Isolation, anticancer potency and camptothecin – producing ability of endophytic fungi isolated from Ixora chinensis

Nhung Thi Doan
VAST Institute of Biotechnology
Dung Thi Le
VAST Institute of Biotechnology
Anh Ngoc Ho
VAST Institute of Biotechnology
Thuong Thi Ho
VAST Institute of Biotechnology
Thao Thi Do
VAST Institute of Biotechnology
Ha Hoang
VAST Institute of Biotechnology
Mau Hung Nguyen
VAST Institute of Biotechnology
Thanh Mai Bui
VAST Institute of Biotechnology
Hoang Ha Chu (chuhoangha@ibt.ac.vn)
VAST Institute of Biotechnology https://orcid.org/0000-0001-6612-7440

Research Article

Keywords: Endophytic fungi, Ixora chinensis, camptothecin, anticancer activity

Posted Date: August 31st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3274704/v1

Abstract

Purpose

Camptothecin (CPT) is an important alkaloid used for anticancer treatment. It is mainly produced by two endangered and overharvested *Camptotheca acuminata* and *Nothapodytes nimmoniana* plants. Endophytic fungi are promising alternative sources for CPT production. This study aimed to evaluate the distribution of fungal endophytes in *Ixora chinensis*, their cytotoxic properties and camptothecin – producing ability.

Methods

In the present study, fungi residing within explants of *Ixora chinensis* were isolated and their CPT-producing capability of their endophytes was verified via TLC, HPLC, LC-HRMS and NMR analyses and compared with standards. In addition, MTT and SRB assays were selected to test the anticancer effect.

Results

The endophytic fungi collection of 62 isolates were assigned to 11 genera, with four common genera (*Diaporthe*, *Phyllosticta*, *Colletotrichum*, and *Phomopsis*) and seven less common genera (*Penicillium*, *Botryosphaeria*, *Fusarium*, *Pestalotiopsis*, *Aspergillus*, and *Didymella*). Moreover, the anticancer activity of extracts was assessed against human lung carcinoma (A549). Among 8 potential extracts, only *Penicillium* sp. I3R2 was found to be a source of CPT, while the remaining seven extracts have not been detected to contain CPT. Thus, other prominent endophytic fungi might be potential candidates of phytochemicals with anticancer properties.

Conclusions

The present study identified novel endophytic fungi from *I. chinensis* as prospective sources for camptothecin and other potential bioactive compounds.

Introduction

Camptothecin (CPT) is an isoquinoline alkaloid first discovered in the stem bark of *Camptotheca acuminate* (Wall et al. 1966). This bioactive compound has demonstrated its potential as an antitumor agent by inhibiting topoisomerase I interaction, ultimately leading to cell death (Wall et al. 1966; Takimoto 2002; Venditto and Simanek 2010; Saravanana and Boopalan 2011; Li et al. 2017; Uzma et al. 2018). Currently, 43 plant species belonging to 8 different families have been shown to be able to produce CPT. Nearly a half of CPT-producing plant species are members of the Icacinaceae family, and 28% belong to the Rubiaceae family. However, the primary commercial production of CPT still relies on *Camptotheca acuminate* and *Nothapodytes nimmoniana* (Kusari et al. 2009).

An estimated 10 million cancer-related deaths were reported in 2020 (Sung et al. 2021), while patients had to face a significant financial burden ranging from $1,500 to $22,000 per year for cancer treatments (Siddiqui and Rajkumar 2012). The cost is believed to continue rising due to the sheared budget for cancer drugs, new research, and clinical trials (Siddiqui and Rajkumar 2012). At present, approximately one ton of raw CPT material is required for the global market each year (Asano et al. 2004). This demand brings *C. acuminate* and *N. nimmoniana* under threat due to overharvesting, driven by the need to fulfill the CPT requirement for the clinical market. It is crucial to explore diverse alternative sources of CPT to prevent these medicinal plants from extinction.

Besides medicinal plants, endophytic fungi are also a potential source of CPT (Amna et al. 2006). In recent decades, many endophytes have been reported to have CPT-producing capabilities such as *Fusarium solani* strain ATLOY-8 (Clarance et al. 2019), *Meyerozyma* sp. OmF3 and *Talaromyces* sp. OmF4 (Aswani et al. 2020), *Diaporthe* sp. F18 (Degambada et al. 2023), *Aspergillus flavus* ER, and *Aspergillus terreus* (El-Sayed et al. 2022). The CPT biosynthesis pathway in endophytes has not yet been reported; however, a recent study has provided evidence to support that some endophytes are CPT producers independent of their host plant (Mohinudeen et al. 2021). These findings have addressed the constraints on CPT resources and cleared doubts about endophytic capabilities. Due to their rapid growth rate and adaptability for large-scale fermentation, their strains can be economically produced. Unfortunately, commercial CPT products from endophytes have not been successful, and endophytic fungi are inconsistent producers with lost or diminished CPT capability across generations, except for the *Alternaria burnii* NCIM1409 strain isolated from *Nothapodytes nimmoniana* (Mohinudeen et al. 2021).

Despite its widespread cultivation or natural growth in Southeast Asia and its frequent use used as a native medicinal plant, there is a lack of available publications about endophytes associated with *Ixora chinensis* plant (Chen et al. 2003; Khare 2007). In addition, the flower extract of *I. chinensis* has been proven to completely inhibit specific tumors (Rastogi et al. 1990; Chatwal 2007). Thus, with the aim of exploring alternative CPT resources, our research identified all endophytes from the *Ixora chinensis* plant and evaluated their CPT-producing potential.

Materials and methods

Sample collection

I. chinensis plants are wild-growing shrubs distributed in Bac Giang Province, Northern Vietnam ($21.^{07^{\circ}}$ – 21.37°N, $105.^{53^{\circ}}$ – 107.02°E). Sample collection was performed during June 2020 at the flowering time of these plants. All parts of the plant (flower, leaf, stem, root) were collected from eight different healthy

trees and deposited in separated zip lock bags and kept at 4°C before being brought to the laboratory for future studies at the National Key Laboratory of Gene Technology, Institute of Biotechnology (IBT)- Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam (Supplementary Figure S1).

Plant material surface sterilization

Shoot and root systems were separated before being washed under running water and then with deionized water to eliminate impurities and debris. Then, the shoot system was divided into three parts: stem, leaf, and flower. Each part underwent a similar surface sterilization process adapted according to a previous study (Li et al. 2017), in which ethanol and sodium hypochlorite were used to abolish the epiphytes. In detail, the plant materials were soaked in 75% ethanol for 3 mins, followed by sodium hypochlorite for 3 mins. These explants were cut into fragments of two to three centimeters and then soaked in 75% ethanol for one minute before being rinsed with sterile water three times for 3 mins each time. Finally, plant fragments were cut into small segments measuring 5 mm (length) x 5 mm (width) for fungal endophyte isolation after being air-dried on sterilized filter paper by sterile flow.

