CHAPTER 1

Introduction

1.1 Rationale

Tomato (*Lycopersicon esculentum* Mill) is valuable vegetable worldwide because they produce various health-promoting related components, inparticular vitamins A, C, E, folate flavonoids, potassium and *β*-carotene (Mangels *et al*., 1993; Beecher, 1998; Leonardi *et al*., 2000; Sahlin *et al*., 2004). Besides, tomato can be consumed as an ingredient in many dishes or drinks and also fresh consumption. Nowadays, tomato in world production is about 100 million tons fresh fruit from 3.7 million ha. The United Nations Food and Agriculture Organization (FAO) reported that a good commercial yield of tomatoes under irrigation between 45 and 65 tons/ha (FAOSTAT, 2011; FAO, 2015). The office of Agricultural Economics (OAE) reported that Thailand had an average planted area for tomatoes of 5624.8 ha (35,154.8 rais), with production of 124,969.2 tons (3,551 kg/rai) and valued at 1,761.4 million baht in last 5 years (from 2010 – 2014) (OAE, 2014). These present statistics indicated that tomatoes are the important crop in Thailand also.

However, *Fusarium* wilt is one of major tomato disease that lead to yield loss in the production. This disease is cited by the soil-inhabiting pathogenic fungus of formae specialis i.e. *lycopersici*, namely *Fusarium oxysporum* f. sp. *lycopersici* W. C. Snyder & H. N. Hans (*Fol*). Usage of resistant cultivars against *Fusarium* wilt seems to be the appropriate solution. On the other hand, a continuous problem occurs when causal pathogen developed new pathogenic races (Jarvis, 1988; Jones *et al*., 1991). The initial symptom of plants afflicted with *Fusarium* wilt is yellowing, that always found in the lowest leaves and often restricted to one side of the plant or a single shoot. Afterwards, infected leaves will be wilt and dead. Wilting progresses up the stem until the foliage is killed and the stem decays (Agrios, 1997).

Recently a number of fumigants and fungicides, such as prochloraz, thiram, thiabendazole and carbendazim, have been used to manage *Fusarium* wilt of tomato, but these fungicides adversely affect other useful soil microorganisms and also pollute the environment. Furthermore, genetic resistance to each of these diseases has been identified (Kawchuk *et al*., 2001; Sela-Buurlage *et al*., 2001). There has been increasing interest in the deployment of non-chemical management strategies including the use of biological control for protection of plants against *Fusarium* wilt. The potential method based on the use of beneficial microorganisms isolated from suppressive soils (Parker *et al*., 1985; Alabouvette *et al*., 1993).

Actinomycetes is beneficial microorganisms belong in a distinct bacteria group that widely spread in nature, especially in natural soils. Actinomycetes are categorized as special interest because they are able to colonize plant tissue and produce spores. These properties lead to a resistant form important for survival in agricultural soil (Banik and Dey, 1982; Mba, 1997; Hamdali *et al*., 2008a, b). Actinomycetes comprise of eight groups with 48 genera, and the special attention given to them in biotechnological applications which is a natural result of their great metabolic diversity, especially in the genera *Streptomyces* (Piret and Demain, 1988). In addition, the development of fungicide-resistant strains of microorganisms and regulations limiting the use of fungicides have reduced the attractiveness of chemical-based control strategies (Johnson and Sangchote, 1994). A substitute method to reduce chemical control extensively investigated for over a decade is the use of antimicrobials (Shimizu *et al*., 2009).

Besides, plant breeding is widely studied nowadays. Using *Fusarium* wilt disease resistance tomato cultivars is a considerable method of economically effective and friendly with environment, but it is not stable over time since the pathogen populations are selected for their ability to overcome; the pathogens is extremely variable. As described, although the use of *Fusarium*-resistant tomato cultivars can provide some degree of control of these diseases, the occurrence and development of new pathogenic races is a continuing problem and currently there is no commercially acceptable cultivars with adequate resistance to *F. oxysporum* f. sp. *radicis-lycopersici* or race 3 of *F. oxysporum* f. sp. *lycopersici* (Javis, 1988; Jones *et al*., 1991). On the other hand,

many recent reports have shown that some of microorganisms can work as a trigger for systemic acquired resistance (SAR). Plant defense against pathogens and insects is mediated in part by an array of constitutive and inducible chemical resistance factors (Duffey and Felton, 1989; Bennett and Wallsgrove, 1994). An effective approach for disease management strategies is represented as plant elicitor (Layon and Newton, 1999). Ross (1961) coined the term "systemic acquired resistance" (SAR) to describe the induction of plant resistance with long-lasting by a pathogen after infection. To be a SAR-inducing compound, the compound that acts as the biological inducer has to elicit protection to the same spectrum of pathogens in the same way. However, the expression has to express the same as biological marker, as well as no anti-microbial activity (Kessman *et al*., 1994).

According to the aforementioned principles, theory and rationale, the remainder of this study will be focused on research designed to stimulate plant self-defense mechanisms on resistance toward the *Fol* pathogen by detection of increased *PR*-proteins after the treatment.

1.2 *Fusarium* wilt disease in tomato

1.2.1 The host

The tomato (*Lycopersicon esculentum* Mill) is a shortlived perennial plant, grown as an annual plant, belongs to the *Solanaceae* or nightshade family which an economically important family of flowering plants with includes a number of important agricultural crops and consists of about 98 genera and some 2,700 species (Went, 1944; Olmstead and Bohs, 2007). The tomato is aboriginal to South America, however tomato is able to grow in temperate climates worldwide. Tomato plant stem is weakly that usually scrambles over other plants. Plant height is normally 1 – 3 m tall. The fruit is an edible, brightly coloured berry depending on the pigment lycopene (usually red). The fruits of wild plants commonly 1 - 2 cm diameter, and usually much larger in cultivated forms. Though it is botanically a berry, a subset of fruit, the tomato is nutritionally categorized as a vegetable.

The tomato is native to western South America and Central America before distributed most widely in Peru and Chili. Then, tomatoes were introduced into Southern Europe in a while the discovery of New World, America (Davies and Hobson, 1981; Knapp, 2002). There are around 7,500 tomato varieties worldwide grown for various purposes. The tomato varieties are roughly divided into several categories, based mostly on shape and size and also commonly classified as determinate or indeterminate. Nowadays, genetic design of a commercial variety that combines the advantages requires fine tuning, but may be feasible (Cocaliadis, 2014). The phylogenetic classification has been recently revised of the Solanaceae and the genus *Lycopersicon* re-integrated into the *Solanum* genus with its new nomenclature. *Solanum* group *Lycopersicon* includes the cultivated tomato (*S. lycopersicum*) and 12 additional wild relatives. *Solanum lycopersicum* is the only domesticated species (Peralta *et al*., 2006; Peralta *et al*., 2006).

In Thailand, farmers popularly use local cultivars, or called 'landraces'. The fruits are small types, including Sida, Sida-Pakchong and also the selected lines Sidathip and L-22 which are well adapted to the rainy season. However, it may be expected that in the coming years F1 hybrid cultivars like Somtam (Eastwest, F1), S16 (TSA, F1), Valentine (Seminis, F1) and Seeda 013 (Chia Tai, F1) will be replaced landraces and local open pollinated. Tomato cultivars of large fruited type are mainly processing, such as VF-134, Roma-VF, Lima-VF, Peto 4165, Peto 4225 and other new commercial varieties (FAO, 2007).

Tomato production fields in Thailand include Northern provinces (Chiang Mai, Chiang Rai, Tak, Lampang, Phayao and Mae Hong Son), Northeastern provinces (Nong Khai, Sakon Nakhon, Nakhon Phamon, Kalasin, Si Sa Ket and Bueng Kan), Central provinces (Saraburi), Western provinces (Phetchaburi, Kanchanaburi, Ratchaburi and Nakhon Pathom) and Southern provinces (Prachuap Khiri Khan and Nakhon Si Thammarat). Among these fields, the provinces with large-scale production are Chiang Mai, Nong Khai, Sakon Nakhon and Nakhon Phamon (Lhakchaiyagul, n.d.; Wuttiwanit, 2002). For Northern provinces, tomatoes were rotating grown between plains and highlands region throughout the year, include San Sai, Fang, Chom Thong, Hot and Omkoi district, Chiang Mai province and Mae Sariang district, Mae Hong Son

province (Lumyong and Inwang, 1984). Tomato contains a numerous health-promoting related components including vitamins A, C, E, folate flavonoids, potassium and β-carotene (Mangels *et al*., 1993; Beecher, 1998; Leonardi *et al*., 2000; Sahlin *et al*., 2004). Tomatoes are botanically; ovary, together with its seeds, flowering plant and scientifically the berry-type fruits of the tomato plant, they can also be considered a culinary vegetable; perspective the tomato is typically served as a meal, or part of a main course of a meal or consumed in fresh consumption, meaning that it would be considered a vegetable or fruit causing some confusion.

1.2.2 The *Fusarium* wilt disease

The *Fusarium* wilt disease of tomato was first described by G. E. Massee (1895) in England. The devastating disease is occurred specifically in tomato, it now occurs worldwide, having been reported in more than 40 countries and particularly severe in countries with warm climate, most prevalent on acid and sandy soils (Cai *et al*., 2003). The loss in yield varies between 10% to 90% depending on the stage of the plant growth at which section occurs and the environmental conditions (Kumar and Sood, 2002; Singh, 2005). In severe cases it may cause up to 80% loss in tomato production (Malhotra *et al*., 1993). The disease directly affects the market value, thus causing severe economic losses. Farmers are losing lots of money due to this disease. The overcome the problem of yield loss, there is need to control this disease.

