CHAPTER 3

In vitro selection of *Streptomyces* strain against *Fusarium* wilt pathogen using dual culture method

3.1 Introduction

Various kinds of microbial antagonists have been investigated as potential antifungal biocontrol agents for plant disease management. Actinomycetes in general *Streptomyces* are one of the important groups of soil microorganisms. They are important producers of bioactive compounds and constitute a potential group of biocontrol agents (Hardy and Sivasithamparam, 1995). The most interesting property of *Streptomyces* is the ability to produce bioactive secondary metabolites such 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). The *Streptomyces* have ability to parasitize and degrade spores or the cell-wall of fungal plant pathogens and it is assumed that nutrients pass from the pathogen to them so fungal growth is inhibited (El-Tarabily *et al*., 1997). The spectrum of parasitism could range from attachment of cell to fungal hyphae so cell-wall degrading enzymes are needed. The *Streptomyces* can produce a variety of extracellular hydrolytic enzymes such as cellulase, chitinase, amylase etc. (Beyer and Diekmann, 1985; Hopwood, 1990).

To select the effective antagonistic strain, the first screening step for biological control program is required. In a primary screen, Weinding (1932) was first introduced one of the conventionally applied screening tests; it called the confrontation test or well known as “dual culture test”, a method that shows the relationship (interactions) between the two organisms involving the stimulation or inhibition of their growth. The

dual culture test is more commonly used for testing fungal antagonism against other filamentous strains. The test is a comprehensive experiment that exhibits overall antagonistic potential of a fungal biological control agent, and can be applied after preliminary fast screening tests. The primary screen is also lead to understand the effect/role of the process of biological control, the necessity of a quantitative method is increasingly felt (Pakdaman *et al*., 2013).

Many biological control agents are found by screening large numbers of microorganisms against plant pathogens *in vitro* using dual culture test. Two-component screening is exclusively related to interaction studies and potential antagonists are typically ranked according to their ability to inhibit the growth of the pathogen expressed by an inhibition-zone. This present study was focused on six *Steptomyces* sp. strains; NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6, that 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 (Suwan *et al*., 2012; Saengnak, 2012). However, only one strain was required for further study. Therefore, the dual culture test has performed reasonably well for screening these *Streptomyces* and based on this aim.

The objectives of this chapter were as follows:-

To select the most effectiveness of *Streptomyces* strains in controlling the pathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* causing *Fusarium* wilt disease of tomato

3.2 Materials and methods

Efficacy of *Streptomyces* on inhibiting the mycelial growth of *Fol*

Pathogenic strain

The pathogenic *Fol* isolate FolCK_117, the most virulent isolate to tomato seedlings cv. Bonny Best, was tested in this experiment. The strain was grown on PDA at RT for 7 days before testing.

Antagonistic strains

Six *Streptomyces* strains; NPS1, NSP2, NSP3, NSP4, NSP5 and NSP6, previously isolated from natural soil samples from Suthep-Pui National Park, Chiang Mai, Thailand by Boonying (2010) and Nuandee (2010). Soil samples were a mixture of soils from 5 holes at a depth of from 0 to 15 cm and put in a plastic bag and were brought to laboratory. Soil samples were air dried at RT for 7 days. To isolate thermotolerant actinomycetes, air dried soil samples were ground and pretreated at 120^{o}C for 1 h (Xu *et al*., 1996) to become fine soil particles ready to use for isolation. Soil extract agar (SEA) was used for isolation which modified from Takefumi *et al*. (2005), prepared as soil solution by using the other organic soil from Suthep-Pui National Park. The soil particles were taken in random and spread over SEA medium and incubated at RT.

The colonies of actinomycetes were observed and transferred to the selective media, casein starch agar (CSA) and yeast starch agar (YSA). Subsequently, the pure culture of actinomycetes was transferred to glucose yeast extract-malt extract (ISP-2) agar plates (Shirling and Gottlieb, 1966). Pure cultures were transferred to Emerson's agar slant (Gottlieb *et al*., 1948) as stock cultures.