Fungal endophyte isolation

Fungal endophytes were isolated according to a previously described protocol (Shweta et al. 2013). After sterilization, the treated explants were placed on PDA (TM media, No. M3D1LT01, India) Petri plates with the addition of antibacterial agents (penicillin 0.05% and streptomycin 0.1%) and cultivated at 28 ± 2°C for 20 days in the dark. The Petri dish was sealed using Parafilm (Bemis-PM966). To confirm the success of the sterilization process, 100 µl aliquots of water from the last washing step were plated on Luria–Bertani (LB) agar and potato dextrose agar (PDA) media for seven days under optimized conditions of 28 ± 2°C. The effectiveness of the surface sterilization protocol was presented by the absence of any observable colonies, indicating that epiphytic microbes were completely removed (Chow, Rahman & Ting 2017). The fungal hyphae emerging from plant tissue were observed every day for 20 days of cultivation to check the growth of fungal endophytic colonies from small explants. The hyphae were subcultured onto a new PDA plate to attain the isolated fungal strains at their early stage. The purified colony was stored in glycerol at 50% (v/v) and deposited at -80°C for long-term storage. The endophytic strain was characterized by analyzing the morphologies of the colony, hyphae, mycelium, and reproduction features through observation and measurement under an optical microscope (Axiostar, Carl Zeiss, No. 45718). Fungal mycelium and spores were stained with lactophenol cotton blue to visualize their structures (Brathen et al. 2015; Impullitti & Malvick 2012).

Fungal DNA extraction and amplification

Genomic DNA of endophytic fungi isolated from *I. chinensis* was extracted by the AllPrep Fungal DNA/RNA/Protein Kit (Qiagen) according to the manufacturer's recommendation. The genomic DNA was used as a template for amplification of the ITS region for taxonomic identification. The primer set (ITS4 forward primer-ITS5 reverse primer) designed by White and his coworkers was used to completely cover the ITS1-5.8S-ITS2 region (White et al. 1990). The PCR reaction comprised 100 ng genomic DNA, 25 µL DreamTaq Green PCR Master Mix (2X), 0.5 µL forward primer, and 0.5 µL reverse primer. Sterile deionized water was added to bring the reaction mixture to a final volume of 50 µl. The PCR programs were set up as follows: preheating at 94°C for 3 min, 35 cycles of 94°C for 30 s, annealing at 58°C for 20 s, extension at 72°C for 1 min, and final extension at 72°C for 10 mins. The resulting PCR products were then subjected to electrophoresis on a 1.5% agarose gel at 80 V for 50 min. Predicted DNA fragments were collected, purified by the GeneJET PCR Purification Kit (Thermo Fisher), and sequenced using the BigDye Terminator Cycle Sequencing Kit v 3.1 kit (Applied Biosystems) on the capillary system ABI Prism 3500 XL sequencer (Applied Biosystems) at the National Key Laboratory of Gene Technology (IBT-VAST, Hanoi, Vietnam).

Molecular phylogenetics of endophytic fungi

The acquired DNA sequences were qualified, and a consensus was constructed using BioEdit software version 7.0.5.3 (Hall 1999). The consensus sequences were then compared to the available fungal sequences by using the online BLAST tool from the NCBI/GenBank database (National Center for Biotechnology Information -http://www.ncbi.nlm.nih.gov/BLAST/).

To determine the taxonomy of the fungal strains, internal transcribed spacer (ITS) regions including 5.8S rDNA from isolates and reference strains were aligned by Clustal X software (Thompson et al. 1997). The neighbor-joining method was used to create a phylogenetic tree (Saitou and Nei 1987). The percentage of replicate trees, displayed adjacent to the branches, represent the associated taxa determined through bootstrap analysis (Felsenstein 1985) with 1000 replicates in Mega X, and the sequences were uploaded to NCBI (Kumar et al. 2018).

Fermentation and extraction of CPT

The liquid fermentation was prepared in two steps. First, the seed medium was incubated with 50 ml liquid medium without agar in a 250 ml flask at 28 ± 2°C on a rotary shaker at 160 rpm for 3 days to inoculate the endophytic fungal strains. Then, the seed liquid was transferred into the fermentation medium to a total of 250 ml under the same conditions for 7 days. After liquid cultivation, the whole culture of each strain was transferred into a 250 mL Erlenmeyer flask and percolated with 100 mL of dichloromethane (DCM) and methanol (MeOH) with a 9:1 (v:v) ratio solvent mixture and sonication (33 MHz, Roop Telesonic, India) at room temperature for 60 mins. The process was repeated twice for complete extraction (Pu et al. 2013). After sonication, the extracts were filtered through 25 µm pored paper and then evaporated to dryness in vacuo (Buchi evaporator system R-300 Rotavapor, V-300 vacuum pumper, B-300 heating bath). To determine CPT content, the concentrate was weighed and transferred into polypropylene microcentrifuge tubes, mixed with HPLC grade DCM/MeOH (3:1, v:v) at a concentration of 50 mg/mL, and vortexed for 30 s, followed by centrifugation at 11000 rpm for 2 mins. The clean supernatants were applied directly onto HPLC (Ran et al. 2017) and TLC screening.

SRB and MTT assays

SRB and MTT assays were carried out to evaluate anticancer activities of the plant extracts and derived compounds based on the optical density (OD) measured when the protein composition of the cells was stained with sulforhodamine B (SRB). The human lung carcinoma A549 cell line was chosen for these experiments. First, the SRB assay was performed by trypsinizing experimental cells to separate them, followed by cell counting in a counting chamber to

adjust the density. Then, 190 µL of cells were added to a 96-well plate for testing. The fermented extracts were dissolved in 100% DMSO to obtain a stock concentration of 20 mg/mL. The samples on a 96-well plate were diluted with cell culture medium (without FBS) into 4 ascending concentrations. Reagents diluted at various concentrations (10 µL) were introduced into the wells of the pre-prepared 96-well plate containing cells. Wells without reagent but supplemented with TBUT (190 µL) and DMSO 1% (10 µL) were used as the no-growth control (day 0). After 1 hour, cells in day 0 control wells were fixed with 20% trichloroacetic acid (TCA).

The MTT assay was conducted in similar manner to the SRB assay, with slight modifications. The fermented extracts were dissolved in 100% DMSO to obtain a stock concentration of 20 mg/mL. The samples on a 96-well plate were diluted with cell culture medium (without FBS) into 4 ascending concentrations. Reagents diluted at various concentrations (10 µL) were introduced into the wells of the pre-prepared 96-well plate containing cells. In the same 96-well plate, the control wells received only 10% DMSO without the extract. Only culture medium without cells was added to the wells, and samples were considered blank wells. The cultured dishes were placed in an incubator at 37°C with 5% CO_2 for 72 hours. After adding 10 mM unbuffered Tris base, the absorbance of each well was measured at 540 nm. The percentage of cell growth inhibition in the presence of reagents was determined using the following formula:

$$\mathbf{Percentageinhibition} = 100\backslash\mathrm{varvec}\% - \frac{\backslash\mathrm{varvec}O\backslash\mathrm{varvec}D\left(\backslash\mathrm{varvec}t\backslash\mathrm{varvec}h\backslash\mathrm{varvec}e\backslash\mathrm{varvec}e\backslash\mathrm{varvec}x\backslash\mathrm{varvec}t\backslash\mathrm{varvec}r\backslash\mathrm{varvec}a\right.}{\backslash\mathrm{varvec}O\backslash\mathrm{varvec}D\left(\backslash\mathrm{varvec}D\backslash\mathrm{varvec}M\backslash\mathrm{varvec}S\backslash\mathrm{varvec}O\right) - \backslash\mathrm{va}}$$

TLC screening of fungal CPT

The presence of active metabolites in the extract was evaluated by TLC. Normal-phase TLC was performed on precoated aluminum plates (TLC silica gel 60 F254 Supelco), dried at room temperature for 30 mins, and activated in an oven at 110°C for the next 30 mins. The solvent system was DCM:MeOH (95:5). Approximately 2 µl of extract (50 mg/mL) was spotted on silica plates. The experimental plates were developed in a chromatographic chamber saturated with 5 ml of the solvent system. Plate development required approximately 5 to 7 mins; plates were then visualized at 254 nm under a UV illuminator (Cleaver). Rf values of bands were recorded using a formula and were compared with a control of 2 µl CPT at 1 mg/ml concentration (Valletta et al. 2007).

