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In vitro and *In vivo* Evaluation of *Streptomyces* Suppressions against Anthracnose in Chili Caused by *Colletotrichum*

(Penilaian Penindasan *Streptomyces* Secara *In vitro* dan *In vivo* terhadap Antraknos dalam Cili Disebabkan oleh *Colletotrichum*)

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ABSTRACT

The isolation of 66 streptomycetes from rhizosphere soil of chili plants was done for their inhibitory activities against three different dominant species of Colletotrichum *namely* C. acutatum, C. gloeosporioides *and* C. capsici. *Twenty one streptomycetes strains were active against at least one of the* Colletotrichum *species. In addition, ten strains that inhibited the* in vitro *growth of* Colletotrichum *species showed chitinase activity. Strain P42, which displayed the highest inhibitory activity against all three anthracnose fungi species and high chitinase activity was tested as biological control agent in a greenhouse study. The strain successfully controlled chili anthracnose disease by significantly reducing the disease severity. Phylogenetic analysis of the 16S rRNA gene sequences showed that strain P42 belongs to the* Streptomyces rochei *clade. The results of the current study showed that rhizosphere-derived soil of chili plants is an important source of bioactive streptomycetes which are antagonistic against* Colletotrichum.

Keywords: Biological control; chili pepper; Colletotrichum*; inhibitory activity;* Streptomyces

ABSTRAK

Enam puluh enam pencilan streptomiset daripada tanah rizosfera tumbuhan cili telah dilakukan saringan aktiviti antimikrob terhadap tiga spesies kulat Colletotrichum *yang berbeza iaitu* C. acutatum, C. gloeosporioides *dan* C. capsici. *Dua puluh satu strain streptomiset didapati aktif terhadap sekurang-kurangnya salah satu spesies* Colletotrichum. *Tambahan pula, sepuluh terikan streptomiset yang mampu merencat pertumbuhan* Colletotrichum *secara* in vitro *juga menunjukkan aktiviti kitinase. Strain P42 yang memaparkan aktiviti perencatan tertinggi terhadap ketiga-tiga spesies kulat kajian dan juga mempunyai aktiviti kitinase tinggi diuji sebagai agen kawalan biologi dalam kajian rumah hijau. Strain P42 ini berjaya mengawal penyakit antraknos cili. Analisis filogenetik gen jujukan 16S rRNA menunjukkan bahawa strain P42 kepunyaan klad* Streptomyces rochei. *Keputusan kajian ini memberikan gambaran bahawa tanah rizosfera tumbuhan cili adalah sumber penting streptomiset bioaktif yang berupaya mengawal pertumbuhan* Colletotrichum.

Kata kunci: Cili padi; Colletotrichum*; kawalan biologi; saringan aktiviti;* Streptomyces

INTRODUCTION

Colletotrichum species are one of the most important plant pathogens that causes anthracnose disease and are effective on decreasing the value of crop yield production between 10 and 80% in tropical developing countries like Pakistan, India, Thailand, Mexico and Malaysia (Freeman et al. 1998; Than et al. 2008). Members of this genus are responsible for causing anthracnose disease in fruits such as chili pepper, papaya, guava and mango (Peres et al. 2002). Three notable *Colletotrichum* species affecting chili farms in Malaysia are *C. acutatum, C. capsici* and *C. gloeosporioides* (Yun et al. 2009). These pathogens are also the main plant pathogens in the South Asia and America continents, particularly in the Asian tropical regions (Pakdeevaraporn et al. 2005). *C. capsici* generally infects mature chili fruits while *C. gloeosporioides* has the ability to infect both green and mature fruits (Pakdeevaraporn et al. 2005). Although synthetic chemicals have been successfully used to inhibit these pathogens, the excessive use of harmful chemicals can pollute the environment. Furthermore, the effectiveness of these chemicals is continuously decreasing due to the development of resistant pathogens. Improvement of alternative control methods including the use of microorganisms as biocontrol has started 25-30 years ago (Pakdeevaraporn et al. 2005). Microorganisms used for biological control usually have the ability to control the pathogenic population by means of physical inhibition or by production of antimicrobial compounds (Cho et al. 2003; Yakoby et al. 2001; Yoshida et al. 2002). Actinomycetes, especially *Streptomyces* is a useful source of antimicrobial compounds (Berdy 2005; Demain & Fang 1995; Taddei et al. 2006). *Streptomyces* are responsible for approximately 60% of biologically active inhibitors that have been implemented for agricultural usage (Ilic et al. 2007). *Streptomyces* strains have been reported to control

plant diseases caused by nematodes, fungi and bacteria, indicating their broad spectrum of activity against other microorganisms. They had been isolated from various types of soil (Kim et al. 1998), rice paddy farmland (Xu et al. 1996), lake mud and water (Jiang & Xu 1996), deciduous forest, tropical forest, wasteland (Xu et al. 1996), cave soils and mangrove mud (Getha & Vikineswary 2002; Vikineswary et al. 1997).

