Endophytic actinomycetes associated with *Cinnamomum cassia* Presl in Hoa Binh province, Vietnam: Distribution, antimicrobial activity and, genetic features

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Endophytic microbes associated with medicinal plants are considered to be potential producers of various bioactive secondary metabolites. The present study investigated the distribution, antimicrobial activity and genetic features of endophytic actinomycetes isolated from the medicinal plant *Cinnamomum cassia* Presl collected in Hoa Binh province of northern Vietnam. Based on phenotypic characteristics, 111 actinomycetes were isolated from roots, stems and leaves of the host plants by using nine selective media. The isolated actinomycetes were mainly recovered from stems (n = 67; 60.4%), followed by roots (n = 29; 26.1%) and leaves (n = 15; 13.5%). The isolates were accordingly assigned into 5 color categories of aerial mycelium, of which gray is the most dominant (n = 42; 37.8%), followed by white (n = 33; 29.7%), yellow (n = 25; 22.5%), red (n = 8; 7.2%) and green (n = 3; 2.7%). Of the total endophytic actinomycetes tested, 38 strains (occupying 34.2%) showed antimicrobial activity against at least one of nine tested microbes and, among them, 26 actinomycetes (68.4%) revealed anthracycline-like antibiotics production. Analysis of 16S rRNA gene sequences deposited on GenBank (NCBI) of the antibiotic-producing actinomycetes identified 3 distinct genera, including *Streptomyces*, *Microbacterium*, and *Nocardia*, among which *Streptomyces* genus was the most dominant and represented 25 different species. Further genetic investigation of the antibiotic-producing actinomycetes found that 28 (73.7%) and 11 (28.9%) strains possessed genes encoding polyketide synthase (*pks*) and nonribosomal peptide synthetase (*nrps*), respectively. The findings in the present study highlighted endophytic actinomycetes from *C. cassia* Presl which possessed...
broad-spectrum bioactivities with the potential for applications in the agricultural and pharmaceutical sectors.

Key Words: antimicrobial activity; anthracyclines; Cinnamomum cassia; endophytic actinomycetes; nonribosomal peptide synthetase; polyketide synthase

Introduction

For thousands of years, medicinal plants have widely been used as natural medicines in the treatment of human diseases (Pan et al., 2014). Nevertheless, medicinal plants are also well known to be the hosts of endophytic micro-organisms (Golinska et al., 2015; Qin et al., 2009). Since this association has formed over the long-term, endophytic microbes might acquire and develop specific genetic determinants to produce bioactive compounds similar to those produced by plant hosts (Alvin et al., 2014; Golinska et al., 2015). Thus, endophytic actinomycetes associated with traditionally used medicinal plants, especially in the tropics, could be a rich source of functional metabolites (Golinska et al., 2015). In the literature, endophytic actinomycetes associated with medicinal plants have shown the ability to synthesize many valuable bioactive compounds, including anticancer, antiviral, antimicrobial, antifungal and antiparasitic agents (Inahashi et al., 2015; Tanvir et al., 2016; Zhang et al., 2014; Zin et al., 2017). A number of the secondary metabolites are novel in structure and have broad-spectrum bioactivities that could be potentially applicable in the pharmaceutical, medical, and agricultural sectors (Golinska et al., 2015; Matsumoto and Takahashi, 2017).

The diversity and bioactivity of endophytic actinomycetes are associated with the genetic background of actinomycetes, plant species and geographic areas (Golinska et al., 2015; Matsumoto and Takahashi, 2017; Qin et al., 2009). Moreover, the distribution of the endophytic actinomycete population also varies according to the different tissues of host plants (Qin et al., 2009; Gohain et al., 2015; Salam et al., 2017). Recently, a number of novels and previously uncultured endophytic actinomycetes with diverse metabolic pathways have been isolated through new isolation approaches and media (Matsumoto and Takahashi, 2017; Qin et al., 2009; Tanvir et al., 2016).

