Diversity and Antimicrobial Activity of Plant Growth Promoting Endophytic Actinomycetes Isolated from Thai Orchids

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ABSTRACT

Thirty-two endophytic actinomycetes isolated from 15 Thai orchids were taxonomically studied based on their phenotypic characteristics and 16S rRNA gene sequence analyses (98.97-100.00%). The isolates were identified as *Streptomyces* including *S. parvulus* (3 isolates), *S. tendae* (2 isolates), *S. ardesiacus* (2 isolates), *S. heilongjiangensis* (2 isolates), and each of *S. daghestanicus*, *S. antibioticus*, *S. malaysiensis*, *S. deserti*, *S. spiralis*, *S. thermoviolaceus* subsp. *apingens*, *S. globosus*, *S. collinus*, *S. olivaceus*, and *S. zaomyceticus*. *Micromonospora* including *M. humi* (2 isolates), *M. maritima* (2 isolates), and each of *M. tulbaghiae*, *M. schwarzwaldensis*, *M. chersina*, *M. chalcea M. citrea*, and *M. aurantiaca*; *Streptosporangium* (2 isolates) including *S. sandarakinum* and *S. pseudovulgare* and an isolate of *Actinomadura hibisca*. *Streptomyces* (7 isolates), *Micromonospora* (7 isolates), and *Streptosporangium* (1 isolate) exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. Indole-3-acetic acid (IAA) production of the isolates ranged from 0.04 to 67.30 µg/mL. Isolates DR10-1 and DR9-7 produced high amounts of IAA (58.03 and 67.30 µg/mL) and were selected for optimization. Maximum IAA values obtained were 284.87 and 132.35 µg/mL, using 0.4% L-tryptophan and pH 7 with incubation at 30°C for 13 days. These two isolates enhanced root length, shoot length, number of roots, and fresh weight of rice seedlings (*Oryza sativa* L. cv. RD49) compared to the control. Results indicated that actinomycetes from Thai orchids were promising sources of antimicrobial compounds and plant hormones for agricultural applications.

1. INTRODUCTION

Actinomycetes are Gram-positive filamentous bacteria having a high guanine (G) and cytosine (C) content in their genomic DNA (Stackebrandt et al., 1997). Research has demonstrated that they are generally safe and beneficial microorganisms in the pharmaceutical and agricultural industries as antimicrobials, antimalarials, anticancer, enzymes, pesticides, and plant growth hormones (Berdy, 2005; Flores-Gallegos and Nava-Reyna, 2019). Plant-associated microbes create substances with significant medicinal potential (Wu et al., 2021), while endophytes are commonly distributed in nature and maintain unique associations with host plants (Nair and Padmavathy, 2014), leading to the development of safer products for the environment and human health. Endophytic actinobacteria have been found in various plants, including the predominant *Streptomyces* and the genera *Micromonospora*, *Microbispora*, *Nocardia*, *Nocardioides*, and *Streptosporangium* (Shimizu, 2011), *Streptomyces platensis* 3-10 from healthy rice (Shakeel et al., 2016) and *Streptomyces* sp. KLBMP 5084 from healthy *Limonium sinense* (Qin et al., 2017).

Dendrobium, is the second largest genus of orchid in the family Orchidaceae (Puchooa, 2004)

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which contains approximately 1,100 species. They are mainly distributed in the subtropical and tropical regions of Asia and Oceania (Xiang et al., 2013). Since ancient times, many *Dendrobium* plants have been used as ingredients for nutraceutical beverages and food products (Bao et al., 2001). Orchids have been shown to generate a wide range of bioactive substances including antibiotics, anticancer, antitumor, antioxidant, and anti-infection agents, demonstrating a crucial impact on drug discovery. The methanol extract from the whole plant of *Dendrobium harveyanum* had a high anti-lipase effect (Maitreesophone et al., 2022), while the crude extract from *D. venustum* showed antimalarial and anti-herpetic activities (Sukphan et al., 2014). The gigantol from *Dendrobium draconis* exhibited pharmacological activity (Charoenrungruang et al., 2014), while moscatilin, gigantol, lusianthridin, and dendroflorin from *D. brymerianum* (Klongkumnuankarn et al., 2015) showed cytotoxicity against human lung cancer cell lines. Dendroparishiol, a new compound from *D. parishii*, has shown high antioxidant activities (Kongkatitham et al., 2018). Recently, orchid-associated microorganisms have been found on orchid roots. They assist plants to solubilize important nutrients, create a variety of metabolites and control phytopathogenic fungi (Herrera et al., 2022). The endophytic bacterium strain PVL1 isolated from the leaf of the orchid *Vanda cristata* promoted the growth of *Cymbidium aloifolium* by IAA (Shah et al., 2021), while fungal endophytes isolated from *Dendrobium officinale* (Wu et al., 2016) and *D. devonianum* (Xing et al., 2011) were great sources for the prevention and treatment of harmful fungi and bacteria.

Plant-microbial interactions have been extensively researched. However, the variety and functional activity of orchid-associated bacteria are little understood. In Thailand, orchid species rich in fungi and endophytic organisms established in the tissues of terrestrial orchids have been found to follow a seasonal rhythm (Chutima et al., 2011). Investigation of endophytic actinomycetes might lead to the discovery of new species, their biological activity and secondary metabolites. In this study, endophytic actinomycetes were isolated from selected Thai orchids and characterized based on their phenotypic, chemotaxonomic and genetic characteristics. Their antibacterial activity and the plant growth promoting IAA production were also evaluated.

2. METHODOLOGY

2.1 Sampling and isolation

Endophytic actinomycetes were isolated from the roots of fifteen species of Thai orchids: *Dendrobium christyanum*, *D. formosum*, *D. kentrophyllum*, *D. findlayanum*, *D. chrysanthum*, *Calanthe cardioglossa*, *D. friedericksianum*, *D. chrysotoxum*, *D. crumenatum*, *D. heterocarpum*, *Coelogyne lawrenceana*, *Eria ornate*, *Cleisostoma rostratum*, *Coelogyne assamica*, and *Pinalia globulifera*. All endophytic actinomycete isolates were characterized based on their phenotypic, chemotaxonomic, and genetic characteristics, as well as their antibacterial activity and indole-3-acetic acid (IAA) production. The orchid roots were prepared according to Tedsree et al. (2021). The suspension of bacteria was serially diluted ten times. Each diluted suspension was applied to a different medium including gellan gum, Gause synthetic No.1 (Gause et al., 1983), glycerol arginine (Arai, 1975), and starch casein (Küster and Williams, 1964) supplemented with nalidixic acid (25 µg/mL) and cycloheximide (50 µg/mL). All the plates were incubated at 30°C for 20 to 30 days. The purified isolates were preserved on ISP 2 and freeze-dried for long-term preservation.

