



Analysis of endophytic fungi in roots of *Santalum album* Linn. and its host plant *Kuhnia rosmarinifolia* Vent.*

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Abstract: *Santalum album* Linn. is an evergreen and hemi-parasitic tree, the heartwood-sandalwood of which was used during a long history in traditional Chinese medicine. *Kuhnia rosmarinifolia* Vent. is a good host for 1- or 2-year-old growing *S. album*. The interaction between *S. album* and *K. rosmarinifolia* is still little known. Many studies have been carried out on a number of plants for identification and diversity of endophytes. In this study, in total 25 taxa of endophytic fungi were isolated from the roots of *S. album* and the roots of *K. rosmarinifolia*. The most frequently isolated genera were *Penicillium* sp. 1 and *Fusarium* sp. 1 in the roots of *S. album* and *K. rosmarinifolia*, respectively. *S. album* is a root parasite of *K. rosmarinifolia*. The interesting result is that they apparently do not share the same endophytic fungi isolates. This study for the first time explored the content of endophytic fungi from *S. album* and *K. rosmarinifolia*, which provides important information for further studies.

Key words: Endophytic fungi, *Santalum album*, *Kuhnia rosmarinifolia*, *Penicillium* sp., *Fusarium* sp.

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1 Introduction

Endophytic fungi are defined as the endosymbionts that live within the plant intercellular and intracellular spaces for at least part of their life cycle without causing apparent harm to their host (Wilson, 1995). Endophytic fungi have been reported to be associated with plants for over 400 million years (Krings *et al.*, 2007), and there are about one million species existing ubiquitously in plants (Shekhawat *et al.*, 2010). Endophytic fungi play important physiological (Malinowski *et al.*, 2004) and ecological (Malinowski and Belesky, 2006; Tintjer and Ruderger, 2006) roles in plant symbiosis, which protect their hosts from infectious agents and stressful environment by secreting bioactive secondary metabolites

(Carroll and Carroll, 1978; Azevedo *et al.*, 2000; Redman *et al.*, 2002; Strobel, 2003; Rodriguez *et al.*, 2004; Márquez *et al.*, 2007). Therefore, the study of endophytic fungus has become one of the popular topics in mycology.

Medicinal plants were used for many years in traditional Chinese medicine. Since the population is increasing, it is not possible to afford plant-based medicine because of the exhaustion of some of the plant resources. Medicinal plants are known for storing endophytic fungi, which are important sources of various secondary metabolites and bioactive compounds valuable for the pharmaceutical industry (Krishnamurthy *et al.*, 2008; Khan *et al.*, 2010). Therefore, it is necessary to explore endophytic fungi in medicinal plants for developing some alternative medicines. *Santalum album* Linn. is an evergreen and hemi-parasitic tree belonging to the Santalaceae family, which is a well known plant distributed in India, Australia, New Zealand, South America, Indonesia, and other countries. It was introduced to

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China in the 1960s and widely cultivated in most tropical and subtropical areas in the 1980s. *S. album*, called “Tanxiang” in Chinese, was recorded in *China Pharmacopoeia* (State Pharmacopoeia Commission of China, 2010) and its heartwood has been found to be particularly effective in activating “Qi” and warming the middle “Jiao”. It can also stimulate the appetite and eliminate pain. In addition, volatile oil extracted from *S. album* roots and heartwood has antiviral activity and antifungal activity (Viollon and Chaumont, 1994; Benencia and Courrèges, 1999; Sindhu et al., 2010).

Xu et al. (2011) reported that *Kuhnia rosmarinifolia* Vent. from a seedling pot can be a good host for 1- or 2-year-old *S. album*. In the wild fields, we observed that 3- or 4-year-old *S. album* was near to *K. rosmarinifolia*, which grew up strongly. We also found that the leaf of *S. album* was yellow without any *K. rosmarinifolia* around, and *S. album* did not develop well. Previous studies investigated endogenous hormone levels and anatomical characters of haustoria in *S. album* seedlings before and after its attachment to *K. rosmarinifolia* (Zhang et al., 2012). There is not enough information on endophytic fungi from *S. album* and its host plants. The endophytic fungi assemblages of parasite relationships have been reported in *Cuscuta reflexa* and its seven angiosperm hosts that showed no overlap between *Cuscuta reflexa* and *Cucurbita maxima* (Suryanarayanan et al., 2000). While *S. album* is a hemi-parasitic plant, it can support nutrients by its own photosynthesis, but *Cuscuta reflexa* cannot.

