CHAPTER 3
ISOLATION AND SCREENING FOR ANTAGONISTIC ACTINOMYCETES AGAINST FUNGAL SEEDLING DAMPING OFF DISEASES IN ECONOMIC PLANT NURSERY

3.1. Abstract

Seedling damping off disease as the one of the most serious problems was found in Economic Plant Nurseries. *Phytophthora infestans* was isolated from the infected tomato plant seedling obtained from an economic plant nursery in Amphoe Pak Chong, Nakhon Ratchasima Province, Thailand. *Pythium aphanidermatum* was also isolated from the infected Chinese spinach seedling with obtained from nursery in Chiang Mai Province, Thailand. Six isolates of soil actinomycetes were screened for antagonistic against *P.infestans* and *P. aphanidermatum*. The isolate S4 isolated from termite mounds at the grove of Amphoe Si-sawat, Kanchanaburi Province, Thailand had promising antagonistic control of the two seedling damping off fungi. The isolate S4 preliminary classified to be the genus *Streptomyces* based on 16s r RNA gene sequencing which had 100% similarity to *Streptomyces fradiae* and *S. rubrolavendulae*.

Keywords : Seedling damping off /. *P. infestans* / *P. aphanidermatum* /*Streptomyces* / Antagonistic activity

3.2. Introduction

The value of vegetable crops in Thailand was estimated to be around 14,561 million baht in 2009, including tomato and chili. The plantation of these economic crops is done by using reliable seedling producers. Therefore, the economic plant nursery business has been increasing. Disease management has become a major concern during the production of vegetable plug transplants.

The seedling damping off disease causes serious problems in economic plant nurseries. Causative pathogenic fungi of seedling damping off disease in plants were reported to be *Pythium* spp., *Phytophthora* sp. (Joo, 2005), *Rhizoctonia solani* (Asaka and Shoda, 1996), *Sclerotium rolfsii* (Errakhi et al., 2009), and *Fusarium oxysporum* (Getha and

Vikineswary, 2002). The pathogen can affect many germinating seeds and young seedlings. In pre-emergence damping-off, seeds are withered or decayed and afterwards the germination is sparse and patchy. In post-emergence damping off, the pathogen infects the root-collar tissue or the roots and, within days, these seedlings become dark and rot nearly to the soil surface at the base of the stem and then wilt and die. These diseases can spread quickly in a few days depending on the environmental conditions of the greenhouse or nursery (Postma et al., 2005).

P. infestans is the most infamous species of genus which caused pre- and post-emergence damping-off and late blight of potato and tomato. Also peppers, melons, pumpkins, citruses, strawberries, chestnuts, and forest trees are affected by *Phytophthora* species such as *P. cambivora, P. hibernalis, P. citrophthora, P. kernoviae, P. capsici, P. cactorum, P. drechsleri* and *P. infestans* (Tyler, 2002; Brasier et al., 2005; Rizzo et al., 2005; Jonsson, 2006; Judelson, 2007; Lamour et al., 2012).

Pythium is an oomycete, a fungal-like organism, commonly found in soil and water as a saprophyte which called water molds. The genus *Pythium* is widely reported as a plant pathogen in pre- emergence plant seedlings and post-emergence plant seedlings (Sanchez and Gallego, 2002). In greenhouses, several *Pythium* are known as a causative to damping off, crown and root rot disease in cucumbers, soybeans, chickpeas, peppers, and tomatoes (Georgakopoulos et al., 2002; Sharma et al., 2007; Chen and Nelson, 2008; El-Tarabily et al., 2009; Kamala and Indira, 2011)

Greenhouses or nurseries are usually used a chemical fungicide, such as metalaxyl, to reduce populations of oomycetes in the soil (Mocioni et al., 2003; Martinez et al., 2005). Chemical fungicides are extensively used in current agriculture and also cause environmental pollution. Nowadays, biological control methods are sought as alternatives to chemical fungicides for controlling plant pathogens. The microorganism simultaneously grows together with pathogenic fungi and produced enzyme or organic compounds for suppression fungal growth. Microbial antagonists have been widely applied for controlling plant diseases such as *Streptomyces* spp., *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Trichoderma* spp.(Postma et al., 2005; Hansen et al., 2010). The variables registered biocontrol products for *Pythium*-causing diseases in Denmark are Mycostop (*S. griseoviridis*) and Supresivit (*Trichoderma harzianum*)

(Hansen et al., 2010). The biological control mechanisms involved can be mutualism, protocooperation, commensalism, neutralism, competition, amensalism, parasitism, and predation (Janisiewicz et al., 2000).

In this study, the major causative fungal pathogens of the seedling damping disease were investigated. The antagonistic soil actinomycetes against the fungal pathogen were screened and testified to be used control the disease in laboratory scale.

3.3. Materials and Methods

3.3.1 Isolation of Plant Pathogenic Fungi causing Seedling Damping Off Diseases in Economic Plant Nursery

1. Isolation and Maintenance of Pathogenic Fungi

The infected seedlings of tomato and Chinese spinach were obtained from nurseries in Nakhon Ratchasima Province and Chiang Mai Province, Thailand, respectively. The stems of seedlings were washed to remove any excess peat moss with sterilized water. Then, the infested plant parts were surface-sterilized using 5% (v/v) hypochlorite for 30 seconds, washed with sterilized water and blot dried on sterile filter paper. The particles of stem and soil sample were placed onto potato-dextrose-agar (PDA) (Appendix A) and incubated at room temperature for 2–3 days. When the mycelium of pathogenic fungi was appeared out of the particles on the media, the mycelium was cut at the edge of colony and placed on a new potato-dextrose-agar plate.

2. Identification of Pathogenic Fungi

The pathogenic fungi was identified based on the colony morphology (mycelium, oogonium and sporangium photographs) compared with the Van der Plaats-Niterink's photograph as a preliminary identification (Plaats-Niterink, 1981).

3.3.2 Isolation and Screening for Antagonistic Actinomycetes against Fungal Seedling Damping Off Diseases

1. Isolation and Maintenance of Actinomycetes

Soil samples obtained from organic-rich soils were collected in polythene bags, and transported to the laboratory. One gram of soil sample was added into 100 ml of selective medium and shaken on a rotary shaker (150 rpm) at 30ºC for 7 days. The actinomycetes were isolated by using the serial dilution standard plate-count technique.

The replicate aliquots (0.1 ml) from the selected dilutions were spread onto the surface of nutrient agar (Appendix B) then incubated at 30ºC for 5 days. The pure cultured of actinomycetes was transferred onto the selective medium and maintained in nutrient broth supplemented with 15% glycerol at -70ºC.

