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Evaluation of *Streptomyces* Sp. Culture Media Extracts for Control of Strawberry Anthracnose Disease

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Abstract Six strains of *Streptomyces* sp.; NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6, were evaluated for their antifungal activities against *Colletotrichum gloeosporioides* isolate Cg_MCL8 (highly resistant to carbendazim fungicide; HR) causing strawberry anthracnose. The bioactive component was produced using an enzyme production medium (EPM) by incubation with shaking for 5 days, and divided into 2 parts: non-filtered culture medium (NF) and filtered culture medium (F).There were no significant difference between inhibitory effects produced by the NF of all strains on the pathogen, but the NSP3 was slightly less than the other five strains of F(64.44-67.40% for NF, and 57.77-63.70% for F) and inhibition of conidial germination(delayed germination) (52.38-67.16% for NF, and 51.10-61.69% for F at 6 h after treatment). Some culture medium extract-treated conidia were morphologically abnormal and were not able to develop into mycelium. Moreover, pathogen growth was inhibited on strawberry leaf surfaces treated with NF or F media extracts. Germ tube germination and appressorium formation of pathogen on the NF of NSP1-treated were also reduced. In addition, the disease index (DSI) was reduced by application of NF extracts (DSI = 1.11-1.33) under greenhouse conditions, and not significantly different from the commercial biofungicide; Laminar®(*Bacillus subtilis* AP-01) (DSI = 1.11-1.33), compared to a DSI of 2.88 for the inoculated control.

Keywords: *Colletotrichum gloeosporioides***,** strawberry, anthracnose**,** filtered and nonfiltered bacterial extracts, biofungicides

Introduction

Several species of the fungal plant pathogen *Colletotrichum* cause strawberry anthracnose, a major disease of this crop. The principal pathogens known to be responsible for the disease are *C. gloeosporioides*, *C. acutatum* and *C. fragariae* (Howard *et al*., 1992; Smith and Black, 1990). The pathogen may attack strawberry crowns, petioles, leaves, fruit trusses, flowers and buds, and fruit (Howard *et al.*, 1992).Both fruit and crown rot severely reduce plant

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stands and yields (Ward and Hartman, 2012).Currently many chemical fungicides are being used to manage the disease. But these fungicides may adversely affect useful soil microorganisms and also pollute the environment. Hence, biological control offers a potential alternative to chemical fungicides (Parker *et al*., 1985).Furthermore, genetic resistance to each of these diseases has been identified (Kawchuk *et al*., 2001; Sela-Buurlage *et al*., 2001). A substitute method to reduce chemical control extensively investigated for over a decade is the use of antimicrobials (Shimizu *et al*., 2009). Biological control methods, based on the use of beneficial microorganisms isolated from suppressive soils, such as *Streptomyces*, which are known to produce a variety of antimicrobial compounds, and from the plant rhizosphere, a potential source of many microorganisms producing novel antimicrobial metabolites (Stackebrandt *et al*., 1992, 1997), represent analternative for protection of plants against anthracnose disease of strawberry (Alabouvette *et al.*, 1993).The objective of this study was the evaluation of soil actinomycetes belonging genus *Streptomyces* as biocontrol agents to manage strawberry anthracnose disease.

Materials and methods

Pathogen

Colletotrichum gloeosporioides isolate Cg_MCL8 (highly resistant to the fungicide carbendazim; HR), present in the culture collection of the Laboratory of the Department of Entomology and Plant Pathology of Chiang Mai University, Thailand, isolated from naturally infected strawberry leaves, was used for experimental inoculations. Cultures were grown on potato dextrose agar (PDA) at room temperature (RT) for 7 days before use.

Antagonists

This study used 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, and identified based on morphological characteristics,chemotaxonomy and analysis of the partial 16S rDNA sequence (Suwan *et al*., 2012). Strains were grown on glucose-yeast-malt extract agar (GYMA) at RT for 10 days.

