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# ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI FROM *Catharanthus roseus* AND *Scutallaria barbata*

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## ABSTRACT

Endophytic fungi, microfungi that internally infect living plant tissues, are reported to have the ability to synthesize many enzymes, plant growth hormones and pharmaceutically active compounds similar to those in their hosted plants. This has opened a potential path of using endophytic fungi as a bioreactor for mass production of bioactive compounds at a lower cost. Therefore, it is necessary to establish a robust procedure for the isolation and identification of potential fungal strains that are capable of producing desired biological compounds. In this study, we reported an effective procedure for surface sterilization of 3 types of tissue samples (root, shoot and leaf) of 2 herbaceous plants (*Catharanthus roseus* and *Scutallaria barbata*) using commercial bleach (5% NaOCl), isolation of endophytic fungi from the sterilized samples and identification of isolated fungal strains by ITS sequencing analysis. A total of 48 endophytic fungi were successfully isolated from plant samples collected from Dan Phuong (Ha Noi), Phu Dien (Ha Noi) and Hai Duong city (Hai Duong). Based on results of morphological observation and ITS sequencing analysis, 48 endophytic fungi were classified to one of the four species, including *Clasdosporium colombiae*, *Cladosporium halotolerans*, *Corynespora cassiicola* and *Albifimbria terrestris*. The potential of the isolated endophytic fungal species for the mass production of pharmacologically active compounds will be investigated in future studies.

**Keywords:** *Albifimbria* sp., *Catharanthus roseus*, *Cladosporium* sp., *Corynespora* sp., *Scutallaria barbata*, endophytic fungi, ITS sequencing, surface sterilization.

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## INTRODUCTION

*Catharanthus roseus*, commonly known as Madagascar periwinkle, is a perennial herbaceous plant in the Apocynaceae family (Ramani & Jayabaskaran, 2008). *Scutallaria babarta* is a shrub herbal plant belonging to the mint family, Lamiaceae (Zhang et al., 2017). For many years, *C. roseus* and *S. barbata* are considered as a natural source of several bioactive compounds with anti-inflammatory and anti-cancer activities (Palem et al., 2015; Tan & Zhou 2001; Yin et al., 2004; Zhang et al., 2017). In *C. roseus*, vincristine and vinblastine are the 2 major vinca alkaloids that are most widely used in the chemotherapeutic treatment of different stages of breast cancer, leukemia and Hodgkin's disease (Palem et al., 2015). Similarly, *S. babarta* extract was reported to have inhibitory effects on the proliferation of human lung cancer cell line A549 (Yin et al., 2004) and ovarian cell line A2780 (Zhang et al., 2017). Reports on the medicinal efficacy of these plants have led to a high demand for their bioactive compounds in the global markets. However, the use of plant as a source for the production of anti - inflammatory and anti-cancer compounds is impeded by several factors, including low yield, time consuming, and high production cost (Palem et al., 2015).

Endophytic fungus is a symbiont that could lives between healthy plant cells without causing any negative effects to their host plants (Jia et al., 2016). These endophytes are reported to have the ability to produce a wide range of enzymes and phytohormones as well as plant secondary metabolites that have anti-inflammatory, anti - bacterial and anti - cancer activities (Palem et al., 2015; Tan & Zhou 2001; Yin et al., 2004; Zhang et al., 2017). More specifically, studies have shown that *Fusarium* endophytes isolated from root of apple plant can produce phytohormone, indole-3-acetic acid (IAA) and gibberellins (GA) (Manici et al., 2015), *Cladosporium* endophytes isolated from *Xylocarpus granatum* can synthesize several indole alkaloids (Peng et al., 2013), *Streptomyces cavourensis* from *Cinnamomum cassia* has antibacterial activity against many species from *Salmonella enterica, Pseudomonas aeruginosa, Staphylococcus epidermidis, Enterobacter aerogenes* and *Proteus vulgaris* (Vu Thi Hanh Nguyen et al., 2017), and *Huperzia serrata* derived *Trichoderma* and *Fusarium* endophytes are capable of inhibiting *Escheria coli, Moraxella cataharrlis, Staphylococus aureus* and *Candida albicans* (Le Thi Minh Thanh et al., 2019). These findings have opened a potential path of using endophytic fungi as a bioreactor for mass production of plant metabolites with pharmacological effects at reduced cost. Therefore, it is necessary to establish a robust procedure for the isolation and identification of potential fungal strains that are capable of producing desired biological compounds.

