

Biodiversity and Antimicrobial Activity of Endophytes

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ABSTRACT

Endophytes are microorganism colonizing healthy plants tissue without causing any apparent symptoms and noticeable injury to the host. Both fungi and bacteria are the most common microbes existing as endophytes and are to be found in virtually every plant on earth. It is also suspected that other type of microorganism's viz. archaebacteria and mycoplasmas can undoubtedly exist in plants as endophytes, but no such evidence for them has yet been explored. In recent year's special attention have been made to endophytic fungi because of its ability to produce good number of new and interesting bioactive secondary metabolites, which are of pharmaceutical, industrial and agricultural importance. Several decades of research and numerous articles on endophytic fungi in plants have resulted in a surfeit of knowledge of the group. The data in part however, has been biased by the methodology used, and one question that should come to our mind is "how much do we really know about fungal endophytes? This review will concentrate on what has been discovered, and what is still unknown about endophytic fungi.

Key words: Endophytic fungi, Biodiversity; Plant inhabitants, Natural products, Bioactive compounds, Biological activities.

INTRODUCTION

Fungi are important components in every ecosystem, intimately associated with crucial processes like the decomposition, recycling, and transportation of nutrients in different environments. It has been estimated that there may be over a million different fungal species on this Earth, of which only a small fraction

[approx. 5%] have been identified.¹ There are also many bacteria that exist as plant endophytes, and indeed in most instances they coexist with endophytic fungi. The existence of endophytes has been known for over one hundred years. They live as imperfect fungi most of the time and have been described as benign parasites or true symbionts.

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It has been suggested that they can influence the distribution, ecology, physiology, and biochemistry of the host plants². Botanists have carried out much research about the relationship of plant endophytes, especially for grasses such as tall fescue, where it has been exhibited that endophytes produce toxins that discourage insects and other grazing animals³. It wasn't until the past decade or so, that endophytes have been extensively studied for their potential as novel sources of effective new drugs. Microbes, both fungi and bacteria, have provided modern medicine or drugs with valuable effective treatments, including penicillin from the fungus *Penicillium notatum*, and bacitracin from *Bacillus subtilis*, a common bacterium. Additionally, taxol, a potent chemotherapeutic agent, is synthesized by an endophyte of the Pacific Yew tree. Endophytes represent a huge diversity of microbial adaptations that have developed in special and sequestered environments. Their diversity and specialized habituation make them an exciting field of study in the search for new medicines or novel drug like molecules. The hunt for new drugs is particularly important in view of the fact that so many microorganisms are developing resistance to some of the current drugs. Endophytic fungi are a group of fungi that colonize living and internal tissues of plants without causing any immediate, overt negative effects⁴.

Recent studies have revealed the ubiquity of these fungi, with an estimate of 1 million species of endophytic fungi residing in plants⁵ and even lichen⁶. Endophytic fungi represent an important and quantifiable component of fungal biodiversity, and are known to affect plant community diversity and structure.⁷ According to¹, only about 100,000 fungal species have been described out of a conservative estimate of 1.5 million. Recent studies of endophytic fungi from tropical and temperate forests support the high estimates of species diversity¹⁰.

What are endophytes?

Much has been written on endophytes, they have been defined in many ways and there

have been many reviews and even books on the subject. So what is the best definition for plant endophytes? The most commonly used definition is that of Petrini¹¹ "All organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host", though, there are many alternatives. The specific term "endophyte" was introduced by De Bary and was for some time applied to "any organisms occurring within plant tissues" or "All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host"¹² or "A group that colonize living, internal tissues of plants without causing any immediate, overt negative effects"⁴ or "Endophytes are any fungi isolated from internal symptomless plant tissues"¹³ or "Fungi and bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease"¹⁴.

History of endophyte studies:

One of the early publications describing an endophytic fungus was by Freeman in 1904, where he has made reference to four other papers on endophytes that were published in 1898. This paper described fungus in Persian darnel-an annual grass that today is considered a troublesome weed by many wheat farmers. In between 1930-1990, several discoveries prompted a series of studies in which similar asymptomatic endophytes were recorded in a wide range of grasses¹⁵. Reports are also available in between 1990-2000 on European endophytes, endophytes of palms, grasses and woody plants¹². Significant contributions to knowledge of endophytes have been published by several authors from 2000-2012¹⁰. Relationship between endophytic fungi and host plant A variety of relationships exist between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic³⁰. Endophytes may produce over abundance of substances of potential use to agriculture, industry and modern medicine such as novel

antibiotics, antimycotics, immunosuppressant and anticancer compounds.²⁹ In addition, the studies of endophytic fungi and their relationships with host plants will shed light on the ecology and evolution of both the endophytes and their hosts: the evolution of endophyte plant symbioses; the ecological factors that influence the direction and strength of the endophyte host plant interaction.²⁴ Since natural products are likely adapted to a specific function in nature, so search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes. Schulz *et al.*²⁵ isolated about 6500 endophytic fungi from herbaceous plants and trees and screened them for biologically active compounds. They found a correlation between biological activity and biotope. They also got a higher proportion of the fungal endophytes, in contrast to the soil isolates, suppressed at least one of the test organisms for antialgal and herbicidal activities. Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance²⁷. The various natural products produced by endophytic fungi possess unique structures and great bioactivities, representing a huge reservoir which offers an enormous potential for exploitation for medicinal, agricultural and industrial uses²⁹.