3.3.3 Antagonistic Test on Dual Culture Agar Plate

The pathogen-antagonist interaction was observed by using a dual culture technique on a solid medium. Six isolates of actinomycetes were streaked on PDA plate as four lines. The plates were then incubated at 30°C for 3 days to allow growth and sporulation of actinomycetes. Afterwards a 4 mm diameter freshly-growing mycelium plug of fungal pathogens was placed on the center of a plate and incubated for another 3 days. The pathogen alone was used as a positive control and the experiments were repeated three times. The non-fungal growth area surrounding the actinomycetes lines were indicated the antagonistic activity as positive result.

3.3.4 Identification of the Selected Actinomycetes

1. Cell and Spore Morphological Characterizations

Morphological characteristics of the best antagonistic actinomycetes were examined by light and scanning electron microscopy. Spore-chain morphology and spore-surface ornamentation were studied by examining gold-coated dehydrated specimens with a model FEI QUANTA electron microscopy.

2. Cultural Morphological Characterizations

The cultural morphological of the selected antagonistic actinomycetes strain S4were carried out by following the standard protocol of the International Streptomyces Project (Shirling and Gottlieb, 1966, 1968a, b). The aerial mass color, substrate mycelium color, and melanin pigments of antagonistic actinomycetes were observed after incubated 14 days at 30°C on standard media including nutrient agar (Waksman, 1961), bennets agar (BNA), yeast extract/malt extract agar (ISP 2), oatmeal agar (ISP3), inorganic salt-starch agar (ISP4), glycerol-asparagine agar (ISP5) (Appendix B).

3. Physiological Characterizations

- The carbon utilization media were modified from Pridham and Gottlieb (1958) (Appendix B). The cellobiose, lactose, arabinose, sucrose, mannitol, manose, and glucose were used as carbon sources for preparing solid media. The antagonistic actinomycetes was streaked and incubated for 14 days at 30°C.The record results were followed as 1.the positive utilization (+), when growing significantly better than growing on the basal medium without carbon but somewhat less than with glucose, 2.utilization doubtful (±), when growing only slightly better than growing on basal medium without carbon and significantly less than with glucose, 3.utilization negative (-), when growing similar to or less than growing on basal medium without carbon.

- The amino acid utilization media was modified from Pridham and Gottlieb, 1958 (Appendix B). L-leucine, L-asparagines, L-arginine mono, L-histidine mono, L-tyrosine, L-serine, L-glutamic acid, L-proline, L-lysine mono, and L-methionine were used as the substrate. The antagonistic actinomycetes was streaked and incubated for 14 days at 30°C. The growth of antagonistic actinomycetes was studied in order to reach a possible classification of actinomycetes.

- The degradation of polysaccharide was examined from the differences media with contained differences substrates. The colloidal chitin agar, chitosan agar, xylan agar, carboxy methyle cellulose agar, and starch agar were prepared (Appendix B). The antagonistic actinomycetes was spotted onto the medium plate and incubated for 5 day at 30°C. Afterwards the colloidal chitin agar, chitosan agar, xylan agar, and carboxy methyle cellulose agar plate were flooded with an aqueous of 1% (w/v) congo red for 15 min and then poured off. The CMC plates were continually washed by flooding 1.0 M NaCl for 15 min. The diameters of the colonies and the clear zone around the colonies on CMC agar were measured. The starch agar was flooded with Gram's iodine solution and observed the blue-black color formed with starch may fade giving a false-positive result of absence of starch. Appearance of a clear zone surrounding the bacterial growth indicates carbon hydrolysis (+) by the organism due to its production of the extracellular enzymes. The lack of a clear zone surrounding the growth indicates that carbon is present and has not been hydrolyzed (-) and the organism did not produce the extracellular enzymes.

Table 3.1 List of microorganisms for testing antimicrobial properties of the actinomycetes

Bacteria	Fungi
Bacillus subtilis	*Fusarium oxysporum*
Bacillus cereus	*Pythium aphanidermatum*
Aeromunas sp.	*Phytophthora infestans*
Listeria monocytogenes	*Rhizoctonia* sp.
Salmonella typhimurium	*Sclerotium rolfsii*
Staphylococcus aureus	*Penicillium* sp.
Escherichia coli	*Trichoderma* sp.
Vibrio harveyi	*Aspergirus niger*
pseudomonas aeruginosa	*A.orysae*

4. Antimicrobial properties

- Microorganisms used for testing antimicrobial properties of the actinomycetes were listed in Table 3.1. All the cultures from the culture collection of The Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi. All bacterial strains were maintained on LB medium (Appendix B) at 4°C. All fungal strains were grown on potato dextrose agar (PDA) for 7 days and maintained in the PDA slant covering with 15 % glycerol at 4°C.

- The actinomycetes was grown in 20 ml nutrient broth with agitation (150rpm) at 30°C for 48 hr used for antagonistic test. All bacterial strains were cultured in LB broth at 37°C for 24 h and then spread on the LB plates. 6 mm in diameter wells were punched in the agar with a sterile tip. 100µl of the selected actinomycetes was directly filled into the wells inoculated with bacteria strains and incubated at 37°C for 24 h. The antimicrobial activity was observed and reported as (+) positive results that mean the inhibition zone were appeared, (-) negative results that mean the inhibition zone was not appeared surround the agar wells.

- The dual culture technique method was used for observing the antagonistic interaction. The selected actinomycetes was streaked on PDA at the distance of 1.5 cm from the edge of the Petri dish. The plates were then incubated at 30°C for 3 days to allow growth and sporulation of the actinomycetes. Afterwards a 6 mm diameter freshly-growing mycelium plug of fungal strain was placed on the center of a plate and incubated for another 3 days. The pathogen alone was used as a positive control and the experiments were repeated three times. The non-fungal growth area surrounding the actinomycetes was indicated the antifungal activity.

5. Molecular Characterizations

- The antagonistic actinomycetes was grown in 20 ml nutrient broth at 30°C for 48 hour under shaking condition (150rpm). DNA was extracted by using the protocol for gram-positive bacteria method modified by (Mehling et al., 1995) (Appendix E).

- The 16S ribosomal RNA gene (16S rRNA) of the organism was amplified by polymerase chain reaction (Appendix D) using the forward primer as 27f (5'GAGTTTGATCCTGGCTCAG-3') and reverse primer as 1525r (5'-AAGGAGGTGATCCAGCC-3') (Brosius et al., 1978). The purified PCR product was stored at –20°C for sequencing analysis.

- The sequencing solution was prepared (Appendix D) and sequenced in ABI PRISM 310 sequencer (Applied Biosystems, USA) by using the primer 27f (5'-GAGTTTGATCCTGGCTCAG-3'), primer 357f (5'CTCCTACGGGAGGCAGCAG-3'), primer 937r (5'-TTGGTGCTGTATGCTGTAACTG-3') and primer 1525r (5'-AAGGAGGTGATCCAGCC-3') (Rintala et al., 2001). The sequence was searched for homology BLASTn in DDBJ nucleotide databases.

