

Chapter 3

Aspect and Prospect of Endophytic Fungi

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Abstract

Endophyte biology has become one of the hot topics of research in view of importance of endophytes in plant protection, natural products and existence of cryptic fungi. Mutualistic association between host plant species and endophytes has considerable importance in plant ecology, community structure, fitness and evolution. Exploration of rare and endangered habitats, habitat-dependent and host-dependent endophytic fungi needs special attention. Understanding the role of entomopathogenic endophytes are of immense value in biocontrol, plant protection and disease management. As endophytic fungal association with plants has evolutionary significance, reduction or elimination of endophytes in plant tissues by agricultural chemicals and human interference may be detrimental. Production, bioprospecting and utilization of bioactive metabolites from endophytic fungi need further investigation. There are several unsettled issues regarding endophytic fungi and their significance in host plant species.

Keywords: *Endophytes, Mutualists, Endophytic fungi, Bioactive metabolites*

Introduction

Historical perspectives of endophyte study have been traced as early as 1898 (see Hyde and Soyong, 2008). De Bary (1866) first introduced the term 'endophytes' and defined as any organisms occurring within plant tissues. Petrini (1991) defined endophytes as all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host. Schulz and Boyle (2005) denote that endophytes are broad range of inhabitants such as bacteria, fungi, algae and insects in healthy plant tissues. Definitions on endophytes have been viewed in different magnitude and revised time to time by several investigators (see Hyde and Soyong, 2008).

Explosion of research on fungal endophytes in the last three decades yielded tremendous results in understanding the diversity, systematics, ecology, evolution, metabolites, biological control and bioprospecting (e.g. Carroll, 1988; Fisher and Petrini, 1990; Petrini, 1991; Sieber, 1989, 2007; Schulz and Boyle, 2005; Arnold, 2007; Jones *et al.*, 2008; Suryanarayanan *et al.*, 2010; Zhang *et al.*, 2009). Besides publications of several books on endophytic fungi (e.g. Bacon and White, 2000; Schulz *et al.*, 2006; Cheplick and Feath, 2009), some issue of journals have been recently dedicated to project the magnitude of research on endophytic fungi (*Fungal Biology Reviews*, 21 (2-3), 2007; *Fungal Diversity*, 33 (6), 2008). The goal of the present chapter is to address briefly some recent developments in diversity, distribution and function of mainly non-clavicipitaceous endophytic fungi and their importance in selected habitats.

Diversity and distribution

Endophytic fungi are polyphyletic group, primarily composed of ascomycetes. Details of diversity, life-history strategy, adaptation and ecological role of endophytic fungi have been well documented by Rodriguez *et al.* (2009). Based on phylogeny and life-history traits, endophytic fungi have been broadly classified into two categories: i) clavicipitaceous (grass-inhabiting); ii) non-clavicipitaceous (non-grass-inhabiting) (Schulz and Boyle, 2005; Rodriguez *et al.*, 2009). Clavicipitaceous endophytes have been studied extensively than non-clavicipitaceous endophytes (e.g. Clay, 1996, 1997). Based on host colonization, mechanism of transmission and ecological functions, the non-clavicipitaceous endophytes have been differentiated into three functional groups: i) the first group establishes in both above- and below-ground tissues of host plants; ii) the second group confined to above-ground tissues; iii) the third group restricted to below-ground tissues. Although, these groups have a broad host range, the pattern of colonization of host tissues differs: i) the first and third groups extensively colonize the plant tissues, while the second group shows highly localized patchy colonization; ii) the second and third groups are transmitted to plant tissues horizontally, while the first group by both vertically and horizontally.

There are some evidences that saprobes are derived from endophytes and they are latent pathogens (e.g. Brown *et al.*, 1998; Sieber, 2007; Duong *et al.*, 2008). If so, Hyde and Soyong (2008) argue that endophytes colonize above-ground tissues are host- or tissue-specific and genus- or family-specific. Schulz and Boyle (2005) hypothesized that regardless of the life-history strategy of the fungal endophyte, the disease does not manifest until fungal virulence and the host defense reaction are balanced. Sieber (2007) suspects that the endophytic pathogens have been co-evolved with their host plant species. Molecular sequence data from 1403 endophytic fungal strains demonstrated increased diversity, incidence and host range from arctic to tropical regions (Arnold and Lutzoni, 2007). In higher latitudes, the endophytic fungi are characterized by a few species belonging to different classes of ascomycetes in contrast to very large number of endophytes by a small number of classes with a wide host ranges in tropics. Sampling endophytic fungi from selected angiosperms of different latitudinal gradient (northern boreal forest to tropical forest) revealed interesting facts: i) higher diversity in tropical angiosperms; ii) colonization of tissue segments decreased from tropics to temperate latitudes; iii) tropics represented by fewer classes with dominance of Sordariomycetes,