In this study, we aim to optimize procedures for: (1) surface sterilization of 3 types of tissue samples, root, shoot and leaf of *Catharanthus roseus* and *Scutallaria barbata* by testing 2 sterilizing agents, commercial bleach and $HgCl_2$, (2) isolation of endophytic fungi from the sterilized samples, and (3) identification of isolated fungal strains by ITS sequencing analysis. Our results showed that a total of 48 endophytic fungal strains were successfully isolated from plant samples collected from Dan Phuong (Ha Noi), Phu Dien (Ha Noi) and Hai Duong city (Hai Duong); they have been identified as endophytic strains of either *Cladosporium halotolerans, Cladosporium colombiae, Corynespora cassiicola* or *Albifimbria terrestris*. The potential of the isolated endophytes as a bioreactor for synthesizing plant secondary metabolites will be investigated in future studies.

## MATERIAL AND METHODS

### Plant material

*Catharanthus roseus* (L.) G. Don of at least one-year old collected from Phu Dien and Dan Phuong, Ha Noi, and *Scutellaria barbata* D. Don of at least one-year old collected from Hai Duong city (Hai Duong) were used in this research. Collected plants were grown in soil pots at the Functional

genome laboratory for later use. Plant species of collected samples were identified by ABI PRISM® 3100 Avan Gentic Analyzer (data not shown) using 3 sets of primers, namely RbcL (F: 5'-ATGTCACCACAAACAGAA AC, R: 5'-TCGCATGTACCTGCAGTAGC), tmL (F: 5'-CGAAATCGGTAGACGCTACG, R: 5'-GGGGATAGAGGGACTTGAAC), and ITS (F: 5'-ACGAATTCATGGTCCGGTGA AGTGTTCG, R: 5'-TAGAATTCCCCGGTT CGCTCGCCGTTAC) (Olmstead et al., 1992; Tarberlt et al., 1991; Sun et al., 1994). Fresh and healthy tissues from shoot, leaf and root were used for the isolation of endophytic fungi.

**Surface sterilization of *C. roseus* and *S. Barbata***

Fresh plant material of *C. roseus* and *S. barbata* was surface sterlized to remove bacteria and exophytic fungi and to ensure the collection of endophytic fungi. Surface sterilization was performed based on the methodologies reported by Robert, Terry (1978) and Arnold et al., (2000) with some minor modifications for optimization.

***Optimized method for surface sterilization based on the methodology of Robert & Terry (1978)***

*C. roseus* and *S. barbata* plants were rinsed with water to remove dirt before shoots, leaves and roots were cut into small segments (approximately 3 cm in length) and separately sterilized. Fresh plant material was washed with sterilized distilled water twice for 1 min each time, ethanol 75% for 1 min, and rinsed with sterilized distilled water. Plant material was then treated with commercial bleach (Javel clean, Ha Noi; 5% NaClO, v/v) on a rotary shaker for 30 min at 150 rpm, washed with sterilized distilled water for 5 min and air dried on sterilized filter paper. Plant material was then transferred onto Luria Broth (LB) agar plates (Himedia, India) supplemented with cefotaxime (200 µg/L; Flamingo Farm, India), and incubated at 25 ± 2 $^{o}$C for 5 days. Post 5-day cultivation, contamination - free plant material was used for isolation of endophytic fungi.

***Optimized method for surface sterilization based on the methodology of Arnold et al. (2000)***

Plant material was surface sterilized as described in previous section, however, mercury chloride ($HgCl_2$; 0.1%) solution was used as the sterilizing agent instead of commercial bleach. Plant material was washed with $HgCl_2$ solution on a rotary shaker for 7 min at 150 rpm. Once finished, the remaining steps were performed as described in 2.2.1.1 section.

