Endophytes inhabit theoretically every plant on the earth. They have gained increased importance after these have been reported as a novel source of potentially useful bioactive molecules e.g., anticancer, immunosuppressive and anti-viral compounds, alkaloids, antibiotics, anti-oxidants, cytochalacins etc. This has raised the hope that medicinally important plants might escape the wrath of mankind in their over exploitation for extraction of bioactive molecules from them and might survive the danger of getting extinct in near future. One of the major problems facing the future of endophyte biology is the rapidly diminishing rainforests, which hold the greatest possible resource for acquiring novel microorganisms and their products. The present review is to consolidate the data available on the role of endophytes producing various important bioactive molecules of therapeutic importance.

Key Words: Endophytic Microorganisms; Bioactive Molecules; Life Cycle; Isolating Endophytes; Eco-friendly; Torreyanic Acid; Cytochalacine; Antibiotics; Alkaloids; Pesticides; Biodiversity.

1 Introduction

The term endophyte refers here to microorganisms mostly fungi inhabiting plant organs without causing apparent harm to their host [1]. Plant tissues remain entire and functional. Since the discovery of fungal endophytes in conifers [2] numerous investigations on their abundance, species composition and response to various environmental factors have been published. Endophytes have one or more varieties of interactions with their host plant: Some fungi are widespread and are found on many different plant species; others are highly specific to a single host in a specific environment. Further, a diverse array of interactions between plant and fungus have been found. Given that a huge array of fungi may be isolated from any one host, it seems possible that endophytes will have one or more arrays of functions, most of which are unknown at present [3]. However, in some thoroughly investigated cases, the fungus-plant relationship has turned out to be mutualistic/symbiotic, as the endophyte acts antagonistically against insect herbivores [4], [5]. Hence, it has been proposed that short-cycle (one or several generations each year) endophytes increase the resistance potential of long-living trees against the effects of short-cycle insects [6].

Endophytic microorganisms are to be found in virtually every plant on earth. These organisms reside in a symbiotic relationship with its host plant, obtaining nutrients from it and in return providing it protection against insect pests. Endophytes are invisible to the naked eye and must be stained and viewed under a microscope to be identified. Of the approximately 300,000 higher plant species inhabiting the world, each individual plant is a host to these unique microorganisms (Strobel, unpublished data). Only a few of these plants (grass species) have been studied relatively complete for their endophytic biology. Consequently, the opportunity to find new and interesting endophytic microorganisms among myriad of plants in different settings and ecosystems is not only very great but also a thrilling challenge.

Plants may benefit from the presence of endophytes in many ways. Potential plant benefits have been examined in only a few cases. *Rhabdocline parkeri* produces a compound that reduces needle attack by borers. Metabolites produced by *Phomopsis* sp in cotton appear to deter larvae of *Helicoverpa* from feeding on leaves. Similar properties have been reported with *Neotyphodium*. In addition, aphids feeding on leaves of cotton may become colonised by *Lecanicillium lecanii*, when conditions permit. Thus the aphid may be killed or it may transfer the fungus to another leaf. In addition, endophytes may upregulate host responses to pathogens and pests. *Chaetomium globosum* has been shown to increase host resistance to rust and tan spot pathogens in wheat. Direct interactions appear to be too small to measure in this case. Presence of *Lecanicillium lecanii* appears to reduce the feeding by aphids from leaves of cotton. The interaction is probably due to induction of host responses, which is perplexing because plant regulatory pathways for responses to insects and pathogens are not thought to be complementary. Endophytes appear to have direct and induced effects on plant responses to biotic agents. The interaction with abiotic agents remains largely unexplored.

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Because of what appears to be their contribution to the host plant, the endophytes may produce ample substances of potential use to modern medicine, agriculture and industry. Novel antibiotics, antifungal agents, immunosuppressant, topoisomerase inhibitors and antineoplastic compounds are only a few examples of what has been found after the isolation, culture, purification, and characterization of some novel endophytes in the recent studies. The potential prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great.

Endophytic fungi are an important, yet relatively unstudied group of microbial plant symbionts. Endophytic fungi live symptomatically, and sometimes systemically, within plant tissues. Endophytes usually inhabit above-ground plant tissues (leaves, stems, bark, petioles and reproductive structures), which distinguishes them from better known mycorrhizal symbionts. The distinction is not firm, because endophytes may also inhabit root tissues. Overall, endophytic fungi are ubiquitous and extremely diverse in host plants. Every plant examined to date harbors at least one species of endophytic fungus and many plants, especially woody plants, may contain literally hundreds or thousands of species.

In the past two decades, a great deal of information on the role of endophytic microorganisms in nature has been collected. The capability of colonizing internal host tissues has made endophytes valuable for agriculture as a tool to improve crop performance. Webber was probably the first researcher who reported an example of protection given by an endophytic fungus to plant by resisting the growth of insects, in which the endophyte Phomopsis oblonga protected elm trees against the beetle Physococneum brevilineum. After four years, Claydon et al. confirmed it and showed that endophytic fungi belonging to the Xylariaceae family synthesize secondary metabolites in hosts of the genus Fagus and that these substances affect the beetle larvae.

