Chapter 4

Endophytic Fungi, A Versatile Organism for Modern Microbiological Research and Industrial Applications

Kalyanaraman Rajagopal[1]; Meenambiga SS[2]; Arulmathi R[2]

[1]*Department of Botany, Ramakrishna Mission Vivekananda College (Autonomous), Mylapore, Chennai- 600004*

[2]*Department of Biotechnology, School of Engineering, Vels University (VISTAS), Pallavaram, Chennai- 600117.*

Correspondance at: *Kalyanaraman Rajagopal, Department of Botany, Ramakrishna Mission Vivekananda College (Autonomous), Mylapore, Chennai- 600004*

E-mail: ammaaappa@gmail.com

Abstract

Endophytic fungi are one of the least studied plant microbe- interactions. In this chapter ecology, taxonomy, bioactive compounds and enzyme production by endophytic fungi were discussed. Finally a general procedure for isolation of endophytic fungi from plant parts is given.

1. Introduction

The word endophyte was primarily coined by De Bary in 1866 (Wilson, 1995). Ever since then it has undergone many subtle changes. Suryanarayanan [2] reported that the horizontally transmitted endophytic fungi establish endosymbiotic relationship with host plants of all lineages. Endophytic fungi occur universally and they have been reported in plants from different ecological niches. Further, these groups of fungi have the ability to produce an array of secondary metabolites showing different bioactivities and resulted in the use of these fungi for biotechnological applications. David *et al*., [3] quantified the associations between fungal endophytes and biocontrol-induced herbivory of invasive purple loosestrife (L.). *Lythrum salicaria* and Suryanarayanan *et al*., [4] studied the role of endophytic fungi as biocontrol agents. Recently, Raman and Suryanarayanan, [5] described that fungus plant interaction influences plant-feeding insects behavior. The endophytic fungi are also involved in multiple balanced antagonisms and it was revealed by Schulz et al. [6]. Szink, et al., [7] showed a new evidence for broad trophic status of leaf endophytic fungi of *Quercus gambelii*. The chemical interactions of endophytic fungi can be exploited as microbial factories for sustainable applications in biotechnology [8].

The term endophyte itself has evolved to accommodate our growing knowledge on these fungi. Ainsworth [9] defined an endophyte as "a plant living inside another organism". Carroll [10] restricted its usage to organisms that cause asymptomatic infections within plant tissues; this description excluded pathogenic fungi. It was further widened by Petrini [11] with an explanation to include all organisms inhabiting plant organs at some time in their life. He further stated that they can colonize internal plant tissues without any obvious damage to the host. This definition would include latent pathogens and epiphytes which are established as endophytes at some stages of their life. Thus, some endophytes residing in plant tissues become pathogenic when their hosts are weakened [12]. Wilson [1] proposed that endophytes are fungi or bacteria which, for their entire or part of life cycle, occupy the living plant tissue. They do not cause any apparent and symptomatic infections within plant tissues but only symptoms of disease.

Sampson [13] reported the occurrence of endophytic fungi in plant tissues such as *Lolium* grass. The presence of endophytes in *Pseudotsuga menziesii* was reported by Bernstein and Carroll [14] and it was considered as a contemporary renaissance of research on endophytic fungi. Apart from diversity of endophytic fungi, the study on the metabolites produced by them offers a diverse knowledge of their pharmaceutical, medical and agricultural importance.

2. Taxonomy of Endophytic Fungi

The endophytic fungi present in plant parts above the ground generally belong to the Ascomycetes, Coelomycetes and their conidial (anamorphic) forms and Hyphomycetes forms lacking a sexual state. Petrini [15] reported that rarely a small amount of Basidiomycetes and Oomycetes occur as endophytes. He also classified the endophytic fungi assemblage of a plant into two broad categories – the non-specific, epiphytic and the true endophytic. Every effort to isolate endophytic fungi from a plant is certain to yield quite a few forms and prevent their explicit classification. Therefore, mycologists take recourse to the culture characteristics of these fungi to make a distinction from one another [16,17].

3. Ecology of Endophytic Fungi

Endophytic fungi are omnipresent. They have been isolated from varied groups of plants such as marine algae [18], mosses and ferns [15] and coniferous trees [19,20]. Some angiosperms such as members of the Poaceae [21,22,23] and Ericaceae [15] have been studied for the incidence of endophytic fungi in them. Investigation on species composition of endophytic fungal communities of many hosts has shown that a large number of endophytic fungal taxa could be isolated from a lone host species [24]. In general, one or a few endophytic fungal species dominate in a host plant [11,17].

