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Use of antagonistic *Streptomyces* spp. against chili wiltdisaese caused by *Fusariumoxysporum*f.sp. *capsici*

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The project was conducted to study the antagonistic activities of six strains of *Streptomyces* spp.; NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6, against the fungal pathogen of chili wilt disease; *Fusariumoxysporum*f.sp.*capsici*. The efficiencies of these *Streptomyces* spp. were no significantly different to inhibit the colony growth of pathogen according to dual culture method, which ranging from 72.18 – 77.87%. Then, these *Streptomyces* spp. were developed on enzyme production medium (EPM) by incubate shaking for 3, 5 and 7 d, and the culture medium of each isolate was then divided into two parts, non-culture medium filtrate (NF) and culture medium filtrate (F). The efficiencies of 5 day-old culture mediums showed significantly highest to inhibit the conidial germination at percentage ranging from 55.41 - 62.74% (for NF) and 45.77 – 56.23 (for F). Moreover, the efficiencies of NF were significantly higher than F in every isolates. In addition, soil treated with NSP2 under greenhouse conditions has significantly reduced chili wilt disease severity in scale of 2.78 while negative control (pathogen inoculation) was found in scale of 5.00.

Keywords: *Streptomyces* spp., *Fusariumoxysporum*f. sp.*capsici*, chili, culture medium

Introduction

Chili is one of Thailand's economic crops. The total area under chili cultivation in Thailand was estimated at 720 sq km (Mustafa *et al.*, 2006). The major disease contributing to the loss in the production of crop is *Fusarium*wilt, which caused by soilborne pathogen *Fusariumoxysporum* W. C. Snyder & H. N. Hans. and one of common disease in crop of Solanaceae: chili, tomato, eggplant, and potato. This disease is one of the important diseases occurring in both temperature and warmer areas. Once a field is infested, the pathogen may survive in the soil for many years. The degree of loss caused by the pathogen varies depending on host cultivar, race of the pathogen and environment

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conditions. This disease causes serious seedling wilting, yellowing and died. *Fusarium* also causes plant to grow abnormally, or uses the plant as agent of the pathogen transmission to other host plants. The pathogen infects young root, growing, developing and spreading in root and stem vessel, inhibiting water and nutrient transport (Miller *et al*., 1986).

Several fungicides were use to control the disease in infested areas. Although, their systemic activity plays a very important role in plant disease control, but these fungicides adversely affect other useful soil microorganisms and also pollute the environment (Parker *et al*., 1985). Furthermore, genetic resistance to each of these diseases has been identified (Kawchuk *et al*., 2001; Sela-Buurlage *et al*., 2001), especially in bezimidazole group, such as carbendazim, benomyl (Brent and Hollomon 1998; Deising *et al*., 2008; Damicone and Smith, 2009). So, biological control methods, based on the use of beneficial microorganisms isolated from suppressive soils, represent an alternative for protection of plants against *Fusarium* wilts (Alabouvette *et al*., 1993).

Actinomycetes are recognized as metabolically and morphologically more complex than sessile bacteria. Metabolically, they are prolific producers of an array of secondary metabolites, including antimicrobial agents, plant growth hormones, and siderophores (Conn *et al*., 2008). Among actinomycetes species, the genera *Streptomyces* member of the order *Actinomyceyales*are gram-positive, mycelia-forming soil bacteria with 69 – 78% guanine-plus-cytosine (G+C) content in their genomes are known to produce a variety of antimicrobial compounds and the plant rhizosphere represents an uncharacterized source of microorganisms producing novel antimicrobial metabolites (Stackebrandtv*et al*., 1997). So, the aim of this study were to evaluate antifungal activities of *Streptomyces* spp. to control *F. oxysporum*f.sp. *capsici*causing *Fusarium*wilt disease of chili.