The best studied endophytic plant/microbes system to date is the grass species/Neotyphodium species relationship (Plate 1). This fungal species provides a host of biologically active compounds including toxic alkaloids such as Peramine (deters insect pests), Loliotrem B (deters larvae feeding) and Ergovaline (provides resistance to black beetle). However, enormous opportunities exist for the recovery of novel forms, including genera, biotypes and species in the myriad of plants yet to be studied. It is now well established that endophytes are a rich and reliable source of genetic diversity and may represent morphological and molecular levels, often have been associated with them novel natural products. However, there has to be a sound and specific rationale for the selection of each plant for endophyte isolation and natural product/secondary metabolite discovery. The most accepted such rationale is the following:

a) Plants from unique environmental settings, especially those with unusual biology and possessing novel strategies for survival;
b) Plants that have an ethanobotanical history or use by the indigenous people that is related to the specific uses or applications of interest i.e., through direct contact with local people or via local literature e.g., healing powers of a botanical source, in fact, may have nothing to do with the natural products of the plant, but of the endophyte inhabiting the plant;

c) Plants that are endemic, having an unusual longevity, or that have occupied certain ancient land mass, such as Gondwanaland, are also more likely to lodge endophytes with active natural products than other plants;

d) Plants growing in area of great biodiversity also have prospects of housing endophytes with great biodiversity e.g., tropical and temperate rainforests.

2 Isolating Endophytes

Endophytes are defined as microorganisms, mostly fungi, colonizing healthy plant tissue without causing overt symptoms in or apparent injury to the host [18]. Many, if not all woody plant stems harbor neutral stem endophytes but because they are symptom-less they are difficult to detect and can only be successfully surveyed by plating out carefully prepared surface-sterilized tissues. For example, a study on the endophytes isolated from *Alnus* xylem and barks tissues in roots and stems yielded 85 different fungal taxa [19]. This is considered a fairly typical taxa for this type of study. Among the other woody plants surveyed for endophytes are *Ilex, Hedera, Ruscus, Ulex, Pinus, Fagus, Juniperus, Fraxinus* and *Quercus*. Members of the Ericaceae (*Rhododendron, Vaccinium*, etc.) also harbor a diverse group of endophytes that have not been thoroughly investigated. Endophytes are generally not considered organ-specific, and it is likely that many of the species isolated from stems also occur in leaves. Most procedures for isolating endophytes are so simple and can be followed by anyone skilled in basic microbiological techniques. One of the critical needs for isolating endophytic fungi is obtaining fresh plant material. The need for preventing desiccation must be balanced against the need for adequate aeration, the former slows tissue death, whereas the latter minimizes the growth of secondary contaminating fungi and bacteria. If plants are to be stored for long periods of time, especially in frost-free refrigerators, tissue desiccation will occur. However, it is possible to isolate a surprising number of fungal species even from desiccated woody tissues after freezer storage of more than a year [18]. One of the critical needs for isolating endophytic fungi is obtaining fresh plant material. Isolation procedures with reference to sterilization methods and parts of plants have been given in Tables 1 and 2.

### Table 1. Sterilization of samples for the isolation of endophytes

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Minutes in NaOCl</th>
<th>Dilution of NaOCl</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lichens</td>
<td>1</td>
<td>1:5</td>
<td>Petrini (1986)</td>
</tr>
<tr>
<td>Mosses</td>
<td>1</td>
<td>1:5</td>
<td>Petrini (1986)</td>
</tr>
<tr>
<td>Ferns</td>
<td>3</td>
<td>1:5</td>
<td>Dreyfuss &amp; Petrini (1984)</td>
</tr>
<tr>
<td>Conifer needles</td>
<td>5</td>
<td>1:2</td>
<td>Carroll &amp; Carroll (1978)</td>
</tr>
<tr>
<td>Conifer twigs</td>
<td>7</td>
<td>1:2</td>
<td>Carroll &amp; Carroll (1978)</td>
</tr>
<tr>
<td>Monocots-leaves &amp; culms (<em>Triticum</em>)</td>
<td>3</td>
<td>1:5</td>
<td>Petrini (1986)</td>
</tr>
<tr>
<td>Dicots (general)</td>
<td>3</td>
<td>1:5</td>
<td>Petrini (1986)</td>
</tr>
<tr>
<td><em>Erica</em> leaves</td>
<td>3</td>
<td>1:5</td>
<td>Petrini (1986)</td>
</tr>
<tr>
<td><em>Erica</em> stems</td>
<td>5</td>
<td>1:5</td>
<td>Petrini (1986)</td>
</tr>
<tr>
<td><em>Rhododendron</em></td>
<td>3</td>
<td>1:5</td>
<td>Petrini (1985)</td>
</tr>
<tr>
<td><em>Vaccinium</em></td>
<td>3</td>
<td>1:5</td>
<td>Petrini (1985)</td>
</tr>
</tbody>
</table>

**Isolation of endophytes:** For surface sterilization of the material following sequential steps are followed: A) first dip the sample in 96% EtOH for 1 min (lichen and mosses for only 30 secs.); B) sterilization in NaOCl; C) second dip in 96% EtOH for 30 secs.

### Endophytes: Eco Friendly

A heap of properties of endophytes have been already elaborated, as endophytes may produce substances of potential use to modern medicine, agriculture, and industry like antibiotics, antimiycotics, immuno-suppressant, and anticancer compounds. So by this we can use the term eco-friendly for endophytes. Here we have tried to exploit the uses of endophytes fully so that more and more consideration to be given to endophytes for the human welfare.

