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# Isolation and Identification of Actinobacteria against *Colletotrichum*, the Causal Agent of Anthracnose Disease of *Mangifera indica* L.

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**Abstract** Actinobacteria are a large group of gram-positive bacteria classified into *Streptomyces* and non-*Streptomyces* genera. *Streptomyces* is commonly found in nature, especially in soil samples collected in different geographic properties. It is well-known in its capability to produce bioactive compounds, including antimicrobials, immunostimulants, anticancer, and antioxidants. The aims of this study were concentrated on the isolation and screening of the antifungal-producing actinobacteria from termite mound soil and wasp soil against *Colletotrichum* using culture-dependent techniques with Sodium Caseinate Agar (SCA) and spread plate technique. A total of 45 actinobacterial strains were isolated and purified for testing of antifungal features. The inhibitory activities against *Colletotrichum* sp. PSRU-NDM65, the plant pathogen of anthracnose disease of *Mangifera indica* L. were tested by dual culture technique on Potato Dextrose Agar (PDA). The results indicated that two isolates, namely TM-A3 and TM-A7, had the highest percentage of inhibition against *Colletotrichum* sp. PSRU-NDM65 growth by 87.79% and 85.81%, respectively. Based on the morphological studies, both actinobacterial isolates produce the long chain of spore and hypha which are fitted with the typical characters of *Streptomyces*. Concomitantly, comparative analysis of 16S rDNA partial nucleotide sequences revealed that TM-A3 and TM-A7 belonged to the *Streptomyces* genus based on 99.7% similarity and 100% identity to *Streptomyces misionensis* and *Streptomyces prasinopilosus*, respectively. In the future, utilization of two *Streptomyces* strains will be applied on farms planted with economical *Mangifera* spp., and other horticulture plants.

**Keywords** Isolation, Identification, *Streptomyces*, *Colletotrichum*, Anthracnose Disease, *Mangifera indica* L.

## 1. Introduction

Actinobacteria are gram-positive bacteria highly distributed in terrestrial and aquatic ecosystems, especially in soils, air, fresh seawaters, deserts and deep-sea sediments [1-4]. Based on of polyphasic studies, most of them were identified as *Streptomyces* produced the bioactive compounds, including antimicrobials, immunostimulants, anti-cancers, anti-parasites, antimalarial drugs, antioxidants, enzymes, bioinsecticides,

biostimulants, biosurfactants, dyes and plant stimulating substances [5-14].

*Streptomyces* is well known biocontrol of phytopathogens in agricultural application, including agronomic crops and horticulture plants. For examples, biocontrol of bacterial canker and speck (*Pseudomonas syringae*), bacterial wilt (*Ralstonia solanacearum*), black rot in crucifers and citrus canker (*Xanthomonas* sp.), Pierce's disease in vineyards, variegated chlorosis of citrus, and almond leaf burns (*Xylella fastidiosa*), bakanae disease of rice, corn ear rot, pine canker (*Fusarium*), gray mold disease (*Botrytis cinerea*), necrotic lesions such as spots or brown/black spots leaf blight (*Alternaria alternate*) and anthracnose strawberry crown rot, banana crown rot, red rot in coffee berries and sugar cane Blotch in cowpea (*Colletotrichum* spp.) [15].

Anthracnose is one of the most important post-harvest diseases in mango (*Mangifera indica* L.) exported by many tropical countries, including Thailand. The causal agent of anthracnose disease is *Colletotrichum gloeosporioides* [16]. *Colletotrichum* is a major phytopathogen that some species are saprophytes and endophytes, including *C. fructicola* and *C. jiangxiense*) [17-18]. Phytopathogenic some strains of *C. gloeosporioides* are responsible for tropical fruit diseases, especially anthracnose of avocado, chilli, citrus, coffee, dragon fruit, durian, guava, mangosteen, rose apple, rambutan, strawberry and mango [19-21].

Nowadays, control of fungal disease, especially anthracnose caused by *C. gloeosporioides*, focuses on using high level of fungicide which could lead to the accumulation of toxic substance and causing fungal resistance to chemical control. There are several reports found that *Streptomyces* spp. could synthesize anti-fungal substances used as biocontrol against *C. gloeosporioides* [22-29]. Organic agriculture using biocontrol is one of interest to reduce the use of chemicals leading to green agriculture and the production of food based on the security of consumers. The chemicals obtained from *Streptomyces* are interesting in the choices for plant pathogen control because these chemicals are bioactive compounds produced by *Streptomyces* that showed the fungal growth inhibition and are eco-friendly to environments and all living organisms.

