

Isolation of Antagonistic Actinomycetes Species from Rhizosphere as Effective Biocontrol Against Oil Palm Fungal Diseases

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Abstract

Antagonistic actinomycetes were isolated from oil palm rhizosphere and tested against oil palm fungal diseases caused by *Curvularia oryzae* PSU-NK1012, *Ganoderma boninense* T1, and *Schizophyllum commune* SK1, that are the causal agents of leaf spot, stem rot, and brown germ diseases, respectively. Three isolates of actinomycetes were selected for inhibition tests against these fungal pathogens. Based on morphological, physiological, biochemical, and molecular properties, the 3 actinomycetes species were identified as *Streptomyces abikoensis* ChM3-1, *Kitasatospora nipponensis* KM6-4, and *S. angustmyceticus* NR8-2, time course of *in vitro* tests showed that these actinomycetes species were capable of inhibiting the growth of oil palm pathogenic fungi. Scanning electron microscope observations revealed that the actinomycetes culture filtrates could degrade the mycelia cell walls of the 3 disease pathogens, possibly due to the production of some antimicrobial agents. The study identified an actinomycetes strain with antagonist activity that could be used for biocontrol of plant pathogenic fungi.

Keywords: Antagonist, biological control, *Elaeis guineensis*, fungal diseases

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a perennial crop cultivated especially in southern Thailand. In these tropical and subtropical areas with long periods of high rainfall, the oil palms are vulnerable to several pathogens. Fungal plant diseases have caused severe losses to the oil palm industry in both quality and quantity, and affect any stage of growth. During the seedling stage, young oil palms are faced with leaf blight and leaf spot diseases caused by *Curvularia* spp. Stem rot is caused by *Ganoderma* spp. which is destructive in the oil palm trunk, while brown germ disease, or seed rot, is caused by *Schizophyllum* spp. common in seeds.

Fungicides are effective in controlling fungal diseases. However, their extensive use leads to resistant fungal strains, and the accumulation of chemicals poses human health risks. Therefore, control by natural antagonistic microorganisms of plant pathogenic fungal diseases is actively pursued as an alternative approach. Among such microorganisms, the *Streptomyces* bacteria are of particular interest, because of their properties [1]. *Streptomyces* are gram-positive bacteria belonging to the actinomycetes family, and are similar to fungi in their filamentous form and found in various environments. Interestingly, *Streptomyces* produce bioactive secondary metabolites, mainly antibiotics and immunosuppressants [2-4]. Some *Streptomyces* species are known to reduce the growth of plant pathogenic fungi [5-7].

To collect candidate isolates of *Streptomyces* for the biological control of oil palm fungal diseases, actinomycetes were isolated from oil palm rhizosphere. Biological control was assessed with dual culture tests, time course inhibitory tests, and scanning electron microscope observation, to assess and select useful bioactivity.

Materials and methods

Sample collection, isolation, cultural and morphological characteristics

The oil palm rhizosphere soils were collected from eight provinces in southern Thailand, including Chumphon, Krabi, Nakhon Si Thammarat, Phuket, Ranong, Satun, Suratthani, and Trang. The soil samples were dried in laboratory conditions at 28 °C for 5 - 10 days. Each soil sample was then separately suspended in sterilized 0.85 % NaCl, shaken well at 120 rounds per minute (rpm) for 24 h at 25 °C, and diluted 10 fold. An amount of 20 µl of each dilution was spread on plates containing glucose yeast-extract malt-extract agar (GYMA) and incubated at 28 °C for 7 - 14 days. Hypha-like colonies were selected and cultured on GYMA slants, stored at 4 °C. Cultural properties were observed after 10 days of culturing on GYMA [8]. For morphological identification, slide cultures were observed by a compound microscope (Olympus, Japan).

Pathogen sources

Oil palm pathogenic fungi were provided by the Culture Collection of the Pest Management Department, Faculty of Natural Resources, Prince of Songkla University. The *Curvularia oryzae* PSU-NK1012 [9], *Ganoderma boninense* T1, and *Schizophyllum commune* SK1, which cause leaf spot, stem rot, and brown germ disease of oil palm, respectively, were selected to screen for antagonistic *Streptomyces* in dual culture tests. The pathogenic fungi were cultured on potato dextrose agar (PDA) for further assays.

