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## ORIGINAL ARTICLE

# Endophytic filamentous fungi from a *Catharanthus roseus*: Identification and its hydrolytic enzymes



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## KEYWORDS

Hydrolytic enzyme;  
Medicinal plant;  
Symbiotic microorganisms,  
Molecular and morphological identification

**Abstract** This paper reported on the various filamentous fungi strains that were isolated from a wild grown *Catharanthus roseus*. Based on the morphological characteristics and molecular technique through a Polymerase Chain Reaction and DNA sequencing method using internal transcribed spacer (ITS), these fungi had been identified as a *Colletotrichum* sp., *Macrophomina phaseolina*, *Nigrospora sphaerica* and *Fusarium solani*. The ultrastructures of spores and hyphae were observed under a Scanning Electron Microscope. The hydrolytic enzyme test showed that all strains were positive in secreting cellulase. *Colletotrichum* sp. and *F. solani* strains also gave a positive result for amylase while only *F. solani* was capable to secrete protease. These fungi were putatively classified as endophytic fungi since they produced extracellular enzymes that allow them to penetrate plant cell walls and colonize with symbiotic properties.

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## 1. Introduction

*Catharanthus roseus* commonly called Madagascar periwinkle is a herbaceous sub-shrub of latex producing plants belonging to the family *Apocynaceae* (Gajalakshmi et al., 2013). This plant is a native to Madagascar but also found in Malaysia, where it is called Kemunting Cina and is popularly employed in landscaping or gardening due to its colorful flowers. This ornamental plant is also reported to be used as anticancer where it produced the alkaloids called vincristine and vinblastine (Balaabirami and Patharajan, 2012). The report

on the medicinal efficacy of this plant incurs the current surge in its global market, and thus the flower of this plant was chosen as a logo for the National Cancer Council Malaysia (MAKNA). However, this plant takes about one year to harvest and leads the high cost of production. Alternative producer of these alkaloids in the short period is required.

In view to the importance of these alkaloids in the medical applications, several studies have been made to discover the potential source for these metabolite. The plant associated microorganisms are believed able to produce similar metabolites as their host plant. Endophytes, microbes that colonize healthy tissues of the plant for at least part of their life cycle without causing any apparent disease symptoms in their host (Petrini, 1991). These endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan and Zou, 2001) including enzymes and plant growth hormones (Carol, 1988). Some of these metabolites are bioactive compounds that demonstrated potent anticancer, antibacterial and antiarthritic activities.

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**Table 1** The morphological appearance and hydrolytic enzyme properties of endophytic fungi strains isolated from different tissues and colors of *Catharanthus roseus* after a week incubated on MEA at 25 °C.

Strain	Host plant	Tissue	Morphological appearance	Hydrolytic enzyme
1	<i>C. roseus</i> (purple)	Leaf	White colored	C, A
2	<i>C. roseus</i> (purple)	Root	Light black colored	C
3	<i>C. roseus</i> (white)	Leaf	White colored	C, A
4	<i>C. roseus</i> (white)	Leaf	Red to green colored (turn the media into red colored)	C
5	<i>C. roseus</i> (white)	Root	White and yellowish colored	C, A, P

Symbols: C = Cellulase, A = Amylase, P = Protease.

**Table 2** Fungal species isolates from different varieties and tissues of *Catharanthus roseus* and respective accession number for their ITS sequences.

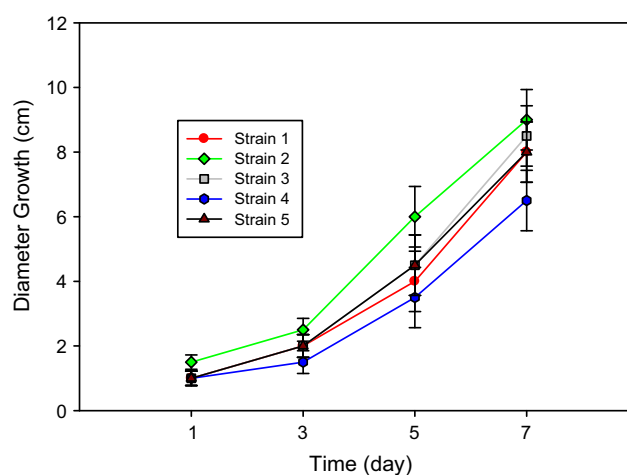
Strain	Host plant	Tissues	Species	Molecular size (bp)	Similarity (%)	Accession no. GenBank	Accession no. ENA
1 & 3	<i>C. roseus</i> (purple & white)	Leaf	<i>Colletotrichum</i> sp.	501	100	JQ 894655.1	HG 938365
2	<i>C. roseus</i> (purple)	Root	<i>Macrophomina phaseolina</i>	521	100	KC 822431.1	HG 938366
4	<i>C. roseus</i> (white)	Leaf	<i>Nigrospora sphaerica</i>	240	99	KC 505176.1	HG 938367
5	<i>C. roseus</i> (white)	Root	<i>Fusarium solani</i>	541	100	JQ 277276.1	HG 938368