The aims of research were to isolate and identify the actinobacterial strains collected from termite mound soil and wasp soil. The selected strains of actinobacteria were tested for biocontrol activity against *Colletotrichum* isolated from anthracnose disease of *Mangifera indica* L. in order to evaluate their potential for application in the mango orchard in the future.

# 2. Materials and Methods

### 2.1. Soil Sample Collection and Isolation of Actinobacteria

The termite mound and wasp soils were randomly collected in Phitsanulok and Sukhothai provinces. Ten grams of soil (1-5 cm in depth of termite mound soil/whole wasp soil without insect) were dried at room temperature followed by performing a 10-fold serial dilution for $10^{-1}$–$10^{-6}$ dilution in distilled water as the diluent. The diluted soil suspension was spread on Sodium Caseinate Agar (SCA) and incubated at 30 ℃ for 5-7 days. The colony with a compact, leathery, cottony, velvety, or powdery character was selected and transferred to the SCA slant. Pure cultures of actinobacteria were preserved as working and long-term stock cultures.

### 2.2. Isolation, Morphological and Molecular Study of the Causal Agent of Mango Anthracnose

*Colletotrichum* causal anthracnose disease of *M. indica* L. was isolated by tissue transplanting technique; the pieces of mango including both disease and healthy tissues were cut into 0.5×0.5 cm and immersed in 3% w/v of NaClO for 3 mins followed by washing twice in sterile distilled water. The piece of mango tissue was placed on sterile filter paper to remove water and then transferred to water agar (WA) and incubated at room temperature for 3-4 days until hypha formed. The hyphal tip was cut and transferred to potato dextrose agar (PDA) and incubated at room temperature for 6-7 days for morphological study; macro and microscopic characteristics (modified from Kongtragoul and Nalumpang, 2010 [30]). The pure living culture was sent out for DNA extraction, PCR amplification, and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using ITS4/ITS5 primers according to Gibthai company protocols. The partial sequence of ITS rDNA was analyzed with MEGA X and blasted in the National Center for Biotechnology Information (NCBI) database.

### 2.3. Determination of Actinobacterial Activity on Inhibition against *Colletotrichum*

The selected actinobacteria were tested against a 7-day-old of *Colletotrichum* dual culture method. Briefly, the fungal colony was cut with cork borer into 5 mm mycelial disc and placed on the center of the PDA plate against the stripe of 5 cm actinobacteria. All treatments have been done in triplicate and incubated at room temperature. After 9 days of incubation, fungal growth was recorded and % inhibition was determined according to a formula in Zhang *et al*. [31].

$$\% \text{ IRG} = (R1\text{-}R2)/R1 \times 100$$

%IRG = percentage of inhibition rate
R1 = Radial of *Colletotrichum* sp. colony in control plate
R2 = Radial of *Colletotrichum* sp. colony in the test plate

### 2.4. Morphological Study and Molecular Identification of Actinobacteria

A morphological study of actinobacteria was performed

based on slide culture technique. Briefly, a small piece of agar was cut into 0.5×0.5 cm, and put on a sterile slide. Then actinobacteria were spotted into the agar edge and covered with cover glass. The slide was incubated with moisture in the sterile petri dish at room temperature for 7 days. After incubation, the slide was performed gram staining and the arrangement of spores was under light microscope according to Taechowisan [32]. The actinobacteria which could inhibit the *Colletotrichum* was identified based on 16S rDNA marker. $1\times10^8$ cell of bacteria was used for DNA extraction with BioFact™ Genomic DNA prep Kit (Biofactory, Korea) according to the manufacturer's protocol (Biofactory, Korea). The genomic DNA was electrophorized in 0.8% TBE agarose gel and visualized under UV light using Gel documentation (Bio-Rad, USA). 30 ng of genomic DNA was used in polymerase chain reaction [33] using universal primer for 16s rDNA gene: 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GTTACCTTGTTACGACTT-3') [34] with BioFact™ *Taq* DNA Polymerase (Biofactory, Korea) according to manufacturer's protocol (Biofactory, Korea).