Frequently, it has been noticed that many endophytes are isolated from the plant species but one or few biotypes of a given isolate will produce the desired biological active compound in culture [20]. A great deal of uncertainty also exists between what the endophyte may produce in culture and what it may be producing under natural endophytic conditions.

There are very valid reasons to believe that some endophytes produce certain phytochemicals, originally characteristic of host plant, which might be related to a genetic recombination of the endophyte with the host DNA in evolutionary time [21]. This argument was also produced to explain the production of taxol by *Taxomyces andreanae* [22]. Thus if the endophyte can produce the same rare or important bioactive compound as their host plants, this would not only reduce the need to harvest slow growing or possibly rare plants but also help preserve world’s ever diminishing biodiversity. Besides, a microbial source of high value product may be easier or economical to produce effectively, thereby reducing its market price.

### 4 Endophytes: Source of Bioactive Molecules

A number of natural products have been reported to have been obtained from endophytes with potential in pharmaceutical and agrochemical arenas [23]. One of the important examples is that of *Cryptosporiopsis*
Vijeshwar Verma et al.

Table 2. Selected examples of isolation procedures for endophytes from woody plants Bills et al., 1996

<table>
<thead>
<tr>
<th>Tissues/Hosts</th>
<th>Dissection of Tissue</th>
<th>Surface Sterilization</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulex-spines, stems</td>
<td>Pines and 2 cm stem section</td>
<td>1 min 96% EtOH, 3 min 3.25% NaOCl, 0.5 min 96% EtOH</td>
<td>MEA</td>
</tr>
<tr>
<td>Fagus-bark</td>
<td>2 mm borer</td>
<td>Propane torch or none</td>
<td>Bark extract, glucose-yeast extract, benomyl MEA</td>
</tr>
<tr>
<td>Alnus-xylem and bark</td>
<td>1 cm stem sections, bark and xylem</td>
<td>35% peracetic acid</td>
<td>Malt extract with 10 mg/l cyclosporin</td>
</tr>
<tr>
<td>Castanea &amp; Quercus-bark</td>
<td>5 mm arch punch</td>
<td>0.525% NaOCl —10 min</td>
<td>Glucose-yeast extract medium</td>
</tr>
<tr>
<td>Picea-mature stems</td>
<td>Sawing followed by chisel extraction</td>
<td>Only extraction tools sterilized</td>
<td>Malt agar slants</td>
</tr>
<tr>
<td>Picea-towits</td>
<td>1 cm segments</td>
<td>sink washing, ultrasound, serial washing</td>
<td>MEA</td>
</tr>
<tr>
<td>Fraxinus, Quercus, Fagus-twiggs</td>
<td>2 cm segments, and xylem cultured separately</td>
<td>bark ethanol flaming</td>
<td>MEA</td>
</tr>
<tr>
<td>Carpinus-bark</td>
<td>1 cm leather punch</td>
<td>0.525% NaOCl for 3 min., flaming in alcohol lamp</td>
<td>MEA + benomyl and surfactants</td>
</tr>
<tr>
<td>Rhizophora-seedlings</td>
<td>1.1 cm cork borer of hypocotyl and radicle</td>
<td>sterile sea-water rinse, 0.1% HgCl₂ in 5% EtOH</td>
<td>Mangrove-sea water agar</td>
</tr>
<tr>
<td>Licuala-leaves</td>
<td>3 mm discs from veins and interveins</td>
<td>1 min 96% EtOH, 10 min 3.25% NaOCl, 0.5 min 96% EtOH</td>
<td>Cornmeal dextrose agar, MEA</td>
</tr>
<tr>
<td>Euterpe-leaves</td>
<td>3 mm discs from veins and interveins</td>
<td>1 min 75% EtOH, 10 min 3.25% NaOCl, 0.5 min 75% EtOH</td>
<td>Cornmeal dextrose agar with 5 mg/l cyclosporin A</td>
</tr>
<tr>
<td>N. foetida-stem</td>
<td>Twig (young &amp; old)</td>
<td>1 min 95% Ethanol</td>
<td>Sabouradu agar</td>
</tr>
<tr>
<td>Phexandrum-rhizomes</td>
<td>1 cm small pieces</td>
<td>1 min 70% Ethanol</td>
<td>Aqueous agar</td>
</tr>
</tbody>
</table>

from cf. quercina (an imperfect stage of Pezicula cinnamomea) isolated from medicinal plant of Eurasia origin Tripterigeum wilfordii [24-26]. The endophyte produces a unique peptide antifungal named cryptocandin containing a number of peculiar hydroxylated amino acids and a novel amino acid, 3-hydroxy-4-hydroxymethylproline. This antifungal peptide is related to known antifungal, the echinocandins and pneumocandins [27]. This peptide is also active against the rice blast Pyricularia oryzae, a worst plant pathogen and is being used as a model to synthesise other antifungal compounds.

