# Post-print: Changes in Endophytic Fungal Community Composition of Two Cultivars of Pogostemon cablin

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## Abstract

To explore the influence of endophytic fungi-Pogostemon cablin interactions on the formation mechanism of host active constituents, two varieties with significant compositional differences, Paixiang and Zhanxiang, were selected as research subjects. The obtained strains were classified using traditional morphological methods, and their taxonomic status was identified and their diversity studied by amplifying rDNA-ITS sequences with fungal universal primers ITS1/ITS4. The results showed: (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. Among these, 1,624 strains were isolated from Paixiang, with 1,319 identified, belonging to 36 genera; 1,446 strains were isolated from Zhanxiang, with 994 identified, belonging to 33 genera. Seven species of unique endophytic fungi were isolated from Paixiang, namely *Epichloe typhina*, *Colletotrichum gloeosporioides*, *Botryosphaeria* sp., *Rhizoctonia* sp., and *Truncatella* sp., with *Phytophthora* sp. and *Sclerophthora* sp. isolated for the first time; these two species belong to the oomycete endophytes. Two species of unique endophytic fungi, *Paecilomyces* sp. and *Cercospora* sp., were isolated from Zhanxiang. (2) The dominant endophytic fungi in both Paixiang and Zhanxiang were the same, 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) The colonization rate of endophytic fungi in both Paixiang and Zhanxiang gradually increased from the seedling to the mature stage, following the order: 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 showed an increasing trend; meanwhile, the average similarity coefficient of

endophytic fungi between the two Pogostemon cablin varieties was 0.86. It is evident that Paixiang and Zhanxiang harbor rich endophytic fungal species, each possessing unique endophytic fungi, and the composition of endophytic fungal communities varies across different growth stages. These research findings lay a foundation for screening active endophytic fungal strains and exploring how endophytic fungi influence the synthesis and accumulation of active constituents 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 interactions between endophytic fungi and *Pogostemon cablin* on the formation mechanism of host active components, we investigated the endophytic fungi of *P. cablin* cv. shipaiensis and *P. cablin* cv. zhanjiangensis, which exhibit significant chemical composition differences. Strains were initially classified using traditional morphological methods, and their rDNA-ITS sequences were amplified using universal fungal primers ITS1/ITS4 to identify their taxonomic status and assess diversity. The results showed that: (1) A total of 3,070 strains were isolated from stem and leaf tissues of *P. cablin* at the seedling, branching, and adult stages using PDA and LBA media. Specifically, 1,624 strains were isolated from *P. cablin* cv. shipaiensis, with 1,319 strains identified across 36 genera, while 1,446 strains were isolated from *P. cablin* cv. zhanjiangensis, with 994 strains identified across 33 genera. Seven endophytic fungi were unique to *P. cablin* cv. shipaiensis: *Epichloe typhina*, *Colletotrichum gloeosporioides*, *Botryosphaeria* sp., *Rhizoctonia* sp., *Truncatella* sp., and for the first time, *Phytophthora* sp. and *Sclerophthora* sp., which belong to Oomycota. *P. cablin* cv. zhanjiangensis yielded two unique endophytic fungi: *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 *P. cablin* cv. shipaiensis, and 10.16% and 8.65% in *P. cablin* cv. zhanjiangensis, respectively. (3) Endophytic fungal colonization rates increased progressively from seedling to adult stage: for *P. cablin* cv. shipaiensis, August (97.78%) > July (72.50%) > May (55.28%); for *P. cablin* cv. zhanjiangensis, August (91.11%) > July (63.06%) > May (46.67%). The average colonization rates were 75.19% and 66.95%, respectively. (4) Endophytic fungal diversity increased with growth stage, with an

average Sorenson similarity coefficient of 0.86 between the two cultivars. These findings demonstrate that both cultivars harbor rich endophytic fungal communities with unique species, and that community composition varies across growth stages. These results establish a foundation for screening active endophytic fungal strains and investigating their influence on the synthesis and accumulation of active components in *P. cablin*.