Agar plug assays

To evaluate the antagonistic effects on the growth of pathogenic fungi, the selected actinomycetes were streaked, while plates without actinomycetes served as negative controls. In total, 156 isolates of actinomycetes were streaked on GYMA, with growth at 28 °C. After 7 days of incubation, a plug (5 mm diameter) from the edge of a 7 days old colony of *C. oryzae*, of *G. boninense*, or of *S. commune* was inoculated on each plate. The colony radii were measured at 7 days of incubation, in this dual test. The experiments were repeated twice. Percentage inhibition of growth (PIG) was calculated with:

$$\% \text{ inhibition} = \frac{R_1 - R_2}{R_1} \times 100 \quad (1)$$

where R1 is the average diameter of the control colonies, and R2 is the average diameter of the dual culture treated colonies [10].

Physiological tests

Catalase activity, utilization of carbohydrates as the source for growth, and hydrolysis of xylan, chitin, casein, tyrosine, xanthine, starch and gelatin of selected actinomycetes were determined using methods described by Liu *et al.* [11]. Growth in the presence of sodium chloride (1, 3, 5, 7 % w/v) was determined on the culture media. The optimal temperature and pH ranges for growth were tested on culture media.

Molecular identification

DNA extraction was conducted as previously described [12,13]. Briefly, the cultured actinomycetes on GYMA were flushed with sterilized DI water, collected on a filter, washed by sterilized distilled water (DW), and kept at -20 °C. The samples were ground with a small mortar and pestle, and lysis buffer was added. The samples were then centrifuged and 3.0 M potassium acetate was added. After the second time of centrifuging (12000 rpm) the supernatant was collected and precipitated by isopropanol. The pellets were washed with 70 % ethanol and dried. The occurrence of DNA was measured by gel electrophoresis.

Amplification of 16S rDNA was done with PCR. The 16S rDNA gene regions were amplified using a BIO-RAD T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). A portion of the 16S rDNA of the actinomycetes isolates was amplified by PCR, using 27F forward (5' AGAGTTTGATCMTGGCTCAG

3') and 1389R reverse (5' ACGGGCGGTGTGTACAAG 3') primers. The PCR was carried out sequentially in a final 50 µl reaction volume containing 10 pmol of each primer, 2× DreamTaq Green PCR Master Mix (Thermo Scientific), and 50 ng of template DNA. An initial denaturation step was 3 min at 94 °C followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, and an extension for 3 min at 72 °C, with a final extension step of 10 min at 72 °C. The PCR products were visualized by agarose gel electrophoresis. The portion of 16S rDNA gene region was sequenced at the Scientific Equipment Center, Prince of Songkla University, Songkhla, Thailand, by automated DNA sequencing with ABI Prism 377 (Applied Biosystems, USA) using the same primers as used in the PCR reaction [14,15].

Time course *in vitro* test

Three selected isolates of actinomycetes (5 % v/v) were cultured on yeast-extract malt-extract broth (GYMB) and shaken at 120 rpm at 25 °C. The culture supernatants were collected at 5 days of incubation. The culture broth was centrifuged (6000 rpm) and the supernatant was collected and filtered on a membrane filter (0.45 µm). The culture filtrates of 3 isolates were mixed with GYMA (agar) in 1:1 ratio. A plug of a pathogen colony (5 mm in diameter), either *C. oryzae*, *G. boninense*, or *S. Commune*, was inoculated onto each GYMA with culture filtrate from an actinomycetes isolate. There were 4 replicates for each isolate in each pathogen (4×3×3 cases). A GYMA with distilled water in place of an isolate served as a negative control. The radial growth of a pathogen was measured on up to 8 days after inoculation. The experiments were repeated twice. The percent inhibition of growth (PIG) was calculated as described by Gamliel *et al.* [16], approximately comparing areal extents of the colonies:

$$\% \text{ inhibition} = 100 - \frac{R^2}{r^2} \times 100 \quad (2)$$

where *R* is the average radius with treatment, and *r* is the average radius in the control group.

Electron microscope assays

Microscopic analysis was conducted as previously described by Sunpapao and Pornsuriya [17]. Mycelia of *C. oryzae*, *G. boninense*, and *S. commune* were fixed by immersion in 2.5 % glutaraldehyde in 1 mol/L phosphate buffer (PB). They were then fixed with 0.1 % osmium tetroxide in PB, and were dehydrated gradually with 50, 70, 80, 95 and 100 % alcohol. The samples were then air-dried before coating with gold/palladium. Finally, the samples were transferred to scanning electron microscope stubs, and three random fields were imaged.