The polymerase chain reaction was performed with the optimal condition according to Lane [34] in a T100™ thermal cycle (Bio-Rad, USA) and then the PCR product was electrophorized in 1.5% TBE agarose gel and visualized under the UV light using Gel documentation (Bio-Rad, USA). The PCR product was purified using BioFact™ Gel & PCR Purification System (Biofactory, Korea) before performing standard sequencing at Bionics (Korea). The partial sequence of 16S rDNA was analyzed with MEGA X [35] and blast in the National Center for Biotechnology Information (NCBI) database followed by phylogeny analysis studied.

# 3. Results

## 3.1. Actinobacteria Isolated From Termite Mound soil and Wasp Soil

Thirty-one and 14 isolates of actinobacteria have been isolated from termite mound (TM) and wasp (W) soils, respectively. Each isolate of actinobacteria is different in colony morphology and also different in color on SCA agar (Table 1).

## 3.2. Morphological and Molecular Study of *Colletotrichum*

The colony of 9-day-old *Colletotrichum* sp. PSRU-NDM65 is grayish-white, brown in color under the colony with round and sharp borders. Microscopic characteristics; brown-septate hyphae. The conidia were 13.0-18.50×3.0-5.0x µm in size (400X magnified), colorless, cylindrical in shape and round at both ends without septa (Figure 1). The morphology of this fungal is conformable with the *Colletotrichum* genus [16]. The nucleotide consensus of ITS rDNA was compared with the NCBI database. The blast search result indicated that the ITS rDNA nucleotide of *Colletotrichum* sp. PSRU-NDM65 was similar to the closest species *Colletotrichum chrysanthemi*, with 99.83% similarity. This *Colletotrichum* is one of several species grouped under the *Colletotrichum acutatum* species complex [36].

**Table 1.** Colony morphology and color on Sodium Caseinate Agar (SCA)

| Strain | Colony morphology | color |
|---|---|---|
| TM-A8, M-A9, TM-A10, TM-A11, TM-B2, TM-D4, W-C1, W-E9 | cottony | black |
| TM-A1, TM-A3, TM-A6, TM-A7, TM-D5, TM-D11, TM-D12, TM-D13, W-C2, W-C3, W-E6, W-E7, W-E8, W-E10 | powdery | brown |
| TM-A2, TM-A4, TM-A5, TM-B1, TM-B3, TM-D6, TM-D8, TM-D14, TM-D15 | cottony | grey |
| TM-B4, TM-B5, TM-D1 W-E 3, W-E4 | leathery | yellow |
| TM-D2, TM-D3, TM-D10, W-C4, W-E1 | cottony | brown |
| TM-D7, TM-D9, W-E2, W-E5 | cottony | white |