**Keywords:** *Pogostemon cablin*, cultivated type, *P. cablin* cv. shipaiensis, *P. cablin* cv. zhanjiangensis, endophytic fungi, community composition

## Introduction

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

*P. cablin* contains up to 37 active chemical constituents, with patchouli alcohol and pogostone being the most abundant. Pogostone content is particularly important for evaluating the genuineness of *P. cablin*. Luo et al. (2001; 2003) found that pogostone levels were significantly higher in shipaiensis and zhiqing compared to zhanjiangensis and nanxiang, with shipaiensis showing the highest content. Based on these chemical differences, comparative analysis of chloroplast genome protein-coding genes—specifically the conserved 18S rRNA gene and the more rapidly evolving *matK* gene—further divided the four types into two chemotypes: a pogostone chemotype (including shipaiensis and zhiqing) and a patchouli alcohol chemotype (including zhanjiangensis and nanxiang). Liu et al. (2002) also demonstrated genetic sequence differences between shipaiensis and zhanjiangensis through 16S rRNA sequence analysis.

Endophytic fungi, which colonize host plant tissues internally and co-evolve with their hosts, form complex symbiotic relationships that influence host constituent accumulation and stress responses [**?**]. Endophytic fungi have been detected in

nearly all studied plants, exhibiting broad distribution, diverse species, and rich biodiversity. They also demonstrate host preference or specificity, as different hosts or growth environments significantly affect endophytic fungal community composition and colonization. Medicinal plants represent an important reservoir for mining active endophytic fungal resources, and the small-molecule secondary metabolites produced through their interactions with hosts play crucial regulatory roles in host growth, systemic defense, and secondary metabolite synthesis [**?**, **?**]. As a genuine medicinal material in Guangdong Province, previous studies have screened active endophytic fungi from shipaiensis and investigated their chemical constituents [**?**], resistance to bacterial wilt [**?**], antitumor properties [**?**], and stress tolerance [**?**]. However, these studies did not account for cultivation type differences or growth stage variations, resulting in considerable variation among the isolated active endophytic fungi. As the two most widely cultivated types of *P. cablin* in Guangdong Province with distinct chemical composition differences, the unclear mechanism underlying active component formation has hindered variety breeding efforts.

*P. cablin* medicinal components are primarily sesquiterpenoids synthesized through the mevalonic acid (MVA) pathway of isoprenoid metabolism. Studies on jasmonic acid synthesis in *Rehmannia glutinosa* revealed that expression of allene oxide synthase (AOS) and 12-oxophytodienoate reductase (OPR) genes is regulated by endophytic fungi, with varying expression levels in 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" formation model [**?**]. Shipaiensis and zhanjiangensis, cultivated in different geographic regions for extended periods, exhibit significant morphological and endophytic fungal community composition changes. Therefore, this study used shipaiensis and zhanjiangensis as materials to investigate community composition and population dynamics of endophytic fungi across different growth stages, laying the groundwork for understanding the role of endophytic fungi in *P. cablin* medicinal synthesis mechanisms.

## Materials and Methods

### 1.1 Material Sources

Shipaiensis samples were collected from the *P. cablin* GAP base of Guangzhou Xiangxue Pharmaceutical Co., Ltd. in Luogang District, Guangzhou (now Luogang Town, Huangpu District). Zhanjiangensis samples were collected from the *P. cablin* planting cooperative in Wutang Town, Suixi County, Zhanjiang. Sampling was conducted across three growth stages: seedling, branching, and adult. Shipaiensis sampling dates were May 10 (seedling stage, PX-1), July 14 (branching stage, PX-2), and August 20 (adult stage, PX-3). Zhanjiangensis sampling dates were May 21 (seedling stage, ZX-1), July 5 (branching stage, ZX-2), and August 7 (adult stage, ZX-3). Thirty healthy plants were randomly

collected at each sampling time using the five-point sampling method. Plants were excavated, cut into sections, sealed in bags, and stored under refrigeration. 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 blocks 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 stirring to ensure full contact with the sterilizing solution. After removal, tissues were rinsed three times with sterile water (2 minutes each), dried with sterile filter paper, and cut into approximately 0.5 cm × 0.5 cm pieces under sterile conditions for later use.

### 1.2 Cultivation and Isolation

From each processed tissue block, 120 pieces were randomly selected and placed on PDA and lima bean agar (LBA) plates. LBA preparation followed the method of Zuo (2004). Controls were prepared by rolling or spreading tissue blocks on medium surfaces for 2 minutes before removal. Plates were incubated at 25°C under constant humidity in darkness and observed every other day. When mycelia emerged around tissue blocks, they were transferred for cultivation and single-spore isolation. Purified strains were numbered and inoculated into test tubes for storage at 2–5°C for taxonomic identification. All experiments were repeated three times.

