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# Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr.

Sukanyanee Chareprasert[1,*], Jittra Piapukiew[2], Surang Thienhirun[3], Anthony J. S. Whalley[4] and Prakitsin Sihanonth[5,*]
[1]*Program of Biotechnology, Faculty of Science, Chulalongkorn University, 10330, Bangkok, Thailand*
[2]*Research Centre for Bioorganic Chemistry, Department of Botany, Faculty of Science, Chulalongkorn University, 10330, Bangkok, Thailand*
[3]*Department of Royal Forest, Forest Products Research Division, 10900, Bangkok, Thailand*
[4]*School of Biomolecular sciences, Liverpool John Moores University, L3 3AF, Liverpool, UK*
[5]*Research Centre for Bioorganic Chemistry, Department of Microbiology, Faculty of Science, Chulalongkorn University, 10330, Bangkok, Thailand*
**Author for correspondence:*

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**Summary**

In order to study the foliar endophytes from teak (*Tectona grandis* L.) and rain tree (*Samanea saman* Merr.) growing in the campus of Chulalongkorn University, healthy leaves were collected at two-monthly intervals during January to December. The number of genera and species, together with their colonization frequency (CF%) in mature teak and rain tree leaves were greater than those in the young leaves. More endophytic isolates in the leaves of both trees were recovered during the rainy season. The fungal genera found in both young and mature teak leaves were *Alternaria*, *Colletotrichum*, *Nigrospora*, *Phomopsis* and mycelia sterilia. *Phomopsis* was the dominant genus in both young (newly emerged) and mature leaves. *Fusarium*, *Penicillium*, *Schizophyllum commune* and members of the Xylariaceae were found only in mature leaves. For the rain tree leaves, species of *Phomopsis* and mycelia sterilia were found in both young newly emerged and mature leaves. *Colletotrichum* and *Penicillium* were found only in mature leaves, whereas *Nigrospora* was found only in young newly emerged leaves. In this study, *Phomopsis* was the dominant genus in the leaves of both tree species. A total of 37 isolates of endophytic fungi isolated from teak and rain tree leaves were tested for the production of antimicrobial activities. Out of these, 18 isolates could produce inhibitory substances effective against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* and 3 isolates inhibited growth of *Candida albicans in vitro*.

## Introduction

Endophytes are defined as fungi colonizing healthy plant tissue without causing overt symtoms in, or apparent injury to, the host (Bills 1996). Until recently most of the research with endophytes has been carried out using plants from temperate regions. However in the last few years it has been found that tropical plant hosts contain a great diversity of endophytic microorganisms, many of them not yet classified and possibly belonging to new genera and species (Azevedo *et al.* 2000). Some of the tropical plants that have been studied for their endophyte associations include tropical palm (Rodrigues 1994; Frohlich *et al.* 2000), *Stylosanthes* (Pereira *et al.* 1993) and tropical fruit trees (Azevedo *et al.* 2000).

In Thailand there have been few reports on endophytic fungi. The only tree species examined have been bamboo (Lumyoung *et al.* 2000), banana (Photita *et al.* 2001), teak (Mekkamol 1998) and wild ginger (Bussaban *et al.* 2001). Teak (*Tectona grandis* L.) is one of the most valuable timber resources in the tropics, it is also one of the most widely studied tropical plants in terms of its ecology and silviculture. This species has been planted in various areas outside of its natural distribution area (Tanaka *et al.* 1998). This investigation concerns the endophytic assemblages of leaves of teak trees growing on the campus of Chulalongkorn University. This research was aimed at the isolation and identification of endophytic fungi found within healthy leaves, comparison of the endophytic assemblages of young and mature leaves and to examine any seasonal effects. In addition

