CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

3.1.1 Collection site

Healthy rachis and leaflets of Thai dwarf fishtail palm were randomly collected from Huay Kog Ma, Doi Suthep – Pui National park, Chiang Mai. The site is located at 1200-1500 m. above sea level, latitude 18.48.00 longitude 98.56.00. The location if covered by an evergreen hill forest located along Doi Suthep-Pui road. The canopy is dense and the palms grow among other plants species (Techa, 2002).

3.1.2 Collection methods

Within 24 hours after the collection, each individual Thai dwarf fishtail palm samples were collected then washed in running tap water for 5 minutes. Each sample was divided into 3 parts; primary rachis, secondary rachis and leaflet. The term 'primary rachis' was applied in this research for the stalk between the trunk and the expanded laminar which referred to the leaf or frond blade. The term 'secondary rachis' was used for the leaf vein. The dimension of primary rachis and secondary rachis length are ca 1.5 cm. The dimension of leaflet is ca 1 × 1 cm which collected from three regions (distal, central and basal).

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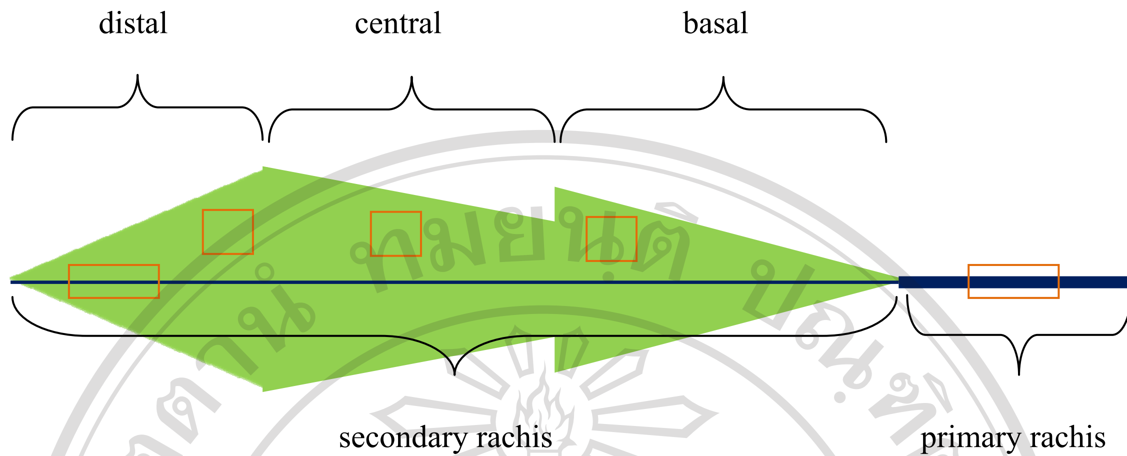

Figure 3 Structure of a typical Thai dwarf fishtail palm frond.

3.2 Isolation of endophytes fungi

A triple surface sterilization technique was conducted according to Rodrigues and Samuels (1990) with some modification. Each sample tissue was sterilized by dipping in 70% ethanol for 60 seconds, in the solution of 3%sodium hypochloride for 8 minutes, and 70% ethanol for 30 seconds and finally, rinsed in distilled water. The tissues were dried on sterilized tissue paper for 4 hours under a laminar flow hood and until they dry, 4 fragments were deposited on a Petri-plate with PDA and PDA containing rose bengal. Each trial was replicated for 3 times. The rose bengal was used for decreasing the growth of fast-growing fungi in order to isolate slow-growing fungi (Fröhlich, 1997). The Petri-dish containing sample tissues was incubated at room temperature. Samples on media were observed daily. Once the fungal hypha grows out from plant tissues, the hyphal tips of the fungi were removed by the sterile cork borer and transferred to another PDA plate. After pure culture isolated, the

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culture was transferred to PDA slant as a stock culture and kept for further examination.

