

## CHAPTER 5

### DISCUSSION

The endophyte assemblages from the same plant species growing in different locations may be quite different. A number of factors might cause this phenomenon including vegetation in the vicinity and environmental condition such as temperature, rainfall and humidity. There can also be effects on the mycoflora resulting from human activities (Mekkamol, 1998) whereas, the endophyte assemblages from the same plant species growing at the same location are generally similar, marked differences in species richness and distribution of selected fungal species can be detected between young and old individuals of the same host (Petrini, 1991).

It is well known that the mycoflora in tropical and subtropical area are more complex and diverse than those in temperate areas (Hyde and Hawksworth, 1997). There are also without doubt large numbers of new fungal species remaining to be discovered, especially in tropical regions. (Wildman, 1997). However, very few studies on endophytes have as yet been done in the tropics. In this investigation of fungal endophytes, both young and mature teak leaves and rain tree leaves were sampled from sites on the campus of Chulalongkorn University, Bangkok. This research is the first known study of rain tree endophytes and therefore most of information presented here is by default novel. Comparison of the data is made with those of Mekkamol *et al.* (1996) and Mekkamol (1998) from their studies on the teak leaves which had been sampled from two geographically separated sites where the trees from one site were growing in a natural forest and from the second site from trees in a plantation. Further comparisons are made with the results of Rodrigues and Samuel (1990), Rodrigues (1994, 1996) and Frohlich, Hyde and Petrini (2000) from their studies on the tropical palms *Licuala* spp. and *E.oleracea*.

The number of isolates from mature leaves of both teak and rain trees is greater than for young leaves (Figure 4.11 and Figure 4.24). This result is in accordance with most of the investigations undertaken with other host plants from other localities in which

older leaves tended to support a greater frequency of internal fungal colonization. Changes in the plant physiology e.g. leaf microenvironment and biochemical contents and production of secondary compounds has been proposed as possible causes for an increase of endophytic colonization as the leaf ages (Espinosa-Garcia and Langenhiem, 1990). In explaining the much higher recovery of endophytic fungi from mature leaves, Rodrigues (1994) suggested that the expanded palm leaves represent a better physical trap for spores than folded leaves. As pointed out by Mekkamol (1998) the mature teak leaves attain a greater size, growing to approximately 40 cm in length and 30 cm at their widest point. The young leaves were around one third the size of the mature leaves. Therefore in teak the mature leaves offer much greater surface area for inoculum capture. In addition, older leaves would have been exposed longer than younger leaves and therefore received higher amounts of inoculum (Wilson and Carroll, 1994).

Climatic conditions may greatly influence the colonization of plants by endophytic fungi (Petrini, 1991). The temporal pattern of infection level was most influenced by environmental condition, which change from wet and moist on the phylloplane to dry and hot with the onset of summer. Infection levels were closely associated with rainfall, and increased as rainfall increased in the early part of the growing season. When rain stops, usually infection levels do not increase (Wilson and Carroll, 1994). In this study, the leaves were collected during January to December for investigation of seasonal effects. There are usually three seasons a year in Thailand such as the winter season from mid-November to January, dry season from February to May and rainy season from June to November (Thienhirun, 1997). The number of fungal isolates from teak and rain tree leaves increased during June to November (rainy season) but decreased during March to April (dry season). The lower number of isolates recovered from trees during the dry season indicating that environmental factors, such as rainfall and atmospheric humidity might influence the occurrence of some endophytic species. Rodrigues (1994) suggested the lower number of isolates recovered during dry season could be related to the effects of water stress. It is known that under water deficit some plants may accumulate non-structural carbohydrates. This accumulation

generally leads to a build up of carbon-based defenses such as tannins, making the plant less susceptible to fungal endophyte colonization during the dry season.