while boreal communities consists of several classes with dominance of Dothideomycetes; iv) the host generalism was dominant in the tropics, whereas strong host-affinity was evident in boreal endophytes (Arnold, 2007; Arnold and Lutzoni 2007). The cultivated endophytes from tropical plant species showed dominance of a large number of rare species, whose host range is not clear (Arnold and Lutzoni, 2007).

World's average fungal resource was estimated by Hawksworth (1991, 2001) as 1.5 million species based on plant and fungus ratio. This estimation was based on plant and fungus ratio (1:6) out of 250,000 plant species. It was initially applied to temperate regions, while investigations on palm fungi in Queensland raised this ratio to 1:26 in the tropics, which has been further updated to 1:33 mainly based on palm fungi in Australia and Brueni Darussalam (Hyde, 1995; Fröhlich and Hyde, 1999). A variety of endophytic fungi have been reported from diverse palms (e.g. Fröhlich *et al.*, 2000; Lumyong *et al.*, 2009) and there is a strong belief that missing fungi are hidden as endophytes. However, the evolutionary origins and diversity of mutualistic endophytes are still not clearly understood.

Terrestrial habitats

Leaves

A wide range of plant species surveyed composed of endophytic fungal symbionts in foliar tissues (Stone *et al.* 2000). Endophytes inhabiting leaves (short-lived, photosynthetically versatile and easily subject to damage by herbivores) are under high selective pressure than those associated with persistent tissues (e.g. bark, xylem or other woody parts) (Arnold, 2007). Arnold *et al.* (2003) suspect that tropical plant species have the potential to develop differential endophytic symbiosis with diverse mycota by horizontal transmission. They have demonstrated that horizontally transmitted endophytes in woody angiosperms play an important role in host defense. On inoculation of endophyte-free leaves of cocoa (*Theobroma cacao*) with endophytic fungus (*Phytophthora* sp.) significantly decreased leaf necrosis and mortality (Arnold *et al.*, 2003). As endophyte in maize, *Fusarium verticillioides* reduced disease severity caused by *Ustilago maydis* (Lee *et al.*, 2009). Similarly, *Fusarium oxysporum* isolated from healthy tissues of tomato seedlings exhibited potent *in vivo* anti-oomycete activity against tomato late blight and *in vitro* anti-oomycete potential indicating its usefulness as biocontrol agent (Kim *et al.*, 2007).

Protection by endophytes was greater in mature than young leaves and *in vitro* experiments revealed that leaf chemistry mediate their host affinity. Thus, leaf chemistry assumes special importance in foliar endophytic assemblage, diversity and function. Due to biochemical diversity among the leaves of different plant species and high degree of competition among fungi to colonize foliage, Arnold (2007) suspects that endophytes of leaves are host-specific or facultative saprotrophs and possess different evolutionary origins than wood-inhabiting endophytes. It is likely the lifestyle of foliar endophytes dependent on nature of plant species such as deciduous or evergreen in contrast to those colonizing woody tissues although both substrates colonized by common endophytes (Arnold, 2007). Horizontal transmission of foliar endophyte (*Phialocephala scopiformis*) from white spruce trees (*Picea glauca*) to its seedlings has been studied recently by Miller *et al.* (2009). Dissemination of endophyte was up to 40% in three years with mean rugulosin (anti-insect toxin) concentration up to 1 µg/g tissue. An interesting perspective has been given by Devarajan and Suryanarayanan (2006) in relation to the role of grasshoppers in the dispersal on non-grass endophytes. Grasshoppers preferred or avoided milkweed leaves covered by spore suspension of *Colletotrichum*. However, spores retained their viability through gut passage suggesting grasshoppers as potential vectors in dissemination of endophytic fungi.

