



Chiang Mai J. Sci. 2018; 45(7) : 2652-2665

<http://it.science.cmu.ac.th/ejournal/>

Contributed Paper

## Characterization, Antifungal Activity and Plant Growth Promoting Potential of Endophytic Actinomycetes Isolated from Rice (*Oryza sativa* L.)

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Received: 1 June 2018

Accepted: 14 August 2018

### ABSTRACT

Actinomycetes are well known as producers of biologically active compounds with high commercial value. They have been isolated from various sources including plant species. In this study, the plant growth promoting traits of endophytic actinomycetes isolated from rice plants were evaluated, as well as their antagonistic activities against rice pathogenic fungi. Healthy rice plants were collected from nine provinces in Thailand. One hundred and ninety-one isolates were able to be recovered from the surface-sterilized roots and stems of the rice plants. Of these, 96 actinomycetes were classified as streptomycetes and 95 strains were non-streptomycetes species. Plant growth promotion assays showed that 79.6% and 30.9% of these isolates could produce siderophore and indole-3-acetic acid, respectively and 42.9% could solubilize phosphate. Potential strains which showed strongly antagonistic against rice pathogenic fungi, *Fusarium oxysporum*, *Rhizoctonia solani* and *Helminthosporium oryzae*, with plant growth promoting activities were selected to determine their effects on root system of rice seedlings *in vitro*. The results showed that the isolates R07-04, R07-06 and S03-26 could increase the number of adventitious roots, but led to shorter seminal roots. However, in the pot experiment, isolate R07-06 could promote rice growth better than the other isolates and significantly enhanced shoot length, root dry weight and shoot dry weight. An identification of the isolate R07-06 using the 16S rRNA gene showed that the strain was most closely related to *Streptomyces lydicus* DSM 40002<sup>T</sup> (100% similarity). These results demonstrate that endophytic *Streptomyces* R07-06 could be a promising strain to develop as an antifungal and plant growth promoting agent for rice plants.

**Keywords:** actinomycetes, antifungal, endophyte, plant growth promotion, *Oryza sativa* L.

## 1. INTRODUCTION

Actinomycetes are Gram-stain-positive bacteria with high G+C genomic content. They are well known as sources of secondary metabolites and have been well characterized in the literature due to their economic importance as producers of two-thirds of the microbial derived antibiotics known today [1]. Actinomycetes are widely distributed in nature, for example, in soil, rhizospheres, sediments, including plant tissues. Endophytic actinomycetes are known to form more intimate associations with plants and colonize plant internal tissue without causing detectable damage to the host plant. Their abilities to promote plant growth and protect host plants from pathogens have been described [2-4].

Rice (*Oryza sativa* L.) is one of the most important agricultural and economical crops in Thailand. Thai people consume rice as a staple food. Moreover, rice is one of the most important grain crop foods, supporting about half of the world's human population. However, rice diseases caused by phytopathogenic fungi, such as blast (*Pyricularia grisea* Sacc.), sheath blight (*Rhizoctonia solani*), brown spot (*Helminthosporium oryzae*) and bakanae (*Fusarium moniliforme*), are one of the major problems in rice cultivation, since they can reduce rice quality and crop yield. Although the use of synthetic chemical pesticides to control these diseases can be highly efficient, it is harmful to humans, causes environmental pollution, and has detrimental effects on a variety of non-target microorganisms. An alternative method to used antagonistic microorganisms alone or antagonistic microorganisms supplemented with chemical pesticides has been reported to minimize the used of agrochemicals in rice plants [5, 6]. The combination of *Streptomyces philanthi* RM-1-138 with chemical fungicide has been proved to increase the protection of rice plants from sheath blight

[6]. Recent reports suggested that endophytic actinomycetes are potential biological control agents and can promote plant growth by producing siderophores, solubilize of insoluble phosphates, as well as by the production of indole-3-acetic acid [2, 7, 8]. Therefore, the present work was carried out to isolate endophytic actinomycetes from the roots and stems of rice plant. The isolates which showed high antagonistic activity against rice pathogenic fungi, as well as *in vitro* plant growth promoting traits, were studied for their ability to promote rice growth.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection and Isolation of Endophytic Actinomycetes

Healthy rice plants (*Oryza sativa* L.) were collected from 22 rice-field sampling sites in Thailand, located in nine provinces, including Ayutthaya, Bangkok, Chiang Mai, Nakornsawan, Nonthaburi, Pathumthani, Petchaburi, Ratchaburi and Suphanburi. Rice plants were pulled out carefully from the soil, placed in plastic bags and transferred to the laboratory for isolation of actinomycetes.