- The phylogenetic analysis was performed using the software package MEGA version 2.1 (Kumar et al., 2001) after multiple alignment of data by CLUSTAL_X (Thompson et al., 1997). A phylogenetic tree was reconstructed using the neighbor-joining method of Saitou and Nei (Saitou and Nei, 1987). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein with 1000 replicates (Felsenstein, 1992). The almost complete 16s

rRNA gene sequence of the actinomycetes was subjected to a BLAST search. Then, the phylogenetic tree of closely sequences of the 16S rRNA gene was constructed.

3.3.5 Comparison Test between Selected Actinomycetes, *S. fradiae* and *S. rubrolavendulae*

S. fradiae NBRC3360 and *S. rubrolavendulae* NBRC13683 from Biological Resource Center (NBRC) were used as the reference strains. Phenotype and genotype comparisons between strain S4, S. *fradiae* and *S. rubrolavendulae* were studied by following the method in Topic 3.3.1-3.3.3. The DNA-DNA hybridization between *Streptomyces* sp. strain S4, reference strains and *E. coli* as negative control were carried out as described by Ezaki (1988) with some modifications (Appendix D). The NIH image software for calculate whole peak was used.

3.4. Results and Discussions

3.4.1 Plant Pathogenic Fungi causing Seedling Damping Off Diseases

1. Pathogenic Fungi causing Seedling Damping Off Diseases from nurseries in Nakhon Ratchasima Province and Chiang Mai Province, Thailand

The major causative pathogenic fungi of seedling damping off disease in plants were reported to be *Pythium* spp., *Phytophthora* sp. (Joo, 2005). In this experiment, the result showed that the plant pathogenic fungus as oomycetes was isolated from the infected tomato plant seedling. It can be grown in PDA and produced white, profusely branching (Figure 3.1A). The plant pathogen fungus, oomycetes was also isolated from the infected Chinese spinach seedling pots obtained from plant nurseries in Chiang Mai Province, Thailand. The mycelium of isolated pathogenic fungi on PDA had white color (Figure 3.1B).

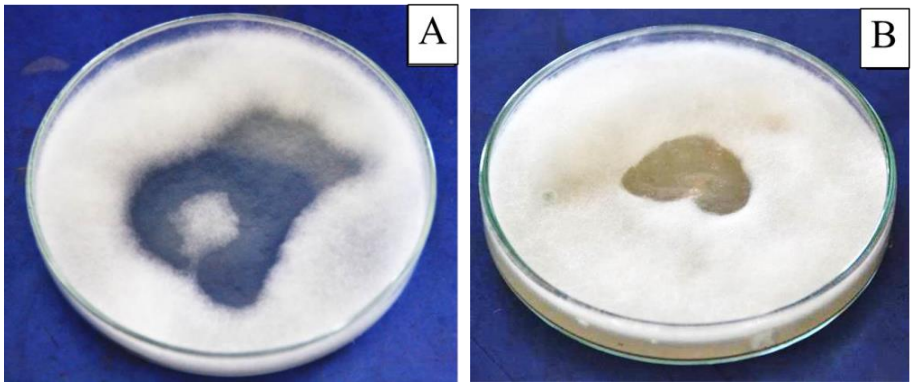

Figure 3.1 The plant pathogenic fungi, oomycetes isolated from the infected tomato seedling (A) and chinese spinach seedling (B).

2. Identification of the isolated plant pathogenic fungi

- **Identification of the pathogenic fungi isolated from tomato seedling**

The isolated pathogens fungi produced aseptate aerial mycelium, sporangia, and oospores. The aseptate mycelium without septum and hyphal swelling were observed under light microscopy. In the presence of light, chlamydospore and sporangia as asexual spore type were observed. The zoospores were in a lemon-shape sporangium (Figure 3.2A). When antheridia was attached to oogonium in the dark with high water contained, the fertilized oospores were produced (Figure 3.2B). *Phytophthora* often called water mold can be grown in wet soil and can spread widely with zoospores in oogonium (Lebreton et al., 1999; Mizubuti et al., 2000; Rubin et al., 2001; Porter and Johnson, 2004; Clement et al., 2010). From the results, the infected tomato plant seedling was *P. infestans* based on the morphological criteria. A pathogenic fungi infected tomato plant seedling was reported to be *P. infestans*, (Cohen et al., 1997; Cohen et al., 2000; Levin et al., 2001; Rubin et al., 2001). Therefore, the pathogenic fungus isolated from infected tomato seedling sample was *P. infestans*.

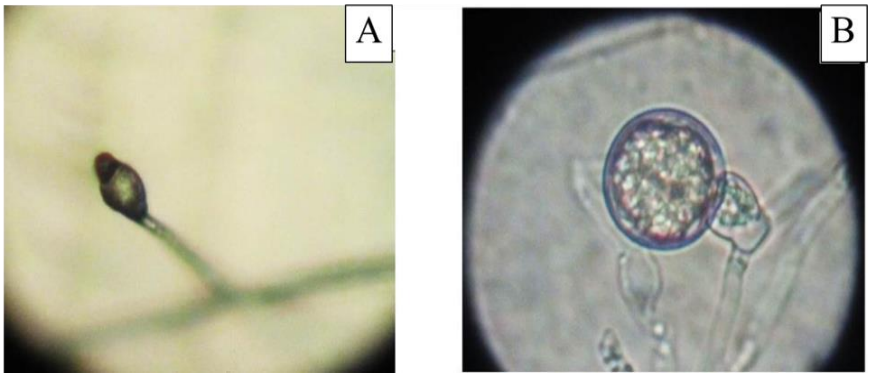

Figure 3.2 *Phytophthora infestans*, (A) Lemon shape sporangia, (B) Oospore.

- **Identification of the pathogenic fungi isolated from infected Chinese spinash**

The pathogenic fungi isolated from infected Chinese spinach had characteristics of genus *pythium*. From morphological identification, the pathogenic fungi formed sporangia as asexual spore which was developed by formation of tube and vesicle at the end of hyaline mycelium on the inverted cover plate which contained water. The zoospores were released from matured sporangium after the wall of sporangia vesicle disappeared under wet condition. The oogonium as sexual spore was also formed by attachment of the oogonium with more than one antheridia cell. The oospore was produced inside a thick smooth wall of spherical oogonium as a zygote. Oospore was formed a germ tube which used for infecting the host tissue or forming the sporangia (Figure 3.3). *Pythium* belongs to a group of fungal-like organism's oomycetes which are called water molds. Based on taxonomy, the morphological of *Pythium* spp. has been formed structures including hyphae (devoid of septa), sporangia, antheridia, and oogonia (van der Plaats-Niterink, 1981).

From the morphological characteristics, the isolated plant pathogen was primary identified to be genus *Pythium.* The results of morphological indicated that the pathogenic was *P. aphanidermatum*.