Vietnam has been recognized as one of the tropical countries with a very high biodiversity of medicinal plant species, accounting for approximately 11% of the 35,000 species of medicinal plants known worldwide (Khanh et al., 2005). Unfortunately, little is known about the distribution, and the potential to produce secondary metabolites, of endophytic actinomycetes associated with medicinal plants (Khieu et al., 2015; Salam et al., 2017). Cinnamomum cassia Presl, a commonly used medicinal plant, is one such example. The present study clarified the distribution and characterization of endophytic actinomycetes from Vietnamese C. cassia Presl.

Actinomycetes which were isolated were then examined for antimicrobial activity against microbial pathogens and the production of anthracycline-like antibiotics. Furthermore, the presence of secondary metabolic biosynthetic genes encoding for polyketide synthase (pks-I and pks-II) and nonribosomal peptide synthetase (nrps) was also determined.

Materials and Methods

Sample collection and isolation of endophytic actinomycetes from C. cassia Presl. The stem, root and leave segments from 5 C. cassia plants were randomly selected from different sites in Hoa Binh province (20°47′21″ N; 105°21′20″ E) of northern Vietnam. The samples were separately placed in plastic bags according to the different plant organs, transported to the laboratory and then processed within 4 hours after collection. The plant voucher specimens were identified as C. cassia Presl species by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology.

Surface-sterilization of plant organs and the isolation of endophytic actinomycetes have been described in previous studies (Li et al., 2012; Qin et al., 2009; Salam et al., 2017). Briefly, the plant organs were firstly washed with sterile distilled water (dH2O), cut into small pieces (1–2 cm), surface-sterilized for 5 min by 15% NaClO, rinsed in 2.0% Na2S2O3 for 2 min, and washed three times with dH2O. The pretreated samples were immersed in 70% ethanol for 7 min, followed by triple washes with dH2O, then dried under laminar flow conditions. Finally, for each plant organ, the sterilized segments were homogenized in sterile dH2O and used for endophytic actinomycete isolation. The supernatants were spread onto nine different agar media previously described (Li et al., 2012; Qin et al., 2009; Salam et al., 2017), including humic acid-vitamin B agar (HV), raffinose-histidine agar (RA), tap water-yeast extract agar (TWYE), International Streptomyces Project 5 (ISP5), trehalose-proline agar (TA), sodium succinate-asparagine agar (SA), starch agar (STA), citrate acid agar (CA) and sodium propionate agar (SPA). All media were supplemented with filter-sterilized mixtures of nalidixic acid (25 mg/mL), nystatin (50 mg/mL), and K2Cr2O7 (50 mg/mL) to inhibit the growth of bacteria and fungi. The culture media plates were incubated at 30°C for 6–8 weeks. The experiments were performed in triplicate. Apparent actinomycete colonies were rapidly picked up and streaked out on ISP2 medium. Pure isolates were recovered and stored in 15% glycerol at –80°C.

Identification of actinomycetes based on morphological characteristics. Actinomycete isolates were tentatively classified by traditional identification methods, according to morphological characteristics, color of aerial mycelium and pigmentation produced on ISP media (Shirling and Gottlieb, 1966; Goodfellow and Haynes, 1984). Isolates were grown on ISP2 agar plates for morphological feature analysis of spores and spore-chains using a BH2 light microscope (Olympus Corporation, Tokyo, Japan) with a magnification of 100x or 400x and a scanning electron microscope JSM-5410 (JEOL, Tokyo, Japan) (Li et al., 2009).
Evaluation of antimicrobial activity. All endophytic actinomycetes were evaluated for inhibitory activity against 9 microbes, including Gram-negative bacteria (Escherichia coli ATCC 11105, Proteus vulgaris ATCC 49132, Pseudomonas aeruginosa ATCC 9027, Salmonella enterica Typhimurium ATCC 14028 and Enterobacter aerogenes ATCC 13048); Gram-positive bacteria (Sarcina lutea ATCC 9341, Bacillus cereus ATCC 11778 and meticillin-resistant Staphylococcus epidermidis ATCC 35984 (MRSE)); and yeast (Candida albicans ATCC 10231), using the agar well diffusion method (Holder and Boyce, 1994). All experiments were performed in triplicate.

