CHAPTER 1

Introduction

1.1 Rationale

Damping-off is a plant disease that demonstrates a very serious problem for crop productions especially in seedling stage. The disease impacts on crop production, such as causing losses of seeds, pre-emergence and post-emergence damping-off, rots of seedlings, roots or basal stalks, and decayed vegetables during cultivation (Abdelzaher *et al*., 2004). The most pathogenic fungi causing this disease belong to the genus *Fusarium, Pythium, Rhizoctonia* and *Sclerotium*. Those fungi are considerated to be serious pathogens because they potentially produce survive propagules in fluctuation condition, high temperature soil, dry soil or soilless media, for years, and again infect crop under favorable conditions (Hasegawa *et. al*., 2006). Wide host range (over 200 genera vegetable species) of pathogens causing economic losses on important crops throughout tropical, sub-tropical and other warm temperate regions (Edmunds and Gleason, 2003; Yaqub and Shahzad, 2005). Because the fungi are destructive and wide host range, the conventional practice to control the disease depends on the use of fungicide. However, widely used and long-term used of synthetic fungicide have tended to increase ecological system impact and have also induced the mutagenesis of plant pathogens. Therefore, the alternative management options are of interest to study and in demand to reduce the effects of using synthetic fungicide. One possibility option is the use of antagonistic microorganisms as biological controls, which are naturally occurring, and their relatively safe status are wide acceptance by consumers (Hasegawa *et. al*., 2006). Biological control agent (BCA) refers to the antagonistic microorganisms that could suppress the activity and population of one or more plant pathogens and also pest (Pal and Gardener, 2006). Endophytic actinomycetes are the promising group of fungus-antagonistic to develop as bioproduct for disease controlling in sustainable agriculture. Endophytic *Streptomyces* is the group that many researchers have interested in studying for the possibility of disease control.

They are well known as potential producer of variety secondary metabolites that have been reported to use as antibiotics, antimicrobials and others therapeutically useful compounds (Crawford *et al*., 1993; Elmer, 2008; Kekuda *et al*., 2010; Chaudhary *et al*., 2013). *Streptomyces* group has also been reported their efficiency to inhibit plant pathogenic fungi and could control several plant diseases. Errakhi *et al*., (2007) reported that biomass inoculum and culture filtrate of antibiotic-producing *Streptomyces* spp. inhibited the growth of *Sclerotium rolfsii* damping-off and also significantly reduced the disease severity. The antifungal activities of *Streptomyces* have also been reported to inhibit the growth of plant pathogenic fungi, such as *Aspergillus flavus*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora cinnamomi*, *Pestalotiopsis sydowiana* and *Rhizoctonia solani* (Shimizu *et al*., 2000; Cao *et al*., 2005; Kavitha *et al*., 2010; Kanini *et al*., 2013). In addition, the bioproducts of potential endophytic *Streptomyces* have also been reported to control plant disease, such as frosty pod rot in cocoa, rice sheath blight and Rhizoctonia damping-off (Arunyanart *et al*., 2001; Sabaratnam and Traquair 2002).

Moreover, some endophytic *Streptomyces* have capacity to colonize plant tissues enhancing the growth of plant and inducing disease resistance in the plant or even inducing drought tolerance (Hasegawa *et al*., 2005; Taechowisan *et al*., 2005). Coombs and Franco (2003) studied the colonization of germinating wheat seed using *Streptomyces* sp. that tagged with the *egfp* gene and they found that endophytic could colonize plant from the early stage of development including embryo, endosperm and emerging radicle. Moreover, many researchers inoculated endophytic *Streptomyces* in plants under axenic condition and observed the colonization of them under SEM and TEM, finding that some of *Streptomyces* mycelia colonized around stomata of leaves, entered substomatal cavities through stomatal openings and grew in intercellular space of the leaves (Nishimura *et al*., 2002; Minamiyama *et al*., 2003; Suzuki *et al*., 2005).

As described above, this research was conducted aiming at the selection of active endophytic actinomycetes isolated from medicinal plants, extraction of antifungal metabolites from the selected isolates and development of bioproduct formulations for controlling damping-off.

1.2 Plant disease

1.2.1 The development of plant disease

Plants become diseased when they are attacked by plant pathogens. At least two components, such as plant, and pathogen are involved and must have interactions in a proper time. Environmental conditions within a favorable range are certainly become a third component for disease develop. Each of the three components can display considerable variability, thus, as one component changes it affects the degree of disease severity. The duration of plants in a susceptible growth stage or weak must occur long time enough for the development and infection of pathogenic pathogens. Similarity, the pathogens must be virulent, abundant and active for infection. Also, the environmental conditions that are suitable for the growth and development of pathogens, such as low temperature, high moisture or windy may be easier for the infection of pathogens and may cause severe diseases. The three components including host plant, pathogen and environment, are called disease triangle. In this study, the most destructive disease in seedling stage, damping-off, is described its host plants, casual pathogenic fungi and the favorable environment condition for disease development.

1.2.2 Damping-off disease: Symptom

Damping-off has been recognized as one of the most serious disease particularly in seedling stage of crops. The disease causes economic losses in various important crop productions. Planting of susceptible crops in damping-off fungi infested soil may lead to the loss of pre-emergence or post-emergence of seeds and/or seedlings. Pre-emergence damping-off results in low seed viability due to seed rot. The infected seeds become soft and turn into brown. In addition, young seedlings may also be easily infected and die shortly after emergence. The symptoms on seedlings are normally occurs at below the soil line as seedling root or basal stalks rot, seedling wilt, or simply collapse and die. Under favorable conditions, susceptible seedlings may be attacked by one or more pathogenic fungi. Moreover, the pathogenic fungi could also infect in all stages of plant growth resulting in delaying of growth and development of crops such as stunting, wilting of a warm day, foliage turn into yellow and fall prematurely, roots tissue turn into gray and water-soaked. These symptoms could cause crop yield loss and also have impact on the quality of crop productivity. The outbreaks of damping-off are triggered

by high temperatures and humidity, water-logged soils, excessive fertilizer, or an excessive seedling rate (Kohmoto *et al*., 1995; Agrios 2005).

1.2.3 The host: Brassica

The genus Brassica is one of 51 genera in the tribe Brassiceae belonging to the crucifer family. Brassica is the most important genus within this tribe. The genus contains 37 different species with an economically importance (Gomez-Campo, 1980). Many of them are temperate crops distributed in different parts of the world, and are crucial importance in Asia. The members of the genus are known as cruciferous vegetables, cabbages or mustard plants. Those are the economically agricultural crops and commonly used for food. The main economic vegetable crops distribute in Asia such as Chinese cabbage, turnip, broccoli, cauliflower and kale, which provide edible leaves, stems, buds, flowers and seed (Rakow, 2004). Because many of them are temperate crops, thus, the growers must have to prevent their crops from the infection of plant diseases, especially damping-off disease. Damping-off is most destructive disease during seedling stage of crops. The infection of the disease causes losing in seed germination, seedling and/or plant growth and development, and thus loses in crop product quality contributed to economic loses.

