# Postprint: Diversity of Endophytic Fungi from Guangxi Oleander and Screening for Inhibitory Activity Against Several Aquatic Pathogenic Bacteria

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## Abstract

Oleander is an important medicinal plant. To investigate the diversity of endophytic fungi in oleander and evaluate the activity of their secondary metabolites, this study isolated and purified endophytic fungi from Guangxi oleander (Nerium indicum), identified them using a combination of morphological and ITS sequence analysis, and screened the antimicrobial activity of endophytic fungal extracts against five indicator strains (including three Vibrio species). The results showed that: (1) A total of 19 endophytic fungal strains were obtained from Guangxi oleander, all belonging to the phylum Ascomycota and encompassing five orders and seven genera, including Colletotrichum, Guignardia, Phyllosticta, Neofusicoccum, Aspergillus, Nothophoma, and Diaporthe. The dominant genera were Colletotrichum (isolation frequency of 36.85%) and Guignardia (isolation frequency of 21.05%), with Colletotrichum mainly distributed in stems and Guignardia exclusively isolated from leaves. (2) Antimicrobial tests demonstrated that jing-117 (Neofusicoccum sp.) and ye-130 (Guignardia sp.) exhibited relatively specific inhibitory effects against Vibrio campbellii; ye-136 (Aspergillus sp.) could simultaneously inhibit Bacillus cereus and Vibrio campbellii; ye-135 (Aspergillus sp.) and jing-116 (Colletotrichum sp.) could only inhibit Bacillus cereus; and ye-134 (Guignardia sp.) showed inhibitory activity against Vibrio alginolyticus. This study, for the first time based on ITS sequences, revealed that endophytic fungi in Guangxi oleander possess relatively rich diversity, and identified several strains with antimicrobial activity whose extracts can inhibit the growth of aquatic pathogenic Vibrio species, demonstrating promising development potential.

## Full Text

# Diversity of Endophytic Fungi Isolated from *Nerium indicum* in Guangxi and Activity Screening Against Several Aquatic Pathogens

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### Abstract

*Nerium indicum* is an important medicinal plant. To investigate the diversity of endophytic fungi associated with *N. indicum* and evaluate the bioactivity of their secondary metabolites, we isolated and purified endophytic fungi from *N. indicum* in Guangxi and identified them using a combination of morphological and ITS sequence analysis. The antibacterial activity of fungal extracts was then screened against five indicator bacteria, including three *Vibrio* species. The results demonstrated: (1) A total of 19 endophytic fungal strains were obtained from Guangxi *N. indicum*, all belonging to the phylum Ascomycota and encompassing 5 orders and 7 genera: *Colletotrichum*, *Guignardia*, *Phyllosticta*, *Neofusicoccum*, *Aspergillus*, *Nothophoma*, and *Diaporthe*. The dominant genera were *Colletotrichum* (isolation frequency of 36.85%) and *Guignardia* (21.05%), with *Colletotrichum* primarily distributed in stems and *Guignardia* exclusively isolated from leaves. (2) Antibacterial assays revealed that strains jing-117 (*Neofusicoccum* sp.) and ye-130 (*Guignardia* sp.) exhibited specific inhibitory effects against *Vibrio campbellii*, while ye-136 (*Aspergillus* sp.) simultaneously inhibited both *Bacillus cereus* and *V. campbellii*. Strains ye-135 (*Aspergillus* sp.) and jing-116 (*Colletotrichum* sp.) only inhibited *B. cereus*, and ye-134 (*Guignardia* sp.) showed inhibitory activity against *Vibrio alginolyticus*. This study is the first to reveal the rich diversity of endophytic fungi in Guangxi *N. indicum* based on ITS sequence analysis and to identify several strains with antibacterial activity that can inhibit the growth of aquatic pathogenic vibrios, demonstrating promising potential for future development.

