CHAPTER 6

Discussion

6.1 General discussion

A total 126 isolates of *Fusarium. oxysporum* f. sp. *lycopersici* (*Fol*) were obtained from *Fusarium* wilt infected vascular of 2 tomato cultivars; cv. 'Cherry' and cv. 'Thomas'. Traditionally, characterization of *Fusarium* species has been based on the published description; distinctive characters of the shapes and sizes of macro- and microconidia, presence and absence of chlamydospores as well as colony appearances, pigmentations and growth rates on agar media (Nelson *et al*., 1994; Leslie and Summerell, 2006). In this study, morphological characteristics of isolated fungi were observed PDA and under microscopic, which all identified as *F. oxysporum* and agreement with previous reports from Nelson *et al*. (1983) and Synder and Hans (2003).

The pathogenicity tests in this study were conducted using the susceptible cultivar to avoid genetic factor of resistant gene. Based on this principle, tomato cv. 'Bonny Best' was selected to be used as standard susceptible host because they do not contain any *Fusarium* resistance gene (Grattidge, 1982; Marlatt *et al*., 1996). The pathogenicity test confirmed that a total 126 isolates of *Fol* were pathogenic to tomato seedling cv. 'Bonny Best', which the isolate *Fol*CK_117 was considered as the most virulent strain and selected to use as pathogenic strain in further experiments. Seedlings inoculated with each isolate of *Fol* showed differentiate virulence level verified from low to very high virulent isolates. When cutting the stem lengthwise with a knife, it showed browning of the vascular system, which is typical characteristic of the disease and generally can be used for its tentative identification. Such pathogens are specific for certain plant hosts and known as 'forma speciales' (Marasas *et al*., 1984; Joffe, 1986; Cartia *et al*., 1988; Rivelli, 1989; Fletcher, 1994; Mushtaq and Hashmi, 1997; Jovicich *et al*., 1999)., the more detailed tests comfirmed them as *F. oxysporum* f. sp. *lycopersici* (Armstrong and Armstrong, 1968; Jones, 1991; Reis *et al*., 2005). The symptoms were

the same as those described by Massee (1895) who first explained the *Fusarium* wilt of tomato in England, and other reports (Walker, 1971; Jones *et al*., 1982; Agrios, 1988; Smith *et al*., 1988; Jones, 1991; Frank, 1998; Messenger and Braun, 2000).

Afterward, some of representative *Fol* was randomly selected to recheck for their pathogenicity, which the compared tests using a resistant cultivar, cv. EWS-37434. Two pathogenicity tests were evaluated as a previous method. The results were similar to the first seedlings test cv. 'Bonny Best', whereas no symptom was found in the resistant cv. 'EWS-37434' seedlings due to resistance gene. These results clearly indicated the suitable of susceptible and resistance cultivars for further experiments.

The forma specialis of F. *oxysporum* is classified on the basis of virulence on a particular host (Correll, 1991). Variation in virulence within a forma specialis has been categorized by signing phenotypes the pathogenic races. Races are defined by their differential interaction with host genotypes, which in some cases are varieties known to carry one or more major genes for resistance (Gordon and Mattyn, 1997). Four specific dominant resistant genes of tomato varieties of *Fol* are known and designed *I*, *I*-1, *I*-2 and *I*-3 and only three races of *Fol* have been found (race1, 2 and 3). The three known races of *Fol* are distinguished by their pathogenicity to varieties with specific dominant resistance genes (Elias and Schneider, 1992). The initiation and development of plant disease is caused by interaction of specific genes for virulence in the pathogen and of specific genes for susceptibility in the host. In this study, race identification of the isolate *Fol*CK_117 was examined for pathogenicity on a set of standard differential tomato varieties. The cv. 'EWS-S' as the susceptible control (susceptible to race1, 2 and 3), while cv. 'EWS-R1' as resistance to race 1, cv. 'EWS-R12.1' and 'EWS-R12.2' as resistance to race 1 and 2, and cv. 'EWS-R123' as resistance to race1, 2 and 3. The results of this study showed that the isolate *Fol*CK_117 are designated as race 2 because this isolate could infect cv. 'EWS-S' (susceptible to race1, 2 and 3) and cv. 'EWS-R1' (resistance to race 1). Huang and Lindhout (1997) reported that tomato lacking *I* genes are susceptible to *Fol*. The *Fol* race 1 attacks only this cultivar, race 2 overcomes resistant varieties which contain the *I* genes (single dominant resistant gene which resists race 1 and the race 3 overcomes the *I*-2 gene that is resistant to *Fol* race 2. The gene *I*-3 has been proposed for resistance to *Fol* race 3. The interaction between these

resistant genes and race is not completely clear. 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; Holguin-Pena, 2005; Sheu and Wang, 2005; Bunyatratchata1 *et al*., 2005; Elena and Pappas, 2006).

