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Antagonistic *Streptomyces* species can protect chili plants against wilt disease caused by *Fusarium*

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The project was conducted to study antagonistic activities of six strains of *Streptomyces* species, named as NSP (1-6) against the fungal pathogen of chili wilt disease, *Fusarium oxysporum* f.sp. *capsici* isolate FoC4. According to the dual culture method, the *Streptomyces* species all inhibited strongly the pathogen colony growth ranging from 75.7 to 81.0%. After that, the bioactive component of *Streptomyces* was produced using an enzyme production medium (EPM) by incubation with shaking for 5 days compared to 7 days, and division into 2 parts: non-filtered culture medium (NF) and filtered culture medium (F). The efficiencies of 5-day-old culture media to inhibit the conidial germination were significantly higher than 7-day-old culture media, which showed inhibition of 53.6 – 62.7% for NF and 45.8 – 56.2% for F. The efficiencies of NF were higher than those of F with all of the isolates. Moreover, Isolate NSP4 showed the highest inhibition of the conidial germination. In addition, application of NSP4 reduced markedly *Fusarium* wilt disease in greenhouse conditions. Application of isolate NSP4 before inoculated pathogen, as prevention method, was significantly reduced disease severity (DS 2.8) higher than applied after inoculated pathogen, as eradication method (DS 3.3), while negative control (pathogen inoculation) was found in maximum scale (DS 5.0).

Keywords: *Streptomyces* sp., *Fusarium oxysporum* f. sp. *capsici*, chili, culture medium

Introduction

The total area of chili cultivation in Thailand has been estimated to be 720 km^2 (Mustafa *et al.*, 2006). The major disease of chili crop is *Fusarium* wilt caused by the soilborne pathogen *Fusarium oxysporum* (W. C. Snyder & H. N. Hans, 1940). It is common disease in *Solanaceae*, e.g. in chili, tomato, eggplant, and potato. 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 the host cultivar, the pathogen subspecies and environmental

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conditions. This disease causes wilting of seedlings, yellowing and finally death. *Fusarium* also causes plant to grow abnormally, or exploits the plant as the 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).

Various fungicides have been applied against *Fusarium* to control the disease in infested areas. While fungicides can play important role in the disease control, they can adversely affect other useful soil microorganisms as well as pollute the environment (Parker *et al*., 1985). Furthermore, formation of genetic resistance toward the fungicides has been identified (Kawchuk *et al*., 2001; Sela-Buurlage *et al*., 2001), especially in the 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 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* is gram-positive, mycelia-forming soil bacteria with 69 – 78% guanine-plus-cytosine (G+C) content in their genomes. The plant rhizosphere represents an uncharacterized source of microorganisms producing novel antimicrobial metabolites (Stackebrandt *et al*., 1997). The aim of the present study was to evaluate antifungal activities of *Streptomyces* sp. to control *F. oxysporum* f.sp. *capsici* causing *Fusarium* wilt disease of chili.

Materials and methods

Pathogen and pathogenicity test

Fusarium oxysporum f.sp. *capsici* was isolated from a chili cultivation by s*plitting* each *stem* into two parts, then placed in a moist chamber, and incubated at the room temperature (RT) for 2 days. The appeared mycelium was 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 served as the inoculating media after boiling, sterilization and packing in polythene film (100 g/bag). *F. oxysporum* f.sp. *capsici* colonies on PDA were cut from a peripheral part of A colony with a cork bore of 5-mm diameter, then transferred to sterilized sorghum seeds in a bag (10 seeds/bag) and mixed well. The inoculated bags were incubated at RT for 14 days in the dark before testing.

All isolates of *F. oxysporum* f.sp. *capsici* were tested for pathogenicity with 10-week-old chili seedling cv. Chomthong 2 by inoculating soil with the pathogen inoculum at rate 100 g/1 kg of soil-compost mixture (3:1) from Mea Hia Agricultural Research, Demonstrative and Training Center, Chiang Mai University, Thailand. The roots of the seedlings were trimmed and transplanted into the inoculated soil, one seedling per pot of 9-cm diameter. The development of the disease was visualized beginning a week after inoculation. The disease was rated on a scale from 1 to 5 as follows: 1 = no symptoms; 2 = slight chlorosis, wilting or stunting of plant; 3 = moderate chlorosis, wilting or stunting of plant; 4 = severe chlorosis, wilting or stunting of plant, and 5 = dead plant (Marlatt *et al.*, 1996). Cultivars with average disease ratings higher than 2.5 were considered susceptible. The most virulent isolate was selected for further experiment. The experiment was done with applying Randomized Completely Block Design (RCB) with three replicates.