16S rRNA gene sequencing and phylogenetic analysis. The genomic DNA extraction and amplification of 16S rRNA gene were performed as previously described (Phi et al., 2010; Salam et al., 2017). PCR amplicons were purified and sent for sequencing at 1st Base Laboratories Sdn. Bhd., Malaysia. For each strain, the 16S rRNA gene sequence was treated and blasted on GenBank database using Blast tool (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) for the identification of the homology species. A neighbor-joining phylogenetic tree based on 16S RNA gene sequences was computed using MEGA7 software (Tamura et al., 2013). The tree branch was supported with a bootstrap of 1,000 replications. The phylogenetic tree was rooted using Bacillus thuringiensis ATCC 10792 (GenBank accession number CP020754) as an out-group.

Screening for secondary metabolic biosynthetic genes. Three sets of degenerate primers: A3F (5’-GCS TAC SYS ATS TAC ACS TCS GG-3’) and A7R (5’-SAS GTC VCC SGT SCG GTA S-3’), K1F (5’-TSA AGT CSA ACA TCG GBC A-3’) and M6R (5’-CGC AGG TTS CSG TAC CAG TA-3’), KSaF (5’-TSG CST GCT TGG AYG CSA TC-3’) and KSaR (5’-TGG AAN CCG CCG AAB CCG CT-3’) were used for amplification of the nmps, pks-I and pks-II genes, respectively (Ayuso-Sacido and Genilloud, 2005; Metsä-Ketelä et al., 1999). PCR compositions and amplification conditions were performed as previously described (Salam et al., 2017).

Screening for anthracycline-producing actinomycetes. The potential for the production of anthracycline-like antibiotics in actinomycetes was screened using the pigment formulating test described previously (Trease and Evans, 1996). The principle of the method is based on the presence of an anthraquinone ring in the chemical composition where the color of anthracycline compounds will be changed from orange to purple when their pH levels are altered from acid to alkaline, respectively. Briefly, actinomycete isolates were grown on ISP2 agar plates at 30°C for 5–6 days. After that, two wells of 6 mm diameter were punched carefully on the culture plate. The first well was loaded with 25 μl of 1 N HCl, while the second one was loaded with 25 μl of 2 N NaOH, then the plates were incubated at 30°C. The change of color around the wells was observed after 10–30 min.

Results

Distribution of endophytic actinomycetes from C. cassia Presl

On the basis of colony morphology and mycelium color, a total of 111 endophytic actinomycetes were obtained on 9 selective media, among which strains were mainly retrieved from stems (n = 67; 60.4%), followed by roots (n = 29; 26.1%) and leaves (n = 15; 13.5%) (Fig. 1A). According to the isolation media, the greatest proportion of endophytic actinomycetes was obtained on CA medium (n = 28; 25.2%), and the lowest number were isolated on HV medium (n = 1; 0.9%) (Fig. 1B). Overall, four media
Antimicrobial activity of endophytes against microbial pathogens

Thirty-eight (34.2%) of the 111 isolates exhibited inhibitory activities against at least one of nine tested microbes, and none of the actinomycetes showed bactericidal activity against *E. aerogenes* (Table 1). The inhibitory effect against bacterial pathogens was highest for *P. vulgaris* (*n* = 28; 73.7%), followed by *B. cereus* (*n* = 27; 71.1%), MRSE (*n* = 26; 68.4%), *S. lutea* (*n* = 25 of each; 65.8%), *P. aeruginosa* and *E. coli* (*n* = 24; 63.2%), and *C. albicans* (*n* = 10; 26.3%), whereas the results were less for growth inhibition against *S. Typhimurium* (*n* = 1; 2.6%).

