DISTRIBUTION AND ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM *Litsea cubeba* (Lour.) Pers. IN NORTHERN PROVINCES OF VIETNAM

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ABSTRACT

Endophytic actinomycetes from medicinal plants have been recently reported as the potential producers of antibiotics, anticancer agents and other bioactive compounds. In this study, the distribution and antimicrobial activity of endophytic actinomycetes isolated from *Litsea cubeba* (Lour.) Pers. were investigated. Consequently, the antimicrobial activities of endophytic isolates against eight pathogens, consisting of *Salmonella enterica* ATCC 14028, *Escherichia coli* ATCC 11105, *Sarcina lutea* CNLM, *Bacillus cereus* ATCC 11778, *Proteus vulgaris* CNLM, *Pseudomonas aeruginosa* CNLM, *Staphylococcus epidermidis* ATCC 12228, *Enterobacter aerogenes* ATCC 13048 were tested. Total 143 actinomycetes strains were isolated from *L. cubeba* tissues, in which 53, 23 and 67 strains were respectively obtained from plant samples collected in Thanh Hoa, Hanoi and Phu Tho provinces. Among media used for isolation, the highest number endophytes were from two main media including HV (18.2 %) and SA (16.1 %). Assessment of strains isolated from three kinds of plant tissues, most of endophytes were from roots (n = 65, 45.5 %) and followed by stems (n = 41, 28.7 %) and leaves (n = 37, 25.9 %). Examination of antimicrobial activity of endophytes revealed that 32.9 % of total strains showed inhibitory activity against at least one of the eight tested pathogenic microorganisms. The obtained results indicated that the *L. cubeba* (Lour.) Pers. could be an extremely rich reservoir for isolation of diverse actinobacteria producing antimicrobial activities and other bioactive compounds.

Keywords: antimicrobial activity, endophytic actinomycetes, *Litsea cubeba*, pathogenic strains.

1. INTRODUCTION

Day by day due to excessive use of antibiotics, the multidrug resistance capacity of pathogens is becoming more and more severe. The scientists all over the world are endeavouricing...
continuously to search new antibiotic compounds in order to tackle this problem. Endophytic microbes, especially actinobacteria appear as a source of novel and active compounds to combat the increasing number of multidrug resistant pathogens. Endophytic actinobacteria produce a number of compounds to maintain symbiotic relationships with host plants, promoting host growth and helping them to survive in host plants. In recent years, endophytic actinobacteria have attracted attention with increasing reports of isolates from a range of plant species, including crop plants as citrus, carrots, potato, tomato, rice and various medicinal plants. In 2009, Qin et al. [1] isolated 2174 strains from medicinal plants collected from tropical rainforest. Among 46 selected isolates, 24 isolates showed antibacterial activity. In 2011, 560 endophytic actinomycetes were isolated from 26 Chinese medicinal plants. Out of 60 isolated endophytic actinomycetes, 59 strains showed the antimicrobial against at least one pathogenic bacteria, Zhao et al. [2]. From the seven Indian medicinal plants, 42 endophytic actinomycetes were obtained and all of isolates displayed moderate inhibitory activities against Staphylococcus aureus and E. coli, Passari et al., 2015 [3]; Saini et al., 2016 [4] isolated 50 endophytic actinomycetes from Syzygium cumini grown in Ludhiana, India, in which 30 % isolates showed antibacterial activities.

Litsea cubeba (Lour.) Pers. so called aromatic Litsea or May Chang, is a traditional herb cultivated in many Asia countries including Vietnam. Citral in the L. cubeba’s essential oil has been well-known as a predominant antibacterial, anticancer and antioxidation compound [5]. Although L. cubeba has been frequently used in our life, the number of researches on the antibacterial activities of L. cubeba has been limited. Therefore, in this study, endophytic actinomycetes were isolated from L. cubeba collected in Thanh Hoa, Hanoi and Phu Tho provinces, Vietnam and the distribution of endophytic bacteria in various parts of plant and their antibacterial activities have been investigated.

2. MATERIALS AND METHODS

2.1. Pathogenic strains

Eight pathogenic strains S. enterica ATCC 14028, E. coli ATCC 11105, S. lutea CNLM, B. cereus ATCC 11778, P. vulgaris CNLM, P. aeruginosa CNLM, S. epidermidis ATCC 12228, E. aerogenes ATCC 1304 were obtained from Institute of Biotechnology, Vietnam Academy of Science and Technology.