Bark and wood

Endophytic fungi in branches of many dicotyledonous trees possess several ascomycetes and basidiomycetes (Chapela and Boddy, 1988). Endophytic fungi in twigs of *Quercus ilex* and dominant fungi include: *Colletotrichum*, *Nodulisporium* and *Phyllosticta* (Fisher *et al.*, 1994). *Aureobasidium*, *Botryosphaeria* and *Cytospora* have

been recovered from stems of *Eucalyptus* (Simeto *et al.*, 2005). Some studies have indicated that the diversity of endophytes in twigs is less diverse than other niches such as bark (Tejesvi *et al.*, 2005). Kumar and Hyde (2004) demonstrated tissue specificity of endophytes in woody angiosperms and conifers.

Endophytic fungal community from the twig xylem of Chinese medicinal plant (*Tripterygium wilfordii*) was most diverse than leaves, bark, root xylem and flowers (Kumar and Hyde, 2004). *Pestalotiopsis cruenta*, *Phomopsis* spp. were dominant in twig xylem and bark. In addition, 13 morphotypes were isolated from twig bark and twig xylem. Analysis of twigs and bark of *Terminalia arjuna*, a tropical woody angiosperm (used as an important ayurvedic medicinal plant) revealed greater diversity during monsoon season (Tejesvi *et al.*, 2005). Endophyte colonization was four-fold higher in bark than twigs. The most dominant endophyte genera were *Chaetomium*, *Myrothecium* and *Pestalotiopsis*. Recently, Gangadevi and Muthumary (2009) demonstrated production of anticancer drug, taxol by an endophytic fungus (*Chaetomella raphigera*) isolated from *Terminalia arjuna*. Freshwater hyphomycetes were also known as endophytes in aerial plant tissues (Sokolski *et al.*, 2006). Several typical freshwater hyphomycetes were colonizers of epiphytic fern (*Drynaria quersifolia*) in tree canopies of the west coast and Western Ghats of India (Sridhar *et al.*, 2006; Karamchand and Sridhar, 2009).

Roots

Leaves, berries, stems and roots of *Coffea arabica* from different geographic regions (Colombia, Hawaii, Mexico and Puerto Rico) yielded 843 isolates of fungal endophytes, which resulted in 257 unique genotypes (Vega *et al.*, 2009). Taxa belonging to *Colletotrichum*, *Fusarium*, *Penicillium* and Xylariaceae were most abundant. Species of dark-septate endophytic fungi are relatively few (Addy *et al.*, 2005), which colonize roots of many plant species and have been recognized as a functional group based on their melanized hyphae (Jumpponen and Trappe, 1998; Jumpponen, 2001, 2003). Although their diversity and ecological functions are not yet clear, it is known that they have a variety of lineages with Pezizales, Helotiales and Pleosporales (Jumpponen, 2001). The dark-septate endophytes are also found in foliar tissues (Jumpponen, 2001; Higgins *et al.*, 2007). These fungi have been examined in some plant species by Suryanarayanan and Vijaykrishna (2001) and Kumar and Hyde (2004). A brief account on colonization of roots by freshwater hyphomycetes is given in subsequent section.

Piriformospora indica is a cultivatable root-inhabiting basidiomycete, which has capability to establish in semi-defined media without a host (Varma *et al.*, 2001). It is known from the roots of a wide range of plant species including trees, agricultural/horticultural/medicinal plants, monocots, dicots and mosses (see Vadassery *et al.*, 2009). It colonizes the roots of *Arabidopsis thaliana* and promotes growth, development and seed production. The cell wall extract of *P. indica* promotes growth of *Arabidopsis* seedlings and also induces intracellular elevation of calcium roots. This fungus is also known to stimulate uptake of nutrients and helps to develop resistance to biotic and abiotic stresses (e.g. Verma *et al.*, 1998; Varma *et al.*, 1999, 2001; Pham *et al.*, 2004; Waller *et al.*, 2005; Shahollari *et al.*, 2007; Sherameti *et al.*, 2008a, 2008b).

Seeds

Relatively, seeds possess less number of endophytic fungi than other tissues probably due to vertical transmission. For instance, Ganley and Newcombe (2006) recovered over 2000 endophytic fungal isolates from foliage of *Pinus monticola*, while only 16 from 750 surface-sterilized seeds. Although foliage of *Theobroma cacao* associated with diverse endophytes, their seeds were devoid of endophytes (Arnold *et al.*, 2003). In contrast to vertical transmission of clavicipitaceous endophytes in grasses, multiple species of endophytes have been documented from seeds of plants other than grasses (Clay and Schardl, 2002; Arnold, 2007). The culture-independent methods (environmental PCR) revealed occurrence of diverse ascomycete endophytes in seeds of *Ceropia insignis* in tropics (Gallery *et al.*, 2007).

