Diversity and antimicrobial activity of culturable endophytic actinobacteria associated with Acanthaceae plants

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ABSTRACT: In this study, a total of 52 endophytic actinobacteria were isolated from 6 species of Acanthaceae plants collected in Thailand. Most actinobacteria were obtained from the root part. Based on 16S rRNA gene analysis and phylogenetic tree, these actinobacteria were classified into 4 families (\textit{Nocardiaceae}, \textit{Micromonosporaceae}, \textit{Streptosporangiaceae} and \textit{Streptomycetaceae}) and 6 genera including \textit{Actinomycetospora} (1 isolate), \textit{Dactylosporangium} (1 isolate), \textit{Nocardia} (3 isolates), \textit{Microbispora} (5 isolates), \textit{Micromonospora} (10 isolates) and \textit{Streptomyces} (32 isolates). The result of antimicrobial activity screening indicated that 8 isolates, including 1 \textit{Actinomycetospora} and 7 \textit{Streptomyces}, exhibited antimicrobial activity against tested microorganisms. In addition, the selected \textit{Streptomyces} sp. 5R010 showed antagonistic activity against fungal plant pathogens including \textit{Fusarium sp.}, \textit{Colletotrichum sp.} and \textit{Sclerotium sp.}. Therefore, this study demonstrated that the Acanthaceae plant species harbored the endophytic actinobacteria which can be used as the source of the antimicrobial compound.

KEYWORDS: endophytic actinobacteria, antimicrobial activity, Acanthaceae, phytopathogenic fungi

INTRODUCTION

Microorganisms, especially actinobacteria, are the primary source of the bioactive natural products which is driving drug discovery [1]. In the past century, numerous actinobacteria have been isolated from soil and used as the producer of key drugs such as actinomycin, avermectin, erythromycin, gentamicin, neomycin, platensimycin, streptomycin and vancomycin. Although many drugs are developed from the actinobacteria, the discovery of novel lead compounds has decreased because of the redundancy of the samples. Consequently, it is extremely necessary to investigate the untapped microorganisms to drive natural product research.

Actinobacteria are well known to contain valuable economically important microorganisms for a long time because of their ability to produce a large number of bioactive secondary metabolites [2]. Actinobacteria are one of the major soil microbiota. However, they are widely distributed in other various environments such as marine sediment, freshwater, insects and plants. In the past decade, the untapped habitats, especially endophytic, have become a promising source of novel actinobacteria [3].

Endophytes are the microorganisms that spend at least parts of their life cycle inside the plant tissues without having a negative impact on the host plants [4]. These microbes, especially actinobacteria, have a massive potential to produce a number of novel compounds that find wide-range application as agrochemicals, antibiotics, immunosuppressants, antiparasitics and anticancer agents [5].

A huge diversity of secondary metabolites of actinobacteria may occur because of the natural adaptation to the environments [6]. Recently, many of novel actinobacteria such as \textit{Asanoa endophytica}, \textit{Phytotoactinopolyspora endophytica}, \textit{Phytohabitans kaempferiae} and \textit{Streptomyces oryzae} have been isolated from various plant species [7–10].
Acanthaceae is a family of dicotyledonous flowering plants containing approximately 210 genera and nearly 4000 species. These plants are widely distributed in tropical and subtropical regions [11]. At present, many plant species in this family, for example Andrographis paniculata, Barlelia lupulina, Clinacanthus nutans and Thunbergia laurifolia, have been used for Thai traditional medicines. However, the actinobacteria associated with this plant family are rarely reported. Therefore, the objectives of this study were to study the diversity of endophytic actinobacteria associated with the Acanthaceae plant and to screen the antimicrobial activity of the actinobacterial isolates.

**MATERIALS AND METHODS**

**Plant collections and isolation of actinobacteria**

Plant samples were collected and planted in the botanical garden of the Department of Biology, Faculty of Science, Ramkhamhaeng University prior to isolation. In this study, 6 species of plants in the Family Acanthaceae including Andrographis paniculata, Asystasia gangetica, Berleria lupulina, Clinacanthus nutans, Justicia subcoriacea and Ruellia squarrosa were collected.