Another very important example of endophyte isolated from the plant source is Pestalotiopsis microsorpa from the bark of Torreya taxifolia producing several compounds having antifungal activities including pestaloside, an aromatic b-glucoside and two pyrones (pestapolyrone & hydroxystapolyprone) having phytotoxics [28]. Also, antifungal activity of Artemisia annua endophyte against phytopathogenic fungi is well recognized [29]. Two new caryophyllene sesquiterpenes (Pestalotiopsins A & B), a new sesquiterpene 2a-hydroxy-dimeninol and a highly functionalized humulane. These caryophyllene sesquiterpenes have also been reported from another endophyte P. microsorpa isolated from Taxus brevifolia [30]. Variations in the amount of bioactive molecules found in these fungi depend upon both the culture conditions and the original plant source from which the endophytes were isolated.

Pestalotiopsis jesteri is another endophytic fungal isolate obtained from a plant species from Papua New Guinea. It produces jesterone and hydroxyjesterone which exhibit strong antifungal activity against a variety of plant pathogenic fungi [31]. Phomopsichalasin is an important metabolite obtained from an endophte Phomopsis species. This metabolite represents the first cytochalasin-type compound with three ring system replacing the cytochalasin macrolide ring. This shows a strong antibacterial activity against Bacillus subtilis, Salmonella gallinarum and Staphylococcus aureus and a moderate activity against Candida tropicalis [32]. A Colletotrichum species isolated from Artimisia annua, a traditional Chinese herb produces metabolites with activity not only against the human pathogenic fungi and bacteria but also metabolites that were fungistatic to plant pathogenic fungi [33].

Besides, there are numerous reports on endophytic bacterial species producing antibiotics and phytotoxic compounds now flooding the literature e.g., ecomycins, representing a family of novel lipopeptides with masses of 1153 ND 1181 Da besides, containing unusual amino acids like homoserine and b-hydroxyaspartic acid [34]. The ecomycins are active against human pathogenic fungi like Cryptococcus neoformans and Candida albicans. Pseudomycins is another group of antifungal...
Endophytes: A Novel Source for Bioactive Molecules

peptides reported from various plant-associated pseudomonads [35, 36]. Pseudomycins are active against a number of important human pathogenic fungi like Cryptococcus neoformans and Candida albicans and also a variety of plant pathogenic fungi including Ceratocystis ulmi and Mycosphaerella fijiensis [36]. Pseudomycins also contain several nontraditional amino acids including L-chlorothreonine, L-hydroxyaspartic acid and both D- and L- diaminobutyric acid.

A number of endophytic streptomycetes have been reported to possess antibiotics e.g., Streptomyces species from Lollium perenne producing methylalbonoursin [37]. Streptomyces species NRRL 30562 from Kennedia nigriscans- an important traditional plant used by Australian aborigines to treat cuts and open wounds produces a host of extremely potent peptide antibiotics[38]. The antibiotics produced from this endophyte is called munumbicins having wide variety biological activities. Streptomyces species NRRL 30566 from Grevillea pteridifolia produces in culture novel antibiotics called kahakumycins that are related to echinomycins [39]. Kakakumycin A significantly inhibited the RNA, protein and cell wall syntheses rates in B. subtilis [39]. However, the effect was lower in DNA synthesis. Recently endophytic streptomycetes have been discovered from the biologically diverse areas like the upper Amazon of Peru yielding streptomycetes species possessing outstanding inhibitory activities against pythiaceous fungi as well as malarial parasite Plasmodium falciparum. The bioactive principle is a mixture of lipopeptides named coronamycin [40]. Endophytic actinomycetes are now being tested regularly and considered for the use in controlling plant diseases [41].

Endophytes have also been reported to produce anticancer principles which are highly useful. Taxol and its derivatives are the first such major bioactive principles reported from an endophytic isolate Taxomyces andreanee from Taxus brevifolii [42]. Pestalotiopsis microspora from Taxus wallichiana, Tubercularia species from Chinese yew T. mairei, Sporormia minima, and Trichothecium species from T. wallichiana have also been shown to produce taxol using 14C precursors[43-46]. This lead to the search for taxol producing endophytes residing plants other than Taxus species even in continents not known to have indigenous Taxus species like South America and Australia. Pestalotiopsis guepini was isolated from Wollemia nobilis (extremely rare and earlier thought to be extinct plant) and it showed to produce taxol[47]. Besides, Scimatocantaruelium tepuense isolated from Maguireothamnus speciosus has also been shown to produce taxol[43-44]. Endophytic fungi making taxol has now been shown to be distributed worldwide and in plants other than yews. The ecological and physiological explanation for the wide distribution of endophytic fungi making taxol seems to be related to the fact that taxol is a potent fungicide against world’s known worst plant pathogens like Pythium and Phytophthora species[48]. Thus these endophytic fungi may be producing taxol and related taxanes to protect their respective host plants from degradation and disease caused by these pathogens. However, taxol production by these endophytes in culture conditions is in the range of sub-micrograms to micrograms per litre. A lot of research work is being undertaken the world over to enhance microbial production of taxol by using activator compounds in the culture medium. Besides, search for the new endophytes producing better quantities of microbial taxol/intermediates continues.

Endophytes encoding important bioactive molecules like camptothecin [8],[49] and podophyllotoxin [50] are reported. The microorganisms involved are novel and have been patented.