The collected whole plants were washed thoroughly under running tap water. The roots and stems were separated into segments 3-5 cm in length before being subjected to a surface sterilization procedure as described previously by Mingma *et al.* [9] with some modifications. Each sample was shaken in 70% (v/v) ethanol for 5 min and 1% (v/v) sodium hypochlorite (NaOCl) for 10 min, and then rinsed with sterile distilled water five times to remove traces of the disinfectant. In order to validate the surface sterilization protocol, an aliquot of 0.2 ml of the final rinsing water was spread on starch-casein (SC) agar plates [10] to observe bacterial and fungal growth after incubation at 28 °C for 24-48 h. The surface-sterilized samples were

crushed in 0.85% (w/v) NaCl solution and 100 µl of plant suspension was spread on SC agar plate supplemented with ketoconazole (100 µg/ml), nalidixic acid (25 µg/ml) and nystatin (50 µg/ml), in order to minimize and inhibit the growth of unwanted bacteria and fungi. The plates were incubated at 28-30 °C. The actinomycete colonies were transferred from isolation plates every week for up to four weeks and purified on glucose yeast extract (GYE) agar. Spores and cells of the pure culture were stored in 20% (v/v) glycerol at -20 °C for long-term preservation.

## 2.2 Determination of the Diaminopimelic Acid (DAP) in Whole-cell Hydrolysates

Diaminopimelic acid (DAP) in whole-cell hydrolysates was detected using modified method according to Hasegawa *et al.* [11]. Two or three colonies of the actinomycetes were hydrolyzed with 6 N HCl and heated by autoclaving at 121 °C for 15 min. Then, the hydrolysates (10-15 µl) were spotted directly on the chromatography paper (3MM Whatman, 20 × 20 cm) together with DAP standard. Ascending chromatography was performed on the solvent system of methanol : distilled water : 10 N HCl : pyridine (80 : 17.5 : 2.5 : 10, v/v) for 2 hr. The spots of DAP isomers (LL- or *meso*- DAP) were visualized by spraying with 0.2% (w/v) ninhydrin in acetone and heated at 100 °C for 5 min.

## 2.3 Taxonomic Characterization of Endophytic Actinomycetes

The actinomycete isolates containing the *meso*-isomeric form of DAP and the isolates with strongly antagonistic activities as well as high plant growth promoting traits were selected for identification based on an analysis of the 16S rRNA gene sequences. The isolates were cultured on GYE agar plate and incubated at 28 °C for five days.

Genomic DNA was extracted and PCR amplification was carried out according to the method described by Mingma *et al.* [9]. The 16S rRNA gene was PCR amplified from genomic DNA by using the primers 27F (5'-AGAGTTTGTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3'). PCR protocol included an initial hot start incubation (10 min at 94 °C) followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 1 min followed by a final extension step at 72 °C for 10 min. The PCR products were purified using a Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taiwan), following the manufacturer's protocols, and sequenced by a commercial sequencing company at 1<sup>st</sup> Base Laboratory (Malaysia) and/or Macrogen (Seoul, South Korea). The EzBioCloud identification service (<https://www.ezbiocloud.net/>) was employed in order to assess the degree of DNA similarity. The 16S rRNA sequences were deposited in the DDBJ Nucleotide Sequence Database (Research Organization of Information and Systems National Institute of Genetics, Shizuoka, Japan) under accession numbers LC011586-LC011697.

## 2.4 Antagonistic Activity Against Rice Pathogenic Fungi

The three rice pathogenic fungi used in this study, *Helminthosporium oryzae*, *Rhizoctonia solani* and *Fusarium moniliforme*, were maintained on potato dextrose agar (PDA; Difco). All 191 endophytic actinomycetes were tested for their antagonistic activity against these fungi, using the dual culture technique as described by Himaman *et al.* [2]. Briefly, an agar plug of well-grown actinomycete (5 mm in diameter) on ISP medium 2 agar [12] was transferred onto PDA plates (3 cm away from the center of the plate) and

incubated at 28 °C for seven days. Then a fungal mycelium disc was placed in the center of the plate and further incubated at 28 °C. PDA plates with a fungal disc in the center of the plate without actinomycetes were served as a control. The experiment was conducted in two replicates. The percentage of inhibition was calculated when the fungal mycelium of the control reached the edge of the plate, using the formula:  $[(r_1 - r_2)/r_1] \times 100$ , where  $r_1$  is the radius of fungal growth in control and  $r_2$  is the radius of fungal growth in the direction of the actinomycete on the tested plates.

## **2.5 Plant Growth Promoting Traits of Endophytic Actinomycetes**

### **2.5.1 Siderophore production**

The actinomycete isolates were screened for siderophore production using a Chrome azurol S (CAS) assay as described by Schwyn and Neilands [13]. Agar discs (5 mm in diameter) from a seven-day-old culture of actinomycete isolates growing on ISP medium 2 were inoculated onto a CAS agar plate and incubated at 28 °C. The experiment was conducted in duplicates. The isolates that produced an orange halo zone around the colony were considered as siderophore-producing isolates. The size of the orange halo was measured and recorded after incubation for five days.