**Keywords:** *Nerium indicum*, endophytic fungi, ITS sequence analysis, *Vibrio*, antibacterial activity

## Introduction

*Nerium indicum* is a traditional medicinal plant belonging to the family Apocynaceae. The genus *Nerium* comprises two species, *N. indicum* and *N. oleander*, with *N. indicum* including the white-flowered cultivar *Nerium indicum* Mill. cv. Paihua. These species exhibit similar medicinal properties and contain abundant bioactive compounds, leading to widespread applications worldwide, particularly in India and China. In traditional Chinese medicine, *N. indicum* has been used to treat various ailments including cardiac diseases, diabetes, asthma, cancer, and epilepsy (Elliott, 2002). Modern pharmacological studies have demonstrated that *N. indicum* possesses multiple bioactive components with antibacterial (Hussain & Gorsi, 2004), antifungal (Hadizadeh et al., 2009), antiviral (Singh et al., 2013), antioxidant (Dey et al., 2012), and antimalarial properties (Sharma et al., 2005).

Endophytic fungi are important components of the plant micro-ecosystem and ubiquitously inhabit healthy plant tissues. These fungi and their secondary metabolites have extensive applications in pharmacology and agriculture (Alurappa et al., 2014). Since the discovery of the anticancer compound taxol from the endophytic fungus *Taxomyces andreanae* in Pacific yew (Stierle et al., 1995), numerous antiviral, antitumor, and antimicrobial compounds have been isolated from plant-associated endophytic fungi. In terms of antimicrobial activity alone, dozens of bioactive compounds have been identified, including altersolanol A, enfumafungin, colletotric acid, jesterone, hydroxy-jesterone, guignardic acid, pestacin, and isopestacin (Gupta et al., 2020), suggesting that endophytic fungi may play a significant role in the synthesis of bioactive substances in medicinal plants.

Previous studies have reported endophytes and their bioactive compounds from *N. indicum* (Huang et al., 2007, 2008; Vallabhbhai, 2008; Ma et al., 2017a, b; Ren et al., 2016; Zheng et al., 2020; Zhu et al., 2020; Ramesha et al., 2013). However, fungal identification in these studies was primarily based on morphological characteristics. Since many endophytic fungi do not produce spores, morphological data alone cannot accurately reflect the true diversity of fungi in *N. indicum*. Furthermore, no studies have employed molecular systematic approaches to investigate the diversity of endophytic fungi in this plant. Additionally, while reported bioactivities of endophytic fungi from *N. indicum* include antitumor, antioxidant, insecticidal, anti-phytopathogenic fungal, and anti-human pathogenic activities, there have been few reports on activity against aquatic pathogens.

Guangxi is located in a subtropical region with favorable environmental conditions that support numerous medicinal plants of high pharmacological value. Investigating the diversity of endophytic fungi in medicinal plants from this region can provide a reference for systematically evaluating the relationship between geographical origin and medicinal efficacy. As a globally distributed medicinal plant, *N. indicum* presents an opportunity to address several questions: What

is the actual diversity of its endophytic fungi? What types of endophytic fungi are present? How are they distributed across different tissues? Moreover, with strict restrictions on antibiotic use in aquaculture and the continuous emergence of new aquatic pathogens and multidrug-resistant strains, endophytic fungi from *N. indicum* represent an important resource for discovering novel antimicrobial compounds. Can we obtain new antimicrobial strains and compounds that inhibit aquatic pathogens? This study aims to isolate and identify endophytic fungi from *N. indicum* in Qinzhou, Guangxi, and screen them for antibacterial activity, with the goal of obtaining strains active against aquatic pathogenic vibrios and laying a foundation for developing novel antimicrobial agents.

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## 1. Materials and Methods

**1.1 Sample Collection and Pretreatment** *Nerium indicum* samples were collected from Qinbei District, Qinzhou City, Guangxi, and identified by experts as *Nerium indicum* from the genus *Nerium*. Healthy fresh stem segments and leaf tissues were selected and washed with tap water to remove surface soil and dust. Surface sterilization was performed in a sterile workstation: samples were first soaked in 75% ethanol for 3–5 minutes, then transferred to 3–5% sodium hypochlorite solution for 30 seconds, and finally rinsed thoroughly with sterile water. The sterilized samples were placed in dry, autoclaved petri dishes in a laminar flow hood to air-dry before use.