Statistical analysis

Prior to such analyses, the growth effects of actinomycetes treatments were normalized to percentage relative to control. The differences between means from actinomycetes treatments and the control were tested for statistical significance by SPSS software. The statistical significance of differences between means for treatment and control groups was determined with Duncan's Multiple Range Test (DMRT) for multiple comparisons [18].

Results

Morphological and physiological characteristics

The 3 actinomycetes isolates produced aerial mycelia consisting of straight chain, smooth surface spores, and the spore mass was whitish-grey on GYMA (**Figure 1**). The isolates ChM3-1 and NR8-2 released soluble yellow-brown pigment on GYMA, while the isolate KM6-4 did not stain the medium (**Figure 1** and **Table 1**). ChM3-1 showed positive catalase activity and reduction of nitrate. This isolate utilized fructose, lactose, mannitol, cellobiose, dextran, and sucrose, while chitin, casein, gelatin, tyrosine, xanthine, and xylan were decomposed. Growth was observed in the presence of 1 - 5 % sodium chloride. The optimal conditions for growth of this isolate were pH 5 - 9 and a temperature was in the range of 28 -

37 °C. The isolate KM6-4 revealed positive catalase activity and possessed reduction of nitrate. It utilized glucose, fructose, galactose, lactose, mannose, mannitol, cellobiose, dextran, and sucrose. Casein, gelatin, xanthine, and xylan were decomposed, while chitin and tyrosine were not decomposed. Growth was observed in the presence of 1 - 3 % sodium chloride. The optimal conditions for growth of this isolate were pH 5 - 11 and a temperature in the range 28 - 37 °C. For the NR8-2 isolate, catalase activity and reduction of nitrate were positive. This isolate utilized glucose, fructose, galactose, lactose, mannose, mannitol, cellobiose, dextran, sucrose, and xylose. Chitin, casein, gelatin, tyrosine, and xanthine were decomposed, while xylan was not. Growth was observed in the presence of 1 - 3 % sodium chloride. The optimal conditions for growth of this isolate were pH 5 - 11 and a temperature was in the range of 28 - 37 °C (Table 1).

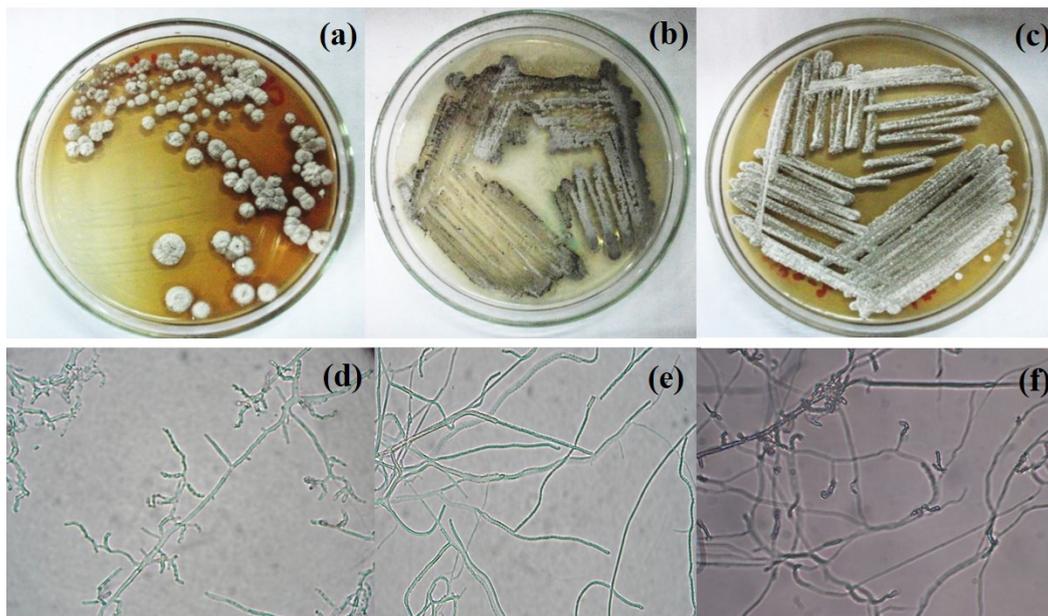


Figure 1 Colony shapes and microscopic morphology of the antagonistic actinomycetes found by screening, namely *Streptomyces abikoensis* ChM3-1 (a and d), *K. nipponensis* KM6-4 (b and e) and *S. hygroscopicus* NR8-2 (c and f).

