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In-vitro evaluation of actinomycetes with plant growth-promoting properties

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Abstract

The aim of this study was to evaluate actinomycetes for plant growth-promoting (PGP) traits using *in vitro* experiments. Actinomycetes were isolated from two different soil rhizospheres and subjected to dual culture experiments in order to determine antimicrobial activity. Subsequently, selected isolates with potent antimicrobial activity were examined further for their ability to biosynthesize indole-3-acetic acid (IAA) and siderophores, solubilize inorganic phosphate, and produce ammonia. Results showed that these strains produced 38.73-211.35 ng/mL IAA in Tryptic Soy Broth (TSB), and solubilized 116.20-319.33 µg/mL inorganic phosphate in National Botanical Research Institute's phosphate growth medium (NBRIP) broth. Additionally, all selected actinomycetes produced siderophores in chrome azurol S (CAS) agar and ammonia in peptone water. Five isolates showed promise as plant growth promoters, with isolates SNN045, SNN056, and SNN059 being the most plausible candidates for agricultural applications.

Keywords: Actinomycetes, Biofertilizer, Plant growth-promoting bacteria

1. Introduction

The current rising demand for food production due to overpopulation has resulted in food insecurity [1]. To meet dietary needs, either genetically modified (GM) crops with increased yields or alternative methods of soil nutrient improvement are essential. Chemical inputs (pesticides and synthetic chemical fertilizers) enhance plant productivity and protect plants from diseases; however, they adversely affect the environment and human health [2].

Organic agriculture, which uses more environmentally friendly, holistic methods, is gaining in popularity [3]. This approach benefits the agroecosystem, sustains biological activity, and increases soil fertility, while limiting the need for chemical inputs and GM organisms [3]. For example, plant growth-promoting rhizobacteria (PGPR), which are abundant in the rhizosphere, facilitate nutrient absorption and activate plant defense mechanisms against pathogens [4]. In agriculture, PGPR have established themselves as safe alternatives to synthetic chemicals and have demonstrated the greatest efficacy in promoting plant growth.

Actinomycetes, in particular *Streptomyces* spp., exhibit the ability to act as plant growth promoters through natural processes such as the production of plant hormones, increase of plant biomass, recycling of organic matter, fixation of N_2, and solubilization of minerals [5]. Actinomycetes have been implicated in plant health status due to their prevalence in healthy soil rhizospheres, as well as their protective abilities [6,7]. Additionally, secondary metabolites produced by actinomycetes demonstrate antimicrobial activity, allowing them to compete with pathogens in the soil rhizosphere. Therefore, actinomycetes are likely to have the ability to act as both effective biocontrol agents and efficient plant growth promoters [8].

In this study, five plant pathogens frequently found in Thailand were used to assess the antimicrobial activity of actinomycete isolates: *Colletotrichum gloeosporioides* DoA c1060, *Phytophthora palmivora* CbP01 and CbP03, *Xanthomonas axonopodis* pv. *Manihotis*, and *X. campestris* pv. *campestris*. *Colletotrichum gloeosporioides* is a fungal pathogen that causes anthracnose in a wide variety of economic crops [9]. and is one of the most prevalent fungal plant pathogens within its genus, especially in tropical regions [9]. *Phytophthora palmivora* is a pathogen that has a serious impact on economically valuable crops such as cocoa, rubber, coconut, and durian [10]. *Xanthomonas axonopodis* pv. *manihotis* is the causative agent of cassava bacterial blight [11], while *X. campestris* causes black rot disease, infecting brassicas worldwide [12].

This study aimed to describe the isolation and characterization of five actinomycetes isolates and evaluate their potential as biofertilizers, in order to better understand their interactions and potentially beneficial effects, prior to conducting plant growth promotion experiments.

