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Isolation and characterization of actinobacteria with antibacterial and plant growth-promoting activities from maoberry cultivated soil in Northeast Thailand

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Abstract A total of five actinomycetes were isolated from 20 soil samples collected after 12 months of paclobutrazol application at maoberry cultivation sites in Sakon Nakhon province, Northeast Thailand. The five actinomycetes isolates, namely SM-11, SM-31, SM-51, SM-52, and SM-53, were identified based on morphological characters, particularly pigment production, and then tested for antibacterial and plant growth-promoting activities. The results showed that the ethyl acetate and ethanolic extracts of the isolate SM-31 both exhibited excellent bacterial activities against *Salmonella typhi* and *Staphylococcus aureus*. Similar results were observed for the ethanolic extract of SM-31. Meanwhile, the isolates SM-11 and SM-31 displayed the best plant growth-promoting activities compared to the other isolates. Based on 16s rRNA gene and phylogenetic tree, the potential isolates belonged to *Streptomyces* (SM-11 and SM-31) and *Amycolatopsis* (SM-51) genera. The isolate SM-11 showed 99.66% similarity to *Streptomyces roietensis* strain *WES2*, *Streptomyces xylanilyticus* strain SR2-123 and *Streptomyces mexicanus* strain NBRC 100915. The isolate SM-31 displayed 99.83% similarity to *S. tibetensis* strain XZ 46, *S. hawaiiensis* strain ISP 5042, and *S. coeruleofuscus* strain CSSP429. Moreover, the isolate SM-51 is mostly related to *Amycolatopsis rhabdoformis* strain SB026 with 99.51% similarity. Overall, this study revealed that the selected actinobacteria recovered from maoberry cultivation sites served as a candidate to be explored as a source of bioactive compounds.

Keywords: Actinobacteria, Antibacterial, Plant growth-promoting bacteria, Cultivated soil

Introduction

Maoberry [*Antidesma thwaitesianum* Müll. Arg.] is a wild plant that is classified in the Phyllanthaceae family. It is a shrub plant with a height of 5–10 m and usually flowers in the rainy season, bearing young fruits that are dark green and turn to orange-red, dark red and deep purple in the fully ripened stage in the late rainy season (Suravanichnirachorn *et al.,* 2018).

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The entire plant of maoberry is of medicinal value due to its antioxidant, anticancer and antidiabetic properties (Jorjong *et al.,* 2015). Maoberry is one of the tropical fruit trees that grow well in a wide variety of soil types, particularly in dipterocarp forests. It is commonly found in Africa, Australia, a group of islands in the Pacific Ocean and tropical Asia. In Thailand, it is typically found and widely grown in the northeastern region of the country (Jorjong *et al.,* 2015; Suravanichnirachorn *et al.,* 2018). The ability of maoberry to grow abundantly in various soil types may be due in part to rhizosphere microbial communities that extend the plant's ability to adapt to diverse environmental conditions (Chen *et al.,* 2020).

Rhizosphere also known as microbe storehouse is the soil zone surrounding plant roots, in which there is intense chemical dialogue between plants and microorganisms (Barra Caracciolo and Terenzi, 2021; Prasad *et al.,* 2019). It is regarded as one of the most complex environments colonized by several classes of microorganisms (Li *et al.,* 2020). In the rhizosphere, plants release root exudates offering a great variety of carbon-rich substances to promote microbial population development (Barra Caracciolo and Terenzi, 2021). Rhizosphere microbiota in turn protect plants against pathogens, improve plant growth by producing phytohormones, and help plants withstand environmental perturbations such as abnormal variations in temperature, drought, and salinity (Lu *et al.,* 2018). In general, microbial communities in the rhizosphere have a close association with plant roots (Li *et al.,* 2020) and their structure and diversity vary considerably across regions, which are mainly driven by environmental factors like soil saturation and nutrient levels (Bickford *et al.,* 2020; Lin *et al.*, 2019).

