CHAPTER 2

Isolation and Morphological Characterization of Damping-off Pathogenic Fungi

2.1 Introduction

Damping-off is a serious disease for vegetable crops, pathogenic fungi of damping-off are soil-borne fungi, such as *Pythium* sp., *Rhizoctonia* sp., *Sclerotium* sp., and *Fusarium* sp. (Filonow and Dole, 1999; El-Tarabily *et al*., 2009). The infestation of those pathogenic fungi are serious problems in every stage of plant growth especially in seedling stage. The fungi are destructive plant pathogens that cause disease symptoms such as losses of seeds, pre-emergence and post-emergence damping-off, rots of seedlings, roots or basal stalks, decayed vegetable during cultivation, and cause serious damage of a wide variety of crops (Abdelzaher *et al*., 2004). Moreover, the reproduction of those pathogens may survive overwintering by persisting in low or high temperature extremes, dry soil or soilless media for years and still cause disease (Hasegawa *et al*., 2005). Wide host range, over 200 genera vegetable species, of pathogens resulted in crop yield loss throughout tropical, sub-tropical and other warm temperate regions (Edmunds and Gleason, 2003; Yaqub and Shahzad, 2005).

The objectives of this chapter were as follows:

1. To isolate the casual pathogenic fungi of damping-off disease including *F. oxysporum*, *P. aphanidermatum*, *R. solani* and *S. rolfsii*
2. To study the morphological characteristics of the pathogenic fungi

2.2 Materials and Methods

2.2.1 Collection and isolation of the pathogenic fungi

Damping-off infected brassica seedlings and rhizospheric soil were collected to isolate pathogenic fungi by three techniques as follow;

Moist chamber culture technique was used to isolate the seedlings infected damping-off. The infected seedlings were washed by running tab water to remove soil particles and was then surface sterilized with 70% aqueous methanol. The seedling was incubated in sterile moist chamber and incubated at room temperature (28-30°C). The fungal hyphae that grew out of the infected areas were transferred to PDA plate by sterile needle. The pathogen was then purified by hyphal tip isolation.

Baiting technique was used to isolate soil infested damping-off. The collected soil was added into the sterile plastic box, moist with sterile distilled water and a piece of sterile potato was then put on the soil surface, lid off the box, and sealed with parafilm to maintain the humidity.

Tissue transplantation was used to isolate from seedlings infested damping-off. The collected seedlings were washed by running tap water to remove soil particle. The isolation was performed in laminar flow chamber. The tissue between infected and healthy areas were cut (5×5 mm) and surface sterilized by soaking in 2% sodium hypochlorite (NaOCl) solution for 3 min. The pieces of plant tissue were then rinsed twice with sterile distilled water for 2 min and dried on sterile paper towel in Petri dish. A piece of dry tissue was transferred to PDA plate, the fungal growth was daily observed and purified by hyphal tip isolation.

2.2.2 Morphological characterization of the pathogens

The isolated damping-off pathogenic fungi were studied their morphological characteristics using slide culture technique. A piece (1×1 mm) of water agar (WA) was placed on the center of prepared slide in Petri dish and the pure fungal hyphae was then cultured in the center of each side WA. The cultured agar was covered with sterile cover slip and incubated at room temperature for 1 to 3 days. The cover slip that showed fungal growth was removed and dyed with lactophenol cotton blue and placed on a new

slide. The prepared slide was observed under compound light microscope and the morphological growth of the fungi was recorded.

2.2.3 Plant pathogenic testing

Pure colonies of the isolated damping-off pathogenic fungi were tested their pathogenicity on Chinese cabbage and tomato seedlings. Each fungus was cut by sterile cork borer (5×5 mm). The culture disc of each pathogen was inoculated on basal stalk of seedlings that were grown on sterile soil for 20 days. The inoculated seedlings were kept in plastic bag to maintain humidity. The seedlings were often observed and recorded the symptoms.

2.3 Results

2.3.1 Morphological characterization of damping-off pathogenic fungi

Four pathogenic fungi were isolated from brassica seedlings, rhizosphere and soil infested damping-off. According to the morphological characteristics, those isolated fungi were determined as *F. oxysporum*, *P. aphanidermatum*, *R. solani* and *S. rolfsii*.