Methods for isolation of endophytes:

In vivo discovery and identification of endophytic microbes is difficult; the endophyte/host relationship tends to be inconspicuous. The microbes reside within intercellular spaces of the tissues and possibly within the plant cells themselves. In this context, structural tools such as Xray and electron diffraction are not very useful, and it remains to be seen whether more specialized analytical methods can be developed that will enable scientists to successfully probe under the outer surface of a plant. Traditionally, staining techniques [e.g. thionin/phenol] have been used so that fungal hyphae could be distinguished from the cell tissues. More recently, once the plant samples (a leaf or stem or part of the root) have been collected, the

associate endophytes are generally encouraged to leave the host and to grow upon Petri plates containing agar medium, and later in nutrient-rich liquid media. Typically, small plant samples (leaf, stem or roots) are collected from the field and stored in plastic bags (preferably cooled) for transportation to a laboratory. Though the plant sample may wither a little if in storage for more than few days, the endophytes present therein appear to be able survive without any obvious adverse effects for several weeks. Care should be taken to restrict the entry of nonendophytic microorganisms into the dead plant sample.

In the laboratory, the plant surfaces are sterilized to remove all microbial epiphytes by soaking in 70% ethanol. Then, with a sterilized knife inside the laminar-flow hood, the outer tissues of the sample are cut so as to expose the interior surface to water agar on a covered culture plate. After a week's of incubation at room temperature, the hyphal tips of fungi and bacterial growth can be seen exuding from the plant sample. The tiny cuts of these growths are then transferred onto new water agar plates or onto more nutrient-rich potato dextrose plates and repetitive re-plating of the microbial colonies is continued until a pure culture is obtained. Identification of endophytes can be done in preliminary level by studying differences in morphology, shape and colour. For bioactivity testing, obtaining a pure fungal or bacterial culture is not that much crucial at first, because in some instances greater activity is found when endophytes stimulate and interact with each other. Endophyte identification the morphological identification of endophytic fungal strains is based on the morphology of the fungal culture colony or hyphae, the characteristics of the spores, and reproductive structures. If these features were distinct³⁰ the identities of some major groups were subsequently verified with molecular methods following the procedures of Promputtha *et al.*²⁸ for inducing sporulation, the fungal strains were separately inoculated on potato dextrose agar, potato carrot agar, and water agar in Petri dishes. Measurements of all fungal characters were made in water mounts,

and the slides were subsequently mounted in lactophenol and sealed with nail varnish or parafilm. All experiments and observations were repeated at least twice. Those cultures that failed to sporulate were grouped as sterile mycelia, and divided into different morph species according to their cultural characteristics. Some fungal isolates could not be identified to the species or genus level. This is a common problem concerning the identification of endophytes²³. However the most new approaching methods to identify endophytic fungi by using polymerase chain reaction (PCR) and subsequent DNA sequencing. The PCR can be performed on cultured endophytes using primers that amplify DNA encoding ribosomal RNA. Identification of fungal endophytes can best be accomplished by amplification of Internal Transcribed Spacer (ITS) regions, which are repeating units of DNA encoding ribosomal RNA. These regions evolve rapidly and there is a very large database of sequences on Genbank and the AFTOL (Assembly of the Fungal Tree of Life) project. Another sequence that is sometimes useful in identification of fungal species is the 18S RNA. There is less diversity in this sequence across species, especially among families, but in conjunction with ITS sequence data. Since many of the fungi isolated from plants are expected to be novel, it is needful to have primers covering as many taxonomic groups as possible. Sequencing the amplified DNA and comparing it with the database can help determine whether or not the specimen is novel; if the sequence is known, then the species can be identified.

Occurrence and biodiversity of endophytes:

The idea that diversity can have strong effects on ecosystem processes was first suggested by Darwin. Many theoretical models and experimental test revealed important functions for diversity²⁴ including the enhancement of primary productivity,²⁵ nutrient retention,²⁶ nutrient flow,²⁷ water availability and resistance to pathogen invasion²⁸. The diversity of endophytes is manifested not only in the specificity of the hosts and their

morphology, but also in the types of benefits that they offer to the host²⁹. Today endophytes have been isolated from all groups of plants range from large trees,¹⁹ palms,^{28,29} sea grasses,²¹ and even from lichens¹⁷. The numbers of strains and species of endophytes vary considerably and generally depend on the intensity of the study. Few scientist conclude that, (i) the numbers of endophytes [strains and species] depends on how much care, time and Petri-dishes are used in a study; a meticulous researcher will laboriously isolate thousands of strains and consequently more species; a lackadaisical researcher will achieve the opposite; (ii) temperate plants yield different communities of endophytes as those from tropical plants; and (iii) different tissues may yield diverse endophyte communities. Other scientists conclude that very little about the role of endophytes is known²¹. Most endophytes isolated to date have been ascomycetes and their anamorphs; however Rungjindamai *et al.*¹³ reported several endophytes may also basidiomycetes. However, their colonization rate and the isolation rate of endophytic fungi from plants varied greatly¹⁴. Some medicinal plants harboured more endophytic fungi than others⁴⁴. Some of the common endophytes not only existed in more plant hosts but also had higher relative frequencies within each of the hosts. In contrast, some other endophytic fungi were detected in only one given plant host¹⁹.