Actinobacteria were isolated from leaves, stems, and roots of each plant sample. Plant samples were washed to remove soil from the samples. The three-step surface sterilization was used to eliminate the surface microbes. Briefly, a 5-min wash in 3% NaOCl, followed by a 1-min wash in 95% ethanol and a final wash with a sterile distilled water 2 times. A 0.5 g of the surface-sterilized materials was aseptically ground with 5 ml of extraction broth volume) of 95% ethanol was added and shook at 180 rpm for 1 h followed by centrifuge at 4500 rpm for 10 min. The supernatant was collected and preserved at −20°C. To prepare the tested disc, the sterile paper disc was dipped into each broth library and air-dried in the biosafety cabinet. The sterile ISP2 broth added with one volume of ethanol was used as the negative control.

Six microorganisms including 3 Gram-positive bacteria: Staphylococcus aureus, Bacillus subtilis and Kocuria rhizophila, and 2 Gram-negative bacteria: Escherichia coli and Pseudomonas aeruginosa, and a yeast, Candida albicans, were used as the tested microorganisms. The tested bacteria and yeast were activated on Mueller-Hinton agar (MHA) and sabouraud dextrose agar (SDA) for 27 h at 37°C and 30°C, respectively. To prepare a microbial suspension, the turbidity of each tested microorganism in normal saline solution was adjusted to 0.5 McFarland standards. Then, the tested bacteria and yeast were swabbed on the surface of MHA and SDA, respectively. The prepared paper disc was put on the surface of media swabbed with the tested microorganisms and incubated for 24 h at 37°C and 30°C for bacteria and yeast, respectively. The inhibition zone was observed and documented.

**Antimicrobial activity screening**

Antimicrobial activity of actinobacterial isolates was determined using the agar disc diffusion method. Briefly, each actinobacterium was cultured in ISP2 broth pH 7.0 in shaking condition at 180 rpm 30°C for 14 days. Then, one volume (equivalent to culture broth volume) of 95% ethanol was added and shook at 180 rpm for 1 h followed by centrifuge at 4500 rpm for 10 min. The supernatant was collected and preserved at −20°C. To prepare the tested disc, the sterile paper disc was dipped into each broth library and air-dried in the biosafety cabinet. The sterile ISP2 broth added with one volume of ethanol was used as the negative control.

**Identification of actinobacteria**

The identification of actinobacteria was performed by 16S rRNA gene analysis. The genomic DNA of actinobacteria was extracted from the mycelia grown in yeast-dextrose broth (1 g glucose; 1 g yeast extract; 100 ml water, pH 7.0–7.2) at 30°C for 3–7 days [16]. The amplification was carried out using standard primers (5’-GAGTTTGATCCTGGCTCAG-3’) and 1530R (5’-GTTACCTTGTACGACTT-3’) with the initial incubation of 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C and followed by a 3 min final extension at 72°C [17, 18]. The nucleotide of the PCR product was sequenced using the sequencing service (Macrogen, Korea). The nucleotide sequence was manually analyzed using BioEdit software (Ibis Biosciences). BLAST was determined using the EzbioCloud database [19]. Phylogenetic analysis was constructed using MEGA 7.0 software [20]. The tree topology was evaluated using the bootstrap test [21].
Curvularia oryzae, Fusarium sp., Lasiodiplodia theobromae and Sclerotium sp.

The selected actinobacterium was cultured on one side of the ISP2 agar plate and incubated at 30°C for 7 days. Then, the 7-day-old of the tested phytopathogenic fungi grown on SDA agar were cut by the cork borer (6 mm in diameter) and transferred to the opposite of the prepared actinobacterium plate and incubated at 30°C for 7–10 days. The inhibition zone around the actinobacterial colony indicated fungal inhibition. The fungi grown on ISP2 agar without actinobacteria were used as the growth control of the fungi.

RESULTS AND DISCUSSION

Diversity of actinobacteria

In this study, 52 actinobacteria were isolated from leaves, stems and roots of 6 species of Acanthaceae plants. In this number, 49 isolates were obtained from roots, followed by 2 and 1 isolate obtained from leaves and stem, respectively. The results of this study are similar to the previous studies showing that nearly all the plants harbor endophytes [22]. Janso and Carter [23] discussed that actinobacteria could be isolated from every tissue type of samples; however, root and bark had the highest isolate-to-sample ratio.