This study had selected six *Streptomyces* strain; NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6, as biocontrol agent, which were previously isolated from natural soil samples from Suthep-Pui National Park, Chiang Mai, Thailand. These six *Steptomyces* strains were previously identified based on morphological characteristics, chemotaxonomy and analysis of the partial 16S rDNA sequence (Boonying, 2010; Nuandee, 2010; Suwan *et al*., 2012; Saengnak, 2012). The quantification of the confrontation or dual culture test in order to get access to analyzable data was a primary inescapable task in the comparison of biological control isolates. In this study, these six *Steptomyces* exhibited antifungal activities against *F. oxysporum* f. sp. *lycopersici* isolate *Fol*CK_117 over 70% inhibition of colony growth and over 60% inhibition of conidia production. The strain NSP3 demonstrated the highest antifungal activities, which was selected to use as antagonistic strain in further experiments. Previous studies, these six *Streptomyces* were shown to owe a great biocontrol potential both *in vitro* and *in vivo* (Boonying, 2010; Nuandee, 2010; Chantima, 2010; Viriya, 2010; Jaiyen, 2010; Mukta, 2010; Saengnak, 2012). Accordingly to the several findings that possessed antagonistic activity of *Streptomyces* spp. were able to against various phytopathogenic fungi (Muntañola-Cvetkovic *et al*., 2000; Sabaratnam and Traquair, 2002; Taechowisan *et al*., 2005; Khamna *et al*., 2009; Boukaew *et al*., 2010; Sowndhararajan and Kang, 2012; Zarandi *et al*., 2013).

The antagonistic *Streptomyces* species has potential involving the production of antifungal compounds (Crawford *et al*., 1993; Ouhdouch *et al*., 2001). This may be achieved by the production of enzymes, which degrades the fungal cell wall, or antifungal compounds (El-Tarabily *et al*., 2000; Errakhi *et al*., 2007; Getha *et al*., 2005;

Goodfellow and Williams, 1983). In this study, mycelia of the pathogenic *Fol*CK_117 in dual culture test plates showed abnormality under compound microscope observation, thus the growth was reduced and colony abnormality when compared to normal mycelia growth on control plate with *Fol*CK_117 alone, suggested that it probably involved chitinase enzyme. The results were supported by Totree *et al*. (2011) that previously examined chitinase activities were found between 0.10 – 0.80 U/ml in the third day of incubation. Moreover, all tested *Streptomyces* were previously proved to produce chitinase, amylase and cellulose (Boonying, 2010; Nuandee, 2010; Saengnak, 2012; Suwan, 2012). These findings were related to other reports that chitinase production was found in *Streptomyces* spp. (Rodriguez-Kabana *et al*., 1983; Nguyen *et al*., 1997; Romaguera *et al*., 1992; Mahadevan and Crawford, 1997). They were also similar to other reports regarding amylase production (Fairbairn *et al*., 1986; Virolle and Bibb, 1988; Mellouli *et al*., 1996; Chakraborty *et al*., 2009) and cellulase production (Wittmann *et al*., 1994; Jang and Chen, 2003; Alam *et al*., 2004; El-Sersy *et al*., 2010).

Plants have endogenous defense mechanisms or latent defensive systems that are induced upon response to attack by insects and pathogens, were activated. Induced resistance in plants refers to a state of heightened defensive capacity created by a prior stimulus. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy (Kuc, 1995; Ramamoorthy *et al*., 2002; McGovern, 2015). 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 development of SAR is associated with the induction of pathogenesis-related (*PR*) proteins (White, 1979). The hypothesis was that the non-pathogenic rhizobacteria have been shown to enhance disease resistance by stimulating the systemic defense pathways (Hammerschmidt 1999). Many reports have demonstrated the efficiencies of *Streptomyces* spp. in controlling plant diseases caused by pathogenic fungi (Abd-Allah, 1995; Yuan and Crawford, 1995; Hardy and Sivasithamparam, 1995; El-Raheem *et al*., 1996; Nemec *et al*., 1996; Singh *et al*., 1999; Abd-Allah, 2001; Getha1 and Vikineswary, 2002; Sabaratnam and