**1.2 Isolation and Purification of Endophytic Fungi** The sterilized stem and leaf tissues were cut into 5 mm × 5 mm pieces using sterile scissors and placed onto PDA medium plates. The plates were incubated inverted at 28°C for 2–4 days, with regular monitoring of fungal growth. Well-growing strains were selected. When hyphae were observed emerging from the tissue edges, hyphal tips were aseptically transferred to purification medium plates. After repeated isolation and purification, strains were inoculated onto PDA slants for preservation and stored at 4–8°C.

**1.3 Morphological Identification** Isolated strains were inoculated onto PDA plates and incubated at 28°C. During the optimal growth period for each strain, colony morphology (color, size, shape, and margin), growth characteristics, mycelia, and spore morphology and surface features were observed and photographed according to the *Manual of Fungal Identification* (Wei, 1997).

**1.4 DNA Extraction** Mycelia were ground in liquid nitrogen to form a homogenate, then 600 L of CTAB extraction buffer was added and the mixture was incubated in a 65°C water bath for 45 minutes. The sample was centrifuged at 12,000 $r \cdot min^{-1}$ for 5 minutes, and the supernatant was collected. Phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed thoroughly, and

centrifuged at 10,000 r • min ¹ for 5 minutes at 4°C. The supernatant was collected, and pre-cooled isoamyl alcohol at -20°C was added and left to stand for several minutes. The mixture was centrifuged at 12,000 r • min ¹ for 5 minutes at room temperature, the supernatant was discarded, and the pellet was washed with 75% ethanol. Finally, 50 L of sterile water was added, and the DNA was stored at -20°C.

**1.5 ITS rDNA Sequence Analysis** After DNA extraction, the fungal ITS rDNA region was amplified using universal primers ITS1 (5 -TCCGTAGGTGAACCTGCGG-3 ) and ITS4 (5 -TCCTCCGCTTATTGATATGC-3 ). The PCR reaction mixture contained: ddH O 20 L, 2×Taq PCR MasterMix 25 L (Beijing Solarbio Science & Technology Co., Ltd.), 10 mol • L ¹ of each primer 2 L, and 10 ng • L ¹ template DNA 1 L. The amplification program was: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. Primer synthesis and sequencing were performed by Beijing Liuhe Huada Gene Technology Co., Ltd. The obtained sequences were subjected to homology searches in the GenBank database using the BLAST program to identify the most similar nucleotide sequences. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0 software.

**1.6 Extraction of Secondary Metabolites and Determination of Antibacterial Activity** Isolated fungi were inoculated into 100 mL of liquid PDA medium and cultured at 26–28°C with shaking at 200 r • min ¹ for 10–20 days until mycelia filled the flask. Mycelia were collected by centrifugation at 8,000 r • min ¹ and dried by air blowing at 20°C. Dried mycelia (0.3 g) were soaked in 5 mL of extraction solvent (ethyl acetate:methanol:acetic acid = 80:15:5). The extract was allowed to evaporate, and the residue was dissolved in 500 L of ethyl acetate. Indicator bacteria *Bacillus cereus*, *Staphylococcus aureus*, and *Vibrio diabolicus* were purchased and maintained in our laboratory, while *Vibrio alginolyticus* and *Vibrio campbellii* were isolated from diseased fish and shrimp. All indicator strains were inoculated into LB liquid medium and cultured overnight at 30–35°C with shaking at 200 r • min ¹. For antibacterial activity testing, 200 L of bacterial culture was mixed with agar using the pour plate method. The filter paper disc method was used to determine antibacterial activity by observing the presence and size of inhibition zones and measuring their diameters (Gong et al., 2018). Ethyl acetate-soaked filter paper discs served as negative controls, while positive controls used filter paper discs soaked in 50 g • mL ¹ kanamycin and chloramphenicol.

