

## Post-print: Variation in Endophytic Fungal Community Composition Between Two Cultivars of *Pogostemon cablin*

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### Abstract

To explore the influence of endophytic fungi-host interactions on the formation mechanisms of active components in *Pogostemon cablin*, two varieties with substantial compositional differences, Paixiang and Zhanxiang, were selected as research subjects. Strains obtained were classified using traditional morphological methods, and fungal universal primers ITS1/ITS4 were employed to amplify rDNA-ITS sequences for taxonomic identification and diversity analysis. The results demonstrated: (1) Endophytic fungi were isolated from stem and leaf tissue blocks of *Pogostemon cablin* at the seedling, branching, and mature stages using PDA and LBA media, yielding a total of 3,070 strains. Specifically, 1,624 strains were isolated from Paixiang, with 1,319 identified strains distributed across 36 genera; 1,446 strains were isolated from Zhanxiang, with 994 identified strains distributed across 33 genera. Paixiang yielded seven species-specific endophytic fungi, namely *Epichloe typhina*, *Colletotrichum gloeosporioides*, *Botryosphaeria* sp., *Rhizoctonia* sp., and *Truncatella* sp., with *Phytophthora* sp. and *Sclerophthora* sp. isolated for the first time, both belonging to Oomycota endophytes. Zhanxiang yielded two species-specific endophytic fungi, *Paecilomyces* sp. and *Cercospora* sp. (2) The dominant endophytic fungi were identical in both Paixiang and Zhanxiang, namely *Alternaria* sp. and *Colletotrichum* sp., with relative isolation frequencies of 9.48% and 7.81% in Paixiang, and 10.16% and 8.65% in Zhanxiang, respectively. (3) From the seedling stage to the mature stage, endophytic fungal colonization rates in both Paixiang and Zhanxiang gradually increased, following the sequence: Paixiang August (97.78%) > July (72.50%) > May (55.28%); Zhanxiang August (91.11%) > July (63.06%) > May (46.67%). The average colonization rate was 75.19% for Paixiang and 66.95% for Zhanxiang. (4) With the extension of the growth period, the diversity of endophytic fungi in both Paixiang and Zhanxiang exhibited an increasing trend, while the average similarity coefficient of endophytic fungi between the two *Pogostemon cablin* varieties was 0.86. These findings indicate that Paixiang and Zhanxiang

harbor rich endophytic fungal species, each possessing unique endophytes, and that the composition of endophytic fungal communities varies across different growth stages. The aforementioned research results establish a foundation for screening active endophytic fungal strains and investigating their influence on the synthesis and accumulation of active components in *Pogostemon cablin*.

## Full Text

# Community Composition Changes of Endophytic Fungi from Two Cultivated Types of *Pogostemon cablin*

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**Abstract:** To explore the influence of endophytic fungi-host interactions on the formation mechanisms of active components in patchouli, we investigated the endophytic fungi of two chemotypes with significant compositional differences: *Pogostemon cablin* cv. shipaiensis (Shipai patchouli) and *P. cablin* cv. zhanjiangensis (Zhanjiang patchouli). Strains were initially classified using traditional morphological methods, and their taxonomic status and diversity were determined by amplifying rDNA-ITS sequences with universal fungal primers ITS1/ITS4. The results showed that: (1) A total of 3,070 endophytic fungal strains were isolated from stem and leaf tissues at the seedling, branching, and adult stages using PDA and LBA media. From Shipai patchouli, 1,624 strains were isolated and 1,319 were identified, belonging to 36 genera. From Zhanjiang patchouli, 1,446 strains were isolated and 994 were identified, belonging to 33 genera. Shipai patchouli harbored seven unique endophytic fungi: *Epichloe typhina*, *Colletotrichum gloeosporioides*, *Botryosphaeria* sp., *Rhizoctonia* sp., *Truncatella* sp., and for the first time, oomycetous endophytes *Phytophthora* sp. and *Sclerophthora* sp. Zhanjiang patchouli yielded two unique species: *Paecilomyces* sp. and *Cercospora* sp. (2) The dominant endophytic fungi were identical in both cultivars—*Alternaria* sp. and *Colletotrichum* sp.—with relative isolation frequencies of 9.48% and 7.81% in Shipai patchouli, and 10.16% and 8.65% in Zhanjiang patchouli, respectively. (3) Colonization rates increased progressively from seedling to adult stage, reaching 97.78% (August) > 72.50% (July) > 55.28% (May) in Shipai patchouli, and 91.11% (August) > 63.06% (July) > 46.67% (May) in Zhanjiang patchouli. The average colonization rates were 75.19% for Shipai and 66.95% for Zhanjiang patchouli. (4)

Fungal diversity increased with growth stage, with an average Sorenson similarity coefficient of 0.86 between the two cultivars. These findings demonstrate that both patchouli types host rich and distinct endophytic fungal communities with dynamic compositional changes across growth stages, providing a foundation for screening bioactive strains and elucidating the role of endophytic fungi in active component synthesis and accumulation.