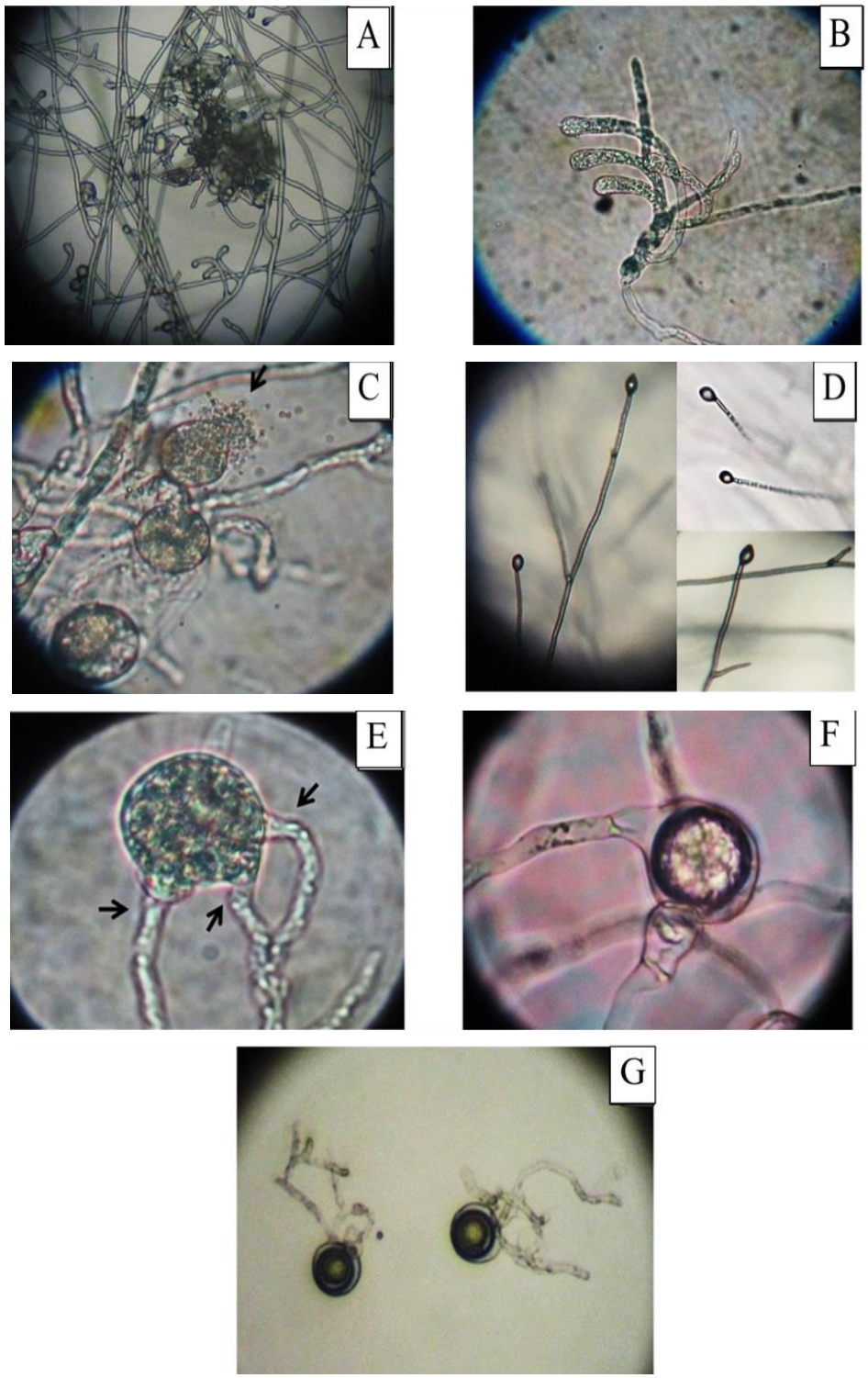

Figure 3.3 The mycelium and the asexual reproduction of *P. aphanidermatum*. Aseptate mycelium (A), Filamentous inflated sporangia (B), The matured sporangium (C) *(Arrow* indicates zoospores), The sporangia (D), Oogonium (E)*(Arrow* indicates antheridia), Oospore (F), The germinating oospores (G).

3.4.2 Isolation of Soil Actinomycetes

Actinomycetes is the major population of soil microorganisms which playing an important role in the soil community as saprophytes. The six isolated of actinomycetes which have different colony appearance were isolated from organic-rich soil samples (Table 3.2). They produced different color colony on nutrient agar (Figure 3.4). Normally, actinomycetes form the branching substrate and aerial mycelium and then developed cross-walls in aerial hyphae and the cells became spores or conidia. Within the developmental life-cycle, they can produce a wide range of secondary metabolites including the hydrolytic enzyme and bioactive compounds especially in sporulation phase.

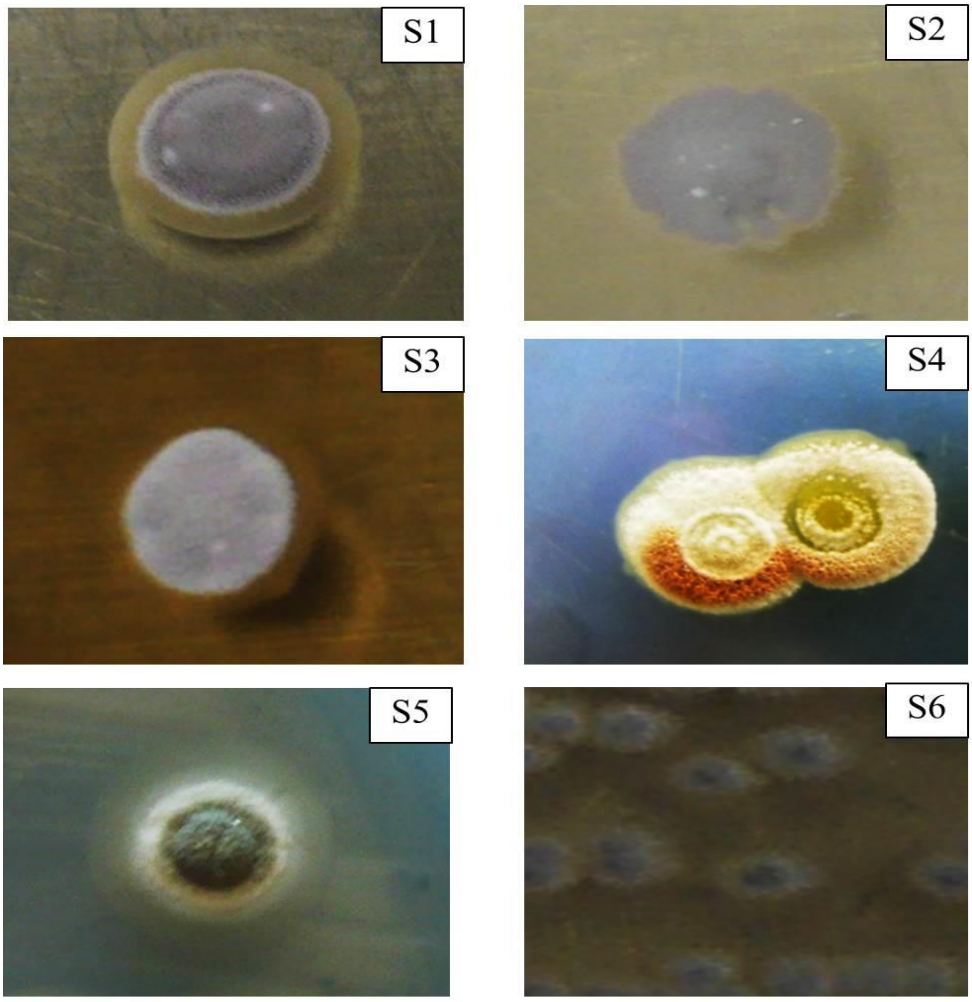

Figure 3.4 The colony of actinomycetes on nutrient agar.