Symptoms

Fusarium oxysporum consists of various formae speciales. These fungi have been characterized as causing these typically symptoms: vascular wilt, yellows, corm rot, root rot, and damping-off. Vascular wilt-causing the fungus *Fusarium oxysporum* is commonly and the most important symptom (Agrios, 1988; Smith *et al*., 1988).

Due to the growth of the *Fusarium* wilt pathogen within the plant vascular tissue, the plant water supply is greatly affected. The plant organism works are blocked the

flow of water by invading the xylem and eventually spreading of the pathogen throughout the plant. A brown discoloration of the vascular system can be used as an aid in diagnosis. On the outer portion of the younger leaves, *Fusarium* wilt symptom is initially appear as slight vein clearing on the outer portion, then epinasty (downward drooping) of the older leaves. After that, plants affected by *F. oxysporum* may wilt and die soon after symptoms appear at the seedling stage. Then, stunting symptom is often appear after vein clearing and leaf epinasty in older plants. Moreover, another symptom is developed, included yellowing of the lower leaves, formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire. The strong recognizance of *Fusarium* wilt is vascular browning discoloration, which extend from roots before up the stem through the branches and into the petioles of the plant. Symptoms can be seen on a single branch, or on several branches on one side of the plant, or on all the lower branches. The yellowing and wilting progress up the plant as the fungus spreads within its host. Yellowed, wilted leaves often dry up and drop prematurely. Eventually the entire plant wilts and dies early, producing few, if any, fruit plant. Further, on older plants, symptoms generally become more apparent during the period between blossoming and fruit maturation (Jones *et al*., 1982; Agrios, 1988; Smith *et al*., 1988; Messenger and Braun, 2000.).

Disease cycle

The disease cycle consists of dissemination, inoculation, infection and pathogen development, symptoms and disease development and pathogen survival (Figure 1.1).

Dissemination: The *Fusarium* wilt can be disseminated in various ways. The pathogen moves infested soil from one area to another by primarily spread over short distances as irrigation or flood water and contaminated farm equipment. They can also spread in soil or infected transplants. Meanwhile, they can contaminated their seeds but scarcely found. Their spores can possible spread by wind, tomato stakes, soil and infested soil adhering to transplants. The pathogen could be propagated long distance through seed and transplants. Local dissemination is by transplants, tomato stakes,

windborne and waterborne infested soil, and farm machinery (Kommedahl *et al*., 1970; Agrios, 1988).

Inoculation: The *Fusarium* wilt pathogen is first attacks and invades the root systems or other underground parts of their host plants through wounds that caused naturally by the growth of young rootlets through the soil by wounds in order roots that are made during transplanting and cultivation; by root-feeding organisms, such as insects or nematode (burrowing, lesion, root knot, sheath, sting, stubby-root and stunt) and by man-made injuries during transplanting, cultivation, harvesting, sorting, and grading. Once within the plant, the fungus grows and multiplies in the vascular system (water- and food-conducting tissues) of the roots. It then moves upward in the plant by conidia (macroconidia and microconidia) that are transported in the sap stream where they become lodged, germinate, and affect new plant parts; or the fungus extends its colonization as it grows in the vascular tissue of the host.

Fusarium wilt is a warm-weather disease, most predominant on acid, sandy soils. The pathogen behaviour is soilborne, it can be inhabited in infested soils for 10 years. The optimum temperature in soil and air for disease is 28°C. The wilt symptom development is delayed when soil temperature is too warm (34°C) or too cool (17 - 20°C). If soil temperatures are optimum but the air temperatures are below the optimum, the pathogen will extend into the lower parts of the stem, but the plants will not exhibit external symptoms.

Factors favouring wilt development are as followed:- 28°C of soil and air temperatures, soil moisture optimum contributing for plant growth, low nitrogen and phosphorus and high potassium conditions, low soil pH, plants preconditioned short day length, and low light intensity. Moreover, micronutrients, phosphorus, and ammonium nitrogen and decreased by nitrate nitrogen lead to pathogen enhancement. The pathogen penetrates plant through root systems before spreading throughout the plant in the vascular system.

Infection and pathogen development: Healthy plants can become infected by the *Fusarium* wilt pathogen if the soil in which they are growing is contaminated with the fungus. Plant root system is invaded by either germ tube or mycelium of the fungus,

before directly infected through the root tips, wounds in the roots, or at the formation point of lateral roots. Then the mycelium grows through the intercellulary root cortex inside the plant and reaches the xylem by invaded the vessels through the xylem's pits. The pathogen mycelium remains on the vessels at this point, and usually advances upwards toward the stem and crown of the plant. As it grows, the mycelium branches and produces microconidia, which are carried upward within the vessel by way of the plant's sap stream. When the microconidia germinate, the mycelium can penetrate the upper wall of the xylem vessel, enabling more microconidia to be produced in the next vessel. The fungus can also advance laterally as the mycelium penetrates the adjacent xylem vessels through the xylem pits (Agrios, 1988).

Once the conidia have penetrated the entire sap system, the pathogen begins to branch outward. The fungus grows from the xylem into the surrounding, dead fleshy tissues and produces many resting spores (chlamydospores). Multi-celled macroconidia are produced on surface lesions once the plant has died. Those spores can germinate directly or produce chlamydospores, which will rest in the soil and start the cycle all over again.

Plant's water supply is considerably affected within the plant's vascular tissue due to the growth of the pathogen. Lack of water effect leads the leaves stomata closed, wilting of leaves, and the plant eventually dies. Likewise, the plant parenchymatous tissue is invaded by pathogen until it finally reaches the surface of the dead tissue. It is at this point that sporulates abundantly (Agrios, 1988). The resulting spores can then be used as new inoculum for further spread of the fungus.

Symptoms and disease development: The normal flow of liquids and nutrients from the roots to the foliage is greatly reduced or stopped because the conducting tissue becomes partially plugged or killed by fungal mycelium and spores, or by the overgrowth of neighbouring cells. Disease and symptom development are extremely dependent upon air and soil temperatures. Symptoms are usually absent or mild at temperatures below 21 - 24°C (70 - 75°F) and are most severe at constant temperatures of 29 - 32°C (80 - 90°F). Toxic substances are believed to be secreted by interaction of the fungus and the host plant. These materials apparently cause the wilting and eventual death of the plant. Plants that are grown at low temperatures may be infected, but wilt

symptoms typically are not observed until the fungus has colonized the underground parts of the plant or the temperatures rise.

Pathogen survival: Some plant pathogenic specific forms of *F. oxysporum* are an abundant and active saprophyte in soil and organic matter. They can survive is soil between crop cycles in infected plant debris because of their saprophytic ability. They survive either as mycelium, or as any types of their three different spores. However, resting structures called 'chlamydospores' are formed within infected plant parts. When the host plant dies or the growing season ends, the pathogen survive as mycelia and chlamydospores, overwintering in dead plant parts. If the soil is warm, the resting pathogen may live in the soil indefinitely in the absence of the host plant, such as in a greenhouse. Chlamydospores can survive up for 30 years by living dormant in the soil or until it is stimulated to germinate by a susceptible host and stimulated to germinate by exudates from the roots of a host plant which they then infect (Agrios, 1988; Smith *et al*., 1988).

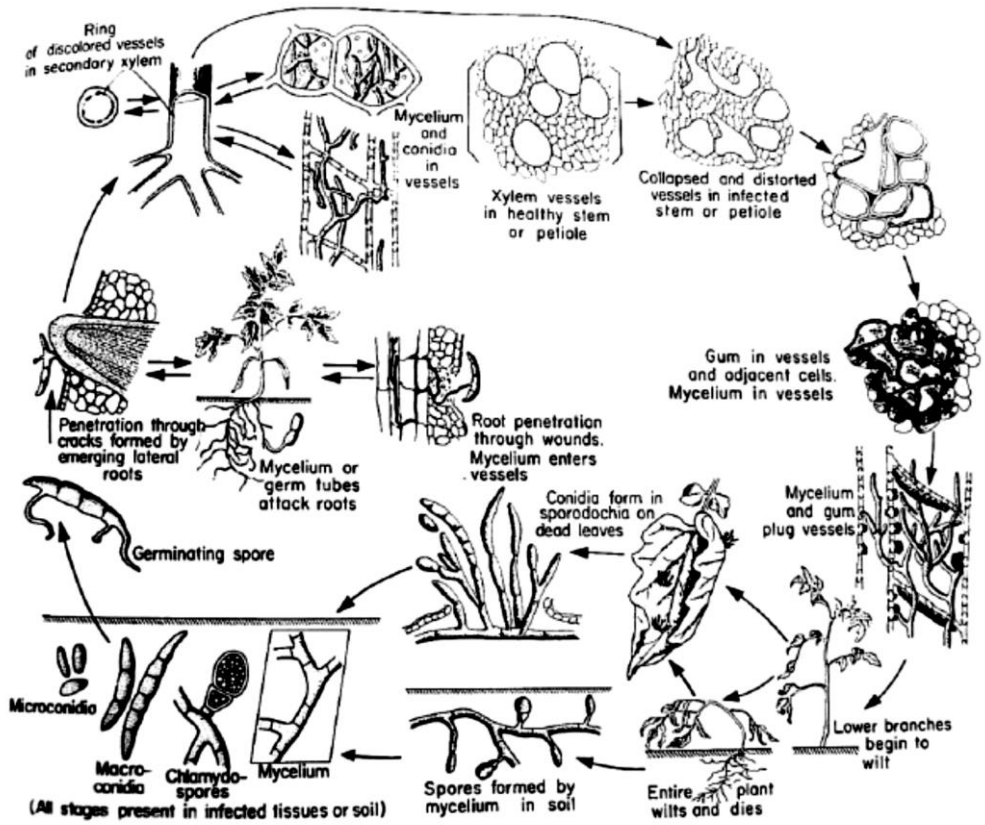

Figure 1.1 Disease cycle of *Fusarium* wilt disease in tomato (Agrios, 1997)

1.2.3 The pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*)

The genus *Fusarium* diversity, cosmopolitan, and responsible for numerous plant diseases, storage rots, and human as well as animal toxicoses and mycoses, are sprecific traits. There have been led the genus *Fusarium* considered as one of the very interesting and important group of fungi(Nelson *et al*., 1981; Nelson *et al*., 1994; Summerell *et al.*, 2003). They is categorized as facultative parasites, meaning they can live as parasites or saprophytes depending on their host. *Fusarium* species are also widely distributed in all major geographic regions of the world (Burgess, 1981; Nelson *et al*., 1994). They are commonly found in soils, and persist as chlamydospores or as hyphae in plant residues and organic matter (Gordon, 1959; Booth, 1971; Burgess, 1981).