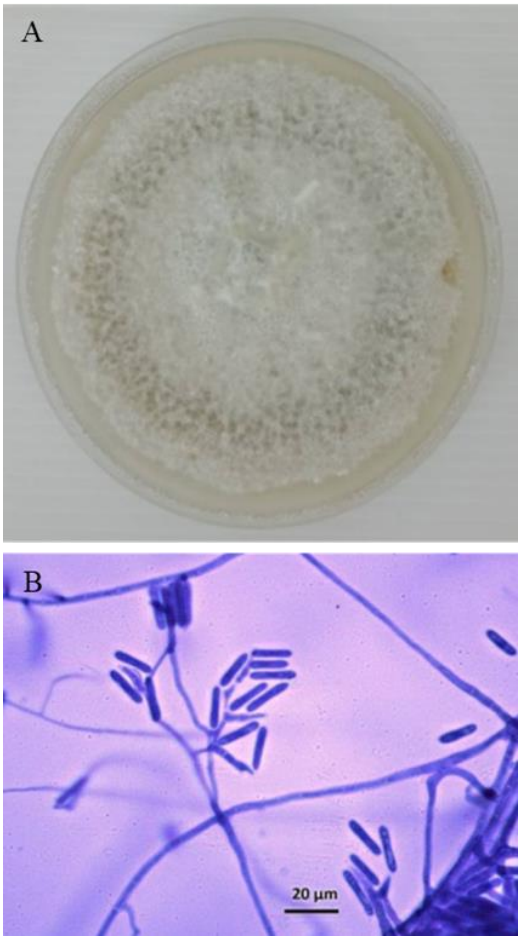


**Figure 1.** Colony (A) and hypha with conidia (B) of *Colletotrichum* sp. PSRU-NDM65

### 3.3. Inhibition Effect of Actinobacteria on Fungal-caused Anthracnose Disease

The inhibition activity of 45 actinobacterial isolates on *Colletotrichum* sp. PSRU-NDM65 was determined using the dual culture method. Strains TM-A3 and TM-A7 showed highly efficient for inhibiting the hypha growth of *Colletotrichum* sp. PSRU-NDM65 ($p$<0.05) as 87.79%, and 85.81%, respectively (Figure 2) and another 7 strains, namely TM-A5, TM-A4, TM-A10, TM-D14, TM-D7, TM-D10 and TM-D6, have the inhibition activity in ranges of 38.28-62.71%. Therefore, TM-A3 and TM-A7 were selected before identify using morphology study and partial sequencing of 16s rDNA gene comparison.

### 3.4. Morphology of Actinobacteria Strains TM-A3 and TM-A7

Two actinobacteria strains of TM-A3 and TM-A7 were performed the morphological study of spore using the slide culture method. The spore of each actinobacteria was produced in different shape; actinobacteria strains TM-A3 and TM-A7 produced the spiral chain of spore (Figure 3) which is conformable with *Streptomyces* [37].

### 3.5. Identification of Actinobacteria

The actinobacterial TM-A3 and TM-A7 strains were phylogenetically identified using partial sequencing of 16S rDNA comparison. PCR product of 1.4 kb length was amplified and performed using Sanger sequencing (Bionics, Korea) before the nucleotide of 16S rDNA was compared with the NCBI database. The result indicated that the 16S rDNA nucleotide of actinobacterial strains TM-A3 was similar to the closest species *Streptomyces misionensis* with 99.70%, whereas strains TM-A7 is 100% identical to *Streptomyces prasinopilosus*. Morphology characteristic analysis of actinobacterial strains TM-A7 was used together with this partial sequence of 16S rDNA analysis to precise the identification results based on of polyphasic approach. The result reveals that morphology characteristics of actinobacteria strains TM-A7 correlated with *Streptomyces prasinopilosus* which is retinaculiaperti spore chains (Figure 3) [37]. The result was supported by the phylogenetic result based on 16S rDNA sequence analysis

The phylogenetic tree was constructed using partial sequence of 16S rDNA of actinobacterial strains TM-A3 and TM-A7, and those of phylogenetically closed *Streptomyces* spp. with the Neighbor-Joining method [38]. *Actinoplanes regularis*, *Micromonospora chokoriensis*, *Nocardiopsis lucentensis*, *Saccharopolyspora rectivirgula* and *Streptosporangium* sp. were used as an out-group (Figure 4). Actinobacterial strains TM-A3 and TM-A7 were categorized into the same clades with these *Streptomyces* spp. with a strong branch support value of 99 and the genetic evolution between actinobacteria strains TM-A3 and TM-A7 presented the score of 0.015 in phylogenetic tree length. The 16S rDNA nucleotides were submitted into the NCBI database with accession no. of MN077150 and MN077162, respectively.