Among the microbial community, actinomycetes have drawn increasing attention over the last few decades because of the ability to thrive profusely in diverse habitats (Khamna *et al.,* 2009) and the potential to produce a myriad of secondary metabolites of economic and biological importance (Poomthongdee *et al.,* 2015; Shamikh *et al.,* 2020). They are a group of Gram-positive bacteria with high guanine–cytosine contents in their genomic DNA, some of which are filamentous with true mycelia (AbdElgawad *et al.,* 2020; Xue *et al.,* 2013). In soil habitats, actinomycetes play an important role in nitrogen fixation, recycling of dead organic matter and phosphate solubilization. Actinomycetes are regarded as plant growth-promoting bacteria due to the beneficial effects on plant growth. These effects can be either direct or indirect (or both). These bacteria indirectly enhance plant growth through inhibiting or preventing the growth of pathogenic or harmful organisms by the production of antimicrobial compounds or by competing with those organisms. Meanwhile, actinomycetes directly influence plant growth by secreting plant growth hormones, growth-promoting compounds or enhance the growth of beneficial microorganisms (Hozzein *et al.,* 2019).

It has been well documented that because of diverse habitat conditions, actinomycetes vary substantially from site to site (Dede *et al.,* 2020; Han *et al.,* 2018; Palla *et al.,* 2018), with their striking differences in biological activities including plant growth-promoting traits (Poomthongdee *et al.,* 2015; Shamikh *et al.,* 2020; Sun *et al.,* 2020). However, the potential effects of actinomycetes isolated from maoberry rhizosphere on plant growth as well as their antibacterial activities are still not yet well studied. For this reason, this study was carried out to isolate and assess the antibacterial activities and plant growth-promoting traits of actinomycetes naturally existing in maoberry cultivated soil in which paclobutrazol was previously applied for 12 months.

Materials and methods

Sample collection and isolation

Soil samples were collected from maoberry cultivated areas (16 ℃51'06.9"N 103 ℃56'56.0"E, 309 m elevation) in Sakon Nakhon Province, Northeast Thailand. The area was treated with paclobutrazol for 12 months before sampling was carried out. Five different soil samples (S1 to S5) were collected at 20 cm in depth and kept in clean polyethylene bags. The samples were air dried and sieved through 100 mesh and stored in a sealed aluminum foil packaging bags at 4 ℃ until use. Briefly, 1 g of the collected soil sample was placed in 99 mL of sterile water, and a soil suspension was prepared by shaking the mixture at 180 rpm for 10 min. Then, 1 mL of this suspension was transferred into 9 mL of fresh sterile water for dilution. Subsequently, the suspension was serially diluted until a 10^{-4} dilution, in duplicates and 100 µL of each dilution from the last three dilutions was plated by spreading on Gauze's synthetic medium no. 1 plates (1 g KNO_3, 0.5 g K_2HPO_4 $3H_2O$, 0.5 g $MgSO_4$ ·$7H_2O$, 0.5 g NaCl, 0.5 g $FeSO_4$ ·$7H_2O$, 20 g starch, 17 g agar, and 1 L distilled water), and incubated at 35 ℃ for 7 days. The colony was selected and cross-streak on Gauze's no. 1, plates incubated at 35 ℃ for 10 days for pure actinobacteria. The morphological identification ant isolated strains were designated SM-11, SM-31, SM-51, SM-52, and SM-53.

Screening for antibacterial activities of isolated strains

The crude extracts of the isolates SM-11, SM-31, SM-51, SM-52, and SM-53 were tested for the antibacterial activities against pathogenic bacteria, namely *E. coli*, *S. aureus*, *S. typhi*, and *K. pneumoniae* using disk diffusion method. The isolates were cultured in oatmeal broth for 27 days to allow for secondary metabolite production. After filtration, actinomycete cells were collected and subjected to extraction using ethyl acetate and

ethanol as solvents. Next, the extracts were evaporated to dryness under a gentle steam of nitrogen gas. Then, 2 mg of each crude extract were dissolved in 1 mL of dimethyl sulfoxide (DMSO). To assess the antibacterial activities of the isolates, each pathogenic bacterium was spread onto the surface of LB agar. Then, filter paper disks impregnated with 10 µL of each crude extract (2 g/L) were placed on the surface, and the size of inhibition zone around the disk was measured after 24-h incubation at 37 ℃. Absolute DMSO served as the controls.