### **2.5.2 Indole-3-acetic acid (IAA) production**

The ability of isolate to produce IAA was assessed using the colorimetric method. The actinomycete isolates were cultured in GYE broth supplemented with 0.2% (w/v) L-tryptophan and incubated by shaking (180 rpm) in the dark at 28 °C for seven days. The cultures were centrifuged at 8,000 rpm for 10 min and 2 ml of supernatant was

mixed with 1 ml of Salkowski's reagent [14]. The mixture was then incubated in the dark at room temperature for 30 min. The development of a pink color indicated production of indole compounds. The mixture was read using a UV-spectrophotometer at 530 nm absorbance. The amount of IAA produced per milliliter of culture ( $\mu\text{g/ml}$ ) was estimated using a standard curve for indole-3-acetic acid (0.5-100  $\mu\text{mol/L}$ )

### **2.5.3 Phosphate solubilization activity**

The ability of isolates to solubilize phosphate  $[\text{Ca}_3(\text{PO}_4)_2]$  was assessed qualitatively using the method described by Pikovskaya [15]. Agar plugs (0.5 mm in diameter) of a seven-day-old culture of actinomycetes were placed on the surface of a Pikovskaya's (PVK) agar plate containing tricalcium phosphate. The plates were incubated in the dark at 28 °C for two weeks. The colonies that produced clear halo zones were considered as phosphate solubilizing isolates. The phosphate solubilization halo and colony diameters were measured and the halo size was calculated by subtracting the colony diameter from the total diameter. The experiments were replicated twice.

## **2.6 Effects of Endophytic Actinomycetes on Rice Roots**

### **2.6.1 Preparation of actinomycetes spore suspension**

Actinomycetes were grown on ISP medium 3 at 28 °C for 14 days. Spores were scraped from the agar surface, suspended in 0.1% (v/v) Tween 20 solution and sieved through sterile cotton wool. Spore density was measured by counting with a haemocytometer and adjusted to  $10^8$  spores/ml in a 10 mM  $\text{MgSO}_4$  solution.

### 2.6.2 Preparation of rice seeds and determination of rice roots morphology

Thai Hom Mali Rice (Thai Jasmine Rice) was used in this study. The rice seeds were surface sterilized for 10 min each in 75% (v/v) ethanol and 3.5% (v/v) sodium hypochlorite (NaOCl) containing 0.1% (v/v) Triton X-100, and then rinsed four-to-five times with sterile distilled water. The sterilized seeds were then soaked in sterile distilled water overnight and pregerminated on moist-sterile tissue paper at room temperature for two days in the absence of light, until the radical roots had emerged approximately 1 cm in length.

The spore suspension of each of the actinomycetes ( $10^7$  spores/ml) was added and mixed thoroughly in sterile half-strength Hoagland's [16] soft agar (0.6%, w/v) contained in glass tubes (200 mm length  $\times$  25 mm diameter). Once the agar had solidified, one germinated seedling was aseptically transferred into each tube. Uninoculated tubes with pregerminated rice seeds were provided to serve as a control. The seedlings were grown at room temperature under the natural photoperiod for two weeks. Four plants were replicated per treatment and the total experiment was repeated twice. The growth of each isolate was observed visually as a turbid zone around the plant roots. The roots morphology of the inoculated rice plants was observed compared to the control.

### 2.7 Plant Growth Promotion Assay in Pot Experiment

The selected actinomycete strains were evaluated for their effect on rice growth in a pot experiment. The rice seeds were surface sterilized and pregerminated as described earlier. The germinated seeds were aseptically inoculated with each isolate by soaking in spore suspension ( $10^7$  spores/ml) and

incubated at room temperature for 2 h. The density of the spore suspensions was verified by serial dilution and plating on ISP medium 2. The inoculated seedlings were planted in a small plastic pot (7.0 cm in height, 4.5 cm in diameter) containing a mixture of autoclaved soil : vermiculite (2 : 1, w/w). The potted plants were grown outdoors with at a temperature between 25 °C and 30 °C under the natural photoperiod and watered daily. Uninoculated plants were provided to serve as a control. Root lengths, shoot length, root dry weight and shoot dry weight were measured for both control and inoculated plants after two weeks of transplantation. The experiment was carried out in a completely randomized design (CRD) with four replications and repeated twice to confirm the results.

### 2.8 Statistical Analysis

The pot experiment data was analyzed using SPSS version 11.5 software (SPSS Inc., Chicago, IL). Duncan test was used to evaluate the significance of difference ( $p < 0.05$ ) between the treatments.

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation of Rice Endophytic Actinomycetes

The validation of the surface sterilization protocol showed that no bacteria or fungi were recovered from the root and stem's final rinsing water on the starch-casein agar plate. This result indicated that the surface sterilization procedure was effective in removing the surface bacteria and fungi.