endophytes from *Samanea saman* Merr. (Rain tree) growing on the campus of Chulalongkorn University were also investigated to compare their endophytic assemblages with those of the teak. This represents the first survey of foliar endophytes from *S. saman*, which is a large leguminous tree found in the tropics and which is well known as a source of a wide range of useful products. The ripe pods can be used as an edible pulp, or they can be dried and ground into a meal for animal feed, and the timber is used in furniture manufacture. It is also valuable as a shade tree in pastures, where it stimulates the growth of grass (Skerman *et al*. 1988). Following the discovery of the production of the anticancer drug taxol by the endophytic fungus *Taxomyces andreanae* Strobel, Stierle & Hess (Stierle *et al*. 1993), there has been a renewed interest in natural products, with endophytic fungi being targeted as a potential source of novel compounds (Huang *et al*. 2001; Strobel 2002; Stinson *et al*. 2003). Consequently as part of this investigation metabolites from a selection of the fungi isolated were tested for any antimicrobial activities against a panel of target microorganisms, including Gram-positive, Gram-negative bacteria and one species of fungus.

## Materials and methods

### *Plant material*

Healthy leaves of teak and rain tree were collected from sites on the campus of Chulalongkorn University, Bangkok. The leaves were collected at two-monthly intervals during January to December, with both young (newly emerged) and mature leaves being taken. Fresh specimens were processed within 24 h following collection.

### *Fungal isolation and culture methods*

Endophytic fungi were isolated following surface sterilization (Mekkamol *et al*. 1996). The leaf sections of the both young newly emerged and mature leaves were surface sterilized by immersing in 95% ethyl alcohol for 30 s followed by immersion in sodium hypochlorite (5% available chlorine) for 5 min and then transferred to 95% ethanol for 30 s. They were finally washed in sterile distilled water and the sterilized leaf pieces were then surface-dried with sterile filter paper and immediately placed on the surface of 2% malt extract agar (MEA) plates supplemented with streptomycin (50 $\mu$g/ml) to prevent bacterial contamination. Plates were incubated at room temperature (25–30 °C) with normal daily light and dark periods. Plates were examined daily for up to 1 month for development of fungal colonies growing out from the leaf segments. Fungi growing out from the leaf tissue were subsequently transferred on to fresh MEA and on to potato dextrose agar (PDA) plates and when found to be pure, on to agar slopes.

### *Identification and nomenclature of organisms*

Sporulation was induced by incubation under near U.V. light (Philips) and identifications made to genus and/or species by the characteristics of their spores and/or other structures. Cultures that failed to sporulate were recorded as sterile mycelia. The microscopic analyses were based on observations with an Olympus CH2 research microscope. Specimens for light microscopy were mounted in water, lactophenol-cotton blue for observation of spores and other characteristics, and then identified. Nomenclature of the fungi follows Sutton (1980) and Barnett & Hunter (1998). Xylariaceous anamorphs were separated from the others for further study by induction of the teleomorphs. Once telemorphs were obtained following the induction method developed by inoculation of sterilized teak branches and incubation upto 10 weeks (Mekkamol 1998), identification to species level was undertaken.

### *Fungal cultivation for production of antimicrobial substance*

Disks were cut from the edge of an actively growing colony on MEA with a flamed cork borer (7 mm diam.) and transferred aseptically into 250 ml Erlenmeyer flasks with cotton plugs containing 100 ml of 2% malt extract broth. All cultures were incubated for 6–8 weeks at room temperature (25–30 °C) under static conditions. The supernatant was then filtered by passing through Whatman No. 4 filter paper and used immediately to test for inhibitory activity.

### *Agar diffusion tests for antimicrobial activity*

The test organisms for the agar diffusion test were two Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923), a Gram-negative bacterium (*Escherichia coli* ATCC 25922) and a fungus [*Candida albicans* (Robin) Berkhout, obtained from Chulalongkorn Hospital]. The test organisms were grown in test tubes containing 5 ml of nutrient broth and incubated at 35–37 °C for 2–8 h. Turbidity was adjusted to correspond with that of a barium sulphate (0.5 McFarland) standard. Adjustments were made with sterile saline or broth to obtain the required density. Sterile cotton applicators were immersed in the inoculum suspension and nutrient agar plates were inoculated by streaking the swab across the entire surface. Wells were cut in the agar with a flamed cork borer (7 mm) and 0.01 ml of the culture fitrates were pipetted into the wells. All plates were incubated at 35–37 °C for approximately 18 h and any inhibition zones measured.

