CHAPTER 3
MATERIALS AND METHODS

3.1 Sampling

Healthy leaves of teak (*Tectona grandis* L.) and rain tree (*Samanea saman* Merr.) were collected from sites on the campus of Chulalongkorn University, Bangkok. The leaves were collected at two month intervals during January to December 1999, with both young and mature leaves being taken. Five individual leaves from each plant species were obtained from five trees at each collecting site. In total 4 areas on each leaf were sampled: 1 from the vein, 1 from the lamina and 2 from the midribs. Each sample of leaf taken measured 1X1 cm^2 and was then divided into 4 equal pieces before sterilisation. There were therefore, 16 sampling units on each leaf measuring 5X5 mm^2. Fresh specimens were processed within 24 hours after collection.

3.2 Media

Malt extract agar (MEA) was selected for the endophyte isolation as being a medium proven to isolate a diverse taxonomic population. Potato dextrose agar (PDA) and Malt extract agar (MEA) were selected for morphology observations and PDA was used to produce the inoculum for teleomorph induction. For the isolation of endophytic fungi, the antibiotic, streptomycin was added at a final concentration of 50 µg/ml to prevent bacterial contamination.

3.3 Fungal isolation and culture methods

Endophytic fungi were isolated using the surface sterilization method by Mekkamol *et al.* (1996). The leaf sections were surface sterilized by immersing in 95% ethyl alcohol for 30 second followed by immersion in sodium hypochlorite (5% available

chlorine) for 5 minutes and then transferred to 95% ethyl alcohol for 30 seconds. They were finally washed in sterile distilled water and the sterilised leaf pieces were then surfaced dried with sterile paper and immediately placed on the surface of 2% malt extract agar plates (supplemented with streptomycin to prevent bacterial contamination). Plates were then incubated at room temperature (25-30°C). Plates were examined more or less daily for up to 1 month for development of fungal colonies growing out from the leaf samples. The fungi growing out from the segments during the incubation period were transferred to fresh PDA medium plate and, if pure, to a fresh agar slope. The fungi were induced to sporulate under near UV light and were identified to genus or species by the characteristics of their spores and /or other structures. Cultures that failed to sporulate were considered sterile mycelium.

3.4 Identification and nomenclature of organisms

3.4.1 Microscopical features

The microscopical analyses were based on observations by light microscopy on an Olympus CH2 research microscope using a 40x dry objective. 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 and Hunter(1987). Xylariaceous anamorphs were separated from others for further study by induction of the teleomorph. Once telemorphs were obtained using the special techniques described below, identification to species level was undertaken. Ascospores in 10% aqueous KOH were observed for dehiscence or indehiscence of the perispore (Mekkamol, 1998). Measurements of ascospores from squash mounts in distilled water of Melzer's iodine reagent were made, Ascospore dimensions were based on 20 fully mature spores, or 50 where new taxa were proposed. The ascus apical apparatus was examined for its amyloid or dextrinoid reaction following mounting in Melzer's iodine reagent and shape and size were determined.

3.4.2 Macroscopical features

Characters such as shape, size, color, type of stromal surface, shape and size of perithecia, type and size of ostioles were studied using stereomicroscope on a Leica MZ6

3.5 Technique for induction of teleomorph

In a study of some European Xylariaceae in culture, Petrini and Petrini(1985), provided a key for identification by a careful comparison of suspected endophytic xylariaceous colonies with single ascospore isolates from known freshly collected xylariaceous teleomorphs. The present study raises the possibility of inducing teleomorphs from pure cultures of anamorphs by inoculating fungi onto wood which is then incubated to produce the teleomorphs. Some cultures isolated had been previously identified as belonging to the genus *Xylaria* on the basis of their upright stromata which were covered with the anamorphs. Although characteristic *Xylaria* – type stromata often developed in culture however they never developed to maturity. Some isolates only produced anamorphic structures. In the absence of a mature telemorph, it is rarely possible to identify these isolates to species and therefore a special technique was developed to induce telemorph formation.

