the mainly clavicipitaceous systemic grass endophytes and the endophytes of trees and shrubs (including the non-clavicipitaceous grass endophytes as well) (Petrini, 1996).

2.1 Grass endophytes

2.1.1 Fungal taxonomy and biology

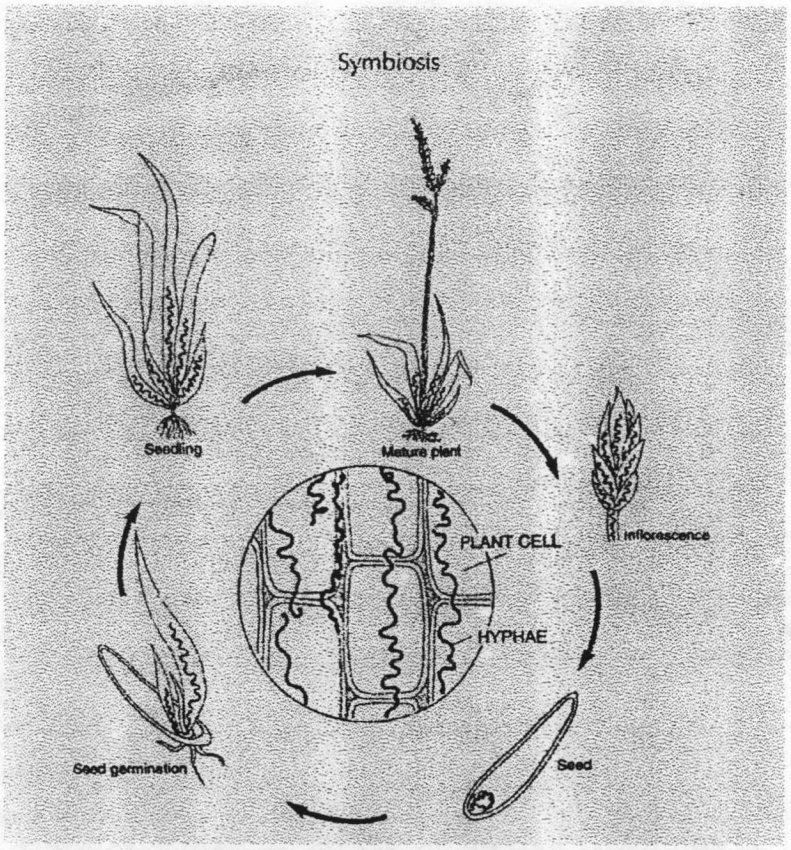
Clavicipitaceous endophytes have been known to exist in grasses since the discovery of an endophyte in seeds of darnel (*Lolium temulentum* L.) by Vogl (1898 cited in Wilson, 1996). The oldest known specimens of darnel with endophytic mycelium were seeds retrieved from a pharaohs tomb in an Egyptian pyramid dating back to 3400 B.C.. Most surveys for clavicipitaceous endophytes have concentrated on wild grasses, cultivated turfgrass and forage grass collections from Europe and North America. Clavicipitaceous endophytes of grasses can be divided into two natural groups: 1) the choke-inducing sexual forms or clavicipitaceous teleomorphic endophytes, and 2) the mutualistic asexual forms or clavicipitaceous anamorphic endophytes which lack a sexual stage and are not considered to induce disease of hosts (Wilson, 1996).

Teleomorphic endophytes are ascomycetes (Clavicipitaceae: tribe Balansieae) that infect members of the grass family (Poaceae), sedge family (Cyperaceae) and rush family (Juncaceae) (Clay, 1989). The Balansieae is one of three tribes within the