The genus *Fusarium* is diverse, widespread and commonly found world-wide. The *Fusarium* comprises a number of fungal species producing characteristically shaped fusoid macroconidia, that are widely distributed in soil and on organic substrates and have for a long time been known as important plant pathogens (Moss and Smith, 1984). The *Fusarium oxysporum* species, causal agents of *Fusarium* wilt disease, were reported from different countries. Such pathogens are specific for certain plant hosts and known as 'forma speciales'; individual isolates within this fungus normally have a narrow host range, and the species is classified into forma specialis based on specific pathogenicity on a host plant. Therefore, *Fusarium* wilt disease of tomato (*Lycopersicon esculentum* Mill) are named as *F. oxysporum* f. sp. *lycopercisi* (Marasas *et al*., 1984; Joffe, 1986; Rivelli, 1989; Fletcher, 1994; Mushtaq and Hashmi, 1997; Jovicich *et al.*, 1999). No sexual stage (telemorph) has been reported for *F. oxysporum* (Booth, 1971).

The genus *Fusarium* was introduced in 1809 (Booth, 1971; Leslie and Summerell, 2006). *Fusarium oxysporum* is one of the most difficult complexes to classify and additional research is needed to build a consistent phylogeny. The classification reported by Snyder and Hansen (1940) as follows:-

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Subclass: Hypocreomycetidae

Order: Hypocreales

Family: Nectriaceae

Genus: *Fusarium*

Species: *F. oxysporum* f. sp. *lycopersici*

Fusarium oxysporum is an anamorphic species circumscribed by different morphological criteria; principally the size and shape of the macroconidia, the presence or absence of microconidia and chlamydospores, colony colour, and conidiophores structure (Nelson *et al*., 1983; Windels, 1992). The mycelia of *Fol* are delicate white to pink, often with purple tinge, and are sparse to abundant. The fungus produces three types of spores: microconidia, macroconidia and chlamydospores (Figure 1.2). Macroconidia (Figure 1.2A), sparse to abundant, are borne on branched conidiophores or on the surface of sporodochia and are thin walled, three- to five-septate, fusoid-subulate and pointed at both ends, have pedicellate base. Three-septate conidia measure 27-46 × 3-5 µm while five-septate conidia measure 35-60 × 3-5 µm. Three-septate spores are more common. These spores are commonly found on the surface of plants killed by this pathogen as well as in sporodochialike groups. Microconidia (Figure 1.2B) are borne on simple phialides arising laterally and are abundant, oval-ellipsoid, straight to curved, 5-12 × 2.2-3.5 µm, and nonseptate. These spores are the type of spore most abundantly and frequently produced by the fungus under all conditions. It is also the type of spore most frequently produced within the vessels of infected plants. Chlamydospores (Figure 1.2C), both smooth and rough walled, are abundant and form terminally or on an intercalary basis. They are generally solitary, but occasionally form in pairs or chains. These spores are either one or two celled and produced either terminally or intercalary on older mycelium or in macroconidia (Nelson *et al*., 1983; Agrios, 1988; Burgess *et al*., 1994; Wong, 2003; Leslie and Summerell, 2006).

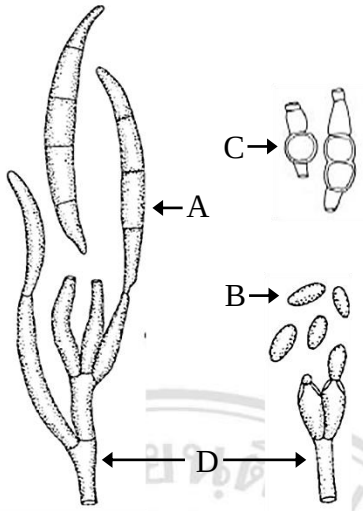

Figure 1.2 Schematic representation of three spore types of *Fusarium oxysporum*; (A) microconidia, (B) macroconidia, (C) chlamydospores and (D) conidiophore (Alexopoulos *et al*., 1996)

Physiology race of *Fol*

The pathogenic fungi of *F. oxysporum* causing wilt have a board range of host plants. However individual strains of the fungus show a high degree of host specificity. Their grouping into forma speciales (sing. forma specialis, f. sp.) based on their host range is very useful to plant pathologists. The same host ranges of isolates are assigned to a forma specialis. *F. oxysporum* is classified over 120 different formae speciales according to their host species (Armstrong and Armstrong, 1981). Isolates from a particular forma specialis can be further subdivided into physiological race with a characteristic pattern of virulence to differential host varieties (Huertas-Gonzalez *et al*., 1999). Three different host-specific races of *Fol* (race 1, 2 and 3) have been identified in Arkansas, USA (Marlatt *et al*., 1996; Cai *et al*., 2003). Race 1 was initially observed in 1886 (Booth, 1971) and race 2 was first reported in Ohio in 1945 (Alexander and Tucker, 1945) and Queensland, Australia in 1978. Race 3 was identified in Australia in 1978 (Grattidge and O'Brien, 1982) and was subsequently reported in several Florida in 1982, California in 1987, Mexico (Davis *et al*., 1988; Elias and Schneider, 1991), and Tennessee in 2001 (Bost, 2001).

All commercially cultivated tomatoes lacking *I*-genes, are susceptible to *Fol*. Resistance to race 1 found in line 160 *Lycopersicon pimpinellifolium* PI79532 that has *I*-gene (Grattidge and O'Brien, 1982). The gene *I* remained effective to race 1 until the early 1970s (Jones and Crill, 1974). Race 2 was reported in Korea and Ohio (Valenzuela–Ureta *et al*., 1996), this race of the pathogen overcomes the resistance of cultivars which showed resistance to race 1. Then *I*-2 gene was found in tomato cv. *L. peruvianum* resistant to both races 1 and 2 (Scott and Jones, 1989). Both gene of *I* and *I*-2 were mapped on chromosome 11 (Laterrot, 1976; McGrath *et al*., 1987). The race 3 was observed in Australia and Florida (Grattidge and O'Brien, 1982). Since the attention have shift to screen for resistance to race 3, which had overcome the resistance to race 1 and 2, respectively. Resistance to race 3 has been observed in genotype of *L. pennellii* that consist of *I*-3 gene (McGrath and Toleman, 1983). Gene of *I*-3 was mapped on chromosome 7 (Bournival *et al*., 1989; Sarfatii *et al*., 1991). Races of *Fol* agents could be distinguished by their differential vilurence on tomato cultivars containing diferent dominant resistance genes (McGrath *et al*., 1987; Mes *et al*., 1999).

Race of *Fol* using a set of the race differential varieties

Fol is classified into forma specialis *lycopersici*, on the basis of its host specificity and the only host on tomato. Isolates of *Fol* has been grouped into three physiological races (race1, 2 and 3) according to the characteristic pattern of virulence to infect a set of differential varieties that carries distinct resistance loci (Reis *et al*., 2005). Four *Fusarium* wilt resistant genes known as *I*, *I*-1, *I*-2 and *I*-3 are screened in wild *Lycopersicon* species. These resistant genes are monogenic specific dominant resistant genes. They were introgressed into commercial tomato varieties. These varieties carrying resistant genes were provided as a set of the race differential varieties for determining the race of *Fol*. A set of tomato varieties consisting of differential tomato varieties has 4 trails; susceptible to all races as cv. 'Bonny Best', resistant to race 1 (owing to the present the locus *I*) such as cv. 'UC82-L', resistance to race 1 and 2 (due to the presence of the loci *I* and *I*-2) such as cv. 'Walter' and the last trail that is resistant to the three races (probably due to the presence of the locus *I*-3) such as cv. 'I3R-1' (Table 1.1).

Table 1.1 Resistance and susceptibility of 4 differential tomato varieties to different races of *Fusarium oxysporum* f. sp. *lycopersici* (modified from Grattidge, 1982; Marlatt *et al*., 1996)

race of *Fol*	standard differential varieties*			
	Bonny Best	UC82-L	Walter	I3R-1
race 1	S	R	R	R
race 2	S	S	R	R
race 3	S	S	S	R

*S = susceptible, R = resistant

This set was used for identification for the pathogenic race of *Fol*. The interaction between resistant genes of host cultivar and pathogenic race is not completely clear. However, a gene-for-gene hypothesis has been proposed for explanning this interation. According to this hypothesis, a race-specific resistance reactionof the host is triggered by an interaction between the product of a dominant resistant gene of the plant and the product of a corresponding, dominant avirulent (*avr*) gene in the pathogen. Grattidge (1982) first reported the occurrence of a third race of *Fol* in Queensland. Afterward, Marlatt *et al*. (1996) used a set of differential tomato varieties for identification of races of *Fol* isolated from the United States, Australia and Mexico, including cv. 'Bonny Best' (susceptible to race 1, 2 and 3), cv 'UC82-L' (resistant to race 1), MH-1 (resistant to race1 and 2) and cv. ' I3R-1 (resistant to race 1, 2 and 3). Moreover, several researchers have reported identification of pathogenic race of *Fol* (Reis *et al*., 2005; Sheu and Wang, 2006; Bunyatratchata1 *et al*., 2005; Elena and Pappas, 2006).