**Isolation and preservation of endophytic fungus from *C. roseus* and *S. Barbata***

Post surface sterilization, shoot, leaf and root tissue samples were grounded, transferred onto potato dextrose agar (PDA) plates (Himedia, India) supplemented with cefotaxime (200 µg/L), and incubated at 25 ± 2 $^{o}$C. Fungal colonies that emerged from the cut surface of plant material were considered as endophytic fungi. They were then transferred onto fresh PDA plates and incubated at 25 $^{o}$C for 10 days. A single colony of each isolated fungus was preserved in a PDA agar slant tube at 4 $^{o}$C and in 25% glycerol at -80 $^{o}$C.

**Extraction of DNA from endophytic fungus**

Fungal mycelia from a single endophytic fungus was inoculated into 100 mL PDA liquid medium and cultured at 25 ± 2 $^{o}$C for 7 days. A hundred (100) mg of fungal mycelia was collected by centrifugation and total DNA was extracted by CTAB method with some minor modifications for optimization (Saghai-Maroof et al., 1984). The concentration and quality of the obtained DNA were checked using a Nanodrop®1000 spectrophotometer and gel electrophoresis. All DNA samples were preserved at -20 $^{o}$C for subsequent experiments.

**Identification of endophytic fungi by sequencing ITS regions**

The internal transcribed spacer (ITS) was amplified from ribosomal DNA (rDNA) using the universal primer ITS1 (forward), 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4

(reverse), 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). PCR was carried on an Eppendorff Mastercycler Pro S (Merck, USA). Each reaction (25 µL) was consist of 8.5 µL of $H_2O$, 2 µL of DNA (30 ng/µl), 2 µL of primer (10 µM), 12.5 µL of 2X PCR master mix. The cycle program for product amplification was 1 cycle of 94 $^oC$ for 4 min, followed by 35 cycles of 94 $^oC$ (denaturation) for 30 s, 55 $^oC$ (annealing) for 30 s, and 72 $^oC$ (extension) for 1 min, and 1 cycle of 72 $^oC$ for 7 min. PCR products were checked by gel electrophoresis. The high quality PCR product was column purified and sequenced using a DNA analyzer ABI 3500 (PE Applied Biosystems). Species identification of isolated fungi was done by blasting the obtained ITS-amplified sequences against the GenBank database (http://www.ncbi.nlm.nih.gov/) using BLASTN online tool (RRID: SCR_001598).

**Construction of unrooted phylogenetic tree**

Unrooted phylogenetic trees were generated by MEGA7 software (RRID: SCR_000667) using sequencing alignments of ITS sequences from isolated fungi and nucleotide sequences in *Albifimbria terrestris* (CBS 126186), *Corynespora cassiicola* (CBS 161.60), *Cladosporium halotolerans* (CBS 119416), *Cladosporium endophytica* (MFLUCC 17-0599), *C. pseudochalastosporoidesi* (CBS 140490), *Corynespora cassiicola* (CBS 161.60), *Cercrospora beticola* (CBS 116456) and *Myrothecium verrucaria* (CBS 253.47). Phylogenetic tree was constructed by maximum likelihood method. The bootstap confidence levels (%) were assessed and ranked as follows: high confidence level (> 85%), average confidence level (65–85%), and low confidence level (< 65%) (Nguyen Duc Anh & Nguyen Giang Son, 2016).

**Statistical analysis**

Analytical data from this study was obtained from at least 3 replicates. Statistical analysis was performed by one-way analysis of variance (ANOVA; RRID: SCR_002427) methods with the *post hoc* Tukey test (*p*-value > 0.05).

## RESULTS AND DISCUSSION

**Optimisation of surface sterilization of *C. roseus* and *S. Barbata***

One of the most critical factors for the successful establishment of an effective endophytic fungi isolation protocol of *C. roseus* and *S. barbata* is the ability to optimize a surface sterilization method. In an attempt to establish a robust surface sterilization protocol, the method of Robert, Terry (1978) and Arnold et al., (2000) were tested on 3 types of plant material, root, stem and leaf, with different periods (Fig. 1).

