Molecular identification of three endophytic fungi from eucalyptus leaves and their pathogenicity

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Abstract. Eucalyptus leaves have antibacterial, antiviral, and antifungal components and have been used to treat colds, influenza, rhinitis, sinusitis, and other respiratory infections. Endophytic fungi can produce secondary metabolites similar to their hosts. The research was performed to assess the pathogenicity of species-specific endophytic fungi isolated from Eucalyptus leaves on Eucalyptus citriodora plantlets through in vitro inoculation and molecular identification. This research was conducted at the Indonesian Spice and Medicinal Crop Research Institute, Plant Protection Laboratory, from July 2020 – May 2021. Isolation of endophytic fungi was done by direct planting method, whereas purification and characterization of fifty isolates were conducted on Potato Dextrose Agar (PDA) media for their activity as antimicrobial. Based on ITS sequence analysis, the DNA sequences of isolate A-TKR1 were identical to Aspergillus flavus, isolate E-TKR3 to Penicillium citrinum, and isolate ECT-TKR7 to Neofusicoccum parvum. The similarity of those isolates was 100%, 99.81%, 99.82%, respectively. The re-isolation analysis of infected tissues showed that A. flavus, P. citrinum, and N. parvum matched Koch's postulates and confirmed endophytes' presence within living tissues.

Keywords: Eucalyptus citriodora, antimicrobial, in vitro, sequence analysis

1. Introduction

Endophytic fungi and bacteria have been found in practically every vascular plant species' tissue and all host plant organs and seeds [1]. Medicinal plants have been identified as a source of endophytes capable of producing novel pharmaceutically important secondary metabolites [2]. A commercially viable multi-beneficial endophyte was utilized as a catalyst for lucrative, environmentally-friendly agriculture[3]. Endophytic fungus generated enzymes and bioactive compounds to compete with pathogens directly for nutrients and space.

These endophytic microbes can produce secondary metabolites in accord with their host plants. This characteristic is very beneficial in supporting the development of health science and medicine. There were about 300,000 types of plants, and each plant contained one or more endophytic microbes consisting of bacteria and fungi [4].

Endophytes of various types have been isolated from their host plants and successfully cultured in an appropriate media. Similarly, the endophytic microbes' secondary metabolites have been isolated and purified, and their molecular structure has been determined. Some endophytic microbes were capable...
of producing antivirals. For example, the endophytic fungus *Cytonaema* sp. could produce cytanic acid metabolites A and B, which had the molecular structure of 1,8 cineol, which possessed anti-corona virus activity. However, several studies on the endophytic fungi diversity, host plant affinity, and environmental and medicinal effects of fungal endophytes have not been deeply explored. Our study was aimed at species-specific endophytic fungi isolated from Eucalyptus leaf parts to evaluate their pathogenicity on *Eucalyptus citriodora* plantlets through in vitro inoculation and molecular identification.

2. Material and method

2.1. Isolation of endophytic fungal strain

Eucalyptus leaves were collected from several areas in West Java and Laing Research Installation, West Sumatra. The isolation method used followed the modified method of [6]. Firstly, the leaves were cut 0.8 cm × 0.8 cm and put in a tightly closed sieve. Furthermore, the leaves were surface-sterilized by washed leaf pieces under tap water for ± 30 minutes. Afterward, leaf samples were soaked in 70% alcohol for 1 minute, then washed with sterile water three times. The pieces of leaves were then dried on tissue paper.

The dried leaf pieces were divided into two parts. The first part was planted directly on the Potato Dextrose Agar (PDA) media, whereas the second part was scraped off its leaf surface and then planted reversely where the scraped part was attached to the surface of the PDA media. Both petri dishes were then incubated at room temperature for 2 days. Afterward, the grown fungi were transferred to new PDA media for purification and molecular identification.