Table 3.2 The colony appearance of actinomycetes isolated from organic-rich soil samples

Soil sample No.	Nature of the sample	Actinomycetes	Aerial mycelium	Substrate mycelium
1. Soil from a wood decay	Loam: organic matter rich soil (organic materials and woody residues) and black in color.	Actinomycetes strain S1	Grey, dense growth with extended white of spore mass	Whitish
2. Soil from the foothill	Sandy loam: Organic matter rich soil (leaf decay) and whitish in color.	Actinomycetes strain S2	Grey, smooth flat colony growth with short aerial mycelium	None
3. Soil from the foothill	Sandy loam: organic matter rich soil (leaf and wood decay) and whitish in color.	Actinomycetes strain S3	Light grey, dense growth with extended grey of spore mass	Whitish
4. Soil from the termite mound	Loam: Organic matter rich soil (lignocellulose and chitin polymers) and maroon in color.	Actinomycetes strain S4	White, dense growth with extended brown of spore mass from the edge to the center of colony	Yellowish
5. Molehill Soil from the forest	Clay loam: organic matter rich soil and maroon in color.	Actinomycetes strain S5	White, dense growth with extended dark green of spore mass from the center of colony to the edge	Whitish
6. Soil from the riverside	Clay: low organic matter and buff color.	Actinomycetes strain S6	grey, small convex colony growth with short aerial mycelium	None

3.4.3 Antagonistic Activity on Dual Culture Agar Plate

Many species of actinomycetes are well known as antagonistic bacteria against several plant pathogenic fungi, especially *Streptomyces* spp. Among six actinomycetes isolates, the strain S4 could inhibit growth of both pathogenic fungi of tomato seedling (*P. infestans*) and pathogenic fungi of Chinese spinach (*P.aphanidermatum*) with strongly reaction. Therefore, the strain S4 was selected to be used as biocontrol agent for controlling seedling damping off disease. The genus *Streptomyces* was reported to be used as biocontrol agents (Raaijmakers and Mazzola, 2012).

Table 3.4 Antagonistic activity of actinomycetes isolates against pathogenic fungi of seedling damping off disease.

Actinomycetes No.	Pathogenic fungi	
	P. infestans	*P. aphanidermatum*
Strain S1	-	-
Strain S2	-	-
Strain S3	-	-
Strain S4	+	+
Strain S5	-	-
Strain S6	-	-

+ positive , - negative

3.4.4 Identification of the Selected Antagonistic Actinomycetes

1. Cell and Spore Morphology of the Selected Antagonistic Actinomycetes Strain S4

The actinomycetes strain S4 stained gram-positive produced both substrate and aerial mycelium (Figure 3.5). Micrographs of strain S4 aerial mycelium were showed as rolled, bent and curled into spiral shape under light microscopy (Figure 3.6A). From electron micrographs, it made clear that the structure of the spores has been developed from the end of the mycelium. Each spore continued a long chain packed and arranged as a spiral approximately four to five spores per a helix (Figure 3.6B). Spores are oval shaped with a width and length about 0.637 microns and 1.246 microns, respectively. The surface of the spore was smoothed with round header and footer. The spores fallen apart when being moved or being exposed. S*treptomyces* is Gram-positive filamentous bacteria

with coiling spore chain (Williams et al., 1983; Waksman and Henrici, 1943). Therefore, the strain S4 was belongs to the genus *Streptomyces* based on the cell and spore morphological characteristics.

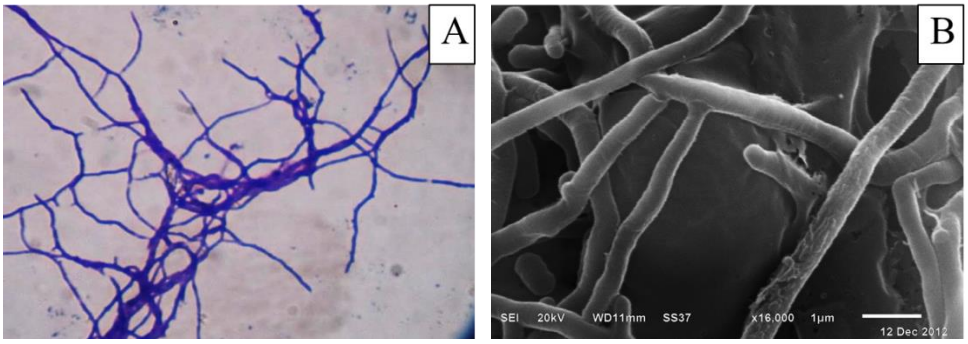

Figure 3.5 Micrographs of actinomycetes strain S4 mycelium, light microscopy (400x) (A), Scanning electron microscopy (B) Bar, 1µm.

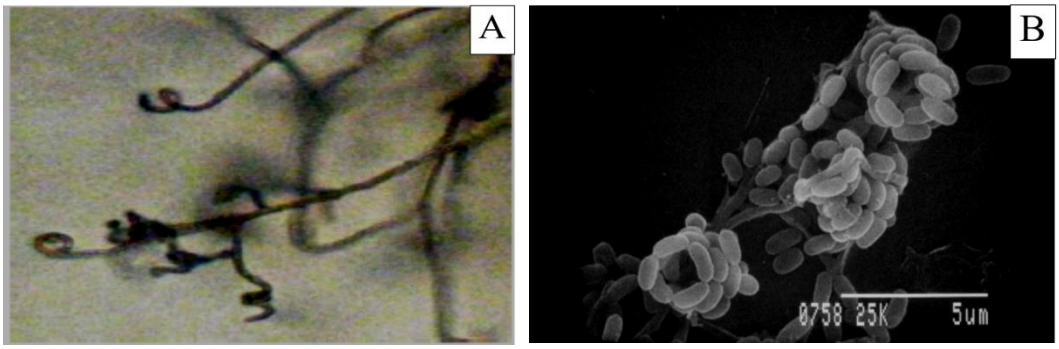

Figure 3.6 Micrographs of actinomycetes strain S4 spores, under light microscopy (400x) (A), Scanning electron microscopy, (B) Bar, 5 µm.