Preparation of culture media extracts

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

Antifungal activities of the culture media extracts

Efficacy on mycelial growth

Inhibition of pathogen growth by the test compounds was carried out on PDA according to the agar well diffusion method (modified from Perez *et al*., 1990). The PDA consists of two layers, the upper layer only being inoculated.Thirty µlof each extract were pipetted into 5-mm-diameter wells.Agar plugs from 7-day-old PDA cultures of *C. gloeosporioides* strains were transferred to the center of the plates, and incubated at RT for 10 days. EPM without *Streptomyces* sp. served as the control. Data were collected as percent inhibition of radial growth (PIRG) modified from Soytong (1989) and Lokesha and Benagi (2007).Three replications were used arranged in a Two-Factors Factorial Design in Completely Randomized Design (CRD).Factor A represented type of culture media extract: A1 = non-filtered culture medium (NF) and A2 = filtered culture medium (F). Factor B represented strain of *Streptomyces* sp.: B1 = NSP1, B2 = NSP2, B3 = NSP3, B4 = NSP4, B5 = NSP5 and B6 = NSP6.

Effect on conidial germination

Colletotrichum gloeosporioides was prepared as conidial suspensions, and adjusted to 1×10^{6} conidia/ml using a haemocytometer. An equal volume (100 µl) of treated conidial suspensions from each culture medium was mixed and spread onto a GYMA plate, then cut into 1×1 cm sections and placed on a microscope slide. Conidial suspensions mixed with equal volumes of EPM served as the control. The slide cultures were incubated at RT and checked for conidial germination at 6, 9, 12, 18 and 24 h. In this context germination was defined as a germ tube that had developed to longer than the cell width. Three

replications were used arranged in aTwo-Factors Factorial Design in CRD. Factor A represented type of culture media extract: A1 = non-filtered culture medium (NF) and A2 = filtered culture medium (F). Factor B represented strain of *Streptomyces* sp.: B1 = NSP1, B2 = NSP2, B3 = NSP3, B4 = NSP4, B5 = NSP5 and B6 = NSP6. To estimate the percent germination, a total of 300 conidia were examined from each treatment (100 per replicate).

Effects of culture media extracts on strawberry leaf anthracnose

Effect on disease development

Thirty µl of each *Streptomyces* sp. extract was applied onto sterile strawberry leaves and incubated at RT for 2 days. Twenty µl of a *C. gloeosporioides* conidial suspension (1×10^6 conidial/ml) was applied to leaves and incubated for 4 days at RT. NF and F extracts without the pathogen and non-treated leaves served as controls. Leaf tissue was examined by cutting sections using a Leica CM1850 freezing microtome. Three replications were done per experiment.

Effect on conidial germination

One ml of extracts from *Streptomyces* sp. strain NSP1, serving as arepresentative strain, was sprayed on strawberry leaves, and incubated for 12 hat RT. Equal volumes of conidial suspensions (1×10^6 conidial/ml) were sprayed on to the leaves. EPM without *Streptomyces* spp. culture media served as the control. The treated leaves were incubated at RT. After 6, 9, 12, 18, 24, 30, 36 and 48 h, treated tissues were covered by clear nail polish, and air dried. The clear covering was removed and transferred to a microscope slide. Three replications were used arranged in a Two-Factors Factorial Design in CRD. Factor A represented the type of culture media extract: A1 = non-filtered culture medium (NF) and A2 = filtered culture medium (F). Factor B represented the strain of *Streptomyces* sp.: B1 = NSP1, B2 = NSP2, B3 = NSP3, B4 = NSP4, B5 = NSP5 and B6 = NSP6.To estimate the percent germination, a total of 300 conidia were examined from each treatment (100 per replicate).