2.2 Characterization of endophytic actinomycetes

Morphological and cultural characteristics of isolates were observed on ISP 2 agar as described previously (Shirling and Gottlieb, 1966). The NBS/IBCC color system was used to determine the colors of aerial mycelia, substrate mycelia and diffusible pigment (Kelly, 1964). Physiological characteristics, including growth at different temperatures (20-45°C), NaCl concentrations (0-10%, w/v), and pH range 4-12 (at intervals of 1 pH unit) were evaluated in ISP 2 broth at 30°C for 14 days. Carbon utilization on ISP 9 supplemented with 1% (w/v) carbon sources, starch hydrolysis, nitrate reduction, milk coagulation and peptonization, and gelatin liquefaction were examined as described by Arai (1975). The method of Staneck and Roberts (1974) was used to analyze the isomers of diaminopimelic acid.

Genomic DNAs of the isolates were generated following the technique of Kudo et al. (1998). The 16S rRNA gene sequence was amplified as reported by Suriyachadkun et al. (2009) and sequenced on a DNA sequencer (Macrogen) using universal primers, 27F forward (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R reverse (5'-TACGGYTACCTTGTTAC

GACTT-3'). Sequence similarity values between the isolates and their related neighbors were calculated using the EzBiocloud service (Yoon et al., 2017) and the Kimura-2-parameter (Kimura, 1980) was used to create a phylogenetic distance matrix. A phylogenic tree was constructed through the neighbor-joining (NJ) technique (Saitou and Nei, 1987) using MEGA 7.0 (Kumar et al., 2016) based on 1000 replications of bootstrap value.

2.3 Evaluation of antimicrobial activity

The culture was cultivated in 10 mL of seed medium No. 301 (2.4% starch, 0.3% peptone, 0.1% glucose, 0.5% yeast extract, 0.3% meat extract, and 0.3% $CaCO_3$, pH 7.0), on a shaker (180 rpm) incubated at 30°C for 3 days. The seed culture of each isolate was inoculated into 10 mL of three production media as ISP 2 (0.4% yeast extract, 1.0% malt extract, 0.4% glucose), medium No. 30 (0.3% peptone, 0.1% glucose, 0.5% yeast extract, 0.3% meat extract, 0.3% $CaCO_3$, 2.4% starch), and 5 mL/L of trace elements containing 1.0 g/L of $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $CoCl_2 \cdot 6H_2O$, and medium No. 57 (0.5% peptone, 2% glucose, 0.5% meat extract, 0.3% dry yeast, 0.5% NaCl, and 0.3% $CaCO_3$). After 14 days, each fermentation broth was extracted with 10 mL of 95% ethanol and shaken at 180 rpm for 2 h before centrifugation at 7,000 rpm for 10 min. The supernatant was collected and 50 µL was applied to each paper disk (6 mm).

A paper disk diffusion technique was used to determine antimicrobial activity (Mearns-Spragg et al., 1998) against *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. Muller-Hinton agar (MHA) and potato dextrose agar (PDA) were used to cultivate the tested bacteria (1×10^8 cells/mL) and yeast (1×10^6 cells/mL), which were incubated for 24 h at 37°C and 30°C, respectively. The inhibition zone (mm) was measured and reported as an antibacterial activity index. All experiments were carried out in triplicate.

2.4 Evaluation of IAA production

All isolates were streaked on ISP 2 agar plates and incubated at 30°C. After seven days, the agar discs containing actinomycete mycelia were transferred to ISP 2 broth (0.2% L-tryptophan, pH 7.0) and incubated at 30°C for seven days with 180 rpm shaking. The culture was centrifuged at 6,500 rpm for five minutes, and the supernatant was used to determine the amount of IAA production. One mL of supernatant was added to two mL of Salkowski reagent (0.5 M of $FeCl_3$ in 35% $HClO_4$ in a proportion of 1:50 (v/v)) and kept in the dark for 30 min (Sameera et al., 2018). A UV-Vis spectrophotometer was used to detect IAA at absorbance 530 nm. The uninoculated medium with reagent was used as a control. The amount of IAA produced per milliliter of culture broth was calculated based on the calibration curve of IAA obtained from standard IAA at different concentrations (0-100 µg/mL). The amount of IAA in the culture was expressed as µg/mL.

2.5 Optimization of IAA production

The potent isolates of IAA production were optimized based on the effects of incubation time, temperature, pH, and L-tryptophan level. Isolates that produced high amounts of IAA were chosen for optimization. The chosen isolates were grown in a 500 mL flask with 100 mL of ISP 2 medium plus 0.2% L-tryptophan with shaking at 180 rpm. Salkowski's method was used to evaluate IAA production. The same cultural conditions as mentioned before were employed. The influence of incubation period on IAA production was investigated at 48 h intervals for 15 days. Results of the concentration of L-tryptophan (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 1.5%), pH (4, 5, 6, 7, 8, 9, and 10), temperature (25, 30, 35, 37, and 40°C) were performed for seven days. The one-factor-at-a-time (OFAT) method was used to optimize all experiments (Czitrom, 1999).

2.6 Plant growth-promoting activity of the isolates

To investigate the impact of IAA produced by the isolates DR9-7 and DR10-1 on seed germination and root elongation in rice (*Oryza sativa* L. cv. RD49), surface sterilization of rice seeds was performed by soaking in 10% sodium hypochlorite (NaOCl) for one minute and 95% ethanol for three minutes, followed by thorough washing in sterile distilled water. The treatment was carried out by soaking rice seeds in a standard IAA solution containing 50 µg/mL and supernatant culture of DR9-7 and DR10-1 with an IAA concentration of 50 µg/mL. The control group was soaked in sterile distilled water. Seeds were placed in sterilized Petri dishes coated with two sheets of filter paper and soaked with 10 mL of sterile distilled water (three replicates, ten seeds/plate). All the Petri dishes were incubated in a chamber with light at 30°C for 16 h daily. Seed germination, root length,

shoot length, number of roots, fresh weight, and dry weight were measured after 7 days.