Taxol, a highly functionalized diterpenoid (Fig.1), is found in each of the world’s yew (Taxus) species[50]. In electrospray mass spectroscopy, taxol usually gives two peaks, one at molecular weight 854 (M + H+), and the other at molecular weight 876 (M + Na+) [51]. This compound is the world’s first billion-dollar anticancer drug, and it is used to treat a number of other human tissue-proliferating diseases. Its cost makes it unavailable to many people worldwide. Therefore, alternative sources are needed, since organic synthesis, while having been accomplished, is not economically feasible [52]. Given the fact that endophytes are virtually universally present in all of the world’s higher plants, it was reasoned that yew trees conceivably might support certain endophytic microorganisms that also make taxol [51]. Thus, if a microbial source of the drug were available it could eliminate the need to harvest and extract the slow-growing and relatively rare yew trees. The price for the drug would also be reduced, since taxol could be produced via fermentation in much the same way that

![Fig. 1: The structure of taxol](image-url)
penicillin is fermented. It was also speculated that the ability of any endophyte to make taxol may have arisen from the exchange of genetic material from the yew tree to one or more microorganisms living in close association with it [51]. By the early 1990s, however, no endophytic fungi had been isolated or were even known from any of the world’s representative yew species. After several years of effort, a novel taxol-producing endophytic fungus, *Taxomyces andreanana*, was discovered in *Taxus brevifolia*[42]. The most critical line of evidence for the presence of taxol in the culture fluids of this fungus, among others, was the electrospray mass spectrum of the putative taxol isolated from *T. andreanana* as well as C-14 labelling studies, which irrefutably showed the presence of fungal-derived taxol in the culture [51]. This early work set the stage for a more comprehensive examination of the ability of other *Taxus* species and other plants to yield endophytes producing taxol. An excellent example of this is the anticancer drug, taxol, which had been previously supposed to occur only in the plant genus *Taxus* (yew). However, taxol has been demonstrated to occur in a number of unrelated fungal endophytes including *Pestalotia, Pestalotiopsis, Fusarium, Alternaria, Pithomyces, Monochaetia* and others. Thus, this report presents information on the presence of taxol among disparate fungal genera, and others. Thus, this report presents information on the presence of taxol among disparate fungal genera, and therefore this report presents information on the presence of taxol among disparate fungal genera, and supports efforts to study fungal endophytes and preserve their associated host plants. An examination of the endophytes of *Taxus wallichiana* yielded *P. microspora*, and a preliminary monoclonal antibody test indicated that it may produce taxol. After preparative thin-layer chromatography, a compound was isolated and shown by spectroscopic techniques to be taxol. This organism from several ^14^C precursors that had been administered to it produced labeled taxol [43-44]. Furthermore, several other *P. microspora* isolates were obtained from bald cypress in South Carolina and were also shown to produce taxol [20]. This was the first indication that endophytes residing in plants other than *Taxus* spp. were producing taxol. Therefore, a specific search was conducted for taxol-producing endophytes in continents not known for any indigenous *Taxus* spp.

From the extremely rare, and previously thought to be extinct, Wollemi pine (*Wollemia nobilis*), *Pestalotiopsis guepini* was isolated, which was shown to produce paclitaxel [53]. Also, quite surprisingly, a rubiaceous plant, *Maguireothamnus speciosus*, yielded a novel fungus, *Seimatoantlerium tepuiense*, that produces paclitaxel. This endemic plant grows on the tops of the tepuis in the Venzuelan-Guyana region in southwestern Venezuela [25],[26]. Furthermore, fungal paclitaxel production has also been noted in a *Periconia* sp., [54] and in *Seimatoantlerium nepalense*, another novel endophytic fungal species [55]. Simply, it appears that the distribution of those fungi making paclitaxel is worldwide and not confined to endophytes of yews.

### 4.1 As a Source of Torreyaic Acid

Torreyaic acid, a selectively cytotoxic quinone dimer (anticancer agent), was isolated from a *P. microspora* strain. This strain was originally obtained as an endophyte associated with the endangered tree *T. taxifolia* (*Florida torreya*) as mentioned above[56]. Torreyaic acid was tested in several cancer cell lines, and it demonstrated 5 to 10 times more potency in those lines that are sensitive to protein kinase C agonists and causes cell death by apoptosis. Recently, a complete synthesis of torreyaic acid has been successfully completed using the application of a biomimetic oxidation-dimerization cascade [57].

### 4.2 As a Source of Cytochalacins

Some of fungal genera such as *Xylaria, Phoma, Hypoxylon, and Chalara* are representative producers of a relatively large group of substances known as the cytochalasins (Fig 2). The Cytochalasins (Greek cytos cell; chalasis, relaxation) are a group of fungal metabolites, related by structure and biological activity. Major biological effects include inhibition of the division of cytoplasm [58] reversible inhibition of cell movement [59] induction of nuclear extrusion [58],[60], inhibition of such processes as phagocytosis [61-63] platelet aggregation and clot retraction. These compounds possess antitumor and antibiotic activities, but because of their cellular toxicity they have not been developed into pharmaceuticals. Three novel cytochalasins have recently been reported from a Rhinocladiella sp. as an endophyte on *Tripterygium wilfordii*. These compounds have antitumor activity and have been identified as 22-oxa-[12]-cytochalasins [64]. Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi, and workers in this field need to be alerted to the fact that redundancy in discovery does occur. Chemical redundancy (dereplication) usually occurs with certain groups of organisms on which previous studies have already established the chemical identity of major

![Fig.2: Different forms of Cytochalasins](image-url)
biologically active compounds. For instance, as with the cytochalasins, they are commonly associated with the xylariaceous fungi. Since their commercial availability in the early 1970’s, this group of compounds has become the subject of intense cytological research.