Effects of culture media extracts as biofungicides under greenhouse conditions

The *Streptomyces* sp. strain NSP1 extracts were tested for efficacy against *C. gloeosporioides* under greenhouse conditions. Inocula of pathogen were

prepared as conidial suspensions (1×10^6 conidial/ml) with Tween-20 (3 drops/10 ml). Sixty-day-old strawberry seedlings were used in this experiment. Fifty ml of the NF were applied to various methods including soil drenching, spraying and combination of soil drenching + spraying. The commercial biofungicide; Laminar® (*Bacillus subtilis* AP-01), was used for comparison. After treatment with the strain extracts or Laminar® for 15 d, strawberry plants were inoculated with the pathogen by spraying10 ml of the conidial suspension/strawberry plant. Pathogen-inoculated and healthy seedlings served as positive and negative controls, respectively. The experiment used three replications and a Randomized Completely Block Design (RCBD). Data were collected at 10 d after inoculation. Disease was rated using a 0 – 4 scale) Dixon, 1981 and Khmel *et al*., 1998), as follows: 0 = no symptoms,1 = 0-25% disease symptoms on seedling, 2 = 26-50% disease symptoms on seedling, 3 = 51-75% disease symptoms on seedling and 4 = 76 – 100% disease symptoms on seedlings.Adisease severity index (DSI) (%) was calculated using Equation 1 for each treatment:

$$DSI = \frac{\Sigma (n_i \times i)}{n \times 5} \times 100 \qquad (1)$$

Where i is the score 1, 2, 3 or 4, n_i is the number of plants in category i and $n$is the total number of plants/treatment.

Results and discussions

Antifungal activities of the culture media extracts

Efficacy on mycelial growth

There were no significant differences between *Streptomyces* strains in terms of inhibitory effects produced by theirnon-filtered (NF) and filtered (F) culture media extracts on the pathogen except in the case of the F extract of strain NSP3 which had a significantly lower inhibitory effect than those of the other strains (Table 1). There were also no significant differences between the NF and F of strains NSP2, NSP4 and NSP6. In contrast, the NF extracts of NSP1, NSP3 and NSP5 were significantly more inhibitory than F extracts, suggesting that NF extracts of those isolates still contained viable *Streptomyces* sp. cells in suspension which could have continued to produce inhibitory compounds to a higher level than the F treatment. Furthermore, the chitinase activities of the F extract of these *Streptomyces* sp. strains were previously measured by Thotree *et al*. (2011). The chitinase enzymes found to be produced

by these strains have the ability to inhibit other fungal pathogens. Therefore, the inhibitory effects of these six *Streptomyces* sp. strains may be due to chitinase activity.

Table 1. Efficacy of culture media extracts of *Streptomyces* strains on inhibition of the mycelial growth of *Colletotrichum gloeosporioides* from strawberry

Strain	Percent inhibition of radial growth[x]		
	NF		F
NSP1	67.40 Aa[y]		59.99 Ba
NSP2	66.66 Aa		63.70 Aa
NSP3	67.40 Aa		33.33 Bb
NSP4	65.92 Aa		59.25 Aa
NSP5	66.66 Aa		57.77 Ba
NSP6	64.44 Aa		58.51 Aa
A (type of culture media extract)		***	$LSD_{0.05}$ = 2.86
B (strain of *Streptomyces* sp.)		***	$LSD_{0.05}$ = 4.95
A*B		***	$LSD_{0.05}$ = 7.00
CV (%)	6.82		

NF and F refer to non-filtered and filtered extracts, respectively.

[x]The agar well method was used. Radial growth was measuredat 10 d after treatment.

[y]Values of each column (a, b) and row (A, B) followed by different letter indicate significantly difference by $LSD_{(P<0.05)}$.

