CHAPTER 3

MATERIALS AND METHODS

3.1 Characteristic and surface appearance of the longan fruit

Longan fruits of Daw cultivar longan (*Dimocarpus longan* Lour. cv. Daw) were harvested monthly from a commercial orchard in Chiang Mai province. Fruits to be used in the study were carefully selected on the basis of uniformity, shape and color, where any blemished or diseased fruits were discarded. Then all selected fruits were transported to the laboratory within one hour after being harvested. Fruit assessment began 14 days after anthesis and continued up to full maturity (194 days after anthesis).

3.1.1 Microscopic level

a. Stereo microscopy

Both pericarp and stem-end surface appearance of longan fruit were examined under an OLYMPUS SZ-PT model stereo microscope.

b. Light transmitted microscopy study

Pericarp anatomy of longan fruit was examined using an OLYMPUS-BHT model light-transmitted microscope. Tissue samples were embedded in paraffin (Appendix C), then semi-thin sections of approximately 20-30µm thick were prepared by cutting with a knife on a microtome before being stained with safranin and fast green (Appendix C).

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3.1.2 Ultrastructure level using a scanning electron microscopy (SEM)

Pericarp surface of longan fruit was examined using a JEOL JSM-5910LV scanning electron microscope (SEM). Tissue specimens of the pericarp were obtained from fruits by sectioning into 0.5 mm square samples before being immersed into chilled 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 7.2 washed with 0.1 M phosphate buffer pH 7.2. Dehydrated the tissue specimens through the series 30%, 5O%, 70%, 80%, 90%, 95%, 100%, 100% acetone, allowing 30 minutes in each before being critical-point dried. The prepared tissue specimens were mounted on copper stubs and sputter coated with gold. The tissue specimens were viewed with the scanning electron microscope.

3.2 Isolation and identification of longan pathogenic fungi

Firstly, fungi were isolated from rotten longan fruits. Tissues taken from the edge of diseased peel and stem-end and then, surface sterilized with 1% sodium hypochlorite for 3-5 minutes before being placed on potato dextrose agar (PDA) in the glass plates. Prepared plates were incubated at the room temperature and observed periodically. During that period, the fungal hypha at the growing edges of fungal colonies from any incubated plates, which the colony appeared to grow, were then transferred aseptically to other dextrose agar plates.

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Under a light microscope, the obtained fungi were identified following Illustrated Genera of Imperfect Fungi (Barnett, 1972), Introductory Mycology (Alexopoulos and Mims, 1979), The Coelomycetes: *Fungi Imperfecti with Pycnidia Acervuli and Stromata* (Sutton, 1980), The Fungi (Ainswort *et al*., 1973) and The Genera of Fungi Sporulating in Pure Culture (von Arx, 1981). Based on characteristic

of colony and spore were identify to genera. Only characteristic of colony and spore were used for identification.

3.3 Pathogenicity of the isolated fungi and disease development

The isolated fungi from 3.2 were tested for their pathogenicity on postharvest longan fruits. Pathogenicity was determined by examination of the events that occur during disease development on inoculated fruit. The healthy fruit was inoculated by dipping them (the fruits) in the spore suspensions prepared from each of the fungal samples. (Otherwise, the peel or stem-end of the fruit was placed on with the mycelial disc obtained from actively growing edges of the fungi samples on the PDA glass plates). Inoculated fruits were packed in foam trays and incubated at ambient temperature for 6 days. The virulent fungi isolates, which cause the most severe symptoms and rot on the experimental fruits rapidly, were selected and stored in PDA slant. For the control group of this experiment, the fruits were dipped in the sterilized distilled water instead of the fungi suspension and packed as above in the foam tray. Preparation details of fungal inocula and the experimental longan fruits are as follows.

a. Preparation of fungal inocula

All of fungal isolates obtained from rotten longan fruits were cultured on PDA. The inocula of these fungi were prepared by has ferring of 0.5 cm-diameter mycelium disc and cultured on PDA for 7 days. Spores formed on PDA were collected from the culture using sterilized distilled water and adjusted concentration to 1×10^6 spores/ml. Germination percentages of conidia on sterilized moister paper disc were examined immediately after each inoculation to verify their viability (≥90%).