Table 1 Morphological, physiological and biochemical characteristics of the actinomycetes isolates, ChM3-1, KM6-4 and NR8-2.

Properties	ChM3-1	KM6-4	NR8-2
Aerial mycelia	Spirales	Rectiflexibiles	Spirales
Soluble pigments	+	-	+
Nitrate reduction	+	+	+
Catalase activity	+	+	+
Starch hydrolysis	+	+	+
Gelatin liquefaction	+	+	+
Carbohydrates utilization			
D-glucose	+	+	+
D-fructose	+	+	+
D-galactose	-	+	+
D-lactose	+	+	+
D-xylose	-	-	+
D-mannose	-	+	+
D-mannitol	+	+	+
L-arabinose	-	-	-
Cellobiose	+	+	+
Dextran	+	+	+
Sucrose	+	+	+
Degradation of			
Xylan	+	+	-
Chitin	+	-	+
Casein	+	+	+
Tyrosine	+	-	+
Xanthine	+	+	+
Gelatin	+	+	+

+, positive

-, negative

Dual culture test

Dilution spread plate cultures from oil palm rhizosphere soil samples showed aerial mycelia, which was considered as actinomycetes. A total of 156 actinomycetes isolates were collected. The primary screening for antagonism against pathogens was based on dual cultures, comparing treated samples with the control. Three *Streptomyces* isolates, ChM3-1, KM6-4, and NR8-2, showed a high > 75 % inhibition of all the oil palm disease pathogens tested (data not shown).

Molecular identification

The amplified portions of 16S rDNA in the PCR products from isolates ChM3-1, KM6-4, and NR8-2 were about 1.5, 1.3 and 1.6 kb in size, respectively (data not shown). The nucleotide sequences of the 16S rDNA portions, from actinomycetes isolates ChM3-1, KM6-4, and NR8-2, were 1049, 917, and 1056 base pairs in size, respectively. These nucleotide sequences were deposited in GenBank with accession numbers AB914562, AB914561, and AB914560, for ChM3-1, KM6-4, and NR8-2 isolates, respectively. The sequences were compared to known actinomycetes sequences in the NCBI (National Center for Biotechnology Information) databases and were found to be 100 % identical to *Streptomyces abikoensis* (JN566040) for ChM3-1 isolates, 99 % identical to *Kitasatospora nipponensis* (JX483762) for KM6-4, and 100 % identical to *S. angustmyceticus* (HQ244457) for NR8-2 isolates. To confirm this genetic relationship to *Streptomyces*, a phylogenetic analysis was conducted using neighbor-joining with 1,000

bootstrap replicates (**Figure 2**), and the 3 actinomycetes isolates of the current study were highly similar to *S. abikoensis* (JN566040), *Kitasatospora nipponensis* (JX483762), and *S. angustmyceticus* (HQ244457). Based on this molecular analysis, the antagonistic actinomycetes isolates from oil palm rhizosphere in Thailand represented *S. abikoensis* (isolate ChM3-1), *K. nipponensis* (KM6-4) and *S. angustmyceticus* (NR8-2).