## 2. Results

**2.1 Morphological Identification of Endophytic Fungi** A total of 19 endophytic fungal strains were isolated and purified from stem segments and leaf surfaces of *N. indicum*, with representative colony morphologies shown in Figure 1. Based on colony characteristics, mycelial morphology, and spore features, preliminary identification was performed according to the *Manual of Fungal Identification*, classifying the strains into 7 genera. Twelve strains were obtained from leaf tissues (63.16%), belonging to *Colletotrichum* spp., *Guignardia* spp., *Phyllosticta* spp., *Neofusicoccum* sp., and *Aspergillus* spp. Seven strains were isolated from stems, belonging to *Colletotrichum* spp., *Nothophoma* sp., and *Diaporthe* sp., accounting for 36.84% of the total isolates (Table 1). These results indicate that endophytic fungi are widely distributed in both stem and leaf tissues of *N. indicum*.

**Figure 1.** Colony morphology of some endophytic fungi isolated from *Nerium indicum*.
A. Strain ye-130; B. Strain ye-134; C. Strain ye-135; D. Strain ye-136; E. Strain jing-116; F. Strain jing-117; G. Strain jing-119; H. Strain jing-180.

**Table 1.** Composition of endophytic fungi in different tissues of *Nerium indicum*

| Tissue | Taxa Number | Isolation Frequency (%) | Total Isolation Frequency (%) |
|---|---|---|---|
| Leaf | *Colletotrichum* spp. | | |
| | *Guignardia* spp. | | |
| | *Phyllosticta* spp. | | |
| | *Neofusicoccum* sp. | | |
| | *Aspergillus* spp. | | |
| Stem | *Colletotrichum* spp. | | |
| | *Nothophoma* sp. | | |
| | *Diaporthe* sp. | | |

**2.2 Molecular Identification of Endophytic Fungi from *Nerium indicum*** PCR amplification and ITS sequencing of the 19 endophytic fungal strains, followed by BLASTn analysis, revealed that all strains belonged to the phylum Ascomycota, encompassing 5 orders and 7 genera, with ITS sequence

similarities to GenBank entries exceeding 98% (Table 2). A phylogenetic tree constructed based on ITS sequences using MEGA 7 software showed six major clades. Strains ye-127, ye-129, ye-130, ye-131, ye-132, ye-133, and ye-134 all belonged to the order Botryosphaeriales, forming a clade with *Guignardia alliacea*, *Guignardia musicola*, and *Phyllosticta fallopiae*. Strains jing-114, jing-115, jing-116, jing-118, jing-179, ye-126, and ye-128 belonged to the order Glomerellales, clustering with *Colletotrichum fructicola* and *Colletotrichum citri-maximae*. Strain jing-117 formed a clade with *Neofusicoccum parvum*. Strain jing-180 belonged to the order Diaporthales and was closely related to *Diaporthe* sp. Strains ye-135, ye-136, and jing-119 formed a large clade, with jing-119 being closely related to *Nothophoma anigozanthi*. Both ye-135 and ye-136 belonged to *Aspergillus*, with ye-135 closely related to *Aspergillus neoellipticus* and ye-136 to *Aspergillus tamarii*.

**Table 2.** BLASTn analysis of ITS sequences of endophytic fungi from *Nerium indicum*

| Strain | Most Similar Type by BLASTn | Accession No. | Maximum Identity (%) |
|---|---|---|---|
| ye-126 | *Colletotrichum fructicola* | NR144783 | |
| ye-127 | *Guignardia musicola* | NR137716 | |
| ye-128 | *Colletotrichum fructicola* | NR144783 | |
| ye-129 | *Phyllosticta capitalensis* | NR144914 | |
| ye-130 | *Guignardia musicola* | NR137716 | |
| ye-131 | *G. alliacea* | AB454263 | |
| ye-132 | *Phyllosticta capitalensis* | NR144914 | |
| ye-133 | *P. capitalensis* | NR144914 | |
| ye-134 | *Guignardia alliacea* | AB454263 | |
| ye-135 | *Aspergillus neoellipticus* | AB250395 | |
| ye-136 | *A. tamarii* | MH854614 | |
| jing-114 | *Colletotrichum fructicola* | NR144783 | |
| jing-115 | *C. fructicola* | NR144783 | |
| jing-116 | *C. citri-maximae* | NR160823 | |
| jing-117 | *Neofusicoccum parvum* | NR119487 | |
| jing-118 | *Colletotrichum fructicola* | NR144783 | |
| jing-119 | *Nothophoma anigozanthi* | NR135992 | |
| jing-179 | *Colletotrichum fructicola* | NR144783 | |
| jing-180 | *Diaporthe* sp. | KU557563 | |