On the basis of BLAST result and phylogenetic tree analysis, actinobacteria obtained in this study were identified and categorized into 4 families (Nocardiaceae, Micromonosporaceae, Streptomycetaceae) and 6 genera including Actinomycetospora (1 isolate), Dactylosporangium (1 isolate), Nocardia (3 isolates), Microbispora (5 isolates), Micromonospora (10 isolates) and Streptomyces (32 isolates) (Figs. 1 and 2, Table 1). Based on this study, the most abundant genus found in Acanthaceae plants were Streptomyces (61%) followed by Micromonospora (19%) and Microbispora (10%) (Fig. 1). The pattern of the diversity of culturable actinobacteria of this study, of which Streptomyces are the predominant species, is similar to the previous report [24]. In 2012, Kim et al [25] isolated 61 endophytic actinobacteria, comprising 15 genera including Streptomyces, Micromonospora, Rhodococcus, Microbispora, Micrococcus, Microbacterium, Streptacidiphilus, Arthrobacter, Dietzia, Kitasatospora, Herbiconix, Mycobacterium, Nocardia, Rathayibacter and Tsukamurella, from the native herbaceous plant species of Korea. In that study, they found that members of the genus Streptomyces comprised 45.9% of the total isolates and were followed by Micromonospora (18.8%). In the study of Janso and Carter [23], 123 isolates of endophytic actinobacteria, including 17 genera, were isolated from the tropical native plants in Papua New Guinea and Mborokua Island, Solomon Island. The community of endophytic actinobacteria may vary according to the host plant. Jiang et al [26] isolated 101 endophytic actinobacteria from 5 different mangrove plants including Avicennia marina, Aegiceras corniculatum, Kandelia obovata, Bruguiera gymnorrhiza and Thespesia populnea. Based on 16S rRNA gene, these actinobacteria were distributed in 15 families and 28 genera including Actinoplanes, Agroccus, Amnibacterium, Brachybacterium, Brenibacterium, Citricoccus, Cartobacterium, Dermacoccus, Glutamicibacter, Gordonia, Isosporicola, Janibacter, Kineococcus, Kocuria, Kyococcus, Leucobacter, Marmoricola, Micrococcus, Microbacterium, Micromonospora, Mycobacterium, Nocardoides, Nocardia, Nocardiosis, Pseudokineococcus, Sanguibacter, Streptomyces and Verrucosispora. In addition, Widiantini and Franco [27] reported that the dominant
Fig. 2 Neighbor-Joining phylogenetic tree based on 16S rRNA gene of the actinobacterial isolates and closely related actinobacterial type strains shown that the isolates were clustered within 4 families and 6 genera. Numbers at the nodes indicate bootstrap values based on 1000 replicates.
endophytic actinobacteria species isolated from rice plants of Australia is *Microbispora*. The variable of endophytic actinobacterial species in the different plants may depend on factors such as host specificity, stage of the host, type of sample, geographical condition, season, surface sterilant, culture condition and selective media [28, 29].

**Antimicrobial activity**

In this study, 8 isolates including 1 *Actinomyces* and 7 *Streptomyces* exhibited antimicrobial activity against tested microorganisms. Most of the active isolates showed antimicrobial activity against Gram-positive bacteria, but no activity was observed.
against Gram-negative bacteria (Fig. 3, Table 1). The antimicrobial activity of endophytic Streptomyces against Gram-positive bacteria has been documented in previous studies. Zhang et al. [30] studied antimicrobial activity of 65 endophytic actinobacteria, isolated from Achyranthes bidentata, Paonia lactiflora, Radix Platycodi and Artemisiae argyi, against penicillin resistant Staphylococcus aureus. They found that 12 strains, the majority of which were Streptomyces spp., showed activity against this pathogen. Although no actinobacteria obtained from this study showed anti-Gram-negative bacterial activity. Mingma et al. [31] isolated 317 actinobacteria from root and rhizospheric soils of leguminous plants, and 64 of the isolates (20.2%) showed antagonistic activity against soybean pathogen Xanthomonas campestris pv. glycine. In addition, 21 endophytic actinobacteria isolated by Jiang et al. [26] showed activity against P. aeruginosa. This evidence showed that anti-Gram-negative bacteria could be observed in some endophytic actinobacteria.

The production of novel antimicrobial metabolites from endophytic actinobacteria has been documented in the various reports. These include maklamicin, misamycin and diastaphenazine.

Maklamicin, a new spirotetronate-class polyketide isolated from Micromonospora sp. GMKU326 — the endophytic actinobacteria from root nodule of the legume Lupinus angustifolius, showed strong to moderate antimicrobial activity against Gram-positive bacteria including Micrococcus luteus, Bacillus subtilis, B. cereus, Staphylococcus aureus and Enterococcus faecalis with MIC values of 0.2, 1.7, 6.5, 13 and 13 µg/ml, respectively [32].