1.2.4 Pathogenic pathogens

Damping-off is horticultural disease that has been considered to be a serious disease for vegetable crops particularly in genus of Brassica. The disease causes by a number of different pathogens and the most destructive soilborne pathogenic fungi are *Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp., *Sclerotium* spp. Those fungi cause economic loss in crop production worldwide (Filonow and Dole, 1999; El-Tarabily *et al.*, 2009). Their morphological characteristics are described below.

1) *Fusarium*

Fusarium oxysporum (Agrios, 1997)

Division Eumycota

Subdivision Deuteromycotina

Class Hyphomycetes

Order Hyphales

Family Tuberculariaceae

Genus Fusarium

Biology

Fusarium is imperfect or asexual fungi that belong to subdivision of Deuteromycetes; mycelium well-developed, septate, branched. Sexual reproduction is rare, lacking or unknown. Asexual spores (conidia) formed on conidiophores existing singly, grouped in specialized structures such as sporodochia and synnemata (Agrios, 2005).

On Potato Dextrose Agar (PDA), the mycelial growth of *Fusarium oxysporum* appears to be white and then color may change ranging from violet to dark purple depending on strains (Smith *et al*., 1988). *F. oxysporum* produces three types of asexual spores including microconidia, macroconidia and chlamydospores. Microconidia are most abundant spores which have one or two celled, frequently produced under various conditions and also are produced within the vessels of infected plants. Macroconidia are three to five celled within crescent shape (pointed and curved toward the ends) which frequently found on the surface of infected plants. Chlamydospores are one or two celled, round, thick-walled spores which are produced either terminally or intercalary on older mycelium or in macroconidia. (Agrios, 2005; Burgess *et al*., 1988).

Disease cycle

F. oxysporum is a saprophyte in soil or organic matter and their propagules are plants pathogenic that are able to survive in soil between crop cycles in infected plant debris as mycelium or its three spore types (Smith *et al*., 1988). The disease cycle of *F. oxysporum* is shown in Figure 1.1. Because its saprophyte in soil, the fungal mycelial may directly penetrate or even sporangial germ tube invades seedling or crop through root tip or lesion. Inside infected plant, the fungal grows through root cortex intercellulary and further invades vessels through xylem's pits. The fungus grows and produces microconidia which are carried upward within the vessel by way of transpiration stream. The microconidia attached upper xylem vessel are able to germinate, penetrate and produce more microconidia to invade the next vessel. The increasing of microconidia within the vessel will interrupt and block up the water uptake. The lacking of water induces the leaves' stomata close then the plant show wilt symptom and eventually dies. The fungus continuously invades within plant tissues and in the point that it can reach the surface of dead plants; it will produce abundant spores as a new inoculum for further spread and infection (Agrios, 2005).

Seeds contaminated with the fungus may show the symptom as pre-emergence or post-emergence. The fungus invades young seedling root emergence causing seedling death. Moreover, fungus contaminated seeds may produce toxins that cause toxicity in animal feeds and human (George *et al*., 1995).

Epidemiology

The propagules of *F. oxysporum* can spread over short distances by irrigation water and/or the contaminated farm equipment. The spread over long distances can be found in the case of infected transplants or in soil and even contaminated seeds. Wind is one of factors for the fungus spread.

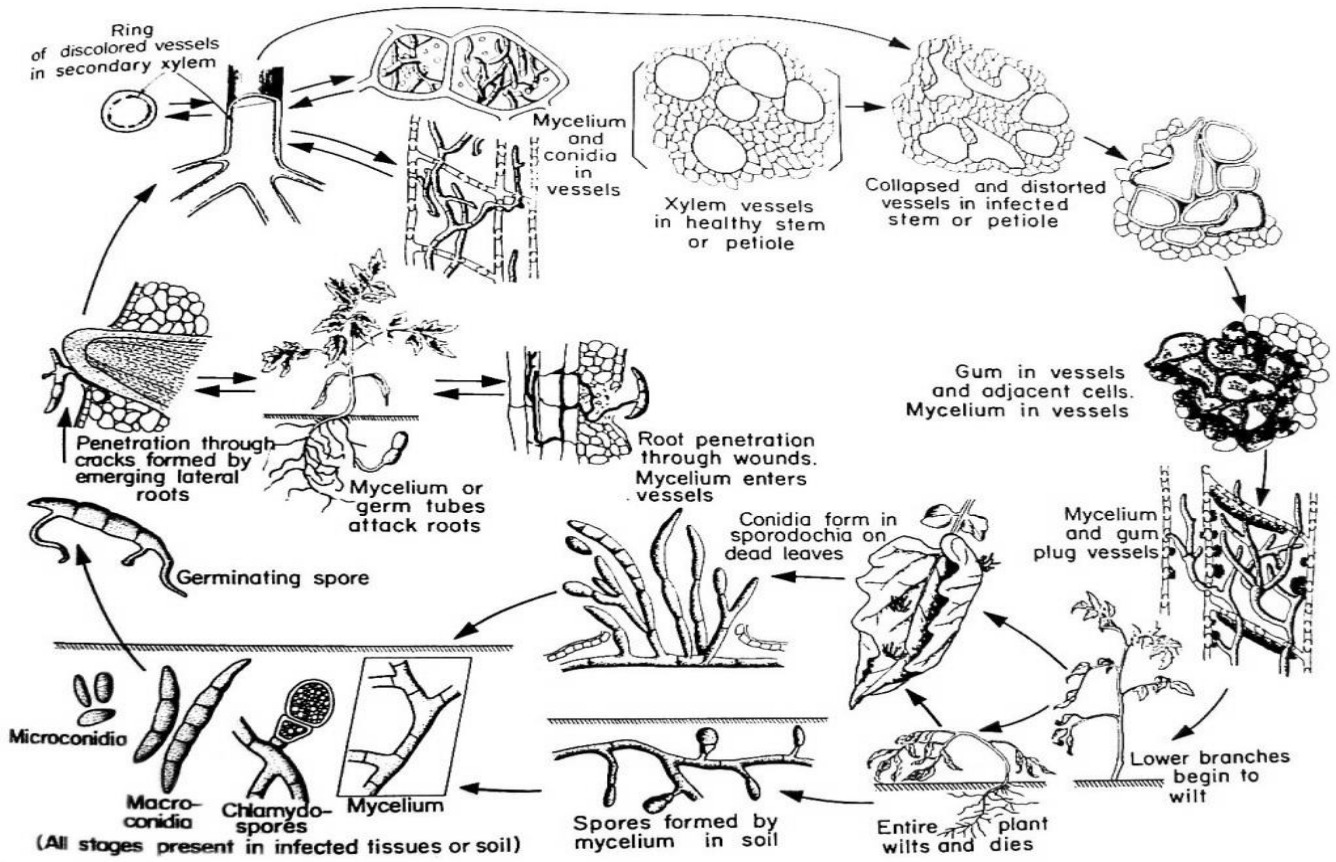

Figure 1.1 Disease cycle of *Fusarium oxysporum* (Agrios, 1997)

2) *Pythium*

Pythium aphanidermatum (Agrios, 1997)

Division Chromista

Subdivision Oomycota

Class Oomycetes

Order Peronosporales

Family Pythiaceae

Genus *Pythium*

Biology

Pythium is commonly referred to water molds, belonging to subdivision of Oomycota, class Oomycetes. The fungi in this class are not true fungi because their cell wall compost of beta-glucans and cellulose rather than chitin as in true fungi, their vegetative stage contain diploid and coenocytic hyphae (Rossman and Palm, 2006). Fungi in this class also have biflagellate zoospores with longer tinsel flagellum directed forward and a shorter whiplash flagellum directed backward. They have zoosporangia as oval or lemon shaped, borne on ordinary mycelium or on sporangiophores. Sporangia in most species are germinated by producing zoospores, but in some cases, they germinate directly and produce a germ tube. Asexual spore (sporangia) may also be effective in long-term soil survival.

