Isolation of Endophytic Fungi from Dracaena cochinchinensis and In Vitro Screening for Antimicrobial Activity (Postprint)

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Abstract

To investigate the resource diversity of endophytic fungi in Dracaena cochinchinensis, preliminarily explore and screen specific strains with antimicrobial activity, and further develop antimicrobial active compounds, endophytic fungi were isolated from stems and leaves of D. cochinchinensis using plant tissue isolation method. The isolates were subjected to 7-day liquid fermentation, and crude extracts were prepared via ethyl acetate extraction. The antimicrobial activity of these fermentation crude extracts was evaluated using the Oxford cup diffusion method against 10 common pathogenic bacteria and 5 clinically drug-resistant strains as targets, and endophytic fungi with relatively good antimicrobial activity were further identified by molecular methods. The results showed that a total of 345 endophytic fungal strains were isolated from stems and leaves of D. cochinchinensis; 294 strains exhibited inhibitory activity against one or more indicator bacteria, among which 84 strains displayed varying degrees of inhibitory activity against the 5 clinically drug-resistant bacteria, accounting for 24.35% of the total isolated strains, and 75% of the endophytic fungi exhibited inhibitory activity against Staphylococcus aureus. These findings indicate that D. cochinchinensis harbors multiple endophytic fungi with antimicrobial activity, laying a foundation for exploration of antimicrobial active components from endophytes of D. cochinchinensis and screening of novel antimicrobial drugs.

Full Text

Preamble

Keywords: *Dracaena cochinchinensis*, endophytic fungi, isolation, antimicrobial activities, fungal identification

The advent of antimicrobial drugs has revolutionized the treatment of infectious diseases and made tremendous contributions to saving human lives. However, this success has also accelerated the development of pathogenic bacterial resistance and the emergence of multidrug-resistant strains (Schmalstieg et al., 2012; Lewis, 2012), such as methicillin-resistant *Staphylococcus aureus* (MRSA). Currently, the rate of new antimicrobial drug development lags far behind the pace of resistance evolution, creating an urgent need to discover compounds with novel mechanisms of action or bioactivities. Sourcing antimicrobial agents from nature—particularly those effective against resistant pathogens—has become a critical avenue for drug discovery. As microbes from conventional environments have been repeatedly studied, leading to increasingly redundant compound discovery, researchers have turned their attention to microorganisms from special ecological niches.

Endophytic fungi are microorganisms that reside in healthy plant tissues without causing disease symptoms, and they are widely distributed across diverse plant species (Tanaka et al., 2002). Plant endophytes represent an important microbial resource, especially since some endophytic fungi have evolved to produce bioactive metabolites identical or analogous to those of their host plants, such as podophyllotoxin, vinblastine, taxol, and curcumin (Stierle et al., 1993; Zhang et al., 2000; Peng et al., 2010; Wang et al., 2017). This suggests tremendous potential for discovering host-identical or host-similar chemical constituents from endophytic fungi. Due to their unique living environments, endophytic fungi possess the capacity to produce structurally diverse bioactive metabolites. To date, various compound types—including alkaloids, steroids, flavonoids, peptides, terpenoids, and cyclopeptides—have been isolated from endophytic fungal metabolites, exhibiting antimicrobial, antitumor, antiviral, and enzyme-inhibitory activities (Xiao et al., 2018). These compounds hold promise for antimicrobial drug development, making the systematic isolation and screening of bioactive compounds from endophytic fungi essential for providing a foundation for novel antimicrobial drug research and addressing the growing problem of pathogenic drug resistance.

Dracaena cochinchinensis (Lour.) S.C. Chen is an evergreen tree belonging to the family Asparagaceae (subfamily Nolinoideae) and genus *Dracaena*, primarily distributed in Yunnan and Guangxi provinces of China. The resin of *D. cochinchinensis* serves as the raw material for the traditional Chinese medicine "Dragon's Blood" (Xue Jie), which demonstrates strong therapeutic effects in promoting blood circulation, removing blood stasis, astringing sores, and stopping bleeding, with loureirin A and loureirin B as its main active components (Cai and Xu, 1979). Due to harsh growing conditions, slow growth, and low yield, wild populations of *D. cochinchinensis* face depletion threats from resin harvesting, leading to its classification as a nationally protected second-grade plant in China. To promote rational resource utilization, recent studies have investigated the antioxidant activity of its main components, screening of resin-inducing agents, and induction of loose callus tissue (Zhou et al., 2015; Wei et al., 2016; Chen et al., 2018). However, research on the antimicrobial ac-

tivity of endophytic fungi from *D. cochinchinensis* remains scarce. This study addresses this gap through the following objectives: (1) isolation and antimicrobial activity evaluation of endophytic fungi from *D. cochinchinensis* to obtain specific strains with potent antimicrobial activity; (2) establishing a foundation for subsequent mining of bioactive compounds from these strains; (3) providing preliminary experimental basis for comprehensive utilization of *D. cochinchinensis* and new drug development; and (4) offering novel strategies for addressing resource scarcity in traditional medicinal plants.