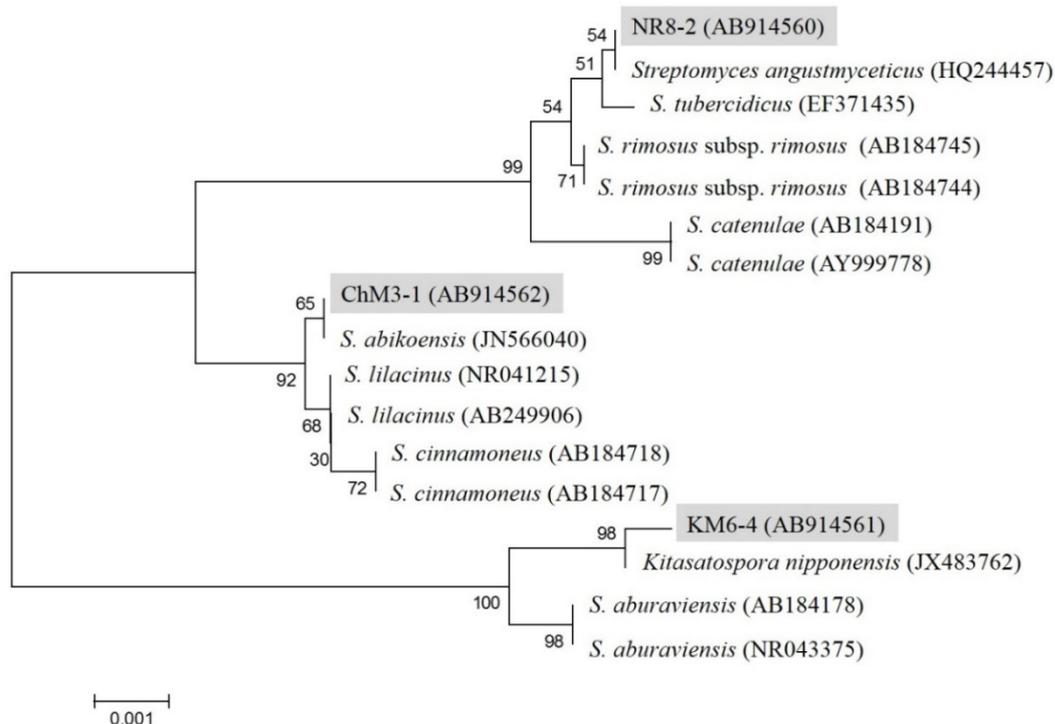


Figure 2 This phylogenetic tree illustrates the relationships of the actinomycetes isolates to select actinomycetes strains sequenced previously. The analysis was based on 16S rDNA sequences, and the tree constructed by the neighbor-joining method with 1,000 bootstrap replicates. Numbers in parentheses are the accession numbers in the GenBank database.

Time course *in vitro* tests

Culture filtrates of the 3 actinomycetes isolates were added to culture media prior to inoculation with an oil palm pathogenic fungus (*C. oryzae*, *G. boninense*, or *S. commune*). Time courses of inhibitory effects on the growth of pathogenic fungi were observed at 1 - 8 days post inoculation (dpi). The effects of the culture filtrates of *S. abikoensis*, *K. nipponensis*, and *S. angustmyceticus*, in controlling the growth of *C. oryzae*, were highest on 5 dpi (91 %), 6 dpi (93 %), and 6 dpi (97 %), respectively (**Figure 3**). For the basal stem rot disease pathogen, *G. boninense*, the culture filtrates had their highest inhibitory effects on 4 dpi (92 %), 5 dpi (90 %), and 4 dpi (93 %), in the same order (**Figure 3**). Also the brown germ disease pathogen was controlled, so that the highest inhibitory effects were on 5 dpi (91 %), 5 dpi (93 %), and 6 dpi (96 %), again in the same order of isolates (**Figure 3**). With further incubation, the growth inhibition again decreased consistently. The inhibitory effects are illustrated in **Figure 3** and summarized in **Table 2**.