2.2. Methods

2.2.1. Sample collection and preparation

Roots, stems, and leaves of L. cubeba were collected from Quang Trung, Ngoc Lac, Thanh Hoa province (20°13’34”N; 105°39’50”E); Ham Lon, Soc Son, Hanoi (21°30’39”N; 105°79’92”E) and Cap Dan, Cam Khe, Phu Tho province, Vietnam (21°24’25”N; 105°04’05”E). The samples were collected and preserved according to the method of Passari et al., 2015 [3]. The tissue segments were surface-sterilized according to the method of Li et al., 2012 [6].

2.2.2. Isolation of endophytic actinomycetes from L. cubeba

Endophytic actinomycetes strains were isolated by using eight isolation media, including CA, HV, STA, TA, SA, SPA [1], TWYE [7], ISP5 [8], according to the method of Li et al.,
Colonies were separated and transferred on YIM38 agar plates and the morphology and mycelium colour were observed after cultivating for two weeks on ISP2 agar plate [9].

2.2.3. Assay of antibacterial activity

The actinobacterial isolates were cultured in YIM38 broth medium for 7 days at 30°C, 200 rpm. After the culture processes, each individual culture broths were centrifuged at 7,000xg for 10 min and then the supernatant was collected and used for antimicrobial screening. Antimicrobial activities of isolates were investigated using the well diffusion agar method described by Hadacek et al., 2000 [10].

3. RESULTS AND DISCUSSION

3.1. Isolation and distribution of endophytic actinomycetes from L. cubeba

Total 143 isolates of endophytic actinomycetes were obtained from roots, stems and leaves of L. cubeba collected from three provinces, Thanh Hoa, Hanoi and Phu Tho with 53 and 67 strains, respectively. As showed in Figure 1, a wide variety of colony morphologies and mycelium colours was observed in all endophytic actinomycetes.

![Figure 1. Colony morphologies of endophytic actinomycetes on SPA (a) and HV (b); ISP2 medium (c,d).](image)

Numerous studies indicated that species distribution and biological diversity of endophytic actinobacteria of medicinal plants are extensively influenced by isolation methods, plant species and sampling geographical area [11]. Gangwar et al., 2014 [12] successful isolated 40 actinomycetes strains from roots, stems, and leaves of Aloe vera, Mentha sp. and Ocimum sanctum. In 2012, Li et al. isolated 228 endophytic strains from 4 Artemisia annua L. grown in Xishuangbanna, Yunnan province, South-West China [6]. The strain number obtained from L. cubeba was relatively high in this study.

3.1.1. Distribution of endophytic actinomycetes on different parts of L. cubeba

A total of 143 strains were found from 3 main parts of L. cubeba. The highest number of isolated strains obtained from roots (n = 65, 45.5 %), followed by stems (n = 41, 28.7 %) and leaves (n = 37, 25.9 %) (Figure 2a). This result was similar to recent studies showing that the number of actinomycetes isolated from roots was regularly higher than that from stems and leaves [11, 12, 13]. Studies on diversity of actinobacteria residing in medicinal plants based on morphology and amino acid composition of the whole-cell extract the percentage of endophytic actinobacteria recovered from different explants, Taechowisan et al., 2003 [14] found 64.2 %
isolates from roots, 29.4 % from leaves, and 6.4 % from stems of 36 different plant species. Verma et al., 2009 [13] reported that among 55 endophytic actinomycetes isolated from 150 Azadirachta indica A. Juss samples in Varanasi (North of India), 54.5 % isolates was found in roots whereas 23.6 % and 21.8 % strains were from stems and leaves, respectively. When isolating endophytic actinomycetes from 36 healthy plants of Aloe vera, Mentha sp. and Ocimum sanctum, Gangwar et al., 2014 [12] found that the maximum endophytic actinobacteria recovered from roots (70 %), followed by stems (17.5 %) and least in leaves (12.5 %). The endophytic actinobacteria are populations derived from the rhizosphere soil bacteria. They tend to grow near water and nutriments transport vessels in plant (roots and stems) which facilitate their nutrients absorbability and metabolism [11]. In addition, high essential oil content in leaves could be negative effect on microorganism life, for example essential oil in L. cubeba accounts for about 1.2 - 2.4 % of total weight [5, 15]. These can explain the higher number of microorganism in root than other parts of plants.

Figure 2. Distribution of endophytic actinomycetes on L. cubeba tissues (a) and on different isolation media (b).

3.1.2. Distribution of endophytic actinomycetes on isolation media

The highest number of isolates was on HV, SA media, which reached 18.2 % and 16.1 % of the total number (n = 143), respectively. This proportion varied in the difference of sampling location. The isolate number was the highest (23.9 %) on HV medium whereas on other media showed no significant difference, ranging from 9.0 to 14.9 % of total number (n = 67) in the L. cubeba obtained from Phu Tho. Similarly, from the sample collected from Thanh Hoa province, the obtained isolates number was highest on SA (18.9 %), and lowest in CA (7.5 %), for the remaining media, the strains number ranged from 11.3 to 15.1 % (n = 53). From the samples collected in Hanoi, there was a significant difference in the number of isolates among different media, the highest was found on TWYE (26.1 %) and the lowest on CA and ISP5 (4.3 %) (n = 23) (Figure 2b).