Materials and Methods

Plant Material

Stems and leaves of *D. cochinchinensis* were collected in January 2016 from Menglun Town, Mengla County, Xishuangbanna, Yunnan Province (sample code B) and from the Xishuangbanna Tropical Flowers and Plants Garden at the Yunnan Institute of Tropical Crops (stem samples coded RJ, leaf samples coded RY). During collection, sterile gloves and tools were used to harvest healthy stem and leaf tissues, which were immediately placed in sterile containers and stored at 4 °C until use. Plant materials were provided and identified by Dr. Cao Yang.

Instruments and Reagents

Instruments: GR-200 analytical balance (Guangzhou Aixin Scientific Instruments), ES-315 autoclave (TOMY), constant temperature incubator (Shanghai Yiheng Scientific Instruments), LRH-250 biochemical incubator (Shanghai Yiheng Scientific Instruments), N-1100 rotary evaporator (Shanghai Ailang Instruments), SW-CJ-2FD clean bench (AIRTECH), electric thermostatic blast drying oven (Shanghai Yiheng Technology). Common reagents and consumables for culture media preparation were purchased from Yayun Biotechnology.

Reagents: Distilled water, anhydrous ethanol, methanol, glacial acetic acid, ethyl acetate, and acetone (all analytical grade). Universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were synthesized by Sangon Biotech (Shanghai). Column-based fungal genomic DNA extraction kits were also purchased from Sangon Biotech (Shanghai).

Indicator Strains

The following test strains were used: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* PA01, *Salmonella typhimurium* 26196, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus albus* 1029, *Mycobacterium smegmatis* 1037, *Acinetobacter baumannii* ATCC 19606, *Enterococcus faecalis* ATCC 29212, three MRSA strains (MRSA 1-3), and two clinical drug-resistant *Candida albicans* strains (Drug-

resistant *Candida albicans* 1-2). All indicator strains were preserved from previous studies in our research group.

Culture Media

For pathogenic bacteria: LB medium containing tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, and water 1 L, pH 7.2–7.6.

For fungi: (1) Sabouraud medium: peptone 10 g, glucose 40 g, agar 15 g, water 1 L, pH 6.0; (2) PDA medium: potato 200 g (boiled for 20 min, filtered, and residue discarded), glucose 20 g, agar 15 g, water 1 L, pH 6.0–7.0; (3) PDB medium: potato 200 g (boiled for 20 min, filtered, and residue discarded), glucose 20 g, water 1 L, pH 6.0–7.0.

Methods

Isolation and Purification of Endophytic Fungi Plant samples were washed under running water, and intact leaves and stems were selected and cut into approximately 2 cm pieces. Surface sterilization was performed by immersing in 50% ethanol for 1 min, then 75% ethanol for 1 min. Roots were treated with 0.10% $HgCl_2$ for 11 min, stems with 0.10% $HgCl_2$ for 7 min, and leaves with 2% NaClO for 5 min, followed by 3–5 rinses with sterile water. Materials were then cut into $ $0.5 cm pieces and placed on PDA medium with the cut surface in contact with the agar. After incubation at 28 °C, emerging fungal hyphae from tissue edges were transferred to fresh PDA medium and repeatedly purified. Strains were deduplicated based on colony morphology, mycelial growth, and pigment production. To confirm successful surface sterilization, 0.2 mL of the final rinse water was plated on PDA medium and incubated under identical conditions; absence of fungal growth after one week verified that isolated strains were true endophytes (Yang et al., 2014; Hou et al., 2015). Purified strains were maintained on 3–5 PDA slants at 4 °C, with detailed records of isolation source and morphological characteristics on solid and liquid media to establish an endophytic fungal repository for *D. cochinchinensis*.