1.2.4 Control of *Fusarium* wilt in tomato

Total investigation total of interactions occurring between plant and pathogen and either eliminate those interactions or tip the balance in favor of the plant are reached to successful disease management program. The keys are reduction of pathogen population densities, called 'inoculum' and/or functionality, which referred to ability to successfully infect the host (McGovern, 2015). Because *Fol* and its many special forms affect a wide variety of hosts, the management of this pathogen is discussed in more

detail in the respective summaries. There are a number of control measures that can be taken when dealing with a *Fusarium* wilt. In general, some effective means of controlling *Fol* include: disinfestation of the soil and planting material with fungicidal chemicals, crop rotation with non-hosts of the fungus, or by using resistant cultivars (Jones *et al*., 1982; Agrios, 1988; Smith *et al*., 1988). The disease control can be achieved by field management. The management must be efficient and cost-effective, as well as safe for consumers, agricultural workers and the environment.

F. oxysporum is a soil-borne pathogen, which can live in the soil for long periods of time, so rotational cropping is not a useful control method. A major challenge in managing *Fol* is their extreme longevity in soil even in the absence of hosts. It can also spread through infected dead plant material, so cleaning up at the end of the season is important.

Improve soil conditions is one control method based on characteristic of *F. oxysporum* that spreads faster through soils having high moisture and bad drainage. Moreover, other control methods are demonstrated, including using resistant varieties, removing infected plant tissue to prevent overwintering of the disease, using soil and systemic fungicides to eradicate the disease from the soil, flood fallowing, and using clean seeds each year. However, applying fungicides restricts by the field environment. Biological control method is difficult because research in a greenhouse and the field have different effects. The best control method found for *F. oxysporum* is planting resistant varieties, although not all have been bred for every forma specialis. Wong (2003) reported that the use of resistant cultivars is mainly control measurement. The control of races 1 and 2 utilizes both polygenic and monogenic resistance while monogenic resistance to race 3 has been developed. Pasteurization of infested soil with steam or fumigants, raise the soil pH to 6.5 - 7.0, and usage of nitrate nitrogen rather than ammonium nitrogen help to reduce the incidence of wilted plants and greatly increases marketable and total yields.

Moreover, agriculturists at Doi Inthanon National Park, Chiang Mai, Thailand, had revealed their behavioral pest management that the chemical control had been suggested as the potential management of *Fusarium* wilt disease of tomato commercial fields. Frequency of commonly used was applied on 5 – 7 days intervals. Metalaxyl

and mancozeb were practically used throughout the seedling period (upto 30-day-old). After transplant, numerous fungicides; cymoxanil, chlorothalonil, antracol, copper, captan, iprodione and iprovalicarb + propineb, were added until harvest (personal communication, 2013).

Additionally, the managements of *Fusarium* wilt disease of tomato were clearly reviewed by McGovern (2015), which covered the subjects as follow:-

Use of resistance cultivar: including traditional breeding, genetic modification (GM), induced resistance and grafting

Use of chemicals: including fungicides, disinfestants (disinfectants), fumigants, anaerobic soil disinfestation (biological soil disinfestation) and plant nutrition and soil chemistry

Biological control

Physical control: including heat method, steam method, solarization, composting and water treatment

Crop rotation/intercropping

Integrative strategies: including biological + chemical and physical + biological or chemical

1.3 Biological control

Biological control (biocontrol, abbreviated synonym) has been widely researched and used in different fields of biology, most notably entomology and plant pathology.

The term of biocontrol is using bioeffector-method using other living organisms of controlling pests, including insects, mites, weeds and plant diseases that involve harnessing disease-suppressive microorganisms to improve plant health (Flint and Dreistadt, 1998). Three different basic types of biological pest control strategies are categorized, including importation (or known as classical biological control),

augmentation and conservation. Predators, parasitoids, and pathogens biological control agents are known as biological control agents that have ability innatural enemies of insect pests. They are most often referred to as antagonistic microorganisms. Biological control agents of weeds include seed predators, herbivores and plant pathogens. Disease suppression by use of biocontrol agents is the sustained manifestation of interactions among the plant (host), the pathogen, the biocontrol agent (antagonist), the microbial community on and around the plant and the physical environment, but typically also involves an active human management role (Handelsman and Stabb, 1996; Pal *et al*., 2006; Chandrashekara and Manivannan 2012).

Modes of action of biological control agents

Microbial antagonism of plant pathogens occurs in several ways. As described above, the most common mechanisms being parasitism and predation, competition for nutrients or space, production of antimicrobial substances and induced resistance. These mechanisms of antagonism has increased and become apparent which often involves the synergistic action of several mechanisms. To eventual improvement and wider use of biocontrol methods, the understanding the mechanisms through which the biocontrol of plant diseases occurs is critical is necessary. The biological control of plant diseases are described by Stirling and Stirling (1997) in following ways.

Competition for nutrients and space: When two or more organisms grow together, they require the same resource for growth and survival resulting in competition. The use of this resource by one organism reduces the amount available to the other. In this way, the nutrient competition is useful for a mechanism of biological control. It is depend on the target type of pathogen.

Production of antimicrobial substances: Most microorganisms produce secondary metabolite when grown in culture, which generally produced in a phase subsequent to growth and not essential intermediaries to the central metabolism. These compounds may be antibiotics or mycotoxins that are toxic to other microorganisms. The antibiotic production mechanism is generally found in the wild type strains with any mutant. This

property has ability to reduce disease. However, it is possible that the mutants have lost other attributes that also contribute to biological control.

Parasitism and predation: The parasitism of one fungus by another is named hyperparasitism or mycoparasitism. It is recognized as morphological disturbance, direct penetration of hyphae and hyphal lysis. Without considering predation of pathogens by other organisms, a biological control of plant pathogens would not be complete. However, the role of these organisms in biological control is still unclear. Many are omnivores and may not have a preference for particular plant pathogenic fungi or bacteria.

Cross protection and induced resistance: Cross protection and induced resistance are confusing that difficult to distinguish clearly. In cross protection, the first organism arrives at an infection site acts directly or indirectly against a pathogen that arrives later. In the same way, induced resistance in form of cross protection is occurred when inoculation plants with restricted pathogens, attenuated pathogens (such as pathogens inactivated by heat treatment) or selected non-pathogens, or treatment with chemical substances which stimulate an immune response. After the pathogen subsequently attacks, they are already operating for host defence mechanisms recognise and respond to this perceived threat. The nature of the immune response is poorly understood, but the concept is not new. However, it provides protection against a broad spectrum of pathogens across many crop species. Nevertheless, the protection is not complete and impractically high quantities of the protectant are required for success.

1.4 Induced resistance

Plant defense mechanisms were activated and demonstrated as natural resistance to pathogens based on the combined effects of natural barriers and inducible mechanisms. Induced resistance in plants refers to a state of heightened defensive capacity created by a prior stimulus. Plants have latent defensive systems. This can be activated towards pathogen attack (Kuc, 1995; Pieterse *et al*., 1998; McGovern, 2015). Chester (1933) has first proposed the term “acquired physiological immunity” since

1930s that is induced protection of plants against various pathogens by biotic or abiotic agents. Then several terms have been used to describe the phenomenon of induced resistance, for instance "systemic acquired resistance" (Ross, 1961), "translocated resistance" (Hurbert and Helton, 1967) and "plant immunization" (Tuzun and Kuc, 1991). However, there are two different types of induced resistance have been extensively studied, as follow;-

Systemic acquired resistance (SAR)

All plants possess active defense mechanisms against pathogen attack and responses induced by gene-for-gene resistance (Flor, 1971). Systemic acquired resistance (SAR) is induced systemically throughout the plant in response to a pathogen infected plant (Ryals *et al*., 1996; Sticher *et al.*, 1997; Durrant and Dong, 2004), conferring a broad-spectrum of pathogen resistance against (Ryals *et al*., 1996; Sticher *et al.*, 1997; Durrant and Dong, 2004). The exposure of a plant to abiotic (chemicals) or biotic (pathogenic and nonpathogenic microorganisms) elicitors are also induced SAR. SAR is labeled with dependent on salicylate (salicylic acid) production, and is associated with the accumulation of pathogenesis-related (*PR*) proteins (McGovern, 2015). Furthermore, induced expression of *PR* genes is served as excellent molecular markers for many researches, which correlates with the activation of defense responses (Glazebrook *et al.*, 1996; Falk *et al.*, 1999; Belfanti *et al.*, 2004).

Induced systemic resistance (ISR)

Exposuring of roots to specific strains of plant growth promoting rhizobacteria (PGPR) is triggered of ISR. There is *Bacillus* spp. and *Pseudomonas* spp. in particularly. Moreover, it has to cause no visible damage to the plant's root system (van Loon *et al*., 1998). ISR is different to SAR because it is dependent on ethylene (ET) and jasmonate (JA). Conversely, it is independent of salicylate, and is not associated with the accumulation of *PR* proteins (Pieterse *et al.*, 1996; Knoester *et al*., 1999; Pieterse *et al*., 1998; van Loon *et al*., 1998; Yen *et al*., 2002 Vallad and Goodman,

2004). ISR was reported as delayed disease symptom development, reduced disease severity and disease incidence in cucumber against *Pythium aphanidermatum* the soil borne pathogen (Zhou and Paulitz, 1994; Liu *et al*., 1995).