In their sexual reproduction, diploid thallus occurs during the development of gametangia. The gametangia of *Pythium* are oogonium (female) and antheridium (male), both gametantia contact and produces thick-walled sexual oospores. Oospores play an importance role for survival overwintering. In appropriate conditions, oospores will germinate by giving rise to a sporangium containing zoospores or to a germ tube, which soon produces a sporangium, depending on the species (Agrios, 2005).

Disease cycle

P. aphanidermatum is the large and diverse genus with a worldwide distribution and a broad host range that is capable of causing serious losses of a number of economic crops in a variety of plant families. The fungus normally exists in soil or water as saprophytes. The infection of the fungus lead to damping-off of seedlings and crow or root rots in mature plants.

Disease cycle of Pythium is shown in Figure 1.2. Their sporangia can germinate directly in the site they attach plant roots. Sporangia also can generate sporangiophores to produce zoosporangia in sack-like structure and release their swimming zoospores, encyst and germinate on plant roots. Once they successfully colonize root tissue and generate hyphae, these hyphae release hydrolytic enzymes to destroy the root tissue and absorb nutrients as a food source. The fungus may also breach the host defense mechanisms and colonize by growing intra- and inter-cellular throughout the epidermal and cortical cells tissue of the roots. Once the fungal grows into cortex, it will disrupt the cytoplasm resulting in swelling and decreasing in electron density of the cell walls. After the colonization of cortex, the increasing of intercellular growth of the fungal continuously invades vascular stele, xylem vessels via pit membrane. A breakdown of the pectin compounds functioning as cementing of cell walls results in cellulose degradation and finally root cortical cells collapse (Kohmoto *et al*., 1995; Agrios, 2005).

Epidemiology

P. aphanidermatum thrives in a moist environment, thus their zoospores swim toward plant root exudates as a spread over short distances. The propagules of the fungus may also spread over by farm equipment, water irrigation, transplants of infected seedlings or infected soil and wind.

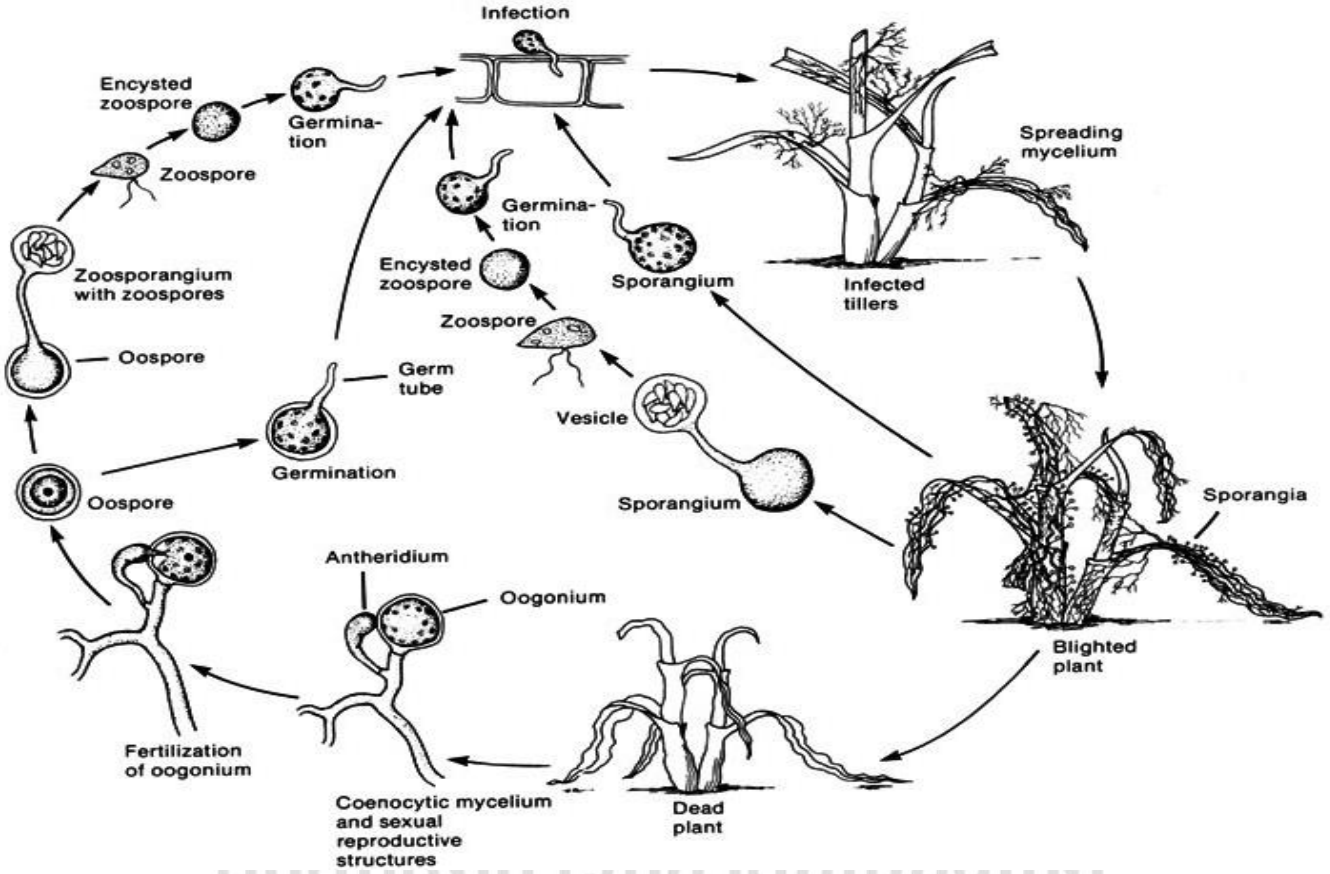

Figure 1.2 Disease cycle of damping-off and seed decay caused by *Pythium* sp. (Agrios, 1997)

3) *Rhizoctonia*

Rhizoctonia solani (Agrios, 1997)

Division Basidiomycota

Subdivision Agaricomycotina

Class Agaricomycetes

Order Cantharellales

Family Ceratobasidiaceae

Genus Rhizoctonia

Biology

Rhizoctonia is soil-borne fungus as non-sporulation. The general characteristics of *Rhizoctonia* are (i) branching near the distal septum of cells in young vegetative hyphae, (ii) formation of a septum in the branch near the point of origin, (iii) constriction of the branch, (iv) dolipore septum, (v) no clamp connection, (vi) no conidia, except monilioid cells, (vii) Sclerotium not differentiated into rind and medulla, and (viii) no rhizomorph. A thick wall monilioid is a small, oval cell that formes in branched chains and the aggregates of these cells are called sclerotia (brown to black). Sclerotia are normally live in soil without the generate of asexual spores. *R. solani* is multi nuclei hyphae cell (4-8 nuclei/cell).