The interactions between *S. album* and *K. rosmarinifolia* are interesting, but still little is known about how *S. album* and *K. rosmarinifolia* are connected with haustoria tissues, which would provide a conduit for diversion of the water and nutrients from the host to the parasite. However, the role of endophytic fungi in the root of the two plants is still unclear. In this study, we examined the hypothesis that the roots of *S. album* and its host *K. rosmarinifolia* are connected with each other by haustorium and consequently the same endophytes would be presented in both roots. We isolated and identified the endophytic fungi in the roots of *S. album* and *K. rosmarinifolia* for diversity determination and to explore the relationship between the endophytic fungi and *S. album* and *K. rosmarinifolia* in roots. To achieve spatial

configuration of endophytic fungi, an anatomical study was undertaken on the two plant roots.

2 Materials and methods

2.1 Source of plant samples

The healthy roots of *S. album* and its host of *K. rosmarinifolia* were collected from Guangdong Province (latitude 23°19'3.65" N and longitude 115°59'27.99" E), China. The plants were identified by Prof. Dr. Shun-xing GUO, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, China. We studied three *S. album*s and three *K. rosmarinifolia*s where each *S. album* was apart by 10–15 m. Five roots with haustoria issues of each *S. album* and *K. rosmarinifolia* were collected. The plant materials were brought to the laboratory in sterile polyethylene bags and stored temporarily in a refrigerator. The endophytic fungi were isolated within 24 h.

2.2 Isolation of endophytic fungi

To eliminate the epiphytic microorganisms, the roots were subjected to a surface-sterilized procedure. Each part of the samples was thoroughly washed under running tap water. Then the samples were sterilized by submerging the part in 75% ethanol for 1 min, followed by immersion in 3% NaClO for 8 min, then repeated in 75% ethanol for 30 s and rinsed three times in sterile distilled water, and the pieces were blot-dried on sterile blotting paper. The roots were cut into 5 mm-length segments. About 4–5 segments were placed into a petri dish (diameter 90 mm) with potato dextrose agar (PDA) medium containing 50 mg/L oxytetracycline and 50 mg/L penicillin (Otero et al., 2002). Samples were incubated at 25 °C in dark conditions (Cui et al., 2010). The fungi were observed every 2 d for at least 3 weeks.

The fungi that grew from the segments were periodically isolated and identified using morphological characteristics by transferring the hyphal tips to the fresh PDA plates without antibiotics. The pure endophytic fungal strains were photographed and preserved in the Laboratory of Mycology, Biotechnology Center, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

The colonization frequency (CF) of an endophyte species in the root tissue was calculated by

$$CF=(N_{col}/N_t)\times 100\%,$$

where N_{col} is the number of segments colonized by each endophyte and N_t is the total number of segments studied (Hata and Futai, 1995).

The contribution to an endophyte assemblage by the dominant endophyte (DE) was calculated as the CF of the DE divided by the sum of CF of all the endophytes in an assemblage ($\times 100\%$). To compare the endophyte assemblage of the host and its hemiparasite combination, a similarity coefficient was calculated as (Carroll and Carroll, 1978)

$$\text{Similarity coefficient}=[2w/(a+b)]\times 100\%,$$

where a is the sum of CF for all fungal species on the host, b is the similar sum for its hemiparasite, and w is the sum of lower colonization frequencies for fungal species in common between the host and the hemiparasite.

2.3 Measurements and analysis

The fresh and clean samples of two plants were obtained from the representative roots with blades and cut into segments (0.5–1.0 cm in length). The root segments were fixed in formalin, dehydrated alcohol, and glacial acetic acid (FAA) fixative for at least 72 h, dehydrated in ethanol at gradient concentrations from low to high, embedded using paraffin, sliced by paraffin sections with a thickness of 10 μm , dyed in safranin-fast green, and then observed under a microscope (ZEISS, AxioImager AI, Germany).