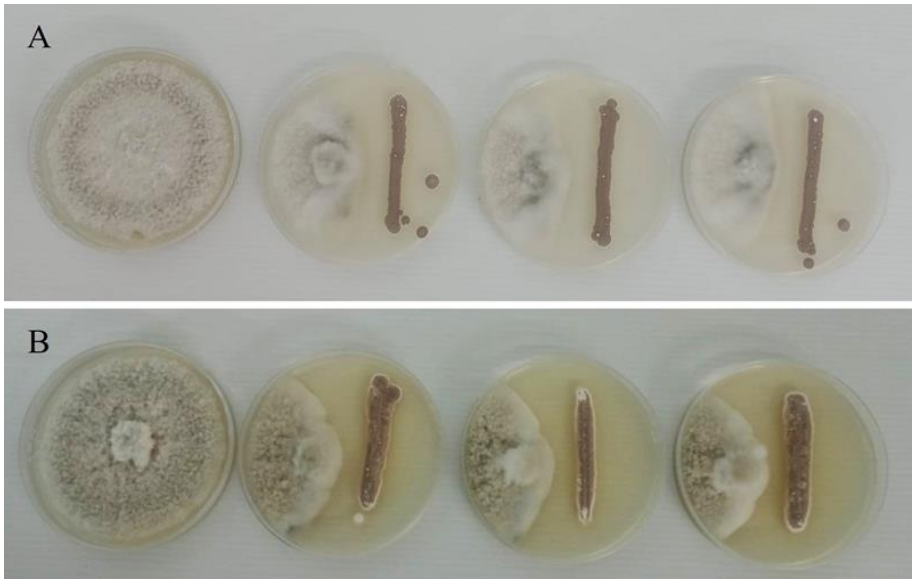


**Figure 2.** The inhibition effect of actinobacteria strains TM-A3 (A) and TM-A7 (B) on hypha growth of *Colletotrichum* sp. PSRU-NDM65 using dual culture method in PDA agar