4.3 As a Source of Antibiotics

Endophytes possess another important property of producing useful antibiotics. In the Northern Territory of Australia, various Aboriginal groups use the ground-up mass of snakevine (Kennedia nigriscans) to promote the healing of skin wounds and infections. The snakevine, known as “mangerporlo” in Dalabon and Mayali, is harvested as a fresh stem piece, placed on some hot coals for a short time (10 min), mashed into a pulp, and then applied as a sticky paste to a cut, wound, or infection. Due to local and much use of that plant, it was taken as to isolate endophytic microorganisms from it with the logic that some of the healing properties of the snakevine may, in fact, be produced as a result of the products of one or more endophytes. In fact, one of the endophytes isolated from this medicinal plant was a Streptomyces sp. This is of particular interest because, as a culture, it was extremely bioactive against a number of test microorganisms. Interest in this endophyte was further piqued because actinomycetes have not been reported to be endophytic on dicotyledonous plants. However, later a Streptomyces sp. was reported on an annual plant -Lolium perenne [37]. This loliun endophyte also produces a weak antibiotic, named methylalbonoursin, which is a diketopiperazine, condensed from leucine and phenylalanine. Streptomyces spp has also been reported to produce novel peptide antibiotics, named munumbicins A, B, C, and D [38]. Munumbicins A, B, C and D are newly described antibiotics with a wide spectrum of activity against many as well as plant pathogenic fungi and bacteria (Table 3), and a Plasmodium sp. These compounds were obtained from Streptomyces NRRL 3052, which is endophytic in the medicinal plant snakevine (Kennedia nigriscans), native to the Northern Territory of Australia. This endophyte was cultured, the broth was extracted with an organic solvent and the contents of the residue were purified by bioassay-guided HPLC. The major components were four functionalized peptides with masses of 1269-6, 1298-5, 1312-5 and 1326-5 Da. Various other related compounds, having bioactivity, with differing masses and lower quantities were also present in the culture broth extract. The munumbicins possessed widely differing biological activities, depending upon the target organism. If citing example, munumbicin B had a minimal inhibitory concentration of 2.5 μg/ml against a methicillin-resistant strain of Staphylococcus aureus, whereas munumbicin A was not active against this organism[42]. However, the most impressive biological activity of any of the munumbicins was that of munumbicin D against the malarial parasite Plasmodium falciparum, having an IC50 of 4.5±0.07 ng ml⁻¹ [19].

Another useful antibiotic reported to be produced by endophyte is named as Cryptocandin A, which is an antifungal peptide and is isolated from endophytic fungus C. quercina. Cryptosporiopsis quercina is the imperfect stage of Pezicula cinnamomea, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from Tripterigeum wilfordii, a medicinal plant native to Eurasia[24-26]. On petri plates, C. quercina demonstrated excellent antifungal activity against some important human fungal pathogens - Candida albicans and Trichophyton spp. A unique peptide antimycotic, termed cryptocandin, was isolated and characterized from C. quercina[24-26]. This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid: 3-hydroxy-4-hydroxy methyl proline (Fig. 3). The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins[27].

4.4 As a Source of Alkaloids

Many grasses harbor fungal endophytes in the genus Neotyphodium, which enhance host fitness, but some also produce metabolites such as ergovaline believed to cause
livestock toxicoses. In *Claviceps* species the first step in ergot alkaloid biosynthesis is thought to be dimethylallyltryptophan (DMAT) synthase, encoded by *dmaW*, previously cloned from *Claviceps fusiformis*. The cloning and characterization of *dmaW* have been reported from *Neotyphodium* sp. isolate Lp1, an endophyte of perennial ryegrass (*Lolium perenne*). The gene was then disrupted, and the mutant failed to produce any detectable ergovaline or simpler ergot and clavine alkaloids. The disruption was complemented with the *C. fusiformis* gene, which restored ergovaline production. Thus, the biosynthetic role of DMAT synthase was confirmed, and a mutant was generated for future studies of the ecological and agricultural importance of ergot alkaloids in endophytes of grasses [65].

4.5 As a Source of Antioxidants

Endophyte also plays an important role in the production of antioxidants. Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *P. microspora*, an endophyte isolated from a combretaceaeous plant, *Terminalia morobensis*, growing in the Sepik River drainage of Papua New Guinea [66],[17]. Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was suspected of antioxidant activity based on its structural similarity to the flavonoids (Fig. 4). Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution[17]. Pestacin was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity [66]. The antioxidant activity of pestacin is believed to have arisen primarily via cleavage of an unusually reactive C—H bond and to a lesser extent, though O—H abstraction[17]. The antioxidant activity of pestacin is at least 1 order of magnitude greater than that of trolox, a vitamin E derivative [66].

4.6 As a Source of Immunosuppressive Compounds

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinol A and B [28]. Subglutinol A and B (Fig. 5) are equipotent in the mixed lymphocyte reaction assay and thymocyte proliferation assay, with a 50% inhibitory concentration of 0.1 µM.