### 1.3 Endophytic Fungi Identification

Morphological identification followed the *Fungal Identification Manual* by Wei (1979), including colony morphology, hyphal characteristics, conidiophore morphology, spore morphology, sporulation structures, and sporulation patterns. For non-sporulating strains, the method of Sutton (1980) was used for sporulation induction before identification. Molecular identification referenced methods by Barnett & Hunter (1987) and Ellis (1988) to determine taxonomic status. Total DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method [**?**]. Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for amplification. PCR products were detected by 1% agarose gel electrophoresis and sent to Guangzhou Tsingke Biotechnology Co., Ltd. for sequencing. Obtained sequences were compared with known sequences in NCBI, and taxonomic status was determined based on sequence similarity, coverage, and morphological characteristics.

### 1.4 Data Processing

Colonization rate measures endophytic fungal abundance in plants, while relative isolation frequency (IF) measures the dominance of specific endophytic

fungi. These were calculated as follows:

Colonization rate = (Number of tissue blocks infected by endophytic fungi / Total number of tissue blocks) × 100%

Relative isolation frequency = (Number of strains isolated from a genus or species / Total number of isolated strains) × 100% [**?**]

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

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

$H = -\Sigma(Pi \times lnPi)$

where Pi represents the percentage of a particular endophytic fungus relative to the total endophytic fungal population.

The Sorenson index (Cs) compares similarity in endophytic fungal composition between two plants:

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

where j represents the number of shared endophytic fungal species between shipaiensis and zhanjiangensis, while a and b represent the total number of endophytic fungal species in each cultivar [**?**].

## Results

### 2.1 Colonization Rate and Strain Isolation

Across three growth stages, 3,070 endophytic fungal strains were obtained from 2,160 tissue blocks of shipaiensis and zhanjiangensis stems and leaves, with 2,203 strains identified. Specifically, 1,319 strains were obtained from shipaiensis and 994 from zhanjiangensis. The average colonization rate was 75.19% for shipaiensis and 66.95% for zhanjiangensis. Results showed that colonization rates increased progressively from seedling to adult stage: shipaiensis, August (97.78%) > July (72.50%) > May (55.28%); zhanjiangensis, 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, shipaiensis showed higher colonization rates than zhanjiangensis. Strain isolation numbers also increased with growth stage: shipaiensis yielded August (702 strains) > July (574 strains) > May (348 strains); zhanjiangensis yielded August (662 strains) > July (478 strains) > May (306 strains). Shipaiensis consistently yielded more strains than zhanjiangensis at each developmental stage [Figure 2: see original paper].

### 2.2 Taxonomic Status of Endophytic Fungi

All molecularly identified strain ITS sequences were submitted to GenBank. Based on morphological characteristics, 1,319 strains were identified from 1,624 shipaiensis isolates, and 994 strains from 1,446 zhanjiangensis isolates, belonging to 40 genera .

Shipaiensis-specific endophytic fungi included *Epichloe typhina*, *Colletotrichum gloeosporioides*, *Botryosphaeria* sp., *Rhizoctonia* sp., and *Truncatella* sp. Using LBA medium, 53 strains of *Phytophthora* sp. and 32 strains of *Sclerophthora* sp. from Oomycota were isolated from shipaiensis for the first time. Zhanjiangensis yielded two unique endophytic fungi: *Paecilomyces* sp. and *Cercospora* sp.

Dominant endophytic fungi were similar between cultivars but showed distinct relative isolation frequencies. The dominant species were *Alternaria* sp. and *Colletotrichum* sp., accounting for 9.77% and 8.17% of identified strains, respectively. Relative isolation frequencies were 9.48% and 7.81% in shipaiensis, and 10.16% and 8.65% in zhanjiangensis. Additionally, *Fusarium* sp., *Penicillium* sp., *Phaeocytostroma* sp., and *Trichoderma* sp. were frequently isolated, with relative isolation frequencies substantially higher than other endophytic fungi .

### 2.3 Diversity and Similarity of Endophytic Fungi

From May to August, endophytic fungal diversity in both shipaiensis and zhanjiangensis increased with growth from seedling to adult stage. Shannon indices for shipaiensis were May 3.17, July 3.28, and August 3.42; for zhanjiangensis, May 3.21, July 3.30, and August 3.35, indicating rich endophytic fungal community diversity in both cultivars .