These six *Streptomyces* were proved their ability to produce cellulose, chitinase and amylase enzyme (Boonying, 2010; Nuandee, 2010; Suwan *et al*., 2012). They were also identified based on morphological study, chemotaxonomic identification and analysis of the partial 16S rDNA sequence (Boonying, 2010; Nuandee, 2010; Saengnak, 2012; Suwan *et al*., 2012). Moreover, they exhibited strong antifungal activities against

various fungal pathogens. Previously studies, these six *Streptomyces* were shown to owe a great biocontrol potential *in vitro* against *Colletotrichum* sp. causing chili anthracnose, and *Cercospora lactucae-sativae* causing lettuce leaf spot (Boonying, 2010; Nuandee, 2010). In addition, they showed over 70% inhibition against other pathogens including *F. moniliforme* causing bakanae disease of rice seeds, *Curvilaria lunata* and *Helminthosporium oryzae* also causing bakanae disease of rice seeds, *F. oxysporum* f. sp. *lycopersici* causing tomato wilt, *F. monoliforme* causing bakanae disease of rice, *Pestalotiopsis* sp. causing strawberry leaf blight, *C. gloeosporioides* causing mango anthracnose, postharvest fungi of fruits and maize (Chantima, 2010; Viriya, 2010; Jaiyen, 2010; Mukta, 2010; Saengnak, 2012; Saengnak *et al*., 2016).

These six *Streptomyces* were grown on glucose-yeast-malt extract agar (GYM) at RT for 20 days before testing.

Antifungal test on Petri dish

Antagonism tests of the *Streptomyces* strains against *Fol* were carried out on Petri dishes containing GYM with the dual culture method (Fokkema, 1978). The *Streptomyces* strains were streaked at 4 cm apart from each others on one side of a Petri dish at a distance of 2.5 cm and incubated for 4 days earlier than the pathogen, presuming the slow growth of these *Streptomyces* in culture and their secondary metabolite production. The 5-mm mycelial discs of 7-day-old *Fol* were placed on the same plate on another side of the each plate at a distance of 1.5 cm after the 4^{th} day. Plates inoculated only with *Fol* were served as controls (Figure 3.1). Paired cultures were incubated at RT for 7 days. Three replications were used arranged in a Completely Randomized Design (CRD), with one plate per replicate. Data were collected as colony diameter (cm) (Figure 3.1) and conidia production counted on a haemacytometer, then transformed to the percent inhibition of colony growth (PIRG) (modified from Soytong, 1989; Lokesha and Benagi, 2007) and conidia production, then descriptive assessment of the antagonistic activity was scaled (Soytong, 1989) based on PIRG as very high antagonistic activity (PIRG > 75%); high antagonistic activity (PIRG 60 to 75%); moderate antagonistic activity (PIRG 50 – 60%) and low antagonistic activity (PIRG < 50%). The most effective *Streptomyces* was selected and used in further experiment.

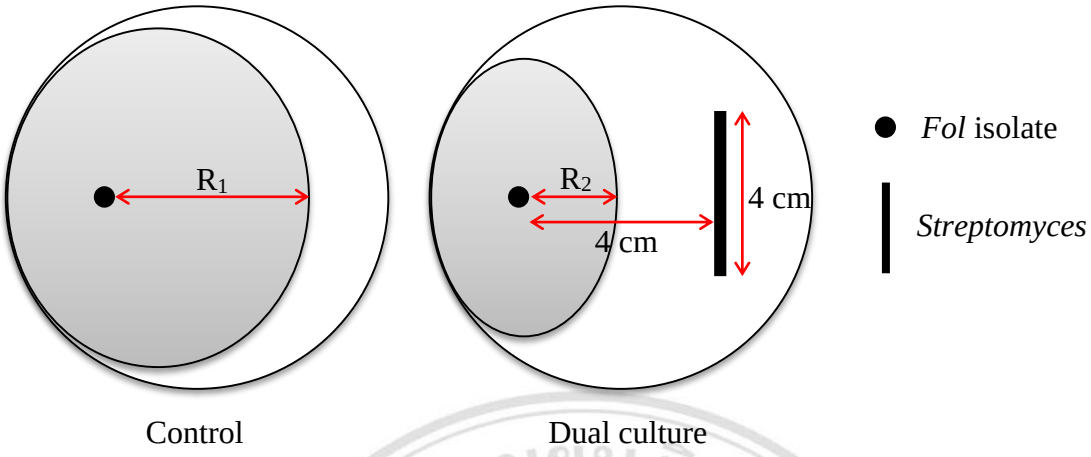

Figure 3.1 Dual culture test layout of *Streptomyces* against colony growth of *Fusarium oxysporum* f. sp. *lycopersici* causing *Fusarium* wilt in tomato on glucose-yeast-malt extract agar