2. Material and methods

2.1 Microorganisms and cultivation condition

Colletotrichum gloeosporioides DoA c1060, and *Xanthomonas campestris* DoA 1896 were purchased from the Department of Agriculture, Ministry of Agriculture and Co-operatives. *Phytopthora palmivora* CbP01, and *Phytopthora Palmivora* CbP03 were kindly provided by Asst. Prof. Dr. Wisuwat Songnuan (Department of Plant Science, Faculty of Science, Mahidol University). *Xanthomonas axonopodis* pv. *manihotis* was isolated by members of our laboratory and identified by Miss Patcharee Laoburin. Fungi and oomycetes were incubated at 30ºC and maintained on Potato Dextrose Agar (PDA) agar plates (g/L: glucose, 20.0; potato extract, 4.0; agar, 15.0). All bacterial pathogens were incubated and maintained (short-term preservation) at 30°C on Müller-Hinton (MH) agars (g/L: beef extract, 2.0; casein hydrolysate, 17.5; starch, 1.5; agar, 15.0). For long term preservation, pure cultures were grown in MH broth (g/L: beef extract, 2.0; casein hydrolysate, 17.5; starch, 1.5) for 1 day and preserved in 20% glycerol at -80ºC. Isolated actinomycetes were incubated at 30ºC and maintained on 301 agar (g/L: calcium carbonate, 4.0; glucose, 1.0; starch, 24.0; meat extract, 3.0; peptone, 3.0; yeast extract, 5.0; agar, 12.0) for short-term preservation. For long-term preservation, the isolated strains were grown in 301 broth (g/L: calcium carbonate, 4.0; glucose, 1.0; starch, 24.0; meat extract, 3.0; peptone, 3.0; yeast extract, 5.0) for 3 days and preserved in 20% glycerol at -80ºC.

2.2 Isolation of actinomycetes

Two soil rhizosphere samples were collected from mulberry and jackfruit trees in Bangkok and dried at room temperature for a week. One gram of each sample was suspended in 9 mL of sterile normal saline solution and serially diluted 10-fold in order to obtain 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions. Subsequently, 0.1 mL of each dilution was spread on Pridham's agar (g/L: glucose, 10.0; starch, 10.0; $(NH_4)_2SO_4$, 2.0; $CaCO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4$, 1.0; NaCl, 1.0; agar, 12.0) and water-proline agar (g/L l in tap water: proline, 10.0; agar, 12.0) supplemented with 25 μg/mL nalidixic acid and 50 μg/mL cycloheximide before incubation at 30°C for 3-7 days. Actinomycetes-like colonies were sub-cultured to obtain pure isolates.

2.3 Determination of antimicrobial activity by co-culture

Isolated strains were grown on 301 agar at 30°C for 7 days. The pathogenic fungus *Colletotrichum gloeosporioides* DoA c1060 and the oomycetes *Phytophthora palmivora* CbP01 and *P. palmivora* CbP03 were cultivated on 301 agar for 7 days, similar to the actinomycete strains. Agar blocks of fungus and oomycete were cut out using an 8 mm cork borer and placed in the center of a 301 agar plate. Agar blocks for six actinomycete strains were cut out using the same type of borer and positioned 2 cm apart around the fungus or oomycete block. The plates were incubated at 30°C for 7 days. Subsequently, the length of the inhibition zones between the fungal and bacterial colonies was determined.

The plant pathogenic bacteria *X. axonopodis* pv. *manihotis* and *X. campestris* pv. *campestris* were cultivated overnight in MH broth at 30°C. The OD_{600} was determined and the concentration was adjusted to obtain an absorbance of 0.25. The bacterial cultures were then swabbed onto MH agar. Agar blocks containing actinomycetes isolates were cut out with a cork borer, as above, and placed on the swabbed MH agar plates. The plates were incubated at 30ºC for 1 day, after which the size of the inhibition zone against the pathogenic bacteria was measured, including the diameter of the agar block.