In this study antagonistic streptomycetes isolated from rhizosphere soils of chili plants were evaluated for the ability to inhibit *C. acutatum*, *C. capsici* and *C. gloeosporioides* under *in vitro* assay. Therefore the aims of this study were to isolate, identify and investigate *in vitro* and *in vivo* antimicrobial activities of soil actinomycetes to control *C. acutatum*, *C. gloeosporioides* and *C. capsici* that cause chilli anthracnose disease. A further investigation was then carried out to evaluate the most potent strain to protect chili plants from anthracnose under greenhouse conditions. The strain was also identified based on its 16S rRNA gene sequences. The nucleotide sequence was deposited in the GenBank database library under the accession number JN967802.

Materials and Methods

Fungal Pathogen

Three different *Colletotrichum* species namely *C. acutatum*, *C. capsici* and *C. gloeosporioides* previously isolated from infected chili pepper (*Capsicum annuum* L. Kulai) were obtained from the Fungal Biotechnology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya. The fungal pathogens were maintained on 2% distilled water agar at 4ºC.

Isolation of Streptomycetes from Soil

Rhizosphere-derived soils (six samples) were collected from a farm planted with chili in Ulu Chuchoh (101º41' E; 2º41' N) and Sungai Burung (101º8' E; 3º48' N) in the state of Selangor, Malaysia. Soil samples were taken at 5-15 cm depth with sterile cork borer from the root area of plants without destroying the plant. The soil samples were air dried for three days and 1 g of the dried soil was suspended in 9 mL normal saline solution (0.9% NaCl, w/v). Serial dilution of suspension was done to achieve final dilutions of 10^{-2} and 10^{-3}. An amount of 0.1 mL of each 10^{-2} and 10^{-3} dilution was spread on starch-casein agar (SCA) (containing 10 g/L starch, 2 g/L KNO_3, 0.3 g/L casein, 0.01 g/L $FeSO_4.7H_2O$, 0.02 g/L $CaCO_3$, 2 g/L K_2HPO_4, 0.05 g/L $MgSO_4.7H_2O$, 2 g/L NaCl and 15 g/L agar) and raffinose-histidine agar (containing 10 g/L raffinose, 1 g/L histidine, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$ and 12 g/L agar). Nalidixic acid (25 µg/mL) and cycloheximide (50 µg/mL) were added to both media to inhibit the growth of Gram-negative bacteria and fungi. Incubation of plates was done at 28°C for 14 days (Labeda & Shearer 1990). Putative streptomycetes were purified on ISP3 (containing 20 g/L oatmeal, 12.5 g/L agar, 0.001 g/L FeSO4.7H2O, 0.001 g/L $MnCl_2.4H_2O$, 0.001 g/L $ZnSO_4.7H_2O$) (Shirling & Gottlieb 1966). Plates were incubated at 28°C for 14 days (Shirling & Gottlieb 1966) and maintained as suspension of spores and mycelial fragments in 30% (v/v) glycerol at -20°C for long-term (Labeda & Shearer 1990) and on ISP3 agar slant at 4°C for short-term preservations.

Purification and Dereplication of Streptomycete Strains

Strains were purified and selected based on phenotypic features such as colony morphology, the colour of aerial and substrate mycelia and pigmentation characteristics on ISP3 medium as suggested by Shirling and Gottlieb (1966).

Screening of Antifungal Activity

A modified 'cross-plug' assay method (Getha & Vikineswary 2002) was used to test the ability of streptomycete strains to inhibit the growth of three species of *Colletotrichum*. Using a sterile cork borer, a 5 mm diameter plug of a seven-day old actinomycete on ISP4 agar was placed upside-down on a potato dextrose agar (PDA) plate and incubated at 28±2°C for five days. Then, a 5 mm diameter agar plug of seven-day old of *Colletotrichum* (*C. acutatum, C. capsici* or *C. gloeosporioides*) was placed 3 cm away from the streptomycete plug. The growth of test fungi (from agar plugs) on PDA without streptomycete, were used as controls. The plates were further incubated at 28±2°C and the inhibition of mycelial growth, if any, was observed after five days. This assay was conducted in three replicates.