Traquair, 2002; Anitha and Rabeeth 2009; Cardoso and De Vasconcellos, 2009; de Oliveira *et al*., 2010; Baharlouei1 *et al*., 2010; Kekuda *et al*., 2013). However, little is known about the ability of *Streptomyces* to trigger SAR in tomato against *Fol.*

The present study was conducted to investigate the accumulation of the encoding of induced *PR* proteins in tomato plants responded towards *Streptomyces* NSP3 challenge inoculated with or without *F. oxysporum* f. sp. *lycopersici Fol*CK_117 causing *Fusarium* wilt. In this study, four *PR* proteins, including *PR-1a, Chi3* encoding acid chitinase, *Chi9* encoding basic chitinase and *CEVI-1* encoding peroxidase, were investigated the upregulation by real-time RT-PCR. The *PR-1* genes have been frequently used as marker genes for SAR in many plant species as previously described by Mitsuhara *et al.* (2008). Additionally, the *PR-3* (*Chi3*) and *PR*-4 (*Chi9*) genes are comprised of chitinases, which well-known that are constitutively expressed at low levels in plants, but are dramatically induced when plants respond to infection by fungal, bacterial, or viral pathogens (Leubner-Metzger and Meins, 1999; Neuhaus, 1999; van Loon, 1999). Chitinases have the potential to hydrolyse chitin, which is a major component of fungal cell walls. Chitin and glucan oligomers released during degradation of fungal cell walls act as elicitors that elicit various defence mechanisms in the plants (Frindlender *et al*., 1993). Whereas, the *PR-9* or peroxidases (*CEVI-1*) are key enzymes in the cell wall building process, and it has been suggested that extracellular or wall-bound peroxidases would enhance resistance in various plant species against phytopathogens by the construction of a cell wall barrier that may hamper pathogen ingress and spread in plant cells. 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; Taheri and Tarighi, 2012). Plant peroxidase produces antimicrobial phenolic compounds in the chemical defense systems against plant pathogens (Kobayashi *et al*. 1994). The present study implied that plants had acquired defense mechanisms to counteract potential pathogens and the strategy involves inducible defense reactions that are activated after elicitor applications within 24 hpi. Gene expression of *PR-1a* was immediately increased to maximum (54.3 fold) after challenge-inoculation with *Fol,* which was in accordance with those already published by Berrocal-Lobo and Molina (2004), van Loon *et al.* (2006), Conn *et al*. (2008) and Silvar *et al*. (2008). Whereas, gene expression of *Chi3*

and *CEVI-1* were also remarkably increased at 3 hpi to 23.2 and 7.6 fold, respectively; while *Chi9* was found to show little variation from 0 – 12 hpi with an average increase of 4.7 fold. These results were revelvant to previously reported by Cachinero *et al*. (2002), Ito *et al*. (2005), Sridevi *et al*. (2008) and Taheri and Tarighi (2012).

Moreover, the present experiment revealed that the treatments of *Streptomyces* NSP3 challenge inoculated with *F. oxysporum* f. sp. *lycopersici Fol*CK_117 greatly exhibited high levels of studied *PR* proteins either seed treatment or soil application. The studied *PR* genes in the plant challenge inoculated with *Fol*CK_117 and NSP3 were responded at the higher level than plants challenge inoculated with NSP3 alone or *Fol*-inoculation plants. Furthermore, no upregulation of *PR* proteins was found in healthy plants. These results indicated that the combination of seed treatment and soil application was more effective for induction and accumulation of these *PR* proteins than one individual method. Related to Ramamoorthy *et al*. (2002) the protective strain *Pseudomonas fluorescens* isolate Pf1 was found to protect tomato plants by exhibited *PR* proteins and phenolics, including PAL, peroxidase, PPO, chitinase and TLP. The accumulation was higher responded after challenge inoculated with the pathogen *Fol*. Results of this study indicated that the selected *Streptomyces* NSP3 served as excellent trigger in defense mechanism against *Fusarium* wilt disease in tomato plants as described by Suwan (2012) that the selected NF-*Streptomyces* NSP-167 (*Streptomyces* NSP3 in this study) might activate the plant defense genes in the absence of a pathogen inoculation, suggesting that are detected as “minor” pathogens which do not trigger a full resistance response on their own, because they do not show pathogenic determinants, and this may result in more effective priming of the defense response against *Colletotrichum gloeosporioides* isolate TPCMCg60 causing chili anthracnose. In the result of this chapter indicated that whole interested *PR* proteins may play an important role in host plant defense These results also suggested that induction of *PR* proteins involved in SAR pathway might have contributed to restriction of invasion of *F. oxysporum* f. sp. *lycopersici* in tomato plants. These results also suggested that *Streptomyces* NSP3 is a strong elicitor of plant defense responses. Understanding the bioactive component of defense induction may lead to a control strategy for *Fusarium* wilt disease in tomato. Therefore, the confirmation of results tested by molecular technique was required.