This result revealed the number strains obtained depend on different isolation media used and sampling location. According to Shirling and Gottlieb (1966) [8], ISP media were the best ones for the growth of actinomycetes, however in this study, isolates obtained from ISP5 occupied only 7 % of the total, lower than other media. This was apparently reasonable when comparing to other recent studies, in which components of media obviously played an important role in isolates’ numbers and detecting new species [16]. Using media with minimal nutrients was not efficient to recover new species. Many studies found that Streptomyces predominated over other actinomycetes species (> 50 % of total), therefore, researches changing the media...
components to obtain new species have been more popular [1, 6]. For example, when using 11 different isolation media, including HV, ISP5 and TWYE, to seek for endophytic actinomycetes from Chinese medicinal plants, Qin et al., 2009 [1] demonstrated that most of isolates grew on basic media such as HV and TWYE. The remains media were not appropriate because its nutrients concentration were too rich which facilitated the growth of bacteria. Coombs and Franco (2003) [7] also agreed that the minimal nutrient media such as HV, TWYE, and YECD (casein hydrolysate yeast extract agar) are most effective for isolating endophytic actinomycetes from wheat (Triticum aestivum L.).

3.1.3. Evaluation of distribution of endophytic actinomycetes by mycelium colour

It was assumed that mycelium colour was the basic criterium to screen endophytic actinomycetes [17]. Fifty-three isolates from Thanh Hoa province clearly divided in three colour groups (out of seven mycelium colour groups), of which 47.2 % was yellow, 45.3 % grey and 7.5 % white. Sixty-seven strains from Phu Tho province presented in four mycelium colour groups including yellow (40.3 %), white (29.8 %), grey (20.9 %) and green (9.0 %). Twenty-three strains from Hanoi, were divided into mycelium five colour groups. They were grey, yellow, white, red, and green with 60.9 %, 17.4 %, 8.7 %, 8.7 % and 4.3% respectively (Figure 3). Gayathri et al., 2013 [18] found that isolates from plants grown in mangroves area processed grey (66.7 %) and white mycelium (33.3 %). Phuakjaiphaeo et al., 2015 [19] also reported there were three kinds of mycelium colour: white (50.0 %), yellow (41.7 %) and grey (8.3 %) from 36 isolates collected from Centella asiatica (L.) Urban. However, the mycelium colour varies depending on medium components, inoculum time and cultivation conditions. The diversity of mycelium colour and isolate number in different parts of L. cubeba. when using different culture media in this study indicated that there was a wide range of endophytic actinomycetes growing in L. cubeba tissues.

![Figure 3. Mycelium colour of endophytic actinomycetes obtained from Thanh Hoa, Hanoi and Phu Tho.](image)

3.2. Screening for antibacterial activity

In recent years, many of novel antibiotics from endophytic actinobacteria of medicinal plants found to be active against pathogenic bacteria. Similar results were obtained from this study. Table 1 shows a large variety of antibiotic resistance of endophytic isolates. Out of 143 strains of isolated endophytic actinobacteria 47 strains (32.9 %) were able to suppress least one of the eight tested pathogenic strains. These strains mostly isolated from Phu Tho (n = 35, 74.5 %), followed by Thanh Hoa (n = 10, 21.3 %) and Hanoi (n = 2, 4.2 %). The number of isolates displayed the highest antimicrobial activities against S. epidermidis ATCC 12228 (n = 34, 23.8 %); followed by against B. cereus ATCC 11778 (n = 23, 16.1 %), P. auroginosa CNLM
(n = 21, 14.7 %); and P. vulgaris CNLM (n = 19, 13.3 %), respectively. Only two strains showed the antimicrobial activities against E. coli ATCC 11105 (1.4 %).

According to Zhao et al., 2011 [20], when testing antimicrobial activity of 60 strains out of 560 isolates from 26 medicinal plants in Panxi, China, 59 isolates killed at least one pathogenic strain. In 2008, Li et al. [21] reported that out of 41 Streptomyces sp. strains isolated from Chinese medicinal plants, 65.9 % were resistant to E. coli, 24.4 % to S. aureus, 31.7 % to S. epidermidis resistibility, 12.2 % to Candida albicans and none showed resistance against Klebsiella pneumoniae. In other study, Li et al., 2009 [9] found that of 228 isolates from Artemisia annua, 31 strains had a wide range of resistance to pathogenic bacteria except E. coli YIM 1011; 19 strains inhibited the growth of grass. In Vietnam, there are very few studies on the endophytic actinomycetes and their antibacterial activity. Therefore the results of this study suggested that L. cubeba is a potential medicinal source of endophytic actinomycetes which can be exploited in many aspects of pharmaceutical application.