Sorenson similarity coefficients varied between cultivars and growth stages. Within shipaiensis, similarity coefficients ranged from 0.87 to 0.97 across three sampling times; within zhanjiangensis, from 0.97 to 0.98. Between shipaiensis and zhanjiangensis, similarity coefficients ranged from 0.75 to 0.86. The highest similarity was observed in zhanjiangensis between May and August (0.97) and July and August (0.98). The lowest similarity occurred between shipaiensis and zhanjiangensis in May (0.75) . These results indicate relatively stable endophytic fungal community composition within each cultivar across growth stages, but significant differences between cultivars at the same growth stage, particularly during the seedling stage.

## Discussion

Endophytic fungal community composition is closely related to host plants, with substantial variation observed among different host genera, and even among congeneric species [**?**]. Host plants exhibit both rich endophytic fungal diversity and selective preferences for colonization. Previous studies indicate that endophytic fungal richness is significantly higher at high latitudes than low latitudes, with Sordariomycetes predominating at high latitudes and Dothideomycetes more

common at low latitudes [**?**]. Common Sordariomycetes endophytes such as *Colletotrichum*, *Phoma*, *Phomopsis*, and Xylariales can be isolated from plant stems and leaves, while *Phyllosticta* occurs only in leaves and *Fusarium* cannot be isolated from leaves [**?**, **?**, **?**].

Our isolation results from shipaiensis and zhanjiangensis showed that 82.23% of endophytic fungi belonged to Sordariomycetes, 10.04% to Dothideomycetes, and 7.73% to Oomycota (Oomycetes). The dominant endophytic fungi in both cultivars were *Colletotrichum* and *Alternaria*, though *Colletotrichum* showed much higher relative isolation frequency in shipaiensis (162 strains) than in zhanjiangensis (86 strains). *Alternaria* was an exceptional case, belonging to Dothideomycetes. Rosa et al. (2009) isolated an *Alternaria* endophyte from Antarctic hair grass (*Deschampsia antarctica*) and suggested that Pleosporales (Dothideomycetes) are less affected by environmental factors and are common endophytes in herbaceous plants. We obtained 125 *Alternaria* strains from shipaiensis and 101 from zhanjiangensis, with minimal difference, indicating that *Alternaria* is less influenced by latitude and likely ubiquitous in herbaceous plants. In addition to the two dominant species, *Fusarium*, *Penicillium*, *Phaeocytostroma*, and *Trichoderma* showed relatively high isolation frequencies. These subdominant endophytes mostly belong to Ascomycota (Sordariomycetes) or mitosporic fungi, commonly existing as saprophytes in various plant tissues.

Medicinal plants have different medicinal parts with varying efficacies. Research suggests that different endophytic fungi exhibit tissue preferences, and community composition varies among plant parts [**?**]. We found that throughout *P. cablin* development, leaf tissues yielded significantly more endophytic fungal species and individuals than stems, with higher community abundance in leaves. Community composition also varied across growth stages, reflecting a dynamic balance between endophytic fungi and host growth. In shipaiensis, *Ascochyta* sp., *Colletogloeum* sp., *Glomerella* sp., *Monochaetia* sp., *Phoma* sp., and *Verticillium* sp. were not isolated during the seedling stage but were obtained in July and August samples with increasing isolation frequencies. This period coincides with the transition from branching to adult stage in shipaiensis, a critical time point for medicinal component accumulation. Similarly, *Arthrocladiella mougeotii* in zhanjiangensis was obtained only 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 within their genera that can cause plant diseases. Although their relative isolation frequencies are lower than other endophytes, their role in influencing *P. cablin* component accumulation cannot be ignored.

Besides shared endophytic fungi, we obtained cultivar-specific endophytes from the 3,070 strains. Shipaiensis yielded seven unique species: *Epichloe typhina*, *Botryosphaeria* sp., *Colletotrichum gloeosporioides*, *Rhizoctonia* sp., and *Truncatella* sp. (Ascomycota), plus *Phytophthora* sp. and *Sclerophthora* sp. (Oomycota). Zhanjiangensis yielded two unique species: *Cercospora* sp. and *Paecilomyces* sp. *Epichloe* is a common endophyte in wild grasses, and its

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 meaningful.

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

This study demonstrates that shipaiensis and zhanjiangensis harbor rich endophytic fungal communities, with higher abundance in shipaiensis. Community composition in these two cultivated types is influenced by host genotype, growth stage, and climatic factors. The community composition and dynamics characteristics provide a foundation for further understanding the pharmacological differences between shipaiensis and zhanjiangensis.

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*Note: Figure translations are in progress. See original paper for figures.*