To observe for morphological changes of the fungal mycelia occurring at the margin of the inhibition zone, three mycelia plugs were cut from the assay plates at different points of the inhibition zones within each plate. Samples were then fixed overnight in 2% (v/v) aqueous osmium tetraoxide at 4°C. Samples were washed three times with distilled water and dehydrated in a cold graded ethanol series (15 min for each step: 30, 50, 70, 80, 90 and 100% v/v), followed by two changes of absolute ethanol. The alcohol was replaced gradually by different mixtures of acetone and ethanol (30:70, 50:50 and 70:30 v/v). Samples were then washed twice in absolute acetone. The dehydrated samples in acetone were critically point dried using liquid carbon dioxide. The dried samples were mounted on aluminium stubs using double-sided adhesive carbon tape, sputter-coated with gold and examined in a JEOL JSM-6400 scanning electron microscope. The strains exhibiting good antifungal activity were selected for further studies.

In addition, seven-day old cultures of each streptomycete strains were transferred into sterile vials containing 2 mL semi-solid SCA (1 g agar/L) and gently mixed to homogenize the suspension. Suspension was adjusted to a concentration of 10^5 cfu/mL. The suspension (25 µL) was then placed as a single drop at the centre of colloidal chitin agar (CCA) plates (Hsu & Lockwood 1975)

in three replicates and incubated at 28±2°C for 14 days. The horizontal and vertical diameter of the clear zone around each drop-colony was measured and averaged.

BIOLOGICAL CONTROL OF COLLETOTRICHUM IN GREENHOUSE CONDITION

Bacterial suspension from a seven-day old pure culture of the most potent strain against *Colletotrichum* species was prepared. Suspension was adjusted to 10^5 cfu/mL. Aerial spores of *Colletotrichum* species were suspended in 10 mL sterile distilled water and filtered through double-layered cheesecloth to remove mycelial fragments. The inoculum concentration of 10^5 spores/mL was prepared using a haemocytometer and immediately used for chili-fruit inoculation in *in vivo* biocontrol assay (Jetiyanon & Kloepper 2002).

Eighty chili plants were numbered and grouped for the eight different treatments (Table 1) in a complete randomized design (CRD). The bacterial suspension was applied by soil drenching when chili plants were 45 days old. The fertilizer (N-P-K 13:13:21) was then incorporated six days after drenching. The 90-days old chili plants with at least four fruits per plant were challenged with either one of the three *Colletotrichum* species namely *C. acutatum*, *C. capsici* or *C. gloeosporioides*, by drenching the pepper fruit with the respective fungal spore suspension. The plants were then maintained in a moist chamber for 24 h and then, transferred to a greenhouse. The *in vivo* biocontrol assay was repeated three times.

Disease severity (DS) was assessed nine days after the fungal spore had been introduced. Two fruit samples were randomly collected from each plant for the fruit symptom evaluation. The severity of the symptom presented was graded (0 = fruit was healthy, 1 = 10% of fruit area was infected, 2 = 25% fruit area infected, 3 = 50% of fruit area infected, 4 = 75% of fruit area infected and 5 = 100% of fruit area infected) (Jetiyanon & Kloepper 2002). Disease severity index was calculated using McKinney's formula (1923), $\mathrm{DSI} = \frac{\sum v \times n}{N \times Z} \times 100$, where v is the disease severity, n is the number of infected plants with disease, N is the total number of plants and Z is the maximum disease severity.

All data were analyzed using analysis of variance (ANOVA) and the treatment means were separated using Fisher's protected least significant difference (LSD) test at p=0.05 using SAS software version 9.1 (SAS Institute, Gary, NC, USA).

DNA EXTRACTION AND 16S RRNA GENE AMPLIFICATION

The biomass for DNA extraction was obtained by growing the streptomycete strain on a non-sporulating agar medium (2% casamino acid, 2% starch, 0.4% yeast extract, 1.8% agar) for seven days at 28±2°C (Ishikawa et al. 2000).