## Results and discussion

The colonization frequency (CF%) from mature leaves of both teak and rain trees is greater than for young

newly emerged leaves (Table 1 and 2). 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 (e.g. Rodrigues 1994; Mekkamol 1998; Frohlich *et al.* 2000; Bussaban *et al.* 2001). 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 a 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 & Carroll 1994).

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 & 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: the winter season from mid-November to January, the dry season from February to May and the rainy season from June to November (Thienhirun 1997). The colonization frequency (CF%) from teak and rain tree leaves increased during June to November (rainy season) but decreased during March to April (dry season) (Tables 1 and 2). The lower number of isolates recovered from trees during the dry season indicated that enviromental factors, such as rainfall and atmospheric humidity might influence the occurrence of some endophytic species. Rodrigues (1994) suggested that the lower number of isolates recovered during the 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. Furthermore dry conditions would result in a reduction in the overall air spora in the vivinity of the plants thus reducing the potential inoculum.

The *Penicillium* sp. was isolated only in the dry season (Tables 1 and 2). This may be because spores of *Penicillium* sp. can survive and even grow in a low water environment or in dry conditions categorizing it as a xerophilic type of fungus. In the rainy season spore inocula of Coelomycetes such as *Phomopsis* spp. produce slimy conidia that are not forcibly released but dispersed by water in various ways. Thus they may be present in higher numbers than spores of the *Penicillium* sp. which was not isolated as an endophyte during the rainy season. Differential susceptibility of leaves could also have caused the high variance in infection levels. Inoculation of greenhouse trees with conidial

*Table 1.* Colonization frequency (CF%) of fungal endophytes isolated from teak leaves

| Endophyte | January–February | | March–April | | May–June | | July–August | | September–October | | November–December | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | Y | M | Y | M | Y | M | Y | M | Y | M | Y |
| *Alternaria* cf. *alternata* (Fr.)Keissler | 10 | 7.5 | – | – | – | – | – | – | 3.8 | – | 13.8 | – |
| *Colletotrichum* sp. T1 | 8.8 | – | – | – | 12.5 | 10 | 12.5 | – | 15 | – | 20 | – |
| *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. | – | – | – | – | – | – | – | – | – | – | 22.5 | – |
| *Fusarium* sp. | – | – | – | – | – | – | – | – | 6.25 | – | – | – |
| *Nigrospora sphaerica* (Sacc.) Morgan | 11.25 | – | – | – | 6.25 | – | 5 | 5.0 | – | 5.0 | 7.5 | 15.0 |
| *Penicillium* sp. | – | – | 25 | – | – | – | – | – | – | – | – | – |
| *Phomopsis* sp. T1 | 26.25 | – | 18.75 | – | – | 15 | 12.5 | 2.5 | 25 | 16.25 | 11.25 | 15.0 |
| *Phomopsis* sp. T2 | – | – | – | – | 18.75 | – | 15 | – | 30 | – | 30 | – |
| *Phomopsis* sp. T3 | 8.75 | – | – | – | 7.5 | – | 5 | 10.0 | 12.5 | – | – | – |
| *Phomopsis* sp. T4 | 16.25 | – | 21.25 | – | – | 7.5 | – | 5.0 | – | – | 17.5 | – |
| *Phomopsis* sp. T5 | – | – | – | – | 13.75 | – | – | – | – | – | – | – |
| *Phomopsis* sp. T6 | 6.25 | – | – | – | – | – | – | – | – | – | – | – |
| *Phomopsis* sp. T7 | 11.25 | – | – | – | – | – | – | – | 5 | – | – | – |
| *Phomopsis* sp. T8 | – | – | | – | | 7.5 | | – | – | – | – | – |
| *Schizophyllum commune* Fr. | – | – | – | – | – | – | 7.5 | – | 6.25 | – | – | – |
| *Xylaria* sp. 1 | – | 5.0 | – | – | – | – | 7.5 | – | – | – | – | – |
| *Xylaria* sp. 2 | – | – | – | – | – | – | 6.25 | – | – | – | – | – |
| *Xylaria* sp. 3 | – | – | – | – | 8.75 | – | – | – | – | – | – | – |
| *Xylaria* sp. 4 | – | – | – | – | – | – | – | – | 12.5 | – | – | – |
| *Xylaria* sp. 5 | – | – | – | – | 10 | – | – | – | – | – | – | – |
| *Xylaria* sp. 6 | – | – | – | – | – | – | – | – | 6.25 | – | – | – |
| *Xylaria* sp. 7 | – | – | – | – | 6.25 | – | 5 | – | – | – | – | – |
| *Xylaria* sp. 8 | – | – | – | – | – | – | 6.25 | – | – | – | – | – |
| *Xylaria* sp. 9 | – | – | – | – | – | – | 10 | – | – | – | – | – |
| *Xylaria* sp. 10 | – | – | – | – | – | – | 3.75 | – | – | – | – | – |
| *Xylaria* sp. 11 | – | – | – | – | 6.25 | – | 10 | – | – | – | – | – |
| *Sterile mycelium* | 37.5 | 6.25 | 12.5 | 10.0 | 22.5 | 15 | 15 | – | 10 | 5.0 | 5 | 10.0 |