The *Penicillium* sp. was isolated only in the dry season. This may be because spores of *Penicillium* can survive and even grow in a low water environment or dry condition, categorizing it as a xerophilic type of fungi. In the rainy season, spore inoculum of Coelomycetes such as *Phomopsis* sp. produce slimy conidia that are not forcibly released but dispersed by water in various ways, may be higher than for the spores of the *Penicillium* sp. which was not found in the rainy season. Differential susceptibility of leaves could also have caused the high variance in infection levels. Inoculation of greenhouse trees with conidial suspensions of the endophyte suggested that differential susceptibility of leaves and in particular the conditions during exposure of the leaves to conidia are both important. (Wilson and Carroll, 1994). On highly hydrophobic leaf surfaces, i.e., with low wettability, water droplets maintain a high contact angle and are readily removed if the leaf is shaken or inclined. The wettability of both spores and leaf surfaces influences the deposition and distribution of spores. The non-wettable spores held on the surface of water droplets were deposited on the hydrophobic leaf surfaces over which they had passed, whereas wettable spores were carried away with the droplets. Furthermore, a wettable surface holds more water droplets and spores, possibly accounting for increased infection efficiencies (Allen *et al.*, 1991).

The endophytic assemblages of the *T.grandis* trees were composed of a number of cosmopolitan species such as *Alternaria* spp. which have been recorded as endophytes in both temperate and tropical areas (Rodrigues and Petrini, 1997), and of fungi such as the xylariaceous anamorphs known to live endophytically in a large number of hosts. There were also a large number of coelomycetous taxa such as *Phomopsis* spp. and *Colletotrichum* sp. The diversity of endophytic fungi isolated from *T.grandis* in Bangkok, Thailand was lower than that of the endophyte mycobiota of *T.grandis* from Chiang Mai, Thailand as found by Mekkamol(1998) (Table 5.1). This may be because the indigenous mycobiota and teak in Chiang Mai are better adapted to

each other than the Bangkok mycobiota is to the trees present in Bangkok which were originally introduced from northern Thailand. On the other hand, since horizontal transmission of fungal endophytes is probably the rule for woody plants, these differences could well be only the result of the absence of inoculum, so that trees planted outside their natural distribution area would tend to become colonized by indigenous fungi such as *Alternaria* spp. A study by Fisher, Petrini and Sutton (1993) confirmed that the endophyte assemblages of trees planted outside their original range consisted of species different from those in native habitats. A number of factors might cause this phenomenon including vegetation in the vicinity and environmental condition such as temperature, rainfall and humidity and perhaps even pollution. Higher colonization rates by endophytic fungi can be observed for samples from homogenous stands with a closed canopy. It is therefore not surprising that studies by Mekkamol (1998) showed that endophytic fungi from teak in the forest had a higher species richness and different diversity.

The current study also resulted in the isolation of Basidiomycetes from mature teak leaves. This was based on the presence of clamp connections or the production of basidiocarps in cultures. These isolates was *Schizophyllum commune*. Whereas in the recent study by Mekkamol (1998) basidiomycetes were not found. Petrini and Carroll (1981) reported that basidiomycetes as a component of an endophytic flora may be more apparent than real, an artifact of isolation and scoring methods used. *Schizophyllum* sp. have been reported as a fungal endophyte of *Eucalyptus nitens* in Australia (Fisher, Petrini and Sutton, 1993) .

Table 5.1 Comparison of endophytic fungi isolated from mature teak leave from Chiangmai and Bangkok

Chiangmai (Mekkamol,1998)	Bangkok(This study)
-	<i>Alternaria</i> sp.*
<i>Cladosporium</i> sp.	-
<i>Colletotrichum</i> sp.*	<i>Colletotrichum</i> sp.*
<i>Curvularia</i> sp.	-
<i>Daldinia eschscholzii</i> *	<i>Daldinia eschscholzii</i>
<i>Diplodia</i> sp.	-
<i>Fusarium</i> sp.	<i>Fusarium</i> sp.
<i>Humicola</i> sp.	-
<i>Hypoxyton</i> cf. <i>anthochroum</i>	-
<i>Hypoxyton</i> cf. <i>subrutilum</i>	-
<i>H. subrutilum</i>	-
<i>H. haematostroma</i>	-
<i>Nemania subannulata</i>	-
<i>Nigrospora</i> sp.*	<i>Nigrospora</i> sp.*
-	<i>Penicillium</i> sp.
<i>Penzigia</i> sp.	-
<i>Pestalotia</i> sp.	-
<i>Phialophora</i> sp.	-
<i>Phoma</i> sp.	-
<i>Phomopsis</i> spp.*	<i>Phomopsis</i> spp.*
Pycnidia*	-
<i>Septonema</i> sp.	-
<i>Septoria</i> sp.	-
<i>Xylaria aristata</i>	-
<i>X. cf. allantoidea</i>	-
<i>X. cubensis</i>	-
<i>X. feejeensis</i>	-
<i>X. grammica</i>	-
<i>X. juruensis</i> var. <i>microspora</i>	-