Misamycin, a new anthracycline antibiotic, was isolated from the culture broth of endophytic Streptomyces sp. YIM66403. The compound exhibited moderate antibacterial activity against S. aureus with MIC value of 64 µg/ml. Besides antibacterial activity, it showed cytotoxicity against various human cell lines including human promyelocytic leukemia HL-60, human hepatoma SMMC-7721, non-small cell long cancer A-549, breast cancer MCF-7 and human colorectal carcinoma SW4801 with IC_{50} values of 15.37, 16.34, 25.98, 20.71 and 9.75 µM, respectively [33].

Diastaphenazine, a new dimeric phanazine, was isolated from the culture broth of endophytic Streptomyces diastacicus subsp. ardesiacus from sterile tissue of Artemisia annua. The compound showed antimicrobial activity against Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and Candida albicans ATCC 10231. In addition, it showed weak cytotoxicity against 5 human tumor cell lines including BGC-823, Hela, HCT116, HepG2 and H460 with IC_{50} values of 14.9, 28.8, 65.2, 82.5 and >100 µM, respectively [34].

In this study, the isolate 5R010, closely related to Streptomyces siyaeasnis NRRL-B5408\(^2\), showed antifungal activity against C. albicans. This isolate was selected to test the antagonistic activity against phytopathogenic fungi.

Antiphytopathogenic fungi activity

Based on the co-cultivation method, the strain 5R010 showed antagonistic activity against Fusarium sp., Colletotrichum sp. and Sclerotium sp., but no activity was observed on Colletotrichum gloeosporioides, Curvularia oryzae and Lasiodiplodia theobromae (Fig. 4). It has been reported in several studies that the endophytic actinobacteria can be used to control plant diseases. Álvarez-Pérez et al. [35] used endophytic actinobacteria isolated from the root system of the grapevine plants, Vitis vinifera, to reduce nursery fungal graft infections caused by Diplodia seriata, Dactylonectria macrodidyma, Phaeomoniella chlamydospora and Phaeoacremonium minimum. Taechowisan et al. [36] reported that 3 endophytic Streptomyces sp. showed strong inhibition for Colletotrichum musae and 5 were very active against Fusarium oxysporum. The Streptomyces strain CEN6, isolated from Centella asiatica, showed good antagonistic activity against Alternaria brassicicola; the pathogen causes leaves spot of cabbage. The fungal treated by this strain showed abnormal characteristics including swelling and frequent septa [37]. The use of endophytic Streptomyces platensis F-1, isolated from Oryza sativa, as biofumigation to control...
Fig. 4 Antagonistic activity of the isolate 5R010 against phytopathogenic fungi (a) *Lasiodiplodia theobromae*, (b) *Curvularia oryzae*, (c) *Fusarium* sp., (d) *Sclerotium* sp., (e) *Colletotrichum gloeosporioides* and (f) *Colletotrichum* sp. The arrows → and ← indicate the colony of isolate 5R010 and fungal pathogens, respectively.

plant fungal disease was reported by Wan et al [38]. The volatile substance produced by the strain F-1 could effectively reduce the incidence and the severity of the disease caused by *Botrytis cinerea*, *Rhi zoctonia solani* and *Sclerotinia sclerotiorum*. Besides the application as biocontrol, the novel antifungal compounds such as dehydroxyaquayamycin B and fistupyrone were isolated from endophytic actinobacteria. Dehydroxyaquayamycin B, a new C-glycosylated benz[a]anthraquinone, was isolated from endophytic *Streptomyces blastomycetica* F4-20. The compound showed fungicidal activity against *Valsa mali*, *Colletotrichum orbiculare* and *Fusarium graminearum* [39]. Fistupyrone, a new microbial compound, isolated from the culture broth of endophytic *Streptomyces* sp. TP-A0569 can inhibit the in vivo infection of the seedlings of Chinese cabbage by *Alternaria brassicicola*, the cause of *Alternaria* leaf spot [40]. The antagonistic activity of the strain 5R010 found in this study revealed that this strain may be used for the fungal biocontrol in the future. In addition, the active compounds produced by this strain should be characterized in further study.

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Authors’ contributions: we declare that the present study was performed by the authors named in this article. W. Phongsopitanun designed the study and performed experiments on isolation, identification of actinobacteria and screening of antimicrobial activity of the actinobacterial isolates; P. Sripreechasak performed experiments on data analysis; K. Rueangsawang and R. Panyawut performed experiments on plant sample collection and identification of the plant species; P. Wittayakhonwut and S. Tanasupawat gave conceptual advice.

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