In summarized, it recognized that SAR is induced by the exposure of root or foliar tissues to abiotic or biotic elicitors, is dependent of the phytohormone salicylate (salicylic acid), and associated with the accumulation of pathogenesis-related (*PR*) proteins. Whereas ISR is induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria, is dependent of the phytohormones ethylene and jasmonate (jasmonic acid), independent of salicylate, and is not associated with the accumulation of *PR* proteins (or transcripts). However, both responses are intertwined molecularly, as demonstrated by their reliance on a functional version of the gene *NPR1* in *Arabidopsis thaliana* (Pieterse *et al*., 1998; van Loon *et al*., 2002), and also effective against a broad spectrum of virulent plant pathogens. The two most clearly defined forms of induced resistance are SAR and ISR (Figure 1.3), which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved, as demonstrated in model plant systems (Ward *et al*., 1991; Uknes *et al*., 1992; Pieterse *et al*., 1996, 1998; Knoester *et al*., 1999; Maleck *et al*., 2000; Schenk *et al*., 2000; van Wees *et al*., 2000; Yen *et al*., 2002).

Over the last 20 years, there have been numerous researches done to demonstrate the treatment of induced resistance elicitors in plant *Fusarium* wilt diseases control. However, the majority researches have been utilized tomato seedlings and plotted plants, few trails have evaluated induced resistance for management of *Fusarium* wilt (McGovern, 2015). Several effectiveness chemical inducers, including Acibenzolar-S-methyl (ASM), compost, JA, SA, Validamycin A and Validoxylamine A, have been identified in managing *Fusarium* wilt disease in tomato, which produced many types of induction products, including alkaline phosphatase peroxidases (POX), polyphenol oxidase (PPO), tyrosine ammonia lyase, chitinases (CHI), glucanases (GLU), lipoxygenase (LIP), phenylalanine ammonia lyase (PAL), SA, peroxidase (POD) and *PR* proteins (Ishikawa *et al*., 2005; Vallad and Huang, 2010; Abdel-Fattah and Al-Amri, 2012; Ferraz *et al*., 2014). Besides, partial of effectiveness microorganisms including non-pathogenic *Fusarium oxysporum* FO47, *Pseudomonas fluorescens* WCS417r., *P.*

fluorescens Pf1, *Bacillus subtilis* EPCO16, *Penicillium oxalicum*, *Streptomyces setonii* UFV 618, *Bacillus cereus* UFV 592 and *Serratia marcescens* UFV 252, have been reported to induce resistance of tomato against *Fusarium* wilt (Duijff *et al*., 1996; De Cal *et al*., 2000; Ramamoorthy *et al*., 2001; Ramamoorthy *et al*., 2002; Ramyabharathi *et al*., 2012; Ferraz *et al*., 2014).

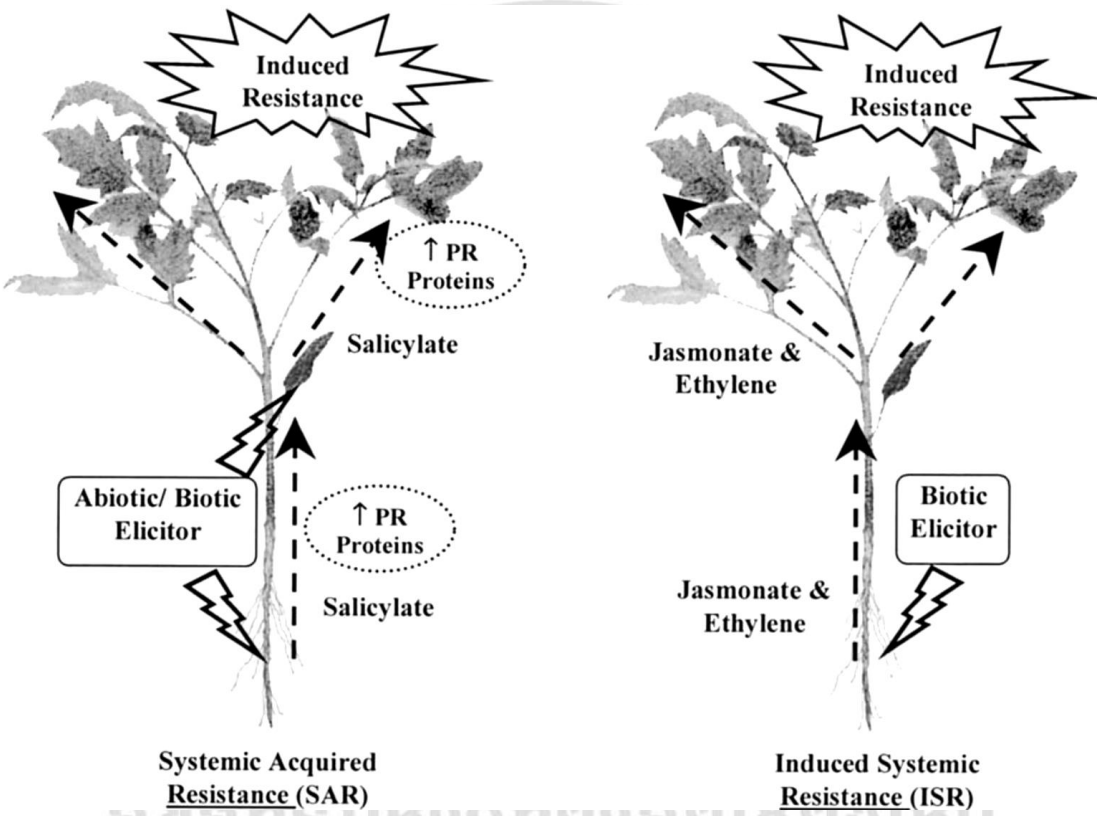

Figure 1.3 A pictorial comparison of the two best characterized forms of induced resistance in plants, including systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Vallad and Goodman, 2004)

1.4.1 The defense related genes

Physical, chemical and biological stresses are categorized as threats of plants. These are examples; such as wounding, exposures to salinity, drought, cold, heavy metals, air pollutants and ultraviolet rays and pathogen attacks, like fungi, bacteria and

viruses. In higher plants, they have a broad range of mechanisms that is a great property to protect themselves (Agrios, 1977). However, reactions of plant to these factors are complicated. They also involve the activation genes that encoding different proteins. Either of biochemical and physiological changes in plants can be induced by these stresses. In particularly, physical strengthening of the cell wall through lignification, suberization, and callose deposition; by producing phenolic compounds, phytoalexins and pathogenesis-related (*PR*) proteins which subsequently prevent various pathogen invasion were studied (Bowles, 1990). As we know, The development of SAR is associated with the induction of *PR* gene expression (White, 1979). Among these, production and accumulation of *PR* proteins in plants in response to invading pathogen and/or stress situation is very important**.**

Pathogenesis-related (*PR*) genes

Activation of defense-related genes is one of the most important in plant defense mechanisms against various pathogens. These genes are encoding various type of defense proteins, called pathogenesis related (*PR*) proteins. van Loon *et al.* (2006) defined the term "pathogenesis related proteins" is microbe-induced proteins and their homologouses to the extent that enzymes, which are generally presented constitutively and only increased during severe infection. After plant tissues responded to pathogen infection, the plant *PR* proteins are induced. Accumulation of *PR* protein is occurred locally of infected and surrounded tissues, and likewise in distance tissues. The first reported of plants that discovered *PR* proteins is tobacco infected by tobacco mosaic virus (van Loon and van Kammen, 1970). Afterthat, these *PR* proteins were discovered in other various plants. *PR* proteins in many plant species are mainly acid-soluble, low molecular weight, and protease-resistant proteins (Leubner-Metzger and Meins, 1999). *PR* proteins may be acidic or basic based on their isoelectric points, but the function of both types is same. Acidic *PR* proteins are commonly located in the intercellular spaces, while basic *PR* proteins are predominantly located in the vacuole (Legrand *et al*., 1987; Niki *et al*., 1998; van Loon and van Strien, 1999). Production of *PR* proteins in the uninfected parts of plants has ability to protect the affected plants from further infection (Ryals *et al*., 1996; Delaney, 1997). Currently the *PR* proteins are

further classified into 17 families (*PR-1* to *PR-17*) (Table 1.2) on the basis of amino acid sequence data, biochemical functions and structural homologies within the groups (van Loon *et al.*, 2006).

Among these *PR* proteins, there are two substantial hydrolytic enzymes, *PR-3* and *PR-4* encoding chitinase and *PR-2* encoding *β*-1,3-glucanases, which are important in most plant species. These enzymes are induced after infection by different type of pathogens. Because chitin and *β*-1,3-glucan are the main structural component fungal pathogen cell wall, therefore these described enzymes are consequently increase and play main role of defense reaction by degrading cell wall of pathogenic fungi. Appearances of *β*-1,3-glucanases and chitinases are concurrently expressed after fungal infection. In addition, resistance elicitors are obtained from the degradation products of the fungal cell wall (Misawa and Kuninaga, 2010). This co-induction of the two enzymes has been described in many plant species, including pea, bean, tomato, tobacco, maize, soybean, potato, and wheat (Mauch *et al*., 1988; Mauch *et al*., 1988; Vogelsang and Barz, 1993; Jach *et al*., 1995; Bettini *et al*., 1998; Lambais and Mehdy, 1998; Petruzzelli *et al*., 1999; Cheong *et al*., 2000; Li *et al*., 2001).