Disease cycle

R. solani is widely distributed plant pathogen. It is the best known to cause various plant disease such as root rot, damping-off and collar rot. The fungus penetrates plants in several ways such as direct penetration the cuticle/ epidermis layer of plants or entry through plant natural opening. The disease cycle of the fungus is shown in Figure 1.3. The hyphae of *R. solani* contact with plant surface and attach to the cuticle with mucilage substances, and align along the junction of epidermal cell walls. These hyphae produce appressorium in form short, swollen side branches, and aggregation of these braches is the initiation of formation of the infection cushion. Penetration of the epidermis occurs from the base of the cushion with infection pegs or hyphal tips, through this way the fungus obtains nutrients from plants (Agrios, 2005). Moreover, the fungus can also produce and release hydrolytic enzymes to breakdown plant cell wall,

then colonize and grow inside dead tissue. The fungal growth produces new inoculums outside or inside the invaded tissue. A new inoculum overwintering and begins disease cycle again when the plants and environmental condition suitable. Various symptoms have associated with the disease such as root rot, stem rot and especially damping-off.

Epidemiology

The fungus survives overwintering in soil and plant debris as sclerotia. These sclerotia can persist in soil for years and again germinate to infect plants. Young and active of the fungus mycelial attacks radicle or hypocotyl of the emerging seedlings causing seedling root rot and die if in severe symptom. The propagules of the fungus spread by the transport of infested soil or plants and also wind.

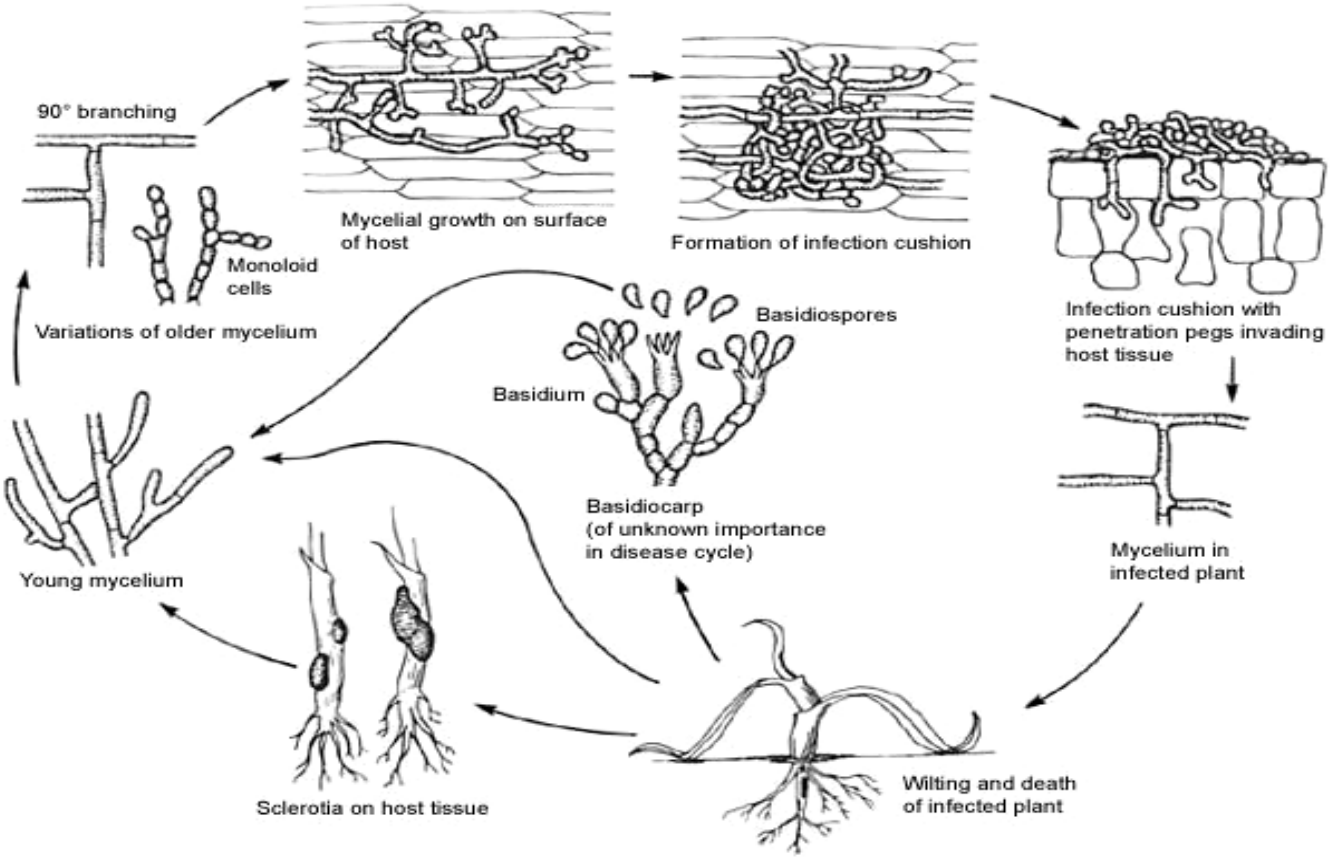

Figure 1.3 Disease cycle of *Rhizoctonia solani* (Agrios, 1997)

4) *Sclerotium*

Sclerotium rolfsii (Agrios, 1997)

Division Basidiomycota

Subdivision Agaricomycotina

Class Agaricomycetes

Order Atheliales

Family Atheliaceae

Genus *Sclerotium*

Biology

S. rolfsii is soil-borne plant fungus, produces a white mycelium with no spore or conidia production. The hyphae of the fungus are multinucleate. The composed of their hyphae form clamp connections and the aggregate of the mycelial also forms sclerotium. *S. rolfsii* typically forms the variation in size, shape, color and number of sclerotium which are host plant dependent. The fungus distributes in warm temperature and subtropical areas of the world. The mycelium germinates eruptive form sclerotium can direct infected plant tissue. The fungus can also produce hydrolytic enzymes to degrade plant cell wall for their penetrated. The infection areas decay and the fungal further produce the mycelial to form sclerotia. *S. rolfsii* is able to survive within a wide range of the environmental conditions. High moisture is required for the fungus germination and the mycelial growth occur rapidly in the continuous light.

Disease cycle

S. rolfsii, as its soil-borne pathogen, germinates mycelia from the sclerotium under the proper conditions toward plant exudates. Disease cycle of Sclerotium is shown in Figure 1.4. The mycelium primarily attacks and infects any part of plant such as roots, stems, leaves, flower and fruits. Dark-brown lesions on plant stem or other parts are the signs of the infection. The lesions usually occur beneath the soil level. The first visible symptom may show as yellow lesions and wilt on leaves of the infected plant. Then the lesions become brown, the fungus produce abundant mycelial as white and further form sclerotia around the infection sites. The young sclerotia of the fungus show as roundish and white, and then the color changes to dark brown. Plant seedlings

are susceptible to infect by this fungus and may die quickly. The infection in other plant stages may show as gradually girdled lesions in dark brown, the lesion is soft but no water-soaked.