Determination of plant growth-promoting activities of isolated strains

The extracellular culture filtrates of the isolates SM-11, SM-31, SM-51, SM-52, and SM-53 were tested for the plant growth-promoting activities. The isolates were grown on actinomycete isolation agar (AIA) and incubated at 35 ℃ for 10 days or until the agar was fully covered by the mycelia. The agar was then dissected into squares (1 cm^2) and transferred into Erlenmeyer flasks containing glucose yeast extract (GYE) broth. The flasks were shaken on a rotary shaker (180 rpm) at 35 ℃ for 10 days. The extracellular filtrate was taken by filtration through No.1 Whatman filter papers. To assess the influence of the filtrates on seed germination, rice seeds were surface sterilized in 3% H_2O_2 for 5 min, washed twice with distilled water, and then 10 seeds sown on AIA previously spread with the filtrates. After 5 days incubation, the germinability of rice seeds was recorded.

Pot experiments were also performed to assess the effects of the filtrates on the growth of rice seedlings. Rice seeds were steeped in water for 24 h to induce root germination, surface sterilized in 3% H_2O_2 for 5 min and then washed thrice with distilled water. Subsequently, the seeds were sown in pots containing sterile soil taken from the same site of maoberry grown area. In the first week, the pots were irrigated daily with 1 mL of the individual filtrates. After that, they were irrigated at 3-day intervals for 21 days with 5 mL of the individual filtrates. Each experiment consisted of 10 pots, each sown with three rice seeds. At the end of the experiment, the number of leaves, shoot and root lengths, and fresh and dry weights were recorded.

Molecular identification

Actinobacteria strains grown on oatmeal agar were used for total genomic DNA extraction (Dees *et al.*, 2013). PCR amplifications of 16S rRNA gene fragments (16S rDNA) were performed in a 100-µL volume of PCR reaction composed of ~100 ng DNA template, 200 µM dNTPs, 1.5 mM MgC_{l2}, 5% DMSO and 2 unit of Platinum® *Taq* DNA Polymerase (Invitrogen™; Thermo Fisher Scientific). The primer sets used in

amplifying and sequencing the isolated strains were Act-235F (5′-CGCGGCCTATCAGCTTGTTG-3′) and Act-878R (5′-CCGTACTCCCCAGGCGGGG-3′) (Stach *et al.,* 2003). The PCR amplification was carried out using the thermal cycler (T100^{TM}; BIO-RAD) and programed as follows: initial denaturation at 95 ℃ for 3 min, denaturing at 95 ℃ for 30 s, primer annealing at 50 ℃ for 30 s and primer extension at 72 ℃ for 1 min. The annealing and extension were repeated for 35 cycles. The final extension was performed at 72 ℃ for 5 min. The PCR reaction products were electrophoresed on a 1% agarose gel containing 1X SYBR™ Safe DNA Gel Stain (Invitrogen^{TM}; Thermo Fisher Scientific) and the DNA bands were visualized under the LED blue light transilluminator (Blupad, Bio-Helix). The PCR products were purified (PureDireX, Bio-Helix) and then sequenced by an automated sequencer. The nucleotide sequence data were submitted to the BLASTN programs search nucleotide database (http://www.ncbi.nlm.nih.gov) within the National Center of Biotechnology Information (NCBI), GenBank. Phylogenetic analyses were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

Isolation and characteristics of actinobacteria isolates

A total of five isolates of actinobacteria were isolated from the soil dilution agar plates. Most of the isolates formed brown-to-black colonies and were able to produce diffusible pigments. The colonies of the isolate SM-11 were dull and dry with green powdery spores and yellowish-green aerial mycelia producing yellowish-green diffusible pigment. The isolate SM-31 formed dull and dry colonies and produced white velvet spores and pink diffusible pigment from substrate mycelia. The isolate SM-51 produced white dull and dry colonies and light brown diffusible pigment. The isolate SM-52 formed dry colonies and produced yellowish-brown powdery spores and yellowish-brown diffusible pigment. The isolate SM-53 produced dry colonies, black velvet spores and substrate mycelia (Figure 1).