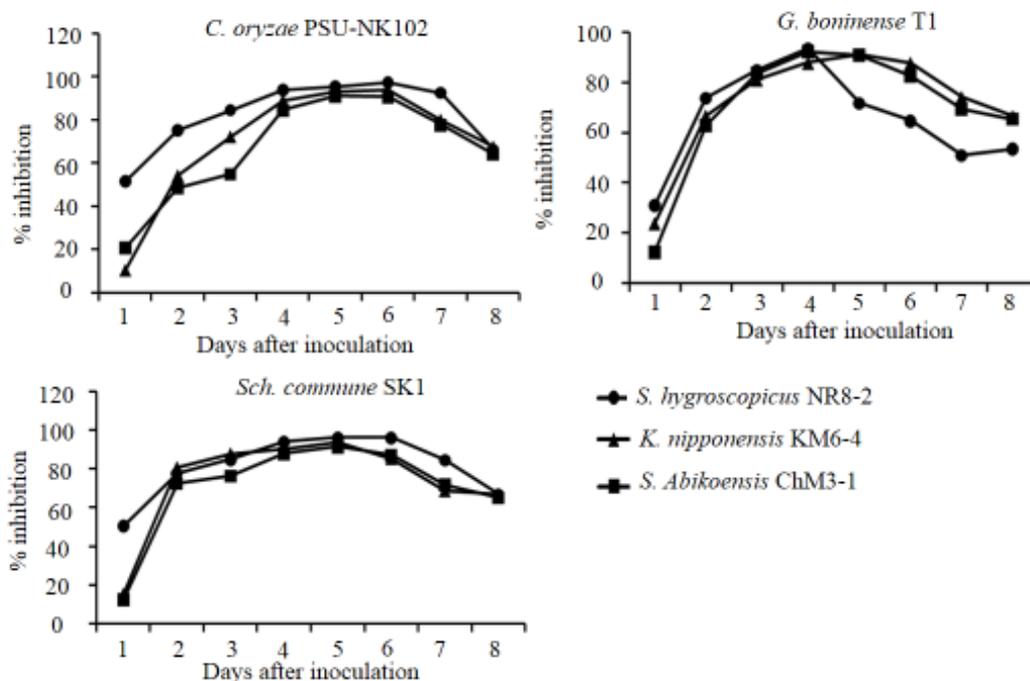


Figure 3 Time courses of growth inhibition by actinomycetes culture filtrates, on the areal colony growth of selected oil palm pathogenic fungi, namely *C. oryzae* PSU-NK1012, *G. boninense* T1, and *S. commune* SK1.

Table 2 Effects of the culture filtrates on the radial growth of oil palm disease pathogen colonies.

Culture filtrates	<i>C. oryzae</i>			<i>G. boninense</i>			<i>S. commune</i>		
	0 days	4 days	8 days	0 days	4 days	8 days	0 days	4 days	8 days
Percentage inhibition [PI±SD] ^a									
ChM3-1	20.94±1.2	84.60±1.6	64.15±2.5	12.21±1.8	92.04±3.5	65.20±2.4	12.35±2.0	87.94±3.6	66.82±4.5
KM6-4	10.48±1.4	89.07±2.6	67.52±3.4	23.69±1.4	87.60±2.4	66.34±2.8	15.32±2.2	90.35±3.2	66.67±2.8
NR8-2	57.70±1.5	93.87±1.7	66.41±2.2	30.76±1.2	93.33±2.2	53.30±3.4	50.38±1.7	93.77±2.5	66.82±2.5

^aControl radial growth of oil palm disease pathogens on Petri-plate, 8.9 cm.

Effects of culture filtrate on mycelial shape

The oil palm disease pathogens were cultured on *S. abikoensis*, *K. nipponensis* and *S. angustmyceticus* culture filtrates, in order to observe the degradation of mycelia. The results showed degraded and swollen mycelia of pathogens when compared with the control (**Figure 4**). The mycelia shape of *C. oryzae* was severely damaged by the culture filtrate of *S. hygrosopicus*, while mycelia wilt was observed when cultured with *S. abikoensis* and *K. nipponensis* culture filtrates. For *G. boninense*, mycelia collapsed when cultured with any of the culture filtrates. The mycelia of *S. commune* were severely damaged by *S. hygrosopicus* culture filtrate, while in other culture filtrates there was wilt (**Figure 4**).