Screening of Dominant Strains Endophytic fungi were cultivated in PDB medium for 7 days, and their fermentation extracts were evaluated for antimicrobial activity using the Oxford cup diffusion method. Dominant strains were selected based on activity, TLC profiles, and metabolite yield.

Fermentation and Extraction: Activated fungal mycelia or spores were inoculated into 100 mL PDB liquid medium and incubated at 28 °C with shaking at 200 rpm for 7 days. Growth characteristics were recorded, then equal volumes of ethyl acetate were added for overnight soaking, followed by three rounds of ultrasonic extraction. Extracts were concentrated under reduced pressure, transferred to brown vials with organic solvent, and weighed after solvent evaporation.

Antimicrobial Activity Assay: Pathogenic bacteria were inoculated into LB liquid medium and cultured at 37 °C, 200 rpm for 12 h in darkness. *Candida albicans* was inoculated into Sabouraud liquid medium and cultured at 28 °C, 200 rpm for 24 h in darkness. Cultures were diluted to 10^6–10^7 cfu·mL^{-1} with appropriate liquid media for use.

The Oxford cup diffusion method (Shao et al., 2012) was employed for antimicrobial testing. Diluted indicator strains were evenly spread on solid medium. Crude extracts were dissolved in acetone to 20 mg·mL^{-1}, and 50 μL was applied per assay. Acetone (50 μL) served as negative control, while antibiotics were used as positive controls (5 μg vancomycin for bacteria, 20 μg amphotericin B for *C. albicans*). *C. albicans* plates were incubated at 28 °C, while bacterial plates were incubated at 37 °C. Inhibition zone diameters were measured after 12 h.

Chemical Diversity Analysis Chemical diversity was assessed by thin-layer chromatography (TLC). Samples were dissolved in methanol to 5 mg·mL^{-1}, sonicated for 30 min, and filtered through a 0.22 μm membrane. Aliquots were spotted on GF254 silica plates (5 cm × 10 cm) and developed with chloroform:methanol (10:1) until the solvent front reached 4 cm. Plates were air-dried and visualized under various conditions. Strains producing samples with numerous bands/spots and distinctive coloration were designated as having high chemical diversity.

Molecular Identification of Dominant Strains Forty strains with broad antimicrobial spectra, strong activity, rich chemical diversity, and high metabolite yield were selected for ITS molecular identification. After 3 days of cultivation, 50–100 mg of mycelia was harvested, and total genomic DNA was extracted using a column-based fungal DNA extraction kit. The ITS region was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

PCR was performed in a 50 μL reaction containing 25 μL TSINGKE Master Mix, 1 μL ITS1, 1 μL ITS4, 2 μL DNA template, and 21 μL ddH_2O. Cycling conditions: initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min; final extension at 72 °C for 8 min (Hou et al., 2015). Amplified products were sequenced by Sangon Biotech (Shanghai).

Sequences were subjected to NCBI BLAST analysis to identify homologous sequences, followed by multiple sequence alignment. A phylogenetic tree was constructed using MEGA 6.0 with the neighbor-joining method (Yang et al., 2014).

Results

Isolation of Endophytic Fungi from *D. cochinchinensis*

A total of 345 endophytic fungal strains were isolated from three *D. cochinchinensis* samples: 152 strains from leaves collected in Menglun Town (coded B1–B152, 44.05% of total), 100 strains from stems of the Tropical Flowers and Plants Garden (coded RJ1–RJ100, 28.99%), and 93 strains from leaves of the same garden (coded RY1–RY93, 26.96%). These results demonstrate that *D. cochinchinensis* harbors a rich diversity of endophytic fungi.

Screening of Dominant Strains

All 345 isolates were evaluated for antimicrobial activity against pathogenic bacteria and fungi, chemical diversity, and metabolite yield to identify superior strains with broad-spectrum activity and high production capacity. Strain B (from Menglun Town leaves) showed the richest metabolite profile with the most TLC bands/spots across all visualization conditions, with 109 strains (71.71%) exhibiting inhibitory activity against at least one indicator organism. Strain RJ (from Tropical Flowers and Plants Garden stems) demonstrated the best and broadest antimicrobial activity, with all isolates inhibiting at least one pathogen. Strain RY (from Tropical Flowers and Plants Garden leaves) yielded the highest metabolite production with good antimicrobial activity, as 80 strains (86.02%) inhibited at least one indicator organism. Among all isolates, the highest inhibition rates were observed against *S. aureus* (75.5%) and *M. smegmatis* (62.61%). The antimicrobial activities of endophytic fungi from different sources against 15 indicator strains are summarized in .