Inhibitory patterns of endophytes against the pathogens were very diverse (Table 1), ranging from one to eight microbes (Fig. 2). Nine isolates showed antagonistic activity against all three groups of pathogenic microbes, notably, the HBQ19 isolate revealed remarkable inhibitory activity against eight microbes. Seventeen isolates exhibited antimicrobial activities against six pathogens, among which 11 exhibited growth inhibition against both Gram-negative/-positive bacteria, but no antimicrobial activity against *C. albicans* was recorded. Meanwhile, six other isolates exhibited inhibitory activities against three pathogenic groups and none of all isolates possessed antibacterial activity against *P. aeruginosa*. Thirteen isolates exhibited inhibitory effects against two to five microbes, while seven isolates were active against only one microbe.
**Biosynthesis of anthracycline-like compounds from antibiotic-producing actinomycetes**

Various reports previously summarized that anthracycline antibiotics biosynthesized by actinomycetes have numerously shown cytotoxicity against human tumors or tumor cell lines (Metsä-Ketelä et al., 2007; Zhang et al., 2017). Therefore, anthracycline antibiotics from actinomycetes have been so far, one of the most effective substances widely used in the treatment of many types of cancer (Minotti et al., 2004). The present study showed that of the 38 antimicrobial-producing actinomycetes, 26 (68.4%) isolates possessed capacity for biosynthesis of anthracycline-like antibiotics (Table 1). Among those, 17 isolates interestingly exhibited antimicrobial activity against at least 3 tested microbes (Table 1).

**Analysis of 16S rRNA gene sequence of antibiotic-producing actinomycetes**

By analysis of 16S rRNA gene sequence, 38 antibiotic-producing actinomycetes could be classified into three different genera, among which *Streptomyces* was the most common genus (*n* = 36; 94.7%), followed by *Microbacterium* (*n* = 1; 2.6%) and *Nocardia* (*n* = 1; 2.6%) (Table 1). Overall, comparison of 16S rRNA gene sequences of endophytic actinomycetes and type strains show high similarity levels (96–100%). Accordingly, phylogenetic tree analysis revealed a main cluster of *Streptomyces* genus and two monophyletic branches corresponding to 2 rare actinomycetes genera (Fig. 3). Notably, among the *Streptomyces* cluster, isolates were distributed in multiple branches. Moreover, 36 *Streptomyces* isolates could be assigned into 25 different species (Table 1). All 16S rRNA gene sequences of 38 actinomycetes were deposited on the GenBank (NCBI) with assigned accession numbers (Table 1).

**Screening of biosynthetic genes of antibiotic-producing endophytes**

Three common genes *pks-I*, *pks-II* and *nrps* coding enzymes involved in secondary metabolite-biosynthesis have been widely proved to associate with the prediction of antibiotic biosynthetic pathways (Ayuso-Sacido and Genilloud, 2005; Metsä-Ketelä et al., 2007). Investigation of three genes in 38 antibiotic-producing actinomycetes found 28 isolates (73.7%) possessing *pks-I* and/or *pks-II* gene in which 17 isolates (44.7%) hold both genes; 11 isolates (28.9%) out of 38 isolates harbored *nrps* gene (Table 1). Taken together, 4 isolates hold all three biosynthetic genes and were identified as 4 different species of *Streptomyces* genus of which 3 inhibited the growth of at least 5 microbes.

**Discussion**

The present study revealed considerable diversity of endophytic actinomycetes associated with the medicinal plant *C. cassia* Presl collected from a mountainous region in Hoa Binh province of Vietnam. Analysis of morphological features revealed a high proportion of 111 isolated actinomycetes from the *C. cassia* host plant. In general, the distribution of isolated endophytic actinomycetes remarkably varies according to the geographic region, plant species, specific plant tissues and isolation media (Gohain et al., 2015; Janso and Carter, 2010; Kaewkla and Franco, 2013; Qin et al., 2009; Salam et al., 2017). For instance, a recent study reported the diversity of endophytic actinomycetes in *Dracaena cochinchenensis* Lour from 4 distinct provinces of China and Vietnam (Salam et al., 2017). The distribution of endophytic actinomycetes was different among the provinces in each country and between the countries. In a large surveillance, Qin et al. (2009) obtained 2,174 endophytic actinomycetes from nearly 90 plants in the tropical rain forest in Xishuangbanna of China (approximately 24 isolates/plant), while Janso and Carter (2010) isolated only 123 actinomycete strains from 113 plants of coastal tropical forests in Papua New Guinea and Mborokua Islands (1 strain/plant). Another study from China, Li et al. (2012) successfully isolated 228 endophytic actinomycetes from *Artemisia annua* plant.