2.7 Statistical analysis

Data were statistically analyzed by one- way analysis of variance (ANOVA) using the SPSS software package (SPSS 28 for Windows). The grouping was performed by Duncan's multiple range tests at $p<0.05$ on each of the significant variables measured. Data were expressed as mean values of triplicates±standard deviation.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of isolates

Thirty-two endophytic actinomycetes were recovered from the roots of Thai orchids. All isolated actinomycetes revealed 4, 12, 8, and 8 isolates from gellan gum, starch casein, glycerol arginine, and Gause No. 1, respectively (Table 1). Based on 16S rRNA gene sequence analysis and phenotypic characteristics, the isolates were classified into four taxa (Table 2) including, *Streptomyces* (Group I, 19 isolates), *Micromonospora* (Group II, 10 isolates), *Streptosporangium* (Group III, 2 isolates), and *Actinomadura* (Group IV, 1 isolate). Most of the Group I isolates (59.5%) had LL-DAP, whereas the remaining 13 isolates (40.6%) carried *meso*-DAP (Williams and Cross, 1971). Similarities of all isolates and closely related isolates ranged between 98.97 and 100%. The NJ-phylogenetic tree based on 16S rRNA gene sequences is shown in Figure 1. The 16S rRNA gene sequences of the isolates were deposited at the NCBI database, with accession numbers listed in Table 1.

Group I contained 19 isolates. The isolates produced spiral spore chains with pale blue to greenish gray on ISP 2 agar after 7 days of incubation (Table 1). The isolates, DR2-3, DR5-2, and DR7-6 were closely related to *S. parvulus* NBRC 13193^T (99.40-99.85% similarity); DR3-5 and CC1-3 were closely related to *S. tendae* ATCC 19812^T (99.93-99.70%); CC1-1 and DR9-7 were closely related to *S. ardesiacus* NRRL B-1773^T (99.92-99.93%); CL1-6 and EO1-13 were closely related to *S. heilongjiangensis* NEAU-W2^T (99.78-99.93%). The isolates DR1-1, DR2-2, DR8-9, DR9-4, DR9-5, DR10-1, DR10-6, DR10-8, CL1-8, and EO1-10 were closely related to *S. daghestanicus* NRRL B-5418^T (99.32%), *S. antibioticus* NBRC 12838^T (99.70%), *S. malaysiensis* NBRC 16446^T (99.92%), *S. deserti* C63^T (99.41%), *S. spiralis* NBRC 14215^T (99.98%), *S. thermoviolaceus* subsp. *apingens* DSM 41392^T (98.97%), *S. globosus* LMG 19896^T (99.93%), *S. collinus* NBRC 12759^T (99.93%), and *S. olivaceus* NRRL B-3009^T (99.85%), *S. zaomyceticus* NBRC 13348^T (99.48%), respectively (Table 1).

Group II contained ten isolates. They produced single spores on mycelium substrate. Colonies on ISP 2 agar were dark purplish red to greenish black. Based on the 16S rRNA gene sequences, the two isolates DR4-1 and CA1-5 were closely related to *M. humi* DSM 45647^T (99.33-99.47%); two isolates, CR1-1 and CA1-9 were closely related to *M. maritima* D10-9-5^T (99.93-100.00%); and isolates DR6-8, CA1-1, YG1-1, YG1-7, YG1-8, and EO1-8 were closely related to *M. tulbaghiae* DSM 45142^T (99.75%), *M. schwarzwaldensis* HKI0641^T (99.92%), *M. chersina* DSM 44151^T (99.85%), *M. chalcea* DSM 43026^T (99.85%), *M. citrea* DSM 43903^T (99.77%), and *M. aurantiaca* ATCC 27029^T (99.49%), respectively (Table 1).

Group III consisted of two isolates, DR9-9 and YG1-5. They produced spherical sporangia in the aerial mycelium. On ISP 2 agar, the isolates were pale purple-pink (Table 1). The isolates DR9-9 and YG1-5 were closely related to *S. sandarakinum* GW-12028^T and *S. pseudovulgare* DSM 4318^T, based on 16S rRNA gene sequence similarity (99.32 and 99.93%), respectively (Table 1).

Group IV contained one isolate, CL1-5. This isolate formed straight chain spores on the tip of aerial mycelium and colonies and was vivid reddish-orange on ISP2 agar plate. Based on 16S rRNA gene sequence analysis, the isolate CL1-5 was 99.85% closely related to *A. hibisca* NBRC 15177^T (Table 1).

In this study, nineteen *Streptomyces* isolates were associated with the eleven orchids *D. christyanum*, *D. polyanthum*, *D. formosum*, *D. kentrophyllum*, *D. findlayanum*, *D. chrysanthum*, *C. cardioglossa*, *D. friedericksianum*, *D. chrysotoxum*, *C. lawrenceana*, and *E. ornate*. Ten *Micromonospora* isolates were found in the six orchids, *D. crumenatum*, *D. heterocarpum*, *C. rostratum*, *C. assamica*, *P. globulifera*, and *E. ornate*. Two *Streptosporangium* isolates were distributed in the two orchids *D. friedericksianum* and *P. globulifera*, and one *Actinomadura* isolate was found in *Coelogyne lawrenceana*. The isolates DR2-3, DR5-2, and DR6-6, all closely related to *S. parvulus* NBRC 13193^T, were found in *D. polyanthum*, *D. kentrophyllum*, and *D. findlayanum*, while the isolates DR3-5 and CC1-3

Table 1. Source, isolate number, cultural characteristics, and the nearest isolate species based on 16S rRNA gene sequence similarity (%)