Antagonistic strains

Six soil actinomycetes strains NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6, present in the culture collection of the Laboratory of the 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* sp. based on morphological study, chemotaxonomic 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.

Antifungal tests on Petri dishes

Antagonism tests of the six *Streptomyces* strains (above) against *F.oxysporum* were carried out on Petri dishes containing glucose yeast malt agar (GYMA, Shirling and Gottlieb, 1966) with the dual culture method (Fokkema, 1978). The isolates of the antagonistic strains were streaked at 3 cm apart from each others on one side of a Petri dish for 4 days earlier than the

pathogen, preassuming the slow growth of these actinomycetes in culture and their secondary metabolite production and incubated at RT. The 5-mm mycelial disc of 7-day-old pathogen was placed on the same dish on anoter side of the dish after the 4th day. Paired cultures were incubated at RT for 7 days. Dishes inoculated only with test pathogens served as controls. The experiment was done using Completely Randomized Design (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 media on conidial germination

Preparation of pathogen inoculum

F. oxysporum f.sp. *capsici* FoC4, 7-day-old culture grown on Petri dishes with PDA at RT, was flooded with a 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. The mycelial suspension was then filtered through a sterile cheese cloth. The concentration of conidia in the suspension was determined with a hemacytometer and adjusted to 1×10^4 conidia/ml.

Preparation of culture media

The strains NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6, grown on GYMA for 10 days at RT, were separately cultured in 250-ml Erlenmeyer flasks containing 50 ml "enzyme production medium" (EPM) modified from Rattanakit *et al*. (2000). The flasks were incubated on a rotating shaker at 2.26 g (35 ^{o}C) either for 5 or 7 days. The culture medium of each of the isolates was divided into two parts: non-filtered culture medium (NF) and filtered culture medium (F). The cultures were centrifuged for 20 min at 4,355 g (4 ^{o}C) and the half of supernatants was collected as the NF. Another half supernatant was then filtrated through membrane filter pore size 0.22-µm (Minisart®) to get the F (Chareunrat, 1999).

Fifty microlitres of the pathogen suspension and each of the 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 cm^2, and then transferred to sterile microscope slides. The control consisted of equal suspension of the pathogen conidia in sterile distilled water. The slides were incubated in a moist chamber for 24 h including three replicate slides for each culture medium type and isolate and checked the conidial germination after 12 h. The germination was defined to be positive if the germ tube had

developed longer than the cell width. The percent of germination of *F.oxysporum* was determined by counting 100 conidia from each isolate under a light microscope with magnification of 40. The conidial suspension without actinomycetes culture medium served as the control. The CRD study was done with three replicas. Factor A represented incubation period of *Streptomyces* sp. culture media: A1 = 5-day-old, and A2 = 7-day-old. Factor B represented type of *Streptomyces* sp. culture media: B1 = NF type, and B2 = F type. Factor C represented isolate of *Streptomyces* sp.: C1 = NSP1, C2 = NSP2, C3 = NSP3, C4 = NSP4, C5 = NSP5 and C6 = NSP6.

Efficiency of Streptomyces sp. NSP4 under greenhouse conditions

Preparation of pathogen inoculum

The most virulent *F. oxysporum* f. sp. *capsici* isolate FoC4 was prepared as described before in the context of the pathogenicity test.

Preparation of Streptomyces sp. inocula

Rice seeds were used as inoculating media according to Fermino (2007) afrter boiling, sterilization and packing in polythene bags (100 g/bag). *Streptomyces* sp. isolate NSP4, grown on GYMA for 10 days at RT, was flooded with sterile distillated water. Culture suspension was collected, and then transferred to sterilized rice seeds (10 ml/bag) and mixed well. The pathogen inocula were incubated in the dark for 14 days before testing.