Screening for antibacterial activities of isolated strains

The crude extracts of five isolates were tested for their antibacterial activities against *S. typhi, S. aureus, K. pneumonia* and *E. coli* using disk diffusion method. The results showed that the ethyl acetate extract of SM-31 could inhibit growth of *S. typhi* and *S. aureus*, with the average diameter of inhibition zone of 11 and 10 mm, respectively. The ethyl acetate extracts of SM-11, SM-51 and SM-52 displayed the inhibitory effects against *S. aureus* only, showing the inhibition zone diameter of 10, 8 and 9 mm,

respectively. Moreover, the ethanolic extracts of SM-31 and SM-52 exhibited the inhibitory effects against *S. typhi* and *S. aureus*, with the inhibition zone diameter of 10-11 mm (Figure 2 and Table 1).

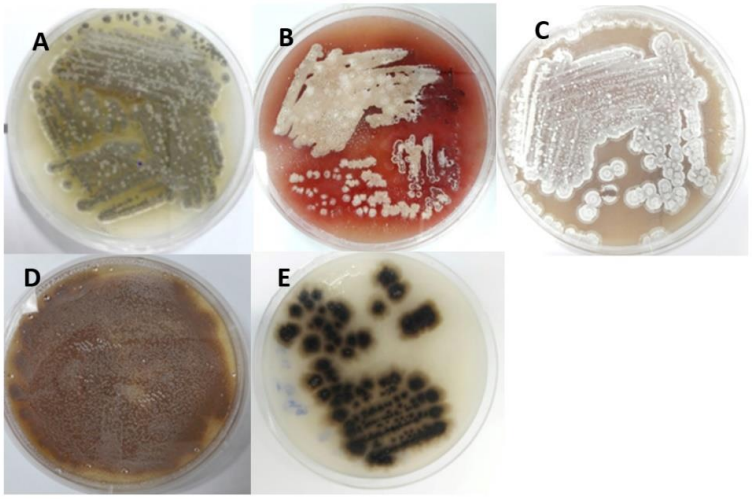

Figure 1. Mycelial morphologies of actinobacteria isolates SM-11 (A), SM-31 (B), SM-51 (C), SM-52 (D), and SM-53 (E)

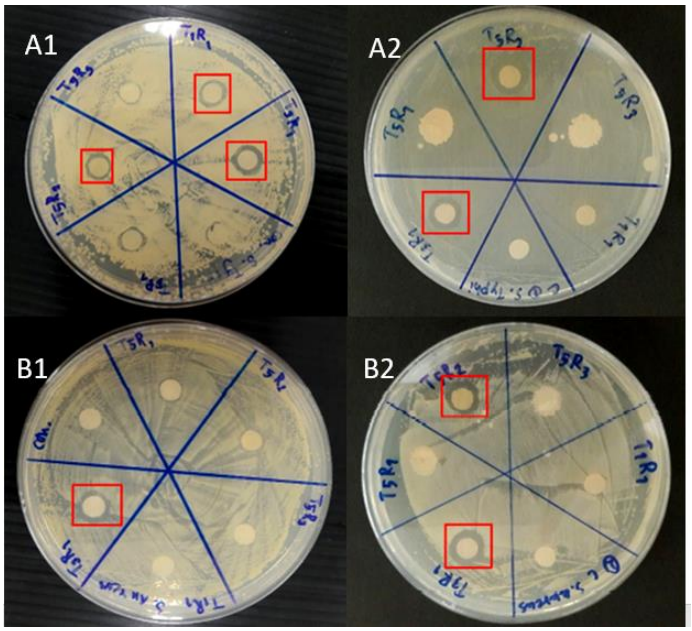

Figure 2. Antibacterial activities of actinobacteria isolates measured in terms of zone of inhibition, (A1) Ethyl acetate extracts of SM-11, SM-31, and SM-52 against *S. typhi*. (A2) Ethanolic extracts of SM-31 and SM-52 against *S. typhi*. (B1) Ethyl acetate extract of SM-31 against *S. aureus*. (B2) ethanolic extracts of SM-31 and SM-52 against *S. aureus*