Fungi is one of endophyte microorganisms that could be found in plant. By having a wide spectacular array of shapes, sizes and colors, this species diversity actually remains unexplored (Srinivasan and Muthumary, 2009). The capability of these fungi to grow and produce their metabolites within 2–4 weeks becomes more economic. Thus, this study was carried out to identify fungi strains that were isolated from a medicinal plant, *C. roseus* through observation of morphological characteristics and molecular technique. The hydrolytic enzymes produced by these strains were also determined. The potential strains will be used for determination of anticancer activity using various cell line cancers in the further study.

## 2. Materials and methods

### 2.1. Plant sample collection, preparation and fungal isolation

The root and leaf of two types of wildy grown *C. roseus* (purple and white) were taken from a peat soil in Alor Setar, Kedah (6.1167°N, 100.3667°E) which is the northern part of Peninsular Malaysia. The fungi strains were isolated according to Robert and Terry (1978). The root samples were cleaned under running tap water to remove the soil particles. The cleaned samples of root and leaf were cut into 1 cm size and surface sterilized was done by immersion in 10% (v/v) bleach for 15 min. Then, it was rinsed using distilled water for 5 min and blotted dry with a sterile paper. The piece of samples was placed on Malt Extract Agar (MEA: OXOID). Plates were incubated at 25 °C and observed daily. The seven days old of fungal culture was cut into small plug of mycelium using sterile borer and transferred to a new plate of MEA. Subculturing was repeated several times in order to get a pure culture. The pure culture was maintained on Potato Dextrose Agar (PDA) slant agar at 4 °C.

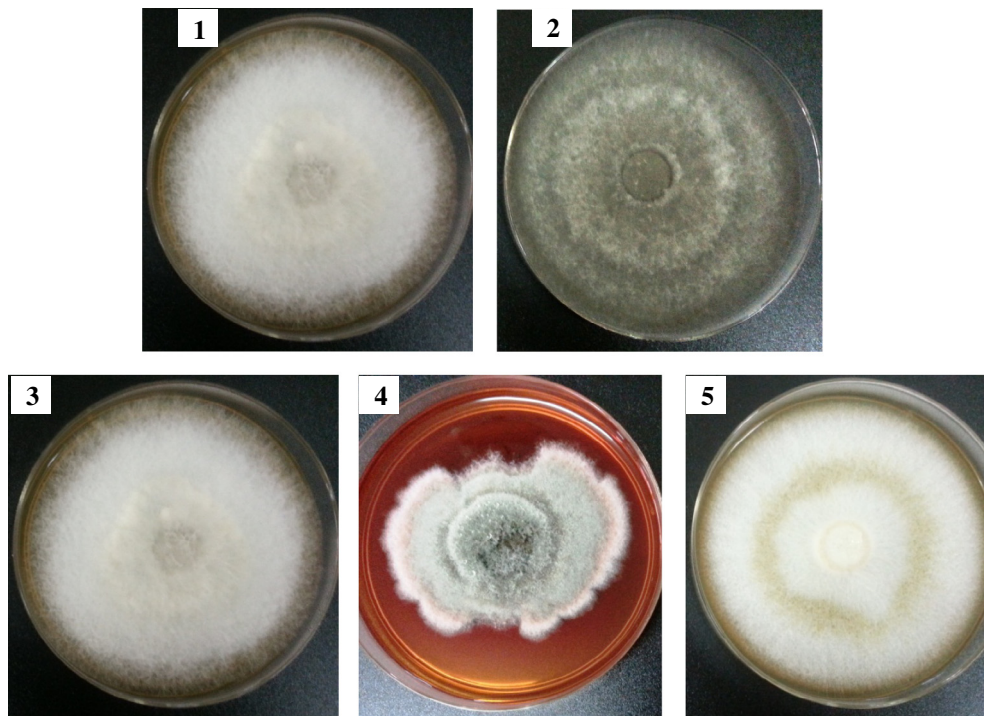
**Figure 1** The growth profiles of fungi strain isolated from *Catharanthus roseus*. All strains were incubated for seven days on MEA at 25 °C.