**Keywords:** *Pogostemon cablin*, cultivated type, Shipai patchouli, Zhanjiang patchouli, endophytic fungi, community composition

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## Introduction

*Pogostemon cablin* (Blanco) Benth., an annual aromatic herb in the Lamiaceae family, is a genuine medicinal material in Guangdong Province and one of the renowned “Four Great Southern Medicinals” of China. It possesses therapeutic effects for resolving dampness, harmonizing the stomach, stopping vomiting, and dispelling summer heat. Patchouli serves as a primary ingredient in numerous proprietary Chinese medicines, and its extracted essential oil is a crucial auxiliary material in the light chemical industry. Traditionally, patchouli has been classified into four cultivation types: Shipai (Guangzhou), Zhixiang (Zhaoqing), Zhanjiang (Zhanjiang), and Nanxiang (Hainan), which exhibit distinct morphological differences, particularly between Shipai and Zhanjiang types. Using ultrathin isoelectric focusing electrophoresis, Xu et al. (2003) categorized these four regional variants into three cultivars: *P. cablin* (Blanco) Benth. cv. shipaiensis, *P. cablin* (Blanco) Benth. cv. gaoyaoensis, and *P. cablin* (Blanco) Benth. cv. zhanjiangensis. Shipai and Zhixiang types constitute the primary sources of medicinal patchouli, while Zhanjiang and Nanxiang types are mainly used for essential oil extraction.

Patchouli contains up to 37 bioactive chemical constituents, with patchouli alcohol and pogostone being the most abundant. Pogostone content serves as a key indicator for evaluating the genuineness of patchouli. Luo et al. (2001; 2003) reported that pogostone levels in Shipai and Zhixiang types were significantly higher than in Zhanjiang and Nanxiang types, with Shipai patchouli showing the highest concentration. Based on these compositional differences, comparative analysis of conserved 18S rRNA genes and rapidly evolving matK genes in the chloroplast genome further divided the four types into two chemotypes: a pogostone chemotype (Shipai and Zhixiang) and a patchouli alcohol chemotype (Zhanjiang and Nanxiang). Liu et al. (2002) also demonstrated genetic sequence differences between Shipai and Zhanjiang patchouli through 16S rRNA sequence analysis.

Endophytic fungi, through long-term colonization of host tissues and coevolution with their hosts, establish complex symbiotic relationships that influence the accumulation of various host components and stress responses. Endophytic fungi have been detected in nearly all studied plants, exhibiting broad distribution,

high diversity, and rich biodiversity. These fungi also display host preference or specificity, as different hosts and growth environments significantly affect endophytic fungal community composition and colonization. Medicinal plants represent an important repository for discovering bioactive endophytic fungi, and the small-molecule secondary metabolites produced through host-fungus interactions play crucial regulatory roles in host growth, systemic defense, and secondary metabolite synthesis.

As a genuine medicinal material in Guangdong, previous studies have screened bioactive endophytic fungi from Shipai patchouli and investigated their chemical constituents, resistance to bacterial wilt, antitumor properties, and stress tolerance. However, these studies did not account for cultivar differences or growth stage variations, resulting in inconsistent findings regarding active endophytic fungi. Shipai and Zhanjiang patchouli, the two most widely cultivated types in Guangdong with marked compositional differences, have unclear mechanisms underlying active component formation, which hinders cultivar selection and breeding. Patchouli's medicinal constituents are primarily sesquiterpenoids synthesized via the mevalonic acid (MVA) pathway of isoprenoid metabolism. Studies on *Rehmannia glutinosa* have shown that endophytic fungi regulate the expression of allene oxide synthase (AOS) and 12-oxophytodienoate reductase (OPR) genes involved in jasmonic acid synthesis, with expression levels varying among different organs. Our research team previously investigated endophytic fungal diversity in *Amomum villosum* from Guangdong and Yunnan provinces, finding that growth environments affect community composition and proposing a "soil environment-endophytic fungal colonization-host component accumulation" model. Shipai and Zhanjiang patchouli, cultivated in different geographic regions for extended periods, have undergone significant morphological and endophytic fungal community changes. This study therefore examined endophytic fungal community composition and population dynamics across different growth stages in these two patchouli cultivars to establish a foundation for understanding the role of endophytic fungi in medicinal synthesis mechanisms.