Biological control by using plant-associated microorganisms is an efficient approach for disease management and regarded as friendly to the environment (Bargabus *et al*., 2003; Tjamos *et al*., 2005). *Sreptomyces* species has been attention to the possibility that they can protect roots by inhibiting the development of potential fungal pathogens. The antagonistic potential, involving the production of antifungal compounds, of streptomycetes isolated from plant rhizosphere soils to pathogenic fungi, involving the production of antifungal compounds, has been reported (Crawford *et al*., 1993; Ouhdouch *et al*., 2001). In this study, the effective biocontrol agent *Streptomyces* NSP3 was tested to confirm result of real-time quantitative PCR (qPCR) using same method. The aim of the present study was to conclude the effect of *Streptomyces* NSP3 on the growth of tomato wilts pathogen as well as their effect on *Fusarium* wilt disease incidence and also plant growth parameters under greenhouse conditions and to confirm the results of previously study. Tomato provides a good example of how the use of biocontrol agents. The resistant to *Fusarium* wilt cv. 'EWS-37434' was selected for comparison to cv. 'Bonny Best' (susceptible to *Fusarium* wilt).

The first step was evaluated effects on tomato seeds germination by the standard roll towel method (ISTA, 1993). Although, the *Streptomyces* NSP3 was not affected on seed germination of both tomato cultivar and also with or without seed treatment of *Streptomyces* NSP3, the vigour index revealed higher potential in treatments of NSP3-treated. This confirmed that *Streptomyces* NSP3 plays role in plant growth promoting effects. These results are in agreements with some other reports about stimulating effects of different plant growth promoting bacteria; several *Streptomyces* species, such as *S. olivaceoviridis*, *S. rimosus*, *S. rochei*, *S. lydicus*, *S. filipinensis* and other *Streptomyces* spp., have the ability to improve plant growth by increased seed germination and root elongation (Tokala *et al*., 2002; El-Tarabily, 2008). The results were related to other protective strain, *P. fluorescens* Pf1 (Ramamoorthy *et al*., 2002).

After applied *Streptomyces* NSP3, *Fusarium* wilt disease severity index (DSI) involved *Fol*CK_117 was demonstrated. Both *Fol*CK_117 and NSP3 had no effect on DSI to tomato plants cv. 'EWS-37434' due to resistance gene. Conversely, there were clearly investigated on the susceptible cv. 'Bonny Best' both naturally and artificial infection. The NSP3 helped delaying of *Fol* infection. In particularly, treatment of

NSP3 capable protected plants with *Fol*-inoculated until harvest, whereas plants with *Fol*-inoculation alone lead to death of all plants within 21 DAT. Accordingly to other reports, *Streptomyces* species such as *S. corchorusii* and *S. mutabilis*, *S. violaceusniger*, and *S. griseus*, have antagonistic potential and play an important role in plant disease reduction (Lahdenperä, 1987; Shanshoury *et al*., 1996; El-Raheem *et al*., 1996; Getha *et al*., 2005; Anitha and Rabeeth, 2009).

According to Weller (1988) who remarked microorganisms that colonize the rhizosphere are ideal for use as biological control agents against soil-borne diseases. The finding in this study clearly demonstrated *Streptomyces* NSP3 has good colonization potential, which has been identified as one of the potential biocontrol agents; they showed colonization ability inner root tissues and potting soil throughout experiment period. Moreover, there were slightly increasing trend occurred. It has been suggesting that when *Streptomyces* NSP3 was applied as seed treatment, the population was start colonized emerging roots of germinating seeds and gradually increased until transplant, which were resemble to other potential strains (Yuan and Crawford, 1995; Long and Xiao, 2003; Li *et al*., 2006; Lian *et al*., 2011; Cheng *et al*., 2014). Potential uses of root-colonizing *Streptomyces* spp. as replacements or supplements for agricultural chemical fungicides have been addressed in many reports (Chet *et al*., 1990; Franklin *et al*., 1989; Lechevalier and Waksman, 1962; Weller, 1988).