Coastal sand dunes

Leaves of *Suaeda fruticosa*, a beach halophyte consists of common endophytic fungi belonging to the genera *Acremonium*, *Alternaria*, *Cladosporium*, *Colletotrichum* and *Fusarium* (Fisher and Petrini, 1987). From 1512 root pieces of plant species belonging to coastal and inland soils, sandy soils and salt marshes of southwest Spain yielded 1830 isolates consisting of 142 identifiable endophytes (57 genera) and 177 morphospecies (Maciá-Vicente *et al.*, 2008). Leaves and rhizomes of grasses belonging to *Ammophila* and *Elymus* from 12 coastal sand dunes of the northern Spain yielded 103 isolates of endophytic fungi (Márquez *et al.*, 2008). Significant inverse relationship has been shown between similarities of endophytic assemblages and distance of host plant species.

In coastal sand dunes of southwest coast of India, two wild legumes (*Canavalia cathartica* and *C. maritima*) colonized by 46 taxa of endophytic fungi and morphospecies (Seena and Sridhar, 2004). Among different age (seed, seedling and mature plant) and tissues (root, stem, leaf, seed coat and cotyledon) classes, the highest number of endophytic fungi was recorded in the seedlings and mature plants with lowest in seeds. *Chaetomium globosum* exhibited single species dominance in root, stem and leaf segments of *C. maritima* and root segments of *C. cathartica*. *Chaetomium* has been considered as a cosmopolitan genus and especially common as endophyte in desert plant species and suspected that it colonizes when exterior conditions are inhospitable (Hoffman and Arnold, 2007)

The genus *Chaetomium* is known to produce a variety of secondary metabolites (e.g. chaetomin, chaetoglobosins, chaetoquadrins, oxaspirodion, chaetospiron, orsellides, chaetocyclinones) (Sekita *et al.*, 1976; Loesgen *et al.*, 2007; Suryanarayanan *et al.*, 2010). Chaetoglobicins are cytotoxins, which are analogs of cytochalasin having inhibitory effect on actin polymerization (Yahara *et al.*, 1982; Suryanarayanan *et al.*, 2010). In addition, *C. globosum* also produces a nematicide called flavipin (Chitwood, 2002). Extensive colonization of root, stem and leaf of *Canavalia* by *C. globosum* in coastal sand dunes indicates its potential role against herbivores and nematodes.

Although beaches comprise of several marine fungi (e.g. saprophytes on woody litter, arenicolous fungi on sand grains), surprisingly only 3% of marine fungi were endophytic in wild legumes (*Canavalia* spp.) (Seena and Sridhar, 2004). Similarly, marine fungi were also less dominant as root endophytes in other coastal sand dune halophytes (Beena *et al.*, 2000)

Aquatic habitats

Freshwater

Currently, freshwater hyphomycetes are also well known endophytes in roots exposed to streams and aerial plant parts without producing disease symptoms (see Baerlocher, 2006; Sokolski *et al.*, 2006). Nemec (1969) reported *Tetracladium marchalianum* as root endophyte of *Fragaria* sp., while Watanabe (1975) found *Tetracladium setigerum* endophytic in roots *Fragaria* sp. and *Gentiana* sp. Many strains of *Gyoefferfyella* have also been isolated from healthy roots of *Picea abies* (see Selosse *et al.*, 2008). Currently, about 50 species of freshwater hyphomycetes are known as endophytes in submerged roots belonging to approximately 25 plant species (angiosperms, gymnosperms and pteridophytes) growing in freshwater and brackish water habitats (e.g. Marvanová and Fisher, 1991; Fisher *et al.*, 1991; Sridhar and Baerlocher, 1992a, 1992b; Marvanová *et al.*, 1992, 1997; Raviraja *et al.*, 1996; Sati and Belwal, 2005; Sati *et al.*, 2008, 2009a, 2009b).