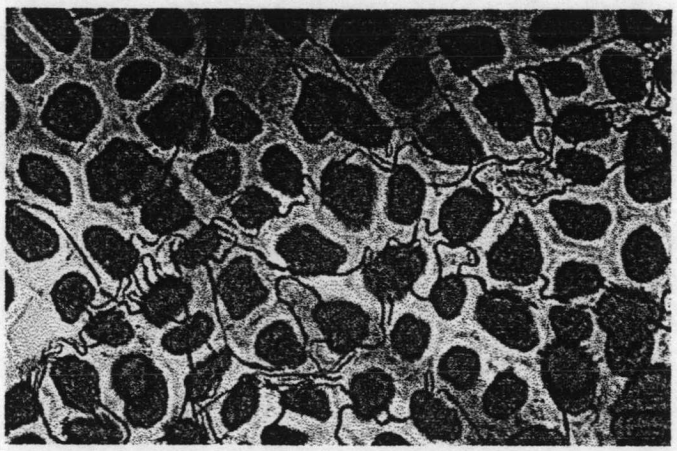
ascomycete subfamily Clavicipitoideae (Clavicipitaceae). The other tribes are Clavicipiteae, containing *Clavicep* spp., and Ustilaginoideae, containing *Ustilaginoidea* spp. (White and Morgan-Jones, 1996). There are five genera (*Atkinsonella, Balansia*, *Balansiopsis*, *Epichloe* and *Myriogenospora* and about 30 species in the tribe (Clay, 1988). These genera are distinguished primarily on the basis of the presence or absence of the macroconidial and microconidial state.

Balansia is the largest genus, with 15-20 species. Unlike *Claviceps*, a related and well known genus of transient ovarian parasites of grass and sedges, the Balansieae fungi are systemic and perennial and most of them are endophytes whose vegetative hyphae are intercelluar and run parallel to long axis of host cells in leaf and stem tissue (Clay, 1988). They produce a sexual stage (teleomorph) of stromata and induce choke diseases which may disrupt reproduction of their hosts (Wilson, 1996). Consequently, infected plants generally are sterile, either due to inhibition of flowering or abortion of developing inflorescences (Clay, 1989).

Anamorphic endophytes are probably asexual derivatives of teleomorphic endophytes, but they lack a sexual stage, rarely sporulate in their hosts, and form mutualistic associations with their hosts. They are imperfect fungi classified in the genus *Acremonium* sect. *Albo-lanosa*, appear to be closely related to the anamorph of *E. typhina*. They are restricted to cool-season grasses, often to genera that also are hosts for *E. typhina*. In culture, many produce conidia similar to those produce by *E. typhina* (Clay, 1989). Unlike the Balansiae, they do not sporulate or produce fruiting bodies on their host plants, but their hyphae occur intercellularly in leaf and stem tissues (Clay, 1988). They are transmitted maternally by vegetative growth of hyphae into ovules and seeds (Figure 2.1). Because the strictly seed-borne endophytes do not produce symptoms on their hosts or suppress flowering and can be vertically transmitted from generation to generation, these anamorphic fungi with affinities to *E. typhina* offer the greatest potential for exploitation as biocontrol agents (Clay, 1989).



(a)



(b)

Figure 2.1 (a) Life cycle of *Acremonium coenophialum*, an endophytic fungus of grasses. The fungus only produces hyphae that grow between plant cells (Paracer and Ahmadjian,2000).

(b) Endophytic hyphae in aleurone layer of perennial ryegrass seed.

At least 80 genera and hundreds of species of graminoids are known hosts for clavicipitaceous endophytes. Clavicipitaceous endophytes of grasses produce a number of biologically active compounds both in *vivo* and in *vitro* that appear to be responsible for their effects on herbivorous animals. Species in the tribe Balansiae are known to produce ergot alkaloids, as is seed-borne endophyte *Acremonium coenophialum* from tall fescue. Their physiological activities against animals are well known from previous research on ergot poisoning by *Claviceps* species. Symptoms of poisoning from infected tall fescue and other grasses resemble the symptoms of ergot poisoning, supporting the idea that they have the same chemical basis (Clay, 1989). Alkaloids produced by such endophytes, including peramine, lolines, indole-diterpenoids and ergopeptines, play a major role in the resistance of infected grasses to a wide range of insect pests (Miles *et al.*, 1998).

2.1.2 Effects of endophytes infection

Although toxicoses induced in domestic herbivores have been known and have been related to grazing fodder for many years, it has only recently been observed that the effects have been correlated with the presence of fungal endophytes in pasture grass populations. The endophytes *A. coenphialum* Morgan-Jones & Gams, from tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) have been a major focus of research associated with toxic syndrome in livestock grazed on infected pastures. Cattle grazing on infected tall fescue plants exhibit toxic effects including slowed weight gain and reduced milk production, increased respiration and body temperature, and gangrene of the extremities. Symptoms are most severe in hot summer months. Ryegrass staggers, a neurological disorder where afflicted animals exhibit severe muscular spasms, occurs in sheep and other domestic animals grazing on endophyte-infected *L. perenne* pastures (Clay, 1989). The total economic loss is hundreds of millions of dollars per year (Clay, 1988).