Materials and methods

Pathogen and pathogenicity test

*Fusariumoxysporum*f.sp.*capsici* was isolated from chili cultivation by s*plitting* each *stem* into two parts, then placed in a moist chamber, and incubated at room temperature (RT) for 2 days. The appeared mycelium were transferred to a potato dextrose agar (PDA) Petri dish, and incubated at RT to get pure culture. Each isolate was transferred onto PDA slants and preserved at 4^{o}C for further use.

Preparation of pathogen inoculum

Sorghum seeds were used as inoculating media by boiling, sterilized and packed in polythene bags (100 g/bag). *F. oxysporum*f.sp.*capsici* grown in PDA was cut from peripheral colony using cock borer (5 mm diameter), then transferred to sterilized sorghum seeds (10 pieces/bag) and mix well. The pathogen inoculums were incubated in the dark for 14 days before testing.

All isolates of *F. oxysporum*f.sp. *capsici* were tested for pathogenicity using 10-week-old chili seedling cv. Chomthong 2 by inoculating soil with pathogen inoculum at rate 100 g/1 kg of soil. Seedling roots were trimmed and transplanted into each pot at the rate single seedling per pot. Beginningaweekafter inoculation,disease will be rated on 1 to 5 scales (Applied from Marlatt*et al.*, 1996), as follow: 1= no symptoms; 2= slight chlorosis, wilting, or stunting of plant; 3= moderate chlorosis, wilting, or stunting of plant; 4= serve chlorosis, wilting, or stunting of plant and 5= dead plant. Cultivars with average disease ratings greater than 2.5 will be considered susceptible. The most virulent isolate was selected for further experiment. The test was done using Completely Randomized Design (CRD) with three replications.

Antagonistic strains

Six soil actinomycetes strains; NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6, present in the culture collection of the Laboratory of Plant Pathology of Chiang Mai University, were previously isolated from natural soil samples from Suthep-Pui National Park, Chiang Mai, Thailand, and identified as *Streptomyces* spp. based on morphological study, chemotaxonomy identification and analysis of the partial 16S rDNA sequence (Saengnak, 2012; Suwan *et al*., 2012), were grown on glucose extract yeast-malt extract agar (GYMA) at RT for 10 days.

Antagonism test

Antagonism test of six *Streptomyces* spp. to pathogen growth were carried on glucose yeast malt agar (GYMA) (Shirling and Gottlieb, 1966) using the dual culture method (Fokkema, 1978). The mycelia plugs of 7-day-old pathogen and antagonistic strains were placed on the same dish 3 cm from each other. Isolate of antagonistic strains were steaked (3 cm on the one side of Petri dishes) 4 days earlier than the pathogen, reflecting the slow growth of these actinomycetes in culture and their secondary metabolite production. The dishes were incubated at RT. Then, a 5 mm of mycelia agar disc from pathogen

cultures were placed away from the disc of actinomycetes isolates on the same dishes. Paired cultures were incubated at RT for 7 days. Dishes inoculated only with test pathogens served as controls. The experiment was done using CRD with three replications. The growth of the pathogen in both the test and control experiments were recorded. Data were collected as percent inhibition of colony growth (PICG) (modified from Soytong, 1989; Lokesha and Benagi, 2007).

Inhibitory effects of culture mediums on conidial germination

Preparation of pathogen inoculum

The pathogens, 7-day-old culture grown on PDA Petri dishes at RT, were flooded with 10 ml of sterile distilled water (Singleton *et al.*, 1992). Mycelia were dislodged by scraping the surface of the agar culture with a sterile loop needle. Suspensions were then filtered through of sterile cheese cloth. The concentration of conidia in the suspensions determined with a hemacytometer and adjusted to 1×10^4 conidial/ml.

Preparation of culture mediums

The strains NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6, grown on GYMA for 10 days at RT, were separately cultured into 250 ml Erlenmeyer flasks containing 50 ml enzyme production medium (EPM) modified from Rattanakit *et al*. (2000). The flasks were incubated continuous shaking on a rotating shaker at 150 rpm and 35^{o}C for 3, 5 and 7 days. The culture medium of each isolate was divided into two parts: non culture medium filtrate (NF) and culture medium filtrate (F). The cultures were centrifuged for 20 min at 6,000 rpm (4^{o}C) and the supernatants were collected as the NF. The supernatant was then filtrated through membrane filter pore size 0.22-µm (Minisart®) to get the F (Chareunrat, 1999).