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b. Preparation of longan fruits for inoculation

The Daw-cultivar longan fruits were used in this experiment. Prior to the inoculation, the fruits were dipped in 70% (v/v) ethanol to reduce the epiphytic microflora. A stem-end and the area of unwounded peel were inoculated with mycelium disc (0.5 cm diameter) or dipped in the spore suspensions before being packed in the foam trays at 25 ^{0}C for 6 days. For the control group of the experiment, the fruits were dipped in sterilized distilled water and also incubated in the foam tray at the same temperature. In addition, each experimental foam tray used was individually wrapped with a clear sheet of plastic film.

3.4 Identification of a rot causing fungal isolate

The most virulent fungal isolate, *Lasiodiplodia* sp. LP20, (which cause the most severe symptoms and rot the experimental longan fruits rapidly) was selected for further experiment, and its species was identified. Morphological characteristics of this species, both on PDA and on the experimental longan fruits were observed under a light microscope and a SEM. DNA sequencing from mycelia of *Lasiodiplodia* sp. LP20 was done by centre for research in fungal diversity. Department of ecology & biodiversity, the university of Hong Kong. At that laboratory the representative of the isolate sequences from the preliminary clades was used to compare with the sequences in the GenBank with a standard nucleotide-nucleotide using BLAST search (Altschul *et al*. 1997). With this information the species name has been confirmed properly.

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3.5 Infection process of *Pestalotiopsis* sp. MLP on the pericarp and stem-end of harvested longan fruits

Before examination of the infection process, it has to estimate the germination period of the fungal inocula (spore).

Spore suspensions were prepared from 10-day old cultures on PDA. The spores were washed and suspended in sterilized distilled water and filtered through cheesecloth to remove most of the mycelia fragments and then adjusted to a concentration of 10^6conidia ml^{-1}. Tween-20 wetting agent was then added to spore suspensions at the ratio of 0.35ml l^{-1}. The preparation of each slide of the experimental fungi isolate was as follows. Firstly, 10 μl of the spore suspensions were dropped onto a millipore filtering-paper placed on a glass slide. The slide was placed on a spreader glass-rod (V-shape) situated in the inoculation plate which was bottom-laid with moist filtering-paper. The plate was incubated at the room temperature. During the incubation, a drop of lactophenol cotton blue was applied onto the millipore filtering-paper every hour periodically to inhibit the growth of the fungi spores for 48 hours. Finally, the cover slips were applied to every slide prepared from the experiment before being examined under OLYMPUS-BHT model light-transmitted microscope. The specimens which the spore with germ tube appeared to be longer than the spore width were accounted as the germinated fungi spores.

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Detection of the infection process

a. Preparation of fungi inocula

Spores for fruit inoculations were obtained from 10 day-old cultures on PDA of *Pestalotiopsis* sp. MLP. The spores were washed and suspended in sterilized distilled water and filtered through cheesecloth to remove most of the mycelia fragments and then adjusted to a concentration of 10^6 spore ml^{-1}. Tween-20 wetting agent was added to spore suspensions at the ratio of 0.35 ml l^{-1}.

b. Preparation of longan fruits for inoculation

The Daw cultivar longan fruits were used in this experiment where the mature fruits harvested from a commercial orchard in Chiang Mai Province. All fruits were transported to the laboratory within one hour after the harvesting.

Prior to the inoculation, fruits were wiped with 70% ethanol to reduce the epiphytic microflora before being placed on sterilized foam trays and allowed to dry. All prepared fruits were dipped in the spore suspensions before being incubated in a humid chamber held at the room temperature. For the control group of the experiment, the fruits were dipped in sterilized distilled water.

c. Longan fruit tissue preparation for stereo microscopy (SM) study

Pericarp surface anatomy of the experimental longan fruits was examined using an OLYMPUS SZ-PT model stereo microscope. In addition, the fruit's tissue samples, for LM and EM studies, were taken from the experimental fruits from 0 to 96 hours after the inoculation.

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d. Longan fruit tissue preparation for light microscopy (LM) study

Pericarp anatomy of the experimental longan fruits was examined using an OLYMPUS-BHT model light-transmitted microscope. The longan fruit tissue for LM study was prepared as follows.