HPLC analysis

Quantification of CPT was performed by following a previously described method (Murthy et al. 2019). Gradient analytical HPLC assays were performed on an Agilent 1100 instrument, and 10 µL of supernatant extract was loaded onto an octadecyl silane (ODS Agilent) (5 µm; Inertsil) column (150 × 4.6 mm). Acetonitrile:water (20:80 to 100:00 in 30 mins) was eluted at a flow rate of 0.6 mL/min, and the alkaloids were detected at 366 nm by a UV detector (DAD). The peak areas corresponding to CPT were integrated by comparison with external standard calibration curves (Pu et al. 2013). HPLC assays of different extracts yielded chromatograms with a retention time of 17.7 min for CPT. Validation of the quantitative method was performed three times with samples. The results of the three injections from the same samples at the five concentrations (0.1–10 mg/mL) showed similar retention times. The standard deviation showed the accuracy of the quantitative method and the ability to determine the CPT content. The concentration of CPT in the cultured liquid was calculated from the same volume of fungal liquid culture.

HRMS and NMR verification of endophytic fungi CPT production

Based on the screening results, potential samples, including the CPT compound, were verified by HRMS analysis using an electrospray ionization (ESI) source in positive mode. Extract samples were dissolve in DCM:MeOH (05:95, v/v) with the concentration 5 mg/ml. The system was set up at 300°C, and high-purity nitrogen was used to construct curtain gas (25 psi) chambers. The capillary voltage was constantly kept at 5500 V. The collision energy was set at 10 V, and the collision energy spread was zero. We used IDA mode to scan mass range from 300 to 400 (m/z). The sample was analyzed by an LC-HRMS system (J'phere column 4.6 ×150 mm, flow rate of 0.5 ml/min, and 60% ACN in water). The data were recorded on an Agilent QTOF 6530. Moleculars of CPT were found using Agilent MassHunter Qualitative Analysis B.07.00.

For NMR verification, a 70 L fermentation tank containing PBD media was prepared, and I3R2 was cultured for 7 days at 180 rpm and 28°C. CPT was extracted using the protocol mentioned above and purified via HPLC system (Agilent HPLC 1290 Infinity II) by collecting the fraction of the peak as CPT. Finally, 4 mg of purified CPT (90%) was dissolved in CDC_{l3} for ^{1}H and ^{13}C structural elucidation. 1D NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) using TMS (tetramethylsilane) as an internal standard.

Preparation of the standard solution of CPT

A stock solution of CPT (Sigma Aldrich, CAS: 7689-04-4) was prepared by dissolving 5 mg of accurately weighed CPT in a DCM:MeOH mixture (3:1, v:v) and bringing the volume to 500 µL with methanol. From this stock solution, standard solutions of 0.1, 0.5, 1, 5, and 10 mg/mL were prepared by transferring aliquots of stock solution to a 1.5 ml microtube and adjusting the volume with a DCM:MeOH mixture (3:1, v:v). The calibration curve was calculated by injecting 10 µL of standard solutions of CPT into the column (Murthy 2019). All analysis experiments were carried out in triplicate. The peaks were detected at 366 nm and the peak areas were recorded. Calibration curves of CPT were prepared by plotting peak area against concentration with a correlation coefficient of R^2 = 0.9999 (Y = 778781X + 33712).

Results

Identification and colony characterization of endophytic fungi in I. chinensis

Fungal endophytes were isolated from explants of *I. chinensis* following the elimination of epiphytes through the surface sterilization process. Sixty-two isolates were obtained from distinct morphological analyses on PDA medium (Fig. 1, Table S1).

Figure 1. Morphological characteristics of isolated endophytic fungi from *I. chinensis*.

(A) Colony morphology of 62 isolated endophytes. (B) Typical colony types, the left side: vegetative hyphae, the right side: aerial hyphae; (B-1): the white cottony type; (B-2): the green powdery type; (B-3): the black spongy type; (C) Septate hyphae; C1: *Phomopsis sp*. I2T2; (C-2): *Diaporthe* sp. I5T3. (D) Conidiophore and conidial structure; D1: *Pestalotiopsis* sp. I1L3; (D-2, D-3): Colletotrichum sp. I1L2; (D-4): *Penicillium* sp. I3R2.

The colony morphological characteristics including aerial mycelia, vegetative mycelia, pigmentation, form, and the margin of the colony, were first used to distinguish and identify the group of fungi. The results showed that 62 isolated fungal endophytes were assigned to four morphotypes, encompassing the green powdery, black spongy, white cottony groups and a group comprising the remaining isolates (Fig. 1). Microfeatures such as hyphal structures, conidiophores, conidia, and spore morphologies were combined with molecular identification to classify isolated endophytic fungi (Fig. 1, Table S1). Hyphal structures showed a septate type with a difference in size, such as 36.3 ± 13×6,3 ± 2,2 µm for the I2T2 strain and 12.3 ± 1.6×5.3 ± 1.1 µm for the I5T3 strain (Fig. 1). The reproduction of fungal endophytes on the PDA plate occurred by fragmentation, as in *Pestalotiopsis sp.* I1L3, or by producing spores from the tips of hyphae (e.g., *Colletotrichum sp.* I1L2) or from conidiospores (e.g., *Penicillium sp.* I3R2) (Fig. 1D).

In addition, the endophytes were identified by ITS sequencing analysis. In detail, ITS consensus sequences containing ITS1, 5.8S and ITS2 regions were blasted against the NCBI database to obtain the closest matches. The results were compared with DNA sequences of types or epitypes and morphology characteristics of the specific taxon. The phylogenetic tree was also constructed using the ITS sequences of endophytic fungal strains (Fig. 2F), and the data were matched with morphological results (Fig. 1, Table S1). Finally, in the present study, 62 endophytic fungal strains isolated from *I. chinensis* were classified into 11 genera (Table S1). The results indicated that most species isolated from *I. chinensis* belong to four common genera: *Diaporthe* (18 strains), *Phyllosticta* (13 strains), *Colletotrichum* (11 strains), and *Phomopsis* (7 strains). For seven less-common genera, the colonization frequency of *Penicillium* was 4 strains, followed by *Botryosphaeria* (2 strains), *Fusarium* (2 strains), *Pestalotiopsis* (2 strains), *Lasiodiplodia* (1 strain), *Aspergillus* (1 strain), and *Didymella* (1 strain) (Table S1). The identified endophytes were consistent between morphological and ITS analyses and were deposited in glycerol (50% v/v) at -80°C at the National Key Laboratory of Gene Technology (IBT-VAST, Hanoi, Vietnam) for long-term storage. In addition, all strains were grown for the collection of crude extracts in the following studies.