1. The culture with young stromata and anamorphs were separated from the others using cultural morphology and characteristics of anamorphs following Thienhirun (1997).
2. The separated isolates were inoculated on to PDA in bottles and incubated for 10 days at room temperature (25-28°C)
3. Twigs* of about 3 cm diameter were freshly cut into 8 cm long pieces. They were put in autoclavable bags and then autoclaved (121°C for 1 hour). One piece of sterilised twig was put vertically on the surface of each culture in the bottle and the cultures were kept at room temperature for 1 month.

4. The twigs that were colonised by the fungi were transferred from the bottles to sterile bags with moist sand at a depth of 4 cm at the bottom. The bags were tied with a rubber band and kept at a temperature of 28-30°C for one to two months. Stromata production was observed at this stage.
5. Teleomorph induction was performed under shade in moist conditions. The twigs with young stromata were transferred to moist sand pots covered with a polyethylene sheet to conserve moisture and watered daily. The infected twigs without any noticeable stromata were incubated further in polyethylene bags for one more month before the bags were cut open horizontally at which stage they were incubated further with water application once or twice a week as necessary.

* twigs used in the experiment were from two different trees species.

1. Teak (*T. grandis* L.)
2. Rain tree (*S. saman* Merr.)

3.6 Isolation of cultures and production of anamorphs

Teleomorphs derived from the wood inoculation techniques were used to isolate cultures in order to make cultural comparison with the initial ones that had been isolated from teak leaves. In general, multispore isolations were made. Colonies were incubated on 9.0 cm Petri dishes at 25-30°C on the bench with alternating light and darkness.

3.7 Fungal cultivation for production of antimicrobial substances

To prepare inocula for fermentation studies, the top third of the agar slants from the culture selected for study were removed and transferred aseptically to Petri dishes of agar media. The plates were incubated at room temperature (25-30°C) for 3-4 days depending on the growth rate. Disks cut with a flamed cork hole borer were transferred aseptically into 250 ml Erlenmeyer flasks with cotton plugs containing 100 ml of the 2%

malt extract medium. All cultures were incubated for 6-8 weeks at room temperature (25-30°C) under static conditions. The supernatant is then sterilized by passing through a membrane filter and tested for inhibitory activity.

3.8 Evaluation of the antimicrobial activity

3.8.1 Test microorganisms

The *in vitro* antimicrobial susceptibility tests were performed using two Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923, two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) and a fungus (*Candida albicans*).

3.8.2 Procedures

a. Preparation of medium

Nutrient agar plates were poured into dishes of 90mm internal diameter. If the plates were not required for immediate use they were stored in a refrigerator and protected from desiccation. They were normally used within seven days, but if kept for a longer period provided, they were subjected to quality control procedures before use. Immediately before inoculation the plates were allowed to reach room temperature and the surface of the plate was dry.

b. Preparation of inoculum

From a pure culture of the pathogen, four or five colonies were taken with a wire loop and transferred onto 5 ml of nutrient broth. Incubation of the broth was at 35-37°C for 2-8 hours. Turbidity was adjusted to the barium sulphate standard. Adjustment were made with sterile saline or broth.

c. Inoculation of the test plate

Sterile cotton applicators were immersed in the inoculum suspension and pressed lightly against the tube wall to remove excess moisture. The agar was inoculated by streaking the swab across the entire surface.This was repeated twice, turning the plate 60 degrees between each streaking. The surface of the medium was allowed to dry for 3-5 minutes.

d. Application of culture filtrate

Wells were made on the agar by removing disks cut with a flamed cork hole borer. 0.01ml of culture fitrate was pipetted into the agar wells. This was absorbed by the media surrounding the wells. All plates were incubated at 35 to 37°C overnight (approximately 18 hours). Inhibition zones around the wells were measured in cm using a ruler.