For testing, fifty microlitre of the pathogen suspension and each culture medium (NF or F) were mixed and spreaded (total volume = 100 µl) onto a papery GYMA plate. The spreaded agar were cut into pieces of approximately 1 x 1 cm, and then transferred to sterile microscope slides. The control consisted of suspensions of the pathogenconidia in sterile distilled water. The slides were incubated in moist chambers for 24 h using three replicate slides for each culture medium type and isolate and checked the conidial germination at 0, 12, and 24 h, respectively. In this context germination was defined as a germ tube that had developed to longer than the cell wide. The percent of germination was determined by counting 100 conidia from each isolate under a

light microscope under high power (×40) for recording the percentage of conidial germination of *F. oxysporum*f.sp.*capsici.* The conidia suspension without actinomycetes culture medium served as a control. The experiment was done by using CRD.

Efficiency of Streptomyces spp. isolate NSP2 under greenhouse conditions

Preparation of pathogen inoculum

*F. oxysporum*f. sp. *capsici* isolate FoC4, the most virulent isolate, were prepared as previously experiment of pathogenicity test.

Preparation of Streptomyces spp.

Rice seeds were used as inoculating media according to Fermino (2007) by boiling, sterilized and packed in polythene bags (100 g/bag). *Streptomyces* spp. isolate NSP2, grown on GYMA for 10 days at RT, was flooded with sterile distillated water. Culture suspension was harvested, and then transferred to sterilized rice seeds (10 ml/bag) and mix well. The pathogen inoculums were incubated in the dark for 14 days before testing.

The pathogen and antaginisticts previously prepared were used for mix soil at rate 100 g/1 kg of soil. Various methods of treatment details includes, were T_1 – soil mixed NSP2 incubated for 4 days, then inoculated pathogen and incubated for 7 days, T_2 – pathogen inoculated in soil incubated for 7 days, then mixed NSP2 and incubated for 4 days, T_3 – soil mixed NSP2 together with inoculated pathogen and incubated for 7 days, C_1 - pathogen inoculated in soil and incubated for 7 days (negative control), C_2 – pot containing sterilized soil (positive control), C_3 – NSP2 mixed soil and incubated for 4 days, C_4 - sterilized ricemixed soil and incubated for 7 days, C_5 - sterilized sorghummixed soil and incubated for 7 days, and C_6– combination of C_4 and C_5, C_4 – C_6 served as inoculating media checked. The 10-week-old chili seedlings cv. Chonthong 2, grown in strilized soil, were used. Seedling roots were trimmed and further transplanted into each pot at the rate single seedling per pot. Beginning a week after inoculation,disease will be rated on 1 to 5 scales (Applied from Marlatt*et al.*, 1996), as previously. The test was done using Completely Randomized Design (CRD) with three replications.

Results

Pathogen

Four isolates of *F. oxysporum*were collected from a chili stem sample showing trypial*Fusarium*wilt symptom, and were code as FoC1, FoC2, FoC3 and FoC4. They were further observed for morphological characterization by growing on PDA for 10 days. The colony characteristic was white cottony colony, and then became purplish-white with aging. Mycelia were branched, septate and hyaline. They formed 3-6 celled hyaline slightly curved conidia with average 29.0-57.0 × 3.5-4.0 µm (Figure 1).