1) *Fusarium oxysporum*

F. oxysporum was isolated by moist chamber culture technique. The pure colony characteristic of *F. oxysporum* on PDA plate was white in initial, but later it became purple. The colony grew fully on PDA plate (9×9 cm) within 5 days at room temperature 28-30ºC. The fungus was dyed with lactophenol cotton blue and observed under light compound microscope. The characteristic of the fungus was septate hyphae, hyaline and produced both of macroconidia and microconidia as shown in Figure 2.1. Macroconidia were fusiform with 3 septate and slightly curved, while microconidia were ellipsoidal with non-septate. The fungus was identified as *F. oxysporum* by comparing the morphological characteristics with Fourie *et al.*, 2011.

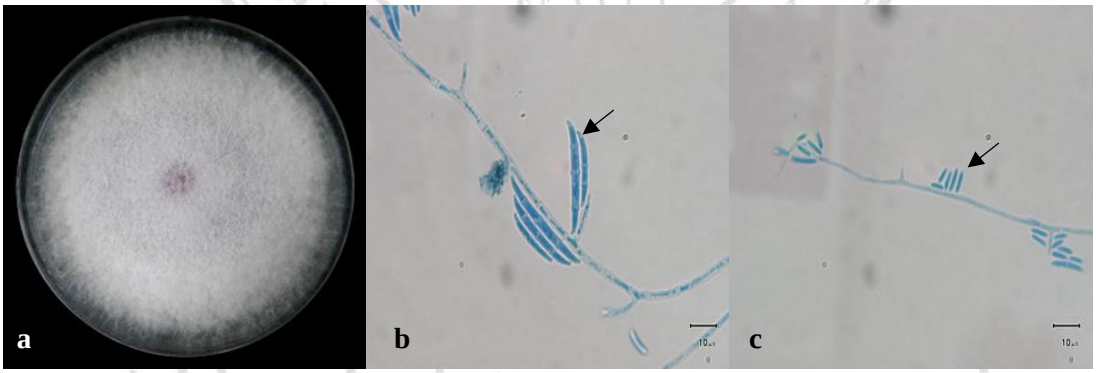

Figure 2.1 The morphological characteristics of *Fusarium oxysporum*
(a) cultural characteristic on Potato Dextrose Agar for 5 days
(b) macroconidia of the fungus
(c) microconidia of the fungus, under light compound microscope at 400 magnification

2) *Pythium aphanidermatum*

P. aphanidermatum was isolated by moist chamber culture technique. The pure colony characteristic of *P. aphanidermatum* on PDA plate was cottony white and rapidly growth. The colony grew fully on PDA plate (9×9 cm) within a day at room temperature 28-30ºC. The fungus was dyed with lactophenol cotton blue and observed under light compound microscope as shown in Figure 2.2. The characteristics of the fungus were non-septate hyphae and hyaline. The fungus produced asexual spores as sporangium that formed a balloon-like, and the production of sexual spore was also observed as oogonium and antheridium. The sexual reproduction, antheridium (male) produced a fertilization tube to enter the oogonium and further formed as zygote spore.