Diversity and distribution of endophytic fungi from different parts of I. chinensis plants

The endophytic population from *I. chinensis* distributed unevenly among various plant tissue types (Fig. 2, Table S1). Endophytes were the most abundant in stem, with 25 strains (40%), followed by leaf with 20 strains (32%). The flowers and roots hosted a minor number of fungal strains at 13% and 15%, respectively (Fig. 2A). In addition, the distribution of specific endophytic species varied in different parts of the plant (Fig. 2B, C, D, E). *Diaporthe sp.* exhibited abundances in root and stem tissues of 56% and 52%, respectively. *Phyllosticta* emerged only in leaf and flower parts. Several genera, such as *Didymella sp.* (12%), *Pestalotiopsi sp.* (10%), *Aspergillus* sp. (11%), and *Lasiodiplodia sp.* (4%) were unique to the flower, leaf, root, and stem parts, respectively.

Figure 2. Diversity and distribution of the endophytic community in *Ixora chinensis* explants (A-E) and phylogenetic tree (F)

SRB and MTT assays

Four extracts of endophytic fungi including I1L2, I1R7, I3R2 and I6T1exhibited anticancer capability with inhibition greater than 50% at a tested concentration of 20 µg/mL in both SRB and MTT assays. However, some extracts only showed anticancer activity in the MTT assay (I3T3, I3T4, I6L2, I8T3). Among the positive strains, the extracts of I1R7, I1L2 and I6T1 indicated the strongest activity, with inhibition percentages of 90, 57 and 59.83%, respectively, in the SRB assay. Meanwhile, the inhibition percentages of these extracts were 100, 84.89 and 71.85% in the MTT assay, respectively (Table 1).

Table 1
Evaluation of the in vitro cytotoxicity of endophytic fungi

Concentration (µg/mL)	I1H1	I1L1	I1L2	I1L3	I1L4
100	32.55 ± 2.37	16.97 ± 0.45	98.29 ± 2.25	70.99 ± 0.63	6.11 ± 0.52
20 (SRB)	3.83 ± 0.34	7.91 ± 0.42	57.04 ± 2.73	21.64 ± 0.94	7.59 ± 0.65
20 (MTT)	17.25 ± 1.82	26.15 ± 0.71	84.89 ± 2.88	19.57 ± 1.4	23.97 ± 0.73
Concentration (µg/mL)	I1R1	I1R7	I2L1	I2L2	I2T1
100	3.76 ± 0.19	98.29 ± 1	14.82 ± 1.2	11.67 ± 1.62	8.66 ± 0.52
20 (SRB)	− 0.99 ± 0	90.13 ± 2.52	7.69 ± 0.36	− 1.29 ± 0.14	3.46 ± 0.16
20 (MTT)	5.36 ± 0.07	100.78 ± 1.89	10.12 ± 0.12	9.62 ± 0.34	21.46 ± 1.13
Concentration (µg/mL)	I2T2	I3L1	I3L2	I3L3	I3R2
100	51.03 ± 2.62	55.85 ± 1.92	63.05 ± 2.96	19 ± 1.15	89.82 ± 2.67
20 (SRB)	12.96 ± 1.26	33.81 ± 2.2	45.94 ± 1.16	16.82 ± 0.12	70.14 ± 2.37
20 (MTT)	43.56 ± 2.9	32.62 ± 1.63	37.49 ± 0.65	37.45 ± 0.47	64.41 ± 1.3
Concentration (µg/mL)	I3T1	I3T2	I3T3	I3T4	I4H1
100	20.9 ± 2.03	75.74 ± 1.41	23.86 ± 1.28	98.46 ± 2.96	7.42 ± 0.9
20 (SRB)	13.99 ± 1.21	0.06 ± 1.79	-1.12 ± 1.93	12.87 ± 0.64	-3.57 ± 0.13
20 (MTT)	16.29 ± 1.09	0.64 ± 0.29	52.64 ± 0.65	57.9 ± 2.67	34.53 ± 3.04
Concentration (µg/mL)	I4H2	I4L1	I4L2	I4T1	I4T2
100	45.79 ± 0.26	5.21 ± 0.19	4.6 ± 0.28	31.59 ± 2.44	2.48 ± 0.25
20 (SRB)	6.42 ± 1.01	-0.24 ± 0.03	-0.67 ± 0.05	13.14 ± 1.08	-3.39 ± 0.15
20 (MTT)	49.89 ± 2.35	34.71 ± 2.12	13.83 ± 1.19	29.42 ± 2.68	9.89 ± 0.44
Concentration (µg/mL)	I4T3	I5L1	I5L2	I5R1	I5T1
100	37.9 ± 3.14	31.95 ± 1.67	16.05 ± 0.28	9 ± 0.55	13.46 ± 1.25
20 (SRB)	3.33 ± 0.13	11.15 ± 0.18	13.05 ± 0.64	0.51 ± 0.04	7.24 ± 0.72
20 (MTT)	9.67 ± 0.63	16.32 ± 1.02	-2.75 ± 0.26	7.68 ± 0.69	45.82 ± 2.36
Concentration (µg/mL)	I5T2	I5T3	I5T4	I5T5	I6H1
100	88.17 ± 2.04	15.38 ± 0.99	6.44 ± 0.24	25.1 ± 1.5	6.28 ± 0.89
20 (SRB)	26.44 ± 2.16	-2.88 ± 0.26	0.29 ± 0.02	4.1 ± 0.41	-1.41 ± 0.13
20 (MTT)	48.12 ± 4.49	10.56 ± 1.09	8.95 ± 0.28	7.14 ± 0.43	-0.41 ± 0.05
Concentration (µg/mL)	I6L2	I6L3	I6R1	I6T1	I6T2
100	95.06 ± 6.22	2.15 ± 0.18	20.5 ± 1.01	92.54 ± 1.68	21.1 ± 2.15
20 (SRB)	32.05 ± 4.95	-0.74 ± 0.12	-1.71 ± 0.15	59.83 ± 1.96	13.16 ± 1.03
20 (MTT)	53.4 ± 0.95	10.37 ± 0.79	-1.09 ± 0.07	71.85 ± 2.31	8.92 ± 0.64
Concentration (µg/mL)	I6T3	I6T4	I6T5	I7H1	I7H2
100	6 ± 0.69	20.41 ± 1.13	30.35 ± 2.79	14.68 ± 0.13	2.16 ± 0.19
20 (SRB)	-1.64 ± 0.15	5.46 ± 0.77	4.83 ± 0.39	0.74 ± 0.3	-0.18 ± 0.02
20 (MTT)	6 ± 0.59	32.75 ± 2.98	10.47 ± 0.51	-2.93 ± 0.23	-8.42 ± 0.07
Concentration (µg/mL)	I7L1	I7L4	I7R1	I7R2	I7R3
100	5.53 ± 0.41	26.41 ± 1.27	2.52 ± 0.25	-1.27 ± 0.16	4.39 ± 0.51
20 (SRB)	0.16 ± 0.93	6.27 ± 0.63	-1.88 ± 0.15	0.49 ± 0.04	0.5 ± 0.04
20 (MTT)	3.34 ± 0.18	30.95 ± 1.45	5.44 ± 0.52	-4.36 ± 0.38	2.16 ± 0.18
Concentration (µg/mL)	I7R4	I7T1	I7T2	I7T4	I8H1
100	39.12 ± 2.48	30.62 ± 2.41	33.33 ± 2.74	48.08 ± 1.53	14.49 ± 0.36