Paraffin section method (Appendix C)

The pericarp tissue of approximately 3 mm long and 1 mm wide were excised from the inoculated areas of the experimental longan fruits. The tissue was paraffin embedded where semi-thin tissue sections of approximately 20-30μm thick were cut with a knife on a microtome before being stained with safranin-fast green.

Clearing method

Pericarp approximately 5 mm long and 5 mm wide were excised from the inoculated areas of fruit. Tissue pieces were boiled in 2:1 mixture of 95% (v/v) ethanol and lactophenol cotton blue according to the method of Shipton and Brown (1962), with the exception that tissue pieces were immered for 5 min instead of 1 min due to the nature of the tissue. Tissue pieces were left in the stain for several days before clearing in a saturated solution of chloral hydrate for 30-60 min.

e. Longan fruit tissue preparation for scanning electron microscopy (SEM) study

The pericarp surface was examined using a JEOL JSM-5910LV model scanning electron microscope (SEM). The tissue pieces were obtained from several experimental longan fruits by sectioning into 0.5 mm square samples and immersed into chilled 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 7.2. They were then washed with 0.1 M phosphate buffer pH 7.2 Dehydrated the tissue specimens through

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the series 30%, 5O%, 70%, 80%, 90%, 95%, 100%, 100% acetone, allowing 30 minutes in each before being critical-point dried. The prepared tissue specimens were mounted on copper stubs and sputter coated with gold. The tissue specimens were viewed with the scanning electron microscope.

f. Longan fruit tissue preparation for transmission electron microscopy (TEM) study

Longan fruit tissue pieces from inoculated areas on the experimental fruits were fixed, dehydrated and embedded in Spurr's resin. Ultra-thin tissue sections of approximately 60-80 nm thick were cut with a knife on an ultramicrotome and put into water. Tissue sections were then mounted on the uncoated 200-mesh hexagonal grids and stained with a saturated solution of uranyl acetate for 20 minutes followed with lead citrate (Coates *et al*., 1993) for 1 more minute. Finally, all prepared tissue sections were studied under a transmission electron microscope (TEM).

3.6 Sulfur dioxide fumigation effect on disease development of harvested longan fruit

3.6.1 Surface appearance changes of longan fruit fumigated with SO_2

The mature longan fruits were firstly harvested and being 20-minute fumigated with burnt sulfur powder at the ratio of 1 gram per 1 kilogram of the fruits (Paull *et al*., 1995). The fruits were incubated in a moist chamber at ambient temperature. The tissue samples were taken from the experimental fruits at 0, 24 and 72 hours after the incubation. The tissue samples were processed according to the methods of Coates *et al* (1993), Tuntipunjaporn (1991) and Vetchagarun (1991). Surface appearance

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changes were examined under both light transmitted microscope and electron microscope.

3.6.2 Effect of SO_2 on disease development on the inoculated longan fruits

The experimental longan fruits were inoculated with the spore suspension of *Pestalotiopsis* sp. MLP: The vilulent fungi isolate which able to cause the most severe symptoms on the experimental longan fruits and placed in a moist chamber at the room temperature for 4 days. Disease developments on the infected fruits were examined, on the inoculated tissues, at 0, 1, 2, 3 and 4 days after the incubation. The tissue samples were randomly collected and prepared for both light microscopy and electron microscopy studies with the same method as mentioned in 3.5. The rest of the inoculated fruits were placed in a moist chamber without SO_2 fumigation for the control group and were randomly collected and examined in the same way as the treatment fruits.

3.6.3 Effect of SO_2 on fungal growth *in vitro*

The virulent fungal isolates, *Pestalotiopsis* sp. MLP and *Lasiodiplodia theobromae* LP20 were cultured on potato dextrose agar (PDA) for 24 hours. After that, the plates of the cultured fungi were fumigated for 20 min with SO_2 (with the same rate as 3.6.1). For the control group of the experiment, the plates of the cultured fungi were not fumigated. Then, the plates were incubated at the room temperature. Mycelia were randomly collected and prepared from the experimental plates for electron microscopy studies. An energy dispersive X-ray analysis (EDX-analysis) program in the SEM was used to detect the presence of S residue on SO_2 treated mycelia. The mycelia of these fungal isolates were also obtained and re-cultured on

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potato dextrose agar (PDA) incubated at the ambient temperature for the recovering observation.

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