One of the main reasons for the recent increase in interest in endophytic fungi has been the realization that endophyte infections have effects the grazing of insect pests. The presence of endophytes makes plant tissues unacceptable and unpalatable to insects such that infected tissue is avoided. Additionally, infected tissues may give rise to toxic effects on the insects causing poor larval growth and development, reduction in reproductive capacity or death of individuals and so having more far-reaching effect on insect populations.

Negative effects of Clavicipitaceous endophytes on insects were first reported from endophyte-infected perennial ryegrass by Prestidge *et al.* (1982 cited in Clay, 1989). Most research has focused on tall fescue and perennial ryegrass and has taken one of three major approaches: correlation of endophytes infection level in the field with diversity and abundance of insects present, laboratory feeding experiments where insects have no choice as to type of plant they feed upon, and laboratory choice experiments where infected and uninfected plants are offered simultaneously and insect responses are observed (Clay, 1989).

A range of species have been reported to be negatively affected by endophyte-infected grasses, including crickets, aphids, armyworms and flour beetles (Clay, 1988, 1989). Much of this information has led to the suggestion that endophytic fungi may be suitable biocontrol agents for the protection of grass species. No-choice laboratory feeding experiments consistently have demonstrated that many grasses are more toxic and result in reduced insect survival, growth and developmental rate compared to uninfected conspecies. In laboratory or greenhouse choice experiments many insects actively discriminated between endophyte-infected and uninfected perennial ryegrass and tall fescue. When given a choice, neonate and fourth-instar larvae of fall armyworm significantly preferred leaves from uninfected perennial ryegrass over leaves from infected plants (Clay, 1989).

Many endophytes are transmitted through the seeds of their hosts. There is evidence that seeds may gain protection from herbivores. Rats fed infected tall fescue

seed exhibited a number of significant physiological abnormalities compared to rats fed uninfected seed (Clay, 1989). Cheplick and Clay (1988) found that survival and population growth rates of flour beetles (*Tribolium castaeum*) on ground seeds of infected perennial ryegrass and tall fescue was significantly lower than on uninfected seed. The anti-herbivore properties of endophyte-infected grasses require careful consideration because they may limit the exploitation of endophytes as biocontrol agents. For example, problems with livestock on endophyte-infected pastures are often seasonal or climate-dependent. Most severe effects of tall fescue and perennial ryegrass are limited to hot summer months while in the rest of the year animal performance is satisfactory (Clay, 1989).

Endophyte-infected grasses may posses other properties that are also of significant value. West *et al.* (1987 cited in Clay, 1989) demonstrated that nematode counts were significantly higher in plots of endophyte-free tall fescue than in plots of infected tall fescue. Resistance to nematodes may be present in a wider variety of endophyte-infected grasses. Infected grasses might also be more resistant to fungal pathogens. Culture studied by White and Cole have demonstrated that there is antagonism between endophytic fungi and common grass pathogens (1986 cited in Clay, 1989). Several researchers have suggested that endophyte-infected tall fescue plants are more drought-tolerant than uninfected plants. Infected plants are able to persist and recover more rapidly than uninfected plants following severe water stress, providing a considerable advantage is environmentally stressful habitats (Clay, 1989).

2.2 Non-grass endophytes

2.2.1 Distribution

Endophytic fungi inhabiting the foliage of woody plants have been far less studied than the endophytes of grasses, although they are usually more diverse and abundant than those in grasses (Feath and Hammon, 1997). Symptomless endophyte infections of plants other than grasses have been known for more than seventy years.

Most investigation on the endophytic fungi of trees and shrubs have been carried out over the last fifteen years, since the presence of symptomless ascomycota and mitosporic fungi infections were demonstrated in the needles of European conifers (Petrini, 1996).