Results showed that surface sterilization using $HgCl_2$ 0.1% was more effective than commercial bleach contains 5% NaClO. Specifically, the rate of contamination of *C. roseus* and *S. barbata* plant tissues treated with $HgCl_2$ dropped significantly from more than 94% to lower than 16% when the treatment time increased from 5 min to 7 min (Figs. 1A, 1C) Plant samples from these 2 treatment periods appeared to be contaminated with different species of bacteria, yeast and fungi, and displayed a proportion of browning and necrotic cells. In contrast, in the 10 min treatment, root, shoot and leaf samples remained fresh and healthy appearance without contamination, which are considered suitable material for isolation of endophytic fungi. Results obtained in this study were consistent with research reported in surface sterilization of a wide range of plant species, including *Zingiber zerumbet* (Nongalleima et al., 2013), Oryza sativa (Ahmad et al., 2016) and Fragaria ananassa (Jan et al., 2013).

Next, surface sterilization by commercial bleach was evaluated on roots, shoots and leaves of *C. roseus* and *S. barbata* (Figs. 1B, 1D). As expected, commercial bleach required a longer period of time to achieve the same effects as in $HgCl_2$ treatment. The rate of contamination was high in all samples (more than 93.7%) under 15 min treatment, significantly reduced in leaf sample (*C. roseus*: 28.3% and *S. barbata*: 28.0%) under 30 min treatment, and free of contamination (0%) under 45 min treatment.