Most of the researches on endophytes have been carried out using hosts from temperate countries, specifically from the Northern Hemisphere and New Zealand. The update data available from tropical regions are scarce. However, these data are showing that tropical plant hosts contain a great diversity of endophytic microorganisms and many of them not yet classified and possibly belonging to new genera and species. In fact potentially, they are of biotechnological importance as new pharmaceutical active compounds, secondary metabolites, biological control agents and other useful characteristics could be found by further exploration of tropical endophytes. A better understanding of plant-

endophyte relationships in tropical conditions can be achieved from these studies. Dreyfuss and Petrini,¹⁴ isolated endophytic fungi from plants belong to family araceae, bromeliaceae and orchidaceae from South America. Later, Rodrigues and Samuels²² isolated, eleven species [One novel species *Idriella licualne*] endophytic fungi from a tropical palm tree growing in the rainforest of Queensland, Australia. The other endophytes found were mostly Xylariaceous fungi. Rodrigues *et al.*⁸ reported a detailed study using 81 isolate with 15 *Xylaria* species from *Euterpe oleracea* plant. Ascomycotina and Deuteromycotina were frequently isolated with *Xylaria cubensis* and *Letendreaopsis palmarum* were the most common endophytic species. Rodrigues²⁵ prepared a review on endophytic communities of palm leaves, mainly from *E. oleracea*. According their report the most common endophytes were *Aspergillus*, *Phomopsis*, *Wardomyces*, *Penicillium* and many unidentified fungus. Also they reported endophytic growth patterns within the leaves of the palm were different from the two species but there was no difference in the infection frequency in between them. The endophytic fungi were isolated from *Trachycarpus fortunei*, a temperate palm species which is outside its natural geographical range²⁸. *Glomerella cingulata* and *Phomopsis* sp. were the dominant species with 75 fertile species were cultured. Hawksworth¹ estimates approximately 6 fungal species per host, which give the figure of 1.5 million species of fungi occurring in the world. If we consider a more realistic figure of 33 fungi species per host than, the global fungal diversity requires revision upwards, being at least 5 times greater than that of 1.5 million³⁰. Endophytic fungi from lemon plant have inhibited the growth of bacteria (*Bacillus* spp.), isolated as endophytes from the same host. In this particular case, fungi and bacteria do not colonize the same regions in the interior of the host or some of the *Bacillus* species are in fact epiphytic, entering occasionally the plant²⁵. These relationships may be important to distinguish endophytes from epiphytes and

to understand the maintenance of a necessary equilibrium between endophytes and latent pathogens, avoiding the emergence of diseases. From banana tree (*Musa acuminata*) growing in three localities of State of Sao Paulo, Brazil, 16 fungal taxa were isolated and the *Xylaria* sp. was the most frequent genus followed by the species *Colletotrichum musae* and *Cordana musae*³⁰. Spontaneous resistant mutants to two fungicides were obtained from the endophytic *C. musae* and equal amounts of mutants and wild-type isolates were also reintroduced in axenic banana plantlets. These were not only successfully reintroduced but also the experiment showed that at least one of the mutants exhibited selective advantage in competition with the wild-type isolate²². Study of these endophytic isolates of *C. musae*, Maccheroni and Azevedo¹⁶ identified at least four individual phosphatase activities under several growth conditions and that may play an important role in phosphate acquisition during the plant colonization and this was the first characterization of the phosphatase system in an endophytic fungus. Medeiros²⁷ isolated endophytic and epiphytic fungi from leaves of cashew tree (*Anacardium occidentale*) growing in four Brazilian Northeastern States. Twenty-one species of endophytic fungi were reported with some quantitative and qualitative differences found from different localities. The species *Colletotrichum gloeosporioides*, *Fusarium solani*, *Pestalotia* sp. and *Phomopsis* sp. were the predominant endophytes. Though *C. gloeosporioides* is a pathogenic fungus for cashew tree, it was found as endophyte and also reported by other authors studying different hosts. An extensive research was carried out using several other fungi isolated mainly as epiphytic in an attempt to control the pathogen *C. gloeosporioides* being a *Trichoderma* strain the most promising one. A study conducted by Rodrigues and Samuels²⁵ revealed that 13 taxa isolated from *Spondias mombin*, collected in the States of Rio de Janeiro and Para, Brazil where *Guignardia* was the dominant endophyte followed by *Phomopsis* species. In Colombia,