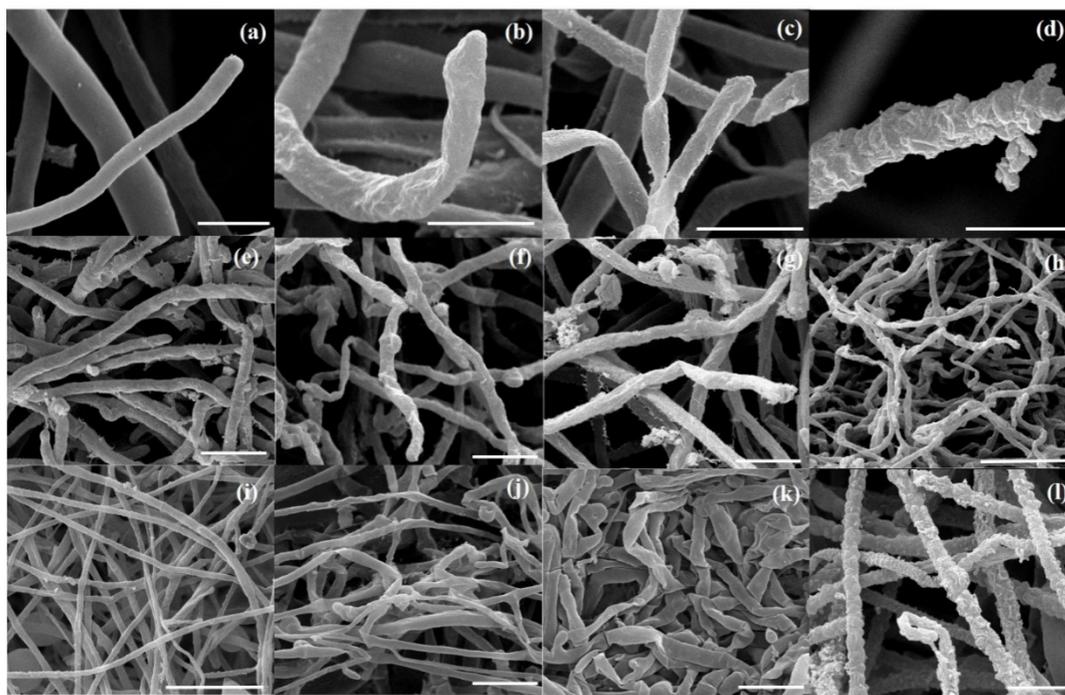


Figure 4 Effects of culture filtrates of *Streptomyces abikoensis* ChM3-1 (b, f and j), *K. nipponensis* KM6-4 (c, g and k), and *S. hygrosopicus* NR8-2 (d, h and l) on the mycelia of *Curvularia oryzae* PSU-NK1012 (a, b, c and d), *Ganoderma boninense* T1 (e, f, g and h), and *Schizophyllum commune* SK1 (i, j, k and l). Culture media without culture filtrate served as a negative control (a, e and i), showing normal mycelia morphologies of the pathogens. (Bar a, b, d = 5 μ m; c, e, f, g, j, k, l = 10 μ m and h, i = 30 μ m).

Discussion

In the present study, one hundred and 56 isolates of actinomycetes were collected from oil palm rhizosphere samples, representing conditions in southern Thailand. The isolates were initially characterized by their morphological, biochemical, physical and molecular properties. By preliminary dual culture tests screening for growth inhibition effects, 3 isolates of actinomycetes were selected to test against *C. oryzae*, *G. boninense*, and *S. commune*. These pathogens cause leaf spot, stem rot, and brown germ of oil palm, respectively. The *in vitro* results strongly suggest that the 3 isolates of actinomycetes could be effective in reducing the plant diseases caused by several fungi. The potential of actinomycetes species to restrict or reduce the severity of plant pathogenic fungi has been observed in prior work; for

instance, the use of *Streptomyces* in controlling the *Fusarium* wilt of cucumber caused by *Fusarium oxysporum* [19], and the control of *Colletotrichum gloeosporioides* and *C. grostidis* in yam [20]. Sadeghi *et al.* [21] reported that *Streptomyces* spp. (S2 and C isolates) could suppress *Rhizoctonia* damping-off of sugar beet. Furthermore, *Streptomyces* spp. could produce chitinase to degrade the cell walls of *C. sublineolum* and *Pythium* sp. [22]. This corroborates our results of 3 *Streptomyces* isolates having potential in the biological control of oil palm disease pathogens. In addition, the culture filtrates of *Streptomyces* affected the mycelial shapes of oil palm disease pathogens, as shown in **Figure 4**. This may be due to secondary metabolites of *Streptomyces* released during its growth. It is known that *Streptomyces* can produce some effective compounds, including siderophores, hydrocyanic acid, cellulases, and proteases, that act against *Fusarium* wilt in green bean [23].

Conclusions

Here, we selected effective actinomycetes from rhizosphere soil of cultivated oil palms, to enable biological control of the causal agents of leaf spot, stem rot, and brown germ diseases of oil palm. The selected *Streptomyces* inhibited the growth of pathogens *in vitro*, as well as degradation of mycelia cell wall. The current study provides a proof of concept *in vitro*, but further studies of both *Streptomyces* and their secondary compounds, leading to effective practical formulations and application techniques, are necessary intermediate steps before eventual field studies.

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