Regarding the distribution of actinomycetes according to the plant tissues, we found the greatest number of endophytic actinomycetes from the stems of *C. cassia* Presl (60.4%). The current findings were different from other studies in which endophytic actinomycetes were mainly recovered from roots compared with stems and leaves (Gohain et al., 2015; Kaewkla and Franco, 2013). This could be explained by different features of the medicinal plants studied. Nevertheless, the proportion of actinomycetes exhibited board-spectrum antimicrobial activity was the highest in the roots (90.9%), followed by the stems (70.0%) and the leaves of *C. cassia* (42.8%). The proportion is significantly different between roots and leaves (*p* < 0.03). Thus, stem and root of *C. cassia* Presl are important sources for recovering valuable antibiotic-producing endophytic actinomycetes.

The media and reliable methodologies for the isolation of endophytes play a very important role in recovering culturable actinomycetes (Kaewkla and Franco, 2013; Qin et al., 2009). In the present study, endophytes were mainly recovered from the 4 isolated media CA, SA, TA and TWYE, which accounted for 77.5% of the total actinomycetes, suggesting that the four above media are

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**Fig. 2.** Inhibitory patterns and number of endophytic actinomycetes against microbes.
Endophytic actinomycetes in *Cinnamomum*

mostly suitable for the isolation of endophytic actinomycetes from *C. cassia* Presl tissues. On the contrary, other media such as RA, STA, ISP5 and SPA were less suitable, particularly the HV medium contributing only 1 isolate. The results obtained in the present study were different compared with previous studies where the HV and RA media were suitable for the isolation of endophytic actinomycetes from other medicinal plants (Kaewkla and Franco, 2013; Li et al., 2012). The differences can result from the different isolation methodologies and media used.

**Fig. 3.** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of 38 endophytic actinomycetes showing the homology with closest type strain sequences.

*Fig. 3.* Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of 38 endophytic actinomycetes showing the homology with closest type strain sequences. *Bacillus thuringiensis* strain ATCC 10792 was used as an out-group. Numbers at branches indicate bootstrap values in 1000 replications. Only bootstrap values greater than 50% are shown in the tree. The bar represents the distance of 0.02 substitutions per nucleotide.
acquiring novel and/or rare strains (Kaewkla and Franco, 2013; Qin et al., 2009).

The antimicrobial activities were systematically evaluated for all of the isolated endophytic actinomycetes. Twenty-seven (71.1%) of 38 antibiotic-producing actinomycetes showed antimicrobial activity patterns against at least three microbes, suggesting that these endophytes could have broad-spectrum antimicrobial activities. Besides, previous studies have led to theories suggesting that potential anti-tumor substances synthesized by actinomycetes are largely due to anthracycline antibiotics (Abdelfattah, 2008; Igarashi et al., 2007; Lu et al., 2017). Here, 26 (68.4%) of antibiotic-producing actinomycetes exhibited the capability of producing anthracycline-like metabolites. Natural anthracycline antibiotics are reported to be produced by the polyketide biosynthesis pathway. We also found high proportions of actinomycetes harboring secondary metabolite biosynthetic genes pks (73.7%) and nrps (28.9%), compared with previous studies (Li et al., 2012; Salam et al., 2017; Zhao et al., 2011). In fact, the polyketide synthases and nonribosomal peptide synthetases consist of a highly-conserved modular enzymatic structure that is encoded by highly similar tandem repeat sequences, spanning 600–700 bp for pks-II, and 1200–1400 bp for both pks-I and nrps genes (Ayuso-Sacido and Genilloud, 2005; Metsä-Ketelä et al., 1999). In many cases, these repeated sequences cause some difficulty for amplification by available primer pairs. This may explain why the pks-I sequence was detected in only 19 of the 26 anthracycline-like metabolites producing isolates.