Effect on conidial germination

Inhibition efficacy on conidial germination by the extracts was related to the colony inhibition; the NF and F extracts of each strain showed no significant difference in inhibition of conidial germination. However, the efficiencies of all strains were reduced by the incubation time (Figure 1). Similarly, germ tube elongation also related to percent colony inhibition, the length of treated-germ tubes was significantly lower than the control (Figure 2). These results indicate that both culture media could inhibit conidia over time in terms of delayed germination. In addition, the morphology of some culture medium-treated-conidia was abnormal and they could not develop into mycelium. Previous studies reported abnormal-appearing conidia of other fungal pathogens when treated by culture media including *Fusarium oxysporum* f. sp. *capsici* causing wilt disease of chili, *F. oxysporum* f. sp. *lycopersici* causing wilt disease of tomato, *Curvularia* sp. and *Helminthosporium* sp. causing dirty panicle disease of rice (Chaisiri, 2010; Jaiyen, 2010; Modted, 2012), etc.

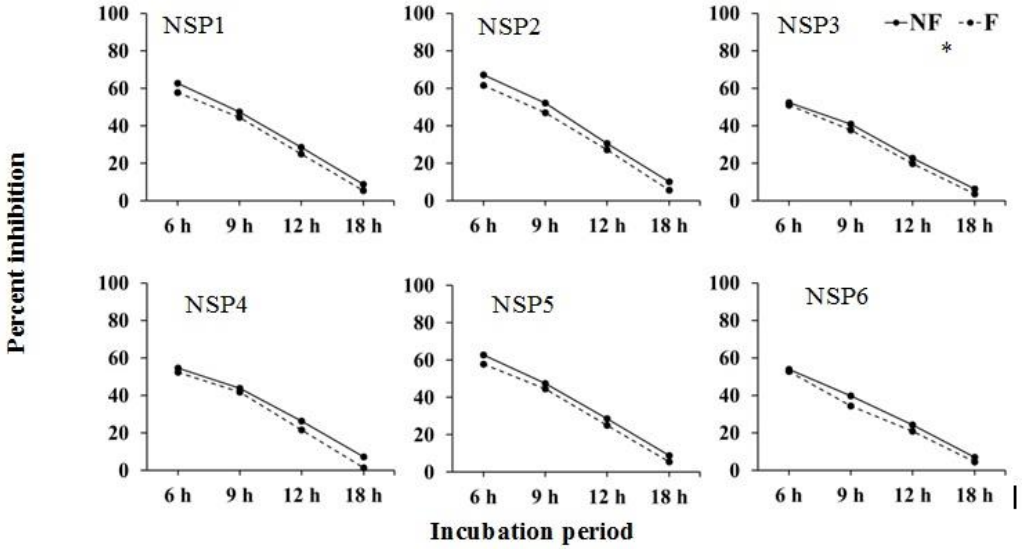

Fig. 1. The effect of culture media extracts of *Streptomyces* sp. strains on the conidial germination of *Colletotrichum gloeosporioides* Cg_MCL8 causing strawberry anthracnose. NF and F refer to non-filtered and filtered extracts, respectively**.**
*At 6, 9, 12 and 18 h after treatment, $LSD_{(P<0.05)}$ = 15.48, 12.48, 12.52 and 8.67, respectively.