2.2. Isolation of fungal DNA for molecular identification.

Endophytic fungi were isolated from eucalyptus leaves that had been purified. The DNA of endophytic fungi was isolated using a modified Doyle method [7]. First, the mycelium was inoculated in 150 mL of Potato Dextrose Broth (PDB) in Erlenmeyer, then grown in a shaker at room temperature for 11 days. Following that, the mycelium was harvested by filtering them through Whatman filter paper. 500 µl of CTAB buffer [CTAB 10% (Sigma H-5882); 2.5 M NaCl; 1 M Tris-HCl pH 8; 0.5 M EDTA pH 8.0; Nuclease-Free Water (Thermo Scientific)] were added to the 0.1 g mycelia, and 2% PVP-40 (Polyvinylpyrrolidone) were crushed by a sterile mortal and placed in 1.5 ml of Effendof.

The microtube was heated in a heat block at 65°C for 60 minutes, and the temperature was reversed every ten minutes. In the microtube, a 500 µl CI solution (Chloroform isoamyl alcohol – 24 : 1, v/v) was vortexed. The samples were centrifuged at room temperature for 10 minutes at 10,000 rpm. The DNA solution, indicated by the clear color at the top, or supernatant, would separate from the chloroform solution and mix with other cells (indicated by the white color in the bottom). The upper portion (500-600 µl) was then transferred to a new microtube, to which cold isopropanol (-20°C) was added as much as 2/3 of the volume of the DNA solution, mixed with its micro-pipet, and reversed to obtain DNA deposits. The DNA solution was then centrifuged at 6,500 rpm for 1-2 minutes, and the supernatant was removed, leaving DNA deposits (the white color) at the microtube's base. The 150 µl DNA deposits were washed twice with 70 percent cold ethanol (-20°C) by centrifuging them for 10 minutes at 12,500 rpm. The DNA pellets were air-dried for 15-20 minutes before being dissolved in Nuclease-Free Water (Thermo Scientific) to obtain a volume of 20-50 µl. For 30 minutes, the solution was incubated at room temperature [7]. The Nano Photometer was used to assess the quality and quantity of DNA (IMPLEN, Korea).
2.3. Amplification of fungal DNA
The extracted DNA was amplified with the SensQuest Labl cycler (Germany), and the ITS gene was amplified with the ITS 4 and ITS1F primers. The total volume of the PCR was 50 µl, which included 2 µl of forward primer, 2 µl of reverse primer, 4 µl of template DNA, 25 µl of Red Mix (BIOLINE), and 17 µl of Aquabides as a solvent. PCR programs, namely (Fungi 1), were run under one cycle of initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation 94°C for 30 seconds, annealing 55°C for 20 seconds, extension 72°C for 1 minute, final extension 72°C for 7 minutes, and final storage temperature 4°C. The universal primary used consisted of two primary pairs, ITS1F as forward primers and ITS4 as reverse primers. The ITS1F primer was (5’-CTTGGTACATTAGAGGAAGTAA-3’) and ITS4 primary was (5’-TCCTCCGCTATTGATATGC-3’). This amplification process was expected to amplify the ITS gene, usually 600 bp in length [8][9]. The amplification results were electrophoresed with agarose and visualized under UV light.

2.4. Visualization of endophytic fungi DNA amplification result.
A PCR machine amplified the endophytic fungi DNA, then was checked by electrophoresis. A 3 µl of 100 bp markers were put into 1.2% agarose gel, combined with 2 µl Red Safe coloring. The same procedure was also conducted for three DNA samples derived from PCR. All material was then checked by electrophoresis for 50 minutes at 50 volts. Subsequently, the agarose gel was washed with sterile distilled water before being visualized with a gel document.

2.5. Sequencing of fungus DNA
The base arrangement that formed the fungus DNA sequence was obtained through sequencing. The sequencing procedure was carried out at Singapore’s first base. The DNA sequencing results were then tracked using the Basic Local Alignment Search Tool (BLAST) data tracking program at the National Center for Biotechnology Information at the National Institute of Health (USA) to determine the percentage homology of the isolates.

2.6. In vitro inoculation for seedling pathogenicity assay
The identified endophytic fungal culture suspension was tested its pathogenicity using pathogenicity assays. One hundred µl of conidia suspension were transferred into bottles of Eucalyptus citriodora plantlet (seedling). The control group was set up in triplicates under the same conditions but added no endophytic fungal cultures. The test co-culturing bottles were moved into the plant growth chamber equipment after the inoculation and kept at 28 °C with light intensity. All the test bottles were examined in 3 days after incubation.