Concentration (µg/mL)	I1H1	I1L1	I1L2	I1L3	I1L4
20 (SRB)	24.74 ± 1.86	24.44 ± 1.35	16.05 ± 1.03	33.08 ± 1.85	6.67 ± 2.06
20 (MTT)	13.91 ± 1.18	7.96 ± 0.31	33.34 ± 1.22	43.64 ± 2.78	20.17 ± 1.69
Concentration (µg/mL)	I8H2	I8L1	I8L2	I8L3	I8T2
100	9.55 ± 0.74	23.56 ± 2.3	5.51 ± 0.49	3.85 ± 0.19	31.35 ± 2.99
20 (SRB)	3.65 ± 0.26	5.67 ± 0.32	2.56 ± 0.33	1.83 ± 0.17	22.12 ± 1.36
20 (MTT)	0.84 ± 0.13	1.71 ± 0.11	5.94 ± 0.22	1.62 ± 0.12	46.62 ± 1.03
Concentration (µg/mL)	I8T3	I8T4	standard CPT		
100	35.58 ± 2.45	38.94 ± 2.31	72.01 ± 2.18		
20 (SRB)	14.42 ± 0.27	5.77 ± 0.27	64.51 ± 1.66		
20 (MTT)	55.21 ± 1.46	33.24 ± 1.87	39.67 ± 1.24		

Screening, quantification and verification of CPT-producing endophytic strains

The crude extracts from the culture liquid of endophytic strains were screened to produce CPT using TLC and HPLC analyses. First, the CPT in the sample was characterized in comparison with standard CPT (Sigma Aldrich). Then, the presence of CPT in extracts was continuously analyzed by HPLC to detect and quantify CPT from fungal endophytes. A standard curve with a correlation coefficient R2 = 0.9999 allowed us to determine the exact CPT content in the extract of endophytes (Figure S2, Table S2). In the present study, a substance with a polarity like that of the CPT standard in the TLC experiment emerged in the extracts. The crude extracts were then subjected to HPLC to give a signal with the same retention time and UV spectrum as the standard CPT R_f of 17.7 mins. These potential endophytic fungi were I1L1, I1L2, I1R1, I2T2, I3L2, I3R2, I3T2, I4H2, I5T3, I6T5, and I8T3. The potential CPT content in endophytic fungi ranged from 2.164 mg/L to 0.055 mg/L (Table 2).

Table 2
The putative CPT concentration (mg/L) was determined from endophytic fungi via TLC and HPLC analysis.

No	Strain	TLC	HPLC	Putative CPT con. (mg/L)
1	I1L1	+	+	0.088
2	I1L2	+	+	0.332
3	I1R1	+	+	0.156
4	I2T2	+	+	2.164
5	I3L1	+	+	0.071
6	I3R2	+	+	0.055
7	I3T2	+	+	0.405
8	I4H2	+	+	0.051
9	I5T3	+	+	0.112
10	I6T5	+	+	N
11	I8T3	+	+	N
N: not determined				

To confirm the presence of CPT in the extracts, the samples were analyzed by an LC-HRMS system. As a result, the presence of CPT in extracts of I8T3 and I3R2 were determined with molecular ions at m/z 349.1237 and 349.1242, respectively. These results were like that of standard CPT with a molecular ion at m/z 349.1228 (Fig. 3). Base on the recovery yield from extract of I3R2 and I8T3, the strain I3R2 was applied in scale up cultivation for further NMR analysis.

Figure 3. Identification of CPT-producing endophytic fungi from the crude extract by HRMS analysis. Standard CPT (A), CPT yield from I3R2 (B) and I8T3 (C)

The ability of the I3R2 strain to produce CPT was reconfirmed by NMR analysis. CPT was isolated and verified with the chemical shifts of ^{1}H and ^{13}C NMR spectra (Figure S3, Figure S4). ^{1}H NMR (600 MHz, $CDCl_3$) d ppm 5.31 (2 H, s, H-5) 8.40 (1 H, s, H-7) 7.94 (1 H, d, *J* = 8.4 Hz, H- 9) 7.66 (1 H, t, *J* = 7.2 Hz, H-10) 7.84 (1 H, t, *J* = 7.2 Hz, H-11) 8.24 (1 H, d, *J* = 9 Hz, H-12) 7.69 (1 H, s, H-14) 0.88 (3 H, t, *J* = 7.34 Hz, H-18) 1.90 (2 H, m, *J* = 7.2 Hz, H-19). ^{13}C NMR (600 MHz $CDCl_3$): 173.95 (C-21), 157.69 (C-17), 152.52 (C-2), 150.15 (C-15), 149.03 (C-13), 146.50 (C-3), 131.06 (C-7), 130.61 (C-11), 128.54 (C-6), 129.88 (C-12), 128.11 (C-9), 128.17 (C-8), 128.02 (C-10), 118.70 (C-16), 98.07 (C-14), 72.76 (C-20), 66.40 (C-17), 50.07 (C-5), 31.66 (C-19), 7.8 (C-18) (Table S3). The NMR spectral data of the isolated compound were in agreement with those of Sigma-grade CPT (Soujanya et al. 2017). These data suggested that the I3R2 endophytic fungi from *I. chinensis* could produce CPT in the fermentation broth during its development.

Discussion

I. chinensis is widely distributed in Southeast Asia and is considered as a promising candidate for its ability to inhibit tumors (Rastogi et al. 1990; Chen et al. 2003; Chatwal 2007; Khare 2007). However, little information has been reported related to this plant or endophytes associated with *I. chinensis*. This is the first study investigating the diverse fungal endophytes within *I. chinensis*, encompassing a total of 62 endophytic strains belonging to 11 genera (Fig. 2, Table S1). The complex of *Diaporthe* and its *Phomopsis* anamorph (*Diaporthe/Phomopsis*) reported in this study were in line with previous research (Dissanayake et al. 2017; Hosseini et al. 2020). The three frequent genera in our collection of isolates - *Diaporthe*, *Phomopsis* and *Colletotrichum* - have also been identified as dominant genera in the endophytic fungal communities of other plants, such as *Nothapodytes foetida*, *Icacinaceae* and *Camptotheca acuminata* (Amna et al. 2006; Pu et al. 2013); (Pu et al. 2015). However, *Trichoderma*, which was commonly found in other plants, was not detected in this study (Lin et al. 2007; Ding et al. 2013). In addition, the results showed that endophytes were abundant in the leaves and stems. *Pestalotiopsis*, *Phomopsis*, and *Didymella* were found only in the leaves, while *Aspergillus* was observed only in the roots (Fig. 2). These findings support the theory that variations in endophyte diversity arise from distinct geographic conditions and specific host plant species (Ding et al. 2013; Yang et al. 2018).

Although eight extracts from endophytic fungi displayed anticancer activity, only two extracts were reported as candidates for camptothecin. Moreover, only the extract from I3R2 contained camptothecin, and the extract from I8T3 might be a camptothecin analog. Since camptothecin is a topoisomerase inhibitor, the extract, which showed robust antitumor capability in the SRB assay are more likely to contain camptothecin. The secondary metabolites in the six prominent extracts could be other active anticancer compounds. Further biochemical experiments are required to identify and classify bioactives. The HPLC results revealed that eleven extracts might contain camptothecin. In this study, TLC and HPLC methods might impose certain limitations on the identification and quantification of compounds produced by endophytic fungi. Strain I2T2 exhibited a peak at a retention time and UV absorption spectrum like that of camptothecin, although the molecular mass peak of camptothecin (Fig. 3) was not detected in the HRMS spectrum. This indicates that, in addition to HPLC, further research is required to confirm the presence of CPT. Only sample from I3R2 was identified as camptothecin by NMR analysis. This emphasizes the vital role of NMR in verifying the compounds present in the extracts.