Based on combined evaluation of antimicrobial activity and TLC profiles, multiple dominant strains with potent activity and rich chemical diversity were identified. The antimicrobial activities of the top 10 selected strains against 15 pathogenic bacteria are presented in . [Figure 1: see original paper] illustrates the inhibitory effects of selected strains against *E. coli*, *M. smegmatis*, MRSA-1, and MRSA-2. [Figure 2: see original paper] shows TLC analysis of fermentation extracts from dominant strains, visualized under UV 254 nm, UV 365 nm, iodine, and sulfuric acid-ethanol staining. The numerous distinctive bands/spots observed under various conditions indicate rich chemical diversity and potential for novel compound discovery.

Molecular Identification of Dominant Strains

Forty bioactive strains were subjected to ITS molecular identification. BLAST analysis of sequencing results against GenBank revealed that these strains belonged to 30 species across 14 genera: *Diaporthe*, *Fusarium*, *Pestalotiopsis*, *Daldinia*, *Cylindrocladium*, *Xylaria*, *Colletotrichum*, *Phomopsis*, *Nectria*, *Nigrospora*, *Periconia*, *Cladosporium*, *Phaeosphaeria*, *Acrocalymma*, plus two species from Sordariomycetes and Pleosporales. Strains B-132, RY-44, RJ-34, B-150, B-76, RJ-44, B-143, and RY-15 showed 100% similarity to

reference strains *Cladosporium* sp. (JQ388271), *Xylaria* sp. (JQ341078), *Acrocalymma* sp. (KU747920), *Phaeosphaeria papaya* (KT224848), *Nigrospora* sp. (FJ527872), Sordariomycetes sp. (HQ130707), *Periconia* sp. (MK367489), and *Pestalotiopsis* sp. (MH445915), respectively. Other strains exhibited 95–99% similarity to their closest matches. Among the 40 bioactive strains, *Fusarium* (6 strains), *Daldinia* (5 strains), *Colletotrichum* (5 strains), and *Diaporthe* (5 strains) were the most abundant, collectively representing over 50% of selected species and constituting the dominant active endophytic fungal populations in *D. cochinchinensis* (, [Figure 3: see original paper]).

The 40 dominant strains obtained in this study were classified into 32 species. Some genera have been previously reported to produce bioactive compounds: for example, *Colletotrichum* sp. from *Artemisia annua* produces colletotricacid with strong antifungal activity (Lu et al., 2000); *Phomopsis longicolla* HL-2232 from mangrove *Bruguiera sexangula* var. *rhynchopetala* produces the antibacterial alkaloid 2-(2'S-hydroxypropyl)-5-methyl-7-hydroxychromone (Song et al., 2015); and *Fusarium* species are known to produce diverse antimicrobial secondary metabolites (Kyekyeku et al., 2017; Liu et al., 2019). These findings suggest that antimicrobial endophytic fungi from *D. cochinchinensis* have significant potential for producing various bioactive substances worthy of further investigation. Notably, we isolated *Acrocalymma* sp. (RJ-34), which exhibited strong inhibitory activity against all 15 indicator pathogens, particularly notable efficacy against three MRSA strains, and displayed rich chemical diversity. Since no secondary metabolites have been reported from this genus, it represents a promising candidate for discovering novel antimicrobial agents and has been prioritized for further metabolite mining.

Some endophytic fungi co-evolving with host plants can produce identical or similar bioactive metabolites. Therefore, loureirin A and B—the main components of Dragon's Blood—were included as standards in chemical diversity assays to identify such specific strains and provide new approaches for *D. cochinchinensis* resource conservation. However, no such strains were detected, possibly due to: (1) low concentrations of target compounds in fermentation broth below detection limits, which could be addressed by scaling up fermentation and employing more sensitive methods (e.g., HPLC); or (2) the fact that some endophytic fungi may not be culturable under standard PDA medium at 28 °C, and even using multiple media cannot guarantee complete isolation of all endophytes. Establishing culture conditions that mimic the host plant's internal environment would be essential for accurately reflecting the biological characteristics of endophytic fungi.

In conclusion, *D. cochinchinensis* harbors a wide distribution of antimicrobial endophytic fungal strains. The selected dominant strains exhibit potent antimicrobial activity, high metabolite yield, rich chemical diversity, and stable culturability, demonstrating significant development potential. Active mining of secondary metabolites from these strains is currently underway.

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