Fisher and Petrini (1989) first demonstrated the endophytic-phase of two typical aquatic hyphomycetes (*Campylospora parvula* and *Tricladium splendens*). Subsequently, Fisher *et al.* (1991) compared endophytic aquatic hyphomycete population in submerged and terrestrial roots and demonstrated higher colonization in submerged (30%) than terrestrial (12%) roots. Submerged macrophytes (e.g. *Potamogeton*, *Ranunculus*, *Apium*) were also constitute major substrate in the absence of submerged leaf litter in streams (Baerlocher, 1992). Studies on root endophytes may reveal additional anamorph-teleomorph connections (Webster, 1992; Sivichai and Jones,

2003). Interestingly, production of teleomorphic state by endophytic *Heliscus lugdunensis* upon subculturing was reported by Sridhar and Baerlocher (1992a). New species of aquatic hyphomycetes (*Filospora fistucella*, *F. versimorpha*, *Fontanospora fusiramosa* and *Tetracladium nainitalense*) have also been described from endophytes of riparian roots (Marvanová and Fisher, 1991; Marvanová *et al.*, 1992, 1997; Sati *et al.*, 2009b).

Brackish and marine

Although saprophytic fungi of mangrove plant species have attracted the attention of mycologists, studies on endophytic fungi have been initiated recently (Suryanarayanan *et al.*, 1998; Suryanarayanan and Kumaresan, 2000; Kumaresan and Suryanarayanan, 2001, 2002; Jones *et al.*, 2008; Sridhar, 2009). Endophytes belonging to the genera *Acremonium*, *Alternaria*, *Cladosporium*, *Colletotrichum* and *Fusarium* were common in mangrove plants (Suryanarayanan *et al.*, 1998, Suryanarayanan and Kumaresan, 2000; Kumaresan and Suryanarayanan, 2001, 2002) and seagrass, *Halophila ovalis* (Devarajan *et al.*, 2002). In addition, *Phomopsis* and *Phyllosticta* were also common foliar endophytes of mangrove plants (Suryanarayanan *et al.*, 1998; Suryanarayanan and Kumaresan, 2000; Kumaresan and Suryanarayanan, 2001, 2002; Ananda and Sridhar, 2002).

Sporormiella minima and *Cladosporium cladosporioides* were major foliar endophytes of mangrove plant species of southeast coast of India. Foliar endophytes were dominated by single species in many mangrove plant species: *Avicennia marina* (*Phoma* sp.), *Bruguiera cylindrica* (*Colletotrichum gloeosporioides*), *Rhizophora apiculata* (*Sporormiella minima*), *Rhizophora mucronata* (*Sporormiella minima*) and *Suaeda maritima* (*Camarosporium palliatum*) (Suryanarayanan *et al.*, 1998; Suryanarayanan and Kumaresan, 2000; Kumaresan and Suryanarayanan, 2001). However, *Lumnitzera racemosa* showed multiple species dominance by *Alternaria* sp., *Phomopsis* sp. and *Phyllosticta* sp. (Kumaresan and Suryanarayanan, 2001). Multiple species dominance of endophytic fungi was also seen in the roots of *Avicennia officinalis*, *Rhizophora mucronata* and *Sonneratia caseolaris* by Ananda and Sridhar (2002).

In mangrove associates (*Acanthus ilicifolius* and *Acrostichum aureum*), 25 endophytic fungi comprising three ascomycetes, 20 mitosporic taxa and two sterile morphotypes were recovered from the West Coast mangrove by Maria and Sridhar (2003). Overall colonization by endophytes was as high as 74.5-77.5%. Out of four tissues screened (leaves, stem, rhizome, root), species richness and diversity were high in stems of *A. ilicifolius* and roots of *A. aureum*. In Mai Po Nature Reserve of Hong Kong, Pang *et al.* (2008) studied endophytic fungal association with bark, woody tissues and leaves of *Kandelia candel.* Endophytic assemblage was similar in bark and wood, but deferred in leaf samples. Dominant and cosmopolitan endophytes were *Guignardia* sp., *Pestalotiopsis* sp., *Phomopsis* sp. and *Xylaria* sp. In mangrove and mangrove associate plant species of the west coast of India, typical marine fungi as endophytes were low (up to 5%) (Ananda and Sridhar, 2002; Maria and Sridhar, 2003; Anita and Sridhar, 2009; Anita *et al.*, 2009).