an endophytic *Phomopsis* sp. was isolated from the woody host of *Cavendishia pubescens*. Pereira *et al.*¹⁸ isolated 13 endophytic fungi from young and old leaves of *Stylosanthes guianensis* widely distributed in the tropical and subtropical regions of South America where most of the isolates were rare except *Glomerella cingulata*, *Phomopsis* sp. and *Xylaria* sp. In *Atriplex vesicatoria* of Eastern Australia, 71 species from 40 genera of fungi were found and *Fusarium* sp. was the most frequent genera. Fisher *et al.*³⁰ isolated 42 different fungal taxa from the host *Gynoxis oleifolia* (Compositae) in Ecuador with twenty-one species occurred in most common. Rodrigues and Dias²⁷ isolated endophytic fungi from two active pastures of tropical grasses *Brachiaria brizantha* cv. *Manrandu* and *B. Humidicola* where imperfect fungi were most frequent occurrence. Silva¹⁰ found 17 different taxa in roots, stems, seeds and leaves of maize, with some unidentified yeast, mycelia sterilia and filamentous fungi. Among these, *Fusarium* was the most common fungi isolated from seeds. Endophytes were isolated from leaves of *Rhizophora apiculata* and *Rhizophora mucronata*, two typical mangrove plants growing in the Pichavaram mangrove of Tamil Nadu, Southern India¹¹. Leaves were sampled during dry and rainy months and quantitatively more endophytes were isolated during the rainy period. However hyphomycetes and sterile forms were the most commonly isolated endophytes¹¹. In Southern Chile, from *Dactylis glomerata*, a new species of endophytic fungi named *Acremonium chilense* was isolated which is highly aggressive and intercellular as other *Acremonium* and conidia are abundantly produced *in vitro*¹³.

Some important discoveries of natural products from endophytes:

Diverse endophytic fungi reside in plants, representing a rich resource of bioactive natural products with potential for exploitation in pharmaceutical and agricultural field²². However, it is thought that most of the endophytic fungal diversity remains to be discovered²⁴. A large number of secondary

metabolites have been extracted and characterized from endophytic microbes and these are detailed with extensive references.⁵ Many of these compounds are biologically active components and the compounds includes alkaloids, flavonoids, steroids, terpenoids, peptides, polyketones, quinols and phenols as well as some chlorinated compounds. Fungal metabolites from endophytes greatly affect the biology of predators. A number of experiments have been conducted by different research groups on the effect of endophyte infection of various grasses on insect and vertebrate herbivores.³ Until 2003 approximately 4,000 secondary metabolites with biological activity had been described from fungi⁵. Most of these metabolites are produced by so called “creative fungi” which include species of *Acremonium*, *Aspergillus*, *Fusarium* and *Penicillium*, but there has been less research on endophytes ability to produce novel metabolites. Schulz *et al.*⁵ isolated around 6500 endophytic fungi and tested their biological potential. They analyzed 135 secondary metabolites and found that 51% of bioactive compounds (38% for soil isolates) isolated from endophytic fungi were new natural products. These workers concluded that endophytic fungi are a good source of novel compounds and that “screening is not a random walk though a forest”. The major query of how microbial endophytes gain access to their host plants has also been the subject of study. Of course, most micorrhizal fungal endophytes and bacterial endophytes from the soil gain access through the roots; but bacterial endophytes are not thought to invade plant tissue directly; instead, they generally tend to enter the plant through natural openings or wounds. The compound taxol (currently used as an anti-cancer agent) was first found in the bark of the Pacific yew tree. The discovery that taxol could be produced by endophytes of the Yew tree (*Taxus* sp.) by Strobel *et al.* lead to an explosion of endophyte studies on Chinese and other medicinal plants.⁴ Taxol has strong anti-fungal properties, and it has been strongly proposed

that its original purpose was as an antifungal agent to protect the plant [and the fungus] from other pathogenic fungi⁶. Another interesting example of the protective power of an endophyte involves an aquatic plant *Rhynchospora penicillata* found in Venezuela. This plant grows in a river system where it is constantly buffeted against the rocks by the force of the water and floating debris. This extreme situation should provide ample opportunity for pathogenic oomycetes (water molds) to enter the plant; but the plant population appeared quite healthy. After extracting and studying the endophytes from this host plant, Strobel⁹ and his colleagues identified a potent antifungal bacterium which was shown to produce oocycin A, a novel and potent antioomycetous compound that obviously provided the plant with the requisite protection from the pathogenic microbes. A previously unknown compound showing significant bio-activity has been extracted and isolated from an endophytic fungus found in the leaves of a plant *Desmodium uncinatum* from the highlands of Papua New Guinea. This plant is used by the indigenous people for healing wounds and body sores. The compound shows anti-fungal, antibacterial and anticancer activity with an IC₅₀ value of 0.9 µg/ml to destroy HeLa cervical cancer cells¹⁶. Few Chinese plants are used as important in the treatment of malaria because they are the hosts of endophytic fungi that produce several metabolites indole as a main component¹⁷.

Methodologies for the isolation of bioactive compounds from endophytic fungi:

The isolation and the purification of bioactive compounds from endophytes happen to be a difficult process. The most common methodology involves the fungal growth in a liquid media and chromatographic techniques such as thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC). Once endophytes are successfully cultured, bioassay guided fractionation of growth media is used to isolate the most promising compounds. At this time it must be emphasized, that having appropriate bio-assays available in order to check the activity

of an endophyte is crucial to the isolation and purification of the active components. The active microbial fungi are transferred onto liquid media (potato dextrose broth) in large flasks and allowed to grow for several weeks. The liquid is filtered before being tested for bio-active compounds and the compounds synthesized by the microbes are extracted from the liquid using various organic solvents. The isolation and purification of the bio-active compounds is attained through chromatographic techniques such as thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC)¹⁸.