In the present study, *Penicillium* sp. I3R2 was reported as a source of CPT, with a concentration of 0.05 mg/L from biomass. The yield was lower than that previously reported for *Fusarium solani* strain ATLOY-8 (Clarance et al. 2019) and *Diaporthe caatingaensis* MT192326 (Dhakshinamoorthy et al. 2021). However, its anticancer activity was more potent than others, such as the *F. solani* MTCC9667 strain from *A. dimidiate* (Pu et al. 2013)d *chrysogenum* (El-Sayed et al. 2022). The difference might depend on the enzyme system involved in the CPT biosynthetic pathway in screening CPT-producing endophytic fungi. In addition, the distinct culture medium caused variations in CPT-producing endophytic fungi (Pu et al. 2013; Bhalkar et al. 2016). Optimized fermentation conditions activated biosynthesis-related genes for CPT biosynthesis, improved the concentration of bioactive compounds, and reduced self-toxicity during fungal metabolism (Singh et al. 2010; Liu et al. 2012). *Diaporthe* sp. F18 produced a 23-fold higher CPT content by using tryptophan in modified SDB medium (Degambada et al. 2023). In addition, the Box–Behnken design for *Fusarium solani* strain ATLOY-8 exhibited 1.4- and 1.2-fold increase in CPT production and biomass yield, respectively (Clarance et al. 2019). Therefore, subsequent studies might be designed to optimize culture conditions to increase the yield of CPT in fungal endophytes from *I. chinensis*.

Conclusion

The endophytic fungi from *I. chinensis* are abundant resources of anticancer compounds for future production of pharmaceutical agents. Sixty-two endophytic fungi were isolated, spanning 11 genera. Antiproliferative activity screening indicated that extracts from eight fungal strains, *Colletotrichum* sp. I1L2, *Aspergillus* sp. I1R7, *Penicillium* sp. I3R2, *Fusarium* sp. I6T1, *Diaporthe* sp. I3T3, *Colletotrichum* sp. I3T4, *Colletotrichum* sp. I6L2 and *Diaporthe* sp. I8T3 displayed strong inhibitory activities against cancer cell line. Camptothecin was found and confirmed in the extract of *Penicillium* sp. I3R2. In addition, the compound derived from the extract of *Diaporthe* sp. I8T3 could be a camptothecin analog. Further phytochemical study is essential to identify and explore secondary metabolites from the remaining six prospective extracts.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The participant has consented to the submission of this article to the journal. We declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. This research and the manuscript were approved by all coauthors.

Availability of data and materials

All endophytic strains from *I. chinensis* were deposited at the National Key Laboratory of Gene Technology (IBT-VAST, Hanoi, Vietnam) under the name and accession number as Supplementary Table S1. The ITS rDNA sequences of their strains were uploaded to the NCBI database by corresponding accession number.

Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Funding

This study was financially supported by the Vietnam Academy of Science and Technology under Grant number TĐCNSH.05/20-22.

Authors' contributions

TND: isolated endophytic fungi, performed molecular identification of fungi, wrote the original draft and edited the manuscript. HH and NAH: performed the extract preparation, TLC, HPLC, HMRS and NMR experiments. TMB and TDL performed the fungal isolation and fermentation experiments and curated the data. TTH contributed to data curation and data analysis and wrote the original draft. MHN: collected samples, identified and classified endophytic fungi. TDT: performed SRB and MTT assays. HHC: contributed to the experimental design, funding acquisition and revised manuscript. All authors commented on previous manuscript versions and read and approved the final manuscript.

Acknowledgments

This study was supported by the project of the Vietnam Academy of Science & Technology (VAST): "Research for the function of camptothecin composition or other substances with cancer activities in labor lever from endophytic fungi on medical plant" (project no. TĐCNSH.03/20-22).

Authors' information

[1]Institute of Biotechnology, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam.

[2]Graduate University of Science and Technology, Hanoi, Vietnam.

References

1. Amna T, Puri SC, Verma V, et al (2006) Bioreactor studies on the endophytic fungus Entrophospora infrequens for the production of an anticancer alkaloid camptothecin. Can J Microbiol 52:189–196. https://doi.org/10.1139/w05-122
2. Asano T, Watase I, Sudo H, et al (2004) Camptothecin Production by in Vitro Cultures of Ophiorrhiza liukiuensis and O. kuroiwai. Plant Biotechnol 21:275–281
3. Aswani R, Jasim B, Arun Vishnu R, et al (2020) Nanoelicitor based enhancement of camptothecin production in fungi isolated from Ophiorrhiza mungos. Biotechnol Prog 36:e3039
4. Bhalkar BN, Patil SM, Govindwar SP (2016) Camptothecine production by mixed fermentation of two endophytic fungi from Nothapodytes nimmoniana. Fungal Biol 120:873–883
5. Chatwal G. (2007) Organic Chemistry of Natural Products, 4th edn. Himalaya Publication House, Mumbai
6. Chen L, Chu CY, Huang M-C (2003) Inflorescence and Flower Development in Chinese Ixora
7. Clarance P, Khusro A, Lalitha J, et al (2019) Optimization of camptothecin production and biomass yield from endophytic fungus Fusarium solani strain ATLOY-8. J Appl Pharm Sci 9:035–046
8. Degambada KD, Kumara PAASP, Salim N, et al (2023) Diaporthe sp. F18; a new source of camptothecin-producing endophytic fungus from Nothapodytes nimmoniana growing in Sri Lanka. Nat Prod Res 37:113–118. https://doi.org/10.1080/14786419.2021.1946535
9. Dhakshinamoorthy M, Kilavan Packiam K, Kumar PS, Saravanakumar T (2021) Endophytic fungus Diaporthe caatingaensis MT192326 from Buchanania axillaris: An indicator to produce biocontrol agents in plant protection. Environ Res 197:111147. https://doi.org/https://doi.org/10.1016/j.envres.2021.111147
10. Ding X, Liu K, Deng B, et al (2013) Isolation and characterization of endophytic fungi from Camptotheca acuminata. World J Microbiol Biotechnol 29:1831–1838
11. Dissanayake AJ, Phillips AJL, Hyde KD, et al (2017) The current status of species in Diaporthe. Mycosphere 8:1106–1156
12. El-Sayed ASA, Zayed RA, El-Baz AF, Ismaeil WM (2022) Bioprocesses optimization and anticancer activity of camptothecin from Aspergillus flavus, an endophyte of in vitro cultured Astragalus fruticosus. Mol Biol Rep 49:4349–4364. https://doi.org/10.1007/s11033-022-07271-x
13. Felsenstein J (1985) Confidence limits on phylogenies: a justification. Evolution (N Y) 39:783–791
14. Hall TA (1999) BIOEDIT: A USER-FRIENDLY BIOLOGICAL SEQUENCE ALIGNMENT EDITOR AND ANALYSIS PROGRAM FOR WINDOWS 95/98/ NT
15. Hosseini B, El-Hasan A, Link T, Voegele RT (2020) Analysis of the species spectrum of the Diaporthe/Phomopsis complex in European soybean seeds. Mycol Prog 19:455–469. https://doi.org/10.1007/s11557-020-01570-y
16. Khare C. (2007) Indian Medicinal Plants—An Illustrated Dictionary. Springer (India) Pvt. Ltd, New Delhi
17. Kumar S, Stecher G, Li M, et al (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547
18. Kusari S, Zühlke S, Spiteller M (2009) An Endophytic Fungus from Camptotheca acuminata That Produces Camptothecin and Analogues. J Nat Prod 72:2–7. https://doi.org/10.1021/np800455b
19. Li F, Jiang T, Li Q, Ling X (2017) Camptothecin (CPT) and its derivatives are known to target topoisomerase I (Top1) as their mechanism of action: did we miss something in CPT analogue molecular targets for treating human disease such as cancer? Am J Cancer Res 7:2350–2394
20. Lin X, Lu C, Huang Y, et al (2007) Endophytic fungi from a pharmaceutical plant, Camptotheca acuminata: isolation, identification and bioactivity. World J Microbiol Biotechnol 23:1037–1040. https://doi.org/10.1007/s11274-006-9329-8