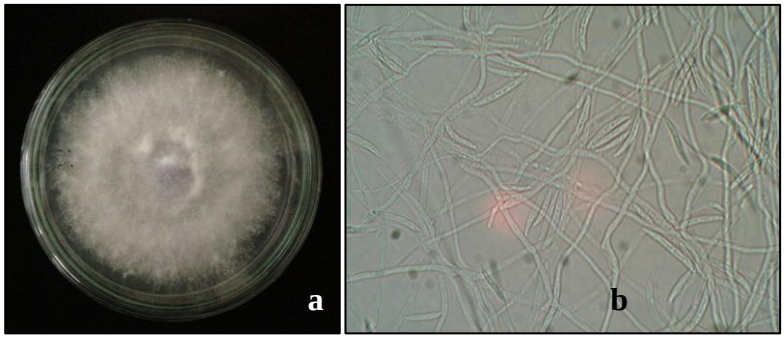


Fig. 1. Morphological character of *Fusariumoxysporum*f.sp. *capsici*isolate FoC4 at 10 days; a. Colony on PDA and b. mycelium and macroconidia (40X)

Pathogenicity test

The pathogenicity test confirmed that FoC1, FoC2, FoC3 and FoC4 isolates of *F. oxysporum* were pathogenic to chili seedlings cv. Chomthong 2. The isolate FoC4 was significantly higest disease index of chili wiltcaused by *F. oxysporum*f.sp.*capsici* (Fig. 2) which categorized as high virulent, and used in next experiment.

Fig. 2. *Fusarium*wilt symptom caused by *Fusariumoxysporum*f.sp. *capsici*isolate FoC4; a. healthy control and b. pathogen inoculation

Antagonism test

Six *Streptomyces* spp. were tested for antifungal activities against *F. oxysporum*f.sp.*capsici* isolate FoC4 on GYMA. There were not significantly different in percenteage inhibition of radial growth of pathogen which ranging from 75.74-80.98% (Table 1).

Table 1. Percentage inhibition of radial growth of six *Streptomyces* spp. against *Fusariumoxysporum*f.sp.*capsici* isolate FoC4 on GYMA at 7 days

Isolate of *Streptomyces* spp.	**Inhibition of radial growth (%)**
NSP1	80.98
NSP2	79.40
NSP3	75.74
NSP4	77.01
NSP5	79.25
NSP6	79.40
F-test	ns
$LSD_{0.05}$	6.26
CV (%)	4.48

ns: non significantly

Inhibitory effects of culture mediums on conidial germination

The 5-day-old ofboth culture mediums (NF and F) was significantly to inhibit the conidial germination of *F. oxysporum*f.sp. *capsici* isolate FoC4 higher than the 7-day-old culture mediums, which ranging from 45.77-62.74% and 24.35-37.08%, respectively. The efficiencies of NF were significantly higher than F in every isolates. In addition, the average percentage of

inhibition indicated that isolate NSP4 was significantly higestto inhibit the conidial germination of pathogen, and followed by the isolate NSP1, NSP2, NSP6, NSP3 and NSP5, respectively (Table 2). However, the efficiencies of all strains were reduced by the incubation time (Fig. 3). Moreover, morphological of some culture medium treated-conidia was found abnormally and cannot be able to develop to mycelium (Fig. 4).

Table 2. Effects of six *Streptomyces* spp. culture mediums (NF and F), incubated for 5 and 7 days, on inhibiting the conidial germination of *Fusariumoxysporum*f.sp.*capsici* isolate FoC4 on GYMA at 12 h after treated