The environmental factors and age of the host tissue are known to influence the distribution of endophytic fungi in a host. The quantity of endophytic fungi obtained from the host tissue decreases with the age [25,26]. Endophytic fungi samples collected simultaneously from *Pinus sp* and *Fagus sp* growing at similar environment and site showed that the endophyte assemblages of these two plants are dissimilar [27]. This shows that endophytic fungi that colonize trees are known to be host specific. Similarly, the endophyte community of a plant is also affected by the quality of air [11]. All these studies show that the endophytic fungi occupy a unique niche. Despite the fact that they may not be exposed to the vagaries of the environment as the Phylloplane fungi are, they have to come across the defense reactions of the host. Hence, their living strategies are likely to be unusual from those of other fungi.

4. Biotechnological Potential of Endophytic Fungi

Endophytic fungi inhabit a very unique and often hostile habitat. Recently they are recognized as sources of new metabolites useful in biotechnology and agriculture [28]. Numerous endophytic fungi cultures produce antibiotic compounds that are active against human and plant pathogens. Fisher *et al.*, [29] showed antifungal and antibacterial activity in more than 30% of the endophytic fungi that they have tested. A broad spectrum antibiotic was isolated by Fisher *et al.*, [30] from an endophyte of *Vaccinium sp*. Seed germination in ivy was influenced by endophytes such as *Aureobasidium pullulans* and *Epicoccum purpurascens* by producing phytohormones [31]. In developed countries, endophytic fungi are viewed as biocontrol agents for plant diseases [32,11,33]. They are always the preferred components of the fungus-plant system and are ideal for experimental manipulations. Research shows that valuable attributes could be introduced into host plants for desirable qualities from endophytic fungi representing natural genomes. Endophytic fungi could also be used as gene vectors and artificially introduced into a population of host plants [31].

Endophytic fungi readily integrate into host systems and hence their associations offer great possibilities for the biocontrol programme [32,33]. Dewan and Sivasithamparam [34] isolated an endophytic fungus from wheat and reported a significant defense against infections by “Take all’ fungus in host plant. Endophytic fungi present in conifer needles produce chemicals that can ward off insect pests. The endophyte-mediated antagonism towards an insect pest in Douglas fir is an excellent example. Their needles harbor an endophytic fungus, *Rhabdocline parkeri* that controls the gall midge, *Contarina* sp [19]. These studies show that endophytic fungi are prospective candidates for biocontrol programme [35,36].

Pharmaceutical and agricultural industries have equally explored the Endophytic fungi [37] representing an untapped pool of secondary metabolites [38,76]. In INBIO project with the collaboration of Merck Research Laboratories, USA and Costa Rica, endophytic fungi from forest trees are screened for novel antibiotics [39,40]. Recently, Li *et al*. [41] reported Taxol, an anticancer drug produced by endophytic fungi isolated from the bark of *Taxus wallachiana* growing in Himalayas of Nepal.

5. Endophytic fungi as a resource of Bioactive Metabolites

This write-up reveals the significance of endophytic fungi from plants as a source of bioactive metabolites. Endophytic fungi produce various metabolites belonging to diverse structural groups like terpenoids, steroids, quinines, phenols, coumarins etc. [42]. They can also be termed as chemical producers inside plants [43,44] . Tejesvi *et al*., (2007) reported that endophytic fungi of medicinal plants produce secondary metabolites which can be studied for curing several diseases. All these studies reveal that Endophytic fungi represent a chemical reservoir of novel compounds such as which offer antimicrobial, antiviral, antifungal, anticancer, antiparasitic, antitubercular, antioxidant, immunomodulatory, insecticidal and many uses in pharmaceutical and agrochemical industries [42].

Fungal endophytes serve as a storehouse for various novel compounds with immense value in agriculture, medicine and various other industries [45]. Owing to their constant interaction with host plants, the secondary metabolites or the bioactive compounds produced by endophytic fungi are unique and distinct compared to those produced by soil fungi or fungi associated with algae [46,47]. Endophyte - plant interactions are different from pathogen - plant interactions. In Endophyte - plant interactions, neither the host nor the fungus gets detrimental effects [48,49,50]. This is beneficial to bioprospectors as the endophytes confer selection pressure to develop novel metabolic pathways due to their sustained and prolonged reactions against the defense mechanism of the host [51,52,53,54,55].