**Figure 2.** Phylogenetic tree of ITS rDNA sequences of endophytic fungi from *Nerium indicum* constructed using the neighbor-joining method.

**2.3 Distribution of Endophytic Fungi in *Nerium indicum* Tissues** Among the isolated endophytic fungi, the dominant genera were *Guignardia* and *Colletotrichum*. *Colletotrichum* comprised 7 strains with an isolation frequency of 36.85%, primarily distributed in stem segments. *Guignardia* comprised 4 strains with an isolation frequency of 21.05%, all isolated from leaf tissues. *Phyllosticta* was represented by 3 strains (15.79% isolation frequency), and *Aspergillus* by 2 strains (10.53% isolation frequency), both exclusively from leaf tissues. Additionally, one strain each of *Neofusicoccum* was isolated from leaves, and *Nothophoma* and *Diaporthe* were isolated from stems (Table 3).

**Table 3.** Distribution of endophytic fungi in different tissues of *Nerium indicum*

| No. | Genus | Species | Leaf | Stem |
|---|---|---|---|---|
| | *Colletotrichum* | *C. fructicola* | | |
| | | *C. citri-maximae* | | |
| | *Guignardia* | *G. musicola* | | |
| | | *G. alliacea* | | |
| | *Phyllosticta* | *P. capitalensis* | | |
| | *Neofusicoccum* | *N. parvum* | | |
| | *Nothophoma* | *N. anigozanthi* | | |
| | *Diaporthe* | *Diaporthe* sp. | | |
| | *Aspergillus* | *A. neoellipticus* | | |
| | | *A. tamarii* | | |

**2.4 Analysis of Antibacterial Activity of Endophytic Fungi** Representative isolated strains were selected for liquid fermentation. Mycelia and fermentation broth were dried and extracted. The extracts were tested against *Bacillus cereus*, *Staphylococcus aureus*, *Vibrio alginolyticus*, *Vibrio campbellii*, and *Vibrio diabolicus*. The results showed that jing-116, ye-135, and ye-136 exhibited strong inhibitory effects against *B. cereus*, with inhibition zones of 7.98 mm, 9.29 mm, and 15.36 mm, respectively. Strain ye-134 showed inhibitory activity against *V. alginolyticus* with an inhibition zone of 6.65 mm. Strains jing-117, ye-130, and ye-136 inhibited *V. campbellii* with inhibition zones of 8.28 mm, 8.33 mm, and 9.74 mm, respectively. These findings indicate that antibacterial endophytic fungi are distributed across different tissues of *N. indicum*, with slightly higher antibacterial activity observed in leaf-derived fungi compared to those from stems.

**Table 4.** Antibacterial activity of isolated endophytic fungi

| Strain Name | *Bacillus cereus* | *Staphylococcus aureus* | *Vibrio alginolyticus* | *Vibrio campbellii* | *Vibrio diabolicus* |
|---|---|---|---|---|---|
| jing-116 | ++ | - | - | - | - |
| jing-117 | - | - | - | ++ | - |
| ye-130 | - | - | - | ++ | - |
| ye-134 | - | - | + | - | - |
| ye-135 | ++ | - | - | - | - |
| ye-136 | ++ | - | - | ++ | - |

Note: ++ indicates inhibition zone diameter > 8.0 mm; + indicates 0.0 mm < inhibition zone diameter < 8.0 mm; - indicates no antibacterial activity; filter paper disc diameter was 6.0 mm.