Table 1.2 Classification of pathogenesis-related proteins (van Loon *et al.*, 2006)

Families	Type member	Properties
PR-1	Tobacco *PR*-1a	Unknown
PR-2	Tobacco *PR*-2	*β*-1,3-glucanases
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco R	Chitinase type I, II
PR-5	Tobacco S	Thuamatin-Like
PR-6	Tomato Inhibitor I	Protinase-Inhibitor
PR-7	Tomato P_{69}	Endoprotinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco "lignin-forming peroxides"	Peroxidase
PR-10	Parsley "*PR*-1"	Riboneclease-Like
PR-11	Tobacco class "V" chitinase	Chitinase, type 1
PR-12	Radish *Rs-AFP*3	Defensin
PR-13	Arabidopsis *THI*2.1	Thionin
PR-14	Barley *LTP4*	Lipid-transfer protein
PR-15	Barley *OxOa* (germin)	Oxalate oxidase
PR-16	Barley *OxOLP*	Oxalate – oxidase – Like
PR-17	Tobacco *PRp27*	Unknown

However, *PR-1* gene expression is induced in response to a variety of pathogens. Therefore, *PR-1* genes have been marked as a dominant group which frequently used as marker genes for systemic acquired resistance in many plant species (van Loon and van Strien, 1999; Mitsuhara *et al*., 2008). The *PR-1* mRNA and/or proteins strongly accumulate in numerous plant species of monocots and dicots upon infection by bacteria, oomycete (Höegen *et al*., 2002) and fungal pathogens (van Kan *et al*., 1992). Although *PR-1* gene, one of the best typical marker of SAR, have been isolated so far, the function is still unknown (van Loon *et al*., 2006).

Besides, *PR-9* encoding peroxidases, a key enzyme in the cell wall building process, is consisted in a large family of enzymes. Peroxidases have very diverse functions and enhance resistance against multiple pathogens with different life styles. They often increase in response to stress and one of the principal roles of peroxidase appears to be cellular protection from oxidative reactions imposed by various stresses (Siegel, 1993). It has been suggested that the construction of a cell wall barrier may block pathogen entry and spread in plant cells when extracellular or wall-bound peroxidases are activated. These occurrence lead enhance resistance in various plant species against phytopathogens. The *PR-9* group contains a specific type of peroxidase that could act in cell wall reinforcement by catalyzing lignification (Taheri and Tarighi, 2011), which occurs as part of the stress response to oxidation stress, salt stress, or wounding, and is accompanied by a rise in peroxidase activity.

Actin

Housekeeping genes are generally considered to be constitutive genes that are required for the maintenance of basic cellular function and are found to be expressed in almost all cells of an organism (Zhu *et al*., 2008). The expression levels of reference genes should remain constant between the cells of different tissues and under different experimental conditions (Thellin *et al*., 1999); lead to decrease noise or erroneous results (Dheda *et al*., 2004). For normalization of gene expression, housekeeping genes are used under the assumption that expression is invariable under different experimental conditions, so that they can serve as internal references. *Actin* or *β-actin* is the most

widely used housekeeping genes on the basis of rather anecdotal evidence of their consistency of expression (Glare *et al*., 2002). They are defined by specific gene promoter elements which determine that they are expressed constitutively in every cell and every condition, most frequently used as control (Thellin *et al*., 1999; Suzuki *et al*., 2000). Actin is commonly used since they are ubiquitously expressed in cells and tissues. Additionally, transcription of these genes is generally resistant to experimental conditions, making them suitable endogenous controls for single gene normalization (van Guilder *et al*., 2008).

1.4.2 Plants defense elicitors

Plants exhibit many defensive processing against pathogen attack. These defense process is occurred by both preexisting (constitutive) and induced defense systems. The discovery of compounds called "elicitor" help the understanding of plant signalling pathway better. Elicitors may be natural or synthetic compounds. But they have similar function in defense responses of plants as induced by the pathogen infection (Hammond-Kosack and Jones, 2000; Gómez-Vásquez *et al*., 2004). For this term, broader definition of elicitors can be included substances of pathogen origin (exogenous elicitors) and compounds released from plants by the action of the pathogen (endogenous elicitors). Different types of elicitors have been characterized, including both of biotic or abiotic (Angelova *et al*., 2006) (Table 1.3). Following elicitor perception, the activation of signal transduction pathways generally lead to act as signals that stimulate the synthesis of natural products, phytoalexins and *PR*-proteins (Benhamou and Theriault, 1992; Ebel and Cosio, 1994; van Loon and van Strien, 1999).

Table 1.3 Elicitors of plants (Angelova *et al*., 2006)

Elicitors						Reported effects on*
Physical elicitors	Injury					P
	Abiotic		Metal ions (lanthanum, europium, calcium, silver, cadmium, oxalate)			Pc
Chemical elicitors	Biotic	Complex composition	Yeast cell wall, Mycelia cell wall, Fungal spores			Pc, F
		Defined composition	Carbohydrates	Polysaccharides	Alginate	Pc, F, B
					LBG	F
					Pectin	Pc, F
					Chitosan	Pc
					Guar gum	Pc
				Oligosaccharides	Mannuronate	F
					Guluronate	F
					Mannan	F
					Galacturonides	Pc
				Peptides	Glutathione	Pc
			Proteins	Proteins	Cellulose, Elicitins, Oligandrins	Pc
			Lipids		Lypopoly-saccharides	Pc
			Glycoproteins		Not characterized	Pc
			Volatiles		$C_6 - C_{10}$	Pc

*P = plants, Pc = plant cell culture, B = bacterial cell culture, F = fungal cell culture

Elicitors may be divided into two groups, which are “general elicitors” and “race specific elicitors”. General elicitors have ability to trigger defense both in host and non-host plants, conversely race specific elicitors has ability in only specific host plant. This result led to disease resistance by induction of defense responses. A complementary pair of genes in a particular pathogen race and a host cultivar determines this cultivar specific (gene-for-gene) resistance (Thakur and Sohal, 2013). At low concentration, elicitors act as signal compounds and sending information for the plant to trigger defense, distinguishing elicitors from toxins, which may act only at higher concentrations and/or affect the plant detrimentally without active plant metabolism (Boller, 1995). Elicitor signal transduction mechanism which activates plant primary immune response is shown in Figure 1.4.

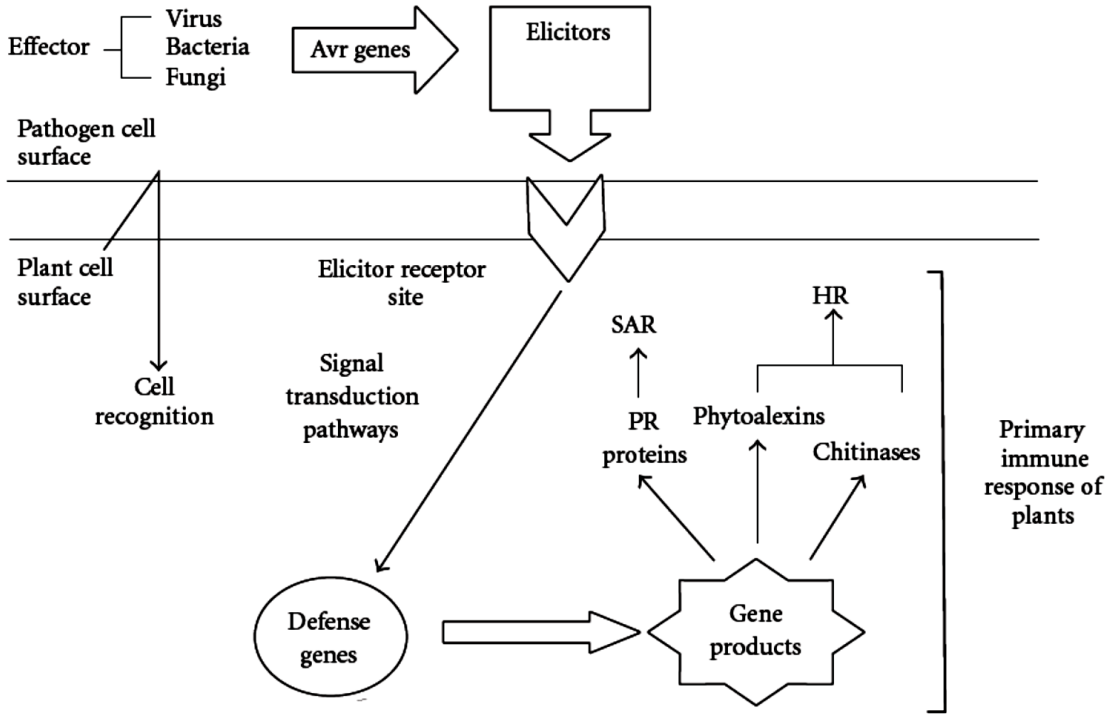

Figure 1.4 Elicitor signal transduction mechanism activating plant primary immune response of plant in plant-pathogen interaction (Boller, 1995)

1.5 Actinomycetes

The classification reported by Buchanan (1917) and Kalakoutskii and Agre (1976) as follows:-

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteria

Subclass: Actinobacteridae

Order: Actinomycetales

Actinomycetes are belonging to subdivision of the gram-positive bacteria phylum and categorized into a group of prokaryotic organisms. Most of them are in subclass Actinobacteridae, order Actinomycetales. Actinomycetes structure is different from

bacteria because their DNA has high G+C content, which found over 55%mol (Stackbrandt *et al*., 1997). Their phenotype is also different, actinomycetes are filamentous bacteria. They often produce two kinds of branching mycelium, aerial mycelium and substrate mycelium. Actinomycetes have been considered as fungi because they have ability to produce spores from the aerial mycelium which is the important part of the organism. For this reason, the name "actinomycetes" is reflected. The name origin was from Greek "akitino" that means ray and "mykes" that means mushroom/fungus, so actinomycete was called ray fungi. Actinomycetes are well known as saprophytic soil inhabitants that most widely distributed group of microorganisms in nature (Takizawa *et al*., 1993). Actinomycetes live in soil are able to produce geosmin, a volatile compound that has specific smell of soil. This compound is literally translates to "earth smell" (Gust *et al*., 2003). This organic substance is contribution of odour that occurs in the air when rain falls after a dry spell of weather. In natural habitats, the genus *Streptomyces* is commonly found in a major component of the total actinomycetes population. Some actinomycete genera are often very difficult to isolate and cultivate due to their slow growth, so they are called rare actinomycetes, including *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineospora, Microbispora, Micromonospora, Nonomuraea,* which (Hayakawa, 2008).