Endophytes can be transmitted from one generation to next through the tissue of host seed or vegetative propagules. Except in the grasses, however, most endophytes appear to be transmitted horizontally, external to host tissue, by spore; climate can greatly influence spore germination and resultant infection frequency of host plants (Carroll, 1988). Such fungi have been isolated from virtually every host and every plant tissue investigated. Among trees these hosts include a wide variety of the Fagaceae, a species of *Carpinus*, species of *Eucalyptus*, the tropical palm *Euterpe oleracea* and a miscellaneous assemblage of other woody species (Bettucci, Alonso and Tiscornia, 1999; Carroll and Carroll, 1978; Fisher, Petrini and Sutton, 1993; Fisher *et al.* ,1994; Hata and Futai, 1996; Johnson and Whitney, 1989a, b,1992, 1994; Muller and Hallaksela, 1998; Petrini and Carroll, 1981; Petrini and Fisher, 1990; Rodrigues and Samuels, 1990; Rodrigues, 1994; Sherwood-Pike, Stone and Carroll, 1986; Suske and Acker, 1987, 1989)

Patterns of distribution have been described on several levels i.e. among diverse stands widely scattered at a landscape level, between geographically close but ecologically distinct stands, among individual trees within a stand, among needles on a single branch, and among truly microscopic sampling units within needles and small patches of bark. At a landscape level, distribution patterns are seen to be determined by host specificity, liquid precipitation, canopy cover and geographic continuity or disjunctness. At the level of the individual stand or tree, height in the crown may become important. At the level of individual branch system, age of substrate appears the most important factor. At a microscopic scale, tissue specificity, leaf topography and phenology of the infection process with respect to leaf development all play a role. When endophytic mycofloras and overall infection levels in a single host species have been compared among trees growing in ecologically diverse sites, one environmental

variable, liquid precipitation (rain, dew, fog interceotion) appears as an important determinant of endophyte infection frequencies (Carroll, 1995).

Carroll and Carroll (1978) showed the infection frequencies of endophytes in Douglas-fir collected over a wide geographical range to be positively correlated with annual precipitation (rain, mist, fog drip) and negatively correlated with elevation. Many endophytes produce masses of slimy conidia, a characteristic often associated with rain dispersal.

Rodrigues (1994) found that leaves were more highly infected when collected during the wet season than during the dry season. These trends come as no surprise because endophytic infection in trees must be initiated through fungal propagules. Endophytic communities of trees of the same species, growing at the same location are generally similar but marked differences in species richness and distribution of selected fungal species can be detected between young and old individuals of the same host (Petrini, 1991).

Studies of fungal endophytes of leaves, xylem and bark of *Eucalyptus nitens* in Australia and England have shown that the geographic origin of the sample often determines the composition of the endophyte assemblages (Fisher, Petrini and Sutton, 1993). Site-related differences may be caused by the density of the stands but trees growing outside their natural distribution area may also tend to become colonized by indigenous fungi and not by their host-specific symbionts (Fisher *et al.*, 1994). A study by Fisher *et al.* (1993) confirmed that the endophyte assemblages of trees planted outside their original range are consist of species different from those in native habitats. Higher colonization rates by endophytic fungi can be observed for samples from homogeneous stands with a closed canopy (Petrini and Carroll, 1981 ; Petrini, Stone and Carroll, 1982).

Several workers have investigated the distribution of endophytes within the crowns of individual trees. Bernstein and Carroll (1977) found no correlation between

height in the crown and endophyte infection frequencies. However, Johnson and Whitney (1989a) showed that infection frequencies decline with increasing height in the crown. The incidence of colonization with different endophytes showed a significant tendency to increase with advancing age of host tissue (Bernstein and Carroll, 1977; Fisher, Anson and Petrini, 1986 ; Petrini and Carroll , 1981).

Rodrigues (1994) found that overall, fungal colonization was positively correlated with leaf age, plant growth stage, site and the interactive effects of growth stage versus season and growth stage versus site. Information on endophyte distribution is based on culture work resulting from experiments in which the physical size of individual sampling units is arbitrarily determined (Carroll, 1995). The available visual evidence suggests that endophytes occupy extremely limited domains within plant tissue and may often be confined to the lumen of single cells (Stone, 1987 ; Suske and Acker, 1987, 1989).