2.4 DNA extraction and polymerase chain reaction (PCR) amplification

Endophytic fungi failing to sporulate were identified using molecular biological analysis of the internal transcribed spacer (ITS) region of rDNA. Molecular methods are useful for evaluating microbial communities' structures and functions. DNA analyses have been applied in different fields for whole communities, bacterial, fungal, and so on. Therefore, a high quality and quantity of DNA is essential. After cultivation for 1 or 3 weeks at 25 °C in the dark, all pure strains were selected for DNA extraction, am-

plification, and sequencing. Primers ITS1 and ITS4 constructed for molecular phylogenetic studies (White *et al.*, 1990) were used to amplify the ribosomal ITS. The partial nucleotide base-pair fragment of the ITS ribosomal DNA (rDNA) gene from the isolated endophytic fungus was amplified using PCR with universal ITS primers ITS1 and ITS4. PCR was carried out as follows: the reaction mixture in a total volume of 25 μl containing 12.5 μl Mix (10 \times buffer (with Mg^{2+}) and 10 $\mu\text{mol/L}$ dNTPs), 9.5 μl double distilled water, 1 μl (10 $\mu\text{mol/L}$) each primer, and 1 μl genomic DNA.

The PCR was done in a thermal cycler with the following conditions: 3 min initial denaturation at 94 °C, 32 cycles of 30 s denaturation 94 °C, 25 s annealing at 55 °C and 30 s elongation at 72 °C, and 7 min final elongation at 72 °C. Single-band PCR products were purified using Watson's PCR purification kit (Watson, China). Sequencing was performed with Big Dye Terminator sequencing kit and an ABI 3730 automated sequencer (Applied Biosystems, USA). All the sequences obtained in this study were submitted to GenBank and the accession numbers of the sequences are JX657338–JX657350 and JX573315–JX573326.

2.5 Sequence analysis

BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>) (Altschul *et al.*, 1997), using ITS1-5.8S rDNA-ITS2 as query sequences, were conducted on all the sequences to check their closest known relatives. The isolates were arranged as the closest to a certain genus and, when identified in a database, the matches were about 95%. However when the similarity was less than 95%, the strain was considered unidentified (Sánchez Márquez *et al.*, 2008).

3 Results and discussion

A total of 68 fungal isolates were obtained from 160 segments (roots) of the two plants, of which 34 from 80 root segments of *S. album* were classified into 13 species, and 24 from 80 root segments of *K. rosmarinifolia* were grouped into 12 species. The dominant endophytic fungi in each host plant were quite different. *Penicillium* sp. 1 was the dominant fungal species in the roots of *S. album* and *Fusarium*

sp. 1 was the dominant species in the roots of *K. rosmarinifolia* (Table 1).

The CF was used for diversity analysis. In our study, all the cultured strains were selected for sequence by the ITS region of nuclear rDNA. Twenty-five fungal species in the roots of *S. album* and *K. rosmarinifolia* were isolated, and 24 species belonged to Ascomycota. One fungal species failing to sporulate was designated as sterile fungus, 22 endophytic fungal species of Ascomycota were classified into four classes: Sordariomycetes (11), Eurotiomycetes (6), Dothideomycetes (4), and Leotiomycetes (1). Four endophytic fungal species belonged to the genus of *Penicillium*, five endophytic fungal species belonged to the genus of *Fusarium*. Only one species

belonged to the group of sterile fungi and thus cannot be identified on the basis of morphology and its sequence reveals 89% identity with *Lophiostoma cynaroidis* in BLAST (Table 2).

A total of 13 fungal species isolates were isolated from the root of *S. album* plants, while *K. rosmarinifolia* plants had 12 fungi species as endophytes. The overall CF was determined as 42.5% on the surface sterilized tissues from *S. album* plants, while overall CF was determined as 30% on the surface sterilized tissues from *K. rosmarinifolia* plants (Table 1). The most frequently isolated genus was *Penicillium* sp. 1 in the roots of *S. album* and *Fusarium* sp. 1 in the roots of *K. rosmarinifolia* (Table 2).

Table 1 Endophyte distribution and percentage of dominant endophyte (DE) in the root of each host species

Host plant	No. of segments examined	No. of total isolates	No. of endophytes species	Total colonization frequency (%)	Dominant endophyte	DE (%)
<i>Santalum album</i>	80	34	13	42.5	<i>Penicillium</i> sp. 1	17.6
<i>Kuhnia rosmarinifolia</i>	80	24	12	30.0	<i>Fusarium</i> sp. 1	16.7

Table 2 Colonization frequencies of endophytes in the roots of *Santalum album* and *Kuhnia rosmarinifolia*