Table 1. Antibacterial activities of actinomycetes isolates against *S. typhi*, *S. aureus, K. pneumonia* and *E. coli*

Crude extracts	**Average diameter of inhibition zone (mm)**			
	S. typhi	***S. aureus***	***K. pneumonia***	***E. coli***
Ethyl acetate extracts				
Control (DMSO)	0	0	0	0
SM-11	10	0	0	0
SM-31	11	10	0	0
SM-51	8	0	0	0
SM-52	9	0	0	0
SM-53	0	0	0	0
Ethanolic extracts				
Control (DMSO)	0	0	0	0
SM-11	0	0	0	0
SM-31	10	11	0	0
SM-51	0	0	0	0
SM-52	11	11	0	0
SM-53	0	0	0	0

Plant growth promotion of isolated strains

Plant growth-promoting activities of actinobacteria isolates were performed to assess the effects of the filtrates on the growth of rice seedlings. It was found that only the isolates SM-11 and SM-31 could improve rice seed germination and the growth of rice seedlings, as shown in Figures 3 and 4, and Table 2. The isolate SM-31 was best for enhancing both rice seed germination and seedling growth, giving the maximum shoot and root lengths of 15.72 and 7.04 cm and the highest fresh and dry weights of 130.62 and 53.68 mg, respectively. By contrast, the isolate SM-53 was least effective in promoting rice seed germination and seedling growth, displaying the lowest shoot and root lengths of 9.57 and 4.56 and the fresh and dry weights of 100.10 and 40.09 mg, respectively.

Table 2. Effect on rice growth parameters of the culture filtrates of actinomycetes SM-11 (A), SM-31 (B), SM-51 (C), SM-52 (D) and SM-53 (E), and control (F)

Isolates	**Growth parameters**			
	Shoot length (cm)	**Root length (cm)**	**Fresh weight (mg)**	**Dry weight (mg)**
Control	10.69	5.78	110.35	45.33
SM-11	11.66	6.84	120.55	50.03
SM-31	15.72	7.04	130.62	53.68
SM-51	10.74	4.84	110.85	46.30
SM-52	11.09	5.02	110.22	45.82
SM-53	9.57	4.56	100.10	40.09