2. Colony Morphology of the Antagonistic Strain S4

The antagonistic strain S4 could grow well on oat meal agar, inorganic salt-starch agar, glycerol-asparagine agar, and nutrient agar, but grow moderately on yeast extract/malt extract agar and grow poorly on bennets agar. The colony of strain S4 was yellow color on yeast extract/malt extract agar and oatmeal agar. The surface of the colony was raise above the surface of the solid medium and had dimpled on the edge of colonies like jagged edges around a soda bottle cap (Figure 3.7A). The color of the colonies was yellow at first, and then gradually changed to white color due to the aerial mycelium production.

The edge of the surface colonies appeared white color first and extended the area into the whole colony which made the whole colony become white (Figure 3.7B). The color of the colony was changed from white to brown color when longer incubation. Colony surface was dry and powdery texture due to spores' production.

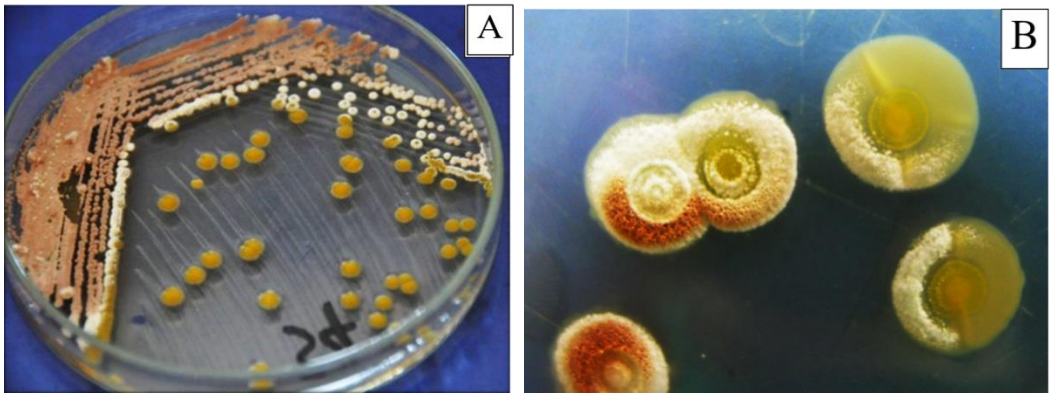

Figure 3.7 The colony of *Streptomyces* sp. strain S4 on nutrient agar.

3. Physiology of the Strain S4

Streptomyces sp. strain S4 could use cellobiose, lactose, arabinose, manose, and glucose except sucrose, mannitol and inositol. The strain S4 could metabolize L-asparagine, L-lysine, and L-methionine. The *Streptomyces* sp. strain S4 could degrade polysaccharide; colloidal chitin, xylan, carboxy methyl cellulose, and starch. The clear zone indicated that *Streptomyces* sp. strain S4 can produce the extracellular enzymes for hydrolyzing all of carbon sources. Genus *Streptomyces* have been previously reported they can produce many biodegradative enzymes such as chitinase, cellulase, and glucanase (Hand and Singh, 1994; Gupta et al., 1995; Lee et al., 2012).

4. Antimicrobial Activity of *Streptomyces* sp. Strain S4

Streptomyces sp. strain S4 antagonistic activity was tested against bacteria: *B. subtilis*, *B.cereus*, *Aeromunas* sp., *L. monocytogenes*, *S. typhimurium*, *S. aureus*, *E.coli*, *V. harveyi*, and *P. aeruginosa*, and fungi: *F.oxysporum*, *P. aphanidermatum*, *P. infestan*, *Rhizoctonia* sp., *S.rolfsii*, *Penicillium* sp., *Trichoderma* sp., *A.niger*, *A.orysae*. Strain S4 showed similar inhibitory effect on both of bacterial and fungal growth. *Bacillus cereus*, *F. oxysporum, P. aphanidermatum, P. infestan, Rhizoctonia* sp., and *Trichoderma* sp. were highly inhibited by *Streptomyces* sp. strain S4 on agar plates. *Streptomyces* is well

known as good antibiotic producer for producing a wide spectrum of antibiotic. The genus *Streptomyces* was reported to be used as biocontrol agents (Raaijmakers and Mazzola, 2012).

5. 16S rRNA Gene of *Streptomyces* sp. Strain S4

Molecular microbial studies based on 16S rRNA gene are generally used for microbial identification and classification. 1388bp of *Streptomyces* sp. strain S4 were investigated in Figure 3.8. The *Streptomyces* sp. strain S4 was highest identities value with *S. rubrolavendulae* and *S. fradiae* (Table 3.5). These sequences were appeared in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB591042.