Fungal strain	Morphological identification	GenBank accession No.	Colonization frequency (%)	
			<i>Santalum album</i>	<i>Kuhnia rosmarinifolia</i>
12144	<i>Chaetomium</i> sp. 1	JX657342	2.50	
12149	<i>Chaetomium</i> sp. 2	JX657346	3.75	
12178	<i>Colletotrichum gloeosporioides</i>	JX573323		2.50
12171	<i>Colletotrichum</i> sp.	JX573319		2.50
12168	<i>Fusarium</i> sp. 1	JX573316		5.00
12170	<i>Fusarium</i> sp. 2	JX573318		2.50
12172	<i>Fusarium</i> sp. 3	JX573320		1.25
12173	<i>Fusarium</i> sp. 4	JX573321		2.50
12213	<i>Fusarium</i> sp. 5	JX573326		1.25
12142	<i>Phomopsis</i> sp. 1	JX657340	3.75	
12167	<i>Phomopsis</i> sp. 2	JX573315		3.75
12212	<i>Peyronellaea</i> sp.	JX573325		2.50
12169	<i>Phoma</i> sp. 1	JX573317		2.50
12211	<i>Phoma</i> sp. 2	JX573324		1.25
12174	<i>Phyllosticta</i> sp.	JX573322		2.50
12147	<i>Aspergillus aculeatus</i>	JX657344	2.50	
12148	<i>Aspergillus</i> sp.	JX657345	2.50	
12139	<i>Penicillium</i> sp. 1	JX657338	7.50	
12140	<i>Penicillium</i> sp. 2	JX657339	2.50	
12143	<i>Penicillium</i> sp. 3	JX657341	6.25	
12145	<i>Penicillium</i> sp. 4	JX657343	3.75	
12216	<i>Myxotrichaceae</i> sp.	JX657347	2.50	
12246	<i>Ascomycota</i> sp. 1	JX657349	2.50	
12247	<i>Ascomycota</i> sp. 2	JX657350	1.25	
12241	Sterile fungi	JX657348	1.25	

The roots of *S. album* and *K. rosmarinifolia* are connected with haustorium (Fig. 1). Fungal colonization was observed both intercellularly and intracellularly and was only involved in the cortical cells of the roots of *S. album* and *K. rosmarinifolia*. In this region, a widespread occupation by intercellular and intracellular hyphae was observed (Fig. 2). The hyphae in the cortex frequently grew into adjacent cortical cells rather than into the cylinder of vascular tissues of two plant roots and occasionally a peloton were observed. We had expected a high similarity in the endophytic fungi in the two roots, but the results were the reverse. We proposed that sandalwood oil in the roots of *S. album* may be a defense against the endophytic fungi in the roots of *K. rosmarinifolia* because of haustoria tissues. In our work, we got the result that there are no similar endophytes when comparing *S. album* with *K. rosmarinifolia* (Table 2). A similar study reported by Suryanarayanan *et al.* (2000) showed that the endophyte assemblages of *Cucurbita maxima* and its *Cuscuta reflexa* parasite overlapped by 0%. These results strongly suggest the existence of some degree of host specificity among endophytic fungi. Zhang *et al.* (2012) firstly reported a relationship between endogenous hormone profiles and structural characters of haustoria before and after attachment to the host *K. rosmarinifolia* in *S. album*. The study also found that many lysosomes occurred in the cells of haustorium. Lysosome contains digestive enzymes which are used to digest invasive microorganisms and break down unwanted or damaged cell organelles, which inhibited endophytes from growing and surviving in haustorium. PDA is the most common medium used for separation of cultivable endophytic fungi. In addition, all endophytic fungi were not separated in the present study because we were only concerned with the cultivable endophytic fungi, which may show a bias in the results. In short, the hormones might act as a web-like set of interactions to regulate the haustorial development of *S. album* and water and nutrient transport in the parasite-host association. However, the endophytic fungi might not act like this.

4 Conclusions

This paper explores the haustorium development and the mechanism of parasitism in the sandalwood