Group	Plant	Isolate No.	Cultural characteristics		% Similarity	Accession No.	Nearest species
			Upper color	Reverse color			
I	*Dendrobium christyanum*	DR1-1[b]	Greenish gray	Light yellow	99.32	LC667376	*S. daghestanicus* NRRL B-5418^T
	Dendrobium polyanthum	DR2-3[b]	Pale blue	Light yellow	99.84	LC667377	*S. parvulus* NBRC 13193^T
	Dendrobium polyanthum	DR2-2[b]	Brilliant greenish blue	Light yellow	99.70	LC667378	*S. antibioticus* NBRC 12838^T
	Dendrobium formosum	DR3-5[b]	Greenish gray	Moderate olive brown	99.70	LC667379	*S. tendae* ATCC 19812^T
	Dendrobium kentrophyllum	DR5-2[b]	Pale blue	Light yellow	99.40	LC667380	*S. parvulus* NBRC 13193^T
	Dendrobium findlayanum	DR7-6[b]	Pale blue	Light yellow	99.85	LC667381	*S. parvulus* NBRC 13193^T
	Dendrobium chrysanthum	DR8-9[b]	Bluish gray	Light yellow	99.92	LC667382	*S. malaysiensis* NBRC 16446^T
	Calanthe cardioglossa	CC1-1[b]	Olive gray	Light olive brown	99.93	LC667383	*S. ardesiacus* NRRL B-1773^T
	Calanthe cardioglossa	CC1-3[b]	Greenish gray	Moderate olive brown	99.93	LC667384	*S. tendae* ATCC 19812^T
	Dendrobium friedericksianum	DR9-4[b]	Greenish gray	Yellowish gray	99.41	LC667385	*S. deserti* C63^T
	Dendrobium friedericksianum	DR9-5[a]	Light olive gray	Yellowish gray	98.98	LC667386	*S. spiralis* NBRC 14215^T
	Dendrobium friedericksianum	DR9-7[a]	Olive gray	Light olive brown	99.92	LC667387	*S. ardesiacus* NRRL B-1773^T
	Dendrobium chrysotoxum	DR10-1[a]	Grayish olive green	Light olive gray	98.97	LC667388	*S. thermoviolaceus* subsp. *apingens* DSM 41392^T
	Dendrobium chrysotoxum	DR10-6[d]	Moderate yellow	Light yellow	99.93	LC667389	*S. globosus* LMG 19896^T
	Dendrobium chrysotoxum	DR10-8[d]	Bluish white	Brownish gray	99.93	LC667390	*S. collinus* NBRC 12759^T
	Coelogyne lawrenceana	CL1-6[c]	Bluish gray	Dark bluish gray	99.93	LC667391	*S. heilongjiangensis* NEAU-W2^T
	Coelogyne lawrenceana	CL1-8[c]	Greenish gray	Moderate olive	99.85	LC667392	*S. olivaceus* NRRL B-3009^T
	Eria ornata	EO1-10[b]	Pale blue	Grayish greenish yellow	99.48	LC667393	*S. zaomyceticus* NBRC 13348^T
	Eria ornata	EO1-13[b]	Greenish gray	Moderate olive brown	99.78	LC667394	*S. heilongjiangensis* NEAU-W2^T
II	*Dendrobium crumenatum*	DR4-1[c]	Greenish black	Greenish black	99.33	LC666836	*M. humi* DSM 45647^T
	Dendrobium heterocarpum	DR6-8[c]	Moderate olive brown	Moderate olive brown	99.75	LC666837	*M. tulbaghiae* DSM 45142^T
	Cleisostoma rostratum	CR1-1[c]	Grayish red	Grayish red	100.00	LC666838	*M. maritima* D10-9-5^T
	Coelogyne assamica	CA1-1[c]	Dark olive brown	Dark olive brown	99.92	LC666839	*M. schwarzwaldensis* HKI0641^T
	Coelogyne assamica	CA1-5[c]	Greenish black	Greenish black	99.47	LC666840	*M. humi* DSM 45647^T
	Coelogyne assamica	CA1-9[a]	Dark grayish red	Dark grayish red	99.93	LC666841	*M. maritima* D10-9-5^T
	Pinalia globulifera	YG1-1[d]	Greenish black	Greenish black	99.85	LC666842	*M. chersina* DSM 44151^T
	Pinalia globulifera	YG1-7[d]	Very dark purplish red	Very dark purplish red	99.85	LC666843	*M. chalcea* DSM 43026^T
	Pinalia globulifera	YG1-8[d]	Brownish black	Brownish black	99.77	LC666844	*M. citrea* DSM 43903^T
	Eria ornata	EO1-8[d]	Deep reddish brown	Deep reddish brown	99.49	LC666845	*M. aurantiaca* ATCC 27029^T

All isolates grew well on ISP 2 agar medium. [a]gellan gum; [b]starch casein gellan gum; [c]glycerol arginine gellan gum; [d]gauze synthetic No.1,

Table 1. Source, isolate number, cultural characteristics, and the nearest isolate species based on 16S rRNA gene sequence similarity (%) (cont.)

Group	Plant	Isolate No.	Cultural characteristics		% Similarity	Accession No.	Nearest species
			Upper color	Reverse color			
III	*Dendrobium friedericksianum*	DR9-9[d]	Pale pink	Pinkish gray	99.32	LC667395	*S. sandarakinum* GW-12028^T
	Pinalia globulifera	YG1-5[d]	Pale purple pink	Pale purple pink	99.93	LC667396	*S. pseudovulgare* DSM 4318^T
IV	*Coelogyne lawrenceana*	CL1-5[c]	Very deep red	Very deep red	99.85	LC667397	*A. hibisca* NBRC 15177^T

All isolates grew well on ISP 2 agar medium. [a]gellan gum; [b]starch casein gellan gum; [c]glycerol arginine gellan gum; [d]gauze synthetic No.1,

Table 2. Differential phenotypic characteristics of the isolates

Characteristic	*Streptomyces*	*Micromonospora*	*Streptosporangium*	*Actinomadura*
	I	II	III	IV
No. of isolates	19	10	2	1
Temperature range, °C	20-37	20-37	25-37	25-37
pH range	5-10	5-8	5-10	5-10
NaCl tolerance				
• 4%	+	-(+4)	w	+
• 6%	+(-3)	-(+2)	-	-
• 8%	-(+8)	-	-	-
Starch hydrolysis				
• Coagulation	-(+3)	-	-	-
• Peptonization	-(+4)	-(+3)	-	-
• Nitrate reduction	-(+5)	-(+4)	+	+
• Gelatin liquefaction	-(+5)	-(+4)	-	-
Utilization of				
• D-Glucose	+(w1)	+(-1)	+	+
• Sucrose	+(w5)	+(-1, w1)	+	+
• Lactose	+	+(-1, w1)	+	+
• Dextrose	+	+(-1, w2)	+	+
• Maltose	+	+(w5)	+	+
• D-Mannitol	+	-(+2, w3)	+	+
• D-Xylose	+	+(-1, w3)	+	w
• Sorbitol	-(+4, w3)	+(-1)	w	+

Note: + positive reaction; w weakly positive reaction; – negative reaction. Numbers in parentheses indicate numbers of isolates showing positive, weak and negative reactions.