Epidemiology

The propagules as sclerotia of the *S. rolfsii* can long-term stay in soil or even overwintering. The fungus may also live in infected tissue or plant debris. The fungus spreads by water irrigation, the transplant of infected soil or seedlings and/or by the contamination of farm equipment. Because its sclerotia are small, round in dark brown, they are easily move and distribute to other areas.

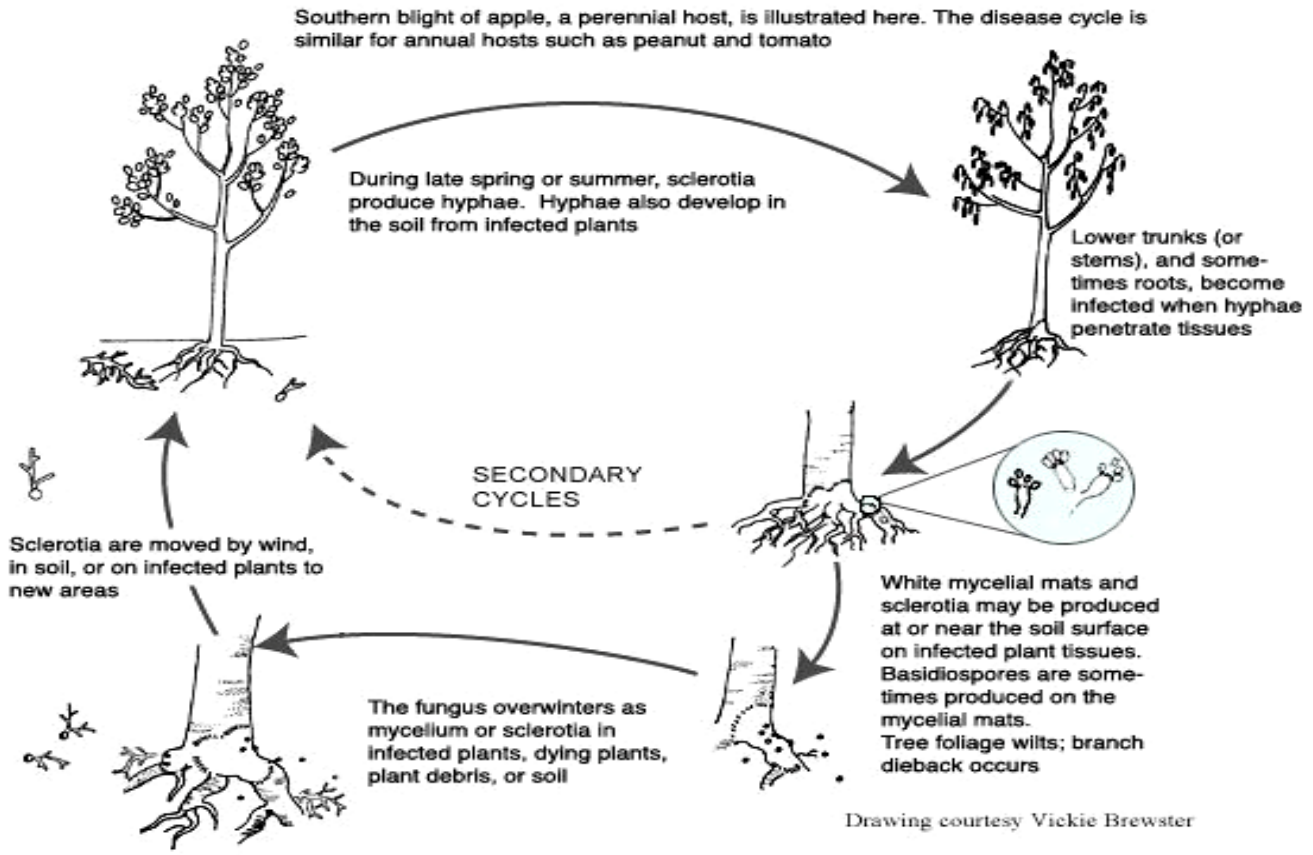

Figure 1.4 Disease cycle of *Sclerotium* sp. (Agrios, 1997)

1.3 Actinomycetes

Actinomycetes are aerobic, spore forming gram-positive bacteria that belong to the order Actinomycetales. Actinomycetes grow as hyphae like fungi, produce branching mycelium (Lechevalier and Lechevalier, 1981; Sprusansky *et al*., 2005). The name of Actinomycetes is derived from Greek “atkis” (a ray) and “mykes” (fungas) and has features of both bacteria and fungi (Das *et al*., 2008). Actinomycetes are characterized by the formation of substrate and aerial mycelium growth. The mycelium is a vegetative that grows in the substrate, while aerial mycelium is produced above the vegetative growth. The hyphae are generally non-septate under certain special conditions, septa may be observed in some forms (Waksman, 1940). Actinomycetes have high genomic (G + C) content and its cell wall consist of a large variety of complex compounds including peptidoglycan, teichoic and teichuronic acid and polysaccharides (Chaudhary *et al*., 2013). It represents one of the largest taxonomic units among the 18 major lineages that currently recognize within the domain bacteria (Ventura *et al*., 2007). The actinomycetes, especially *Streptomyces*, are potential producers of antibiotics and of other therapeutically useful compounds including antibiotics, antitumor agents, immunosuppressive agents and enzymes. These metabolites are known to possess antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti-algal, anti-helmintic, anti-malarial and anti-inflammatory (Chaudhary *et al*., 2013; Kekuda *et al*., 2010; Ravikumar *et al*., 2011). Moreover, several strains of actinomycetes have been found to protect plants against plant diseases.

1.3.1 Classification/ Taxonomy

Actinomycetes are primarily classification based on their occurrence in nature such as pigment formation upon complex organic media, aerobiosis, proteolytic action, colony formation, or cultural characters upon synthetic media. Although, these systems show the primarily ecological or physiological in nature at present time, it may be insufficient for the purpose of separation actinomycetes.

Actinomycetes are characterized by the formation of normally branching threads or rods, frequently giving rise to a typical mycelium which is unicellular, especially during the early stages of growth. The hyphae are generally non-septate, under certain special condition, septa may be observed in some forms. The mycelium is either

vegetative and/or growing above the vegetative growth. Actinomycetes reproduce through special sporulating bodies or from parts of the vegetative mycelium. The spore bearing hyphae are produced on the mycelium either singly and monopodially, or in broom-like or cluster-like formations, or in verticilliate-like tufts or whorls upon the mycelium. The sporophores vary from long to very short forms which may be produced singly on the tips of side branches or in chains attached directly to the mycelium (Waksman, 1940).

The Bergey's Manual of Systematic Bacteriology-2nd edition (Williams *et al*., 1989) for actinomycetes classification reported that phylum actinobacteria is divided into 6 classes namely Actinobacteria, Acidimicrobiia, Coribacteriia, Nitriliruptoria, Rubrobacteria and Thermoleophilia. The class Actinobacteria is further divided into 16 orders which are Actinomycetales, Actinopolysporales, Bifidobacteriales, Catenulisporales, Corynebacteriales, Frankiales, Glycomycetales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales and Incertae sedis.