Seaweeds and seagrasses

Live seaweeds and seagrasses constitute suitable substrates for colonization of fungi (Sugano *et al.*, 1994; Nielsen *et al.*, 1999; Devarajan *et al.*, 2002; Zhang *et al.*, 2009). Zuccaro *et al.* (2003, 2008) reported endophytic fungi from *Fucus serratus*. The endophytic fungus, *Mycophycias ascophylli* has been repeatedly isolated from thallus of *Ascophyllum nodosum* (Stanley, 1991). It grows mutually with *A. nodosum* and *Pelvetia canaliculata* (Kohlmeyer and Kohlmeyer, 1979; Kohlmeyer and Volkmann-Kohlmeyer, 1998; Ainsworth *et al.*, 2001) and remains associated with algal host throughout its life cycle (Stanley, 1991). Raghukumar (1996) has demonstrated association of several marine algae with endophytic fungi in Indian coast. For example, *Cladophora* sp. and

Rhizoclonium sp. were frequently associated with the chytrid *Coenomyces* sp. Similarly, many filamentous fungi and thraustochytrid endophytes were isolated from *Centroceras clavulatum*, *Gelidium pusillum*, *Padina tetrastomatica*, *Sargassum cinereum*, *Valoniopsis pachynema* and *Ulva fasciata* (Raghukumar *et al.*, 1992; Raghukumar, 2008). Thraustochytrids and labyrinthulids (e.g. *Aplanochytrium minutum*) were often isolated from surface sterilized seaweeds (*P. tetrastomatica* and *S. cinereum*) (Sathe-Pathak *et al.*, 1993). Up to 26 species of endophytes have been reported in the seagrasses such as *Thalassia testudinum*, *Zostera japonica* and *Z. marina* (Alva *et al.*, 2002).

Endophytes from marine algae and seagrasses produce a variety of metabolites (see Raghukumar, 2008; Zhang *et al.*, 2009). Some novel metabolites were obtained from algal taxa belonging to different geographic origin (Schulz *et al.*, 2008). Marine fungus, *Dendryphiella salina* was associated with brown seaweeds is known to produce a range of bioactive compounds (Guerrero *et al.*, 1989). The obligate marine fungus *Aschochyta salicorniae* associated with *Ulva* sp. showed anti-plasmodial activity against *Plasmodium falciparum* (Osterhage *et al.*, 2000). However, many interesting algal-fungal associations have not been investigated in view of bioactive metabolite perspective.

Entomopathogens

Research on entomopathogenic endophytic fungi is fairly recent. Webber (1981) seems to be the first investigator to report elm tree protection by endophytic fungus (*Phomopsis oblonga*) against the beetle (*Physocnemum brevilineum*). Azevedo *et al.* (2000) extensively reviewed subsequent two decades of research on entomopathogenic endophytic fungi. Important and widely distributed endophytic fungi serve as entomopathogens include: *Beauveria* spp. (endophytic in maize, potato, cotton, tomato, cocoa, *Pinus*, banana, coffee) (Evans *et al.*, 2003; Ownley *et al.*, 2004; Arnold and Lewis, 2005; Ganley and Newcombe, 2006; Akello *et al.*, 2007; Posada *et al.*, 2007; Vega *et al.*, 2008), *Paecilomyces farinosus*, *P. varioti* and *Verticillium lecanii* (Petrini, 1981; Bills and Polishook, 1991; Ananda and Sridhar, 2002). Endophytic fungi are beneficial to host plant species to prevent herbivory and induce tolerance to stresses (e.g. heat, salt, disease, drought) and also increase below- and above-ground plant biomasses (Waller *et al.*, 2005; Tejesvi *et al.*, 2007). Survey of entomopathogenic fungal endophytes yielded several potential biocontrol agents against pests and possess potentialities to develop as future mycopesticides (see Kaewchai *et al.*, 2009). Vega *et al.* (2008) raised an interesting question: whether the metabolites of entomopathogenic fungal origin inoculated to agronomically important plants enter the food chain?

Natural products

Medicinal plants

Based on traditional morphological techniques, Huang *et al.* (2008) isolated 1160 endophytic fungi from 29 Chinese medicinal plants. Among these endophytes, *Alternaria*, *Colletotrichum*, *Phoma*, *Phomopsis*, Xylariales and sterile morphotypes were dominant. Medicinal plants of the Western Ghats of India endowed with a variety of endophytic fungi (e.g. Raviraja, 2005; Krishnamurthy *et al.*, 2008; Naik *et al.*, 2008). From 9000 leaf segments of 15 medicinal shrubs from seven locations of Western Ghats, 6125 fungal endophytes were isolated by Naik *et al.* (2008). Hyphomycetes and coelomycetes were dominant than mucorales and sterile morphotypes. *Alternaria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Penicillium*, *Phyllosticta* and *Xylaria* were most frequently isolated fungi. Number of isolates obtained during the winter season were significantly more than monsoon and summer seasons.