Actinomycetes have been recognized as a rich source producer which consist of important natural products, especially antibiotics. There are approximately 10,000 antibiotics that have been found. Meanwhile, almost half of them are produced by soil *Streptomyces* (Lazzarini *et al*., 2000). Based on this property, the actinomycetes are well known as a group of filamentous, gram-positive bacteria that produce many useful secondary metabolites, including antibiotics and enzymes (Williams *et al*., 1993). They are a group of branching unicellular organisms which reproduce either by fission or by means of special spores or conidia. They are closely related to the true bacteria, frequently, they are considered as higher filamentous bacteria. They usually form a mycelium which may be of a single kind designated as substrate (vegetative) or of two kinds, substrate (vegetative) and aerial (sporogenous). They are usually placed in a separate order, the *Actinomycetales*, which is said to be distinct from the Eubacteriales or the true bacteria.

The actinomycetes are generally recognised to represent a large and a heterogeneous group of microorganisms, comprising several genera and numerous species. They vary greatly in their morphology, physiology and biochemical activities. They play an important role in nature by bringing about the decomposition of complex plant and animal residues and the liberation of a continuous stream of available elements, notably carbon and nitrogen essential for fresh plant growth.

Actinomycetes are that group of intracellular branching organisms which reproduce either by fission or by means of spores or conidia. From an ecological point of view, actinomycetes stand in an intermediate position between the fungi and the bacteria in terms of numerical frequency of occurrence in various biotypes. They are closely related to the true bacteria and frequently they are considered as higher filamentous bacteria (Wlodzimier *et al*., 1979). The outstanding fungal characteristic is a morphological one, the possession of a true branching mycelium. In addition to possessing mycelium, actinomycetes may also show strong parallels with the true fungi in their production of sporangia and motile spores. However, mycelium diameter and spore size is of a lower order of magnitude in actinomycetes as compared with the fungi, averaging 1µm only. They usually form a myceliurn,which may be of a single kind designated as substrate (vegetative), or of two kinds,substrate (vegetative) and aerial mycelium. They produce a wide variety of spore types which includes the endospore, long regarded as the typical spore structure of eubacterials (Waksman, 1959). Some genera such as *Streptomyces* and *Micromonospora* form an extensive branched mycelium composed of individual hyphae, sub divided by infrequent cross walls. The actinomycetes are predominantly aerobic, heterotrophic and saprophytic. They have a cell wall structure characteristic of bacteria and frequently show the presence of lytic viruses (actinophages).

Actinomycetes occur in a wide range of environments in which they have the ability to grow on most naturally occurring substrates (Wlodzimierz *et al*., 1979; Goodfellow *et al*., 1989). Some are parasitic like *Dermatophilus* or some have symbiotic associations like *Frankia* and therefore have well developed ecological niches. However, vast majority of actinomycetes are saprophytes in soil, water, composts and other habitats, which form the bulk of many isolates obtained for

scientific study or commercial exploitation. Despite the intensive study of such actinomycetes *in vitro*, there is generally a surprising lack of information about the macro- or micro-niches that they occupy in natural environment or their activities within them.

1.5.1 The genus *Streptomyces*

The genus *Streptomyces* belongs to group Streptomycetes (one of 8 divided group of actinomycetes families) according to the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The classification reported by Waksman and Henrici (1943) as follows:-

Kingdom: Bacteria
Phylum: Actinobacteria
Class: Actinobacteria
Subclass: Actinobacteridae
Order: Actinomycetales
Family: Streptomycetaceae
Genus: *Streptomyces*

The genus *Streptomyces* was proposed by Waksman and Henricii (1943) and was placed in the family Streptomycetaceae on the basis of morphology and cell wall properties. Incorporation of molecular biology into classification changed early numerical systems using phenotypic characters (Anderson and Wellington, 2001). The family Streptomycetaceae are commonly aerobic actinomycetes forming a nonfragmenting substrate mycelium which may bear spores (*Microellobosporia flytrosporangium*, *Kitasatoa*, occasionally *Streptomyces*), and in most genera a well-developed aerial mycelium bearing uniserial chains of arthrospores enclosed within a thin fibrous sheath. Most spores are non-motile but zoospores can be formed in short rows within the swollen sheath or club shaped vesicle (*Kitasatoa* and *Elytrosporangium*). The cell wall is Type I (Constituent: glycine and *LL*- DAP or

diaminopimelic acid). The family contains the genera: *Chainia*, *Elytrosporangium*, *Kitasatoa*, *Microellobosporia*, *Streptomyces* and *Streptoverticillium*.

Stackebrandt *et al*. (1997) proposed a classification system in which phylogenetically neighbouring taxa at the genus level are clustered into families, suborders (related organisms ranking between order and family), orders (comprise of related families), subclasses (related organisms ranking between class and order), and a class (comprised of related orders) using 16S rDNA phylogenetic analysis and the presence of taxon-specific 16S rDNA signature nucleotides regardless of the phenotypic properties used in describing the taxa in the past. For example, prior to this, *Streptomyces* and *Streptoverticillium* were 2 separate genera (Anderson and Wellington 2001). Members of *Streptoverticillium* were later linked to the *Streptomyces lavendulae* species group through immunodiffusion assay (Anderson and Wellington 2001). Similarities between the 2 were confirmed by physiologic tests (Anderson and Wellington 2001). It was later concluded from 16S and 23S rRNA comparison that *Streptoverticillium* could be treated as a synonym of *Streptomyces* (Anderson and Wellington 2001).

Streptomyces are gram positive aerobic bacteria belonging to the phylum Actinobacteria (Stackebrandt *et al*., 1997). They have a DNA G+C content of 69-78 %mol (Anderson and Wellington, 2001). They are in many ways similar to filamentous fungi, growing as branching hyphae that form a vegetative mycelium and disperse through spores formed on specialized reproductive structures called aerial hyphae, which emerge from the colony surface into the air (Hopwood, 2007). They are able to colonize the soil by growing as a vegetative hyphal mass that can differentiate into spores that assist in dispersal and persistence. The spores are a semidormant stage in the life cycle and can persist in soil for a long time (Kieser *et al*., 2000). The spores can withstand low nutrient and water availability unlike the mycelial stage that is sensitive to drought (Kieser *et al*., 2000).

Chemotaxonomic and phenotypic properties are employed in defining the genus *Streptomyces*. The major emphasis is now on 16S rRNA homologies in addition to cell wall analysis and fatty acid and lipid patterns. Detecting the presence of *LL*-diaminopimelic acid (*LL*-DAP) (a stereoisomer of diaminopimelic acid) as the

diamino acid in the peptidoglycan is one of the quickest methods for identification to the genus level (Kieser *et al*., 2000). Many studies have attempted to use sequence data from variable regions of 16S rRNA to establish taxonomic structure within the genus, but the variation was regarded as too limited to help resolve problems of species differentiation (Witt and Stackebrandt, 1990; Stackebrandt *et al*., 1992). The genus *Streptomyces* has been subjected to several systematic studies over the past 30 years but the identification of unknown isolates is still difficult. The International *Streptomyces* Project in 1964 established a number of standard phenotypic criteria to help in species characterization. However, the criteria turned out to be too minimal, and the 16 proliferation of species continued with no real attempt to compare species thoroughly with one another. Williams *et al*. (1983) came up with a numerical taxonomic system that allowed for comparison of many phenotypic traits concurrently. Species with similar phenotypic properties were clustered together and treated as a single species resulting in the reduction of the large number of described species (Williams *et al*., 1983).

Life cycle

Streptomycetes structure is similar to fungi as branching mycelium, filamentous arrangement of cells form a network called a mycelium. *Streptomyces* is represent as the best arguably studied genus of actinobacteria because they have complex developmental life cycles (Flärdh and Buttner, 2009) and produce numerous secondary metabolites that are used in human medicine as anti-infectives, antitumour and immunosuppressant drugs (Challis & Hopwood, 2003). *Streptomyces* also has ability to metabolize many different compounds, including sugars, alcohols, amino acids, and aromatic compounds by producing extracellular hydrolytic enzymes. Their metabolic diversity is due to their extremely large genome which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs.

Furthermore *Streptomyces* also resemble fungi in their elaborate life cycle other than their cellular structure. As shown in Figure 1.5, life cycle of *Streptomyces* starts with spore germination and outgrowth of a feeding substrate mycelium. Meanwhile,

DNA replication occurs without cellular division during the vegetative growth stage development. At this stage, filamentous structure is developed. When *Streptomyces* responded to nutritional and other stress signals, their reproductive aerial hyphae are produced undergo cell division to form spores. The spores are produced in aerial filaments rise above the colony; they are called sporophores. Antibiotic production is usually linked to differentiation, although not all *Streptomyces* antibiotics are produced in the stationary phase. Because of the complex *Streptomyce*s life cycle that resembles to multicellular eukaryotes, it enables researchers to study the development of these more complex systems using a simpler system as described above (Seipke *et al*., 2012).