## Materials and Methods

### Sample Collection and Preparation

Shipai patchouli was collected from the GAP base of Xiangxue Pharmaceutical Co., Ltd. in Luogang District, Guangzhou (now Luogang Town, Huangpu District). Zhanjiang patchouli was obtained from the Patchouli Planting Cooperative in Wutang Town, Suixi County, Zhanjiang City. Samples were collected at three growth stages: seedling, branching, and adult. Shipai patchouli sampling dates were May 10 (seedling, PX-1), July 14 (branching, PX-2), and August 20 (adult, PX-3). Zhanjiang patchouli sampling dates were May 21 (seedling, ZX-1), July 5 (branching, ZX-2), and August 7 (adult, ZX-3). Thirty plants were sampled at each stage using the five-point sampling method. Healthy plants were randomly excavated, transported back to the laboratory, cut into sections, sealed in bags, and stored under refrigeration. All materials were identified as

*Pogostemon cablin* by Professor He Hong of Guangzhou University of Chinese Medicine.

From each sample, five stem segments were randomly selected. After leaf removal, stems were washed with water and cut into 5 cm lengths, while leaves were cut into 2.5 cm × 2.5 cm pieces. Tissue segments were immersed in 75% ethanol for 3 minutes, rinsed three times with sterile water, then soaked in 0.1% mercuric chloride with Tween-20 (2 minutes for stems, 3 minutes for leaves) with continuous agitation to ensure complete contact. After removal, segments were rinsed three times with sterile water (2 minutes each), dried with sterile filter paper, and aseptically cut into approximately 0.5 cm × 0.5 cm pieces for further processing.

### Culture and Isolation

From each processed tissue batch, 120 segments were randomly selected and placed on PDA and Lima Bean Agar (LBA) plates. LBA preparation followed the method of Zuo (2004). Control plates were prepared by rolling or spreading tissue blocks on the medium surface for 2 minutes before removal. Plates were incubated at 25°C under constant humidity in darkness and examined every other day. When hyphae emerged from tissue blocks, they were transferred to fresh media for purification via single-spore isolation. Purified strains were numbered and stored in test tubes at 2-5°C for taxonomic identification. All experiments were repeated three times.

### Endophytic Fungal Identification

Morphological identification followed Wei (1979) *Identification Manual of Fungi*, examining colony morphology, hyphal characteristics, conidiophore morphology, spore morphology, sporulation structures, and sporulation patterns. Non-sporulating strains were induced to sporulate using the method of Sutton (1980) before identification. Molecular identification followed Barnett & Hunter (1987) and Ellis (1988) to determine taxonomic status. Total DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Wang et al., 2015). The primer pair ITS1 (5' -TCCGTAGGTGAACCTGCGG-3' ) and ITS4 (5' -TCCTCCGCTTATTGATATGC-3' ) was used for amplification. PCR products were detected by 1% agarose gel electrophoresis and sequenced by Tsingke Biotechnology (Guangzhou). Obtained sequences were compared with known sequences in NCBI GenBank, and taxonomic status was determined based on sequence similarity, coverage, and morphological characteristics.

### Data Processing

Colonization rate measures endophytic fungal abundance in plants, while relative isolation frequency (IF) measures the dominance of specific fungal groups. These were calculated as follows:

Colonization rate = (Number of infected tissue blocks / Total number of tissue

blocks)  $\times$  100%

Relative isolation frequency = (Number of strains of a genus or species / Total number of isolated strains)  $\times$  100% (Yuan et al., 2011).

Pearson correlation coefficient analysis in SPSS 21.0 was used to analyze correlations between colonization rates and relative isolation frequencies in the two patchouli cultivars. When variances were homogeneous, one-way ANOVA with Turkey HSD test was used for significance testing ( $P < 0.05$ ). Levene's test assessed variance homogeneity. Differences were considered significant at  $P < 0.05$ .