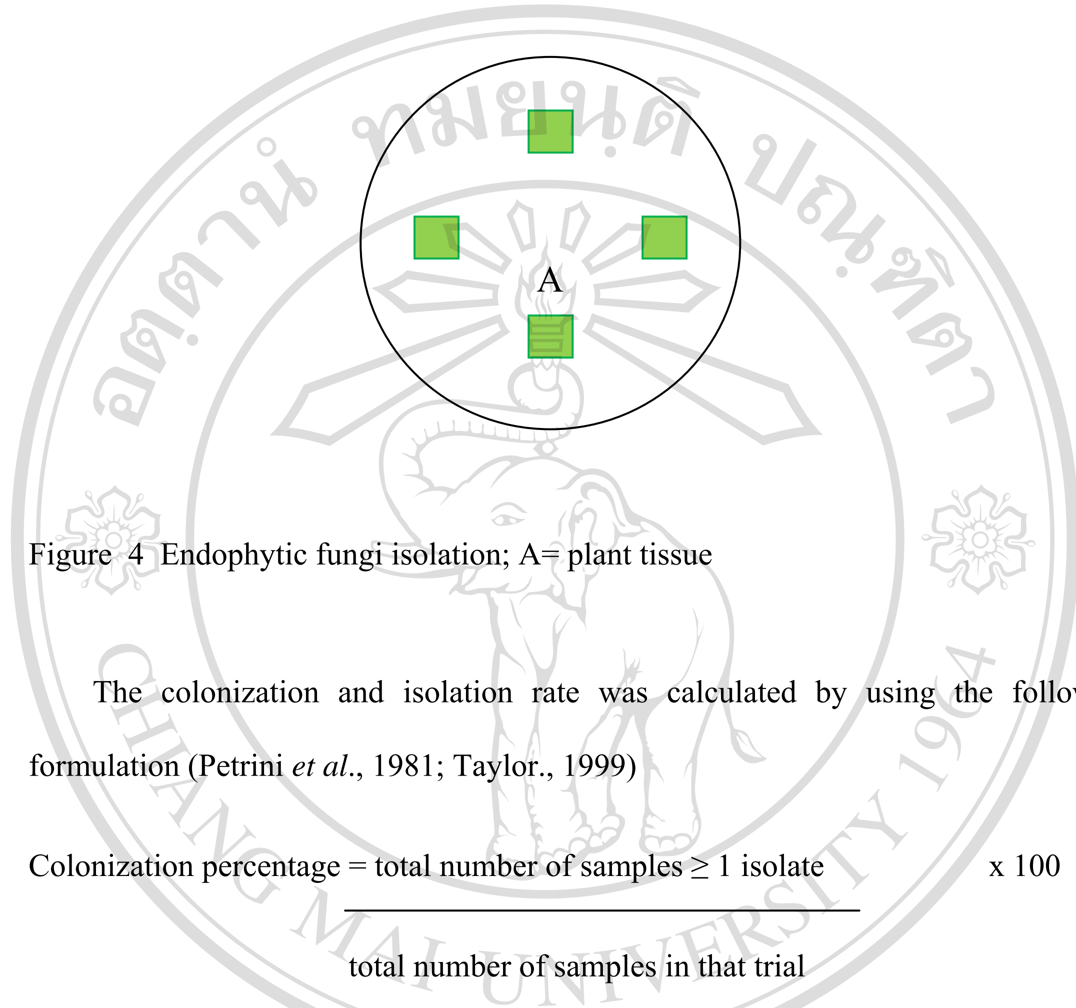

Figure 4 Endophytic fungi isolation; A= plant tissue

The colonization and isolation rate was calculated by using the following formulation (Petrini *et al*., 1981; Taylor., 1999)

$$\text{Colonization percentage} = \frac{\text{total number of samples} \geq 1 \text{ isolate}}{\text{total number of samples in that trial}} \times 100$$

$$\text{Isolation rate} = \frac{\text{total number of isolates yielding in given trial}}{\text{total number of sample in that trial}}$$

Colonization rate are widely used in the literature and are usually multiplied by 100 and expressed as percentages (Petrini *et al*., 1981). Isolation rates cannot be expressed as percentages, they used as a measure of fungal richness in a given trial/plant/ tissue. The significance of two factors; part of tissue and medium will be determined by analysis of variance (ANOVA) (Gomez and Gomez, 1984).

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3.3 Identification of endophytic fungi

Some endophytic fungi were successeful to produce conidia on PDA and PDA containing rose bengal. If the isolates did not produce conidia, they could not be able to be identified. So two methods by growing endophytic fungi on malt extract agar and using filter paper method were carried out to induce the production of conidia, fruiting body and other necessary structure for identification.

3.3.1 Sporulation on malt extract agar (MEA)

The endophytic fungi from the stock culture was subcultured on MEA medium plate and incubated at room temperature for 14 days in the dark. The cultures were observed under stereomicroscope for the morphology characters; mycelium, conidia shape, conidia color, conidiogenous cell (Ellis, 1971; Carmichael, 1980; Sutton, 1980).