Active metabolites

About 4,000 biologically active secondary metabolites have been described from fungi (mainly from *Acremonium*, *Aspergillus*, *Fusarium* and *Penicillium*) (Dreyfuss and Chapela, 1994; Hyde and Soyong, 2008). Endophytes are a good source of diverse novel metabolites and secondary metabolites including plant protectants (e.g.

xanthenes, phenols, isocoumarins, perylene derivatives, quinones, furandiones, terpenoids, depsipeptides, cytochalasins) (see Schulz *et al.*, 2002; Schulz and Boyle, 2005; Raj and Shetty, 2009). Endophytes have been screened for many novel metabolites such as antibiotics, anti-cancer drugs (e.g. paclitaxel, Hsp 90 inhibitors, sequoiatones, camptothecin), lactones, enalin, colletotrichic acid, myrocin and apiosporic acid, phomopsilactone, cyclopentanoids, (+)-ascoschin; (+)-ascodiketone, chaetocyclinones, pestalothols, isofusidienols and naphthoquinone spiroketals (see Suryanarayanan *et al.*, 2010). Schulz *et al.* (2002) studied 6,500 endophytic fungi for their natural products and found 51% out of 135 metabolites as new bioactive compounds. Several endophytic fungal metabolites mimic the structure and function of host compounds (e.g. gibberellins: MacMillan, 2002; taxol: Stierle *et al.*, 1993; subglutinols: Lee *et al.*, 1995; Strobel, 2002). Such mimicking ability of associated endophytic fungi has been suspected to genetic recombination of the endophytes with the host during evolution of mutualism (Tan and Zou, 2001).

The US patents on active metabolites of endophytic fungal origin elevated steeply after discovery of taxol producing fungus, *Taxomyces andreanae* (Priti *et al.*, 2009). Certain metabolites produced by endophytic fungi are same or similar to that of host plant species (e.g. anticancer drug taxol by *Taxomyces andreanae* associated with yew plants *Taxus brevifolia*) (Stierle *et al.*, 1993). Similarly, endophytic fungus, *Pestalotiopsis microspora* associated with an endangered tree species, *Torreya taxifolia* produced torreyanic acid, which is a powerful apoptotic drug (Lee *et al.*, 1996). Endophytes have the ability to produce several compounds produced by the host plants (e.g. gibberellins, subglutinols) (Lee *et al.*, 1995; MacMillan, 2002; Strobel, 2002). Endophytic fungi derived from the oil palm (*Elaeis guineensis*) were also major source of several bioactive compounds and their association reduced the root decay (Rungjindamai *et al.*, 2008).

There seems to be multiple factors (e.g. host, tissue, age, season, environment, geographical location) influence the metabolite production by endophytic fungi (Moricca and Ragazzi, 2008; Shwab and Keller, 2008). Thus, there are failures to obtain commercially exploitable quantity of natural products from isolated endophytic fungi (Li *et al.*, 1998; Young *et al.*, 2006; Szewczyk *et al.*, 2008; Priti *et al.*, 2009). Priti *et al.* (2009) argue that the endophytic fungal metabolites are the product of interaction of host and fungus, which are regulated by several host and environmental factors. Lack of such host stimulus *in vitro* might attenuate the ability of endophytic fungi to generate desired metabolites. There is a need to develop appropriate methods to induce endophytic fungi to produce required metabolites *in vitro*.

Evaluation

Endophytic fungal evaluation in plant species needs special attention to avoid contaminants. Different methods of surface sterilization of plant tissues and isolation of endophytic fungi have been documented by Gallo *et al.* (2008). Schultz *et al.* (1998) advocated assessing sterilized tissue pieces by imprinting on a medium to ascertain efficiency of surface sterilization. Surface sterilization will be assumed effective only when no fungi grow on the medium on imprinting. It is also useful to evaluate fungi associated with plant species in both surface sterilized and unsterilized segments (Anita and Sridhar, 2009; Karamchand *et al.*, 2009). Similarly, Suryanarayanan *et al.* (2009) proposed that evaluation of endophytes should include different substrates to arrive at near-real species diversity.