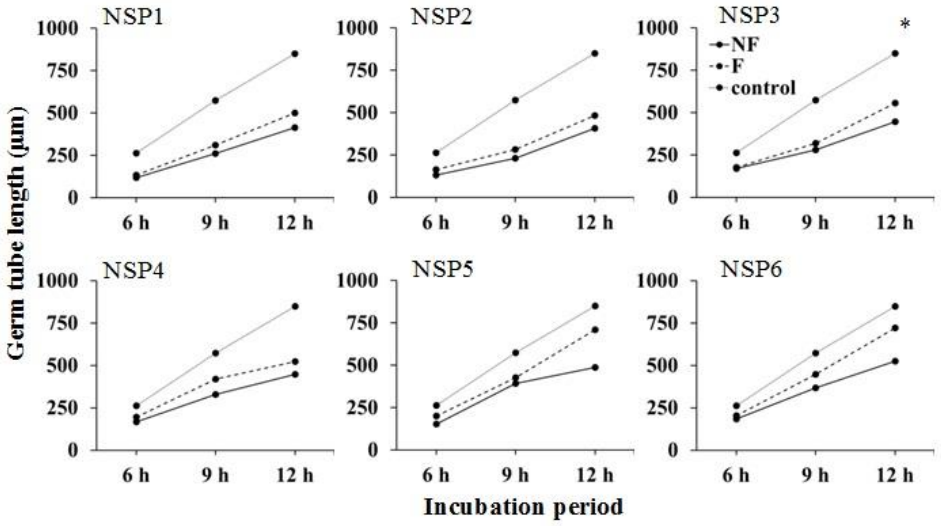

Fig. 2. Effect of of *Streptomyces* sp. strains on germ tube elongation of *Colletotrichum gloeosporioides* Cg_MCL8 causing strawberry anthracnose. NF and F refer to non-filtered and filtered extracts, respectively**.**
*At 6, 9 and 12 h after treatment, $LSD_{(P<0.05)}$ = 20.74, 44.78 and 45.53, respectively.

Effects of culture media extracts on strawberry leaf anthracnose

Effect on disease development

Conidia of pathogen formed germ tubes on the surface of plant tissue after 3 d (Figure 3A). At the site of penetration, tissue color at invasion area changed from green to brown, and finally died (Figure 3B).After that, asexual structures, acervuli, were formed and colonized under the cuticle or epidermis, and then formed conidia on conidiophores (Figure 3C). Curry *et al*. (2002) reported that germ tubes of *Colletotrichum* spp. began to be formed at 16 h after inoculation and that systemic severity gradually increased over a 3 d period. On the other hand, only inhibited conidia of pathogen were found on surface of plant tissue of NF- and F-treated leaves (Figures 3D and 3E) so the penetration process did not occur. In addition, tissue deterioration was not observed (Figures 3F and 3G) similar to the non-inoculated (Figure 3H). Loqman *et al*. (2009) found that six strains of *Streptomyces* spp. and three strains of *Micromonospora* spp., isolated from the rhizosphere soil of *Vitis vinifera* L. sampled from four Moroccan areas, had*in vitro* inhibitory effects on*Botrytis cinerea* causing gray mold of grape. Moreover, pathogen growth was stopped when plant tissues were treated with these strains.