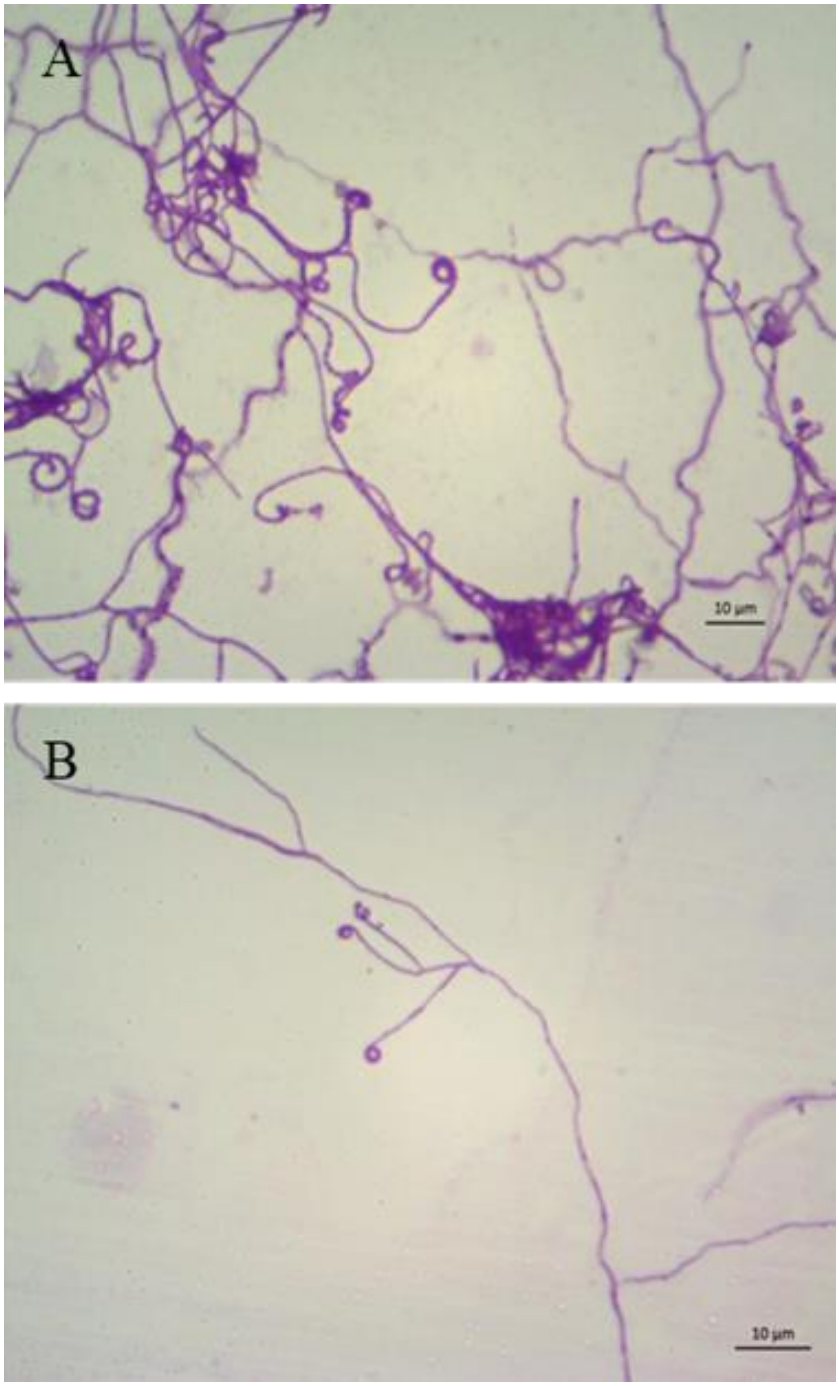


**Figure 3.** Morphology of actinobacteria strains TM-A3 (A) and TM-A7 (B)

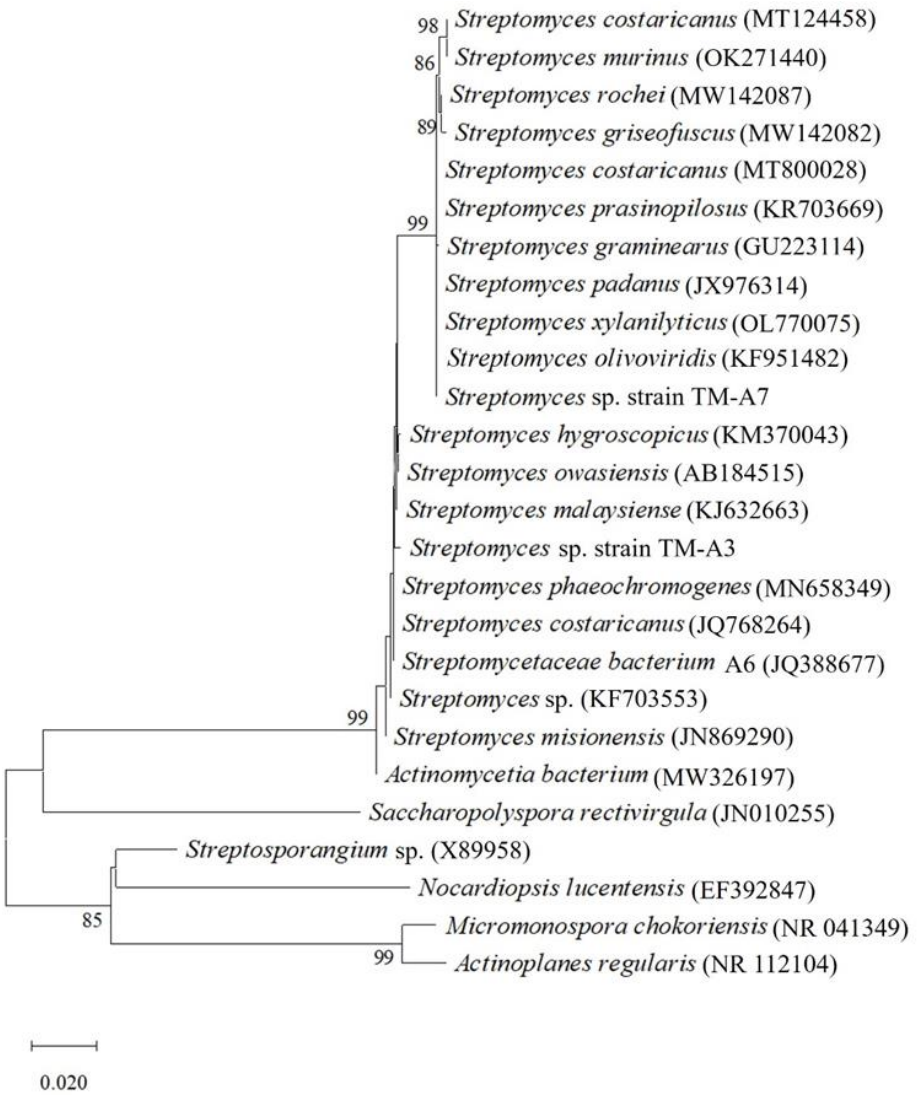


**Figure 4.** Phylogenetics tree of actinobacteria strains TM-A3 and TM-A7 based on partial sequence of 16S rDNA using Neighbor-Joining method; *Actinoplanes regularis*, *Micromonospora chokoriensis*, *Nocardiopsis lucentensis*, *Saccharopolyspora rectivirgula* and *Streptosporangium* sp. were an out-group