A total of 191 endophytic actinomycetes were recovered from the rice roots and stems as shown in Table 1. The highest number of endophytic actinomycetes was recovered from rice plants collected from Petchaburi sample 4 (27 isolates), followed by Chiang Mai sample 2 and Nakornsawan

(24 isolates each). The majority of isolates were recovered from root samples (68%; n = 130), while the remaining 32% (n = 61) were recovered from stem tissues. This result is in agreement with the studies reporting that the roots present a better habitat for endophytic actinomycetes than the other parts of the plant [17]. The relatively abundant

diversity in the roots may be due to their direct contact with the soil. However, there were five samples from which actinomycetes could neither be isolated from the roots nor from the stems. This was due to a fungal contamination covering the surface of the isolation plate, even though an antifungal antibiotic was added to the isolation medium.

**Table 1.** Sources and numbers of actinomycetes isolated from roots and stems of rice (*Oryza sativa* L.).

Sampling location	Number of actinomycetes		Total isolates
	Stems ( <i>LL/meso</i> )**	Roots ( <i>LL/meso</i> )**	
Ayutthaya sample 1	2(0/2)	2(0/2)	4
Ayutthaya sample 2	0(0/0)	0(0/0)	0
Bangkok	2(1/1)	8(7/1)	10
Chiang Mai sample 1	0(0/0)	10(10/0)	10
Chiang Mai sample 2	6(2/4)	18(11/7)	24
Nakornsawan	1(0/1)	23(17/6)	24
Nonthaburi	0(0/0)	2(1/1)	2
Pathumthani sample 1*	0(0/0)	0(0/0)	0
Pathumthani sample 2*	0(0/0)	0(0/0)	0
Petchaburi sample 1	8(2/6)	12(5/7)	20
Petchaburi sample 2	0(0/0)	8(2/6)	8
Petchaburi sample 3	0(0/0)	0(0/0)	0
Petchaburi sample 4	7(0/7)	20(9/11)	27
Petchaburi sample 5	12(2/10)	1(1/0)	13
Ratchaburi	1(1/0)	4(3/1)	5
Suphanburi sample 1	13(11/2)	1(1/0)	14
Suphanburi sample 2	0(0/0)	5(2/3)	5
Suphanburi sample 3	0(0/0)	0(0/0)	0
Suphanburi sample 4	2(0/2)	11(2/9)	13
Suphanburi sample 5*	1(0/1)	0(0/0)	1
Suphanburi sample 6	4(2/2)	1(1/0)	5
Suphanburi sample 7	2(0/2)	4(3/1)	6
Total	61(21/40)	130(75/55)	191

\* Rice plants were in the flowering stage onward

\*\* *LL*-isomer and *meso*-isomer containing isolates



In this study, it was observed that most of the endophytic actinomycetes were obtained from young rice plants. In addition, only a small number of endophytic actinomycetes were obtained from rice in the flowering stage onwards. This may be because, in the flowering stage onwards, rice has many decayed and dead roots. Coombs and Franco [18] also reported that the younger rice (within 11 weeks of germination) allowed for a higher percentage recovery of endophytic actinomycetes, representing 77% than the older ones. Mahaffee and Kloepper [19] reported that the endophytic bacterial communities in plants such as rice and cucumber change with the growth stages of the plant since the internal environment of the plant seems to change as the plant develops. There have been reports that the population structures of endophytic bacteria are influenced by other factors, such as crop rotation, chemical composition of soil and plant pathogens. Moreover, significant differences in the populations of endophytic bacteria can be linked to rice cultivar, age, tillage and environment conditions [20].

### 3.2 Determination of the 2,6 Diaminopimelic Acid (DAP) in Whole-Cell Hydrolysates and Taxonomic Characterization Using the 16S rRNA Gene

The determination of DAP has been used to separate the actinomycete genera into 'streptomycete' and 'non-streptomycete' groups. In this study, 96 isolates (50.3%) contained the *LL*-isomeric form of DAP and belonged to the streptomycete group within the genus *Streptomyces*. The remaining isolates (95 isolates, 49.7%) contained the *meso*-isomeric form of DAP and belonged to the non-streptomycete group. Based on an analysis of the partial 16S rRNA gene,

these non-streptomycetes belonged to 10 genera distributed among six families, including *Micromonosporaceae* (51 isolates, 53.7%), *Nocardiaceae* (eight isolates, 8.4%), *Nocardioideaceae* (one isolate, 1.1%), *Pseudonocardiaceae* (seven isolates, 7.4%), *Streptosporangiaceae* (22 isolates, 23.1%) and *Thermomonosporaceae* (six isolates, 6.3%). Members of the genus *Micromonospora* were found in the highest percentage (53.7%), followed by *Microbispora* (16.8%), *Nocardia* (7.3%), *Actinomadura* (6.3%), *Pseudonocardia* (6.3%), *Nonomuraea* (5.2%), *Actinomycetospira* (1.1%), *Kribbella* (1.1%), *Rhodococcus* (1.1%) and *Sphaerisporangium* (1.1%). These results indicate that rice plants represent a rich reservoir for diverse genera of non-streptomycetes. The gene sequences of these isolates were deposited in the DDBJ database under accession numbers LCO11586-LCO11697.