Anti-bacterial assay:

Two simple methods can be utilized for testing of crude filtered growth media for antibacterial properties; the first is a paper disc method and the second involves a liquid assay. For the former, a bacterial “lawn” of fast-growing organism is generated in a Petri dish containing Mueller Hinton agar using sterile glass beads. Then paper discs are placed on this lawn and small aliquots of filter-sterilized endophyte growth media are pipetted onto the discs. Several other different media samples can be ran concurrently and activity is determined by a visual zone of inhibition surrounding the discs. The liquid antibacterial method involves addition of pathogenic bacteria in a broth to a 96-well plate and adding endophytic media at varying concentrations to the wells and inhibition of growth in the wells is determined by optical transmittance through the wells.

Anti-fungal assay:

Again there are two possible approaches to an antifungal assay. First in a competitive assay, two fast growing fungi (e.g. *Pythium* and *Geotricium*) are placed on opposite sides of a Petri dish and a plug containing a drop of the endophyte liquid is placed at the centre of the plate and if the endophyte has any anti-fungal activity, the centre spot will have a strong zone of inhibition adjoining it. In a non-competitive assay, the liquid extracted from the endophyte is placed in agar well plate and *Geotricium* or *Pythium* is added to the wells. The wells should be inspected at 24 hr intervals and the

growth of the added fungus compared with that in a control medium. Similarly the test may be tested with other fungal species.

Cytotoxicity assay:

This assay measures the ability of the plant extract to inhibit the growth of cancer cells *in vitro*. The immortalized HeLa cervical cancer cells [or any other available cancer cells] can be used for this purpose. The cells are incubated for 24 hr in 96-well plates before the plant extract is added. Sulfurhodamine is added as a dye and the colour identifies living cells as opposed to those that have been killed by the extract applied. The same procedure can then be repeated using normal cells in order to compare the efficacy of the drug against cancer cells.

Structural studies:

After obtaining a sample of pure bio-active material, the final and ultimate goal is to determine its structure and functional group. The two main structure determining techniques used for this work are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). Both of these methods have seen vast improvements in sensitivity and technological advances over the past one decade and when taken in tandem they represent very powerful means of solving unknown molecular structures. Addition to these, X-ray diffraction is also available as a powerful method if the sample can be crystallized. High resolution mass spectrometry can also, in most instances, provide the molecular mass to such a precision that the molecular formula can also be efficiently determined. The exact number of hydrogen and carbon atoms can usually be verified from 1D NMR data and various 1D and 2D experiments can give information about connectivity's between magnetic nuclei in the sample, elucidating the full structure. It should be noted in to the mind however that not all experiments lead to the discovery of a totally new chemical compound; occasionally the structural probing reveals a known bioactive chemical, although its source might be novel.

Importance of endophytes:

Endophytes are saprobic decomposers:

Several recent studies have explored relationships between endophytes and their role as saprobes¹². The evidence is circumstantial; however, it seems likely that some (or many) saprobes are derived from endophytes.¹⁹ If this hypothesis is correct and saprobes are derived from endophytes then it is more likely that they would be host or tissue specific. Endophytes may have developed intimate relationships with their hosts during evolution and may be host or even tissue specific¹⁰. Several studies provide evidence to support the hypothesis that saprobe host specificity in plants is dependent on internal endophytes, while reports are available that host components may regulate the endophytes¹¹. Whatever the reason it is clear that many endophytes in leaves (and woody tissues) are host, host genus or host family specific²⁰ and that this specificity must depend on factors such as initial endophyte colonization and/or substances within leaves and wood.¹⁴ Some endophytes are known to be mutualists and latent pathogens¹⁰.

Endophytes as producer of Antibiotics:

Antibiotics are defined as low-molecular-weight organic natural products made by microorganisms that are active at low concentration against other microorganisms¹⁵. Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents including, phyto-pathogens, bacteria, fungi, viruses, and protozoans that generally affect humans and animals. *Cryptosporiopsis quercina* is the imperfect stage of *Pezizula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It has been isolated as an endophyte from *Tripterygium wilfordii*, a medicinal plant native to Eurasia¹⁵. On Petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens *viz.* *Candida albicans* and *Trichophyton* sp. A unique type of peptide antimycotic termed cryptocandin was isolated and characterized from *C. Quercina*¹⁷. This compound contains a number of peculiar hydroxylated amino acids

and a novel amino acid: 3-hydroxy-4-hydroxy methyl proline. The bioactive compound is related to the known antimycotics, echinocandins and pneumocandins¹⁶. Cryptocandin is also active against a number of phyto-pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. The cryptocandin and its related compounds are currently being considered for use against a number of fungi causing diseases of skin and nails. Cryptocin, a unique tetramic acid, is also produced by *C. Quercina*⁶. This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant-pathogenic fungi⁶. *P. viridiflava* is a member of a group of plant-associated fluorescent bacteria. It is putatively associated with the leaves of many grass species and is located on and within the tissues¹⁸. The ecomycins represent a family of novel lipopeptides. Besides common amino acids like alanine, serine, threonine, glycine and some unusual amino acids are also involved in the structure of the ecomycins which includes homoserine and β -hydroxyaspartic acid. This ecomycin is active against human-pathogenic fungi such as *Cryptococcus neoformans* and *Candida albicans*. Another group of antifungal compounds is the pseudomycins which is produced by a plant associated pseudomonad⁷. The pseudomycins represent a family of lipopeptides that are active against variety of plant and human pathogenic fungi. Some of the notable target organisms include *C. albicans*, *C. neoformans* and a variety of plant pathogenic fungi including *Ceratocystis ulmi* (the Dutch elm disease pathogen) and *Mycosphaerella fijiensis* (the causal agent of Black Sigatoka disease of banana)⁸. The key conserved part of the pseudomycins is a cyclic nona-peptide. The terminal carboxyl group of L-chlorothreonine closes the macrocyclic ring on the OH group of the N-terminal serine. The pseudomycins contain several nontraditional amino acids such as L-chlorothreonine, both D- and L-diaminobutyric acid and L-hydroxy aspartic acid. The molecules are candidates for use in human medicine especially after structural modification has successfully