21. Liu B, Hui J, Cheng Y-Q, Zhang X (2012) Extractive fermentation for enhanced production of thailandepsin A from Burkholderia thailandensis E264 using polyaromatic adsorbent resin Diaion HP-20. J Ind Microbiol Biotechnol 39:767–776. https://doi.org/10.1007/s10295-011-1073-x
22. Mohinudeen IAHK, Pandey S, Kanniyappan H, et al (2021) Screening and selection of camptothecin producing endophytes from Nothapodytes nimmoniana. Sci Rep 11:11205. https://doi.org/10.1038/s41598-021-90778-3
23. Pu X, Chen F, Yang Y, et al (2015) Isolation and characterization of Paenibacillus polymyxa LY214, a camptothecin-producing endophytic bacterium from Camptotheca acuminata. J Ind Microbiol Biotechnol 42:1197–1202. https://doi.org/10.1007/s10295-015-1643-4
24. Pu X, Qu X, Chen F, et al (2013) Camptothecin-producing endophytic fungus Trichoderma atroviride LY357: isolation, identification, and fermentation conditions optimization for camptothecin production. Appl Microbiol Biotechnol 97:9365–9375
25. Rastogi RP, Mehrotra BN, Sinha S, et al (1990) Compendium of Indian Medicinal Plants: 1960-1969. Central Drug Research Institute and Publications & Information Directorate, New Delhi
26. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454
27. Saravanana P, Boopalan E (2011) Occurrence of Camptothecin an Anticancer Drug FromIxora coccinea Linn. Intl J Appl Biol 2:30–34
28. Siddiqui M, Rajkumar S (2012) The high cost of cancer drugs and what we can do about it. Mayo Clin Proc 87:935–43
29. Singh MP, Leighton MM, Barbieri LR, et al (2010) Fermentative production of self-toxic fungal secondary metabolites. J Ind Microbiol Biotechnol 37:335–340. https://doi.org/10.1007/s10295-009-0678-9
30. Soujanya KN, Siva R, Mohana Kumara P, et al (2017) Camptothecin-producing endophytic bacteria from Pyrenacantha volubilis Hook. (Icacinaceae): A possible role of a plasmid in the production of camptothecin. Phytomedicine 36:160–167. https://doi.org/https://doi.org/10.1016/j.phymed.2017.09.019
31. Sung H, Ferlay J, Siegel RL, et al (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 71:209–249. https://doi.org/10.3322/caac.21660
32. Takimoto CH (2002) Camptothecins. In: Bertino JRBT-E of C (Second E (ed) Encyclopedia of Cancer. Academic Press, New York, pp 301–309
33. Thompson JD, Gibson TJ, Plewniak F, et al (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
34. Uzma F, Mohan CD, Hashem A, et al (2018) Endophytic Fungi-Alternative Sources of Cytotoxic Compounds: A Review. Front. Pharmacol. 9:309
35. Venditto VJ, Simanek EE (2010) Cancer therapies utilizing the camptothecins: a review of the in vivo literature. Mol Pharm 7:307–349. https://doi.org/10.1021/mp900243b
36. Wall ME, Wani MC, Cook CE, et al (1966) Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from Camptotheca acuminata1,2. J Am Chem Soc 88:3888–3890. https://doi.org/10.1021/ja00968a057
37. Yang G, Li P, Meng L, et al (2018) Diversity and communities of culturable endophytic fungi from different tree peonies (geoherbs and non-geoherbs), and their biosynthetic potential analysis. Brazilian J Microbiol 49:47–58. https://doi.org/https://doi.org/10.1016/j.bjm.2018.06.006

Figures

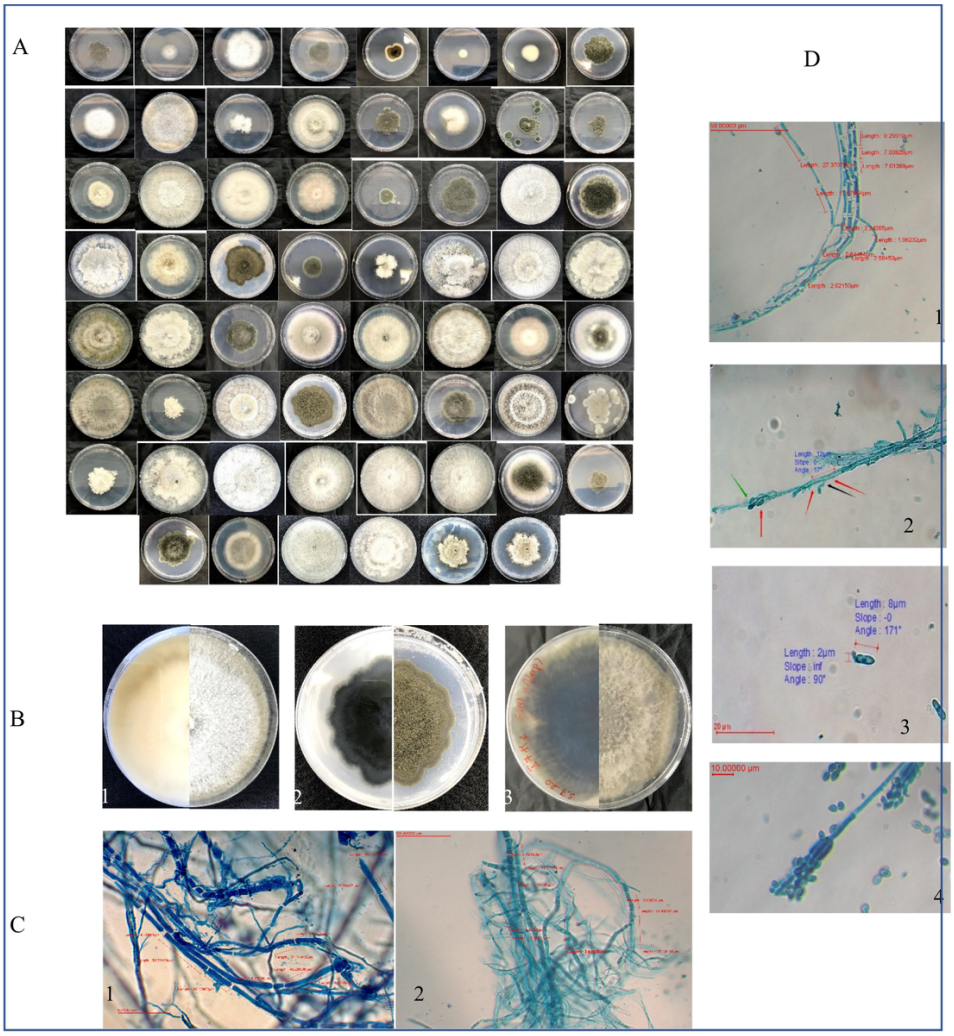

Figure 1

Morphological characteristics of isolated endophytic fungi from *I. chinensis*.