Formula of percent inhibition of colony growth (PIRG) (modified from Soytong, 1989; Lokesha and Benagi, 2007)

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100$$

R_1 = radial growth of *Fol* in control plate

R_2 = redial growth of *Fol* in dual culture plate

Descriptive assessment of the antagonistic activity (Soytong, 1989)

> 75%	very high antagonistic activity
> 60-75%	high antagonistic activity
> 50-60%	moderate antagonistic activity
≤ 50%	low antagonistic activity

Statistical analysis

Data were computed analysis of variance (ANOVA). Treatments mean were compared using Fisher's Least Significant Difference (LSD) at *P = 0.05*.

3.3 Results

Six *Streptomyces* strains exhibited little different antifungal activities against *F. oxysporum* f. sp. *lycopersici* isolate *Fol*CK_117 on GYM. The colony growth of *Fol*CK_117 in dual culture plates were measured in range of 1.33 to 1.90 cm, while in control plate was 6.50 cm. The conidia production was also inhibited. The conidia in dual culture plates were counted in range 2.0×10^5 to 6.6×10^5 conidia/ml while in control plate was 18.4×10^5 conidia/ml (Figure 3.2).

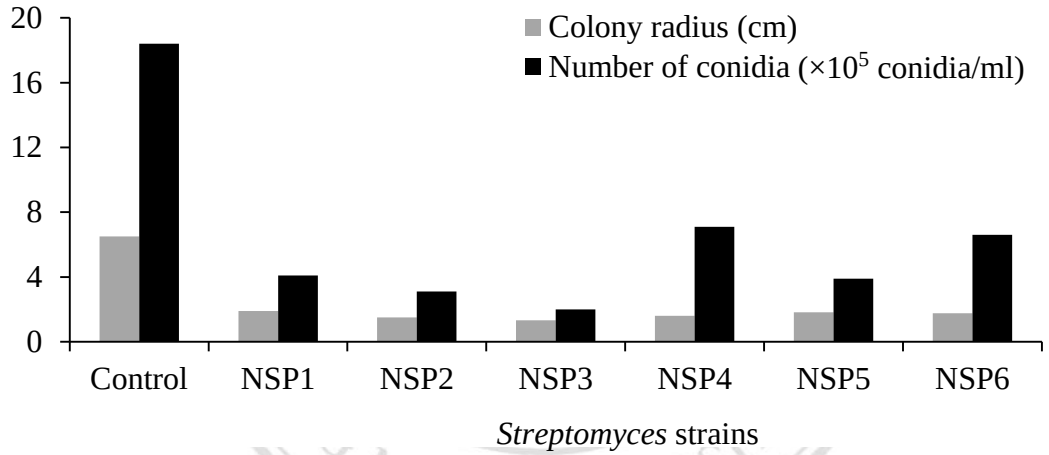

Figure 3.2 Efficacy of *Streptomyces* species on the colony growth and conidia production of *Fusarium oxysporum* f. sp. *lycopersici* isolate *Fol*CK_117 causing *Fusarium* wilt in tomato on glucose-yeast-malt extract agar at 7 days

The transformed data to PIRG showed that the *Fol* colony growth was inhibited in range of 70.77 to 79.50%. The NSP3 showed significantly highest to inhibit the colony in percent of 79.50% which categorized in group of very high antagonistic activity, and followed by NSP2 (76.90%) and NSP4 (75.37%) which also categorized in group of very high antagonistic activity. The NSP1, NSP5 and NSP6 inhibited the colony growth of 70.77, 71.80 and 72.80% respectively, which categorized in group of high antagonistic activity. For conidia production, the percent inhibition was found in range of 61.60 to 89.00%. The NSP3 and followed by NSP2 showed significantly highest to

inhibit the conidia production in percent of 89.00 and 83.20% respectively, which categorized in group of very high antagonistic activity and related to PIRG. The NSP1 and NSP5 were also categorized in group of very high antagonistic activity, which gave the percent inhibition of 77.57 and 79.04% respectively and followed by the group of high antagonistic activity; NSP4 (61.62%) and NSP6 (79.04%) (Table 3.1). However, 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. Branchy hyphae of the *Fol*CK_117 was generally shown in pair culture with the NSP3, when compare to normal elongation hyphe of the *Fol*CK_117 alone (Figure 3.3).