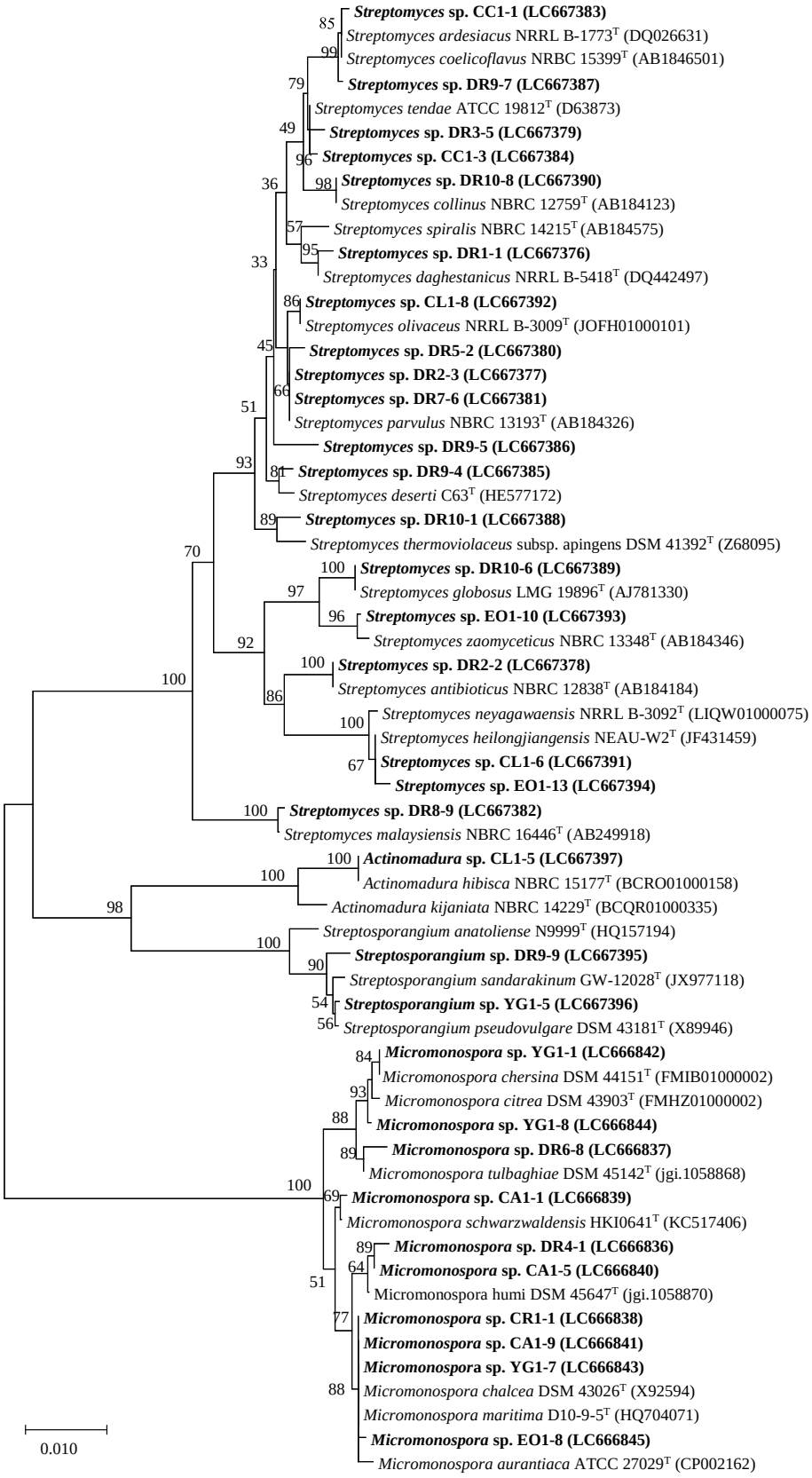

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between the 32 isolates and related type strains. Numbers at branch nodes indicate bootstrap percentages derived from 1000 replications. Bar, 0.01 substitutions per nucleotide position.

closely related to *S. tendae* ATCC 19812^T were presented in *Dendrobium* and *Calanthe*. *Streptomyces* and *Micromonospora* isolates were found in *E. ornate*, while *Streptomyces* and *Streptosporangium* isolates were found in *D. friedericksianum*. These results indicated that various associated bacteria were extensively distributed among the host plants.

Most of the isolates (59.38%) belonged to the genus *Streptomyces*, previously identified as a dominating organism in sugar cane roots (Sinma et al., 2015), *Citrus reticulata* L. (Shutsrirung et al., 2013), and *Acacia auriculiformis* (Bunyoo et al., 2009) in Thailand. Our result was similar to previous research that found *Streptomyces* strains in plant roots (Taechowisan and Lumyong, 2003; Gangwar et al., 2012; Shan et al., 2018). Actinobacteria were prominent in the roots and stems of *Neottia ovata* (50.02 and 48.47%, respectively) and in the seeds of *Spiranthes spiralis* (48.95%) (Alibrandi et al., 2020). Endophytic actinomycetes were distributed in plant roots, where water and nutrients were absorbed (Passari et al., 2015). Many plant species had diverse strains of *Microbispora* (Bunyoo et al., 2009) and *Micromonospora* (Kuncharoen et al., 2019), including novel species of *Amycolatopsis dendrobii* from the root of *Dendrobium heterocarpum* Lindl. (Tedsree et al., 2021) and *Streptomyces radicis* from the roots of plants (Kuncharoen et al., 2022).

3.2 Antimicrobial activity

Fifteen isolates (46.87%) showed antimicrobial activity against at least one of the six tested bacteria (Table 3). Five isolates of *Streptomyces* and five isolates of *Micromonospora* inhibited *S. aureus* ATCC 25923 when cultivated in all production media. The isolate DR8-9 presented the highest antimicrobial activity against *S. aureus* ATCC 25923 (24.32±1.01 mm) when cultivated in 57 media. Four isolates of *Streptomyces*, five isolates of *Micromonospora*, and one isolate of *Streptosporangium* inhibited *K. rhizophila* ATCC 9341. Isolate DR6-8 showed the highest activity (24.91±0.94 mm), while isolates DR2-2 and DR4-1 showed 22.76±0.58 and 19.65±0.58 mm, respectively. Five isolates of *Streptomyces* and four of *Micromonospora* were active against *B. subtilis* ATCC 6633 when grown in all media. Isolate DR7-6, which was closely related to *S. parvulus* NBRC 13193^T, showed the highest activity (20.00±0.58 mm) in medium No. 30. Isolates DR8-9 and DR1-1 inhibited *C. albicans* ATCC 10231 in all production media, including DR2-2 in ISP 2 medium. The isolate DR1-1 was closely related to *S. daghestanicus* NRRL B-5418^T and showed the highest activity against *C. albicans* ATCC 10231 (21.90±0.77 mm) on medium no.30. Three isolates DR1-1, DR2-2, and DR8-9 were active against the gram-negative bacteria *P. aeruginosa* ATCC 27853, while isolate DR2-2 inhibited this bacterium when cultivated on all production media. Interestingly, DR2-2 was closely related to *S. antibioticus* NBRC 12838^T and inhibited all indicator organisms, including *Escherichia coli* ATCC 25922 (Table 3).