Genomic DNA was isolated using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instruction. The 16S rRNA gene were amplified using primers 27f and 1525r (Lane 1991). Amplifications were performed using SwiftTM Maxi Thermal Cycler (Esco, Singapore) in a final volume 50 µL containing 20 ng genomic DNA, 2.0 mM MgCl2, 1× *Taq* buffer with KCl, 0.2 mM of each dNTP, 0.4 µM each primer and 2.25 U recombinant *Taq* DNA polymerase (Fermentas). Thermal cycling was carried out as follows: pre-denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 56ºC for 1 min and extension at 72°C for 1 min. The final extension was at 72°C for 10 min and the reaction mixture was stored at -20°C until use. The 1.5 kbp amplified 16S rRNA gene fragment was purified using a QIAquick gel extraction kit (QIAGEN, Germany) before being sent for sequencing.

16S RRNA GENE SEQUENCING AND PHYLOGENETIC ANALYSIS

The 16S rRNA gene fragments were sequenced using ABI 3730XL automated sequencer by First Base (Malaysia). The 16S rRNA gene sequences (more than 1300 bp) were checked and aligned with comparative sequences of reference strains retrieved from the GenBank database through the EzTaxon server (Kim et al. 2012) using Chromas version 2.33 (Technelysium Pty. Ltd. Australia) and MEGA 4.0 (Tamura et al. 2007), respectively. An unrooted phylogenetic tree was constructed based on the neighbour-joining method (Saitou & Nei 1987) via evolutionary distances calculated with the Jukes-Cantor

TABLE 1. Treatment group of chili plants in *in vivo* biocontrol assay

Treatment group[a]	*Colletotrichum* spore suspension	Bacterial suspension
1 (negative control)	Absent	Absent
2 (positive control)	Absent	Present
3	Present (*C. acutatum*)	Absent
4	Present (*C. acutatum*)	Present
5	Present (*C. capsici*)	Absent
6	Present (*C. capsici*)	Present
7	Present (*C. gloeosporioides*)	Absent
8	Present (*C. gloeosporioides*)	Present

[a]: Each treatment group contained ten replicates (bags); one plant per bag. Control treatment groups were treated with sterile distilled water in place of the bacterial or fungal spore suspension

model (Jukes & Cantor 1969) using MEGA 4.0 (pairwise deletion option) (Tamura et al. 2007). The phylogenetic tree was statistically tested using bootstrap phylogeny analysis with 1000 replications.

Results and Discussion

Isolation of Actinomycetes

Rhizosphere soil of chili plants was used for isolation of actinomycetes because of abundance and diversity which were almost twice in comparisons with non-rhizosphere associated soils (Crawford et al. 1993). In this study, a total of 82 putative actinomycetes were isolated from six rhizosphere soils (three sample from each) collected from chili plants from Ulu Chuchoh and Sungai Burung in Selangor, Malaysia. The actinomycetes from each location were broadly classified as either streptomycetes or non-streptomycetes based on phenotypic characteristics (Table 2). A total of 66 putative streptomycete strains with aerial and substrate mycelium and 16 non-streptomycetes strains with only substrate mycelium were isolated and purified. Streptomycetes were notably dominant in the rhizosphere soil samples collected from different sites in the selected chili farms. The predominance of streptomycetes in many actinomycete populations also had frequently been reported (Wang et al. 1999; Xu et al. 1996).

Furthermore, the use of antagonistic actinomycetes for the biological control of root-attacking fungi, Gupta et al. (1995) reported similar activity against several phytopathogenic fungi and Saadoun et al. (2000) against several food-associated fungi and moulds. These findings have shown the potential of actinomycetes as biological control agents.

Bioactivity of Streptomycetes Against *Colletotrichum*

In the present study, 31.8% of tested streptomycete strains showed antagonism against one or more test fungi. Among the 66 streptomycete strains, 21 strains (31.8%) were active against at least one of the *Colletotrichum* (Table 3). From these 21 strains, 16 (24.2%) showed very strong to moderate inhibition against one or more test fungi and ten strains (15.2%), showed inhibitory activity, at varying degrees, against all three *Colletotrichum* species tested. Previously, 33% of selected *Streptomyces* isolated from a Malaysian coastal mangrove area showed antagonism to fungal plant pathogens (Vikineswary et al. 1997).

The inhibition observed in this study may be due to the antifungal compounds or chitinase produced by *Streptomyces* (De & Chandra 1983; Yeo et al. 1994). Links et al. (1957) described an antifungal antibiotic from a soil isolate of *Streptomyces* that caused bulging at the hyphal tips of several fungi. The site of action of these antibiotics was related to fungal cell wall biosynthesis (Satomi et al. 1982). There are many reports related to antibiotic substances that induced malformation such as stunting, distortion, swelling and hyphal protuberances of fungal germ tubes (Getha & Vikineswary 2002). The production of antibiotics has been suggested as the principal mechanism of action of many actinomycetes in biological control (Jones & Samac 1996).