– , not detected

M, mature leaves; Y, young leaves

*Table 2.* Colonization frequency (CF%) of fungal endophytes isolated from rain tree leaves

| Endophyte | January–February | | March–April | | May–June | | July–August | | September–October | | November–December | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | Y | M | Y | M | Y | M | Y | M | Y | M | Y |
| *Colletotrichum* sp. S1 | 5.0 | – | – | – | 10.0 | – | 10.0 | – | 20.0 | – | 30.0 | – |
| *Nigrospora sphaerica* (Sacc.) Morgan | – | – | – | – | – | 5.0 | – | 5.0 | – | 1.25 | – | – |
| *Penicillium* sp. | – | – | 12.5 | – | – | – | – | – | – | – | – | – |
| *Phomopsis* sp. S1 | – | – | – | – | – | – | – | – | – | – | 11.25 | – |
| *Phomopsis* sp. S2 | – | – | – | – | – | 5.0 | – | – | – | 1.25 | 7.5 | – |
| *Phomopsis* sp. S3 | – | – | – | – | 6.25 | – | 10.0 | – | 12.5 | 10.0 | 22.5 | – |
| *Phomopsis* sp. S4 | 7.5 | – | 7.5 | – | 7.5 | 5.0 | 20.0 | – | 15.0 | – | 7.5 | – |
| *Phomopsis* sp. S5 | 15.0 | – | 5.0 | – | 11.25 | – | 25.0 | – | 15.0 | – | 22.5 | 5.0 |
| *Phomopsis* sp. S6 | 22.5 | – | – | – | – | – | – | – | 22.5 | – | – | 5.0 |
| *Phomopsis* sp. S7 | – | – | – | – | – | – | – | – | – | – | 8.75 | – |
| Sterile mycelium | 25.0 | – | – | – | 12.5 | 10.0 | 15.0 | – | 10.0 | – | – | – |

–, not detected
M, mature leaves; Y, young leaves

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 & Carroll 1994).

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 were later identified as *Schizophyllum commune*. Whereas in the study by Mekkamol (1998) basidiomycetes were not found. Petrini & 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. A *Schizophyllum* species has been reported as a fungal endophyte of *Eucalyptus niten* in Australia (Fisher *et al*. 1993).