2.4 Quantification of indole-3-acetic acid (IAA)

To quantity IAA, the selected strains were inoculated in 5 mL The International Streptomyces Project (ISP) -2℃ for 3 days, followed by cultivation in 100 mL Tryptic Soy Broth (TSB) medium for 7 days, with shaking at 170 rpm. Subsequently, the supernatant was adjusted to pH 2.5 with 4 N HCl, and IAA was extracted three times using 100 mL ethyl acetate. High-performance liquid chromatography (HPLC) was carried out using the Agilent HP1100 system (Hewlett-Packard) with a C18 reverse phase column (Cadenza CD-C18 reverse phase symmetry column, 4.6×210 mm, 3.5 µm pore size). A UV/VIS detector (280 nm) was used to determine the quantity of IAA [13]. The temperature of the column was maintained at 40°C. The flow rate was 1 mL/min, and the solvent system was 20% CH_3CN/ 0.1% HCOOH.

2.5 Quantification of solubilized phosphate

The selected strains were inoculated in 5 mL ISP-2 broth and shaken at 200 rpm, for 3 days at 30°C. Next, the cultures were inoculated in 100 mL of National Botanical Research Institute's phosphate growth medium (NBRIP) broth (g/L: glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g) (Nautiyal, 1999) and shaken at 170 rpm, for 5 days at 30°C. After cultivation, the level of solubilized phosphate was determined [14] and the pH of each sample was measured.

2.6 Ammonia production assay

The selected strains were evaluated for ammonia production in peptone water (g/L: peptone 10.0; NaCl 5.0). Freshly grown cultures were inoculated in 5 mL peptone water and incubated at 30°C for 7 days. Nessler's reagent (0.5 mL) was added to each bacterial suspension. The formation of an orange precipitate was considered a positive result for indicating ammonia production.

2.7 Siderophore production assay

Bacterial isolates were examined for siderophore production on chrome azurol S (CAS) agar plates, as described by Schwyn and Neilands [15]. The actinomycete strains were inoculated on CAS agar and incubated at 30°C for 3 days. Development of a yellow-orange halo around the colony was considered a positive indicator of siderophore production.

2.8 Evaluation for physiological traits

2.8.1 Salinity

The selected actinomycetes were streaked onto ISP-2 agar containing NaCl at concentrations of 0%, 4%, 7%, 10%, and 13%. The plates were incubated at 30°C for 7 days and observed for growth at the end of the incubation period.

2.8.2 pH

The selected actinomycetes were streaked onto ISP-2 agar adjusted to pH 5, 7, 9, and 11. The plates were incubated at 30°C for 7 days and observed for growth at the end of the incubation period.

2.8.3 Temperature

The selected actinomycetes were streaked onto ISP-2 agar**.** The plates were incubated at 20°C, 30°C, 40°C and 50°C for 7 days and observed for growth at the end of the incubation period.

2.9 Morphological characterization

The selected actinomycetes were streaked onto ISP-2 agar and incubated at 30°C for 7 days to observe culture characteristics, e.g., color of aerial and substrate mycelia, and pigmentation.

2.10 Molecular identification of the selected strains by 16S rRNA gene sequencing

Bacterial biomass of each selected isolates was prepared by growing in 301 medium at 30°C for 3 days, with shaking at 200 rpm. Pellets were harvested by centrifugation at 8000 rpm for 10 min prior to suspending in Type 1 water. The washing procedure was repeated twice to ensure that the broth was completely removed. The cells were then resuspended in 500 µL 1X Tris-EDTA (TE) buffer (1 L; 10 mL 10 mM Tris-HCl, pH 8; 10 mL 1 mM Na2EDTA, pH 8; 980 mL Type 1 water) before being broken down using a tissue grinder. The DNA concentration of each sample was diluted to approximately 100 ng/mL. DNA amplification was performed using 2X PCR Master mix Solution (i-StarTaq) in a PCR thermal cycler (Perkin Elmer GeneAmp PCR System 2400 Thermal Cycler). Universal primers 11F (5' AGTTTGATCATGGCTCAG 3') and 1540R (5' AAGGAGGTGATCCAGCCGCA 3') were used for PCR amplification. PCR of the desired fragments (~1,500 bp) was confirmed by agarose gel electrophoresis (1% w/v); samples were stained with 6X DNA loading dye (Thermo Scientific). PCR products were purified using a PCR purification kit (PureLink Quick Gel Extraction and PCR Purification Combo Kit, Invitrogen) according to the manufacturer's instructions. The purified PCR products were commercially sequenced at Macrogen, Korea. To identify closely related species, obtained sequences were compared to similar sequences in the EzBioCloud database (https://www.ezbiocloud.net/identify).