\* found in both of young leaves and mature leaves.

Table 5.1 (continued)

Chiangmai (Mekkamol,1998)	Bangkok(This study)
<i>Trichoderma</i> sp.	-
<i>Mycelia sterilia</i>	<i>Mycelia sterilia</i>

\* found in both of young leaves and mature leaves

*Phomopsis* spp.. was the dominant taxon in young and mature teak leaves. Whereas Mekkamol (1998) found that the members of the Xylariaceae dominant in mature leaves but *Phomopsis* and *Colletotrichum* were more dominant in the young leaves. In this study it was found that members of Xylariaceae were the second most frequently isolated fungi. Rodrigues and Samuels (1990) isolated endophytic fungi from leaves of a fan palm (*Licuala ramsayi* (Muell.) Domin.) that was growing in a lowland tropical rain forest in Queensland, Australia and found that most of their isolates were anamorphs of xylariaceous fungi. Later Rodrigues (1994) isolated endophytic fungi from healthy foliage of another palm (*Euterpe oleracea* Mart.) that was growing in periodically inundated rain forest in the Brazilian Amazon. The Xylariaceae were more highly encountered in living leaves of *E.oleracea* than any other family (Rodrigues, 1994) and in another study a species of *Xylaria* was frequent in leaves of *Stylosanthes guianensis* (Pereira *et al.*, 1993). It is difficult to explain the occurrence of members of the Xylariaceae as common endophytes of teak leaves (Mekkamol, 1998), since no species of *Xylaria* are known to cause disease of teak. On the other hand, endophytic Xylariaceae are active producers of lignolytic enzymes and many occur as their teleomorph on woody substrata. This led Carroll and Petrini (1983) to hypothesis an active involvement of xylariaceous endophytes in the senescence process. Certainly the early presence of Xylariaceae in the host would give them a definite edge over other potential saprobes and allow them a quick colonization of the dead and decaying tissues. Such ecological adaptation would testify to a subtle ecological adaptation of xylariaceous fungi to their host, in which they would survive latently until the appropriate conditions favourable for growth and reproduction would set in (Petrini, Petrini and Rodrigues, 1995). The development of Xylariaceae in natural forest is very dependent on the onset of the rainy season and it is not until late July that teleomorphic material

becomes evident and then much of the material does not develop mature stromata with the discharge of ascospores. This could explain the high presence of members of the Xylariaceae in mature leaves of teak when sampled in November (Mekkamol, 1998). However, in this study it was found that the Xylariaceae could be isolated during May to December. Most of endophytic inoculum species consists of sexual or asexual spore that are dispersed by wind, rain and insects (Helander *et al.*, 1994). The occurrence of member of the Xylariaceae during May to October may be a result of asexual spores or the long term survival of ascospores. In November to December, only *D.eschscholzii* was isolated whereas the other member of the Xylariaceae were not found. This could be explained since *D.eschscholzii* is one of the large members of the family and produces a high inoculum of ascospores.