Endophytic actinomycetes have been reported for their antibacterial activity against pathogenic bacteria (Taechowisan and Lumyong, 2003; Passari et al., 2015). In this study, both *Streptomyces* and *Micromonospora* isolates inhibited *S. aureus*, *B. subtilis*, and *K. rhizophila*. Our results concurred with Musa et al. (2020) who discovered that 54 of 126 endophytic actinobacteria strains were resistant to at least one or more indicator species. Notably, the majority of *Streptomyces* strains exhibited antagonistic activities. Rao et al. (2015) found that all *Streptomyces* strains from *Combretum latifolium* showed significant antimicrobial activity against both bacterial and fungal pathogens. This study reported on the incidence of possible endophytic actinomycetes that suppress pathogenic bacteria.

3.3 Indole-3-acetic acid production

Twenty-two isolates (68.75%) were able to produce indole-3-acetic acid (IAA) (Table 4). In Group I *Streptomyces*, 15 isolates produced IAA ranging from 10.59±0.45 to 67.30±1.00 µg/mL. Isolate DR10-1 was closely related to *S. thermoviolaceus* subsp. *apingens* DSM 41392^T and produced the highest IAA (67.30±1.00 µg/mL), followed by DR9-7 closely related to *S. ardesiacus* NRRL B-1773^T and DR2-2 closely related to *S. antibioticus* NBRC 12838^T at 58.03±0.16 and 52.39±0.89 µg/mL, respectively. Five of ten isolates of Group II *Micromonospora* produced between 18.22±0.84 and 44.77±0.54 µg/mL of IAA. Isolate CA1-5 closely related to *M. humi* DSM 45647^T showed the highest IAA production (44.77±0.54 µg/mL) followed by isolate YG1-7 closely related to *M. chalcea* DSM 43026^T (30.91±0.80 µg/mL). Only one Group III *Streptosporangium*, DR9-9, closely related to *S. sandarakinum* GW-12028^T produced maximum IAA of 43.97±0.30 µg/mL (Table 4).

Table 3. Antimicrobial activities (inhibition zone, mm in diameter) of Group I *Streptomyces*, Group II *Micromonospora*, and Group III *Streptosporangium* isolates cultivated in ISP 2, 30, and 57 media.

Isolate No.	*S. aureus* ATCC 25923			*K. rhizophila* ATCC 9341			*B. subtilis* ATCC 6633			*P. aeruginosa* ATCC 27853			*C. albicans* ATCC 10231		
Medium	ISP 2	30	57	ISP 2	30	57	ISP 2	30	57	ISP 2	30	57	ISP 2	30	57
Group I															
DR1-1	-	-	-	-	-	-	-	-	-	18.79± 0.56^{a}	-	-	20.60± 0.58^{a}	21.90± 0.77	20.37± 1.00
DR2-2	16.98± 0.57^{c}	16.43± 1.00^{c}	17.98± 0.58^{b}	21.18± 1.00^{b}	21.43± 1.01^{b}	22.76± 0.58^{b}	16.66± 1.00^{b}	18.47± 0.57^{b}	18.25± 0.58^{a}	9.13± 1.00^{c}	9.57± 0.67	10.08± 0.66	17.23± 0.61^{b}	-	-
DR5-2	15.24± 1.00^{d}	14.98± 1.00^{c}	9.84± 0.50^{de}	11.23± 1.01^{f}	15.89± 1.00^{d}	16.95± 1.01^{d}	16.51± 0.79^{b}	17.84± 0.86^{b}	13.85± 1.00^{c}	-	-	-	-	-	-
DR7-6	21.22± 1.00^{b}	19.11± 1.00^{b}	18.01± 1.00^{b}	-	-	-	19.87± 1.01^{a}	20.00± 0.58^{a}	18.80± 0.57^{a}	-	-	-	-	-	-
DR8-9	22.64± 0.75^{a}	21.79± 0.57^{a}	24.32± 1.01^{a}	15.66± 0.57^{d}	15.35± 1.00^{de}	18.52± 1.00^{c}	14.53± 0.67^{c}	14.35± 0.76^{c}	15.95± 0.99^{b}	-	-	9.36± 0.58	12.76± 0.59^{c}	13.01± 0.57	12.37± 0.57
DR9-5	11.56± 1.00^{e}	12.44± 0.61^{c}	12.46± 1.01^{c}	-	-	-	-	-	-	-	-	-	-	-	-
DR10-1	-	-	-	8.07± 0.58^{g}	8.17± 0.81^{g}	7.97± 0.84^{h}	9.55± 0.61^{d}	9.62± 1.01^{ef}	10.74± 1.00^{d}	-	-	-	-	-	-
Group II															
DR4-1	8.39± 0.56^{fg}	9.54± 0.58^{d}	9.79± 1.00^{de}	19.65± 0.58^{c}	19.11± 0.57^{c}	17.77± 0.58^{cd}	9.90± 0.57^{d}	11.68± 1.01^{d}	11.94± 0.99^{d}	8.28± 0.60^{c}	-	-	-	-	-
DR6-8	9.67± 0.58^{f}	9.90± 0.58^{d}	10.42± 0.62^{de}	24.91± 0.94^{a}	24.91± 1.53^{a}	24.24± 0.99^{a}	10.36± 0.58^{d}	10.78± 0.85^{de}	11.29± 0.72^{d}	-	-	-	-	-	-
CR1-1	9.46± 0.57^{f}	9.47± 0.53^{d}	11.20± 0.58^{cd}	10.45± 0.88^{f}	11.36± 1.00^{f}	12.34± 1.00^{f}	-	-	-	14.55± 1.02^{b}	-	-	-	-	-
CA1-1	-	-	-	-	-	-	-	-	-	9.72± 0.53^{c}	-	-	-	-	-
CA1-5	7.88± 0.99^{g}	8.63± 0.75^{d}	9.39± 0.60^{e}	15.79± 0.99^{d}	16.60± 0.79^{d}	16.75± 0.73^{d}	9.06± 0.58^{d}	8.84± 0.56^{f}	9.06± 0.99^{e}	-	-	-	-	-	-
YG1-1	17.91± 0.58^{c}	17.92± 0.58^{b}	18.87± 0.59^{b}	13.86± 1.00^{e}	13.91± 0.58^{e}	14.68± 0.57^{e}	7.32± 1.00^{e}	8.23± 1.00^{f}	7.84± 1.00^{e}	-	-	-	-	-	-
YG1-8	-	-	-	7.85± 0.50^{g}	7.46± 1.00^{g}	-	-	-	-	-	-	-	-	-	-
Group III															
DR9-9	-	-	-	10.32± 0.57^{f}	10.15± 0.58^{f}	9.85± 1.01^{g}	-	-	-	-	-	-	-	-	-

Group IV *Actinomadura*, CL1-5, did not exhibit antimicrobial activity.