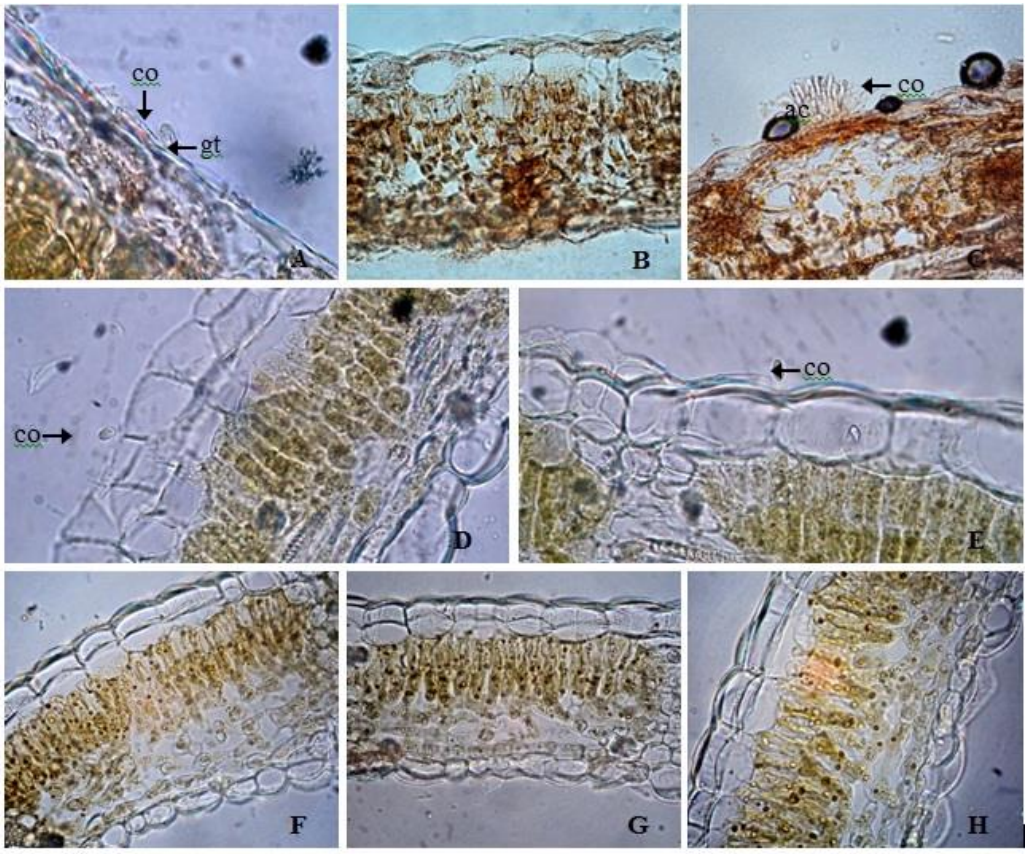

Fig. 3. Light micrographs (40X) of samples from strawberry leaves; (A) germ tube formation of *Colletotrichum gloeosporioides* Cg_MCL8, (B) dead cells at invasion areas, (C) acervulus formation on dead cells at invasion area, (D) strawberry leaf treated with non-filtered culture medium extract of *Streptomyces* sp. strain NSP1 before pathogen inoculation, (E) strawberry leaf treated with filtered culture medium extract of *Streptomyces* sp. strain NSP1 before pathogen inoculation, (F)strawberry leaf treated with non-filtered culture medium extract of *Streptomyces* sp. strain NSP1, (G) strawberry leaf treated with filtered culture medium extract of *Streptomyces* sp. strain NSP1 and (H) non-inoculated control
co = conidium, gt = germ tube and ac = acervulus

Effect on conidial germination

Germ tube germination of *C. gloeosporioides* on strawberry leaves began at 18 h after inoculation, while appressorium formation clearly began at 24 h after inoculation.At 18 h after treatment, germ tubes of NF- and F-treatedconidia wereslightlyshorter than control ($LSD_{(P<0.05)}$ =4.10) (Figure 4). Twenty four h after treatment, NF- and F-treatedgerm tubes were significantlydifferent than the control ($LSD_{(P<0.05)}$ =10.00, 7.11 and 6.88, respectively) and distinctly different at48 h after treatment ($LSD_{(P<0.05)}$ = 9.32).

co

Similarly appressorium formation in NF- and F-treatedconidia was significant different than the control at every period after treatment ($LSD_{(P<0.05)}$ =8.43, 6.60, 8.36 and 6.03, respectively) (Figure 4). However, the level of inhibition varied with type of culture medium extract; the NF gave significantly higher degree of inhibition than F which corresponded with the experiment on GYMA previously presented. Moreover, the percentages of germ tube germination and appresorium formation of EPM-treated conidia were not significantly different than the control (data not shown), demonstrating that all inhibitory effects resulted from *Streptomyces* strain NSP1.

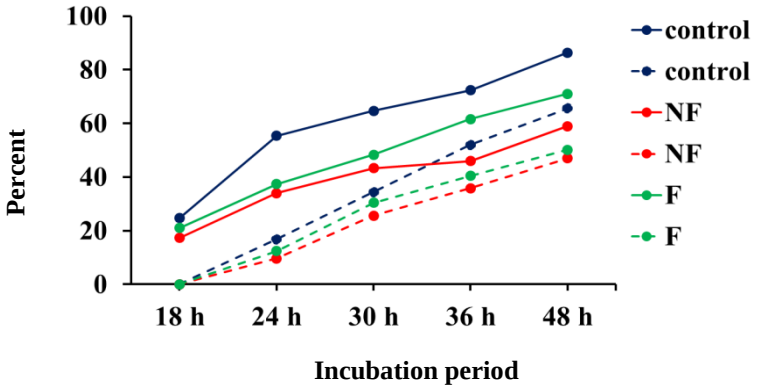

Fig. 4. Effect of culture media extracts of *Streptomyces* strains on germ tube germination (solid line) and appressorium formation (dashed line) of *Colletotrichum gloeosporioides* Cg_MCL8 causing strawberry anthracnose.NF and F refer to non-filtered and filtered extracts, respectively**.**