*Phomopsis* and *Colletotrichum* species which were found in young leaves probably originate from conidia which are produced rapidly in response to the onset of the rainy season. *Colletotrichum* and *Phomopsis* have been reported as endophytes in conifer needles in Pacific Northwest (Carroll and Carroll, 1978), needles of the genus *Pinus* in Japan (Hata and Futai, 1996), healthy leaves of Amazonian palm *Euterpe oleracea* (Rodrigues, 1994) and the healthy wild banana (*Musa acuminata*) at Doi Suthep Pui nation park, Thailand (Photita *et al.*, 2001). Balasundaran *et al.* (1995) reported the leaf spot diseases of teak caused by *Phomopsis* spp., *Colletotrichum gloeosporioides*, *Alternaria* sp. and *Curvularia* sp. Their presence as endophytes is interesting as they may be considered as latent pathogens. Endophytes often reside within the plant tissues for most of their life cycle and become manifest as host symptoms after being triggered by the appropriate ecological or physiological stimuli. The presence of latent pathogens as endophytes in apparently healthy plants has been reported several times (Petrini, 1991), thus making the distinction between latent pathogen and endophyte blurred (Carroll, 1988)

The endophytic assemblages of rain tree leaves were composed of *Colletotrichum* sp., *Nigrospora* sp., *Penicillium* sp., *Phomopsis* spp. and mycelia sterilia. Species of *Phomopsis* were the most frequency isolated endophytes of leaves. A

number of these genera have been reported previously as endophytes in needles or evergreen leaves (Fisher *et al.*, 1993, 1994; Hata and Futai, 1996; Mekkamol, 1998; Pereira *et al.*, 1993; Rodrigues, 1994) and have also been found in teak leaves (Table 5.2). *Phomopsis* spp. was the dominant group in the both of teak and rain tree leaves. In general, coelomycetes are widespread colonizers of plant tissue, in which they can be present either as pathogens or endophytes (Petrini, 1986 cited in Fisher *et al.*, 1994). The diversity of endophytic fungi isolated from rain tree leaves was lower than that of the endophyte mycobiota of teak leaves (Table 5.2). This may be because the sterilization time and the concentration of ethanol and sodium hypochlorite had some effect on the endophyte mycobiota of rain tree leaves. The choice of sterilization time, concentration and volume will be dictated by the thickness of sample, the relative permeability of its surface and the texture of its surface (Bills, 1996). Thus thin delicate leaves require shorter treatment than robust leaves. It is surprising that the members of Xylariaceae were only found in teak leaves even though the trees were growing in the same area. This may be because the leaves of rain tree produce some toxic substances that inhibited the germination of conidia or ascospores of members of the Xylariaceae.

Table 5.2 Comparison of endophytic fungi isolated from teak leaves and rain tree leaves.

Teak leaves	Rain tree leaves
<i>Alternaria</i> sp.	-
<i>Schizophyllum commune</i>	-
<i>Colletotrichum</i> sp.	<i>Colletotrichum</i> sp.
<i>Daldinia eschscholzii</i>	-
<i>Fusarium</i> sp.	-
<i>Nigrospora</i> sp.	<i>Nigrospora</i> sp.
<i>Penicillium</i> sp.	<i>Penicillium</i> sp.
<i>Phomopsis</i> spp.	<i>Phomopsis</i> spp.
<i>Xylaria</i> spp.	-
<i>Mycelia sterilia</i>	<i>Mycelia sterilia</i>