Table 3.1 Efficacy of *Streptomyces* species on the inhibition of the colony growth and conidia production of *Fusarium oxysporum* f. sp. *lycopersici* isolate *Fol*CK_117 causing *Fusarium* wilt in tomato on glucose-yeast-malt extract agar at 7 days

Streptomyces isolate	Percent inhibition[1/]					
	colony growth			conidia production		
NSP1	70.77	C	+++[2/]	77.57	C	++++
NSP2	76.90	B	++++	83.20	B	++++
NSP3	79.50	A	++++	89.00	A	++++
NSP4	75.37	B	++++	61.62	D	+++
NSP5	71.80	C	+++	79.04	C	++++
NSP6	72.80	C	+++	64.03	D	+++
F-test	***			***		
$LSD_{0.05}$	3.63			3.70		
CV (%)	3.48			2.74		

[1/] The mean of three replications were analyzed by ANOVA (1 plate/rep). The differences between treatments in the column are indicated by upper case letters. Significant treatment effects were determined by LSD at $P \leq 0.05$.

[2/] Scale of antagonistic activity; ++++ = very high (PIRG > 75%); +++ = high (PIRG > 60-75%); ++ = moderate (PIRG > 50-60%); + = low (PIRG ≤ 50%)

*** significantly different at $P \leq 0.001$

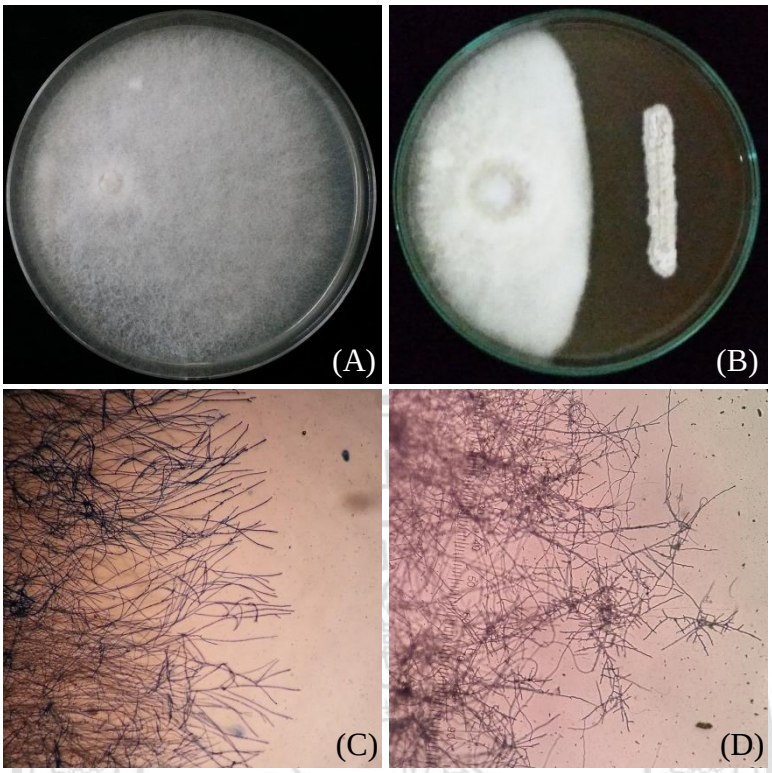

Figure 3.3 Dual culture test between antagonistic *Streptomyces* strain NSP3 and *Fusarium oxysporum* f. sp. *lycopersici* isolate *Fol*CK_117 causing *Fusarium* wilt in tomato on glucose-yeast-malt extract agar at 7 days; (A) control plate, (B) dual culture plate, (C) normal elongation hyphe of *Fol*CK_117 and (D) abnormal branchy hyphae of *Fol*CK_117