Shannon index ( $H'$ ) reflects endophytic fungal species diversity and was calculated as:

$$H' = -\sum(P_i \times \ln P_i)$$

where  $P_i$  represents the proportion of a particular endophytic fungus among all isolates.

The Sorenson index ( $C_s$ ) compares endophytic fungal composition similarity between two plants:

$$C_s = 2j / (a + b)$$

where  $j$  is the number of shared endophytic fungal species, and  $a$  and  $b$  are the total numbers of endophytic fungal species in each patchouli cultivar (Spellerberg & Fedor, 2003).

## Results

### Colonization Rate and Strain Isolation

Across three growth stages, 3,070 endophytic fungal strains were obtained from 2,160 tissue blocks of Shipai and Zhanjiang patchouli stems and leaves, with 2,203 strains successfully identified (1,319 from Shipai and 994 from Zhanjiang). The average colonization rate was 75.19% for Shipai and 66.95% for Zhanjiang patchouli. Results showed that colonization rates increased progressively from seedling to adult stage: Shipai patchouli exhibited August (97.78%) > July (72.50%) > May (55.28%), while Zhanjiang patchouli showed August (91.11%) > July (63.06%) > May (46.67%). Chi-square tests revealed significant differences ( $P < 0.05$ ) [Figure 1: see original paper]. At all three sampling stages, Shipai patchouli demonstrated higher colonization rates than Zhanjiang patchouli. Strain isolation numbers also increased with growth stage: Shipai yielded August (702 strains) > July (574 strains) > May (348 strains), while Zhanjiang produced August (662 strains) > July (478 strains) > May (306 strains). Shipai patchouli consistently yielded more isolates than Zhanjiang at each developmental stage [Figure 2: see original paper].

### Taxonomic Status of Endophytic Fungi

All molecularly identified strain ITS sequences have been submitted to GenBank. Based on morphological characteristics, 1,319 strains were identified from 1,624

Shipai isolates and 994 strains from 1,446 Zhanjiang isolates, representing 40 genera .

Shipai patchouli hosted seven unique endophytic fungi: *Epichloe typhina*, *Colletotrichum gloeosporioides*, *Botryosphaeria* sp., *Rhizoctonia* sp., *Truncatella* sp., plus two oomycetous species—*Phytophthora* sp. (53 isolates) and *Sclerophthora* sp. (32 isolates)—isolated via LBA culture. Zhanjiang patchouli contained two unique species: *Paecilomyces* sp. and *Cercospora* sp.

Dominant endophytic fungi were similar between cultivars but showed distinct relative isolation frequencies. *Alternaria* sp. and *Colletotrichum* sp. were predominant in both, accounting for 9.77% and 8.17% of identified strains, respectively. Specifically, relative isolation frequencies were 9.48% and 7.81% in Shipai, and 10.16% and 8.65% in Zhanjiang patchouli. Secondary dominant fungi included *Fusarium* sp., *Penicillium* sp., *Phaeocystostroma* sp., and *Trichoderma* sp., with relative isolation frequencies substantially higher than other taxa .

### Diversity and Similarity of Endophytic Fungi

From May to August, endophytic fungal diversity in both cultivars increased with growth stage. Shannon indices for Shipai patchouli were 3.17 (May), 3.28 (July), and 3.42 (August), while Zhanjiang patchouli showed 3.21 (May), 3.30 (July), and 3.35 (August), indicating rich community diversity .

Sorenson similarity coefficients varied among sampling periods. Within-cultivar similarity ranged from 0.87–0.97 for Shipai and 0.97–0.98 for Zhanjiang. Between-cultivar similarity ranged from 0.75–0.86, with the lowest coefficient (0.75) observed between Shipai and Zhanjiang in May, while the highest within-cultivar similarity was 0.98 for Zhanjiang between July and August . These results indicate relatively stable endophytic fungal communities within each cultivar across growth stages, but significant compositional differences between cultivars at the same stage, particularly during the seedling phase.

### Discussion

Endophytic fungal community composition is intimately related to host identity, with substantial variation observed among different host genera and even among congeneric species. Host plants exhibit both rich fungal diversity and selective preferences for colonization. Research indicates that endophytic fungal richness is significantly higher at high latitudes than low latitudes, with Sordariomycetes predominating in high-latitude regions and Dothideomycetes more common in low-latitude areas. Common Sordariomycetes endophytes such as *Colletotrichum*, *Phoma*, *Phomopsis*, and Xylariales can be isolated from both stems and leaves, whereas *Phyllosticta* occurs exclusively in leaves and *Fusarium* cannot be isolated from leaves.