Effects of culture media extracts as biofungicides under greenhouse conditions

At 10 d after inoculation with the pathogen, the NF culture medium extract of *Streptomyces* sp. strain NSP1 produced a disease index reduction equivalent to the commercial biofungicide, Laminar® (*Bacillus subtilis* AP-01) (Table 2). Application methods, including soil drenching, spraying and combination of soil drenching + spraying, were not significant different. Moreover, EPM had no effect disease severity reduction, correspondingwith the experiment on strawberry leaves previously presented.Furthermore, this experiment indicates the practicality of applying a culture medium extract of *Streptomyces* sp.for disease prevention. The NF culture medium extract applied as a spray to leaf surfaces could certainly have had a direct inhibitory effect on the pathogen as shown in the experiments indicated above.However, the disease index reduction resulting from soil drenching, where there was no

direct contact of the NF extract with the leaves or pathogen, suggests that the extract may also indirectly inhibit *C. gloespoioides* by the induction of resistance in the plant.Many recent reports have shown that some microorganisms can elicit systemic acquired resistance (SAR), which maybe another mode of action of NSP1. Plant elicitors represent a new approach to integrated disease management strategies (Lyon and Newton, 1999).Moreover, plants also respond to a variety of chemical stimuli produce by soil- and plant-associated microbes. It has been suggested that the actinomycetes may cause plant defense responses in soybeansmost likely as the result of an induction or promotion of the phenylpropanoid pathway (Al-Tawaha *et al*., 2006).

Future researchshould evaluate the development of these six *Streptomyces* sp. strains as ready-to-use biofungicides. Moreover, their inhibitory effects on other plant pathogens should be studied. The six *Streptomyces* sp. strains examined in this study appear to be potential biological control agents against strawberry anthracnose caused by *C.gloeosporioides.*

Table 2. Efficacy of a non-filtered (NF) culture medium extract of *Streptomyces* sp. strain NSP1 on the severity of strawberry leaf anthracnose caused by *Colletotrichum gloeosporioides* Cg_MCL8 under greenhouse conditions at 10 dafter pathogen inoculation

Treatments	Disease index[x]
non treated control	0.00 d[y]
inoculated control	2.88 a
soil drenching with EPM	2.55 ab
spraying with EPM	2.44 b
combination of C_3 + C_4	2.44 b
soil drenching with Laminar®	1.33 c
spraying with Laminar®	1.44c
combination of C_6 + C_7	1.22 c
soil drenching with NSP1-NF	1.22 c
spraying with with NSP1-NF	1.33 c
combination of T_3 + T_4	1.11 c
F-test	***
$LSD_{0.05}$	0.37
CV (%)	13.34

[x]Disease was rated using a 0 – 4 scale at 10 d after inoculation.A disease severity index (DSI) (%) was calculated by DSI = $[\Sigma (n_i \times i) \times 100]/(n \times 5)$ for each treatment.
[y]Different letters following means indicate that they are significantly different by $LSD_{(P<0.05)}$.

Conclusion

The present study described the ability of six *Streptomyces* sp. strains to inhibit the strawberry anthracnose fungal pathogen, *Colletotrichum gloeosporioides*. The application of non-filtered culture medium (NF) and filtered culture medium (F) extracts showed good *in vitro* antifungal properties. Their bioactive component may represent an alternative resource for the biocontrol of plant diseases and could provide an interesting lead for further development of novel fungicides.

Acknowledgements

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