Moreover, ten (47.6%) of the 21 active strains demonstrated chitinase activity by producing clear zones on colloidal chitin media ranging from 10-75 mm in diameter. They arbitrarily can be placed into three groups: Five strains with high chitinase activity (45-75 mm), three strains with moderate activity (30-40 mm) and two strains with low activity (10-30 mm) (Table 3). The two most active strains were strain P42 and strain P8 with a clear zone diameter of 75 and 73 mm, respectively (Figure 1). The chitinase-producing actinomycetes could be used as biological control agents because chitin is a major constituent of the pathogens' cell wall (Hsu & Lockwood 1975). Therefore, the production of this enzyme would be useful for selection of actinomycetes as potential biocontrol agents against this pathogen (El-Tarabily et al. 2000). This shows the effectiveness of chitinase as a potential fungicidal mechanism against mycelial germination and growth of *Colletotrichum*. The strains with high levels of chitinase production are more antagonistic to fungus compared with lower producers of chitinase. High levels of chitinase activity of the strains P8 and P42 *in vitro* corresponded with high levels of antagonistic activity against mycelium growth as well as fungicidal effect against *Colletotrichum*. However, high levels of chitinase activity of strains P46, P121 and P128 were not accompanied by strong inhibition of fungal growth. This may due to selective anti-fungal properties of bacterial chitinase (De Boer et al. 1998). The non-chitinase producers like strain P3 on the other hand showed a weak fungistatic inhibition (×<15 mm) of fungus mycelia growth. This weak inhibition may be due to

TABLE 2. Distribution of representative groups of putative actinomycetes isolated from various chili rhizosphere-derived soils

Source of sample	Streptomycetes[a]	Non-streptomycetes[b]	Total isolates
Ulu Chuchoh chili rhizospheric soil	16	4	20
Sungai Burung chili rhizospheric soil	50	12	62
Total isolates	66	16	82

[a]Colonies forming substrate and aerial mycelia with powdery aerial spore mass. [b]Colonies forming only substrate mycelia, small and compact, waxy or slimy, orange to orange-brown to black in colour

TABLE 3. Inhibition of *in vitro* growth of *Colletotrichum* and chitinase activity of selected streptomycetes

Strains	Degree of inhibition[a]			Chitinase activity[b]
	C. acutatum	*C. capsici*	*C. gloeosporioides*	
P3	1	1	1	-
P6	0	0	2	-
P8	3	3	3	+++
P10	0	2	2	-
P11	2	1	2	++
P42	4	4	4	+++
P43	3	3	1	-
P44	1	2	2	-
P45	0	1	1	-
P46	0	1	0	+++
P54	1	0	2	-
P75	0	0	2	-
P77	1	4	1	++
P78	0	2	1	+
P79	1	1	2	++
P83	2	2	2	-
P94	1	2	1	-
P98	0	2	1	+
P101	0	1	0	-
P121	0	1	1	+++
P128	1	0	2	+++

[a]4 – very strong inhibition, x>23 mm
3 – strong inhibition, 20 mm <x<23 mm
2 – moderate inhibition, 15 mm <x<20 mm
1 – weak inhibition, x<15 mm
0 – no inhibition

[b]+++ high activity (45-75 mm clear zone diameter)
++ moderate activity (30-40 mm clear zone diameter)
+ low activity (10-30 mm clear zone diameter)

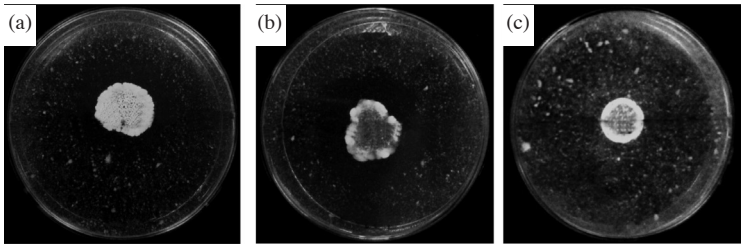

FIGURE 1. Colony growth of two chitinolytic streptomycetes (strains P8 and P42) and a non-chitinolytic streptomycete (strain P3) on CCA media showing clear zones around the colonies as an indicator of chitinase activity. (a): strain P8; (b): strain P42; (c): strain P3

other inhibitory mechanisms produced by non-chitinolytic strains (De Boer et al. 1998; Skujins et al. 1965). Hence, the non-chitinase and weak chitinase producers should not be over-looked as some may be good biological agents against some other plant pathogenic fungi.