The pathogen and antagonistic microbes were mixed with soil-compost mixture (3:1) from Mea Hia Agricultural Research, Demonstrative and Training Center, Chiang Mai University, Thailand, in a proportion of 100 g/1 kg of soil. Various methods of treatment details were designed: (i) soil mixed with NSP4 inoculum and incubated for 4 days, followed by inoculation with the pathogen and incubated for 7 days (T_1); (ii) the pathogen was inoculated in soil and incubated for 7 days, then mixed with NSP4 inoculum and incubated for 4 days (T_2); (iii) soil was mixed with NSP4 together with inoculated pathogen and incubated for 7 days (T_3); (iv) pathogen inoculated in soil and incubated for 7 days as negative control (T_4); (v) pot containing sterilized soil as positive control (T_5); (vi) NSP4 inoculum mixed soil and incubated for 4 days (T_6); (vii) sterilized rice mixed soil and incubated for 7 days (T_7); (viii) sterilized sorghum mixed with soil and incubated for 7 days (T_8); (ix) combination of T_7 and T_8 (T_9); (x) $T_7 – T_9$ served as the inoculating media to be checked. The 10-week-old chili seedlings cv. Chonthong 2, grown in strilized soil, was used. Seedling roots were trimmed and further transplanted into pots contained one

per pot. One week after inoculation, the development of a disease was estimated on a scale from 1 to 5 (Applied from Marlatt *et al.*, 1996), as previously described. The test was done using RCB with three replicates.

Statistical analyses

Treatment means were statistical compared using the Least Significant Difference (LSD) at $P \leq 0.05$.

Results

Pathogen

Four isolates were collected from vegetative chili samples showing typial *Fusarium* wilt symptoms, and were coded 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 (Figure 1a), and then became purplish-white with aging. Mycelia were branched, septate and hyaline. They formed 3-6 celled hyaline slightly curved conidia (Figure 1b) with average 29.0-57.0 × 3.5-4.0 µm.

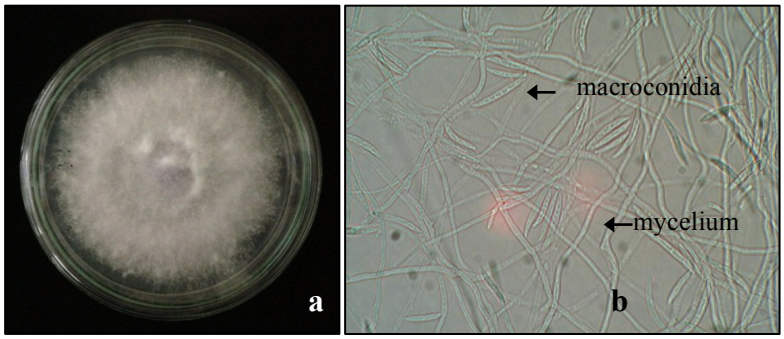

Fig. 1. Morphological character of *Fusariumoxysporum* f.sp. *capsici* isolate FoC4 at 10 days; a. Colony on PDA and b. mycelium and macroconidia (40X)

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 possessed clearly the highest disease index of chili wilt (Fig. 2) with DS = 5.0. This isolate was selected for the more detailed studies.

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

Antagonism test

Six *Streptomyces* sp. were tested for antifungal activities against *F. oxysporum* FoC4 grown on GYMA. There were no significant differences in the inhibition between the species (Table 1).

Table 1. Percentage inhibition of radial growth of *Fusarium oxysporum FoC4* caused by the six studied *Streptomyces* species on GYMA at 7 days. Radial growth means colony size of *F. oxysporum*

Isolate of *Streptomyces* sp.	**Inhibition of radial growth (%)**
NSP1	81.0[1]
NSP2	79.4
NSP3	75.7
NSP4	77.0
NSP5	79.3
NSP6	79.4
F-test	ns[2]
$LSD_{0.05}$	6.3
CV (%)	4.5

[1]average of three replicates
[2]ns: non significant according to the Least Significant Difference (LSD) at $P \leq 0.05$.

Inhibitory effects of culture media on conidial germination

The 5-day-old of both 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 media, ranging from 45.8 – 62.7% and 24.4 – 39.7%, respectively. The efficiencies of NF were significantly higher than F in

every isolates. In addition, the average inhibition indicated that isolate NSP4 caused the strongest inhibition to the conidial germination followed by the isolates NSP1, NSP2, NSP6, NSP3 and NSP5 (Table 2). Moreover, morphology of some conidia treated with the culture media appeared abnormal and could not develop to mycelium, while the mycelium of untreated conidia was elongated (Fig. 3).