4.7 As a Source of Antiviral Compounds

An endophytic fungus also plays a role in the inhibition of viruses. Two novel human cytomegalovirus protease inhibitors, cytonic acids A and B, have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures as p-tridepside isomers were elucidated by mass spectrometry and NMR methods [67]. It is apparent that the potential for the discovery of compounds, from endophytes, having antiviral activity is in its infancy. The fact, however, that some compounds have been found is promising. The main limitation in compound discovery is probably related to the absence of appropriate antiviral screening systems in most compound discovery programs.

4.8 As a Source of Insecticides and Pesticides

In the early 80’s it was demonstrated that the presence of endophytic microorganisms in their respective hosts could result in the reduction of insect attacks [4]. demonstrated the existence of plant protection against
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insects provided in which the endophyte Phomopsis oblonga protected elm trees against the beetle Physococcus brevilineum. It was suggested that the endophytic fungus P. oblonga was responsible for reducing the spread of the Dutch elm disease causal agent Ceratocystis ulmi by controlling its vector, the beetle P. brevilineum. The author associated the repellent effect observed toward the insect to toxic compounds produced by the fungi. This was confirmed four years later by other authors [15], who showed that endophytic fungi belonging to the Xylariaceae family synthesize secondary metabolites in hosts of the genus Fagus and that these substances affect the beetle larvae.

Insect toxins have also been isolated from an unidentified endophytic fungus from wintergreen (Gaultheria procumbens). The two new compounds, 5-hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl)benzofuran and 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl) benzofuran, both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm [68]. Another endophytic fungus, Muscodor vitigenus, isolated from a liana (Paullina paullinoides), yields naphthalene as its major product. Naphthalene, the active ingredient in common mothballs, is a widely exploited insect repellent. M. vitigenus shows promising preliminary results as an insect deterrent and has exhibited potent insect repellency against the wheat stem sawfly (Cephus cinctus) [69], [70]. As the world becomes wary of ecological damage done by synthetic insecticides, endophytic research continues for the discovery of powerful, selective, and safe alternatives.

The work showing the importance of endophytic fungi to the control of agriculture pests, was curiously an indirect demonstration that the endophyte, by blocking insect gall formation, also was able to control a disease caused by a fungus [4-5]. Several other examples were analyzed in which endophytic fungi block or alter insect’s larvae installation and, by doing so, repress potential vectors to disseminate diseases. Other interesting effect of endophytes over insect galls was seen in oak, where apparently dormant endophytic fungi become active with gall formation and, by destroying the leaves, also kill the insect, protecting, therefore, their hosts. This process was described in three associations between endophytes and insects, one of them involving the fungus Gloeosporium quercinum and the insect Neuroterus numismalis [71].

Assis et al. [72] isolated endophytic microorganisms from cabbage growing in the State of Pernambuco, Northeast of Brazil. The method applied for endophyte isolation was the sonication. Seven isolated species were evaluated for antagonistic activities against the causal agent of cabbage black rot disease Xanthomonas campestris pv. campestris. Two isolates, Alcaligenes piechaudii and Kluyvera ascorbata were effective in reducing the disease incidence under greenhouse and field conditions.

The ability of endophytic fungus to resist insects, induce weight loss, growth and development reduction and even to increase pest death rate, was correlated with toxin production. In several cases, it was shown that the mode of action of certain fungi was based on the capability to render the plant unpalatable to several types of pests like aphids, grasshoppers, beetles, etc [6], [73], [74]. Endophytic fungi may indirectly affect seed dissemination by insects, especially ants. In Festuca, seeds infected with certain endophytes are discarded after being collected and, therefore, favour plant dissemination [75].

5 Molecular Biology and the RDT: Tool of Endophytes

Endophytes performing a useful role in inhibiting the insect pests has been shown using molecular biology and the recombinant DNA technology. More recently, recombinant DNA technology has been applied to improve endophytic microorganisms, aiming to the introduction of new characteristics of agronomic interests as biological control of pests [76-77] described the introduction of a heterologous gene in an endophytic microorganism with the purpose of insect control. This was achieved through the secretion of an insecticidal toxin in the host plant. He used the endophyte C. xyli subsp. cynodontis, a gram positive, xylem-inhabiting bacterium, capable of colonizing several plant species. The commercial product received the designation INCIDE. This bacterium received a gene from other bacterium, Bacillus thuringiensis, which is able to produce the d-endotoxin active against insects in nature, especially against Lepidoptera and Coleoptera. Therefore, the genetically modified bacterium is able to secrete toxin inside the plant, protecting it against attacks of target insects.

Endotoxin gene from B. thuringiensis has also been introduced into nitrogen-fixing bacteria from the genus Bradyrhizobium. In this case, the engineered bacterium was introduced into roots of Cajanus cajan, improving nitrogen fixation and protecting the host against Rivelia angulata larvae [78]. Other reviews on the use of genetically modified endophytes may be consulted for more details on the subject [79-81].

Several genes from endophytic fungi and related to toxin production are now being cloned and studied in depth [82] cloned a gene coding for a DMAT Synthase (dimetilail tryptophane synthase) that is responsible for the first step in the synthesis of ergot by the fungus
Claviceps purpurea. Genes related to the latter were identified in the endophytic fungi Balanisia obtecta and Neotyphodium spp., known to produce, respectively, ergobalansine and ergovaline [83] also cloned a gene (DMATS) of C. purpurea involved in the ergot synthetic pathway in planta [84] found in Epichloë a cluster containing genes for the synthesis of mycotoxins like paxiline and lolitem B.