All isolates exhibited no antimicrobial activity to *E. coli* ATCC 25922 except for isolate DR2-2 that showed 16.41±0.58, 17.26±0.53, and 18.52±0.52 when cultivated in ISP 2, 30, and 57 media, respectively.

Data are expressed as mean±standard deviation (SD), including the disc diameter (6 mm).

Different superscripts in the same row indicate significant differences ($p<0.05$).

Table 4. IAA production of Group I *Streptomyces*, Group II *Micromonospora*, Group III *Streptosporangium*, and IV *Actinomadura* isolates.

Group	Isolate No.	IAA (µg/mL)
I	DR1-1	22.04 ± 0.89^{i}
	DR2-3	52.36 ± 0.86^{c}
	DR2-2	52.39 ± 0.89^{c}
	DR3-5	19.67 ± 1.00^{j}
	DR5-2	51.54 ± 0.84^{c}
	DR7-6	45.94 ± 0.67^{d}
	DR8-9	6.95 ± 0.65^{q}
	CC1-1	10.59 ± 0.45^{o}
	CC1-3	11.92 ± 0.79^{m}
	DR9-4	32.61 ± 0.68^{f}
	DR9-5	15.62 ± 0.72^{l}
	DR9-7	58.03 ± 0.16^{b}
	DR10-1	67.30 ± 1.00^{a}
	DR10-6	2.30 ± 0.16^{st}
	DR10-8	6.47 ± 0.16^{q}
	CL1-6	8.72 ± 0.28^{p}
	CL1-8	0.78 ± 0.24^{wx}
	EO1-10	14.17 ± 0.33^{m}
	EO1-13	18.45 ± 0.37^{k}
II	DR4-1	29.13 ± 0.47^{h}
	DR6-8	0.04 ± 0.36^{x}
	CR1-1	18.22 ± 0.84^{k}
	CA1-1	7.33 ± 0.84^{q}
	CA1-5	44.77 ± 0.54^{e}
	CA1-9	21.54 ± 0.78^{i}
	YG1-1	1.63 ± 0.70^{tu}
	YG1-7	30.91 ± 0.80^{g}
	YG1-8	2.84 ± 0.73^{s}
	EO1-8	4.80 ± 0.54^{r}
III	DR9-9	43.97 ± 0.30^{e}
	YG1-5	1.13 ± 0.90^{v}
IV	CL1-5	14.48 ± 0.83^{m}

Different superscripts indicate significantly different ($p<0.05$) mean±SD.

In this study, *Streptomyces* showed the highest IAA production. Several previous studies demonstrated that IAA production of actinomycetes from different crops differed by species and strains. *Streptomyces* sp. En-1 from *Taxus chinensis* (Lin and Xu, 2013) and *S. rochei* ERY1 from *Eryngium foetidum* L. showed the ability to produce IAA (Suwitchayanon et al., 2018).

3.4 Optimization of IAA production

The two isolates DR9-7 and DR10-1 showed the highest IAA production after the screening was optimized at different factors. IAA production of isolates DR9-7 and DR10-1 started after three days and peaked at 106.32±2.04 and 298.12±4.19 µg/mL after 13 days (Figure 2 (a)). *S. atrovirens* ASU14 exhibited the maximum IAA value when optimization at 30°C for 13 days (Abd-Alla et al., 2013). Maximum IAA production of the two isolates was observed in medium containing 0.4% L-tryptophan (Figure 2 (b)). When the concentration of L-tryptophan increased from 0.1 to 0.4%, IAA production of DR9-7 and DR10-1 increased to maximum levels at 94.35±1.56 and 123.14±4.17%, respectively. Results indicated that different amounts of L-tryptophan had variable influence on IAA production, with tryptophan as an important element in increasing IAA production.

Highest concentration of IAA was obtained from isolate DR10-1 at pH 7 (82.76±1.22 µg/mL) and DR9-7 cultivated at pH 7 and 8 at 54.08±0.59 and 54.71±0.25 µg/mL, respectively. IAA levels of DR9-1 and DR10-1 decreased when the pH value was less than 6 and greater than 8, (Figure 2 (c)). *Streptomyces* and other actinomycete strains grew slowly in acidic or basic environments; therefore, pH levels are important for IAA synthesis (Shirokikh et al., 2007). Our findings concurred with Goudjal et al. (2013), who showed that pH 7 was optimal for IAA production of *Streptomyces* sp. PT2 . Isolates DR9-7 and DR10-1 produced the highest IAA when grown at 30°C (Figure 2 (d)). However, there was no significant change in IAA generation of DR10-1 at 35°C compared to 30°C. When temperature exceeded 30°C, IAA production of DR9-7 and DR10-1 decreased. A temperature of 30°C was found to be optimal for this investigation. *Streptomyces* sp. CMU H009 produced the largest IAA when cultivated at 30°C (Khamna et al., 2010). Accordingly, OFAT optimization experiments showed that the highest IAA production required cultivation in ISP 2 broth with 0.4% L-tryptophan, pH 7 at 30°C for 13 days. Maximum IAA values of DR10-1 and DR9-7 were 284.87±8.24 and 132.35±9.39 µg/mL, respectively.

3.5 Plant growth-promoting activity of the isolates

The effects of IAA in DR9-7 and DR10-1 supernatant cultures on rice seed germination, root length, and shoot length were determined. Rice seeds soaked under various conditions exhibited significant differences in root lengths and quantity of roots compared to the controls (Table 5). Treatments with supernatant DR9-7 had the greatest influence on seedling root length, with no significant differences

identified between supernatant DR10-1 and standard IAA. Supernatant of isolate DR10-1 showed the highest number of roots, whereas other treatments showed no significant differences. Fresh and dry weight of seedlings after treatment with DR9-7, DR10-1 and standard IAA were significantly different compared to the control. However, all treatments had no effect on seed germination. Our study results related to root growth of the host plants, as reported by Etesami et al. (2015). IAA-producing bacteria isolated from orchid rhizoplanes of *Dendrobium moschatum* (Tsavkelova et al., 2007) and *Cymbidium eburneum* (Faria et al., 2013) improved symbiotic seed germination. Our isolates indicated the presence of IAA production as a good option for use as plant growth enhancement in both economic and agricultural systems.