3.3.2 Sporulation induction of endophytic fungi by using filter paper technique

Filter papers were used as the material to induce the sporulation (Dhingra and Sinclair, 1994). Filter paper was cut into 8 pieces and steriled at 180°C for 2 hours by hot air oven. Water agar medium was placed with the plug of a 7 day-old pure culture of endophytic fungi disk at the centre of agar plate and then placed the sterile filter paper fragments close to the endophytic fungi disk. All agar plates were sealed and incubated at the room temperature in 12 hours light/12 hours dark cycle. Plates held in the dark were wrapped in aluminium foil. Productions of fruiting body were checked using stereomicroscope after 8 weeks, sterile isolate were identified as morphotype.

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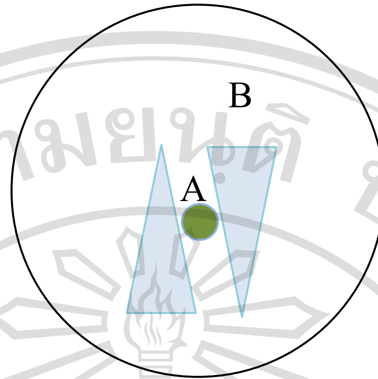

Figure 5 Sporulation induction technique; A = Endophytic fungi, B = Filter paper

3.4. Antagonistic activity test

Petri dishes (9 cm) containing sterile PDA were inoculated with a 0.5 cm plug of a 7 day-old pure culture of endophtyic fungi and the test pathogen *Sclerotium* sp. from chilli, *Phytophthora* sp. from tomato and *Curvularia senegalensis* from seed of *Borassus flabellifer* L. The distance between the pathogen and antagonist was 4 cm. Negative controls dishes were inoculated with pathogens and a water agar plug.

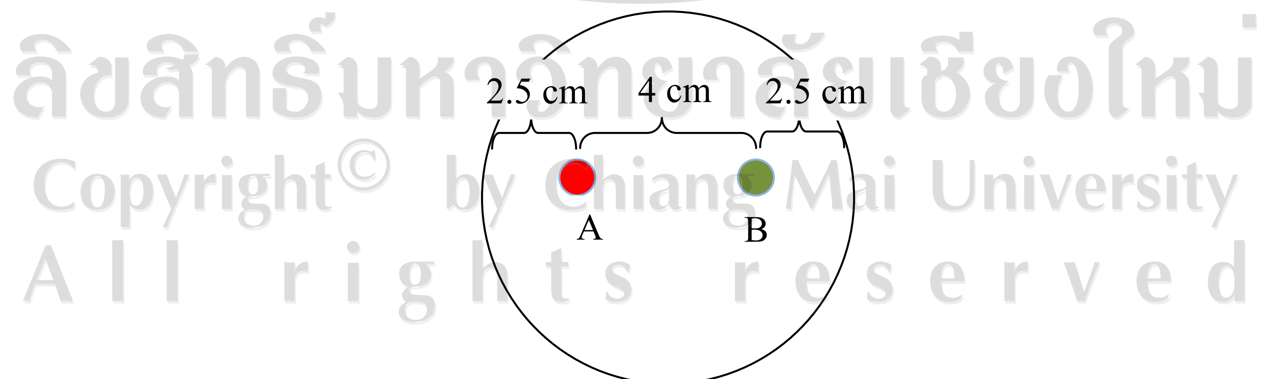

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Figure 6 Dual culture technique; A = Pathogen, B = Endophytic fungi

All Petri-dishes were incubated at room temperature. The experiment was replicated four times. Radial growth of pathogens mycelium was recorded by measuring colony diameter at the pathogen in control plate reach the water agar plug. The antagonism index (AI) will be assessed according to the following formulation.

$$AI = \frac{(RM - rm) \times 100}{RM}$$

Where; RM = ray of the pathogen colony in the control plate

rm = ray of the pathogen colony towards the antagonist

From the calculation of the antagonism index (AI) were evaluated for the antagonistic potential (Khemmuk, 2004).

Where; >75%= very high

61- 75% = high

51- 61% = moderate

<51% = low

The interactions between pathogens and endophytic fungi in dual culture were examined daily. Antagonisms towards pathogens were scored using the Badalyan (2002) rating scale according to 3 types (A, B and C) and 4 subtypes (CA1, CA2, CB1 and CB2) (Reaves and Crawford 1994; Badalyan *et al.*, 2002; Badalyan *et al.*, 2004; Campanile *et al.*, 2007).

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Where, A = deadlock with mycelia contact

B = deadlock at a distance

C = replacement, overgrowth without initial deadlock

CA1 = partial replacement after initial deadlock with mycelia contact

CA2 = complete replacement after initial deadlock with mycelia contact

CB1 = partial replacement after initial deadlock at a distance

CB2 = complete replacement after initial deadlock at a distance

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