3.4 Discussion

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. This present study was focused on six *Steptomyces* sp. strains; NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6, that 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 (Suwan *et al*., 2012; Saengnak, 2012). In this study, 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 was demonstrated the highest antifungal activities, which found 79.50 and 89.00% inhibition of colony growth and conidia production, respectively. Previously studies, these six *Streptomyces* were shown to owe a great biocontrol potential *in vitro* against *Colletotrichum* sp. causing chili anthracnose, and *Cercospora lactucae-sativae* causing lettuce leaf spot (Boonying, 2010; Nuandee, 2010). In addition, they showed over 70% inhibition against other pathogens including *F. moniliforme* causing bakanae disease of rice seeds, *Curvilaria lunata* and *Helminthosporium oryzae* also causing bakanae disease of rice seeds, *F. oxysporum* f. sp. *lycopersici* causing tomato wilt, *F. monoliforme* causing bakanae disease of rice, *Pestalotiopsis* sp. causing strawberry leaf blight and *C. gloeosporioides* causing mango anthracnose (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, including *Diaporthe arctii*, *Phomopsis longicolla* (Muntañola-Cvetkovic *et al*., 2000), *Rhizoctonia solani* (Sabaratnam and Traquair, 2002; Sowndhararajan and Kang, 2012), *Alternaria brassicicola, A. porri*, *C. gloeosporioides*, *F. xysporum*, *Penicillium digitatum* and *Sclerotium rolfsii* (Khamna *et al*., 2009), *C. musae*, *F. oxysporum* (Taechowisan *et al*., 2005), *Sclerotium rolfsii* (Boukaew *et al*., 2010) and *Magnaporthe oryzae* (Zarandi *et al*., 2013), etc.

Streptomyces species has gained 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). This may be achieved through by the production of enzymes, which degrade 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, all tested *Streptomyces* were previously proved to produce chitinase, amylase and cellulose (Boonying, 2010; Nuandee, 2010; Saengnak, 2012; Suwan *et al*., 2012). These findings were related as other reported that found chitinase production by *Streptomyces* spp. (Rodriguez-Kabana *et al*., 1983; Nguyen *et al*., 1997; Romaguera *et al*., 1992; Mahadevan and Crawford, 1997). They were similarity to others reports of 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). 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 was probably involve chitinase enzyme. The results were supported by Thotree *et al*. (2011) that previously examined chitinase activities from the culture medium filtrate (F) of these six *Streptomyces* species against chili anthracnose caused by *C. gloeosporioides*. The third day of incubation, they showed chitinase activity between 0.10 – 0.80 U/ml. Most probably, however, other inhibitory agents were also combined to produce such powerful effects. Presence of antibiotics could be easily confirmed by applying culture media which have been heated to destroy the enzyme activities.

The studied *Streptomyces* did not affect only by inhibiting the mycelial growth, but also reduced the germination of conidia. Some conidia created abnormal shapes and could not develop to mycelium. Previous studies reported abnormal-appearing conidia of other fungal pathogens when treated by these *Streptomyces*, including *F. oxysporum* f. sp. *capsici* causing wilt disease of chili, *F. oxysporum* f. sp. *lycopersici* causing wilt disease of tomato, *Curvularia* sp., *Helminthosporium* sp. causing dirty panicle disease of rice, *Alternaria* sp. causing calyx end rot disease of Asian pear, *Pestalotopsis* sp. causing scab disease of guava, *Acremonium* sp. and *Fusarium* sp. causing storage fungi of maize (Chaisiri, 2010; Jaiyen, 2010; Modted, 2012; Saengnak *et al*., 2016), etc.

3.5 Conclusions

The *Streptomyces* NSP3 had the strongest inhibitory effects on *in vitro* growth of *F. oxysporum* f. sp. *lycopersici* isolate *Fol*CK_117, which found 79.50% and 89.00% inhibition of colony growth and conidia production, respectively. Based on results obtained in this study, the NPS3 was selected to represent as a biocontrol agent in further studies. Biocontrol of the *Fol*CK_117 with *Streptomyces* NSP3 would lead to more successful control.