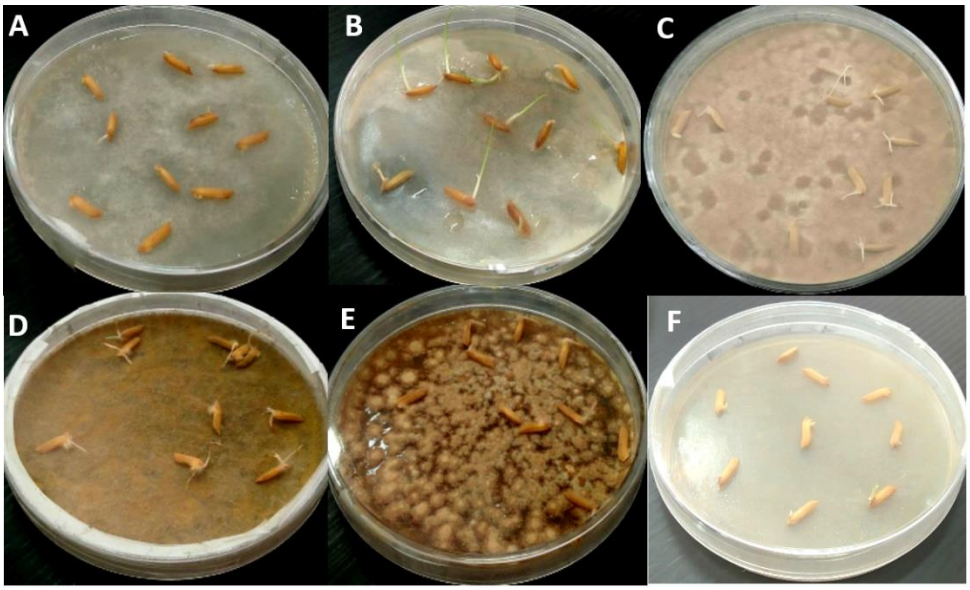

Figure 3. Effect on rice seed germination of the culture filtrates of actinobacteria isolates SM-11 (A), SM-31 (B), SM-51 (C), SM-52 (D) and SM-53 (E), and control (F)

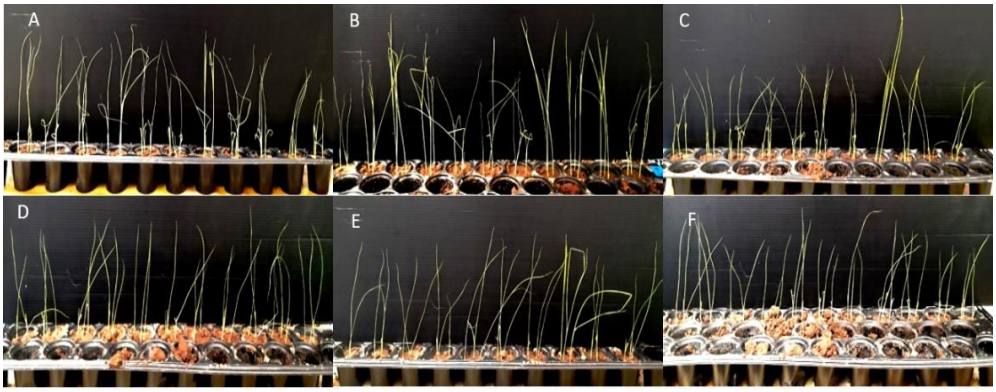

Figure 4. Effect on rice growth parameters of the culture filtrates of actinomycetes SM-11 (A), SM-31 (B), SM-51 (C), SM-52 (D) and SM-53 (E), and control (F)

Molecular identification of actinobacteria isolates

After total genomic DNA extraction, the 16S rDNA fragments were PCR amplified and the PCR reaction products were electrophoresed on a 1% agarose gel containing SYBR™ Safe DNA Gel Stain. As depicted in Figure 5, by using 1 kb DNA ladder as a reference band, only three isolates

(SM-11, SM-31, and SM-51) were positive PCR results, each giving a band of 643 bp DNA size on the gel. The other two isolates (SM-52 and SM-53) were negative. The 16S rDNA sequences of the three isolates were compared to those of known 16S rDNA sequences using the BLASTN programs search nucleotide database within the National Center of Biotechnology Information (NCBI), GenBank. The results showed that three of the isolates belong to *Streptomyces* (SM-11 and SM-31) and *Amycolatopsis* (SM-51) genera. As presented in Figure 6 and Table 3, the isolate SM-11 showed 99.66% similarity to *S. roietensis* strain WES2, *S. xylanilyticus* strain SR2-123 and *S. mexicanus* strain NBRC 100915. Meanwhile, the isolate SM-31 displayed 99.83% similarity to *S. tibetensis* strain XZ 46, *S. hawaiiensis* strain ISP 5042, and *S. coeruleofuscus* strain CSSP429. Moreover, the isolate SM-51 is mostly related to *A. rhabdoformis* strain SB026 with 99.51% similarity.