### 2.2. Identification of fungi

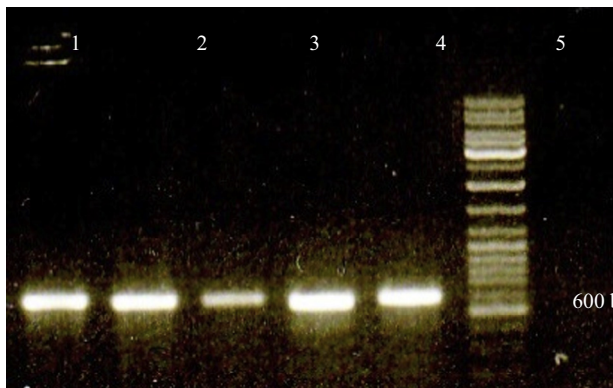
The isolated fungi were identified based on the morphology and cultural characteristics. It included the colors of spores and growth profiles of the colonies. The ability in producing hydrolytic enzymes such as cellulase, amylase and protease was tested using plate assay method (Stewart et al., 1982; NCCLS, 1997; Namasivayam and Nirmala, 2013).

#### 2.2.1. Scanning Electron Microscope

The microstructures of spore and hyphae configuration of these fungi were investigated from seven days old fungal cul-



**Figure 2** Morphological appearances of fungi isolated from *Catharanthus roseus* tissues. Strains 1 and 3 were from the leaf of both varieties. Strain 2 from root of the *Catharanthus roseus* (purple) and strains 4 and 5 from leaf and root *Catharanthus roseus* (white) respectively.



**Figure 3** The amplification of DNA from pure culture of fungi isolated from *Catharanthus roseus* using the primer set ITS1/ITS4. 1 and 3 = *Colletotrichum* sp., 2 = *Macrophomina phaseolina*, 4 = *Nigrospora sphaerica*, 5 = *Fusarium solani*. M = 100 bps DNA marker.

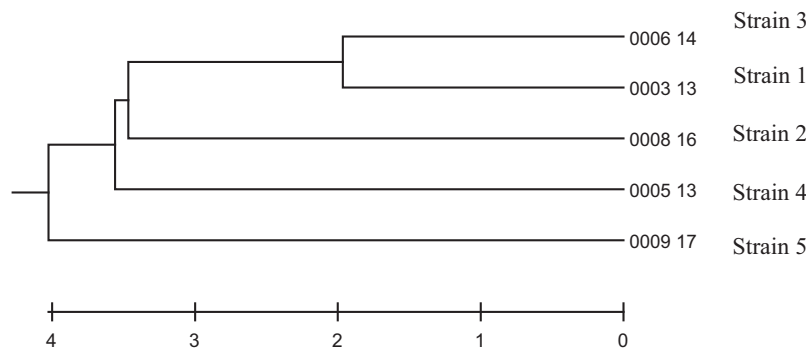
ture. The specimen was cut into a small size (3 mm) and soaked in 8% (v/v) of Glutaraldehyde and Sorenson's Phosphate Buffer (1:1) for 1 h. Then, the samples were washed using distilled water and Sorenson's Phosphate Buffer (1:1). After that the samples were fixed into 4% (v/v) of osmium and distilled water (1:3) for 14 h. Next, all the samples were dehydrated in an ascending series of ethanol from 10% to 90% (v/v) for 15 min each. Then the samples were soaked twice in 100% (v/v) of ethanol (15 min each) before transferred into intermediate fluids of ethanol-acetone mixture with ratio of 3:1, 1:1, and 1:3 for 20 min each. Finally the samples were soaked into

100% acetone four times (20 min each) before proceeding to a Critical Point Drying (CPD) phase. The samples were coated with gold in an ion-coating and viewed under a Scanning Electron Microscope (JEOL JSM-6400).