## 3. Discussion and Conclusion

This study isolated 19 endophytic fungal strains from leaves and stems of *N. indicum*. Morphological and ITS sequence analyses revealed they belong to 7 genera, with dominant taxa including *Colletotrichum*, *Guignardia*, and *Phyllosticta*, along with *Neofusicoccum*, *Aspergillus*, *Nothophoma*, and *Diaporthe*. While *Colletotrichum* has been reported in previous studies of *N. indicum* endophytes (Huang et al., 2007; Ramesha et al., 2013), the other six genera have rarely been mentioned, likely because *Guignardia*, *Phyllosticta*, *Neofusicoccum*, *Aspergillus*, *Nothophoma*, and *Diaporthe* produce few spores, making accurate morphological identification difficult. For example, Huang et al. (2007) isolated 42 endophytic fungi from *N. indicum* and identified only *Colletotrichum*, *Chaetomium*, and *Cladosporium* based on morphology. Similarly, Ramesha et al. (2013) isolated 28 endophytic fungi from *N. indicum* in India and described only *Fusarium semitectum*, *Alternaria*, *Colletotrichum gloeosporioides*, and *Mycelia sterilia*—taxa relatively easy to identify morphologically. Our study combined morphological and molecular approaches to accurately identify the fungal taxa and their tissue distribution, providing the first precise reference data for endophytic fungal diversity in *N. indicum*.

We identified six active strains (jing-116, jing-117, ye-130, ye-134, ye-135, ye-136), most of which with strong antibacterial activity (ye-130, ye-134, ye-135, ye-136) were isolated from leaves. This is notable because cardiotonic glycosides and triterpenoid saponins—major bioactive compounds of *N. indicum*—are primarily concentrated in leaves, suggesting that endophytic fungal activity may be associated with host plant characteristics (Radu & Kqueen, 2002). This implies that isolating endophytic fungi from medicinally important plant tissues may yield higher chances of discovering active strains (Gouda et al., 2016). Additionally, multiple fungal strains within the same genus showed strain-specific

antibacterial activity. For instance, ye-127, ye-129, ye-130, and ye-132 had identical ITS sequences and all belonged to *Guignardia*, yet only ye-130 exhibited antibacterial activity. Similarly, jing-115 and jing-116 (both *Colletotrichum* with identical ITS sequences) differed in activity, with only jing-116 showing inhibition. This strain-specificity is consistent with findings by Phongpaichit et al. (2006) and suggests that increasing the number of isolates during endophyte screening would enhance the probability of obtaining more antibacterial strains.

Among the antibacterial strains identified, ye-134 (*Guignardia* sp.) inhibited *Vibrio alginolyticus*, while jing-117 (*Neofusicoccum* sp.), ye-130 (*Guignardia* sp.), and ye-136 (*Aspergillus* sp.) inhibited *Vibrio campbellii*. *V. campbellii* is a newly identified pathogenic vibrio that has emerged in recent years (Dong et al., 2017), highlighting the promising application potential of our findings. While antibacterial compounds against *Vibrio anguillarum*, *Vibrio parahaemolyticus*, and *Vibrio harveyi* have been reported from *Aspergillus* species (Guo et al., 2016; Zhu et al., 2018; Guo et al., 2019), no anti-vibrio compounds have been documented from *Neofusicoccum* or *Guignardia*. *Neofusicoccum* is a plant pathogen comprising approximately 50 species, from which nine classes of compounds have been identified, including cyclohexenones, 5,6-dihydro-2-pyrones, mycotoxins, naphthoquinones, phenols, alcohols, and sesquiterpenes (Salvatore et al., 2021). *Guignardia* species have also been reported to exhibit antibacterial activity as endophytes in other plants (Gong et al., 2014). Future studies focusing on the active compounds from ye-134, ye-130, and jing-117 may lead to the discovery of novel antimicrobial agents against aquatic pathogens.

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