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1 ACCCACTTCG GTGGGGGATT AGTGGCGAAC GGGTGAGTAA CACGTGGGCA
51 ATCTGCCCTG CACTCTGGGA CAAGCCCTGG AAACGGGGTC TAATACCGGA
101 TACGACCACT TCAGGCATCT GATGGTGGTG GAAAGCTCCG GCGGTGCAGG
151 ATGAGCCCGC GGCCTATCAG CTAGTTGGTG AGGTAACGGC TCACCAAGGC
201 GACGACGGGT AGCCGGCCTG AGAGGGCGAC CGGCCACACT GGGACTGAGA
251 CACGGCCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGCACAATGG
301 GCGAAAGCCT GATGCAGCGA CGCCGCGTGA GGGATGACGG CCTTCGGGTT
351 GTAAACCTCT TTCAGCAGGG AAGAAGCGAA AGTGACGGTA CCTGCAGAAG
401 AAGCGCCGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGGCGCAA
451 GCGTTGTCCG GAATTATTGG GCGTAAAGAG CTCGTAGGCG GCCTGTCACG
501 TCGGATGTGA AAGCCCGGGG CTTAACCCCG GGTCTGCATT CGATACGGGC
551 AGGCTAGAGT TCGGTAGGGG AGATCGGAAT TCCTGGTGTA GCGGTGAAAT
601 GCGCAGATAT CAGGAGGAAC ACCGGTGGCG AAGGCGGATC TCTGGGCCGA
651 TACTGACGCT GAGGAGCGAA AGCGTGGGGA GCGAACAGGA TTAGATACCC
701 TGGTAGTCCA CGCCGTAAAC GTTGGGAACT AGGTGTGGGC GACATTCCAC
751 GTCGTCCGTG CCGCAGCTAA CGCATTAAGT TCCCCGCCTG GGGAGTACGG
801 CCGCAAGGCT AAAACTCAAA GGAATTGACG GGGGCCCGCA CAAGCGGCGG
851 AGCATGTGGC TTAATTCGAC GCAACGCGAA GAACCTTACC AAGGCTTGAC
901 ATACACCGGA AACACCCAGA GATGGGTGCC CCCTTGTGGT CGGTGTACAG
951 GTGGTGCATG GCTGTCGTCA GCTCGTGTCG TGAGATGTTG GGTTAAGTCC
1001 CGCAACGAGC GCAACCCTTG TCCCGTGTTG CCAGCAGGCC CTTGTGGTGC
1051 TGGGGACTCA CGGGAGACCG CCGGGGTCAA CTCGGAGGAA GGTGGGGACG
1101 ACGTCAAGTC ATCATGCCCC TTATGTCTTG GGCTGCACAC GTGCTACAAT
1151 GGCCGGTACA AAGAGCTGCG ATACCGCAAG GTGGAGCGAA TCTCAAAAAG
1201 CCGGTCTCAG TTCGGATTGG GGTCTGCAAC TCGACCCCAT GAAGTCGGAG
1251 TCGCTAGTAA TCGCAGATCA GCATTGCTGC GGTGAATACG TTCCCGGGCC
1301 TTGTACACAC CGCCCGTCAC GTCACGAAAG TCGGTAACAC CCGAAGCCGG
1351 TGGCCCAACC CCTTGTGGGA GGGAGCTGTC GAAGGTGG
```

Figure 3.8 Nucleotide sequence of 16s rRNA gene of *Streptomyces* sp. strain S4.

Table 3.5 Gene bank accession numbers along with the alignments of sequences obtained with reported 16S rRNA gene sequences in the gene bank and highest similarity with different *Streptomyces* species.

Accession No.	Strain	Score (bits)	Identities (%)	AA
JF915304	*S. fradiae* 16S ribosomal RNA	2752	100	1388/1388
AB184463	*S. rubrolavendulae* 16S rRNA	2746	100	1388/1388
AB184585	*S. thermolilacinus* 16S rRNA	2712	99	1383/1388
AY999767	*S.coeruleoprunus* 16S ribosomal RNA	2678	99	1369/1375
HM041901	*S. indiaensis* 16S ribosomal RNA	2668	99	1376/1386
AJ007403	*S. somaliensis* 16S ribosomal RNA	2640	98	1374/1388
EU841628	*S. variabilis* 16S ribosomal RNA	2561	98	1357/1376
EU593715	*S. piloviolofuscus* 16S ribosomal RNA	2561	98	1357/1376

6. Phylogenetic Tree of *Streptomyces* sp. Strain S4

The phylogenetic analysis is used to estimate the relationship between closely sequence species and reconstructed the branch of tree. The phylogenetic tree of *Streptomyces* sp. strain S4 16S rRNA gene sequence was demonstrated in Figure 3.9 at the same distinct sub-clade with *S. fradiae* and *S. rubrolavendulae* with 100% similarity.

3.4.5 Characteristic Comparison of *Streptomyces* sp. strain S4, *S.fradiae,* and *S. rubrolavendulae*

Phenotypic comparison of *Streptomyces* sp. strain S4, *S. fradiae*, and *S. rubrolavendulae* demonstrated the difference among these recognized *Streptomyces* species (Table 3.6-3.8). The recent studies of Waksman and Curtis (1961) indicated that *S. fradiae* had straight and no spiral spores as rod or oval-shaped. Its optimum temperature is 25°C and can produce antibiotic including neomycin and fradicin. *S. rubrolavendulae* was first isolated by Yen and Lu (1964) and *S. rubrolavendulae* had yellow to brown color substrate mycelium and yellow-pink aerial spore mass. These characteristics were different from *Streptomyces* sp. strain S4.

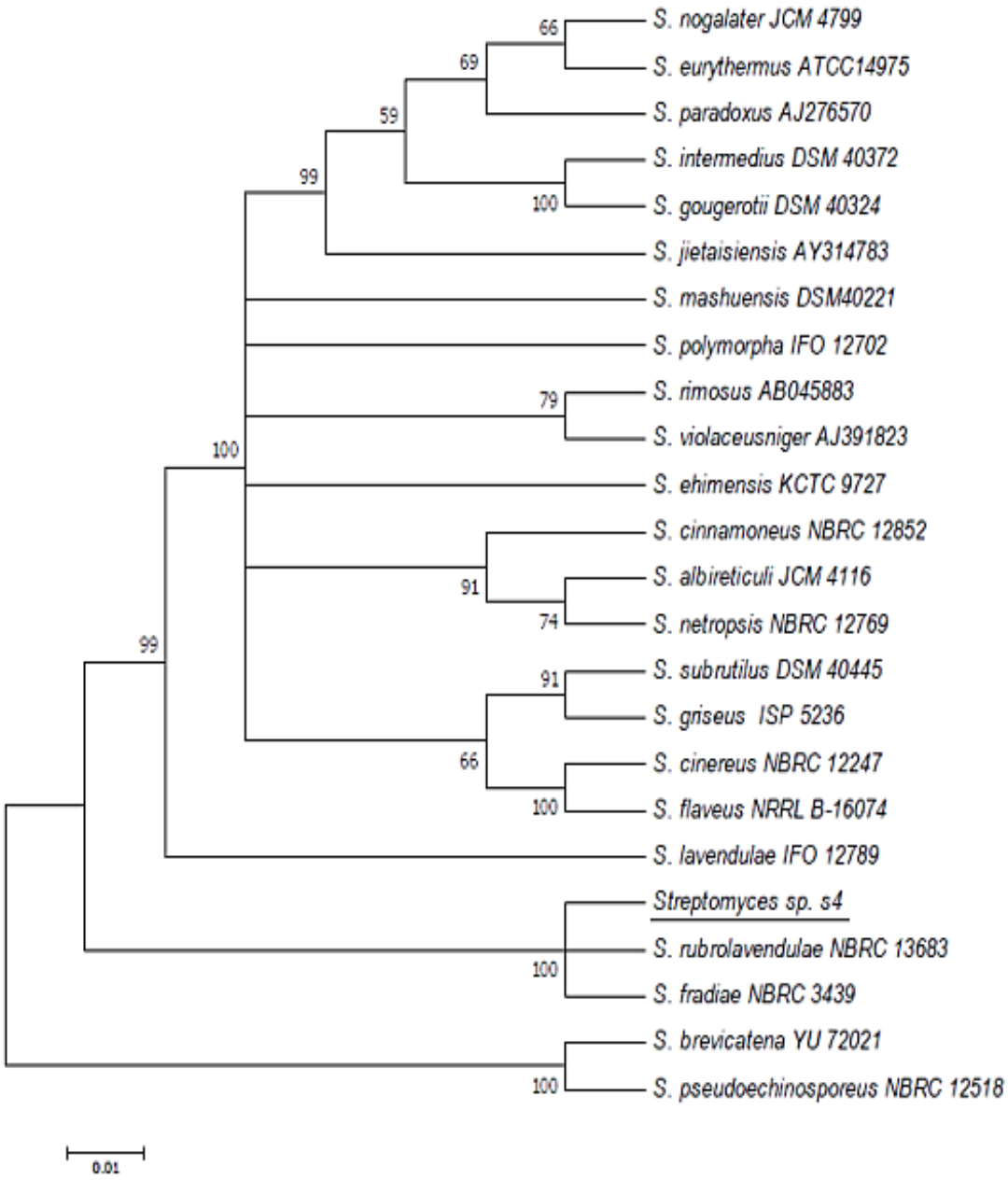


Figure 3.9 Phylogenetic tree of 16S rRNA gene sequences between *Streptomyces* sp. strain S4 and members of genus *Streptomyces*. Bar 0.01 substitutions per nucleotide position.