Class Streptomycetales is a group that comprises a large member of microorganisms among those classes and also their diversity of compounds productions are rich source of natural products, especially clinically useful antibiotics, antimetabolites and antitumor agents. The microorganisms appeared in this class are aerobic, gram-stain-positive, non-acid-alcohol-fast actinomycetes that form an extensively branched substrate mycelium which rarely fragments. At maturity, the aerial mycelium forms chains of three to many spores. The organisms produce a wide range of pigments, which are responsible for the colors of the substrate (primary mycelium) and aerial mycelia (secondary mycelium). Wall of cells from the substrate mycelium contains either LL- or *meso*-diaminopimelic acid (A_2pm) as the predominant diamino acid; aerial or submerged spores contain LL-A_2pm. Whole-organism sugar profiles may contain major amounts of either galactose or galactose and rhamnose. Lipid profiles typically contain hexa- and octa-hydrogenated menaquinones with nine isoprene units as the predominant isoprenologs, diphosphatidylglycerol, posphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides as major polar lipids, and complex mixtures of saturated, and iso- and anteiso-fatty acids. Mycolic acids are not

present. The substrate mycelia are approximately 0.5-1.0 μm in diameter and the differentiation starts with the formation of aerial hyphae which grow in to the air away from the surface of the colony (Figure 1.5). The aerial hyphae undergo septation into uninucleoid compartments and metamorphose into chains of spore (sporophores). The number of spores in a chain and spore-chain types are species dependent (Goodfellow *et al*., 2012). Spore ornamentation is either smooth, spiny, rugose or hairy and the spore chain morphology of Streptomyces are divided into three types namely rectiflexibiles (RF), retinaculiaperti (RA) and spirales (S) (Figure 1.6).

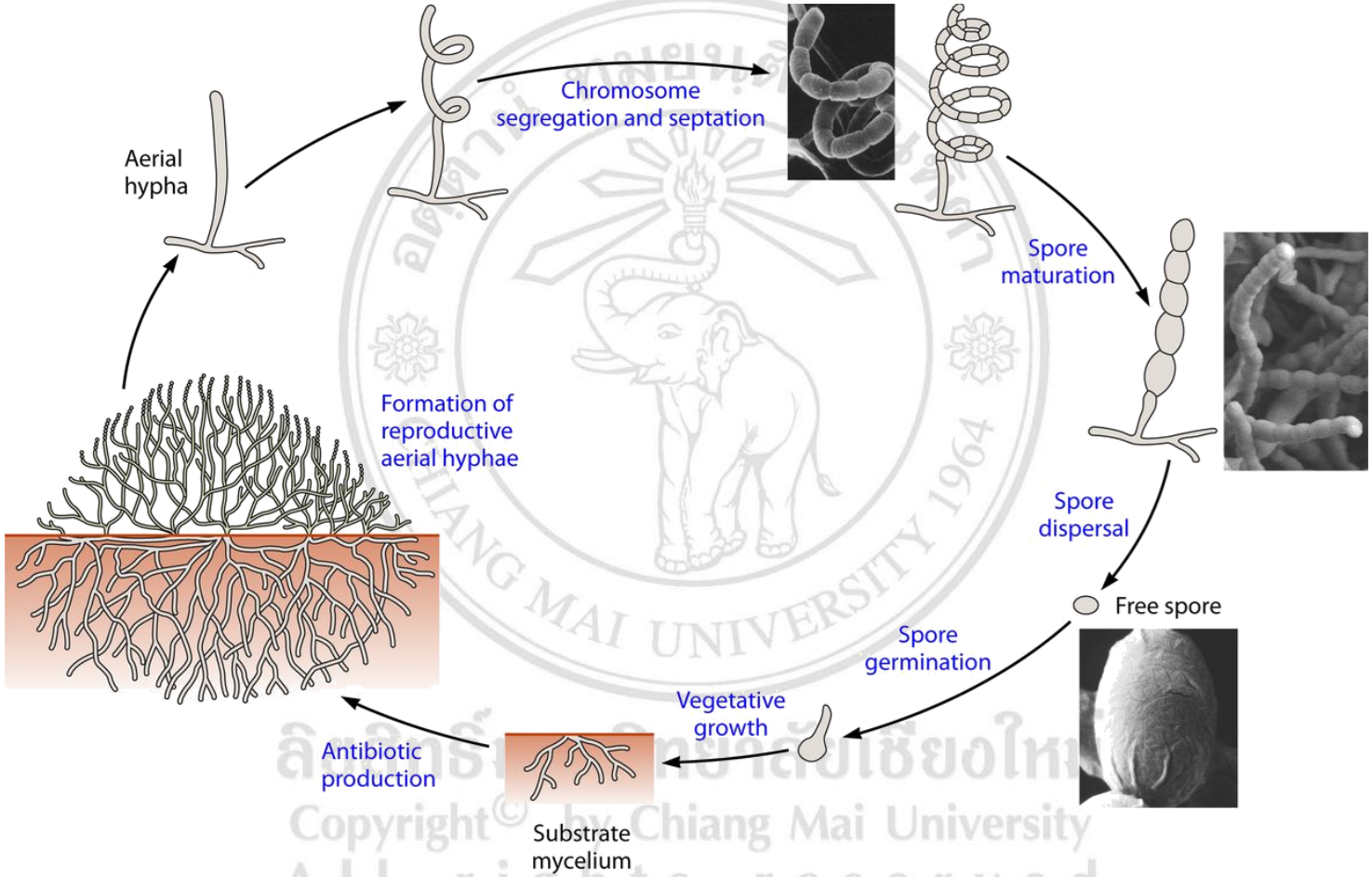

Figure 1.5 Life cycle of actinomycetes in genus *Streptomycetes* (Barka *et al*., 2016)