A well-known anticancer drug paclitaxel was initially produced from the yew (*Taxus brevifolia*) species. Paclitaxel prevents polymerization of tubulin molecules during the process of cell division and is helpful in treating a number of tissue proliferating diseases in human [56,57]. The usefulness of paclitaxel led to the extensive research on endophyte producing the compound which was identified as Taxomyces andreanae from the plant *T. brevifolia* [58]. Anticancer activity of the endophytic fungi is based on the bioactive compounds present in it. The anticancer potential of endophytic fungi has been studied extensively between 1990 and 2013 in which the endophytes *Pestalotiopsis* and *Aspergillus* have been explored for their anticancer compounds [59].

Suryanarayanan *et al*., [60] screened the anti-cancer activity of 110 isolates of endophytic fungi in mouse fibroblast cell line L-929. Using the organic solvent extracts of the endophytes isolated, the cytotoxic effects were assayed using MTT colourimetric assay. Endophytic microbial products act as potent antimicrobial agents against various human pathogens. The endophyte Pestalotiopsis neglecta was explored for its antimicrobial property against the human pathogens. The phenolic compound tyrosol produced by the endophyte *Diaporthe helianthi* isolated from the plant *Luehea divaricata* was studied for its antagonistic effects on pathogenic strains of bacteria [61]. Singh and Kaur [62] isolated thirty-six endophytic fungi from *Acacia nilotica* and screened them for the production of alpha amylase and glycosidase inhibitors. The endophyte *Aspergillus awamori* isolated from *Acacia nilotica* possesses anti-diabetic property. Over 75% of the antibacterial drugs in clinical use are of natural origin and most of them are obtained from fungal sources. Antioxidant activity was observed in the compounds pestacin and isopestacin produced by endophyte *Pestalotiopsis microspora* isolated from *Terminalia morobensis*. The antioxidant activity was due to the structural similarity of isopestacin with flavonoids [63].

In fungi, the genes coding the enzymes required for secondary metabolic pathways are present as gene clusters situated in the same locus [64,65]. These gene clusters are co-expressed and evolve rapidly through horizontal gene transfer and multiple rearrangements. These are conducive for novel chemical synthesis by fungi [66]. It is necessary to screen fungi under different culture conditions for its novel bioactive production. Culture conditions such as growth media composition, pH, temperature, incubation time and the addition of various growth regulators can influence the secondary metabolite profile of the endophytic fungi and also aid in the synthesis of new products [67]. For instance, Squalestatin S1, produced by a Phoma sp. acts as an inhibitor of squalene synthase which is essential for the biosynthesis of cholesterol. Several fungal species produce squalestatin and it is produced in higher quantities in Phoma sp. Parra *et al.*, [68] optimized the composition of fermentation medium for increased squalestatin production.

6. Anticancer and Antioxidant Compounds

Endophytic fungi have been considered as a source of anticancer compounds since the million dollar drug discovery Taxol from the endophytic fungus Taxomyces andreanae from the bark of *Taxus brevifolia* [58]. In the current chapter only few examples of anticancer compounds have been given in (**Table-1**). Antioxidants defend the cells from the harm induced by unstable free radicals in the cells. The Free radicals cause or induce diseases like cancer and degenerative diseases like Alzheimer's etc. However the fact is, only few antioxidant compounds are reported and approved. The need of the hour is to search for new, effective and eco-friendly antioxidants and endophytic fungi are one of the most promising sources. Few antioxidant compounds reported in Table 1.

7. Immunomodulatory Compounds

Immunomodulatory compounds are produced by several microbes. In recent years, fungi are viewed as promising producers. Immunomodulatory compounds are either immunosuppressive or immunoregulatory drugs [42]. There is a huge demand for immunosuppressive compounds and endophytic fungi are the producers of several immunosuppressive compounds. Some of the major immunosuppressive compounds listed in Table 2.

8. Endophytic Fungi as Source of Industrial Enzymes

The production of extracellular enzymes such as cellulases, pectinases, esterases, amylases, proteases, tyrosinases, tanneases, chitinases, L-asparaginase by few endophytic fungi has been studied by substrate utilization test and isozyme analysis [69]. The production of growth hormone Indole Acetic Acid (IAA) has been demonstrated in endophytic fungi such as *Aureobasidium pullulans* and *Epicoccum purpurascens* [70]. A Sterile endophytic fungi and *Fusarium* sp isolated from *Azadirachta indica* produced IAA in culture [33]. Carroll and Petrini [71] investigated the capability of endophytic fungi to tolerate or metabolize phenolics and other defense chemicals of the host tissues. Some endophytic fungi are known to produce compounds that interfere with plant cell division [17].