In addition, the DNA-DNA hybridization (DDH) of strain S4 to *S. fradiae* and *S. rubrolavendulae* was 58.36% and 47.71%, respectively. Therefore, the strain S4 could be a different genomic species of *S. fradiae* and *S. rubrolavendulae*. According to Stackebrandt (1991), the relatedness value in different genomic species was lower than 70% (Stackebrandt et al., 1991). The DNA-DNA hybridization using whole-genome molecular technique could give the different results from using partials DNA. Goris recommend that 70% of DDH could be standard value for considering genomic species (Goris et al., 2007).

Table 3.6 The cultural characters of *Streptomyces* sp. strain S4, *S. fradiae*, and *S. rubrolavendulae*.

Medium	**Mycelium***	**Strain:**		
		1	**2**	**3**
Yeast extract/malt extract agar (ISP 2)	Growth	Moderate	Good	Good
	AM	White	White	White to Brown
	SM	Yellow	White	White
	SP	Yellow	White	-
Oatmeal agar (ISP 3)	Growth	Good	Poor	Moderate
	AM	Brown	White	White Brown
	SM	Yellow	Grey	Ivory
	SP	Yellow	-	-
Inorganic salt-starch agar (ISP 4)	Growth	Good	Poor	Moderate
	AM	White Brown	-	White Brown
	SM	Yellow	White	Ivory
	SP	-	Yellow Brown	-
Glycerol-asparagine agar (ISP 5)	Growth	Good	Moderate	Moderate
	AM	White	-	White
	SM	Yellow	Yellow	Yellow
	SP	-	-	-
Bennets agar (BNA)	Growth	Poor	Good	Moderate
	AM	White	Brown	White
	SM	Yellow	Green Grey	Yellow
	SP	-	-	-
Nutrient agar (NA)	Growth	Good	Good	Good
	AM	White to Brown	White to Brown	White to Brown
	SM	Yellow	White Grey	White
	SP	-	-	-

*AM, Aerial mycelium; SM, substrate mycelium; SP, soluble pigment

Strains: 1, *Streptomyces* sp. strain S4; 2, *S. fradiae* (NBRC3360); 3, *S. rubrolavendulae* (NBRC13183)

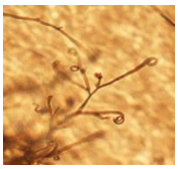
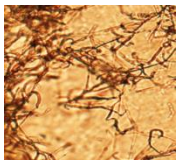
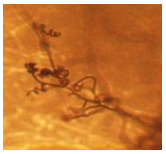
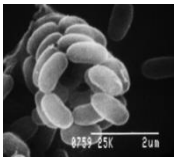
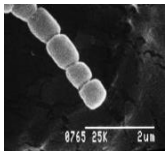
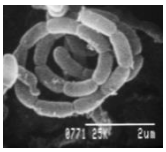
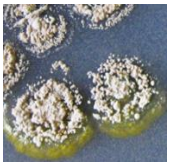
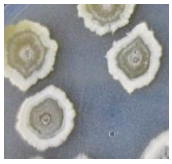
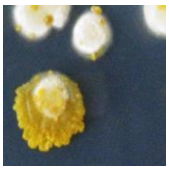
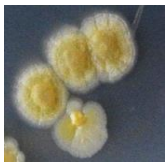
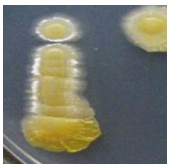
Table 3.7 Physiology and biochemical properties of *Streptomyces* sp. strain S4, *S. fradiae*, and *S. rubrolavendulae*.

Test	Strain:		
	1	**2**	**3**
Form:			
Size	Medium	Medium	Medium
Surface	Rough	Smooth	Rough
Texture	Powdery	non-Powdery	Powdery
Margin	Irregular	Irregular	Irregular
Elevation	Raised	Flat	Raised
Colony morphology	Brown	White	Brown to White
Reverse side colour	Brown	White	Brown to White
Spore chain	SP	SC	SP
Spiral size	2.562 μm	-	3.18μm
Assimilation of :			
Lactose	+	-	-
Arabinose	+	-	+
Sucrose	-	+	-
L-Leucine	-	-	+
L-Asparagine	+	-	-
L-Lysine	+	-	-
L-Methionine	+	-	-
Degradation of :			
Xylan	+	-	+
Cassava Starch	+	+	(+)
Cellulose	+	+	(+)
Growth at/in			
15°C	+	-	-
45°C	+	-	-
NaCl (%, w/v)	≤ 5.0	≤ 6.0	≤ 6.0
pH 4	+	-	-
pH 13	+	-	+
Antibiosis against :			
B. cereus	+	(+)	(+)
F.oxysporum	+	(+)	(+)
Pythium sp.	+	(+)	-
Tricoderma sp.	+	(+)	-

+, Positive utilized; -, negative, not utilized ;(+), Weak Positive utilized
Strains: 1, *Streptomyces* sp. strain S4; 2, *S. fradiae* (NBRC3360); 3, *S. rubrolavendulae* (NBRC13183)

SC, straight chains; SP, spiral chains

Table 3.8 Morphology of *Streptomyces* sp. strain S4, *S. fradiae*, *S. rubrolavendulae*

Observation	Strain S4	*S. rubrolavendulae*	*S. fradiae*
Light microscopy			
Scanning electron microscopy			
Yeast extract –malt extract agar			
Oatmeal agar			
Inorganic salts-starch agar			
Glycerol-asparagine agar			

3.5 Conclusion

The seedling damping off disease fungi were isolated from the infected tomato and Chinese spinach seedlings from economic plant nurseries in Thailand. The pathogenic fungi were identified to be *P. infestans* and *P. aphanidermatum*. Among six soil actinomycetes isolates, *Streptomyces* sp. strain S4 could inhibit mycelium growth of both *P.infestans* and *P. aphanidermatum*. The genotypic and phenotypic datas of strain S4 is clearly differentiated from the reference strains, *S. fradiae* and *S. rubrolavendulae* which had 100% similarity of 16S rRNA. Therefore, we will propose this organism to be a new species of genus *Streptomyces.*