2.7. Re-isolation and examined of endophytic fungi from seedlings
All the samples (seedlings) were washed with sterilized distilled water to eliminate surface fungal growth. After sterilized, 70% alcohol was added into the sample bottles for 30 s and mixed using hand shaking, which was then washed twice with sterilized distilled water under laminar airflow. Tissue paper sterilized in oven at 160 °C for 2 h was used for cutting the samples into smaller pieces. The samples (seedling) were moved onto the potato dextrose agar media plates. Ten pieces of samples were transferred onto each Petri plate with the surface touching the media. The plates were incubated for five days at 28°C in the incubator. The fungal hyphal tip arising from the samples was evaluated after the fifth day of incubation following [10] modification.
3. Results and discussions

3.1 Results

3.1.1 Potential endophytic fungi from Eucalyptus lea. As a result of the isolation, purification, and characterization process, three potential endophytic fungi would be further evaluated to determine their species (Figure 1).

![Colonies of 3 days-potential endophytic fungi on PDA media as seen from: A. top and B. bottom.](image)

The potential endophytic fungus E.TKR3 was isolated from Eucalyptus spp. from Laing, West Sumatra (ECL), A.TKR1 was isolated from the *E. piluraris* from Bukit Unggul Lembang, West Java (EA), while TKR7 was isolated from *E. citriodora* from Tahura West Java (ECT) (Figure 2). Based on the phylogeny tree, the E.TKR3 and A.TKR1 isolates were in the same group and differed from ECT.TKR7. The ECT.TKR7 isolate was in the same group as *Neofusicoccum parvum* isolated from Eucalyptus grown in several countries such as Mexico, Venezuela, the USA, and Uruguay.

![The leaf morphology of Eucalyptus leaves contained potential endophytic fungi from several locations: (A) Laing, West Sumatra (ECL); (B) Bukit Unggul Lembang, West Java (EA), and (C) Tahura Bandung, West Java (ECT).](image)
3.1.2. Molecular identification

The total quantity of DNA of E-TKR3, A-TKR1, and ECT-TKR7 was 6459.0 ng/ul, 953.15 ng/ul, and 2187.4 ng/ul, respectively. Amplification of fungi DNA of those three samples produced a DNA band measuring 600 bp (Figure 3). Because the cycle was shorter than the others, PCR program number 1 was used for the following sequencing.

![Figure 3](image)

**Figure 3.** Visualization of three endophytic fungi DNA from Eucalyptus leaves on agarose gel 1.2% produce a DNA band measuring 600 bp. Note: K = negative control; M = marker 100 bp; Isolate A = E-TKR3; B = A-TKR1; C = ECT-TKR7.

The BLAST results showed the similarity level of the ITS gene nucleotide sequences from the three isolates with the fungal ITS gene nucleotide sequences registered in GenBank. The DNA sequences of the A-TKR1 isolate were identical to *Aspergillus flavus* with a 100% similarity. The E-TKR3 isolate was identical to *Penicillium citrinum* with a similarity of 99.81%, and isolate ECT-TKR7 was identical to *Neofusicoccum parvum* with a similarity of 99.82% (Figure 3).

![Figure 4](image)

**Figure 4.** Phylogenetic analysis based on ITS DNA sequences of three endophytic fungi from Eucalyptus leaves and their similarity with fungal isolates from the Gene Bank. The phylogenetic tree was attained using the UPGMA method.
3.1.3. Re-Isolation and Examined of Endophytic Fungi from Seedlings

Based on the results of Koch’s Postulate test on *Eucalyptus citriodora* planlets, the three isolates could re-isolated from leaf tissue after 3 days inoculation while in the control treatment that no fungus was found [Figure 5].