# 4. Discussion

A total of 45 actinobacterial strains were collected from both termite mound soil (31 strains) and wasp soil (14 strains). To test the antagonistic relationship between our actinobacterial isolates and the *Colletotrichum* sp. PSRU-NDM65 strain causing anthracnose disease isolated from *Mangifera indica* L., the dual culture technique was applied [39]. As the results obtained, nine actinobacterial strains isolated from termite mound soil, namely TM-A3, TM-A7, TM-A5, TM-A4, TM-A10, TM-D14, TM-D7, TM-D10 and TM-D6, presented the inhibition activities on *Colletotrichum* sp. PSRU-NDM65 with 38.28-87.79% inhibition. On the other hand, no inhibition effect was found from any actinobacterial strains isolated from wasp soil. Strains TM-A3 and TM-A7 had the highest inhibition activity on *Colletotrichum* sp. PSRU-NDM65 ($p$<0.05) as 87.79%, and 85.81%, respectively. These strains were identified based on polyphasic approach, such as morphological study and partial sequencing of 16S rDNA comparison. The results revealed that strains TM-A3 and TM-A7 were members of *Streptomyces*.

Based on morphological characteristics, two strains produce hypha together with long chain spore production which are key characteristics of the *Streptomyces* genus. Phylogenetic identification based on analysis of the partial 16S rDNA sequences confirmed that the two strains are the closest species of *Streptomyces* [39].

*Streptomyces* synthesizes a variety of metabolites, including primary and secondary metabolites. The *Streptomyces* enzymes, including proteases, cellulase, chitinase, β-glucanase, and chitosanase, were found to be degradative activities to fungal cell wall of *C. gloeosporioides* [40-43]. It is possible that TM-A3 and TM-A7 controlled the hyphal growth of *Colletotrichum* sp. PSRU-NDM65 via degradation of fungal cell wall. As identification result mentioned above, TM-A3 presented a phylogenetic relationship close to *S. misionensis* that

produced the metabolites against *C. gloeosporioides* as described [44]. The actinobacterial strain TM-A7 can inhibit *C. gloeosporioides* as same as in the previous experiment [45]. As the report of Saadouli and colleagues [46], *S. misionensis* V16R3Y1 isolated from the date palm rhizosphere (southern Tunisia) presented a broad range of antifungal activity against *Fusarium oxysporum*, *Aspergillus flavus*, *Penicillium expansum*, *Aspergillus niger*, *Candida albicans*, *Candida metapsilosis*, and *Candida parapsilosis*. Additionally, Torabi and research team reported the inhibitiory effect of *S. misionensis* on *Paecilomyces formosus* as the causal agent of dieback and canker diseases of pistachio [47].

To the results of Xu and his colleagues, S. prasinopilosus could inhibit C. gloeosporioides and other fungal species, such as Rhizoctonia solani, Pyricularia grisea, Fusarium oxysporum f. sp. niveum, F. oxysporum f.sp. vasinfectum, and Penicillium italicum [48]. Hong-Thao and co-workers [49] isolated endophytic actinomycetes (TQR12-4) from orange fruit and found the inhibitory effect on Colletotrichum truncatum, Geotrichum candidum, F. oxysporum, and F. udum. Additionally, S. prasinopilosus Pn-TN2 isolated from termite nest sample. It showed a broad range of antimicrobial activity against bacterial and fungal strains. Results showed that this strain possessed inhibition rate antifungal activity by 80% against Fusarium oxysporum, 61% against Fomitopsis palustris, and 62% against Trichoderma viridae [50].

As our results mentioned above strains TM-A3 and TM-A7 isolated from termite mound soil had the highest inhibition activities on *Colletotrichum* sp. PSRU-NDM65 compared with other strains of actinobacteria collected in this study. In the future, these strains should be used for preparation of cell-free extract and analysis of chemical compounds found in the extracts is carried out to show the point of view in biotechnological applications.

## 5. Conclusions

Forty-five strains of actinobacteria were isolated from termite mound and wasp soils, however, only 2 strains of TM-A3 and TM-A7 isolated from termite mound soil are effective inhibiting *Colletotrichum* sp. PSRU-NDM65 growth. The actinobacterial strain TM-A3 was phylogenetically close to *S. misionensis*, and TM-A7 was 100% identical to *S. prasinopilosus*. This study will help apply in disease control and reduce chemical use in agriculture together with the field study performing.

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