2.11 Statistical analysis

The data are presented as mean values ± standard deviation. Results were analyzed by one-way analysis of variance (ANOVA), combined with Tukey post-test using the SPSS statistical package (version 18.0 for Windows; SPSS Inc., Chicago, IL, USA). The level of statistical significance was considered at a *P*-value of < 0.05.

3. Results and discussion

3.1 Selected actinomycetes with antimicrobial activity

Actinomycetes have been recognized as biocontrol agents based on several mechanisms, including the production of antibiotics and degrading enzymes to suppress plant-pathogenic fungi and bacteria [16-18]. Their broad spectrum of antimicrobial activity enables them to compete with other microorganisms in soils with limited nutrients while promoting plant health [19].

A total of 137 actinomycetes were obtained from soil rhizospheres, 28 of which demonstrated antimicrobial activity. However, only five strains, designated SNN002, SNN045, SNN052, SNN056, and SNN059 were selected for further testing due to their potent antimicrobial activity (Table 1). Isolates SNN045, SNN056, SNN059 were all effective at inhibiting five indicator microorganisms. Strain SNN002 was active only against pathogenic bacteria (producing a large inhibition zone), while SNN052 showed only antifungal activity and not antibacterial activity.

Table 1 Antimicrobial activity of actinomycete strains.

	Actinomycete strain (SNN)				
	002	045	052	056	059
Antifungal activity					
C. gloeosporioides DoA c1060	-	+	+	+	+
P. palmivora CbP01	-	+	+	+	+
P. palmivora CbP03	-	+	+	+	+
Antibacterial activity					
X. axonopodis pv. *manihotis*	+++	+++	-	+++	++
X. campestris pv. *campestris*	+++	+++	-	+++	++

Note: Antifungal activity: (-) No inhibition zone, (+) inhibition zone detectable. Antibacterial activity: (-) No inhibition zone, (+) 5-10 mm, (++) 11-20 mm, (+++) > 20 mm.

3.2 *IAA production*

All five isolates were able to produce IAA, within the range 38.73-211.35 ng/mL (Figure 1). Strain SNN045 produced the highest concentration (211.35 ng/mL), followed by SNN056, SNN052, SNN002, and SNN059, which produced 149.41, 106.83, 52.26, and 38.73 ng/mL, respectively. IAA is a key regulator of root systems that is commonly produced by actinomycetes as an exogenous hormone and has a positive effect on plant growth [20]. The selected strains produced significantly more IAA than *Streptomyces* sp. 3s (20 ng/mL) [21]. Although high concentrations of IAA (4.38 ng/mL) have been shown to inhibit root formation in *Arabidopsis thaliana* [22], the amount of IAA produced by bacteria in their natural environment has never been determined.