**Table1.** Antimicrobial activities of endophytic actinomycetes from L. cubeba against pathogenic strains.

<table>
<thead>
<tr>
<th>Pathogenic strains</th>
<th>Antagonistic activity of actinomycetes against different pathogenic bacteria</th>
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<tbody>
<tr>
<td></td>
<td>Number of strains</td>
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<tr>
<td>Gram (-)</td>
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<tr>
<td><em>Escherichia coli</em> ATCC 11105</td>
<td>2</td>
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<tr>
<td><em>Proteus vulgaris</em> CNLM</td>
<td>19</td>
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<tr>
<td><em>Salmonella enterica</em> ATCC 14028</td>
<td>9</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em> CNLM</td>
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<td><em>Enterobacter aerogenes</em> ATCC 13048</td>
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<tr>
<td>Gram (+)</td>
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<tr>
<td><em>Sarcina lutea</em> CNLM</td>
<td>3</td>
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<tr>
<td><em>Staphylococcus epidermidis</em> ATCC 12228</td>
<td>34</td>
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<tr>
<td><em>Bacillus cereus</em> ATCC 11778</td>
<td>23</td>
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</table>

**4. CONCLUSION**

The diversity and antimicrobial activity of endophytic actinomycetes isolated from L. cubeba (Lour.) Pers. grown in Thanh Hoa, Hanoi and Phu Tho provinces of Vietnam were investigated. Of 143 isolates, 47 strains (32.9 %) show inhibitory activities against at least one of eight tested pathogenic strains. Endophytic actinomycetes from L. cubeba (Lour.) Pers. are a potential source of antimicrobial agents and new bioactive compounds. Further studies in identifying strains and functional genes associated to antibiotic production are consequently conducting.

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TÔM TÁT

PHÂN BỐ VÀ KHẢ NĂNG KHÁNH KHUẨN CỦA XÀ KHUẨN NỘI SINH TRÊN CÁY MANG TANG (Litsea cubeba (Lour.) Pers.) PHÂN LẬP TẠI MIỀN BẮC VIỆT NAM

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Xà khuẩn nội sinh trong các cây được liệu được đánh giá là một trong những nguồn vi sinh vật có tiềm năng tổng hợp chất kháng khuẩn và kháng ung thư cao. Trong nghiên cứu này, chúng tôi tiến hành phân lập và đánh giá sự phân bố của xà khuẩn nội sinh trên cây Màng Tang (Litsea cubeba (Lour.) Pers.) trên 8 môi trường khác nhau. Khả năng kháng 8 chúng gây bệnh gồm Salmonella enterica ATCC 14028, Escherichia coli ATCC 1105, Sarcina lutea CNLM, Bacillus cereus ATCC 11778, Proteus vulgaris CNLM, Pseudomonas aeruginosa CNLM, Staphylococcus epidermidis ATCC 12228, Enterobacter aerogenes ATCC 13048 của các chúng xà khuẩn phân lập được khảo sát. Trong số 143 chúng xà khuẩn nội sinh được phân lập từ cây Màng Tang, 53 chúng phân lập được từ Thanh Hóa, 23 chúng từ Hà Nội, 67 chúng từ Phú Thọ. Ti lệ xà khuẩn phân lập cao nhất trên các môi trường HV, SA lần lượt là 18,2 % và 16,1 % trên
tổng số chúng xã khuân. Xạ khuân được phân bố nhiều nhất trong rõ với 45,5 % trên tổng số xã khuân phân lặp, trong khi độ trong thân và lá số lượng xã khuân không khác nhau nhiều, lần lượt đạt 28,7 % và 25,9 %. Khảo sát khả năng kháng khuân cho thấy: 47 chúng (32,9 %) kháng ít nhất một chúng vi sinh vật kiểm định, trong đó chiếm đa số là các chúng từ Phú Thọ (74,5 %), tiếp đến là các chúng đến từ Thanh Hóa (21,3 %) và Hà Nội (4,2 %). Nghiên cứu cho thấy sự phong phú của xã khuân nội sinh trong cây Màng Tang và khả năng kháng khuân của chúng rất đa dạng, có tiềm năng trong việc sàng lọc các hợp chất có hoạt tính sinh học mới.

Từ khóa: Màng Tang, Litsea cubeba, hoạt tính kháng khuân, vi sinh vật gây bệnh, xã khuân nội sinh.