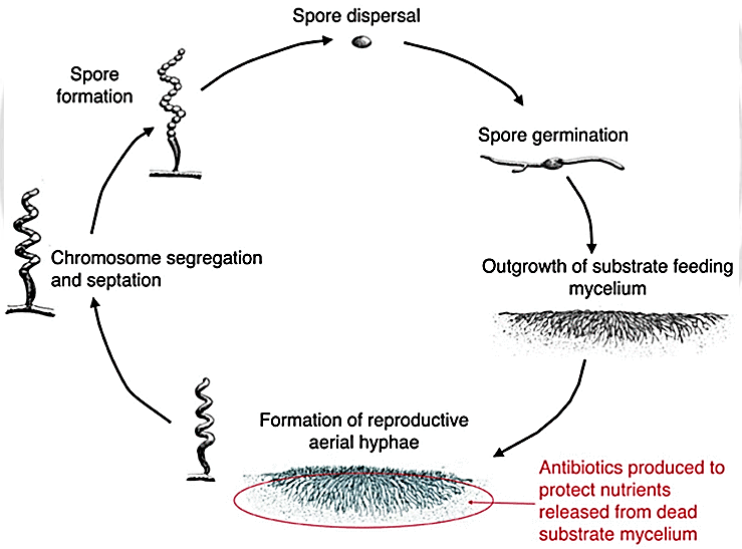

Figure 1.5 Life cycle of *Streptomyces* species (Seipke *et al*., 2012)

Properties and mechanisms

The ability of *Streptomyces* to produce bioactive secondary metabolites is the most obviously property. Various bioactive secondary metabolites are identified as antifungals, antivirals, antitumoral, anti-hypertensives, and mainly antibiotics and immunosuppressives (Omura *et al*., 2001; Patzer and Volkmar, 2010; Khan, 2011), which are biologically active compounds with high commercial value and important applications in human and livestock medicine and agriculture (Watve *et al*., 2001;

Berdy, 2005). Recent reports by Watve *et al*. (2001) have also shown that this group of microorganisms still remains an important source of antibiotics, immunosuppressant, extracellular hydrolytic emzymes, plant growth promoters and siderophores. These characteristics, as well as their ability to with stand desiccation and high temperatures as spores; make them attractive as biological control agents.

Parasitism and production of extracellular hydrolytic enzymes

The *Streptomyces* have ability to parasitize fungal plant pathogens; it is assumed that fungal growth is inhibited because nutrients pass from the pathogen to them. The *Streptomyces* can also degrade spores or the cell-wall of the pathogen by cell wall degrading enzymes (El-Tarabily *et al*., 1997), which is compatibility to pathogen cell wall structure. Many types of extracellular hydrolytic enzymes are produced by the *Streptomyces*, such as cellulase, chitinase, amylase etc. (Beyer and Diekmann, 1985; Hopwood, 2007). A role of cell wall degrading enzymes involved to control of plant pathogen that producing *Streptomyces* has been reported. Singh *et al*. (1999) used a chitinolytic *Streptomyces* sp. to control cucumber wilt caused by *Fusarium oxysporum* and chitinase produces by *S. hygroscopicus* APA14, isolated from crop rhizosphere soil from center of Thailand, showed activity against *Collectotrichum gloeosporioides* and *Sclerotium rolfsii* (Prapagdee *et al*., 2008). Moreover, the cellulase-producing strain from another actinomycetales were reported. *Micromonospora carbonacea* was used for control *Phytophthora cinamomi* (root rot of *Banksia grandis* Willd.) (El-Tarabily *et al*., 1997) and ß-1,3-glucanase-producing actinomycetes were used for control of *Phytophthora fragariae* var. *rubi* (raspberry root rot) (Valois *et al*., 1996).

1.5.2 Application

***Streptomyces* species as biotic elicitors**

The impact of antagonistic *Streptomyces* sp. depends on multiple synergistic strategies, including a direct interaction with the pathogenic partner (antibiosis), as well as indirect mechanisms like the degradation of quorum-sensing signal molecules

(Park *et al*. 2005). *Streptomyces* sp. can also induce systemic and localized resistance to plant pathogens and improve plant growth and metabolism (Conn *et al*. 2008; Lehr *et al*. 2008). According to Conrath *et al.* (2002) who suggested that the nonpathogenic bacteria prime the plant for accelerated and enhanced response to a second stress stimulus, such as a pathogen. Besides, Beausejour *et al.* (2003) reported that *Streptomyces melanosporofaciens* strain EF-76 protect potato tubers against common scab, elicits plant defense mechanisms and systemic resistance. Moreover, Conn *et al.* (2008) described that resistance induced by the gram-positive bacterium *Streptomyces* sp. strain EN27 against *Erwinia carotovora* and *F. oxysporum* in *Arabidopsis* was shown to be associated with SA signaling (van der Ent *et al*., 2009). Related to Baz *et al*. (2012) found new insights are thus provided into the interaction mechanisms between *Streptomyces* sp. and plants. Results lead to induce defence responses. Recently, Kurth *et al*. (2014) found that *Streptomyces* strain AcH 505 elicited a systemic defense response in oak upon oak powdery mildew infection. Furthermore, Srivastava *et al*. (2015) described role of *Streptomyces rochei* strain SM3 that triggered the ethylene (ET) responsive ERF transcription factor (*CaTF2*) under the challenged conditions, led to induce stress tolerance in chickpea against *Sclerotinia sclerotiorum* and NaCl.

Streptomyces species as control agents

Abd-Allah (2001) described *Streptomyces plicatus* as a model of biocontrol agent that selected from 372 isolates. The strain represented the best chitinase producer that had a highly significant inhibitory effect on spore germination, germ tube elongation and radial growth of *Fusarium oxysporum* f. sp. *lycopersici*, *Altrernaria alternata* and *Verticillium albo-atrum*, the causal organisms of *Fusarium* wilt, stem canker and *Verticillium* wilt diseases of tomato. Moreover, application of *S. plicatus* to the root system of tomato plants before transplantation markedly protected tomato plants against the tested phytopathogenic fungi *in vivo*. Accordingly, to the finding of Sabaratnam and Traquair (2002) that possessed antagonistic activity of *Streptomyces* spp. were able to against various phytopathogenic fungi, *Diaporthe arctii, Phomopsis longicolla* (Muntañola-Cvetkovic *et al.*, 2000), *Rhizoctonia solani* (Sabaratnam and Traquair,

2002), *Sclerotium rolfsii* (Boukaew *et al.*, 2010), *Colletotrichum musae*, *Fusarium oxysporum* (Taechowisan *et al.*, 2005), *Pythium* sp. (Muiru *et al.*, 2008), and phytopathogenic bacteria, *Ralstonia solanacearum* causing wilt of tomato (Tan *et al.*, 2006), bacterial wilt (*Ralstonia solanacearum*) and root and stem rot of chili pepper (*Sclerotium rolfsii*) (Boukaew *et al.*, 2010). Additionally, Bonaldi *et al*. (2011) had studied on abilities of endophytic *Streptomycetes*. Twenty-six *Streptomyces* spp. strain showed different patterns of antifungal activity *in vitro* against soil-borne phytopathogenic fungi belonging to six genera: *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Rhizoctonia* and *Sclerotinia*. Moreover, seed-dressing and seedcoat method were further applied for protecting plants *in vivo* that improved percent germination, lower saprophyte contamination and increase elongation of main roots in seedlings. Recently, Alekhya and Gopalakrishnan (2014) had characterized antagonistic *Streptomyces* against fungal pathogens of chickpea and sorghum that showed broad-spectrum antagonistic activity. Besides, they had abilities to produce siderophore, chitinase, cellulase, lipase, and protease with some exceptions, including hydrocyanic acid and indole acetic acid, indicated plant growth promotion potential.

1.6 The objectives of this study

The main objectives were as follows:-

1. To select the most effectiveness of *Streptomyces* strains in controlling the pathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* causing *Fusarium* wilt disease of tomato
2. To induce expression of pathogenesis-related (*PR*) proteins in tomato plants against *Fusarium* wilt by *Streptomyces*
3. To study the application of *Streptomyces* in controlling *Fusarium* wilt disease of tomato plants under greenhouse conditions

1.7 Education/application advantages

Development of the consistent application method of *Streptomyces* will be useful for controlling *Fusarium* wilt disease of tomato caused by *F. oxysporum* f. sp. *lycopersici*. Inducible plant immunity is the effective method to protect plants during pathogen penetration. Systemic acquired resistance (SAR) is a natural, inducible plant defense response, which is long-lasting and effective against a broad spectrum of pathogens.

The consistent method will be useful for other studies with various plant varieties and effective microorganisms. The methods will lead to decrease for disease management, which is also important to other agrosystems because it minimizes the dependence on pesticides. Moreover, it would greatly benefit consumer's health as well as product marketability. Hence, this approach can be exploited as it is a natural, safe, effective, persistent and durable alternative to decrease chemical pesticides for controlling plant diseases.

1.8 Scope of this study

The thesis is consisted of 4 main parts. Part 1 (Chapter 2) describes collection, isolation, morphological characterization and pathogenicity testing of the pathogen *F. oxysporum* f. sp. *lycopersici* (*Fol*) causing *Fusarium* wilt in tomato. Part 2 (Chapter 3) evaluates *in vitro* selection of the most effective *Streptomyces* (from 6 strains; NSP 1-6). Part 3 (Chapter 4) studies on induction of pathogenesis-related (*PR*) genes expression in tomato plants after treatment with *Streptomyces* responsed to the pathogen *Fol*, and part 4 (Chapter 5) evaluates the potential of selected *Streptomyces* by repeating Part 3 and analyzing until maturity.