removed mammalian toxicity⁹. Although the pseudomycins are effective against a number of ascomycetous fungi, still they being considered for agricultural use. Ambuic acid, an antifungal agent which has been described from several isolates of *Pestalotiopsis microspora* found as representative isolates in many of the world's rain forests¹⁰. In fact, this compound and another endophyte product, terrein have been used as suitable models to develop new solid-state nuclear magnetic resonance (NMR) tensor methods to assist in the characterization of molecular stereochemistry of organic molecules¹¹. Phomopsichalasin, a secondary metabolite from an endophytic *Phomopsis* sp., which represents the first cytochalasintype compound with a three-ring system replacing the cytochalasin macrolide ring. This particular metabolite mainly exhibits antibacterial activity in disk diffusion assays against *Bacillus subtilis*, *Salmonella enterica* and *Staphylococcus aureus*. It also displays a moderate activity against the yeast *Candida tropicalis*.¹³ An endophytic *Fusarium* sp. from the plant *Selaginella pallescens*, collected from Guanacaste Conservation Area of Costa Rica, was also screened for antifungal activity. CR377, a new pentaketide antifungal agent was isolated from the culture broth of the fungus and showed potent activity against *C. Albicans*¹⁴. Colletotric acid, a metabolite of an endophytic fungus *Colletotrichum gloeosporioides*, in *Artemisia mongolica*, demonstrated antimicrobial activity against bacteria as well as against the fungus *Helminthosporium sativum*¹⁷. Another *Colletotrichum* sp., an endophyte isolated from *Artemisia annua*, synthesized bioactive metabolites that showed varied antimicrobial activity as well. *Artemisia annua* is a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different regions. The *Colletotrichum* sp. located in *A. Annua* produced not only metabolites with activity against human-pathogenic fungi and bacteria but also metabolites that were fungistatic to

plant-pathogenic fungi¹⁰. *A. annua* is also producing antimalarial compounds. Munumbicins an antibiotic from *Streptomyces* sp. possess widely differing biological activities, depending on the target organism. This broad-spectrum demonstrates activity against Grampositive bacteria such as *Bacillus anthracis* and multi drug resistant *M. tuberculosis* and also some other drug-resistant bacteria. However, the most impressive biological activity of munumbicins is antimalarial activity against *Plasmodium falciparum*¹⁵. An endophytic streptomycete (NRRL 30566), from a fernleaved *Grevillea* tree (*Grevillea pteridifolia*) growing in the Northern Territory of Australia, produces, novel antibiotics called kakadumycins¹⁶. Each of these antibiotics contains alanine, serine and an unknown amino acid. Kakadumycin A has wide-spectrum antibiotic activity similar to that of munumbicin D, mostly against Gram-positive bacteria and it generally displays better bioactivity than echinomycin. Kakadumycin A and echinomycin are related by virtue of their very similar chemistries (amino acid content and quinoxaline rings) but differ slightly with respect to their elemental compositions, biological activities and aspects of their spectral qualities¹⁶. This is yet another example of an endophytic actinomycetes having promising antibiotic properties.

Antiviral compounds:

Another fascinating use of antibiotic products from endophytic fungi is the inhibition of viruses. Cytonic acids A and B two novel human cytomegalovirus protease inhibitors have been isolated from the solid state fermentation of the endophytic fungus *Cytonaema* sp. Their structures isomers were elucidated by mass spectrometry and NMR methods as p-tridepside¹⁷. The fact, however, that some compounds have been found is promising and limitation in compound discovery is probably related to the absence of appropriate antiviral screening systems in most compound discovery programs.

Volatile antibiotics from endophytes:

Muscodor albus is a newly described endophytic fungus obtained from small limbs

of *Cinnamomum zeylanicum*. This Xylariaceae (non-sporeproducing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds.¹⁹ The majority of these compounds have been identified by gas chromatography-mass spectrometry and then ultimately made into an artificial mixture. This artificial mixture mimicked the antibiotic effects of the volatile compounds produced by the fungus and was also used to gain positive identification of the ingredients of the fungal volatile compounds¹⁹. Individually each of the five classes of volatile compounds produced by the fungus had some inhibitory effect against the test bacteria and fungi; but none was lethal. However, collectively they acted synergistically to cause death in a broad range of plant- and human-pathogenic fungi and bacteria. The ecological implications and potential practical benefits of the “mycofumigation” effects of *Muscodor albus* are very promising given the fact that soil fumigation utilizing methyl bromide. The potential use of mycofumigation may soon be a reality. In fact this organism is already on the market for the decontamination of human wastes. Using *M. albus* as a screening tool, it is now possible to isolate other endophytic fungi that produce volatile antibiotics. A newly described *Muscodor roseus* was twice obtained from tree species growing in the Northern Territory of Australia and this fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus*²⁰. In addition, for the first time, a nonmuscodor species, a *Gliocladium* spp. was discovered to be a volatile antibiotic producer. These volatile components of this organism are totally different from those of either *M. albus* or *M. roseus*. The most abundant volatile inhibitor is annulene, formerly used as a rocket fuel and discovered for the first time as a natural product in an endophytic fungus²¹. The bioactivity of the volatile compounds of *Gliocladium* sp. is not as comprehensive as those of the *Muscodor* species²¹.

Endophytic fungal products as anticancer agents:

Paclitaxel and some of its derivatives represent the first major group of anticancer agents that is produced by endophytes. Paclitaxel, a highly functionalized diterpenoid is generally found in each of the world's yew (*Taxus*) species. The mode of action of paclitaxel is to preclude tubulin molecules from depolymerising during the processes of cell division²². This compound is the world's first anticancer drug to make billion-dollar. It is used to treat a number of human tissue-proliferating diseases. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. But, after several years of continuous effort, a novel paclitaxel-producing endophytic fungus *Taxomyces andreanae* was discovered in *Taxus brevifolia*.²³ Later, Wollemi pine [*Wollemia nobilis*], *Pestalotiopsis guepini* shown to produce paclitaxel.²⁴ Also, quite surprisingly *Maguireothamnus speciosus* (Rubiaceae) yielded a novel fungus, *S. tepuiense* that produces paclitaxel. This endemic plant grows on the tops of the Tepuis in the Venezuelan-Guyana region in South Western Venezuela.²⁵ Furthermore, fungal paclitaxel production has also been noted in a *Periconia* spp.²⁶ and in novel endophytic fungal species *Seimatoantlerium nepalense*²⁷. Simply, it appears that the distribution of those fungi making paclitaxel is worldwide. The ecological and physiological explanation for the wide distribution of fungi that make paclitaxel seems to be related to the fact that paclitaxel is a fungicide and the organisms with the most sensitivity to it are plant pathogens such as *Pythium* spp. and *Phytophthora* species²⁸. These pathogens are some of the world's most dangerous plant pathogens and are strong competitors with endophytic fungi for niches within plants. In fact, their sensitivity to paclitaxel is based on their interaction with tubulin in a manner identical to that in rapidly dividing human cancer cells²². Other species, producing paclitaxel belong to *Sporormia minima*, *Trichothecium sp.*, *Tubercularia wallechiana* and *Corylus avellana*^{29,30}. Torreyanic acid, a selectively cytotoxic quinone dimer

(anticancer agent),³¹ confirmed 5 to 10 times more potency in several cancer cell lines, and are sensitive to protein kinase C agonists and causes cell death by apoptosis. Very recently a complete synthesis of torreyanic acid has been successfully completed using the application of a biomimetic oxidation dimerization cascade system³². Fungus such as *Xylaria*, *Phoma*, *Hypoxylon* and *Chalara* species are representative producers of a relatively large group of substances known as the cytochalasins³³. These cytochalasins group compounds possess antitumor and antibiotic activities, but due to their cellular toxicity, they have not been yet developed into pharmaceuticals.

Products of endophytes as antioxidants:

Two compounds pestacin and isopestacin have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from *Terminalia morobensis*, which is growing in the Sepik River drainage of Papua New Guinea shown antimicrobial as well as antioxidant activity^{12,14}. Isopestacin was suspected of antioxidant activity based on its structural similarity to the flavonoids. Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution¹⁴. Pestacin was later described from the same culture fluid which is occurring naturally as a racemic mixture and also possessing potent antioxidant activity¹². Proposed antioxidant activity of pestacin arose primarily via cleavage of an unusually reactive C-H bond and to a lesser extent, though O-H abstraction¹⁴. The antioxidant activity of pestacin is higher than that of trolox, a derivative of vitamin E.¹²

Products of endophytes with insecticidal activities:

Several endophytes are known to have anti-insect properties. In the early 80's the specialized literature published the first reports showing that endophytic microorganisms, in these case fungi, could play an important role inside plants. In between 1981 to 1985, which is considered a historical period to this research field, it was demonstrated the