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 (Blodgett *et al*. 2000; Kumaresan & Suryanarayanan 2001; Ragazzi *et al*. 2003), and of fungi such as the xylariaceous anamorphs known to live endophytically in a large number of hosts (Frohlich *et al*. 2000; Suryanarayanan *et al*. 2002). There were also a large number of coelomycetous taxa such as *Phomopsis* spp. and a *Colletotrichum* sp. The endophytic assemblages of rain tree leaves were composed of a *Colletotrichum* spp., *N. sphaerica*, a *Penicillium* sp., *Phomopsis* spp. and mycelia sterilia. Species of *Phomopsis* were the most frequency isolated endophytes. A number of these genera have been reported previously as endophytes in needles or evergreen leaves (Bussaban *et al*. 2001; Kumaresan & Suryanarayanan 2001; Hata *et al*. 2002; Suryanarayanan *et al*. 2002).

*Table 3.* Antimicrobial activities of fungal endophytes

| Endophyte | Host | Inhibition test organisms | | | |
|---|---|---|---|---|---|
| | | *B. subtilis* | *S. aureus* | *E. coli* | *C. albican* |
| *Colletotrichum* sp. T1 | *T. grandis* | – | + + | – | – |
| *Daldinia eschscholzii* | *T. grandis* | – | + + | – | + |
| *Fusarium* sp. | *T. grandis* | + | + | – | – |
| *Phomopsis* sp. T1 | *T. grandis* | – | + | – | – |
| *Phomopsis* sp. T4 | *T. grandis* | + + | + + | – | – |
| *Phomopsis* sp. T8 | *T. grandis* | + | – | – | – |
| *Xylaria* sp. 1 | *T. grandis* | + | – | – | + |
| *Xylaria* sp. 2 | *T. grandis* | + + | + + | – | + |
| *Xylaria* sp. 5 | *T. grandis* | + | + | + | – |
| *Xylaria* sp. 7 | *T. grandis* | + | + | + | – |
| *Xylaria* sp. 8 | *T. grandis* | + | – | – | – |
| *Xylaria* sp. 10 | *T. grandis* | + + | + | – | – |
| *Xylaria* sp. 11 | *T. grandis* | + | – | – | – |
| *Colletotrichum* sp. S1 | *S. saman* | + | + + | – | – |
| *Phomopsis* sp. S2 | *S. saman* | + | + + | – | – |
| *Phomopsis* sp. S5 | *S. saman* | + | + + | – | – |
| *Phomopsis* sp. S6 | *S. saman* | – | + | – | – |
| *Phomopsis* sp. S7 | *S. saman* | + + | – | – | – |

Activities were classfied according to the diameter of the clear zones around the point of application of the sample + + , clear zone more than 15 mm ; + , clear zone less than 15 mm ; –, no clear zone

The number of genera and species of endophytic fungi isolated from rain tree leaves was lower than that of the endophyte mycobiota of teak leaves. 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 which are less robust than those of the teak or it may be a reflection on the surface characteristics of the leaves making them less accessable to the endophytic propagules. 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.

A total of 37 isolates of endophytic fungi isolated from teak leaves and rain tree leaves were tested for antimicrobial activity, as an indication of their capability to produce secondary metabolites of potential therapeutic interest. There were 18 endophytic isolates which could produce inhibitory substances and inhibited growth of bacteria, and three of these, *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm., *Xylaria* sp. 1 and *Xylaria* sp. 2 inhibited growth of the pathogenic yeast, *C. albicans* (Table 3). Most of the antimicrobial activities were specific for Gram-positive bacteria. Only two isolates were found to produce inhibitors of the resistant Gram-negative bacteria selected. Almost all of the endophytes that exhibited any antimicrobial activity inhibited the growth of *B. subtilis* and *S. aureus*. *Escherichia coli* was inhibited only by the compounds produced by *Xylaria* sp. 5 and *Xylaria* sp. 7 (Table 3). Endophytic fungi are now recognized as potential producers of novel secondary metabolites (Huang *et al.* 2001; Strobel 2002). Our observations 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.

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