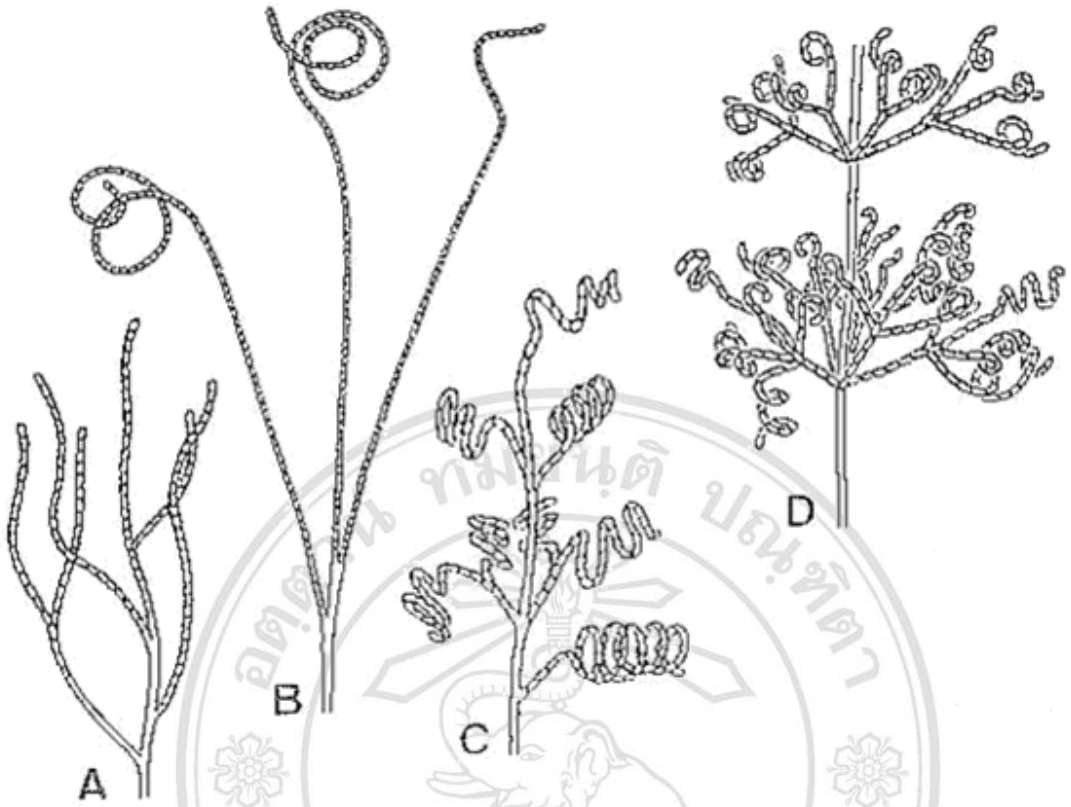

Figure 1.6 Spore production in long chains of Streptomyces (Miyadoh *et al.* 1997)
(a) rectiflexibilies type (b) retinaculiaperti type
(c) spira type (d) verticillati type

1.3.2 Endophytic actinomycetes

Endophytes are microbes that colonize living, internal tissues of plant without carrying any immediate over negative effects as inclusive and widely accepted definition of endophytes by Bacon and White (2000). Endophytes usually draw nutrition and protection from host plants and in return, confer enhanced fitness to the host by producing a variety of bioactive metabolites and providing protection for the plant (Strobel and Daisy, 2003). Plants growing in areas of great biodiversity usually have the prospect of harboring endophytes with great biodiversity. The internal plant tissue is a biologically complex and distinct microhabitat within the terrestrial ecosystem because of its varying content of alkaloids, terpenoids, steroids and chromatic compounds. Thus, healthy plant tissues represent an untapped reservoir or novel endophytic microorganisms producing bioactive metabolites (Strobel and Daisy, 2003). Endophytic

microorganisms have demonstrated to improve and promote growth of host plants as well as reduce disease symptoms by producing a variety of bioactive metabolites such as phytohormone, nitrogen fixation, antibiotic, siderophores, nutrient competition and induction of systemic disease resistance (Hallmann *et al*., 1997 and Hasegawa *et al*., 2006).

Medicinal plants have been generally used to isolate endophytic actinomycetes with the purpose that it may associate with the production of vary bioactive metabolites (Strobel *et al*., 2004). Nearly 300,000 plant species on the earth, each individual plant is considered to host one or more type of endophytes creating an enormous biodiversity (Strobel and Daisy 2003). Qin *et al*. (2011) reported that many endophytic actinobacteria, especially those from medicinal plants possess the ability of inhibiting or killing a wide variety of harmful microorganisms like pathogenic bacteria, fungi and viruses. Moreover, they also have capable colonize in plant roots is ideal for use as a biocontrol agent against soil-borne diseases because seeds and roots are the main tissues that require protection from soil-borne pathogens (Weller, 1988). In addition, it has been reported that if a useful endophytic actinomycete isolated from a field-grown plant can successfully colonize tissue-cultured seedlings of a plant, the seedlings could become resistant to various plant pathogens (Shimizu *et al*., 2000, 2001).

The colonization of endophytic actinomycetes have been studied and reported by numerous researchers. Endophytic actinomycete R-5 could colonize around stomata of leaves, entered substomatal cavities through stomatal openings, and continued to grow in intercellular spaces of the leaves after inoculation on the surface of tissue-culture rhododendron (Minamiyama *et al*., 2003; Suzuki *et al*., 2005). The endophytic actinomycetes could be reisolated from leaves and stems of tissue-cultured seedling within a few days after being placed on the surface of the culture medium (Shimizu *et al*., 2000, Nishimura *et al*., 2002 and Suzuki *et al*., 2005). Besides, Coombs and Franco (2003) coated germinating seeds of wheat with GFP-expressing endophytic *Streptomyces* sp. and detected its localization in the seeds using fluorescent microscopy. The result showed that the strain only found in the embryo and around the break in the seed husk after 1 day and GFP-expressing microcolonies of the strain were seen more frequently in the embryo tissue after 3 days. Colonies were seen in close proximity to

the plant cell walls during intercellular growth and also in the emerging radical of the embryo and in the endosperm. Their observations show that the endophytic actinomycetes are able to associate with its host at a very early stage in the development of the plant.

Many of endophytic actinomycetes have been isolated from tissue of healthy plant and identified as Streptomyces species. Streptomyces endophytic actinomycetes colonized plant and induced disease-resistant seedling by activating the expression of defense genes in plant defense mechanism or even enhanced drought-tolerance of seedling by inducing structural modifications of cell walls in the seedling. (Shimizu *et al*., 2005). *Streptomyces padanus* strain AOK-30 induced callose accumulated in cell wall of tissue-cultured seedling that interfere water loss through by enhancing osmotic pressure (Hasegawa *et al*., 2005).

1.3.3 Importance: Produce natural products

Streptomycetes have a capacity to produce a variation of secondary metabolites that exhibit a wide variety of biological activity (Qin *et al*., 2011). The biological activity produce by Streptomyces such as antibiotics, antitumor, anti-infection agents, plant growth promoters and enzymes and those may contribute to the plants by promoting growth and enhancing their ability of withstanding the environmental stresses. Many researchers isolated endophytic *Streptomyces* from living plants and some of those are extracted the bioactive compound from their culture filtrate to suppress phytopathogens such as novobiocin and cedarmycins (Sasaki *et al*., 2001), alnumycin (Bieber *et al*., 1998), actinomycin X_2 and fungichromin (Shimuzu *et al*., 2004). Particularly, the peptide antibiotics; coronamycin, exhibited activity against phytopathogenic *Pythium* spp. and it is a novel fungicide candidate (Ezra *et al*., 2004). Sharifi *et al.* (2007) reported that 43 of 178 actinomycete isolates showed inhibition growth of the pathogen in culture plates and two of the most active isolates exhibited biological control of the pathogen under greenhouse conditions. Taechowisan and Lumyong (2003) isolated 59 endophytic actinomycetes from the roots of *Zingiber officinale* and *Alpinia galanga* and their antifungal compounds showed the activity to against *Colletotrichum musae* and *Fusarium oxysporum* and 9 isolates against *Candida albicans*.

Endophytic actinomycetes have been shown to protect plants against different soil-borne plant pathogens including *Fusarium oxysporum* (Cao *et al*., 2005) *Rhizoctonia solani* and *Verticillium dahliae* (Krechel *et al.*, 2002) and *Gaeumannomyces graminis* var. *tritici* and *R. solani* (Coombs *et al*., 2004), *Plectosporium tabacinum* and *Pythium aphanidermatum* (El-Tarabily, 2003; El-Tarabily *et al*., 2009), *R. solani* and *Sclerotium rolfsii* (Elad *et al*., 1982). Moreover, some endophytes and bacteria provide beneficial effects on host plants by producing plant growth regulators (Ting *et al*. 2008; Raja *et al*. 2008). Igarashi *et al*. (2002) isolated pteridic acids A and B, auxin-like compound, from the fermentation broth of endophytic *Streptomyces hygroscopicus* TP-A045.