Table 2. Effects of six *Streptomyces* sp. culture media (NF and F), incubated for 5 and 7 days, on inhibiting the conidial germination of *Fusarium oxysporum* isolate FoC4 on GYMA inspected after 12 h of tratment

Isolate of *Streptomyces* sp.	**5-day-old**		**7-day-old**		**Mean**
	NF[1]	F	NF	F	
NSP1	62.7	53.2	34.9	31.1	45 AB
NSP2	58.0	51.7	36.6	31.7	44 B
NSP3	53.6	46.4	28.4	26.1	39 D
NSP4	58.8	56.2	37.1	33.9	46 A[2]
NSP5	56.4	45.8	27.3	24.4	38 D
NSP6	60.0	48.6	32.4	39.7	43 C
Mean	58.3 a	50.3 b	33.8 c	29.5 d	42.7
A (incubation period)		***	$LSD_{0.05}$ = 1.0		
B (type of culture medium)		***	$LSD_{0.05}$ = 1.7		
C (isolate of *Streptomyces* sp.)		***	$LSD_{0.05}$ = 1.0		
A*B		ns			
A*C		***	$LSD_{0.05}$ = 1.4		
B*C		ns			
A*B*C		ns			
CV (%)		4.9			

[1]NF = non-culture medium filtrate, F = culture medium filtrate

[2]Average of three replicates. The differences between isolate of *Streptomyces* sp. in the row and type in the column of each pair are indicated by upper and lower case letters, respectively. The different letters differ by LSD at $P \leq 0.05$.

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

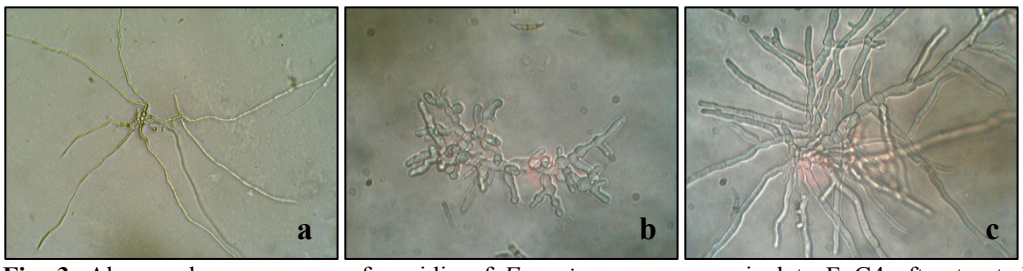

Fig. 3. Abnormal appearances of conidia of *Fusarium oxysporum* isolate FoC4 after treated with culture media (NF and F) of isolate NSP4 on GYMA for 12 h; a. control (10X), b. treated NSP4-NF (40X) and c. treated NSP4-F (40X)

Efficiency of Streptomyces sp. isolate NSP4 under greenhouse conditions

Treatment of mixing NSP4 in soil before inoculation the pathogen gave the lowest disease severity (DS 2.8) of *Fusarium* wilt causing *F. oxysporum* f.sp. *capsici* isolate FoC4, followed by treatment of mixing NSP4 in soil after inoculation the pathogen (DS 3.3) and treatment of mixing NSP4 in soil together with inoculation the pathogen (DS 3.7), respectively when compared to pathogen inoculated control (DS 5.0). The NSP4 wad no any effect, which gave disease severity in scale 1.7 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 1.0 (Table 3; Fig. 5).

Table 3. Efficacy of *Streptomyces* sp. isolate NSP4 on *Fusarium* wilt disease of chili seedlings cv. Chonthong 2 under greenhouse condition

Treatments	**Disease severity scale**[1]
soil mixed NSP4 before inoculated pathogen	2.8 C
soil mixed NSP4 after inoculated pathogen	3.3 BC
soil mixed NSP4 togerther with inoculated pathogen	3.7 B
pathogen inoculated in soil	5.0 A[2]
sterilized soil	1.0 D
NSP4 mixed soil	1.7 D
sterilized rice mixed soil	1.0 D
sterilized sorghum seed mixed soil	1.0 D
sterilized rice + sterilized sorghum mixed soil	1.0 D
F-test	***
$LSD_{0.05}$	0.8
CV (%)	28.2

[1]Disease severity (DS) was scored at 10 days after inoculation. 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.