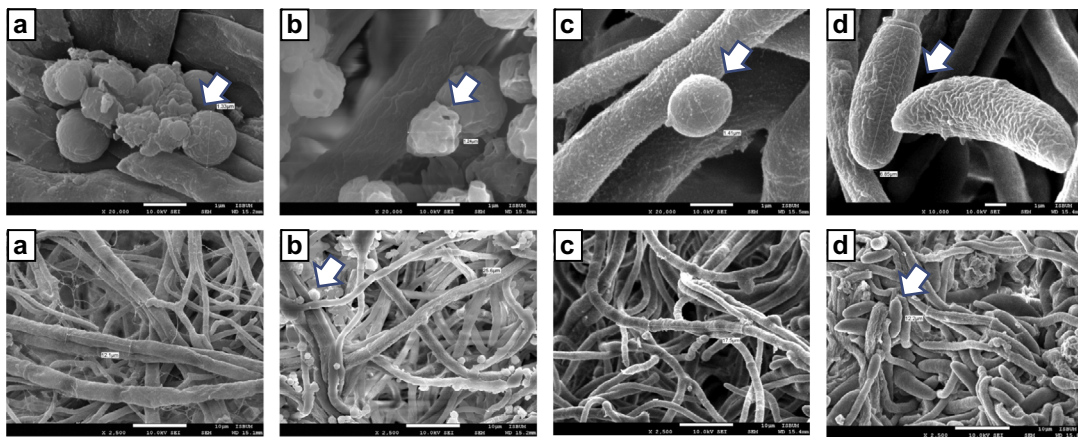
#### 2.2.2. DNA extraction and PCR amplification

Deoxyribonucleic Acid (DNA) was isolated from fresh mycelia taken from the surface plate of each endophytic fungi. The DNA was recovered from scrapped mycelia using i-Genomic BYF DNA Extraction Mini Kit (Intron Biotechnology, Inc.) according to manufacturer's instructions. The quality of genomic DNA was evaluated using a Nanodrops spectrophotometer (Thermo Scientific) and followed by 1% (v/v) agarose gel electrophoresis.

The Polymerase Chain Reaction (PCR) amplification was conducted using primer Internal Transcribed Spacer Ribosomal DNA (ITS rDNA). ITS 1F (forward primer 5' CTT GGT CAT TTA GAG GAA GTA A 3') and ITS 4 (reverse primer 5' TCC TCC GCT TAT TGA TAT GC 3'). The 20  $\mu$ l PCR reaction mixture contained 4  $\mu$ l of buffer, 1.2  $\mu$ l of  $MgCl_2$ , 2  $\mu$ l of dNTP, 0.6  $\mu$ l of primer F, 0.6  $\mu$ l of primer R, 10.4  $\mu$ l of distilled water and 1  $\mu$ l of DNA. The PCR was performed using 2720 PCR Thermo Cycler followed the standard procedure: initial denaturation at 94 °C for 1 min followed by 35 cycles for each denaturation (94 °C for 1 min), annealing (51 °C for 1 min) and extension (72 °C for 1 min). The last stage was the final extension at 72 °C for 8 min and cooling to 10 °C. Then, these PCR products were analyzed on electrophoresis using 1% (v/v) agarose gel that was run at 100 volts, 400 mA for 30 min. The gel was stained with ethidium bromide and a band was photographed on a UV light transilluminator.



**Figure 4** Neighbour-joining tree of all endophytic fungi isolated from *Catharanthus roseus* which was produced in MEGA 5. Strains 1 and 3 were *Colletotrichum* sp., and strains 2, 4 and 5 were *Macrophomina phaseolina*, *Nigrospora sphaerica* and *Fusarium solani* respectively.



**Figure 5** The scanning electron micrographs of endophytic fungi (A) *Colletotrichum* sp., (B) *Macrophomina phaseolina*, (C) *Nigrospora sphaerica* and (D) *Fusarium solani* showed a various shapes of spores (arrow) and hyphae. The spores and hyphae were observed under 20,000 $\times$  and 2500 $\times$  objectives respectively.

The amplicons were cleaned after ascertaining their integrity before automated sequencing directions. The sequences were then aligned using the data from a GenBank at the NCBI (<http://www.ncbi.nlm.nih.gov>) and the most identical sequences of fungi were identified.

### 2.3. Assessment of fungal growth

A 1 cm diameter agar disk from the margin of seven days old growing colony of each isolate grown at 25 °C was used to centrally inoculate each replicate. The plates were incubated at the same condition above and the experiment consisted of three replicates. Assessment of growth was made daily during the seven days incubation period. For each colony, the mean radial mycelia growth was calculated by measuring two different colony radii on each of the three plates. The growth was corrected by subtracting the 1 cm diameter of the original plug of inoculum and plotted against time.