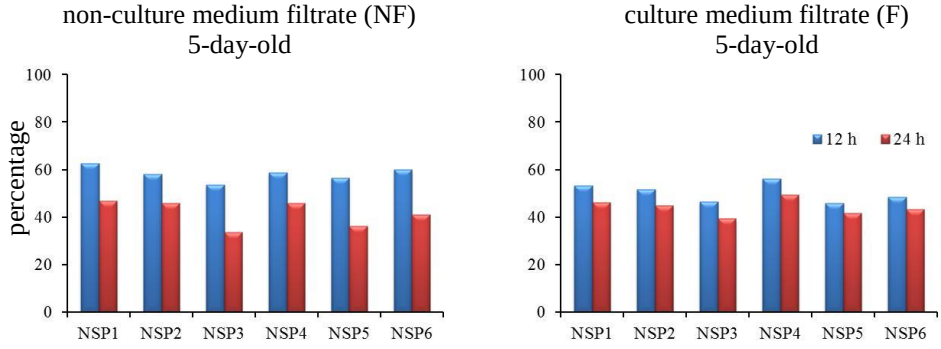
Isolate of *Streptomyces* spp.	**5-day-old**		**7-day-old**		
	NF	F	NF	F	Mean
NSP1	62.74	53.16	34.86	31.10	45.46 AB
NSP2	57.98	51.72	36.58	31.68	44.49 B
NSP3	53.63	46.37	28.38	26.10	38.65 D
NSP4	58.77	56.23	37.08	33.87	46.49 A
NSP5	56.36	45.77	27.26	24.35	38.44 D
NSP6	60.01	48.57	32.38	29.64	42.64 C
Mean	58.25 a	50.30 b	32.76 c	29.46 d	42.70

A (incubation period)	***	$LSD_{0.05}$ = 0.99	
B (type of culture medium)	***	$LSD_{0.05}$ = 1.71	
C (isolate of *Streptomyces* spp.)	***	$LSD_{0.05}$ = 0.99	
A*B	ns		
A*C	***	$LSD_{0.05}$ = 1.39	
B*C	ns		
A*B*C	ns		
CV (%)	4.87		

NF = non-culture medium filtrate, F = culture medium filtrate

The difference between isolate of Streptomyces spp. in the row and type in the column of each pair are indicated by upper and lower case letters, respectively. The different letters are significantly different by LSD $_{(P<0.05)}$

ns: non significantly *** significantly different at $P<0.001$



non-culture medium filtrate (NF) 7-day-old culture medium filtrate (F)7-day-old

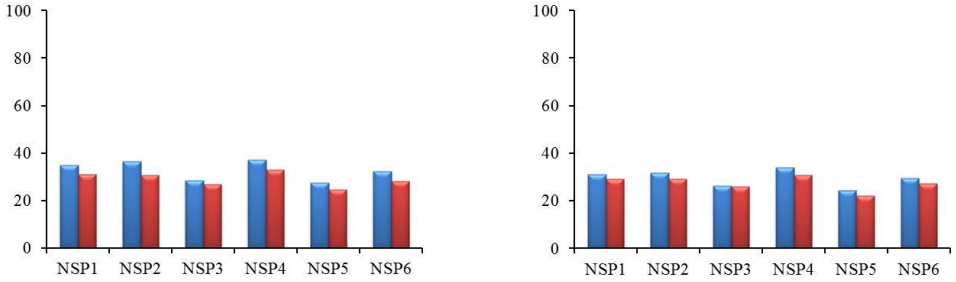


Fig. 3. Effects of six *Streptomyces* spp. culture mediums (NF and F), incubated for 5 and 7 days, on inhibiting the conidial germination of *Fusariumoxysporum*f.sp.*capsici* isolate FoC4 on GYMA at 12 and 24 h after treated

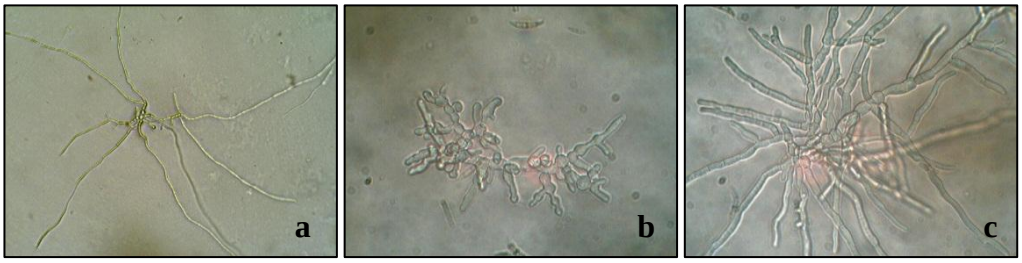


Fig. 4. Abnormally-appearance conidia of *Fusariumoxysporum*f.sp.*capsici* isolate FoC4 after treated with culture mediums (NF and F) of isolate NSP2, incubated for 5 days, on GYMA for 24 h; a. control (10X), b. treated NSP2-NF (40X) and c. treated NSP2-F (40X)