Bisseger and Sieber have studied the microdistribution of bark endophytes in coppices of European chestnut in Switzerland, suggesting that finer subdivision of the patches might reveal even more heterogeneity in endophyte distribution (Carroll, 1995). Species composition of endophyte population of white fir needles and the relative occurrence of each fungal species is dependent on the forest management type applied to the site studied. Clear-cutting and plantations tend to reduce the transmission of endophytic fungi. Air pollutants apparently also affect species composition and frequency of colonization by a given species in endophyte communities (Petrini, 1991). Helander *et al.* (1994) has shown that endophyte colonization was reduced on pines treated with spring water acidified with either sulphuric acid alone or in combination with nitric acid.

2.2.2 Fungal Diversity

The fungal taxa of non-clavicipitaceous endophytes belonging to the Ascomycota, Basidiomycota, Deuteromycota. Representative of these fungal groups are shown in Table 2.1

Table 2.1 Fungi isolated as endophytes from different host plants.

Fungi	References
Ascomycota	
Chaetomium spp.	2,5,6,7
Daldinia escholizii	11
Gelasinospora spp.	5
Guignardia spp.	12
Hypoxylon spp.	11
Nemania subannulata	11
Pezicula spp.	13
Phomatospora spp.	10
Pleospora spp.	5, 6, 7
Sporormiella spp.	5, 6, 7, 12
Sordaria spp.	6, 7, 14
Xylaria spp.	2, 4, 6, 7, 11,12, 13, 15
Deuteromycota	
Coelomycetes	
Colletotrichum spp.	2, 7, 8, 11, 12,15
Coniothyrium spp.	3, 5, 7
Cryptosporiopsis spp.	7, 8, 14
Diplodia spp.	11
Pestalotia sp.	11
Pestalotiopsis spp.	2, 6
Phoma spp.	2, 7, 11, 14
Phomopsis spp.	2,4,5,6,7,8,11,12,15
Phyllosticta spp.	1, 4, 7

Table 2.1 (continued)

Fungi	References
Hyphomycetes	
Acremonium spp.	2, 3
Alternaria spp.	2, 3, 5, 6, 7, 9, 12
Arthrinium spp.	8
Aureobasidium spp.	2, 6, 7, 9, 14
Cladosporium spp.	2, 3, 4, 5, 6, 7, 9, 11
Curvularia spp.	2, 11, 12
Drechslera spp.	12
Fusarium spp.	2, 5, 6, 7, 11, 14
Geniculosporium spp.	4, 5, 7
Gilmaniella spp.	6
Hormonema spp.	6, 9, 13
Humicola spp.	11
Idriella spp.	15
Nigrospora spp.	2, 6, 7, 8, 11, 12
Nodulisporium spp.	7, 9, 12, 13, 14
Penicillium spp.	2, 6, 7
Periconia spp.	7, 12
Phialophora spp.	2, 5, 9
Ramularia spp.	5
Trichoderma spp.	2, 6, 11
Agonomycetes	
Mycelia sterilia	2, 3, 5, 6, 7, 9,11,12
Rhizoctonia spp.	5
Basidiomycota	
Schizophyllum commune	6
Luellia sp.	13

References: 1, Bernstein and Carroll, 1977; 2, Bettucci *et al.* 1999; 3, Cabral *et al.* 1993; 4, Carroll and Carroll, 1978; 5, Fisher *et al.* 1986; 6, Fisher *et al.* , 1993; 7, Fisher *et al.* 1994; 8, Hata and Futai, 1996; 9, Helander *et al.* , 1994; 10, Lodge, Fisher and Sutton, 1996; 11, Mekkamol, 1998; 12, Pereira *et al.* 1993; 13, Petrini and Carroll, 1981; 14, Petrini and Fisher, 1990; 15, Rodrigues, 1994

Many species of endophytes will not sporulate in agar culture. The time needed for sporulation may be weeks or months. Therefore isolates need to be identified and characterized as soon as possible. Because fungal names are tied to sporulating structures and because the majority of plant-inhabiting fungi have been named based on structures produced on the host, examination of the host for fungi is a logical starting point. If an extended investigation of a single host plant is anticipated, then before starting isolations, it may be worthwhile to gather data on what fungi should be expected. Collections of recently dead twigs, stems, leaves and litter should be examined for sporulating structures.