The *Colletotrichum* used in this study differed greatly from each other in their sensitivity to antifungal effects of streptomycetes but strain P42 showed very strong antagonism towards all *Colletotrichum* tested.

Different modes of inhibition were observed against all test fungi when fungal mycelia were examined under scanning electron microscope (Figure 2). Hyphal tip lysis, folding back, stunted mycelium, disintegrated hyphae and curling of hyphae were observed on affected fungal growth. However, not all modes of inhibition were frequently observed for each fungus.

IN VIVO BIOCONTROL ASSAY

Greenhouse experimental trials were implemented to support the *in vitro* results of the most potent strain (P42). Strain P42 was selected for greenhouse study in several treatment groups based on its strong inhibitory activity and high chitinase production. In the presence of *Colletotrichum* (treatments 4, 6 and 8), inoculation with strain P42 significantly (p=0.05) decreased disease severity and increased fresh fruit weight of chili compared with treatments 3, 5 and 7 (*Colletotrichum* only) (Table 4). In the absence of *Colletotrichum* (treatments 2, positive

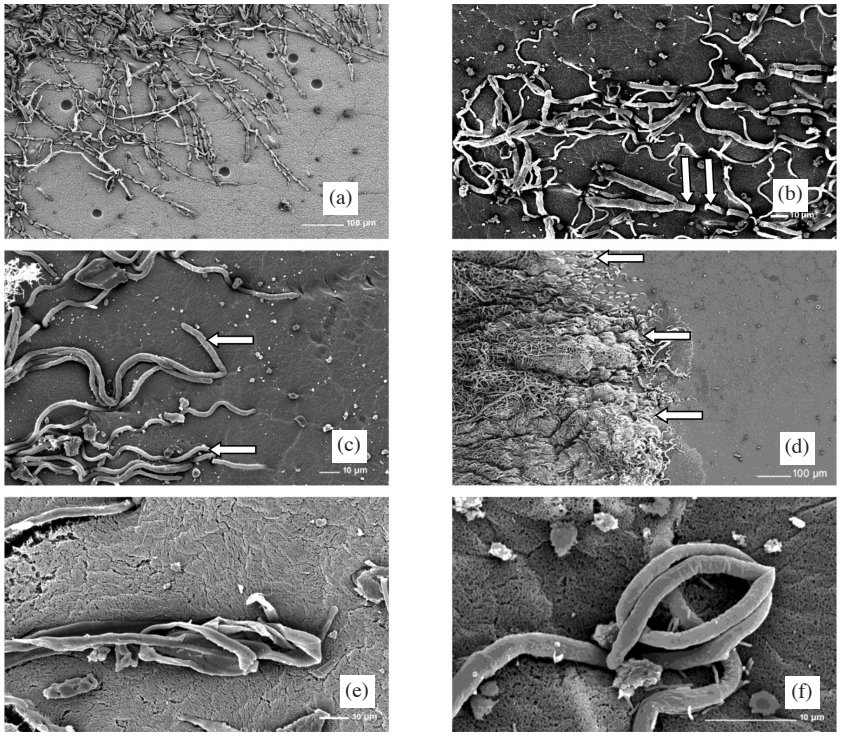

FIGURE 2. Representative scanning electron micrographs showing various modes of fungal inhibition. (a) Control plate hyphae with normal radial growth, (b) fragments of disintegrated hyphae in *C. capsici* caused by strain P8, (c) folding back and lysed hyphal tip in *C. gloeosporioides* caused by strain P8, (d) stunted mycelia in *C. acutatum* caused by strain P42, (e) folding back and lysed hyphae in *C. gloeosporioides* caused by strain P42, (f) curling of hyphae in *C. acutatum* caused by strain P42

TABLE 4. Summary of disease severity and fresh weight of chili fruits in the *in vivo* biocontrol assay