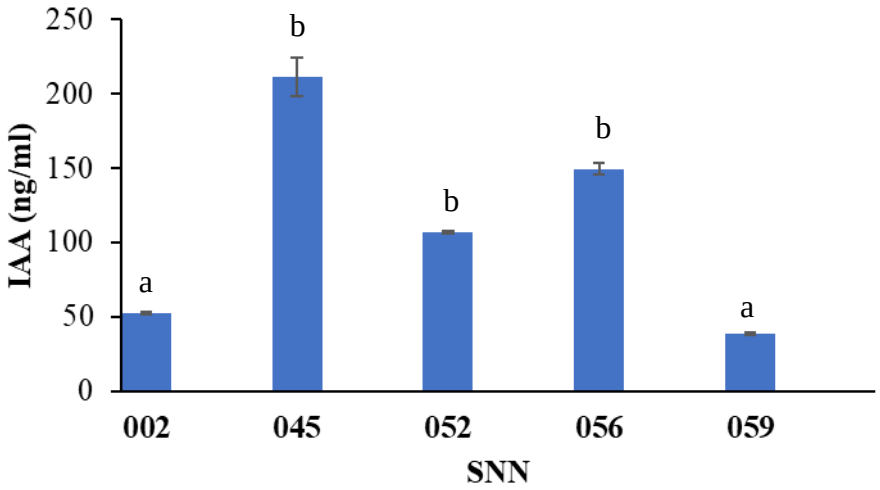

Figure 1 IAA concentrations produced by five actinomycetes isolates in TSB medium. The results shown are the means of three replicate values ± SD. a and b represent significant differences according to the Tukey test ($p < 0.05$).

3.3 Phosphate solubilization and ammonia and siderophore production

Phosphate solubilization by the isolates was examined via quantitative analysis. The isolates were able to solubilize 116.20-319.33 µg/mL phosphate, while the pH of the culture medium decreased sharply for all isolates (Table 2). An extremely high phosphate concentration was determined for the isolate SNN059 (319.33 ± 3.09 µg/mL) with a significant reduction in pH to 4.44 ± 0.07. Phosphorous is the major limiting factor for plant growth. It has been reported that actinomycetes improve phosphate solubilization through acidification or the production of organic acids such as gluconic acid and malic acid [23]. This increase in phosphate solubilizing activity coupled with the steep decline in culture broth pH therefore represents a noteworthy result. A high level of phosphate solubilizing activity (323 µg/mL) has been reported as a result of excretion of gluconic acid by *Streptomyces* sp. CTM396 into NBRIP medium [24]. In the current study, relatively high levels of solubilized phosphate were quantified in the culture broth of strain SNN056 (303.13 µg/mL) and SNN059 (319.33 µg/mL).

Table 2 Phosphate solubilization and ammonia production by actinomycete strains.

Strain	Phosphate solubilization		NH_3 production
	Solublized phosphate (µg/mL)	pH	
SNN002	292.71 ± 7.42^a	4.35 ± 0.03^a	+
SNN045	273.61 ± 4.87^a	4.66 ± 0.02^b	+
SNN052	116.20 ± 3.52^b	4.85 ± 0.03^c	+
SNN056	303.13 ± 9.08^a	4.67 ± 0.06^b	+
SNN059	319.33 ± 3.09^a	4.44 ± 0.07^a	+

The results shown are the means of three replicate values ± SD. a, b, c represent significant differences according to the Tukey test (P < 0.05). **(+),** positive result; (-), negative result.

Organic nitrogen in the soil can be converted into ammonia (NH^{4+}) by ammonifying bacteria, providing nitrogen to plants [25]. Peptone water broth was used to qualitatively investigate ammonia production. Production of ammonia was detected in all isolates, as indicated by the presence of an orange precipitate.

As shown in Figure 2, orange halo zones appeared around all isolates, indicating the ability to produce siderophores. Microbial siderophores are organic chelating agents capable of binding to ferric iron (Fe^{3+}) [26], which facilitate iron movement from the soil into the plant and exhibit antagonistic activity against pathogens [27].

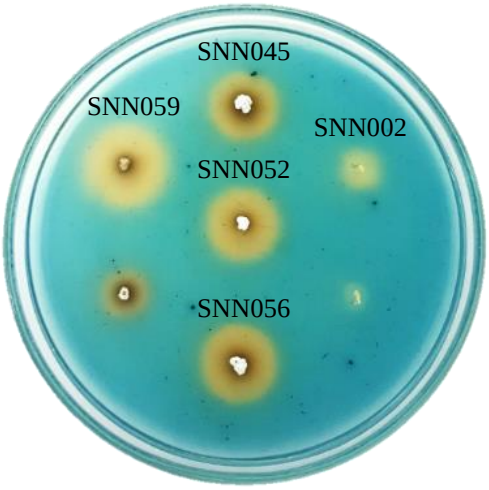

Figure 2 Halo zone around colonies indicating siderophore production.