Coelomycetes will be among the most frequent and the most problematic taxa encountered. The identification of endophytic coelomycetes is especially frustrating because scarcely any cultural data is available for many of the predominant taxa. Pleomorphy among pycnidial, sporodochial, hyphomycetous and yeast-like states is common. If possible, identification should be carried out from structures developing on host material because descriptions of the majority of these fungi are available only from sporulating structures collected in nature(Bills, 1996).

Another major group that is consistently encountered in most plants is the Xylariales. The identification of Xylariaceous endophytes is difficult because they very seldom form the teleomorph in culture. A correct identification of endophytic xylariaceae is problematic even after direct comparison with cultures derived from ascospores of known teleomorphs (Petrini and Petrini, 1985) and a combination of morphological characters with biochemical analyses is probably the only way to achieve a satisfactory identification of species (Rodrigues and Petrini, 1997). Isozyme analysis

has proven to be a useful method to investigate relationships within and among populations of fungi. Brunner and Petrini (1992) have used isozyme electrophoresis for taxonomy of some *Xylaria* sp. and xylariaceous endophytes.

Fungal secondary metabolites are also potential taxonomic markers, especially if production of these compounds can be shown to be consistently associated with more established taxonomic traits (Christensen *et al.*, 1993). Information of rDNA sequences is becoming an important tool in addition to conventional morphological data such as spore formation. In particular base sequences of 18s rRNA genes were useful to identify non-spore forming fungi, for most of fungal endophytes are non-spore forming and conventional taxonomic keys are not therefore available (Yoshida *et al.* , 1999)

2.2.3 Effects of endophyte infection and secondary metabolites were produced by endophytic fungi

Studies of agricultural grasses generally showed that endophytic fungi negatively affect vertebrate and invertebrate macroherbivores (Clay, 1988). Early studies of woody plants also indicated that endophytes confer resistance to macroherbivores, particularly sedentary, internally feeding insects. For example, in 1981, Webber showed that an inner bark endophyte in the genus Phomopsis increased mortality and deterred oviposition of a scolytid beetle on Dutch elm trees. The endophyte was antagonistic toward the *Ceratosistis ulmi* fungus fed upon by the bark beetle larvae (Wilson and Carroll, 1997). Sherwood-Pike *et al.* (1986) found that the endophyte *Rhabdocline parkeri* infecting needles of Douglas fir had negative effects on species of gall midge of the genus *Contrarinia*. Similarly, the endophyte *Discula quereina* increased mortality of the gall-former *Besbicus mirabilis* in Garrey oak (Faeth and Hammon, 1997). Other correlations between fungal infection and insect mortality within galls are known for *Adelges abietis* on white spruce, with galls infected by *Cladosporium sphaerospermum* (Carroll, 1988).

In 1989, Petrini *et al.* found that the endophytic fungi *Phyllosticta* sp. and *Hormonema dematioides* were dominant in the balsam fir *Abies balsamea.* The first fungus colonized preferentially galled needles attacked by *Paradiplosis tumifes*, being a candidate for biological control (Azevedo *et al.*, 2000). In the black spruce *Picera mariana*, Johnson and Whitney (1994) found that 21 hypha extracts out of 100 were toxic to *Christoneura fumiferana,* and extracts of fungi isolated from older leaves were more effective than young ones in cell culture. The dry weight and development of larvae fed on hyphae extracts were reduced.

Since these pioneering work in the field, the capacity of an endophytic fungus to repel insects, induce weight loss, growth and development reduction and even to increase pest death rate, was correlated with toxin production. For instance, in 1986, Miller showed that the protection of Canadian fir against the spruce budworms resulted from the production of toxic secondary metabolites by endophytic fungi (Azevedo *et al.*, 2000). Clark, Miller and Whitney (1989) showed in balsam fir *Abies balsamea* and red spruce *Picera rubens*, that from 900 samples of fungal isolates, five strains produced toxic substances and three of those produced powerful toxins that, once extracted and given to insects, caused death and decreased development rate of spruce budworm larvae.

In 1992, Calhoun *et al.*, for the first time, identified toxic products synthesized by endophytic fungi in woody plants and that were able to modify growth and death rates in larvae of the spruce budworm *C. fumiferana* feeding on balsam fir. The endophytes in this case were identified as *Phyllosticta* and *Hormonema dematiodes* and the toxic compounds were mainly heptelidic acid and rugulosine (Azevedo *et al.*, 2000).