Treatment group	DS[a]	DSI (%)	Fruit fresh weight (g)[a,b]	Fruit death[c]
1[d] (negative control)	0.0C	0	11.92 ± 0.39B	0
2 (positive control)	0.0C	0	13.09 ± 0.29A	0
3	4.1A	83	3.78 ± 0.93D	30
4	1.4B	29	11.60 ± 0.61B	0
5	4.3A	87	3.15 ± 0.63D	24
6	1.7B	34	9.64 ± 1.45C	0
7	4.5A	90	3.05 ± 0.63D	30
8	1.8B	36	9.87 ± 1.43C	0

DS, disease severity; DSI (%), disease severity index; LSD, least significant difference.
[a]: Means with the same letter within a column are not significantly different ($P = 0.05$) according to Fisher's protected LSD test.
[b]: Values are means ± standard deviation of average fruit fresh weight from thirty plant replicates repeated in three independent experiment.
[c]: Total number of dead chili fruits
[d]: 1: Chilli plants only (No strain P42 nor *Colletotrichum* sp.); 2: Chilli plants with strain P42 only; 3: Chilli plants in presence of *C. acutatum* spores but no strain P42; 4: Chilli plants in presence of *C. acutatum* spores and strain P42; 5: Chilli plants in presence of *C. capsici* spores but no strain P42; 6: Chilli plants in presence of *C. capsici* spores and strain P42; 7: Chilli plants in presence of *C. gloesporioides* spores but no strain P42; 8: Chilli plants in presence of *C. gloesporioides* spores and strain P42

control), strain P42 was able to significantly (p=0.05) promote the increment of fresh fruit weight of chili fruits in comparison with treatment 1 (negative control). Though the presence of strain P42 decrease the severity of symptom in treatments 4, 6 and 8, however, the fresh fruit weight in treatment 6 and 8 was not significantly (p=0.05) maintained in comparison to treatment 1. However, dead fruits were only detected in fungal inoculated plants with the absence of strain P42 treatments.

Anthracnose symptoms in plants inoculated with *Colletotrichum* was significantly suppressed when plants were treated with strain P42. The anthracnose disease symptom was expressed severely in non-treated plants after nine days of inoculation of fruits with the pathogen. The *in vivo* biocontrol assays showed that there was an average disease severity reduction from 75 to 10% in term of fruit area destruction when treated with strain P42. This provided preliminary evidence that strain P42 has the

ability to interfere with anthracnose disease cycle and may be a potential biocontrol agent.

Furthermore, *in vitro* primary screening showed that strain P42 was highly active against the tested *Colletotrichum* and the *in vivo* biocontrol assay demonstrated the suppression of disease severity, thus suggesting but not proving that the mechanism of control is direct antagonism. Furthermore, strain P42 could have also induced systemic resistance against the three *Colletotrichum* as the strain P42 was drenched into the soil while the *Colletotrichum* was inoculated directly onto the fruits. This observation suggested that antagonistic effect of strain P42 in the rhizosphere was sufficient to reduce the disease severity.

According to Bell and Mace (1981), antifungal metabolites produced by antagonists could reduce invasion and subsequent development of pathogens and thus play a role in plant resistance. However, relying solely on the antifungal metabolites might not be effective as the active compound have to be harvested at the right time and at concentration sufficient to protect the infection sites from the pathogen (Marois 1990). Henceforth, inoculation of live streptomycetes cell would offer better protection prior to pathogenic invasion and provide continuous suppression of disease symptom.

To date, several *Streptomyces* antibiotics, which are known to be active against fungal pathogens, have been isolated (Ezziyyani et al. 2007). In the present study, it was speculated that the antifungal activity of *Streptomyces* strain P42 could have been one of the major factors that may induce the reduction in incidence of chili anthracnose disease by *Colletotrichum* as supported by the *in vitro* screening.

Besides antibiosis, competition and mycoparasitism have been described as mechanisms that may be involved in biological control of plant pathogens by biocontrol agents (Cook & Baker 1983). Therefore, in this study strain P42 was prepared and inoculated into the soil (rhizosphere area) when the chili plants were 45 days old. This was done to facilitate the adherence and adaptation of strain P42 to the soil that would allow better competitive edge over other microorganisms of the basal flora of the plant rhizosphere (Ezziyyani et al. 2007)

Even though it is always difficult to anticipate the biocontrol activity of a given strain from laboratory to natural environment (Hermosa et al. 2000), the results of the current study noticeably showed the potential of strain P42 for controlling chili anthracnose disease caused by *Colletotrichum*.