In agreement with global studies on actinomycetes from different plants, Streptomycices serves as the dominant genus that accounts for 90% of the total isolates from the C. cassia Presl (Qin et al., 2009). Nevertheless, other studies found less than 30% of endophytic actinomycetes are recovered from different medicinal plants belonging to Streptomyces genus (Li et al., 2012; Zhao et al., 2011). All these findings suggest that the genetic diversity of endophytic actinomycetes is strongly associated with specific plant species and thus, endophytes could have a different capacity to acquire important hereditary features from the plant hosts. In the present study, the Streptomycices genus was dominant, 25 distinct Streptomyces species were assigned, suggesting the high diversity among isolates from this genus. The phylogenetic tree exhibited multiple-phylogenetic branches supporting theories that the isolates have evolved from different ancestors. Importantly, the Streptomyces genus accounts for about 70% of the natural products in the pharmaceutical market (Bull and Stach, 2007).

Since Cinnamomum species are mainly distributed in India, China and South-East Asia countries, recent studies have focused on the study of isolation and antimicrobial activity of endophytes from the plant. For instance, a study from Malaysia found that an endophytic fungus Phoma sp., isolated from Cinnamomum mollissimum, exhibited antifungal activity and cytotoxicity against P388 cancer cells (Santiago et al., 2012). The compound 5-hydroxyxuramulin isolated from the Phoma fungus showed strong antifungal activity against Aspergillus niger (IC$_{50}$ of 1.56 µg/ml) and was cytotoxic against murine leukemia cells (IC$_{50}$ of 2.10 µg/ml). Another study has found a new endophytic actinomycete strain designated as Streptomyces rochei Ch1 from Cinnamomum sp. in Cherapunji rainforest, North-East India (Joy and Banerjee, 2015). Although the strain exhibited broad-spectrum antibacterial activity against eight pathogens of both Gram-positive and Gram-negative bacteria, nevertheless, bioactive compounds were not isolated yet. Similarly, a study from Philippines reported that an endophytic fungus Fusarium sp. 2 isolated from Cinnamomum mercedarii possessed antimicrobial activity against four different pathogenic bacteria (Marcellano et al., 2017). Recently, Vu et al. (2018) isolated and elucidated structures of 5 bioactive metabolites from endophytic Streptomyces cavourensis YBQ59 associated with Cinnamomum sp. in Yen Bai, Vietnam. The compounds revealed not only remarkably antimicrobial activity against MRSA, but also a strong cytotoxicity effect against human cancer cell lines (Vu et al., 2018).

In the last decade, many novel antibiotics have been isolated from endophytic actinomycetes, such as munumbicins which have been isolated from a culture broth of Streptomycices sp. NRRL 30562 (Castillo et al., 2002), alnumycin from Streptomycices sp. DSM 11575 (Bieber et al., 1998), spoxazonicins from Streptosporangium oxazolinicum K07-0460T (Inahashi et al., 2011), stenothricin and bagremycin from Nocardia caishijiensis SORS 64b (Tanvir et al., 2016). Interestingly, some of the novel antibiotics exhibited strong effects against multiple-drug resistant infectious pathogens (Castillo et al., 2002; Tanvir et al., 2016). In addition, novel anthracycline-like antibiotics have been isolated from endophytic actinomycetes (Abdelfattah, 2008; Igarashi et al., 2007; Lu et al., 2017). So far, clinical anti-tumor drugs produced by actinomycetes officially released to the market consisting of doxorubicin, aclarubicin, daunomycin and doxorubicin are antibiotics belonging to anthracyclines. The results from the present study highlight the potential for the isolation of novel and valuable secondary metabolites from the actinomycetes in C. cassia Presl.

In conclusion, the present study is the first report about the distribution and several bioactivities of endophytic actinomycetes associated with the medicinal plant C. cassia Presl, collected from Hoa Binh province, Vietnam. Many of these endophytes displayed broad-spectrum antimicrobial activities, which implies a potential for agricultural, pharmaceutical and medicinal applications. Further studies focus on the isolation and determination of chemical structures of bioactive compounds produced by potential actinomycete strains.

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Conflict of Interest

The authors declare no competing interest.

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