Bills *et al.* (1992) also detected the existence of tremorgenic mycotoxins, paspalitrem A and C in tropical woody plants infected with an endophytic fungus from the *Phomopsis* sp. Polishook *et al.* (1993) reported the isolation and antibiotic activity of preussomerin D from the *Hormonema dematioides* Lagerbery & Melin recovered from

living plant tissue of a coniferous tree. Endophytic *Pezicula* species were isolated from living branches of ten deciduous and coniferous trees and were shown to produce fungicidally active metabolites (mycorrhizin and cryptosporiopsin) that were toxic to pathogens of their hosts (Schulz *et al.*, 1995).

In Xylariaceous fungi, many of which live endophytically, secondary metabolites produced by representatives of at least one third of these genera have been isolated and identified. The major compounds, which are produced in static culture, can be grouped as dihydroisocoumarins, punctaporonins, cytochalasins, butyrolactones and succinic acid derivatives (Whalley and Edwards, 1995).

Taxol, an important and expensive anticancer drug, was originally isolated from *Taxus brevifolia* (Pacific yew). The discovery of *Taxomyces andreanae*, a fungal endophyte which was isolated from the phloem (inner bark) of the Pacific yew was the first demonstration that any organism other than the host plant could produce taxol. With the discovery that certain endophytic fungi are able to produce taxol has come the possibility that a cheaper and more widely available product may eventually be available via industrial fermentation (Li, Sidhu, Bollon *et al.* , 1998; Li, Sidhu, Ford *et al.* , 1998; Stierle, Strobel and Stierle, 1993).

Recently, Lu *et al.* (2000) reported that a *Colletotrichum* species, an endophyte from *Artemisia annua* can produce in vitro metabolites that were shown to be antimicrobial. These findings suggested the possibility that the endophytic *Colletotrichum* sp. in *A. annua* could protect the host by producing metabolites, which may be toxic or even lethal to phytopathgens. On the other hand, the fact that the endophyte *Colletotrichum* sp. can also produce plant regulators such as IAA. This raises the possibility that the presence of the fungus in *A. annua* could regulate the growth of the host.

2.3 Isolation technique of endophytic fungi

Most procedures for isolating endophytes are comparatively simple and routine for one skilled in basic plant pathological or microbiological techniques. However, handling and maintaining the often hundreds of isolates, characterizing the isolates taxonomically, and quantitatively interpreting the results can be burdensome and overwhelming. The technique and materials used for isolation, maintenance identification and preservation of endophytes of grasses have been reviewed recently by Bacon in 1990. The isolation and identification methods for endophytes of non-grass phanerogams was discussed in the last review by Petrini in 1986 (Bills, 1996).

The logistics of moving fresh plant materials to culturing facilities while maintaining tissue in good condition can be a formidable job when working with plants from remote areas. Precautions must be taken in the shipment of living plant material for culture isolation so the need to prevent desiccation and tissue death is balanced with the requirement for proper ventilation. Aeration maintains respiration, prevents over humid conditions and suppresses growth of epiphytic fungi and bacteria. Additionally, there always exists the possibility that the tissue of interest could have been infected by fungal propagules after their collection but before the surface sterilization procedure. Ideal conditions for germination of epiphytic caulo – or phylloplane fungi can be created by maintaining materials in humid environments at ambient temperatures. Therefore, prolonged transport in sealed plastic bags should be avoided if possible (Bills, 1996).

Bernstein and Carroll (1977) recommended transporting conifer foliage in unclosed bags for local transport. It is very important to always select vigorous and disease-free plant material for isolation work, as this prevents the isolation of localized pathogenic endophytic organisms. The isolation of several taxa of fungi may present some problem, since fast growing species prevent the growth of other species. However, selection of the appropriate incubation temperature and media can circumvent this problem.

Sterilization methods continue to vary widely, but, the preferred method is a three–step ethanol, sodium hypochlorite (NaOCl), ethanol treatment. The choice of sterilization

times, concentrations, and volumes will be dictated by the thickness of sample, the relative permeability of its surface and the texture of its surface. Serial washing offers the advantage of eliminating the penetrating and killing effects of sterilizing chemicals. Pre-washing with tap water can help reduce the time needed for surface sterilization. This is especially important if very tiny fragments of tissue are being used. (Bills, 1996).