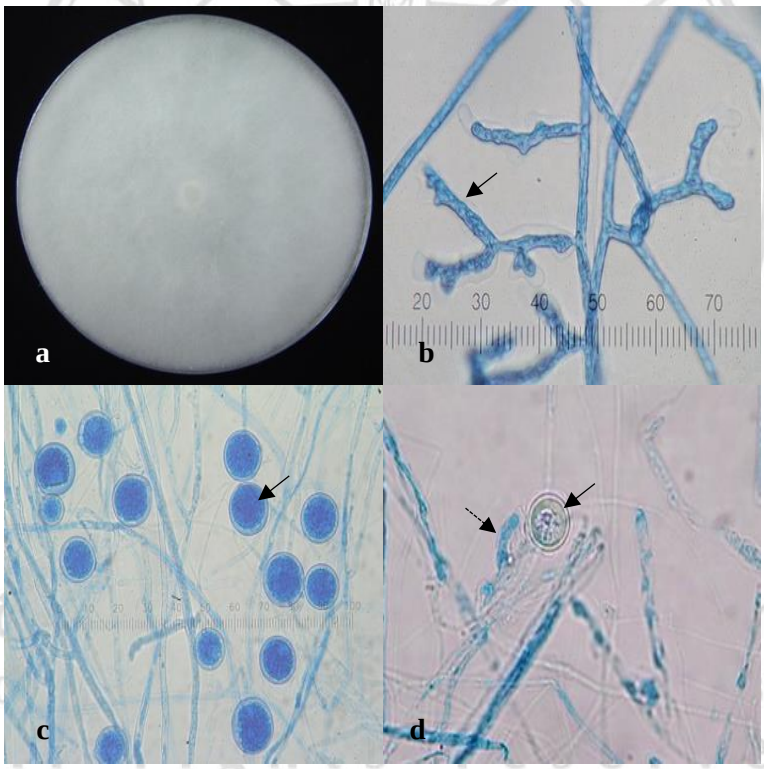

Figure 2.2 The morphological characteristics of *Pythium aphanidermatum*
(a) cultural characteristic on Potato Dextrose Agar for a day
(b) asexual reproduction, sporangia, of the fungus
(c) asexual reproduction, zoosporangia, of the fungus
(d) sexual reproduction, oogonium (solid line) and antheridium (dash line), under light compound microscope at 400 magnification

3) *Rhizoctonia solani*

R. solani was isolated by tissue transplant method. The pure colony characteristic of *R. solani* on PDA plate was light brown colony, rough mycelium, and hyphal aggregated. The colony grew fully on PDA plate (9×9 cm) within 5 days at room temperature 28-30ºC. The fungus was dyed with lactophenol cotton blue to observe under light compound microscope as shown in Figure 2.3. The characteristics of the fungus were septate hypha, hyaline and branched at approximately right angles to the main hypha. The hyphal septate was slightly constrict at the junction.

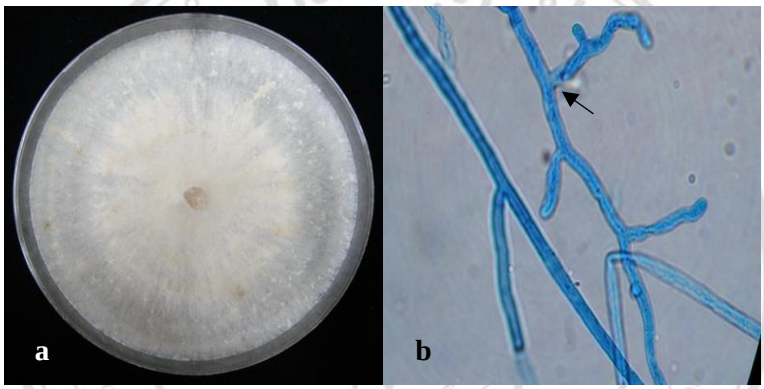

Figure 2.3 The morphological characteristics of *Rhizoctonia solani*
(a) cultural characteristic on Potato Dextrose Agar for 5 days
(b) the approximately right angles branched of the fungal hyphae under light compound microscope at 400 magnification

4) *Sclerotium rolfsii*

S. rolfsii was isolated by baiting technique. The pure colony characteristic of *S. rolfsii* on PDA plate was rough white mycelium, fluffy, hyphal aggregated into small knots called sclerotia. The colony grew fully on PDA plate (9×9 cm) within 3 days at room temperature 28-30ºC. The young sclerotia were initially white and then became light brown and brown, like cabbage seeds (Figure 2.4).

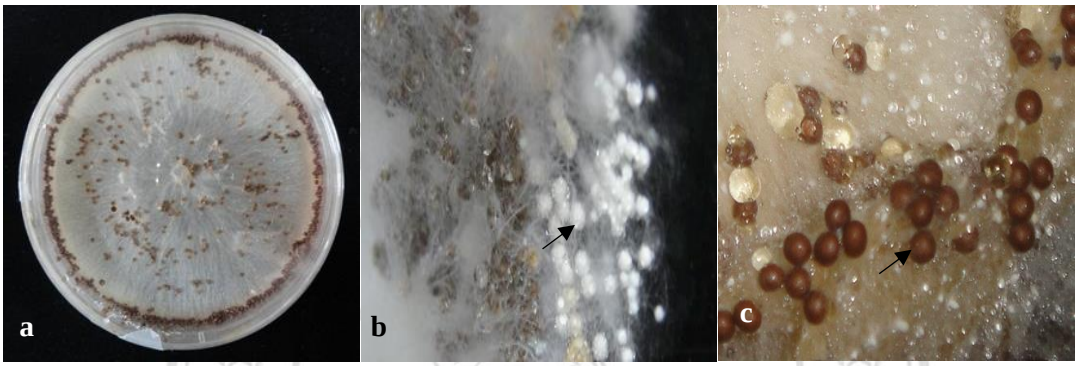

Figure 2.4 The morphological characteristics of *Sclerotium rolfsii*
(a) cultural characteristic on Potato Dextrose Agar for 7 days
(b) the fungus produced young sclerotia in white
(c) mature sclerotia in brown.