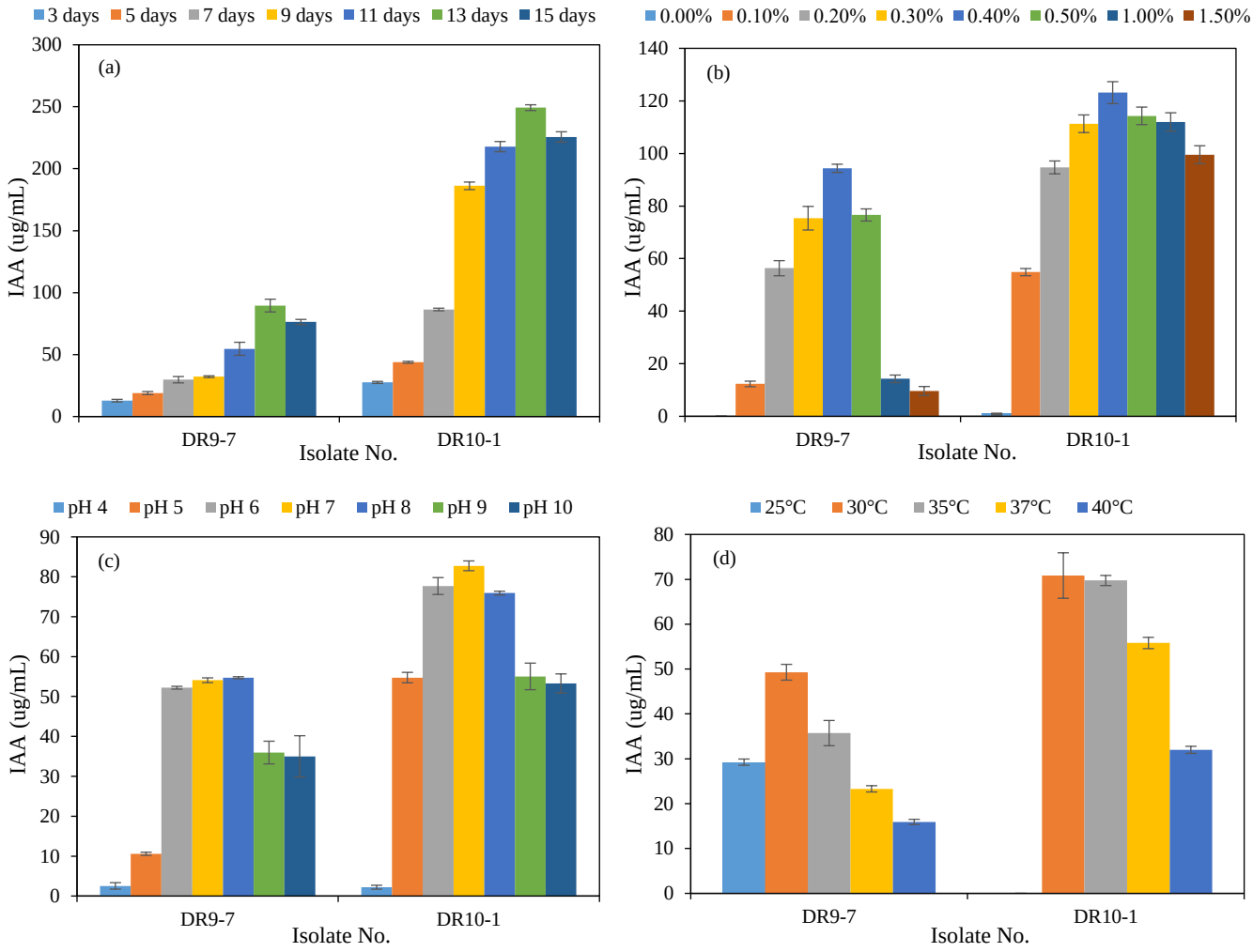

Figure 2. (a) Effect of incubation time (day), (b) Effect of L-tryptophan concentrations, (c) Effect of pH, and (d) Effect of temperature on IAA production by the selected isolates. Vertical bars represent standard deviation from triplicate experiments.

Table 5. Effect of isolates DR9-7 and DR10-1 on the growth of rice (*Oryza sativa*)

Isolate No.	Growth parameters of actinomycetes treated rice					
	Root length (cm)	Shoot length (cm)	Number of roots	Seedling fresh weight (g)	Seedling dry weight (g)	% Seed germination
DR9-7	6.90±0.52^{a}	3.55±0.96^{c}	3.00±1.05^{b}	0.93±0.07^{a}	0.33±0.03^{a}	100
DR10-1	4.85±0.34^{b}	6.25±0.81^{a}	4.30±0.82^{a}	0.81±0.07^{a}	0.30±0.05^{ab}	100
IAA	4.85±0. 24^{b}	3.85±0.94^{c}	3.40±0.52^{b}	0.78±0.08^{a}	0.31±0.06^{ab}	100
Control	3.30±1.27^{c}	4.95±0.65^{b}	3.20±0.63^{b}	0.56±0.07^{b}	0.27±0.04^{b}	100

Different superscripts indicate significantly different ($p<0.05$) mean±SD.

4. CONCLUSION

Endophytic actinomycetes are mainly distributed in the root system and xylem tissues of host plants. The major role of these bacteria is to improve the health and growth of the host plant by producing beneficial metabolites to control plant infections from pathogens and promote the growth of host plants. This study concluded that 19 *Streptomyces* isolates were associated with the roots of the Thai orchid species *D. christyanum*, *D. polyanthum*, *D. formosum*, *D. kentrophyllum*, *D. findlayanum*, *D. chrysanthum*, *C. cardioglossa*, *D. friedericksianum*, *D. chrysotoxum*, *C. lawrenceana*, and *E. ornate*. Ten *Micromonospora* isolates were associated with *D. crumenatum*, *D. heterocarpum*, *C. rostratum*, *C. assamica*, *P. globulifera*, and *E. ornate*, while 2 *Streptosporangium* isolates were found in *D. friedericksianum* and *P. globulifera* and an *Actinomadura* isolate was found in *C. lawrenceana*. Our *Streptomyces*, *Micromonospora* and *Streptosporangium* isolates exhibited significant antimicrobial activities against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. Only *Streptomyces antibioticus* DR2-2 inhibited all tested pathogens, while two endophyte isolates DR10-1 and DR9-7 showed high IAA activity that promoted the number of roots, shoot length, root length and fresh weight of rice seedlings. This is the first report on the diversity, antimicrobial and plant-growth-promoting properties of endophytic actinomycetes associated with the root of Thai orchids.

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