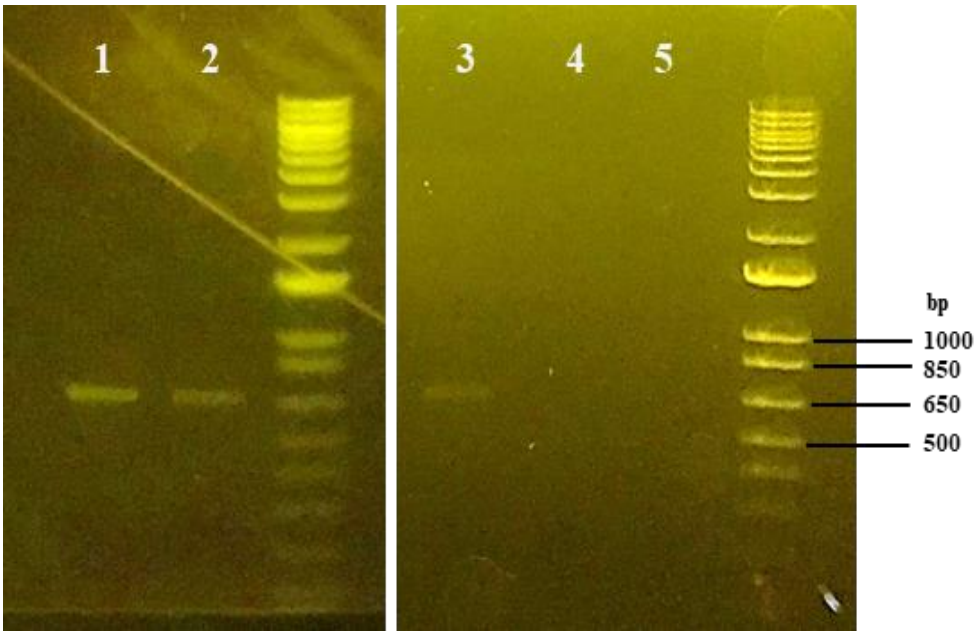

Figure 5. Agarose gel electrophoresis of the PCR products. Lane 1 = SM-11, Lane 2 = SM-31, Lane 3 = SM-51, Lane 4 = SM-52, and Lane 5 = SM-53

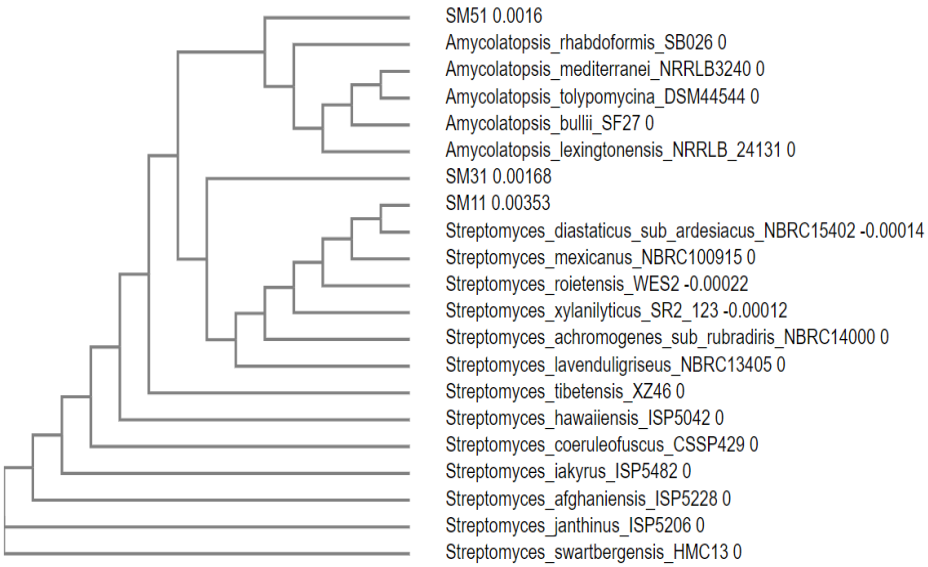

Figure 6. Phylogenetic tree of 3 isolated strains from maoberry rhizosphere and related Actinobacteria based on the 16S rDNA gene sequence. Sequences of 18 type strains were obtained from the GenBank