Efficiency of Streptomyces spp. isolate NSP2 under greenhouse conditions

Treatment of mixing NSP2 in soil before inoculation the pathogen gave the lowest disease severity (2.78) of *Fusarium*wilt causing *F. oxysporum*f.sp.*capsici* isolate FoC4, followed by treatment of mixing NSP2 in soil after inoculation the pathogen (3.33) and treatment of mixing NSP2 in soil together with inoculation the pathogen (3.67), respectively when compared to pathogen inoculated control (5.00). The NSP2 wad no any effect, which gave disease severity in scale 1.67 and no significantly differed when compared to treatment of sterilized soil. Similarly, there were not any effects of sterilized rice or sorghum to disease severity, which found in scale 1.00 (Table 3; Fig. 5).

Table 3. Efficacy of *Streptomyces* spp. isolate NSP2 on *Fusarium*wilt disease of chili seedlings cv. Chonthong 2 under green house condition

Treatments			Disease severity scale
T_1	-	soil mixed NSP2 before inoculated pathogen	2.78 C
T_2	-	soil mixed NSP2 after inoculated pathogen	3.33 BC
T_3	-	soil mixed NSP2togerther with inoculated pathogen	3.67 B
C_1	-	pathogen inoculated in soil	5.00 A
C_2	-	sterilized soil	1.00 D
C_3	-	NSP2 mixed soil	1.67 D
C_4	-	sterilized ricemixed soil	1.00 D
C_5	-	sterilized sorghum seed mixed soil	1.00 D
C_6	-	sterilized rice+ sterilized sorghum mixed soil	1.00 D
F-test			***
$LSD_{0.05}$			0.85
CV (%)			30.05

The difference between treatments in the row are indicated by upper case letters.
The different letters are significantly different by LSD $_{(P<0.05)}$
*** significantly different at $P<0.001$

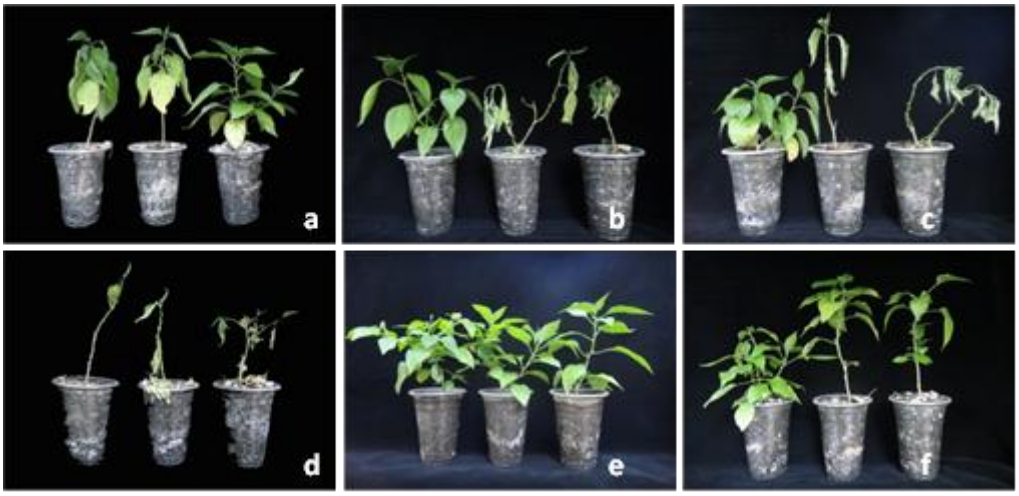


Fig. 3. Effects of *Streptomyces* spp. isolate NSP2 on reduction *Fusarium*wilt symptom cuased by *Fusariumoxysporum*f.sp. *capsici*isolate FoC4; a. treatment of soil mixed NSP2 before inoculated pathogen, b. treatment of soil mixed NSP2 after inoculated pathogen, c. soil mixed NSP2togerther with inoculated pathogen, d. pathogen inoculated in soil, e. sterilized soil and f. NSP2 mixed soil