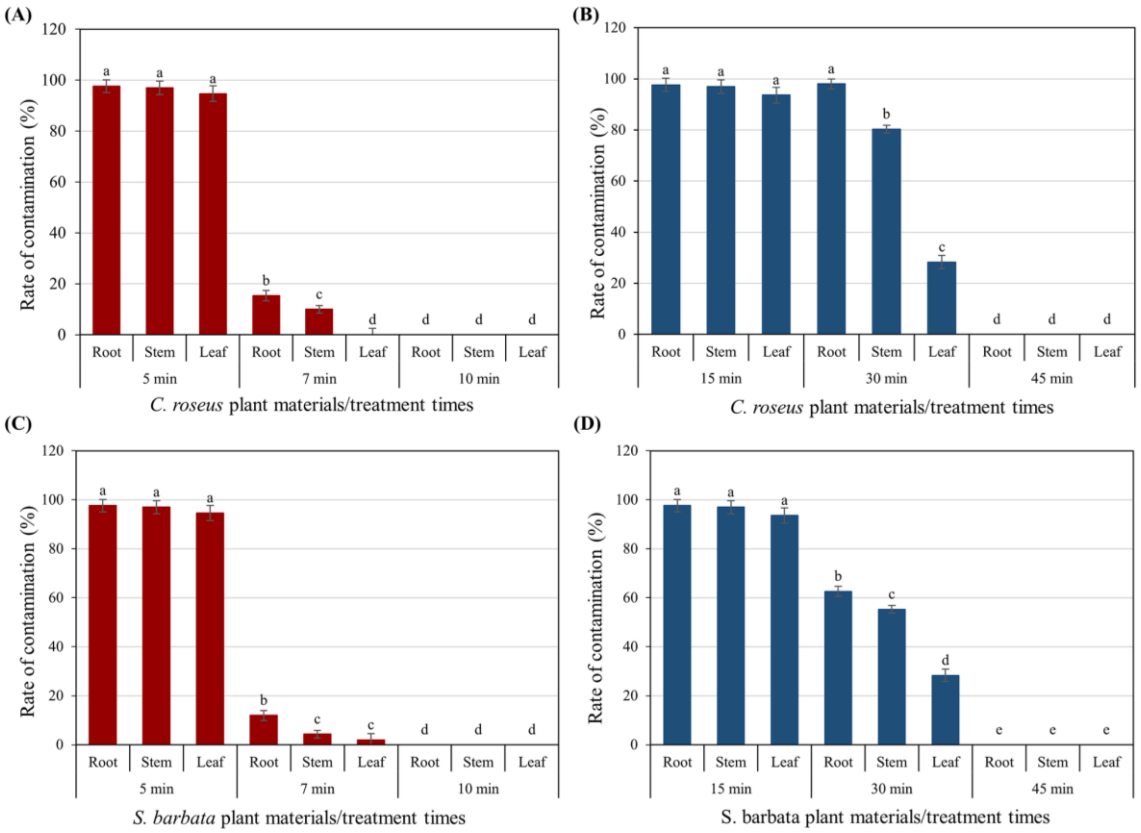


*Figure 1.* Rate of contamination of *C. roseus* and *S. barbata* plant materials treated by (A, C) $HgCl_2$ 0.1% and (B, D) commercial bleach (5% NaClO) based on the methodology of Arnold et al. (1978), and Robert & Terry (2000), respectively. (A, C) Treatment of plant material with $HgCl_2$ requires a shorter time, having 10 min the optimal time for treatment of all samples. (B, D) Surface sterilization by commercial bleach requires 45 min to effectively decontaminate plant tissue samples

Taken together, the optimal treatment time for surface sterilization of *C. roseus* and *S. barbata* plant tissues using $HgCl_2$ and commercial bleach was 10 min and 45 min, respectively. However, mercury chloride is an extremely toxic chemical that requires a complicated safety procedure of handling and disposal, therefore, commercial bleach was chosen as a sterilizing agent in this study.

**Isolation of endophytic fungi from *C. roseus* and *S. Barbata***

A total of 48 endophytic fungi was isolated from *C. roseus* (43 colonies) collected from Dan Phuong, Phu Dien (Ha Noi), and *S. barbata* (5 colonies) from Hai Duong city (Hai Duong) by the established surface sterilization and isolation protocols (Fig. 2). Based on the morphological observation, 48 endophytic fungi were then divided into 5 groups, namely DP, PD, HP, PT and BR (Table 1).

Specifically, the fungal strains in the DP group (Group 1) displayed a puffy and elevated surface, opaque gray to black in color, and a cotton-like mycelium network (Fig. 2A). They also showed a faster growth rate than other groups; the surface of the medium plate (approximately 10 cm in diameter) was entirely covered 6−7 days after inoculation. In group 2, PD fungal strains appeared to have a small (2–3 cm in diameter) irregular shape colony, undulated margin, wrinkle and elevated

surface, olive gray in color, and velvet-like mycelium network (Fig. 2B). In group 3, HP endophytes displayed a large circular colony (7−8 cm in diameter), crateriform surface with greenish-black and grey in color, and a cotton-like mycelium network (Fig. 2C). The PT endophytic fungi in group 4 showed a different appearance from other groups. The revealed morphological features of PT endophytic fungi included white and yellow in color, irregular colony (3–4 cm in diameter) with undulated margin, wrinkle and raised surface and velvet-like mycelium network (Fig. 2D).

*Table 1.* Morphological characteristic of 5 endophytic fungal strains

| No. | Fungal strains | Colony diameter | Colony color | Colony morphology |
|---|---|---|---|---|
| 1 | DP1 | 10 cm | Opaque gray to black | Puffy and elevated surface and cotton-like mycelium network |
| 2 | PD2 | 2–3 cm | Olive gray | Irregular shape colony, undulated margin, wrinkle and elevated surface and velvet-like mycelium network |
| 3 | HP-L1 | 7–8 cm | Greenish-black and grey | Large circular, crateriform surface, and cotton-like mycelium network |
| 4 | PT-T12 | 3–4 cm | White and yellow | Irregular colony with undulated margin, wrinkle and raised surface and velvet-like mycelium network |
| 5 | BR2 | 3–5 cm | Olive gray and greenish-black | Circular shape with undulated margin, wrinkle and elevated surface and velvet-like mycelium network |