[2]Average of three replications. Means with the same common upper letters in each column are not significantly different according to LSD at $P \leq 0.05$.

*** significantly different at $P < 0.001$

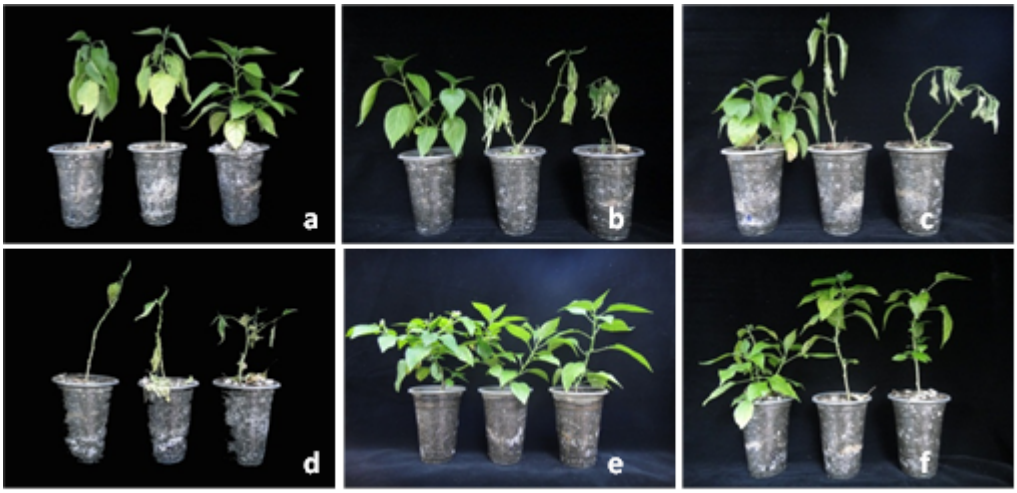

Fig. 4. Effects of *Streptomyces* sp. isolate NSP4 on reduction *Fusarium* wilt symptoms caused by *Fusarium oxysporum* FoC4; a. treatment of soil mixed NSP4 before inoculated pathogen, b. treatment of soil mixed NSP4 after inoculated pathogen, c. soil mixed NSP4 together with inoculated pathogen, d. pathogen inoculated in soil, e. sterilized soil, and f. NSP4 mixed soil

Discussion

F. oxysporum isolate FoC4 was isolated from chili stems. It showed typical symptoms of *Fusarium* wilt disease, and was assed to be the most virulent isolate among those studied ones causing wilt of chili. 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. *capsici*.

The present study focused on genus *Streptomyces* which usually produces antifungal compounds and extracellular hydrolytic enzymes, chitinase and beta-1,3-glucanase (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* sp., was previously 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 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* species against chili anthracnose caused by *Colletotrichum gloeosporioides*. The third day of incubation, the isolate NSP4 showed significantly highest chitinase activity (0.15 U/ml), followed by NSP2, NSP6, NSP1, NSP5 and NSP5 (0.11, 0.10, 0.80, 0.79 and 0.51 U/ml, respectively). Therefore, the inhibitory activities of these six *Streptomyces* species against *Fusarium* may involve chitinase enzyme. 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 six *Streptomyces* species 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. In experiments done in greenhouse conditions, *Fusarium* wilt disease was significantly reduced with preventive treatment with no adverse effects on plants. The results clearly show that the studied *Streptomyces* specias are very promising as biocontrol agents in practise. Next studies should be extended to develop appropriate storable formulations which will be as ready-for-use form followed by their testing in field conditions.

Conclusion

This present study showed a strong protection of chili plants against *Fusarium* wilt disease caused by *F. oxysporum* f.sp. *capsici* by biocontrol agents produced by antagonistic *Streptomyces* species. These effects wwere shown both *in vitro* and in greenhouses. The studied *Streptomyces* species either separately or as a ixture can be the basis of developing new effective biocontrol agents to be used for organic chili. The studies on the actual biocontrol mechanisms remains to be studied in the future.

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