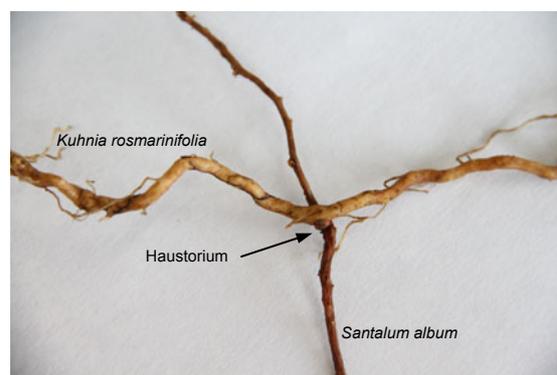
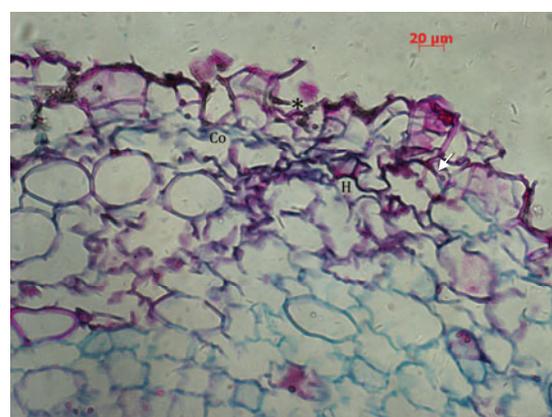
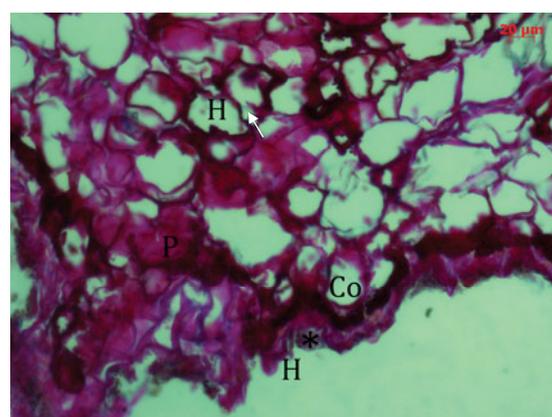


Fig. 1 Haustorium links roots of *Santalum album* and *Kuhnia rosmarinifolia*



(a)



(b)

Fig. 2 Distribution of endophytic fungi in the roots of *Kuhnia rosmarinifolia* (a) and *Santalum album* (b)

Examples of transverse sections of *K. rosmarinifolia* (a) and *S. album* (b) roots. Cortex of the roots of *K. rosmarinifolia* (a) and *S. album* (b), showing the infected place (*) and the infected cell in cortex. Arrow represents the hyphal coils formed by hyphae infected into the cortex cell from one to another, and peloton formed by the fungi colonization in cortex cells. Co: cortex; H: hyphae; P: peloton

tree. We reported for the first time that there were different endophytes between *S. album* and *K. rosmarinifolia* in roots. Moreover, endophytic fungi were isolated firstly from *S. album* and *K. rosmarinifolia*. Endophytic fungi in the roots of *S. album* and *K. rosmarinifolia* are not the same and are only involved the cortical cells of the roots of *S. album* and *K. rosmarinifolia*, which provides new information for further studies.

Compliance with ethics guidelines

Si-sheng SUN, Xiao-mei CHEN, and Shun-xing GUO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要:

本文题目: 檀香及其宿主植物假蒿根中内生真菌的研究

Analysis of endophytic fungi in roots of *Santalum album* Linn. and its host plant *Kuhnia rosmarinifolia* Vent.

研究目的: 檀香 (*Santalum album* Linn.) 为檀香科 (Santalaceae) 檀香属 (*Santalum*) 树种, 属于常绿半寄生小乔木, 其心材为传统名贵中药。檀香与其宿主植物假蒿通过根端的吸盘相连, 探讨檀香与其宿主之间内生真菌的关系具有重要意义。

创新要点: 假蒿 (*Kuhnia rosmarinifolia* Vent.) 是幼龄檀香很好的寄主。目前已经对许多药用植物的内生真菌的分离和鉴定进行了大量研究, 但是关于檀香和假蒿之间关系的研究报道较少。本研究首次探讨了檀香及其宿主植物假蒿根中内生真菌的数量和种类, 为进一步研究两者之间的关系提供一些有用的信息。

研究方法: 采用组织块分离法从檀香的根和其宿主植物假蒿的根部材料分离内生真菌, 并采用形态学鉴定和分子鉴定手段对分离的内生真菌进行鉴定。

重要结论: 从檀香的根和其宿主植物假蒿的根中 160 个组织块中分离和鉴定了共 25 个真菌类群, 其中青霉属真菌 *Penicillium* sp. 1 为檀香根中优势菌, 镰孢属真菌 *Fusarium* sp. 1 为假蒿根中优势菌。特别有意思的是两种植物根中没有分离到明显相同的内生真菌。

关键词组: 檀香; 假蒿; 内生真菌; 青霉属真菌 (*Penicillium* sp.); 镰孢属真菌 (*Fusarium* sp.)