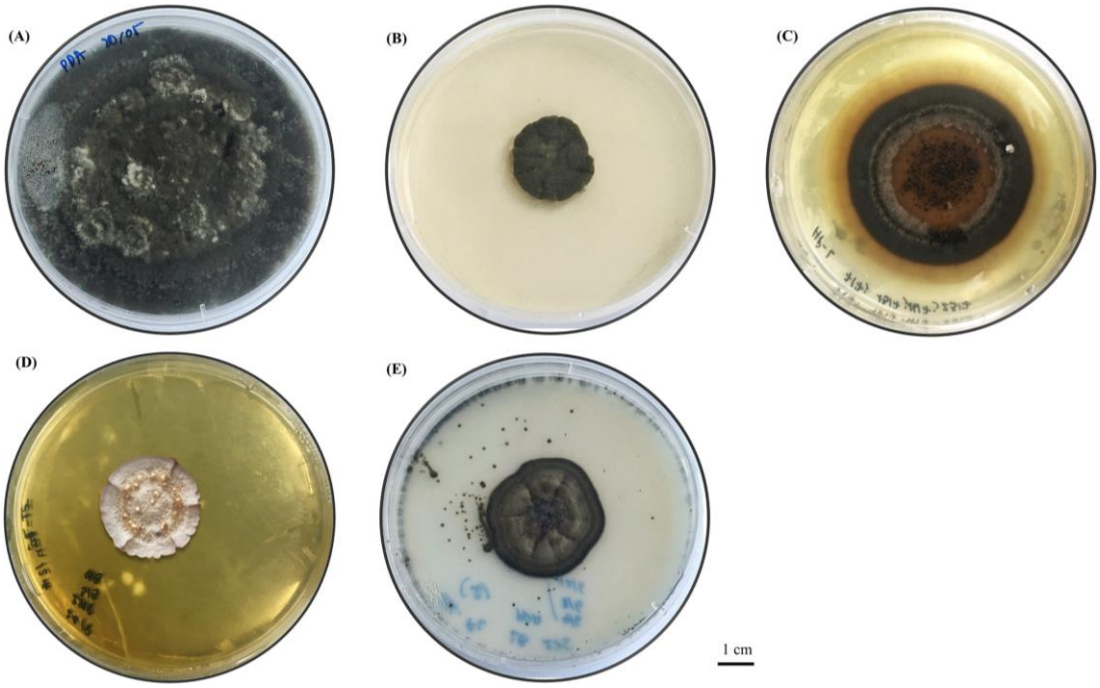


*Figure 2.* Morphological appearance of endophytic fungi isolated from *C. roseus* and *S. barbata* tissues. Fungal strains (A) DP1, (B) PD2, (C) HP-L1 and (D) PT-T12 strains were isolated from *C. roseus* collected from Dan Phuong and Phu Dien, Ha Noi. (E) BR2 strain was isolated from *S. barbata* at Hai Duong

In group 5, the BR fungal colonies showed a similar appearance to those in group 2, displaying a circular shape (3–5 cm in diameter) with undulated margin, wrinkle and an elevated surface, olive gray and greenish-black in color, and velvet-like mycelium network (Fig. 2E). Post morphological observation, a representative fungal strain from each group was selected for species identification by ITS sequencing. The selected strain from each group was named as followed: Group 1: DP1; Group 2: PD2; Group 3: HP-L1; Group 4: PT-T12, and; Group 5: BR2.

**Identification of fungal species by ITS sequencing**

Post isolation of endophytic fungi, the 5 representative strains were subjected to molecular identification based on ITS rDNA sequence analysis. Prior to the amplification of ITS regions, the quality of extracted total DNA was evaluated by gel electrophoresis. A single and distinct band at 550 bp was observed for each sample, a result that strongly suggests that intact and high quality DNA had been obtained from all fungal samples (Fig. 3A).