9. In Silico Studies on Endophytic Fungal Secondary Metabolites

Using modern computational methods, the bio-macromolecule can be designed to bind with the identified target to change a particular biochemical process. When a three dimensional structure of a protein is known, it becomes easy to identify a compound that binds with its active site using modeling techniques [72,73]. Several compounds of natural origin have excellent therapeutic uses against various pathogens. Screening a large number of compounds is a complicated step in research. This is possible by virtual screening of a large number of compounds using modern computational tools and molecular data banks [74]. In silico studies on endophytic fungal secondary metabolites is less explored. Kandasamy *et al*., [75] reported the in silico study on metabolites from *Trichoderma* sp. against 4,5-Diarylisoxazole HSP90 Chaperone, a skin cancer protein. The compounds from *Trichoderma* sp. were also tested for their drug likeness by Lipinski's rule.

Table 1: List of some of the anticancer and antioxidant compounds produced by endophytic fungi from various hosts

S.No.	Endophytic Fungus	Host Plant	Compound
1.	*Taxomyces andreanae*	*Taxus brevifolia*	Taxol
2.	*Pestalotiopsis microsporum*	*Torreya taxifolia*	Torreyanic acid
3.	*Entrophospora infrequens*	*Nothapodytes foetida*	Camptothecin
4.	*Fusarium solani*	*Camptotheca acuminata*	Topotecan

5.	*Aspergillus fumigates* *Philalocephala fortinii* *Fusarium oxysporum*	*Juniperus communis* *Podophyllum peltatum* *Juniperus recurva*	Podophyllotoxin
6.	*Leaf Endophyte*	*Mimusops elengi*	Ergoflavin
7.	*Mangrove Endophyte*	*Mangroves*	Secalonic acid D
8.	*Rhinocladiella sp*	*Tripterygium wilfordii*	Cytochalasin Cytochalasin H Cytochalasin J Epoxycytochalasin
9.	*Chaetomium globosum*	*Imperata cylindrica*	Chaetoglobosin
10.	*Mycelia Sterilia*	*Catharanthus roseus*	Vincristine
11.	*Fusarium oxysporum*	*Annona squamosa*	Polyketide
12.	*Alternaria sp*	*Taxus cuspidata*	Paclitaxel
Antioxidant Compounds			
13.	*Pestalotiopsis microspora*	*Terminalia morobensis*	Pestacin Isopestacin
14.	*Cephalosporium sp.*	*Sinarundinaria nitida*	Isobenzofuranone
15.		*Trachelospermum jasminoides*	Graphislactone A
16.	*Fusarium sp.*	*Cajanus cajan*	Cajaninstilbene acid
17.	*Xylaria sp.*	*Ginkgo biloba*	Phenolics
18.			Flavonoids
19.	*Chaetomium sp.*	*Nerium oleander*	Flavonoids
			Phenolic acids

Table 2: List of major Immunosuppressive compounds produced by endophytic fungi from various hosts

S.No.	Endophytic Fungus	Host Plant	Compound
1.	*Cytonaema sp.*	*Medicinal Plant*	Cytonic acid A & B
2.	*Unidentified endophyte*	*Quercus coccifera*	Torreyanic acid
3.	*Pullularia sp*	*Unidentified tree*	Pullularins A-D
4.	*Pestalotiopsis theae*	*Unidentified tree*	Pestalotheol-C
5.	*Aspergillus fumigates* *Philalocephala fortinii* *Fusarium oxysporum*	*Juniperus communis* *Podophyllum peltatum* *Juniperus recurva*	Podophyllotoxin
6.	*Leaf Endophyte*	*Mimusops elengi*	Ergoflavin