Our results showed that the most common actinomycete genus found in the tissue of the rice plants collected from every sampling site was *Streptomyces*, followed by *Micromonospora*. The predominance of genus *Streptomyces* sp. among the endophytic actinomycetes found in the present study was in conformity with the other reports on endophytic actinomycetes obtained from rice [7, 21-22] and other plants [2-3, 23]. The genus *Micromonospora* is one of the endophytic actinomycetes that has long been recognized as an important producer of a secondary metabolite inferior to *Streptomyces* [1]. Various species of *Micromonospora* were acknowledged to live inside the plant tissue as endophytes [24]. Tian *et al.* [22] identified 38 representative actinomycetes isolated from the stems and roots of rice plants. The 16S rRNA gene sequence analyses showed that *Streptomyces* was the most frequently found with a strain of *Nocardioidea*. Likewise, Gangwar *et al.* [23] successfully

isolated 45 actinomycetes, which belonged to the genera *Streptomyces*, *Saccharopolyspora*, *Actinopolyspora*, *Nocardia* and *Micromonospora* from surface sterilized tissue of rice. Moreover, Kampapongsa and Kaewkla [7] isolated 116 endophytic actinomycetes from the roots, stems, leaves and leaf sheaths of jasmine rice KDML 105 (*Oryza sativa* L.), growing in Roi-Et Province, Thailand. Of these, 63 isolates belonged to the genus *Streptomyces* (54.3 %), 50 isolates to *Microbispora* (43 %) and three to *Kineococcus* (2 %).

The discovery of new taxa of actinomycetes from rice plants has also been reported. For example, *Actinophytocola oryzae* gen. nov., sp. nov., was isolated from the roots of Thai glutinous rice plants [25] and *Actinoallomurus oryzae* sp. nov. from the roots of Thai jasmine rice plants (*Oryza sativa* L.) [26]. In addition, two isolates from the present study had been described as a novel species of *Streptomyces oryzae* sp. nov. and *Sphaerisorangium rufum* sp. nov. [27, 28]. These findings reveal that rice plants are still an interesting source for discovering new taxa of actinomycetes.

### 3.3 Antagonistic Activity of Isolates Against Rice Pathogenic Fungi

The antifungal activity of all actinomycetes against the rice fungal pathogens *Fusarium oxysporum*, *Helminthosporium oryzae* and *Rhizoctonia solani* was investigated using an *in vitro* dual culture technique. The results showed that 46 isolates (24.1%) displayed antagonistic activity against at least one tested fungus. Twenty-six isolates showed antagonistic activity against *F. oxysporum*, 24 isolates against *H. oryzae* and 25 isolates against *R. solani*. Of the active strains, 36 isolates (78.3%) belonged to the genus *Streptomyces*, followed by *Micromonospora* (five isolates, 10.9%) and one isolate each for the genera *Actinomadura*, *Microbispora*,

*Nocardia*, *Nonomuraea* and *Pseudonocardia*.

Based on percentage of inhibition, 10 isolates (5.2%) were found to be active against all tested fungi. All of these 10 isolates belonged to the genus *Streptomyces*. *Streptomyces* sp. S12-10 was able to inhibit all the tested fungi, with the highest percentages of inhibition against *F. moniliforme* (100% inhibition), *H. oryzae* (81.3% inhibition) and *Rhizoctonia solani* (76.3%). *Streptomyces* sp. R07-04 displayed the maximum percentage of inhibition against *R. solani* (100% inhibition) and could inhibit both *H. oryzae* (77.5%) and *F. moniliforme* (77.5%) (Table 2). However, 31.4% of the *Streptomyces* isolates in this study did not show any antifungal activity towards any test organisms; even so, they might produce other useful compounds. Antagonistic activities of endophytic actinomycetes against rice pathogenic fungi have been reported in many studies and a number of the biologically active endophytic actinomycetes belonged to the genus *Streptomyces* [3, 7, 21]. The antagonistic activity displayed in this study further indicates that endophytic actinomycetes hosted by the roots and stems of rice plants are a key source of bioactive compounds. The ability of isolates to inhibit the growth of fungal pathogens is an implication of the secondary metabolites secreted by actinomycetes and some of the metabolites of endophytic actinomycetes may be a resource for new fungicides.

### 3.4 *In vitro* Plant Growth Promoting Traits

#### 3.4.1 Siderophore production

Regarding plant growth enhancement, siderophores production is focused on its ability to promote plant growth by capturing ferric iron in soil and provide nutrients to stimulate plant growth [2]. Assessing the siderophore production activity of 191 isolates showed that 152 isolates (79.6%) were able to produce siderophores on CAS



agar by forming an orange halo around the colonies. Of these, 104 strains were isolated from the roots, while 48 strains were isolated from the stems. Around half of the siderophore-producing isolates (52.6%) showed an orange halo diameter of less than 20 mm on the CAS agar. However, seven isolates (3.7%) showed high siderophore production with an orange halo diameter larger than 40 mm. In addition, 57.9% (88 isolates) of the siderophore-producing strains belonged to the genus *Streptomyces*, followed by *Micromonospora* (26 isolates, 17.1%), *Microbispora* (12 isolates, 7.9%) and *Nocardia* (six isolates, 3.9%). Interestingly, all of the isolates belonging to the genus *Actinomadura* had the ability to produce siderophore. One member of this genus has been reported to produce a novel siderophore, namely madurastatin, which showed an antibacterial activity against *Micrococcus luteus*. Previous studies reported that siderophore-producing streptomycetes could inhibit the growth of phytopathogens through competition for iron in plant rhizosphere soils [29].