3.3 Growth of actinomycetes under conditions of variable salinity, pH, and temperature

The growth of potential isolates under different conditions is summarized in Table 3. Apart from isolates SNN002 and SNN059, all isolates were able to tolerate salinity up to 7%, while growth was observed at pH 5-11 for all strains except SNN022. Additionally, temperatures between 20°C and 40°C were found to be optimal for all isolates except SNN002, which grew poorly at 40°C. One of the key reasons for the capacity of actinomycetes to tolerate a wide range of conditions is their distribution in a variety of habitats, which has necessitated adaptation to living at high temperatures and in, saline, acidic, or alkaline soils [28]. When introduced into the rhizosphere of the host, such characteristics help actinomycetes compete with the natural microflora [28]. The salt and pH levels of the soil rhizosphere are crucial elements affecting the ability of bacteria to compete and survive in the rhizosphere. As a result, microorganisms should be selectedbased on their tolerance for high salinity and pH [28].

Table 3 Growth of actinomycetes under conditions of variable salinity, pH, and temperature.

Condition		SNN				
		002	045	052	056	059
Salinity (%)	0	+	+	+	+	+
	4	-	+	+	+	-
	7	-	+	+	+	-
	10	-	-	+	+	-
	13	-	-	-	-	-
pH	5	-	+	+	+	+
	6	+	+	+	+	+
	7	+	+	+	+	+
	9	+	+	+	+	+
	11	+	+	+	+	+
Temperature (℃)	20	+	+	+	+	+
	30	+	+	+	+	+
	40	+	+	+	+	+
	50	-	-	-	-	-

(+), growth observed; (-), no growth observed.

3.4 Morphological characteristics and 16S rRNA gene identification of the selected strains

Three of the five selected actinomycetes displayed similar features on ISP2 and ISP4 agars, while only strain SNN002 was unable to produce aerial mycelium on either agar (Table 4).

The obtained 16S rRNA gene sequences were identified by comparison with sequences in the EZbioCloud database. The most closely related strains are reported in Table 5. All selected strains were identified as belonging to the genus Streptomyces. Strain SNN002 shared 98.65% identity with *Streptomyces bambusae* T110^T, and strain SNN059 showed 99.86% similarity with *Streptomyces globosus* LMG 19896^T. Strain SNN045 (99.79% similarity), SNN052 (100% similarity) and SNN056 (99.86% similarity) were all found to be closely related to *Streptomyces parvulus* NBRC 13193^T.

Table 4. Morphological characteristics of the selected strains on ISP2 and ISP4 agars.

Characteristics	Strain (SNN)									
	022		045		052		056		059	
	ISP2	ISP4	ISP2	ISP4	ISP2	ISP4	ISP2	ISP4	ISP2	ISP4
Color of substrate mycelium	cream	cream	gray	gray	light brown	cream	yellow	yellow	beige	beige
Color of aerial mycelium	-	-	pale yellow	pale yellow	white	white	white	dusty gray	sandy brown	sandy brown
Diffusible pigment	-	-	yellow	yellow	brown	-	yellow	yellow	-	-

Table 5. Identification of the selected strains based on 16S rRNA gene sequencing.

Strain	Length (bp)	Similarity (%)	Closest strain
SNN002	1,405	98.65	*Streptomyces bambusae* T110^T
SNN045	1,424	99.79	*Streptomyces parvulus* NBRC 13193^T
SNN052	1,404	100	*Streptomyces parvulus* NBRC 13193^T
SNN056	1,399	99.86	*Streptomyces parvulus* NBRC 13193^T
SNN059	1,404	99.86	*Streptomyces globosus* LMG 19896^T

4. Conclusion

The antimicrobial activity, plant growth-promoting (PGP) properties, and growth conditions of actinomycete strains SNN045, SNN056, and SNN059 demonstrate the potential for positive effects on plant development and survival in a highly competitive environment. These microorganisms have the potential to act as high performance biofertilizers; however, there is a need for *in vivo* investigations such as pot experiments and field studies in order to ascertain their true potential for plant growth promotion.

5. Acknowledgements

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