Table 3. 16S rDNA sequence analysis of Actinobacteria isolated from maoberry rhizosphere

Isolates	**Strains**	**identity (%)**
SM-11	*Streptomyces roietensis* strain WES2	99.66%
	Streptomyces xylanilyticus strain SR2-123	99.66%
	Streptomyces mexicanus strain NBRC 100915	99.66%
SM-31	*Streptomyces tibetensis* strain XZ 46 *Streptomyces*	99.83%
	hawaiiensis strain ISP 5042	99.83%
	Streptomyces coeruleofuscus strain CSSP429	99.83%
SM-51	*Amycolatopsis rhabdoformis* strain SB026	99.51%
	Amycolatopsis mediterranei strain NRRL B-3240	99.18%
	Amycolatopsis tolypomycina strain DSM 44544	99.18%
SM-52	PCR Negative	
SM-53	PCR Negative	

Discussion

The use of actinobacteria for improving soil fertility and plant production is an attractive strategy for developing sustainable agricultural systems due to their effectiveness, eco-friendliness, and low production cost (AbdElgawad *et al.,* 2020). Several studies have been carried out to isolate actinobacteria in various extreme and underexplored environments in many parts of the world including Thailand in the last few decades (Ganesan *et al.,* 2017; Kuncharoen *et al.,* 2019; Ouchari *et al.,* 2019). However, there is no report on isolation of actinobacteria from the rhizosphere of mulberry-grown fields in Sakon Nakhon, Northeastern Thailand. Hence, an attempt has been made to isolate actinobacteria from this unexplored region to find novel species. A total of 20 rhizosphere soil samples were collected to isolate actinobacteria and only five isolated strains were chosen to test their antibacterial and plant growth-promoting activities. Morphological examinations clearly indicated that these isolates belong to the Streptomyces and Amycolatopsis genera. The isolates differed in the color of the substrate and aerial mycelia and all the isolates chosen produced diffusible pigments.

Many studies have revealed that actinobacteria produce a broad range of secondary metabolites including important antibiotics (Charousová *et al*., 2017; Singh *et al*., 2016). In this regard, the crude extracts of the five actinobacteria isolates were tested for their potential to inhibit the growth of the four selected pathogenic bacteria. The results unravelled that none of the isolates exhibited antibacterial activities against *K. pneumonia* and *E. coli*, which might be due to the low concentrations of the extracts. However, the crude extracts from some isolates could inhibit the growth of *S. typhi* and *S. aureus*, suggesting that these isolates could be utilized as antibacterial agents.

In addition of producing many metabolites values such as antibiotics, actinobacteria are involved in diverse processes that improve soil health and fertility, including nitrogen fixation, phosphate solubilization, organic matter cycling, compost piles stabilization and plant and animal residues degradation (Hozzein *et al.,* 2019). In this study, the five isolates were assessed for their ability to enhance seed germination and growth of rice seedlings. The results showed that only the isolates SM11 and SM31 could improve the growth of rice seedlings, with the highest improvement observed for the isolate SM31.

According to 16s rRNA gene and phylogenetic tree, three of the isolates belong to Streptomyces (SM-11 and SM-31) and Amycolatopsis (SM-51) genera. The other two isolates were PCR negative, which might be using inappropriate primers. The most critical step for accurate rDNA amplicon analysis was still the choice of primers (Klindworth *et al*., 2013). Based on phylogenetic tree, the isolate SM-11 showed 99.66% similarity to

S. roietensis strain WES2, *S. xylanilyticus* strain SR2-123 and *S. mexicanus* strain NBRC 100915. The isolate SM-31 displayed 99.83% similarity to *S. tibetensis* strain XZ 46, *S. hawaiiensis* strain ISP 5042, and *S. coeruleofuscus* strain CSSP429. Moreover, the isolate SM-51 is mostly related to *A. rhabdoformis* strain SB026 with 99.51% similarity. These results revealed that the actinobacteria strains recovered from maoberry soil rhizosphere served as a good candidate to be explored as a source of bioactive compounds.

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