#### 3.4.2 Indole-3-acetic acid (IAA) production

The screening for IAA production showed that 58 endophytic actinomycete isolates (30.4%) produced IAA and 39 of these belonged to the genus *Streptomyces*. Endophytic actinomycetes isolated from various plants have been reported to produce plant growth regulators, especially IAA [4, 30]. In the present study, the range of IAA production of the isolates was 3.18-53.43 µg/ml. The highest rate of IAA production found in isolate S19-30, which belonged to the genus *Actinomycetospora*, followed by isolates S03-08 and R13-11,

which belonged to the genus *Streptomyces*.

#### 3.4.3 Phosphate solubilization

Phosphate solubilization is also one of the microbial mechanisms to improve phosphorus availability to plants. In this study, a qualitative estimation of phosphate solubilization by endophytic actinomycetes grown on Pikovskaya's medium (PVK) containing tricalcium phosphate was conducted. Eighty-seven isolates (45.5%) were observed to solubilize phosphate, since they formed a clear zone around the colony on the Pikovskaya's medium. Eighteen isolates (9.4%) were able to produce a zone of clearing  $\geq 5$  mm on a tested agar plate. The rest of the isolates were either non-phosphate-solubilizing or produced a very small clear zone. Most of the phosphate-solubilizing isolates were members of the genus *Streptomyces* (73.6%) and the remaining isolates belonged to the genera *Actinomadura*, *Actinomycetospora*, *Kribbella*, *Microbispora*, *Nocardia* and *Pseudonocardia*. Several studies have shown that phosphate-solubilizing actinomycetes can solubilize the fixed soil phosphate and applied phosphates, resulting in higher crop yields [31].

#### 3.5 Effects of Potent Endophytic Actinomycetes on Rice Roots

Four active isolates, namely R07-04, R07-06, S03-26 and S12-10, were selected for further study based on their strongly antagonistic characteristics toward rice fungal pathogens (*R. solani*, *F. moniliforme* and *H. oryzae*) *in vitro*, as well as their plant growth promoting traits, such as indole-3-acetic acid production, siderophore production and phosphate solubilization activity (Table 2).

**Table 2.** 16S rRNA gene sequence similarities of active actinomycete isolates and their activities to inhibit rice fungal pathogens, including the production of plant growth promoter.

Isolate	The most closely related strain	Similarity (%)	Percentage of fungal inhibition			Siderophore production <sup>a</sup>	IAA production (µg/ml)	Phosphate solubilization <sup>b</sup>
			1	2	3			
R07-04	<i>Streptomyces anandii</i> NRRL B-3590 <sup>T</sup> (AY999803)	99.9	77.5	77.5	100	++++	-	++
R07-06	<i>Streptomyces hydicus</i> DSM 40002 <sup>T</sup> (AJ621611)	100	72.5	63.8	70	++	12.15	+
S03-26	<i>Streptomyces parvulus</i> NBRC 13193 <sup>T</sup> (AB184326)	99.6	66.3	58.8	72.5	+++	7.62	+
S12-10	<i>Streptomyces iranensis</i> HM 35 <sup>T</sup> (FJ472862)	99.3	100	81.3	76.3	++++	9.81	+

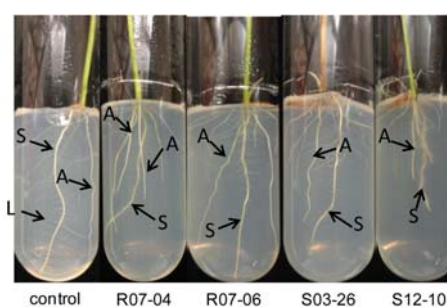
1. *Fusarium moniliforme*; 2. *Helminthosporium oryzae*; 3. *Rhizoctonia solani*

<sup>a</sup> Orange halo diameter +, <20 mm; ++, 20-30 mm; +++, 31-40 mm; +++++, >40 mm