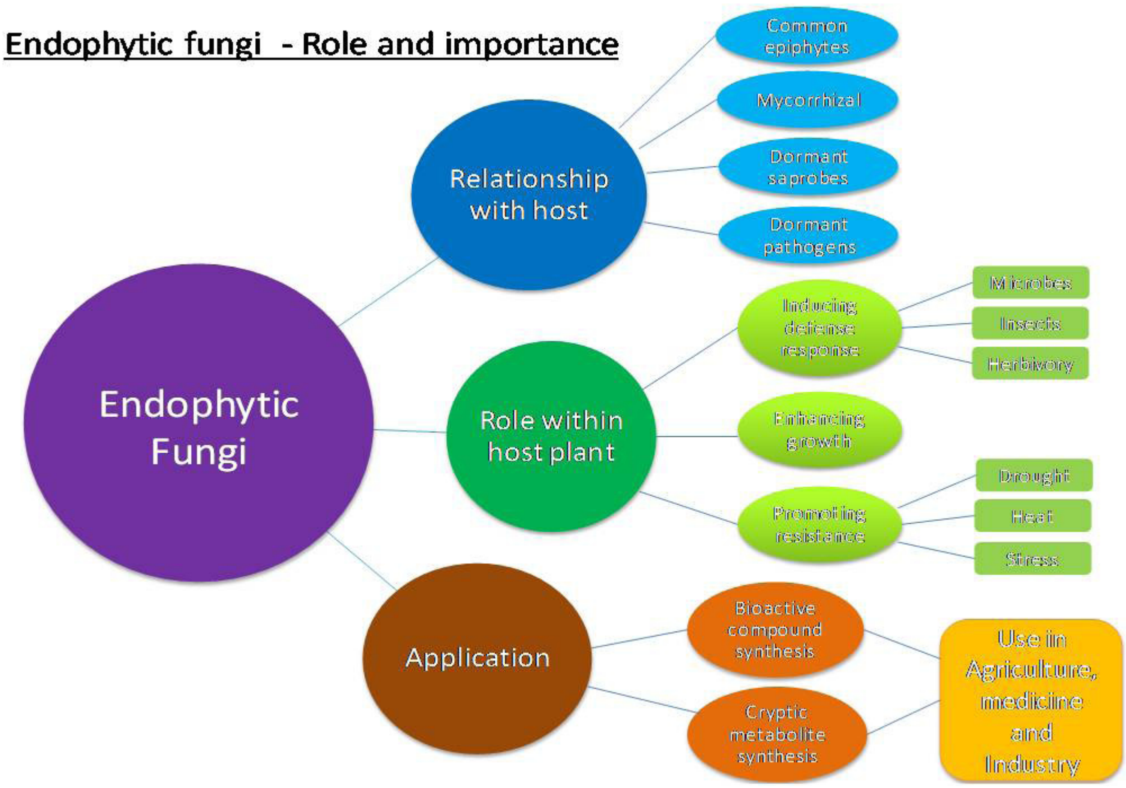


Figure 1: General procedure for the isolation of Endophytic Fungi from various tissues of plant

Sterilization of various plant segments

Collect undamaged and healthy plant tissues-Leaf/Petiole/Stem/Bark/Seed

⇩

Wash thoroughly in running tap water

⇩

Cut Leaf/Petiole/Stem/Bark/Seed into 5-8 mm segments

⇩

Surface-sterilize the segments using in 70% ethanol for 30 seconds and immerse in 4% Sodium hypochlorite for 30 seconds and rinse in autoclaved double distilled water

⇩

Place the segments (4-6) in Petri Plate containing suitable media with a antibiotic

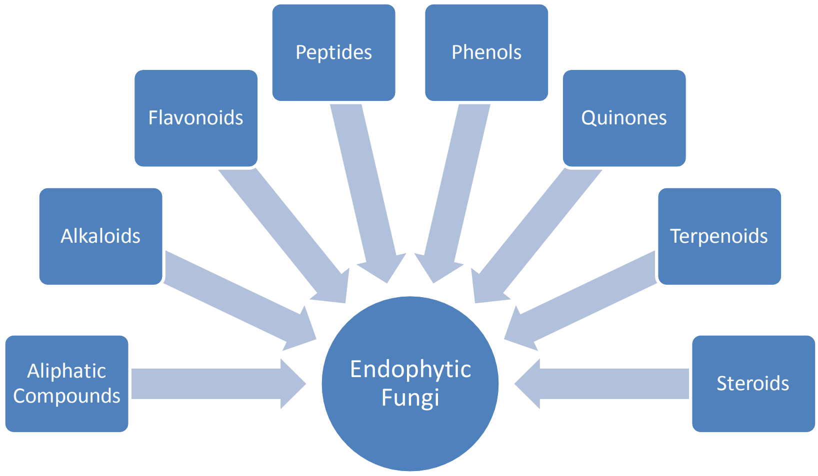
⇩

Incubate the inoculated plates in light chamber for 3-4 weeks for the growth of endophytic fungi

⇩

Identify the endophytic fungi by Conventional (Morphological/Conidial/Fruit body structures) and Molecular Method (ITS primers)

Fig 2: Production of Various Chemical Groups by Endophytic Fungi



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