## Induction of teleomorph formation

Taxonomic studies on xylariaceous fungi have focussed mainly on the teleomorph stage. Cultural studies are frequently undertaken whenever possible to study anamorph-teleomorph connections. On the other hand studies on the anamorph stage are often restricted to selected genera such as *Biscogniauxia*, *Daldinia*, *Hypoxylon*, *Rosellinia*, or *Xylaria*. Even with information on the anamorph identification to species is usually very difficult and furthermore in many genera only fragmentary information is available (Petrini, Petrini and Rodrigues, 1995). The Xylariaceae are well known as endophytes, especially in tropical regions, and identification of xylariaceous endophytes is often difficult since they fail to produce suitable diagnostic features and only very infrequently form their teleomorph in culture (Whalley, 1996). Teleomorphs in culture have been recorded only for *Anthostomella* spp., four species of the *H. serpens* complex, *Rosellinia diathrausta* (Rehm) L.E. Petrini., *Rosellinia thelena* (Fr.) Rabh., and a *Xylaria* sp. (Petrini, and Rodrigues, 1995). In spite of this the pioneering work of Petrini and colleagues (e.g. Petrini and Petrini, 1985; Petrini, Petrini and Rodrigues, 1995) has resulted in the development of keys and the necessary information to enable confident identification to be made to generic level for temperate isolates. The situation regarding tropical endophytic Xylariaceae is much more complex however, as a result of their abundance and impressive diversity (Rodrigues and Samuels, 1990; Whalley, 1993). It is doubtful whether differentiation of species on the basis of cultural and anamorphic features alone will ever be possible since differences between individual species are often insufficient to allow for absolute identifications to be made (Petrini, Petrini and Rodrigues, 1995). However, studies with *Xylaria* indicate that a combination of morphological characters with biochemical analyses might enable satisfactory identification to be made (Brunner and Petrini, 1992). There are also indications that secondary metabolite profiles from endophytic isolates might be matched with those obtained from cultures derived from teleomorphic material thus enabling identification (Whalley and Edwards, 1995). Inoculation experiments to produce the teleomorphs provide another, but long term, alternative (Mekkamol, 1998).

Most members of *Xylaria* can be recognized in culture mainly by their white, flat and silky mycelium and the formation of hyphal strands, often black or brown, usually upright stromatal tissue develops. Generally it is possible to segregate cultures into genera on the basis of overall cultural characteristics and type of anamorph when this is produced. For example *Xylocladium* anamorph appears exclusive to *Camillea* whilst *Geniculosporium* anamorph links with *Nemania* species. (Petrini, Petrini and Rodrigues, 1995). *Nodulisporium* anamorphs are characteristic for *Hypoxylon*, *Daldinia* and many species of *Biscogniauxia* (Callan and Rogers, 1986; Gonzalez and Rogers ; 1993). Only a small number of species produce species-characteristic morphological features in culture (Brunner and Petrini, 1992). *Xylaria cubensis* is, however, one species which produce a flabelliforme anamorph in culture which appears to be a species characteristic (Mekkamol, 1998).

In an attempt to resolve identification problems a technique to induce the production of the sexual state (teleomorph) by inoculation of pure culture into sterilized stem sections of teak and other woody hosts, followed by incubation under controlled conditions in the laboratory or incubation in the field has also been undertaken. In the recent study by Mekkamol (1998), mature teleomorphs were produced in over 60% of the isolates. Species of *Xylaria* were obtained together with *D.eschscholzii*, *N.subannulata* and 5 species of *Hypoxylon* whereas in this study, only *D.eschscholzii* developed to maturity; *Xylaria* species failed to developed to maturity. This was probably because the inoculated wood which failed to develop stromata in the bottles or only produced immature stromata had not been transferred to a natural forest as recommended by Mekkamol(1998). Moisture is a very important ecological condition required by most xylariaceous fungi, because a moist habitat is essential for mycelial growth and development of fruiting structures (Petrini, Petrini, Rodrigues, 1995). Mekkamol(1998) found that sometimes when inoculated wood was transferred to the forest they failed to develop further because the specimens dried out, were destroyed by termites, or taken by monkeys. However, by placing inoculated twigs in damp sand either in bags, open or sealed, or in earthenware pots it was possible to produce mature stromata. In some species, stromata and teleomorph stage developed but required

longer incubation periods to reach full maturity. If the material became too dry development ceased immediately

### Antimicrobial testing

Endophytic fungi are now reconized as potential producers of novel secondary metabolites. Fisher, Anson and Petrini have been reported antibacterial or antifungal activity for more than 30 % of endophytic isolates from ericaceous plants (Rodrigues and Petrini, 1997). We tested for the production of antimicrobial activities against some Gram positive, Gram negative bacteria and fungi. This observation suggested that endophytic fungi of *T.grandis* and *S.saman* may have pharmaceutical potential. The role of these endophytes within *T.grandis* and *S.saman* is still unknown. Benefits to the host plant such antagonism towards pathogenic fungi or decreased susceptibility to phytophagous insects could be speculated. Investigations on the interactions of *T.grandis* and *S.saman* and its endophytes would be the next direction for future research.