<sup>b</sup> Clear zone size +, 1-4 mm, ++, > 4 mm

The effects of these isolates on rice's roots morphology and development were assessed. The results showed that all of the isolates could colonize rice roots. A turbid zone was visually observed around the roots of the rice seeds inoculated with all the isolates, when compared with the control. Moreover, the two-week-old rice seedlings inoculated with isolate R07-04, isolate R07-06 and isolate S03-26 contained a greater number of adventitious roots when compared to the control but produced shorter seminal roots with fewer lateral roots (Figure 1). The seminal roots of rice plants are important for the establishment of seedlings, the lateral roots strongly determine the plant's survival and productive capability and adventitious roots play important roles in nutrient and water uptake. The mechanism by which the isolates enhanced the rice seedlings' growth could possibly be its plant growth promotion attributes; however, in the Hoagland's soft agar used in this study, Fe, P and NH<sub>4</sub><sup>+</sup> also existed in soluble form. Therefore, IAA production may be the only mechanism enhancing the rice seedlings' growth. The positive effect of IAA depends

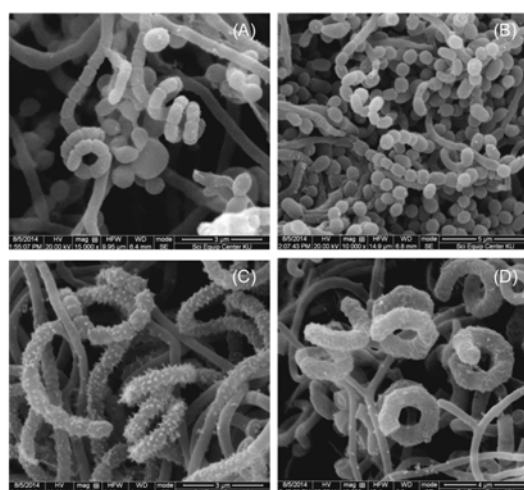
on the amount of IAA produced and an IAA over production is considered to give deleterious effect to plants [32]. Additionally, there are other factors which affect the growth of rice plants, such as gibberellins and cytokinin. On the other hand, the growth of seminal roots treated with isolate S12-10 was highly retarded, resulting in shorter and thicker roots compared with the control (Figure 1) and found to be deleterious to the rice plants.



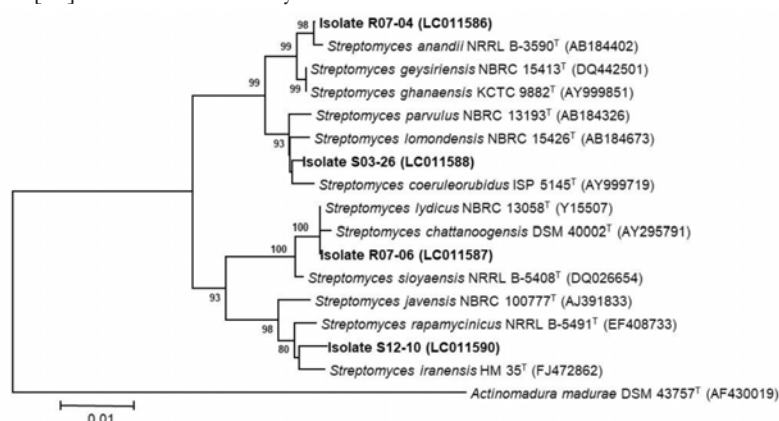
**Figure 1.** Visual differences in roots morphology. Uninoculated rice seedlings and rice seedling inoculated with active selected isolates grown in half-strength Hoagland's solution containing 0.6% agar in the natural photoperiod for 2 weeks. S, seminal roots; L, lateral roots; A, adventitious roots.

Identification of these four isolates based on an analysis of the 16S rRNA gene sequence showed that isolate R07-04 (1412 nt) and isolate R07-06 (1406 nt), which were isolated from roots, were most closely related to *Streptomyces anandii* NRRL B-3590<sup>T</sup> (99.9% similarity) and *Streptomyces lydicus* DSM 40002<sup>T</sup> (100% similarity), respectively. Isolate S03-26 (1402 nt) and isolate S12-10 (1413 nt), which were isolated from stems, were most closely related to *Streptomyces parvulus* NBRC 13193<sup>T</sup> (99.6%)

and *Streptomyces iranensis* HM35<sup>T</sup> (99.3%), respectively (Table 2). The spore chain morphology of these active isolates, observed using a Quanta 450 FEI scanning electron microscope (SEM), is shown in Figure 2. The phylogenetic relationship of isolates R07-04, R07-06, S03-26 and S12-10 with their closely related type strains in the genus *Streptomyces* are shown in Figure 3. All the isolates were clustered with their closest members from blast results.



**Figure 2.** Spore chain morphology of isolates R07-04 (A), R07-06 (B), S03-26 (C) and S12-10 (D) under scanning electron microscope (SEM, Quanta 450 FEI) after growing on ISP medium 3 [12] at 28 °C for 7 days.



**Figure 3.** Phylogenetic tree based on nearly completed 16S rRNA gene sequences showing the relative positions of isolates R07-04, R07-06, S03-26, S12-10 and their closest type strains of *Streptomyces* species. The tree was constructed using neighbour-joining (NJ) method. Bar indicates the nucleotide substitutions per site.