2.3.2 Pathogenicity testing

The isolated pathogenic fungi were tested their plant pathogenicity on Chinese cabbage and tomato seedlings. A culture disc of each pathogen such as *F. oxysporum*, *P. aphanidermatum*, *R. solani*, and *S. rolfsii* was inoculated on seedling stalk and incubated in plastic bag that contained sterile distilled water to keep moist. Results showed that the pathogens caused disease symptoms on Chinese cabbage and tomato seedlings. The symptoms showed as water-soaked lesion in light brown, the infected seedlings were then collapse and dead (Figure 2.5 and 2.6). The fungi grew and infected seedlings were observed as their white mycelia coverage basal stalks, especially *S. rolfsii* that showed rough mycelia and produced young sclerotia in white. In addition, all pathogens continuously infected seedlings until seedling die and produced their reproductive on plant debris for further infection.

Figure 2.5 Disease symptom of damping-off of Chinese cabbage seedlings caused by 4 pathogenic fungi compared with untreated control
(a) Untreated Chinese cabbage seedlings (b) *Fusarium oxysporum*
(c) *Pythium aphanidermatum* (d) *Rhizoctonia solani*
(e) *Sclerotium rolfsii*

Figure 2.6 Disease symptom of damping-off of tomato seedlings caused by 4 pathogenic fungi compared with untreated control
(a) Untreated tomato seedlings (b) *Fusarium oxysporum*
(c) *Pythium aphanidermatum* (d) *Rhizoctonia solani*
(e) *Sclerotium rolfsii*

2.4 Discussion

Damping-off is destructive disease on crop production. The casual pathogenic fungi of damping-off such as *Fusarium* sp., *Pythium* sp., *Phytopthora* sp., *Rhizoctonia* sp., and *Sclerotium* sp. cause pre-emergence and post-emergence in seedling stage and have a wide host range (Abdelzaher *et al*., 2004). In this study, the pathogenic fungi *F. oxysporum*, *P. aphanidermatum*, *R. solani*, and *S. rolfsii* were isolated and characterized the morphological characterization (Figure 2.1-2.4). *F. oxysporum* produces both macroconidia (3 septate) and microconidia (no septate) (Fourie *et al.*, 2011); *P. aphanidermatum* produces oogonia in spherical to limoniform shape (Nzungize *et al*., 2011); and *R. solani* produces 90 degree angles of hyphal branches from the parent hypha and a constriction of the cell at the base of each branch (Agrios, 2005).

The strong pathogenicity of the isolated fungi on seedlings of cabbage and tomato are shown in Figure 2.5 and 2.6. These destructive fungi have a wide host range over 200 genera vegetable species throughout tropical, sub-tropical, and other warm temperate regions (Edmunds and Gleason, 2003; Yaqub and Shahzad, 2005). The infection of the fungi on seed germination causes water-soak symptom at the stem based of seedlings, seedlings collapse and dead (Awasthi, 2015), leading to economic losses in crop production worldwide.

The present study provided different methods to isolate the fungi because of their different growth characteristics, different type of the propagules, and difference in proper environmental conditions (Agrios, 2005). The isolated fungi in this Chapter were used as test fungi to evaluate the effects of antifungal metabolites produced by endophytic actinomycetes in the next Chapter.

2.5 Conclusion

Four pathogenic fungi were isolated from seedling and soil infected damping-off, and identified as *F. oxysporum*, *P. aphanidermatum*, *R. solani* and *S. rolfsii*. These fungi caused damping-off disease on the inoculated Chinese cabbage and tomato seedlings. Different fungi showed different symptoms on host plants. Pure colony of the fungi was cultured on PDA in test tubes, kept at 4ºC, and re-cultured for the next experiments.