Discussion

*F. oxysporum*isolate FoC4 was isolated from chili stem showing typical symptom of *Fusarium*wilt disease, and categorized to be the most virulent

isolate causing wilt of chili. These pathogens are plant pathogens 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 pathogenicity test comfirmed the pathogen was *F*. *oxysporum*f.sp. *capsici*.

This study interested biocontrol agents belonging genus *Streptomyces* spp., which usually producing of antifungal compounds and extracellular hydrolytic enzymes, chitinase and beta-1,3-glucanase (Valois *et al*., 1996; Mahadevan and Crawford, 1997; Trejo-Estrada *et al*., 1998; Singh *et al*., 1999; El-Katatny *et al*., 2001; Ouhdouch*et al*., 2001; Getha and Vikineswary, 2002; Fguira *et al*., 2005; Taechowisan *et al*., 2005; Mukherjee and Sen, 2006), various antibiotics, including 3-ethyl,3-methyl heptane and Diisodecyl ether (Nandhini and Selvam, 2013) aminoglycosides, macrolides, beta-lactams, peptides, polyenes, polyether, tetracyclines, etc. (Omura and Tanaka, 2002). Moreover, these six *Streptomyces* spp., used in present study, had previously showed great biocontrolpoteintial*in vitro*, which completely (100%) against *Colletotrichum*s pp. causing chilli anthracnose and *Cercosporalactucae-sativae* causing lettuce leaf spot (Boonying, 2010; Nuandee, 2010), and moreover, they showed over 70% inhibition against another pathogens including *F. moniliforme*causing bakanae disease of seed rice, *Curvilarialunata* and *Helminthosporiumoryzae* causing bakanae disease of seed rice, *F. oxysporum*f.sp. *lycopersici* causing tomato wilt, *F. monoliforme* causing bakanae disease of rice,*Pestalotiopsis*sp.causing strawberry laef blight and *C. gloeosporioides*causing mango anthracnose (Chantima, 2010; Viriya, 2010; Jaiyen, 2010; Mukta, 2010; Saengnak, 2012). Furthermore, Totree*et al*. (2011) had previously examined chitinase activities from the culture medium filtrate (F) of these six *Streptomyces* spp. against chili anthracnose caused by *Colletotrichumgloeosporioides*. The third day of incubation, the isolate NSP4 showed significantly highest chitinaseactivitiy (0.15 U/ml), followed by NSP2, NSP6, NSP1, NSP5 and NSP5, which found 0.11, 0.10, 0.80, 0.79 and 0.51 U/ml, respectively. So, the bioactive compounds of these six *Streptomyces* spp., used in present study, may be due to chitinase enzyme.

These six *Streptomyces* spp. had not effected to only inhibitiong the mycelial growth, but they also inhibited conidial of pathogen in term of germination. Some of morphological characteristic treated conidia found abnormal and could not be able to develop to mycelium. In greenhouse conditions, *Fusarium*wilt disease was reducedin prevention treatment. Moreover, there were not any effects to plant. These results indicated that this research is suscessfully used *Streptomyces* spp. to control disease and would be feasible to extend. Next study may be developing these strains as biofungicide

to be ready-for-use form, and testing against more pathogens, especially in the field.

Conclusion

This present study described protection of chilli plants by antagonistic *Streptomyces* spp. against *Fusarium*wilt disease caused by *F. oxysporum*f.sp. *capsici*, and showed good antifungal properties both *in vitro* and greenhouse conditions. Their bioactive component may provide an alternative resource for the biocontrol of plant diseases and they could be an interesting lead compound for further development of novel fungicides.

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