Interestingly, one of our active isolates exhibited a 100% sequence identity to *Streptomyces lydicus* DSM 40002<sup>T</sup>. This isolate produced a spiral spore chain of round, oval or short rod spores with a smooth surface (Figure 2). It has been reported that *Streptomyces lydicus* WYEC108 produce a number of extracellular chitinases, a hydrophilic antifungal compound and siderophores [29]. Importantly, this strain is used for the control of diseases, including foliar diseases, botrytis fruit rot and powdery mildew in greenhouse crops, under the trade name Actinovate, which is a registered trademark of Novozymes. According to the above results, isolates R07-04, R07-06 and S03-26 could be promising strains to develop as bioinoculants for rice plant. Therefore, these three isolates were further selected to promote rice seedling growth in a pot experiment.

### 3.6 Plant Growth Promotion in a Pot Experiment

Several studies reported plant growth promoting activities of endophytic

actinomycetes on seedlings. Goudjal *et al.* [3] reported the isolation of endophytic actinomycetes from the roots of native plants of the Algerian Sahara. Treated tomato seedling with strains CA-2 and AA-2 exhibited resulted in a significant increase in the seedling fresh weight, the seedling length and the root length of the seed-treated seedlings compared to the control. Therefore, the plant growth-promoting effects of isolates R07-04, R07-06 and S03-26 were investigated. The results showed that isolate R07-06 significantly enhanced shoot length (13.6%), root dry weight (11.2%) and shoot dry weight (14.8%) over the uninoculated control (Table 3). Furthermore, among the three isolates tested, isolate R07-06 also caused a greater increase in root and shoot growth than the other two isolates. Isolates S03-26 and R07-04 showed a significant decrease in all the parameters. The results of the present experiment confirm the plant growth-promoting abilities of isolate R07-06 to increase the shoot length, and root and shoot dry weight of rice seedling.

**Table 3.** Evaluation of active *Streptomyces* strains for their plant growth promotion potential in rice seedling after growing for 2 weeks in pot experiment.

Treatment	Root length (cm) <sup>a</sup>	Shoot length (cm) <sup>a</sup>	Root dry weight (mg plant <sup>-1</sup> ) <sup>a</sup>	Shoot dry weight (mg plant <sup>-1</sup> ) <sup>a</sup>
R07-04	9.65 ± 0.99 <sup>c</sup>	18.91 ± 0.49 <sup>b</sup>	93.2 ± 5.77 <sup>c</sup>	110.3 ± 5.00 <sup>c</sup>
R07-06	11.06 ± 0.68 <sup>ab</sup>	22.11 ± 0.41 <sup>a</sup>	110.1 ± 0.00 <sup>a</sup>	149.3 ± 5.00 <sup>a</sup>
S03-26	10.79 ± 0.45 <sup>b</sup>	17.00 ± 0.53 <sup>c</sup>	93.9 ± 5.00 <sup>c</sup>	102.1 ± 5.00 <sup>c</sup>
Control	11.84 ± 0.91 <sup>a</sup>	19.45 ± 0.59 <sup>b</sup>	99.0 ± 0.00 <sup>b</sup>	130.1 ± 12.58 <sup>b</sup>

Data are mean of four replications.

<sup>a</sup> Abbreviation: Average ± standard deviation error from four replicate samples

Data followed by the same letter in a column are not significantly different ( $p < 0.05$ ) from each other according to Duncan test.

#### 4. CONCLUSION

The aims of this present study were to isolate endophytic actinobacteria from the roots and stems of rice plants and to investigate their antimicrobial activities against rice fungal pathogens, including their ability to promote the growth of rice plants. It was evident from the results that the roots of rice plants contain high diversity of endophytic actinomycetes compared to rice leaves. The 16S rRNA gene sequence analysis of 95 endophytic non-streptomycetes showed that they were distributed among 10 genera, within six families. Within the non-streptomycete group, *Micromonospora* spp. was found to be the most abundant in the roots and stems of rice plants.

The endophytic *Streptomyces* strain R07-06, was a potential isolate that showed high antifungal activity, as well as the ability to produce siderophores and solubilize phosphate. Moreover, this isolate significantly enhanced the shoot length, root dry weight and shoot dry weight of rice plants over an uninoculated control. The 16S rRNA gene sequence and morphological characteristics of isolate R07-06 showed that it exhibited a 100% sequence identity to *Streptomyces hydicus*. It has been reported that the *Streptomyces hydicus* strain WYEC 108, a soil actinomycete, possesses a fungal disease suppressant [29]. Moreover, this strain is a biocontrol formulation registered in the USA as Actinovate®. Therefore, strain R07-06, which is an endophytic *Streptomyces*, could be a promising candidate to help improve the growth of rice plants, which may provide a new approach to its use as a biocontrol.

#### ACKNOWLEDGEMENTS

This research has been supported by Center of Excellence on Biodiversity (BDC), Office of Higher Education Commission (Project Code BDC-PG1-160003). We

gratefully acknowledge Prof. Dr. Savitree Limtong of Kasetsart University, the director of research program. Ratchanee Mingma is grateful to the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

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