## 3. Results and discussions

A total of five strains of filamentous fungi were isolated from leaf and roots of both varieties of *C. roseus*. Based on qualitative techniques, all strains showed positive results on

a test for extracellular enzymes. Cellulase was present in all strains while amylase only was found in strains 1, 3 and 5. Strain 5 is a sole producer for protease (Table 1). Cellulases and amylases are hydrolytic enzymes that degrade cellulose and starch materials respectively. It is secreted by microorganisms for utilizing that materials as substrat for their growth. Meanwhile, proteases are enzymes, with catalytic function to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids from the plant component. The release of extracellular hydrolytic enzymes, leads these fungi to penetrate and colonize plant roots and debris, including decomposition of organic substrates. Thus, it could compete for nutrients from the host plant and produce secondary metabolites toxic to plant pathogen. Study on the fungal growth on the MEA plate showed the rapid growth after 3 days of cultivation. The observation was made only for seven days since the mycelia of most strains were compact and covered the entire Petri dish within a week except strain 4. The mycelia growth curves of all strains are shown in Fig. 1.

All fungi strains were identified based on the morphological appearance on the surface of solid medium (Fig. 2) and observation of microstructures under SEM. Identification of fungal strains was further confirmed by molecular technique. The high quality DNA extracted from these fungi was used for PCR by using ITS1 and ITS4 as a primer and a marker

(100 bps). The PCR amplification of ITS regions successfully amplified the genomic DNA of the fungi (Fig. 3). These DNA nucleotide sequences have been identified and deposited in European Nucleotide Archive (ENA) European Nucleotide Archive (ENA; <http://www.ebi.ac.uk/ena/>) with the accession number (Table 2). The result showed four different species of fungi were successfully identified and was confirmed through neighbour-joining tree using Molecular Evolutionary Genetics Analysis (MEGA) (Fig. 4).

Strains 1 and 3 were identified as *Colletotrichum* sp. has been isolated from both host plant's leaf. This white colored fungus was also found in *Cephalotaxus hainanensis* (Xuan et al., 2012). Both of *C. roseus* and *C. hainanensis* plants produced alkaloids that had been used for anticancer and this fungus is expected could generate constituents which are similar to the host plants (Stierle et al., 1993). Jin-Long et al. (2011) also reported that due to the complex chemical constituents of medicinal plants more fungi with the novel and functional metabolites of pharmaceutical importance were isolated from the medicinal plant. Strain 2 with light black colored was identified as *Macrophomina phaseolina*. This soilborne fungus (Endraki and Banihashemi, 2010; Arias et al., 2011) was isolated from the root of *C. roseus* (purple). Fungus 4 was identified as *Nigrospora sphaerica* is the imperfect fungus that was found in *C. roseus* (white) leaf. It has a unique appearance by changing the color from white to dark green. It is also able to secrete the metabolites that change the plate culture medium from brownish to red color. This fungus was reported earlier as an endophytic or pathogenic fungus (Zhang et al., 2009) and only a few literatures on this fungus. Fungus 5 with a new moon shape of spore that was isolated from a white *C. roseus*' root was identified as *Fusarium solani*. The round shape of spore was observed from *Colletotrichum* sp. and *N. sphaerica* while the spores of *M. phaseolina* were alike a raspberry shape. All the fungi had a septate hyphae range from 12 µm to 25.6 µm (Fig. 5).

*M. phaseolina*, *N. sphaerica* and *F. solani* were reported as pathogens of many agricultural crops and plants (Anandi et al., 2005; Siavosh and Seyed, 2012). This *M. phaseolina* causes seedling blight, root rot, and charcoal rot of more than 500 crop and non-crop species. It has a very wide distribution covering most of the tropics and subtropics, where high temperatures and water stress occur during the growing season. Initial infections occur at seedling stage but remain latent until the plant approaches maturity. Plants may wilt and die.

However, in this study, these three fungi may play an important role as endophytes. It is probably due to the flexibility of *C. roseus* in producing antimicrobial activity. Balaabirami and Patharajan (2012) reported that the leaf extract of *C. roseus* gave a positive result for antimicrobial test against *Aspergillus niger*. It was indicated that these fungi helped to synthesize bioactive agent that could be used by plants for defense against other pathogens (Selim et al., 2012). Thus, when these fungi became endophytes, it is believed to carry out a resistance mechanism in order to overcome a pathogenic invasion by producing secondary metabolites bearing antimicrobial activity (Selim et al., 2012).

#### 4. Conclusion

In summary, we successfully identified the putative endophytic fungi isolated from different tissues of wildy grown *C. roseus*

(purple and white) using a morphology and a molecular technique. The ITS 28s ribosomal RNA gene showed that the species were *Colletotrichum* sp., *M. phaseolina*, *N. sphaerica* and *F. solani* based on the sequences from a GenBank of BLAST system. All strains possess hydrolytic enzymes for digestion and penetration of plant cell wall.

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