existence of plant protection against herbivore insects given by endophytic microorganisms. The basic nature of protection and the variables involved in the process were also initially addressed. Webber and Gibbs¹⁵ was probably the first researcher to report an example of plant protection giving by an endophytic fungus *Phomopsis oblonga* protected elm trees against the beetle *Physocnemum brevilineum* by reducing the spread of the elm Dutch disease causal agent *Ceratocystis ulmi*. The author associated the repellent effect observed towards the insect to toxic compounds produced by the fungi. Other earlier observations on the control of insect-pests by endophytic fungi are those of Funk *et al.*¹⁶ which is showing protection of the perennial ryegrass *Lolium perenne* against the sod webworm. Later, Gaynor and Hunt¹⁷ observed in several ryegrasses that high fungi infection is correlated with a decrease in the attack frequency of the Argentine stem weevil, *Listronotus bonariensis*. Later, Barker *et al.*¹⁸ and Prestidge *et al.*¹⁹ also observed that plants free of endophytic fungi of the genus *Acremonium*, are severely attacked by this insect. Lasota *et al.*¹⁰ had also found that death rate of the Homoptera *Adelges abietis* in *Picea glauca*, when galls are infected with the endophytic fungus *Cladosporium sphaerosperum*. Later, in *Lolium perenne* weight gain and survival of the insect pest-*Spodoptera frugiperda*, were affected by endophytic fungi- *Balansia cyperii*²⁷. In the same year, Latch *et al.*¹⁷ reported two endophytic fungi, *Acremonium lolii* and *Gliocadium* sp. affecting insect pest in other species of *Lolium*. Ahmad *et al.*¹⁴ verified similar effects of the same fungi over the grasshopper *Acheta domesticus*. Similarly, Johnson *et al.*¹⁵ used choice tests, which showed that insects would feed on endophyte-free *Festuca* plants rather than on infected samples. Another endophytic fungus, *Muscodor vitigenus*, isolated from *Paullina paullinioides*, yields naphthalene, is a widely exploited insect repellent¹⁶. Nodulisporic acids, novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the

blowfly, by activating insect glutamate-gated chloride channels. In recent years, a number of alkaloids have been discovered from endophytic fungi in plants, which exhibited excellent insecticidal activities. Recently, Biils *et al.*¹⁸ isolated a novel species of fungus *Hypoxyton pulicidum* producing a pantropical insecticide.

Antidiabetic agents from endophytes:

A nonpeptidal fungal metabolite [L-783] was isolated from an endophytic fungus *Pseudomassaria* sp. collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo.¹⁹ This compound acts as insulin mimetic and unlike insulin, is not destroyed in the digestive tract. Oral administration of L-783,281 to two mouse models of diabetes resulted in significant lowering of blood glucose levels. These interesting results may lead to new therapies for diabetes¹⁹. Recently Dhankhar and Yadav¹⁵ investigated new antidiabetic drugs from fungal endophytes such as *Aspergillus* sp., *Phoma* sp. and unidentified species; those significantly reduce blood glucose level by glucose tolerance test. By GCMS analysis the main constituents were found to be 2, 6-di-tert-butyl-p-cresol and Phenol, 2, 6-bis [1, 1-dimethylethyl]-4-methyl.

Immunosuppressive compounds from endophytes:

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients and in near future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin dependent diabetes. An endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces¹⁵ subglutinol A and B¹¹ act as the immunosuppressive. In the study conducted by these authors suggest that Subglutinol A and B are more potent in the thymocyte proliferation assay in compared to immunosuppressant drug cyclosporine. Due to the lack of toxicity associated with subglutinols A and B they suggest that it should be explored in greater detail²⁴. An aromatic β glucoside, Pestalosite and two pyrones: pestalopyrone and hydroxypestalopyrone isolated from *P.*

Microspora possess phytotoxic properties¹². A newly described species of *Pestalotiopsis* named, *Pestalotiopsis jesteri*, collected from the Sepik River area of Papua New Guinea, which produces jesterone and hydroxy-jesterone, exhibit antifungal activity against a variety of plant-pathogenic fungi¹⁴.

Why are endophytes so charming?

Hyde and Soyting¹⁵ proposed five statements why the endophytes are so important;-

1. Studies provide high taxon diversity; can be completed in the relative comfort of a laboratory with minimal fieldwork, and use a well established traditional methodology that any motivated student can follow.
2. Most sporulating isolates are relatively easily identified [at least to genus] as they belong to less than 50 characteristic genera.
3. Various methodologies can be applied to mycelia sterilia to promote sporulation; alternatively molecular methods can be utilized to identify these relatively fast growing morphotypes.
4. Sophisticated statistics can be applied to the isolates which “appear” to have been derived from single random units and will satisfy the demands of any unforgiving non-fungal ecologist.
5. The relatively fast growing and “highly” diverse endophytes provide ideal tools for screening and novel compound discovery and they can easily be lodged in culture collections.

CONCLUSION

Endophytic fungi are a rich and reliable source of novel natural compounds with interesting biological activities, a high level of biodiversity and may also produce several compounds of pharmaceutical significance, which is currently attracting worldwide scientific investigations toward isolation and exploration of their biotechnological promise. They represent a relatively unexplored ecological source, and their secondary metabolism is particularly active because of their metabolic interactions with their hosts. In nature, plants seem to be in a close interaction with endophytic fungi. A complete study on

the endophytes of higher plant species with special reference to medicinal plant has not been studied. Out of the 10,000 important medicinal plants very less plants had studied for their endophytic micro flora. So, rapid research should be required to study them because disappearance of plant species will also disappears the entire suite of associated potential endophytes. By collecting, cataloguing and exploiting endophytic microorganisms throughout the world may offer opportunities in the field of agriculture, industry and medicine. The challenge and goal is to be able to manage microbial communities to favour plant colonization by beneficial endophytic microorganism. The contributions of this research field may have economic and environmental impacts. Further research at the molecular level in this field is necessary for a better understanding of the host endophyte interaction which may lead to a quicker recognition of genetically particular and/or host gene carrying endophytes.

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