Our isolation results from Shipai and Zhanjiang patchouli revealed that 82.23%

of endophytic fungi belonged to Sordariomycetes, 10.04% to Dothideomycetes, and 7.73% to Oomycota. The dominant fungi in both cultivars were *Colletotrichum* and *Alternaria*, though *Colletotrichum* showed substantially higher relative isolation frequency in Shipai (162 isolates) than in Zhanjiang (86 isolates). *Alternaria* was an exceptional case as a Dothideomycetes fungus. Rosa et al. (2009) isolated an *Alternaria* endophyte from Antarctic hair grass (*Deschampsia antarctica*) and suggested that Pleosporales fungi within Dothideomycetes are minimally affected by environmental factors and are common endophytes in herbaceous plants. We obtained 125 *Alternaria* isolates from Shipai and 101 from Zhanjiang patchouli, with minimal difference, indicating that *Alternaria* is broadly distributed in herbaceous plants and less influenced by latitude. Secondary dominant fungi including *Fusarium*, *Penicillium*, *Phaeocytophthora*, and *Trichoderma* mostly belong to Ascomycota Sordariomycetes or mitosporic fungi, commonly existing as saprophytes in various plant tissues.

Medicinal plants have distinct medicinal parts with varying therapeutic efficacy. Research suggests that different endophytic fungi exhibit tissue preferences, and community composition varies among plant organs. We observed that leaf tissues yielded significantly more endophytic fungal species and individuals than stems throughout patchouli development, with higher community abundance in leaves.

Community composition varies across host developmental stages, reflecting a dynamic equilibrium between endophytic fungi and host growth. In Shipai patchouli, *Ascochyta* sp., *Colletogloeum* sp., *Glomerella* sp., *Monochaetia* sp., *Phoma* sp., and *Verticillium* sp. were not isolated during the seedling stage but were recovered in July and August samples with increasing isolation frequencies. This period coincides with the transition from branching to adult stage—a critical time point for medicinal component accumulation. Similarly, *Arthrocladiella mougeotii* in Zhanjiang patchouli was only obtained during July–August growth, with no colonization during the seedling stage. These “temperature-sensitive endophytes,” which colonize later under temperature influence, include some pathogenic species that can cause plant disease. Although their relative isolation frequencies are lower than other endophytes, their role in influencing patchouli component accumulation cannot be ignored.

Beyond shared endophytic fungi, we identified cultivar-specific taxa among the 3,070 isolates. Shipai patchouli contained seven unique endophytes: four Ascomycota species (*Epichloe typhina*, *Botryosphaeria* sp., *Colletotrichum gloeosporioides*, *Rhizoctonia* sp., *Truncatella* sp.) and two oomycetes (*Phytophthora* sp. and *Sclerophthora* sp.). Zhanjiang patchouli contained two unique species: *Cercospora* sp. and *Paecilomyces* sp. *Epichloe* is a common endophyte in wild grasses whose metabolites affect host seed germination and active component accumulation. Further investigation of these temperature-sensitive and cultivar-specific endophytes in host active component synthesis mechanisms is particularly significant.

In addition to host genotype, environmental conditions influence endophytic fungal community composition and abundance. Across all three growth stages, Shipai patchouli exhibited higher endophytic fungal abundance than Zhanjiang patchouli in terms of colonization rate, isolate numbers, and diversity indices. However, both cultivars showed similar trends: colonization rates were higher in summer than spring, and Shannon indices ( $H'$ ) increased progressively (adult stage > branching stage > seedling stage). These results align with Lü et al. (2014) regarding seasonal variation in endophytic fungal communities of the medicinal herb *Atractylodes lancea*. Conversely, Gao et al. (2005) found greater endophytic fungal diversity in spring than summer for *Hovenia acerba*, suggesting that annual herbs and woody plants may exhibit different seasonal community dynamics.

This study demonstrates that Shipai and Zhanjiang patchouli harbor rich endophytic fungal communities, with higher abundance in Shipai patchouli. Community composition in both cultivars is influenced by host genotype, growth stage, and climatic factors. The characterized community structures and dynamics provide a foundation for understanding the pharmacological differences between Shipai and Zhanjiang patchouli.

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