(A) Colony morphology of 62 isolated endophytes. (B) Typical colony types, the left side is vegetative hyphae, the right side is aerial hyphae; (B-1): the white cottony type; (B-2): the green powdery type; (B-3): the black spongy type; (C) Septate hyphae; C1: *Phomopsis* sp. I2T2; (C-2): *Diaporthe* sp. I5T3. (D) Conidiophore and conidial structure; D1: *Pestalotiopsis*sp. I1L3; (D-2, D-3): *Colletotrichum*sp. I1L2; (D-4): *Penicillium* sp. I3R2.

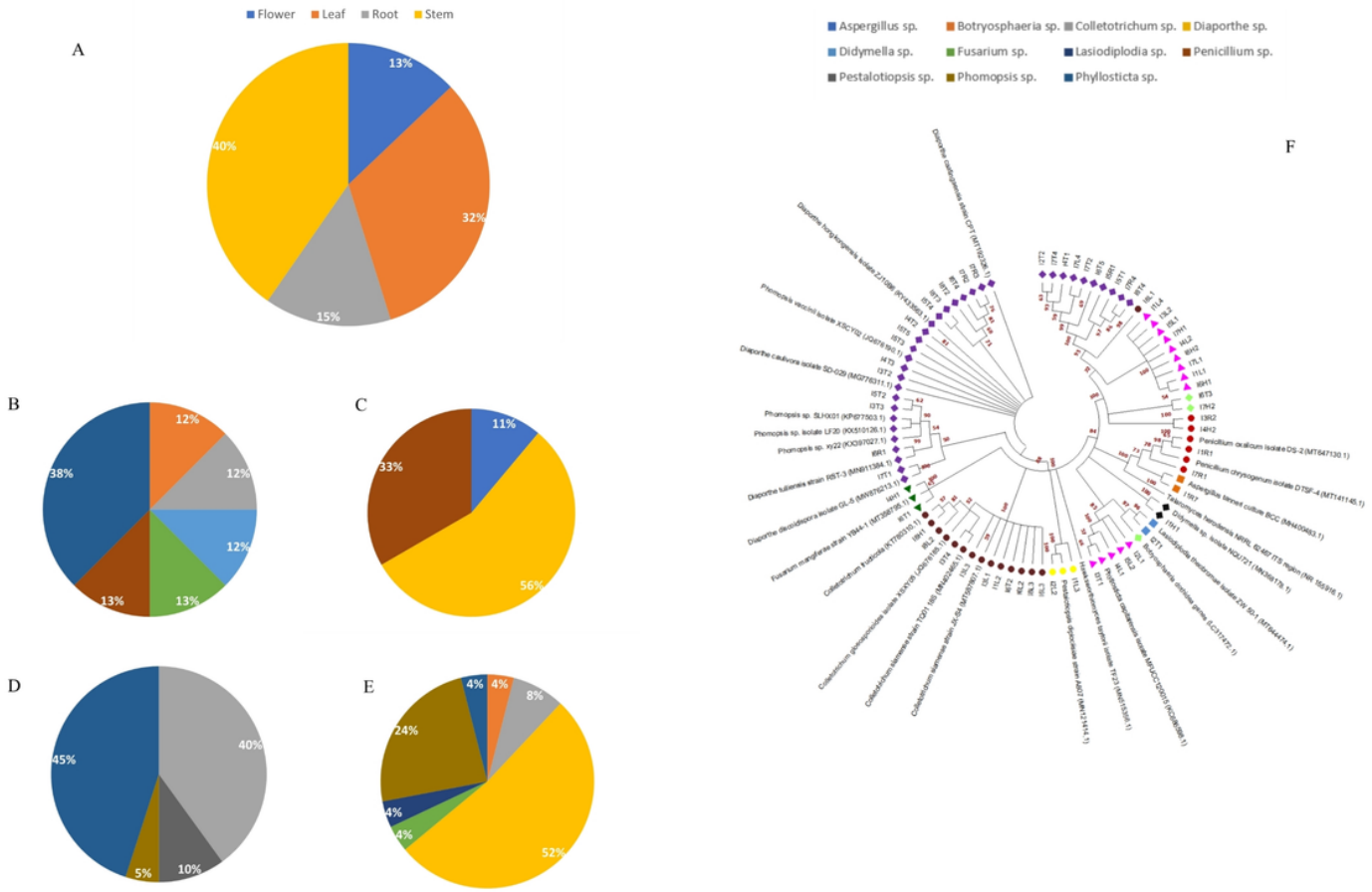

Figure 2

Diversity and distribution of the endophytic community in *Ixora chinensis* explants (A-E) and phylogenetic tree (F)

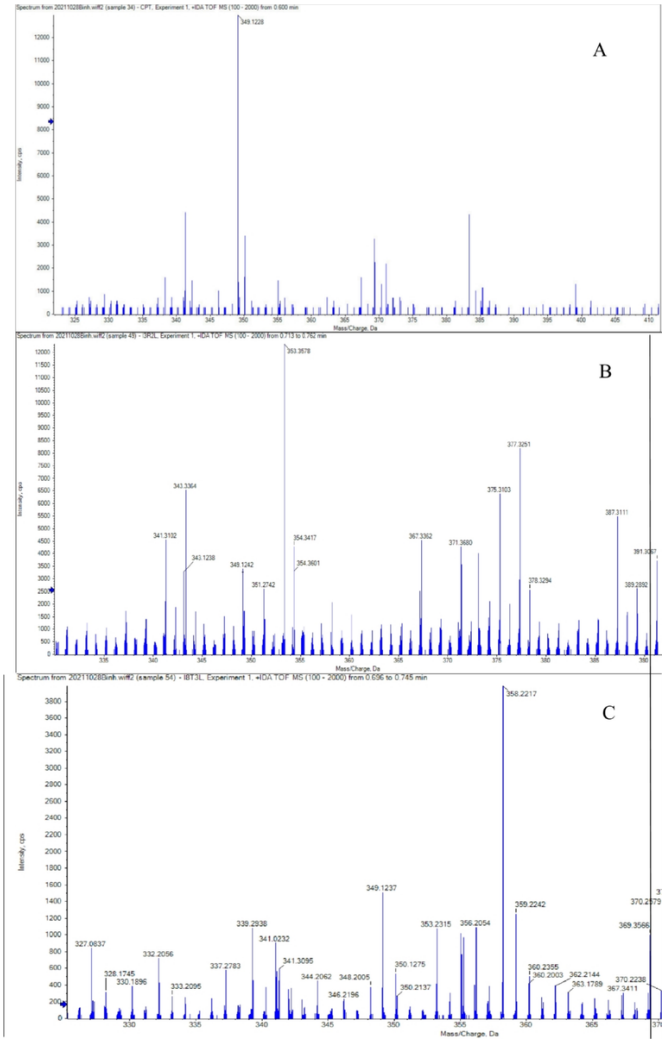

Figure 3

Identification of CPT-producing endophytic fungi from the crude extract by HRMS analysis. Standard CPT (A), CPT yield from I3R2 (B) and I8T3 (C)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementFigures.docx
- SupplementTables.xlsx