6 Biodiversity of Endophytes

Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land’s surface, yet they harbor more than 60% of the world’s terrestrial biodiversity [85]. As such, one would expect that areas of high plant endemicity also possess specific endophytes that may have evolved with the endemic plant species.

Tropical rainforests are a remarkable example of this type of environment. 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 structures and biologically active compounds [86-87] described a metabolic distinction between tropical and temperate endophytes through statistical data which compares the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other tropical substrata. This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes.

7. Present Status of Endophytes at RRL (Jammu)

For the last three years, we are working on endophytes and during this period we isolated two novel endophytic fungi, which form an alternative source of two important anticancer drugs i.e, Camptothecin and Podophyllotoxin from two different plant species [7], [8], [49].

Camptothecin (CPT) is a monoterpenoid indole alkaloid originally isolated from Camptotheca acuminata Decne, a deciduous tree native to south China, that has gained great attention for its significant antitumor activities in experimental studies [88]. Irinotecan (CPT-11) [89-91] and topotecan (TPT) [92-94] two watersoluble derivatives of CPT, have gained approval by the Food and Drug Administration of the United States of America (FDA) for treating colorectal and ovarian cancer. Other camptothecins—such as 9-amino-camptothecin (9AC), 9-nitrocamptothecin (9NC), and 7-(4-methyl piperazino-methylene)-10,11-ethylenedioxy-campto-thecin (GG211) have also showed remarkable potential in the treatment of carcinoma [95-98]. Camptothecins are lauded as one of the most promising anticancer drugs of the twenty-first century [99].

Nothapodytes foetida is well known to produce an important anticancer alkaloid Camptothecin. The endophytic fungus (Entrophospora infrequens) isolated from it produced Camptothecin under culture conditions. This fungus belongs to the family Phycomycetes [7].

Lignans are very well known to play an important role in plant defense. Lignans have antibiotic, antioxidant and pesticidal properties and therefore confer protection against pathogens in plants. Presently, no prokaryotic cells are able to produce lignans.

The natural lignan podophyllotoxin is a dimerized product of two phenylpropanoid moieties which occur in a few plant species. Since podophyllotoxin is too toxic for the treatment of neoplastic diseases in humans, it is used as a precursor for the chemical synthesis of semi-synthetic antineoplastic drugs, etoposide and teniposide, which are being successfully applied as antitumor agent [100]. The availability of this lignan is becoming increasingly limited because of the scarce occurrence of its natural sources and also because synthetic approaches for its production are still commercially unacceptable.

An endophytic fungus Trametes hirsuta isolated from Podophyllum species, is a novel potential alternative source of podophyllotoxin and related aryl tetralin lignans [8].

8 Prospects of Further Research in Endophytes

A comprehensive study on the endophytes of any individual rainforest higher plant species has not been done, much less a study on any individual plant in its entirety, from its complete root system to its stems, petioles, leaves and flowers. The prospects of finding endophytes (fungi and bacteria) that are specific to any given higher plant or even occurring only in a local region in a forest seem great, given the paucity of work in this area.

As endophytes are the source of various bioactive molecules, the production of these molecule can be enhanced by molecular and recombinant DNA technology tools. New strategies, such as bioconversion using precursors to target compounds and stimulation of production using elicitors, may be developed to enhance secondary metabolite production.

In his in vivo experiments with Pestalotiopsis microspora [101] suggested that the plant might have
acquired the genes for paclitaxel from the fungus. This finding has important implications in the biology of *P. microspora* Ne32 since it explains at least one mechanism by which new DNA can be captured by the organisms, eventually expressed and replicated. Such a mechanism may begin to explain how the enormous biochemical variation may have arisen in this fungus [20]. Also, this initial work represents a framework to aid in the understanding of how this fungus may adapt itself to the environment of its plant hosts and suggests that the uptake of plant DNA into its own genome may occur.

9 Conclusions
As we have tried to elaborate the various properties of the endophytes in this review, there is need to highlight those properties and being a producer of various important bioactive molecules these microorganisms should be grown at pilot scale instead of sacrificing the already diminishing forests. Endophytes include fungi that have one or more of a variety of interactions with their host plant: some fungi are widespread and are found on many different plant species; others are highly specific to single hosts in single environments. Further, a diverse array of interactions between plant and fungus have been found. Given that a huge array of fungi may be isolated from any one host, it seems possible that endophytes will have one or more of a wide array of functions, most of which are unknown at present. The mechanisms through which endophytes exist and respond to their surroundings need to be better understood in order to predict as to which type of plants should be harnessed for the isolation of relevant endophytes. This may facilitate the product discovery processes. Certainly, one of the major problems facing the future of endophyte biology and natural-product discovery is the rapid diminishment of rainforests, which hold the greatest possible resource for acquiring novel microorganisms and their products. Countries need to establish information bases of their biodiversity and at the same time begin to make national collections of microorganisms that live in these areas. Endophytes are only one example of a life form inhabiting plant species threatened with extinction. The problem should be one of immediate concern to the entire world.

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