Methodological constraints to study endophytic fungi and necessity to adapt traditional and molecular techniques have been addressed by Hyde and Soyong (2008).

Endophytic fungi within leaves were ascertained by detecting β -D-glucans of fungal cell walls by Johnston *et al.* (2006). Fastidious endophytes may need different media than routine media for isolation (Guo *et al.*, 2001). Similarly, it is necessary to isolate slow-growing endophytes by preventing growth of fast-growing endophytes using differential media (Hyde and Soyong, 2008). Most endophyte isolation exercises usually result in recovery of several sterile morphotypes. A method to promote

sporulation of palm-derived sterile morphotypes has been developed by Guo *et al.* (1998) and achieved sporulation of isolates up to 83.5% against 47.8% on conventional incubation on MEA agar medium. Some studies employed molecular techniques to place sterile morphotypes of endophytic fungi for appropriate taxonomic placement (Promputtha *et al.*, 2005; Wang *et al.*, 2005). Arnold (2007) advocated establishing repository of unknown cultures of endophytic fungi for future use. Interestingly, Hambleton and Sigler (2005) erected a new genus with three new taxa from sterile morphotypes based on gene sequence data. There may be several uncultivable endophytes in live plant tissues and appropriate to assess them by molecular techniques. Techniques like DNA cloning, DGGE, T-RFLP and stable isotope profiling (SIP) have been attempted by several investigators in order to detect total fungal communities in plant tissues (e.g. Nikolcheva and Baerlocher, 2005; Vandenkoornhuyse *et al.*, 2002; Seena *et al.*, 2008; Tao *et al.*, 2008). These methods are useful to evaluate endophytic fungal diversity more precisely.

Systemic fungicide treatment as foliar spray helps to obtain endophyte-free plants for experimental purpose (Cheplick, 1994; Hill and Brown, 2000). Recently, a systemic fungicide, hexaconazole [2-(2,4-dichlorophenyl)-1-(1H-1,2,5-triazol-1-yl)hexan-3-ol] treatment qualitatively and quantitatively decreased foliar endophytes of mango trees (*Mangifera indica*) (Mohandoss and Suryanarayanan, 2009). Elimination or differential/selective elimination of endophytes by fungicide treatment will be valuable to understand the role of endophytes in host susceptibility or resistance to diseases. Similarly, temperature treatment also reduced the endophytes of seeds in grass species, *Elymus virginicus* (Rudgers and Swafford, 2009) and proved to be an useful method to study the importance of vertically transmitted endophytes.

Outlook

According to Schulz and Boyle (2005) the endophytic continuum is a developmental and evolutionary process. Relatively, endophytes of foliar tissues have been studied extensively than other tissues. Are foliar endophytes transient flora? If so, what are the means by which such fungi will be attracted or accommodated by the host plant species for their benefits through horizontal transmission? Still there are several unsettled questions about the diversity, distribution, evolution and functions of endophytic fungi. Some fungi have multiple ecological roles such as endophyte, pathogen (opportunistic?) and or saprotroph (e.g. *Chaetomium globosum*, *Paecilomyces varioti*) (Ananda and Sridhar, 2002; Seena and Sridhar, 2004; Arnold *et al.*, 2007; Naik *et al.*, 2007; Vega *et al.*, 2008). Are such fungal features overlapping? How these traits switchover? Is it depending on the substrate or environmental conditions? Possibly, fungicide and temperature treatments to reduce/eliminate endophytic fungi differentially/selectively in live tissues might facilitate to prove Koch's postulates to understand host range, pattern of colonization, mode of transmission (vertical or horizontal), anthropogenic/environmental influences, host vulnerability, impact of pathogens and ecological functions of endophytic fungi.

It is interesting to understand whether non-sporulating endophyte morphospecies also produce novel metabolites. For instance, rugulosin, a metabolite of a nonsporulating spruce endophyte is active against the spruce budworm (Miller *et al.*, 2009). Are sporulating and non-sporulating endophytes producing novel metabolites in dual culture *in vitro* due to mutual competition? It is worth understanding the impacts of host species and environmental factors in stimulating endophytic fungi to produce secondary metabolites for bioprospecting purpose.

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