Surface–sterilized plant material may be examined with a light microscope for the occurrence of internal hyphae. Fungal endophytes are stained with an aniline blue–lactic acid stain. Another stain used to detect endophytic fungi within plant tissue is rose bengal (Bacon and Hinton,1997). Media used for culture of phytopathogens will be equally applicable to endophytic isolates. Generally prevalent among studies of endophytes are various formulations of malt agar. When handling many unknown species derived from isolation from vegetative growth a useful approach is screen each isolate on several media simultaneously (e.g. cornmeal agar, oatmeal agar, V8 juice agar, etc.) in 60 mm plates. Antibacterial antibiotics should always be included in any primary isolation medium for fungi. Oxytetracycline, chlorotetracycline streptomycin sulfate, and novobiocin have been used most frequently for endophyte isolation.

2.4 Antibiotics

Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: antibiotic, which are natural substances produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized. A hybrid substance is a semi synthetic antibiotic, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desirable properties (Kenneth, 2002).

2.4.1 Definition of antibiotics

Antibiotics are substances produced by microorganisms that kill or inhibit other microorganisms. Antibiotics may have e.g. antibacterial, antifungal, antiviral, antiparasitic or even anticancer activity (Kenneth, 2002).

2.4.2 Desirable properties of a clinically useful antibiotic are as follow (Kenneth, 2002):

1. It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
2. It should be nontoxic to the host and without undesirable side effects.
3. It should be non-allergenic to the host.
4. It should not eliminate the normal flora of the host.
5. It should be able to reach the part of the human body where the infection is occurring.
6. It should be inexpensive and easy to produce.
7. It should be chemically stable.
8. Resistance by microbes is and remains uncommon

2.4.3 Type of antibiotics (Kenneth, 2002)

The great number of diverse antibiotics currently available can be classified in different ways, e.g., by their chemical structure, their microbial origin, or their mode of action. They are also frequently designated by their effective range. Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of bacteria or other microorganisms that are affected by a certain antibiotic are in expresses as its spectrum of actions. Antibiotics effective against prokaryotes which kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be broad spectrum. If effective mainly against Gram-positive or Gram-negative bacteria,

they are narrow spectrum. If effective against a single organism or disease, they are referred to as limited spectrum.

2.4.4 Sources of antimicrobials (Kenneth, 2002)

Antimicrobials are derived primarily from three major sources:

- Molds or fungi
- Bacteria
- Synthetic or semi-synthetic processes

Penicillium and *Cephalosporium*: produce Beta-lactam antibiotics: penicillin, cephalosporin, and their relatives. Actinomycetes, mainly *Streptomyces* species: produce tetracyclines, aminoglycosides (streptomycin and its relatives), macrolides (erythromycin and its relatives), chloramphenicol, ivermectin, rifamycins, and most other clinically useful antibiotics that are not beta-lactams. *Bacillus* species, such as *B. polymyxa* and *Bacillus subtilis* produce polypeptide antibiotics (e.g. polymyxin and bacitracin), and *B. cereus* produces zwittermicin.

These organisms all have in common that they live in a soil habitat and they form some sort of a spore or resting structure. It is not known why these microorganisms produce antibiotics but it may rest in the obvious : affording them some nutritional advantage in their habitat by antagonizing the competition; or the subtle: acting as some sort of hormone or signal molecule associated with sporulation or dormancy or germination. Antibiotics are secondary metabolites of microorganisms and they are produced at the same time that the cells begin sporulation processes. Antibiotics tend to be rather large, complicated, organic molecules and may require as many as 30 separate enzymatic steps to synthesize. The maintenance of a substantial component of the bacterial genome devoted solely to the synthesis of an antibiotic leads one to the conclusion that the process (or molecule) is important, if not essential, to the survival of these organisms in their natural habitat. Most of the microorganisms that produce

antibiotics are resistant to the action of their own antibiotic, although the organisms are affected by other antibiotics, and their antibiotic may be effective against closely related strains.

Synthetic antimicrobials, e.g., the sulfonamides, have always constituted an important source of antimicrobials. Semi-synthetic antimicrobials are those derived from chemical modifications of naturally occurring antibiotics. This constitutes an ever more important group of antimicrobials as new drugs, with special properties, are developed.