1.4 Bioproducts for plant diseases controlling

The worldwide efforts in the search of natural products for the crop protection market have progressed significantly. Actinomycetes, especially those belonging to the genus Streptomyces, appear to be good candidates to new approaches to control plant disease (Doumbou *et al*., 2012). The formulation of biological control agents (BCA) involves in the incorporation of BCA's active propagules with carrier substances to accommodate various application and delivery requirement of the BCA in greenhouses and fields. Many researchers have studied and formulated bioproducts from antagonistic strains to control plant diseases as follow.

Filonow and Dole (1999) formulated and applied used of *Actinoplanes* strain W257 as granules and root dip to control *Pythium* root rot of geraniums and poinsettias in the greenhouse. They found that their bioproducts have had the same effective to control the pathogen as metalaxyl fungicide.

Sabaratnum and Traquair (2002) developed three kind formulations of bioproducts; alginate beads, durum flour granules, and talcum powder, to suppress damping-off caused by *Rhizoctonia solani* in tomato. All formulations showed the different percentage of disease reduction which talcum powder formulation delivered to tomato seeds as a seed-coating was the most effective biocontrol treatment.

Chung *et al.* (2004) applied used of 1.0% granulate biofungicide included *Streptomyces* to soil infested *R. solani* that planting cabbage seeds and the results showed that the colonization percentage of pathogen was significantly reduced in cabbage seeds.

Jobin *et al.* (2004) formulated spore of *S. melanosporofaciens* EF-76 as spore-loaded chitosan beads by using entrapment technique and used against common scab disease. The results showed significantly higher proportion (92%) of healthy progeny tubers when treated with 0.25 g of bioproduct per seed tuber.

Khan *et al.* (2011) developed seed treatment bioproduct by adding 1 part of stock culture (sawdust: soil: 5% molasses, 15:5:1 (w/w)) of the agent with 20 parts of carrier (flyash: soil: 5% molasses mixture, 5:3:1 (w/w)) to control *Fusarium* wilt and root knot disease. For the result, the bioproduct showed greatly reduced the soil population of the pathogens.

In addition, the use of actinomycetes as biological control have been reported to significantly increased plant growth and reduced damping-off disease by many researchers (El-Tarabily *et al*., 2009; Sharifi *et al*., 2007; Jones and Deborah, 1996). The type of bioproduct formulations are decided by the site of action and the target pathogens of the microbial biological control agents and the delivery requirements are the major deciding factors in determining the type of formulation (Green *et al*., 1998; Lewis, 1991; Lumsden *et al*., 1995). The feasibility of using a microbial biological control agent (BCA) in greenhouses or the field is determined largely by its formulation, shelf life, and delivery technologies (Sabaratnam and Traquair, 2002).

1.5 The objects of this study

1. To isolate and select the effective endophytic actinomycetes from medicinal plants for controlling of the causal damping-off pathogenic fungi; *F. oxysporum*, *P. aphanidermatum*, *R. solani* and *S. rolfsii*

2. To extract antifungal metabolites from culture filtrates of the selected endophytic actinomycete isolates and determine their inhibitory activity on the damping-off pathogenic fungi

3. To determine the antifungal metabolites on controlling damping-off disease caused by *P. aphanidermatum* and *S. rolfsii*

4. To study the colonization of the selected endophytic actinomycete isolates on Chinese cabbage seedlings

5. To formulate bioproducts from the selected endophytic actinomycete isolates and their antifungal metabolites

1.5.1 Usefulness of the Research (Theoretical and/or Applied)

1. Providing the information of the effective endophytic actinomycete isolates isolated from medicinal plants for inhibition growth of damping-off pathogenic fungi and controlling damping-off disease

2. Utilization of the effective endophytic actinomycete isolates or its antifungal metabolites on controlling damping-off disease

3. Providing the possibility of the colonization of endophytic actinomycete isolates on non-host plant for the further develop and useful the isolates to induce damping-off disease resistance or tolerance of seedlings

4. Utilization of the developed bioproducts as an alternative disease management in sustainable agriculture.

1.6 Research scope

The research scopes of this thesis are divided into 3 major parts. Part 1 describes the isolation and identification of both damping-off pathogenic fungi and endophytic actinomycete isolates and study their effectiveness on controlling the fungi (Chapter 2, Chapter 3 and Chapter 4). Part 2 describes the effectiveness of antifungal metabolites produced by the selected endophytic actinomycete isolates on controlling damping-off disease and the possibility to colonized inside non-host plant of the selected isolates and their capacity to promote plant growth in seedling stage (Chapter 5 and Chapter 6). Part 3 describes the development of formulations of bioproducts from the selected isolates and/or its crude of antifungal metabolites for the further utilization as biological control of damping-off disease (Chapter 7).

Part 1: Effects of endophytic actinomycetes on controlling damping-off pathogenic fungi

In this part, damping-off pathogenic fungi were isolated from brassica infested damping-off and/or soil infested damping-off. The pathogens were isolated and purified on PDA (Potato Dextrose Agar) for the further experiments. Endophytic actinomycetes were isolated from medicinal plants that collected from Chiang Mai, Thailand. The purified endophytic actinomycetes were determined their inhibitory activities on the growth of the pathogenic fungi. The inhibition percentages were calculated and the effective endophytic actinomycete isolates were selected to study their morphological and physiological characteristics and to identify the taxonomic position by 16S rRNA.

Part 2: The inhibitory activity of antifungal metabolites on damping-off pathogenic fungi and the colonization of selected endophytic actinomycetes on non-host plant

The effective endophytic actinomycete isolates were extracted their antifungal metabolites and determined the ability to control damping-off of Chinese cabbage seedlings. Antifungal metabolites of the selected isolates were extracted from their culture filtrates using ethyl acetate as a solvent for partition. The crude extracts of antifungal metabolites were determined minimum inhibitory concentrations (MIC_{90} values) that inhibited the pathogenic fungi by 90% inhibition, using soaking method. The crude extracts of the antifungal metabolites at MIC values were used for the

evaluation of their ability to control damping-off disease in seedling stage of Chinese cabbage. In addition, the selected isolates were further studied their possibility to colonize inside non-host plant tissues. The colonization was determined by coating Chinese cabbage seeds with dry spore mass of selected endophytic actinomycete isolates. The selected isolates were re-isolated from the cotyledon, hypocotyl and root parts of the treated seedlings to evaluate the most living parts of colonization. The growth of treated seedlings was also measured to determine the capacity of selected endophytic actinomycete isolates to promote plant growth. Colonization of the selected isolates on the treated seedlings was observed by scanning electron microscope (SEM).

Part 3: Formulation and development of bioproducts for controlling damping-off disease

The selected endophytic actinomycete isolates were formulated from the spore mass of the selected isolates and their antifungal metabolites. Bioproducts were developed by considering the purpose of using, cost of products, simplicity of the production process, storage life of the selected isolates in the products and convenient use.