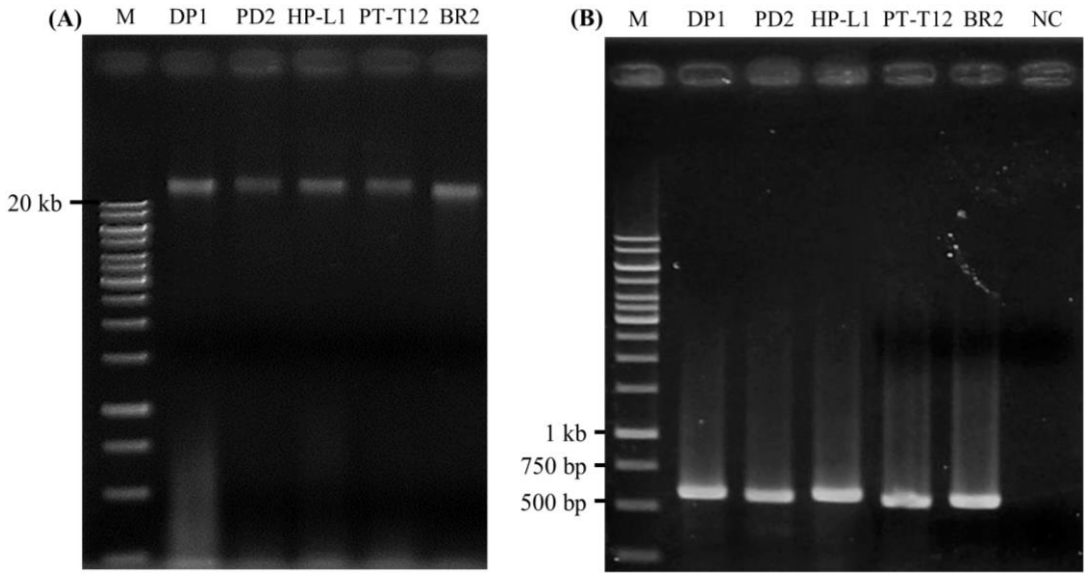


*Figure 3.* Molecular analysis of endophytic fungi isolated from *C. roseus* and *S. barbata.* (A) Evaluation of DNA quality extracted from DP1, PD2, HP-L1, PT-T12 and BR2. (B) PCR products amplified from DNA of DP1, PD2, HP-L1, PT-T12 and BR2 using universal ITS1 and ITS4 primers (550 bp). M: Marker - GeneRuler 1 kb DNA Ladder, ThermoScientific; NC: negative control; DP1, PD2, HP-L1, PT-T12, BR2: isolated endophytic fungi

Those DNA samples were then used as a template for amplification by ITS1 and ITS4 primers. The PCR amplicons of 5 fungal strains were sequenced and the obtained sequencing data were then blasted against reference sequences on the GenBank database for species identification (Fig. 3B). BLASTN results revealed that DP1 strain returned a high similarity of 99.2% with *Cladosporium colombiae*, PD2 and BR2 strains were clustered to *Cladosporium halotolerans* with the similarity of 100 and 99.4%, respectively, HP-L1 strain was a *Corynespora cassiicola* species (100% similarity), and PT-T12 strain showed 100% similarity to *Albifimbria terrestris* (Table 2).

Based on data derived from BLASTN analysis, an unrooted phylogenetic tree was also constructed using the maximum likelihood method with a bootstrap value of

1000 replications. Consistent with the results of ITS sequencing, the unrooted phylogenetic tree clearly showed that 5 isolated fungal strains and 7 reference strains were grouped into 4 branches (Fig. 4). PD2 and BR2 strains formed a branch with 2 reference strains *C. halotolerans* and *C. endophytica*. The 2 strains were more closely related to *C. halotolerans* with the similarity value of 100% and 99.4%, respectively. This relationship was also reflected by the similarity in mophorlogy observed from the 2 strains with *C. halotolerans*.

*Table 2.* Identification of fungal species isolated from *C. roseus* and *S. barbata*

| No. | Plants | Tissues | Strains | Sequence length (bp) | Species and GenBank ID | Similarity (%) |
|---|---|---|---|---|---|---|
| 1 | *C. roseus* | Leaf | DP1 | 522 | *Cladosporium colombiae* (CBS 274.80B) | 99.2 |
| 2 | *C. roseus* | Root | PD2 | 518 | *Cladosporium halotolerans* (CBS 119416) | 100 |
| 3 | *C. roseus* | Leaf | HP-L1 | 539 | *Corynespora cassiicola* (CBS 161.60) | 100 |
| 4 | *C. roseus* | Shoot | PT-T12 | 511 | *Albifimbria terrestris* (CBS 126186) | 100 |
| 5 | *S. barbata* | Root | BR2 | 521 | *Cladosporium halotolerans* (CBS 119416) | 99.4 |