**Figure 5.** A. *Penicillium citrinum* (E.TKR3), B. *Aspergillus flavus* (A.TKR1), C. *Neofusicoccum parvum* (ECT.TKR7), D. *Eucalyptus citriodora* seedling after inoculated by E-TKR3 isolate, E. *E. citriodora* seedling after inoculated by A.TKR1 isolate, F. *E. citriodora* seedling after inoculated by ECT.TKR7 isolate, G. Control without inoculated of fungus, and H. *E. citriodora* seedling without inoculated by fungus isolate.

3.2 Discussion

The ITS sequence analysis was generally used to identify fungi to the species level [11]. The ITS section has the highest probability of identifying a broad range of fungi with the most obvious barcode gaps in interspecific and intraspecific variations [12]. ITS rDNA test of endophytic fungi from 174 medicinal plants showed that they were identified as Alternaria, Phomopsis, Sporidesmium, Paecilomyces, and Fusarium [13]. Guo et al. (2000) used ITS rDNA section sequences to identify Valsaceae, Mycosphaerellaceae, Xylariaceae, Clypeosphaeriaceae, and Dothideales from endophytic fungal isolates of *Livistona chinensis* [14].

Several studies have revealed that *Penicillium citrinum* was an endophytic fungus isolated from *Azadirachta indica* [15], *Ocimum tenuiflorum* [16], and wheat [17]. In addition, *P. citrinum* strain KACC43900 has been patented as an organic fertilizer and was known to produce gibberellins [18]. In contrast, *Penicillium citrinum* was reported as a pathogen on *Allium sativum* [19].

Another fungus in the Penicillium genus has been found to secrete brefeldin A, a metabolite that has been shown to inhibit the dengue virus, the Japanese encephalitis virus, and the Zika virus [20].
Kojic acid (an antibacterial metabolite) was discovered in the leaves of the mangrove plant *Sonneratia alba* isolated from the high yielding endophytic fungus *Aspergillus flavus* on Timor Island, Indonesia [21]. Kojic acid has been widely used in the cosmetic and pharmaceutical industries, particularly for UV B protection, in addition to its antibacterial properties. Another study found that treating human papillomavirus 18-transformed HeLa cells with gliotoxin derived from a marine *Aspergillus* sp. caused apoptosis, effectively preventing HPV proliferation [22].

Many woody plants around the world have been documented to have *Neofusicoccum parvum* as an economically important pathogen, including pomegranate, Eucalyptus, and walnut, with host spread in 90 plant species. *N. parvum*, for example, has been linked to stem cancer in pomegranate plants in Iran [23]. In Iran, it has also been identified as a pathogen that causes disease in *Vitis vinifera, Actinidia deliciosa, Cupressus sempervirens, Ginkgo biloba, Juglans regia, Rubus fruticosus*, and *Salix sp.* [24].

However, it has also been reported that 20 isolates including 7 species of *Neofusicoccum* produced 91 chemical compounds. These compounds were classified based on their structure in nine groups: cyclohexene, 5,6-dihydro-2-pyranone, fatty acids, mellein, myrtocommulones, naphthalenones, naphthoquinones, phenols and alcohols, sesquiterpenes [25].

Although many chemical compounds are found in the endophytic fungi from this study, none of them contain compounds commonly found in the leaves of the Eucalyptus plant, such as citronellal in *E. citriodora* [26][27] or 1,8 cineol in other *Eucalyptus* spp [28, 29].

4. Conclusions
Isolation of endophytic fungi was done by direct planting method, whereas purification and characterization of fifty isolates were carried out on Potato Dextrose Agar (PDA) media for their activity as antimicrobial. Based on ITS sequence analysis, the DNA sequences of isolate A-TKR1 were identical to *Aspergillus flavus*, E-TKR3 to *Penicillium citrinum*, and ECT-TKR7 to *Neofusicoccum parvum*. The similarity of those isolates was 100%, 99.81%, 99.82%, respectively. The findings of reisolate examination of infected tissues revealed that *A. flavus, P. citrinum*, and *N. parvum* met Koch's postulates indicated endophytes existed inside living tissues.

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Contributorship
RN, G and HN have role as main contributors, whereas NB and IR were co-contributors

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