In addition, the stimulating of other growth parameters and yields of tomatoes in this study at harvested day (70 DAT), including plant height, root length, aerial fresh weight, aerial dry weight, root fresh weight, root dry weight and fruit weight, were excessively observed after treated with *Streptomyces* NSP, which showed in other reports (Anitha and Rabeeth, 2009; Registeri *et al*., 2012; Jog *et al*., 2012). Despite their enormous potential in biocontrol, preliminary evidence of their capacity to enhance plant growth as PGPR, the *Streptomyces* NSP3 has not been well investigated specifically for their use as PGPR. Nevertheless, the findings of the present study demonstrated that the *Streptomyces* NSP3 significantly enhanced all the agronomic observations under greenhouse conditions and identified as a potential biocontrol strain.

Hence, using of the NSP3 for control of *Fusarium* wilt disease of tomatoes in field may be feasible and practical.

6.2 Conclusions

1. A total of 126 *F. oxysporum* f. sp. *lycopersici* isolates were obtained from eleven commercial fields of 2 tomato cultivars; cv. 'Cherry' and cv. 'Thomas', at Doi Inthanon National Park, Chiang Mai, Thailand.

2. The pathogenicity test on tomato plants was confirmed that *F. oxysporum* f. sp. *lycopersici* isolate *Fol*CK_117 exhibited very high virulence on tomato seedling cv. 'Bonny Best'. Moreover, the *Fol*CK_117 was identified as race 2 virulent phenotype.

3. The statistical analysis showed antifungal activity of six *Streptomyces* strains (NSP1 – 6) against *Fol*CK_117 by using dual culture method and the results showed that isolate NSP3 had the strongest inhibitory effects in the inhibitory activity on mycelia growth and also decreased sporulation of the tested fungus.

4. The activation of four plant defense related genes was investigated. The results implied that accumulation of *PR-1a* was found the highest level, followed by *Chi3* encoding acidic chitinase, *Chi9* encoding basic chitinase and *CEVI-1* encoding peroxidase within 24 h. The results implied that these *PR* proteins appeared earlier and accumulated to higher levels when plants were treated with the NSP3 and challenged inoculated with the *Fol*CK_117 which were compared to non-treated plants or those treated with *Streptomyces* NSP3 or the pathogen *Fol* alone.

5. Combination of seed treatment and soil application of *Streptomyces* NSP3 is more effective for accumulation of these *PR* proteins than one method alone.

6. Tests of *Streptomyces* NSP3 were assessed for their efficiency in controlling *Fusarium* wilt incidence in tomato plants, with comparison of cv. 'Bonny Best (susceptible to *Fusarium* wilt) and cv. 'EWS-37434' (resistant to *Fusarium* wilt). The NSP3 significantly reduced the disease incidence on the susceptible cv. 'Bonny Best'.

7. *Streptomyces* NSP3 had ability in colonization inner root tissue and potting soil. Moreover, they were slightly increased throughout experiment period.

8. *Streptomyces* NSP3 increased in plant growth parameters of both cultivars at harvesting, increasing in plant height, root length, aerial fresh weight, aerial dry weight, root fresh weight, root dry weight and fruit weight.

6.3 Future Perspectives and Recommendations

1. The six effective *Streptomyces* spp., especially NSP3, should be strongly confirmed as new species.

2. Other genetic markers of defense mechanism as elicitor, such as other *PR*-proteins or phytoalexin, etc., should be additional detect to induce disease immunity. Moreover, the role and significance of defensive mechanism activated during *Streptomyces* NSP3 interaction is still unclear, the *Streptomyces* might be altered or activated the defensive mechanism which underlying cross-talk process and going to be investigated.

3. *Streptomyces* NSP3 should be further studied for other applicabilities, including siderophore synthesis, nitrogen fixation, solubilization and phytohormone synthesis, of minerals to make them available for plant uptake and use or facilitates plant uptake of soil nutrients, for specifically identification as PGPR.

4. Remaining *Streptomyces* spp. should be studied based on this study as the experimental model.

5. *Streptomyces* NSP3 should be investigated in the fields with various crops and developed to be the commercial products.