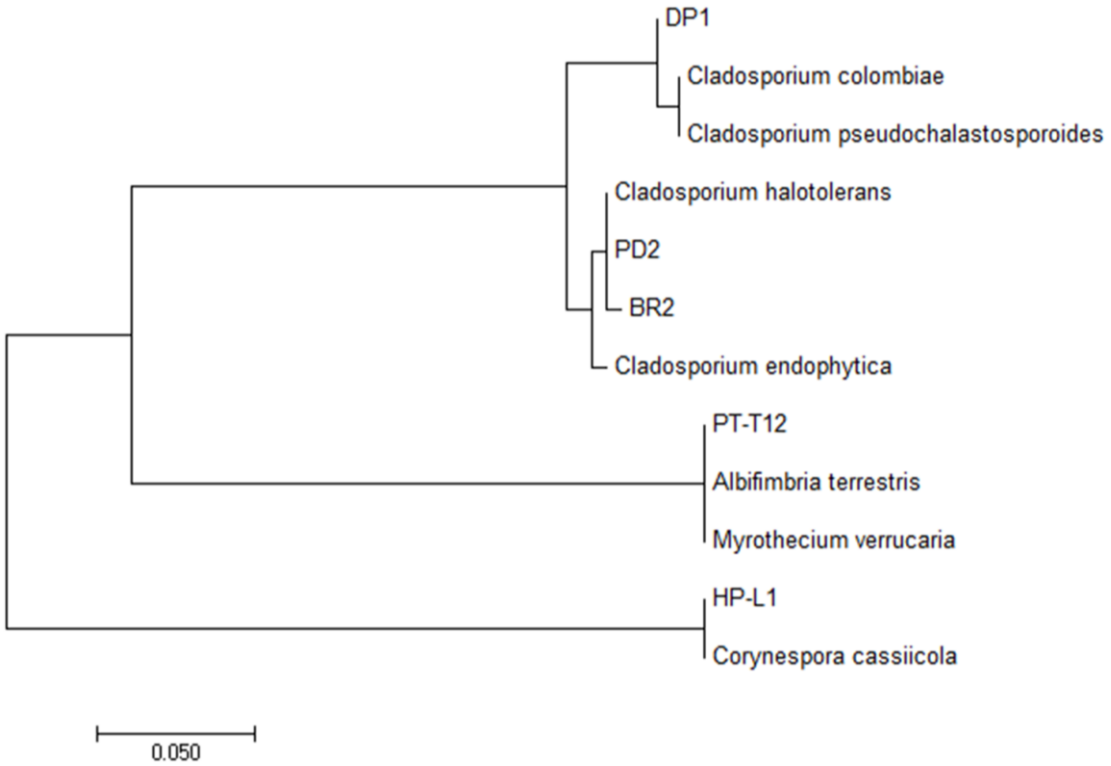


*Figure 4.* Unrooted phylogenetic analysis of *Cladosporium* sp., DP1, PD2 and BR2. The tree was constructed by the maximum likelyhood method using the nucleotide sequence of *Cladosporium* sp. and the ITS sequence of 3 endophytic fungi. A bootstrap value (%) is displayed by a number on each node

DP1 strain formed a separate branch with *C. colombiae* and *C. pseudochalastosporoides*. Based on morphological observation reported by Schubert et al. (2009), DP1 strain was identified as *C. colombiae*. Similar morphological observation was conducted with the endophytic strain PT-T12, and this strain was identified as a *Albifimbria terrestris*. Consistent with ITS sequencing analysis, the HP-L1 strain formed a separated branch with *Corynespora cassiicola* (Fig. 4).

## CONCLUSION

In conclusion, forty-eight endophytic fungal strains were successfully isolated from leaf and root tissues of wildly grown *C. roseus* and *S. barbata* using the established surface sterilization and isolation procedure. The fungal strains were identified to have a close relationship with 4 fungal species, including *A. terrestris*, *C. colombiae*, *C. halotolerans*, and *C. cassiicola*, based on morphological observation and ITS sequencing analyses. The results in this study have provided a platform for further study on the isolation and selection of endophytic fungi with potential for the production of pharmacologically active compounds.

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