MOLECULAR PHYLOGENY OF STRAIN P42

Calculation of pairwise sequence similarity was used to identify strain P42 by using global alignment algorithm, implemented in the EzTaxon-e server (Kim et al. 2012). Strain P42 belongs to the genus *Streptomyces* and has 100% similarities with the sequences in the *Streptomyces* clade 119 which includes the *S. rochei*, *S. enissocaesilis*, *S. plicatus* and *S. vinaceusdrappus* and form distinct cluster from the nearest clade 118 (Figure 3). Although many of the molecular phylogenetic clades are consistent with previously described morphological and numerical taxonomic studies, however, due to insufficient variation present in the 16S rRNA gene sequence, there are members of the same clade within the genus *Streptomyces* that lack distinct branching in the phlogenetic analysis (Labeda et al. 2012). Furthermore many type strains within the

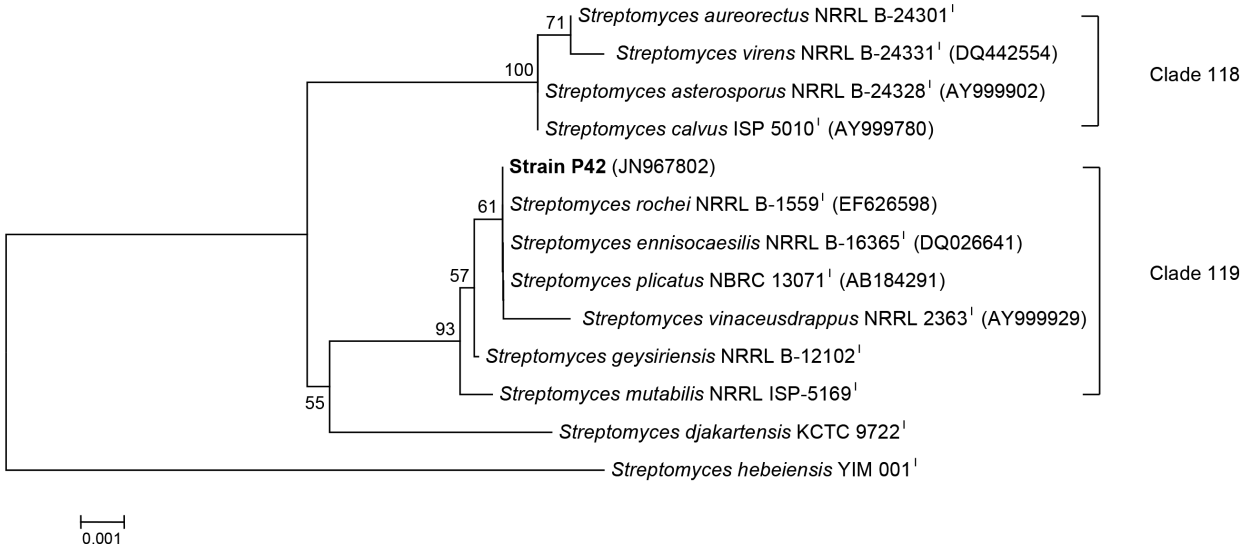

FIGURE 3. Neighbour-joining tree based on partial 16S rRNA gene sequences showing relationship between strain P42 and representative streptomycetes of clade 118 and 119 (Labeda et al. 2012). The numbers at the nodes indicate the levels of bootstrap analysis with 1000 replications; only values above 50% are shown. *Streptomyces hebeiensis* YIM 001^T (AY277529) was used as an outgroup. GenBank accession numbers are in parentheses following species or strain names. The scale bar indicates 0.001 substitutions per nucleotide position

genus *Streptomyces* could not be distinguished solely by phenotypic approach. Bergey's manual of systematic bacteriology (1989) had placed the members of clade 119 as synonymous species in its identification matrix. Therefore, identity validation of a given strain can only be resolved with additional genotypic and phenotypic methods.

CONCLUSION

In this study, streptomycetes were isolated from rhizosphere soils of chili plants. The streptomycetes possess a range of inhibitory activity against *C. acutatum*, *C. capsici* and *C. gloeosporioides* under the *in vitro* assay. In addition, some of these strains showed chitinase production. The selected *Streptomyces* strain P42, identified to belong to the *Streptomyces rochei* clade, had shown the potential ability in protecting chili plants from anthracnose under greenhouse conditions and could be used as promising biocontrol agents of anthracnose disease in chili plants. However, further biotechnological work and more field trials would need to be carried out to make it more effective and stable to be used in the control of *Colletotrichum* and chili anthracnose disease.

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